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The Combi-Targeting Concept: A Novel Tumour Targeting Strategy

by

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Abstract

Over the past two decades, novel targets for anticancer agents have been identified. One such target, the epidermal growth factor receptor (EGFR) that is overexpressed in a large number of carcinomas including breast, ovarian, and prostate, is a marker for tumour invasiveness and poor prognosis. Agents of the quinazoline class have been developed that block EGFR-mediated signaling and induce antitumour activity in the clinic. However, the major deficiency of these compounds is that they are cytostatic agents that induce reversible antiproliferative activities. To circumvent these problems, we designed a novel tumour targeting approach termed "the combi-targeting" concept. This theory is based on the fundamental premise that compounds capable of interfering with multiple targets in the cancer cells are more efficient antitumour agents than the single-targeted ones. Thus, the "combi-targeting" concept proposes the design of molecules termed "combi-molecules" or TZ-*I* to not only bind to the tumour target but also to be allowed to degrade to another inhibitor *I* of the same target + a DNA damaging species TZ, leading to small molecules capable of repetitively blocking one target while damaging another. Using EGFR as a tumour target, the first TZ-*I* prototype SMA41, an anilinoquinazoline containing a triazene tail at the 6-position, was synthesized. We demonstrate herein that the compound enters the cell by passive diffusion, degrades under physiological conditions to yield an intact TK inhibitor ("SMA52") (*I*) + a short-lived DNA-damaging methyldiazonium species (TZ). In the EGFR-expressing human tumour cells, SMA41 (TZ-*I*) behaved as a binary targeted drugs with ability to: a) inhibit EGF-induced receptor autophosphorylation, cell cycle progression and growth, b) induce dose-dependent DNA damage, and c) inhibit cell proliferation with greater potency than

the released reversible inhibitor (*I*) both *in vitro* and *in vivo*. This work represents the first demonstration of the feasibility of a small molecular system "programmed" to bind to a validated tumour target, degrade to a second inhibitor of the same target and another species capable of damaging a secondary target (*e.g.* DNA). Thus, studies performed within the framework of the "combi-targeting" concept have perhaps given rise to the first oncogene-targeted unimolecular mimic of classical multidrug combination therapy.

Résumé

Au cours des deux dernières décennies, de nouvelles cibles permettant le développement de nouveaux antinéoplasiques ont été découvertes. L'une de ces cibles, le facteur de croissance de l'épiderme communément appelé « EGFR » est surexprimé dans un nombre important de néoplasmes tels les cancers du sein, de l'ovaire et de la prostate. Son expression est associée à la capacité invasive de la tumeur et conduit à des pronostics plutôt négatifs. Récemment des agents capables de bloquer l'action de l'EGFR et ainsi induire une activité antitumorale *in vitro* et en clinique, ont été développés. Cependant, les inhibiteurs de l'EGFR étant des agents cytostatiques, la réversibilité de leur effet demeure un obstacle important. Pour contourner ce problème, nous avons récemment conçu une nouvelle stratégie de ciblage dénommée «ciblage combiné». Cette théorie est fondée sur le principe suivant: les molécules conçues pour interagir avec de multiples cibles cellulaires devraient être des agents antitumoraux plus efficaces que celles dirigées vers une cible antitumorale unique. La vérification des postulats de la théorie de « ciblage combiné» est basée sur la conception d'une molécule dénommée « combi-molécule » ou TZ-*I* ayant la propriété de non seulement se lier à une cible mais aussi de s'hydrolyser pour donner lieu à la formation de deux autres entités antitumorales : l'une (*I*) possède une affinité pour la même cible que TZ-*I* et l'autre TZ est dirigée vers une autre cible intracellulaire. Cette stratégie permet de créer des molécules capables d'induire un blocage à répétition de la même cible tout en endommageant une autre différente de celle-ci. Après avoir choisi l'EGFR comme cible antitumorale, nous avons synthétisé le premier prototype de TZ-*I*, SMA41, une triazene accrochée à la position 6 d'un quinazoline. Nous avons ainsi démontré que SMA41 pénètre la cellule par diffusion

passive, se dégrade dans le milieu biologique pour générer SMA52 (un autre inhibiteur *I*) + l'ion diazotiure de methyle pourvu de la capacité de cibler l'ADN. Dans les cellules surexprimant l'EGFR, nous avons démontré que la SMA41 s'est comportée comme un agent à double cible, ayant la propriété: 1) d'inhiber les effets d'autophosphorylation de l'EGFR, la progression du cycle cellulaire et finalement la prolifération induite par le facteur de croissance de l'épiderme (EGF) et 2) d'induire une activité antitumorale supérieure à celle de SMA52 (*I*) tant *in vitro* qu'*in vivo*. Le présent travail présente la première démonstration de la faisabilité d'une petite molécule « programmée » pour se lier à une cible antitumorale, pour être convertie en un autre inhibiteur de la même cible et en un agent dirigé vers une autre cible telle l'ADN. Ainsi, les études exécutées dans le cadre du concept de «ciblage combiné» ont peut-être donné lieu à la première émulation unimoléculaire de la thérapie de combinaison classique.

This thesis is dedicated to my grandmother, the late Mrs. Dorothy Cyr, who perished from the terrible disease that is the focus of this thesis.

May our scientific endeavours be successful.

Acknowledgements

Above all others, my supervisor (Dr. Bertrand Jean-Claude) has provided me with guidance and his limitless knowledge throughout this degree. Not only has he offered tremendous support and inspiration, but he has also served as a trusted confidante. He corrected my path whenever I wavered, and helped reaffirm the adage "try and try again." Without him I would not be writing these words, and for this I will be eternally grateful.

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Thanks to my friends, near and far. You make life a whole lot brighter.

Lastly I would like to thank my loving parents, Robert and Linda Matheson, and my brothers, Chris and Brent, for always being there for me. I would also like to thank my caring and patient boyfriend, John Adams, for his support.

Foreword

This thesis is presented in manuscript-based form, in accordance with the terms listed by the Faculty of Graduate and Postdoctoral Studies. The following excerpt is taken from their Guidelines for *Thesis Preparation*:

As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a table of contents, a brief abstract in both English and French, an introduction which clearly states the rational and objectives of the research, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis.

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List of Abbreviations

AGT	O6-alkylguanine DNA alkyltransferase
BER	base excision repair
BG	benzylguanine
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
HPLC	high pressure liquid chromatography
HRG	heregulin
HRP	horse radish peroxidase
I	inhibitor
MMR	mismatch repair
MTIC	5-(3-methyltriazen-1-yl) imidazole-4-carboxamide
MTZ	mitozolomide
PBR	peripheral benzodiazepine receptor
PBS	phosphate-buffered saline
PGT	poly (L-glutamic acid-L-tyrosine, 4:1)
RTK	receptor tyrosine kinase
SRB	sulforhodamine B assay
TEM	temozolomide
TK	tyrosine kinase
TZ	methyldiazonium

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CHAPTER 1

INTRODUCTION

1.1 Preface

Over the past 20 years, cancer chemotherapy has slowly been evolving. Initially, treatments were limited to the use of cytotoxic drugs with deleterious side effects. These drugs were designed to hinder the growth of rapidly dividing cells, and eventually reduce tumour progression. Although the use of non-selective drugs remains customary, new methods are being developed to overcome their adverse characteristics. Some examples of modern approaches include the design and synthesis of drug conjugates (Langer *et. al.*, 2001; Moody *et. al.*, 2002), antibody-directed enzyme prodrug therapy (ADEPT) (Bagshawe, 1993), and the development of inhibitors of tyrosine kinase involved in cancer cell proliferation (Ward *et. al.*, 1994; Rewcastle *et al.*, 1995).

Acquired drug resistance mediated by DNA repair enzymes imposes another limit on effective chemotherapy with DNA-directed cytotoxic drugs, and is perhaps one of the most common causes of treatment failure (Baer *et al.*, 1993; Aebi *et al.*, 1997; Gerson, 2002; Phillips and Gerson, 1999). To avoid this problem, several distinct drugs with different mechanisms of action are usually administered. For example, patients with Hodgkin's lymphoma are given ABVD (Adriamycin, Bleomycin, Vinblastine, and Dacarbazine) combination therapy (Fung and Nademanee, 2002). Moreover, inhibitors of resistance-associated proteins are being designed (Cai *et al.*, 2000; Wedge *et al.*, 1997; Dolan and Pegg, 1997). Some such inhibitors are currently in clinical trials in combination with classical alkylating agents (Spiro *et al.*, 1999). However, the future of this approach is dampened by the lack of selectivity of inhibition of DNA repair enzymes.

Thus novel chemotherapeutic strategies are required to: a) selectively reduce tumour progression, b) reduce the toxicity of conventional cancer treatment, and c) overcome

drug resistance. This thesis focuses on a novel theory termed the Combi-targeting concept that proposes a new therapeutic approach whereby two anticancer drugs with different mechanisms of action are combined in one molecule to create a tandem method to circumvent the problems associated with a lack of chemosensitivity and acquired chemoresistance.

1.2 Attempts to improve cancer drug selectivity

1.2.1. Antibody-directed enzyme prodrug therapy (ADEPT)

ADEPT, as illustrated in Figure 1.1, was designed to enhance drug selectivity by linking an enzyme to an antibody against a tumour-associated antigen [see (a)] (Bagshawe *et al.*, 1988; Bagshawe, 1993). Following administration of this antibody-enzyme complex, that concentrates the enzyme catalytic activity at the site of the tumour, the patient is given a prodrug [see (b)] designed to be activated by the latter enzyme. Theoretically, this would localize [see (c)] the active chemotherapeutic drug at the tumour site thereby enhancing its target selectivity.

Despite being, *a priori,* an extremely attractive approach ADEPT presents several disadvantages. Firstly, tumour-associated antigens are rarely limited to cancer cells. Some antigens are indeed expressed at higher levels in malignant tissue, such as carcinoembryonic antigen (CEA) in colon cancers, but a bystander effect may occur whereby neighbouring (sometimes normal) cells are damaged (Springer and Niculescu-Duvaz, 1995; Springer *et al.*, 1995). Secondly, the cytotoxic drug of choice must have an optimal half-life (*i.e.* neither too short, nor too long) to be effective. In addition, the

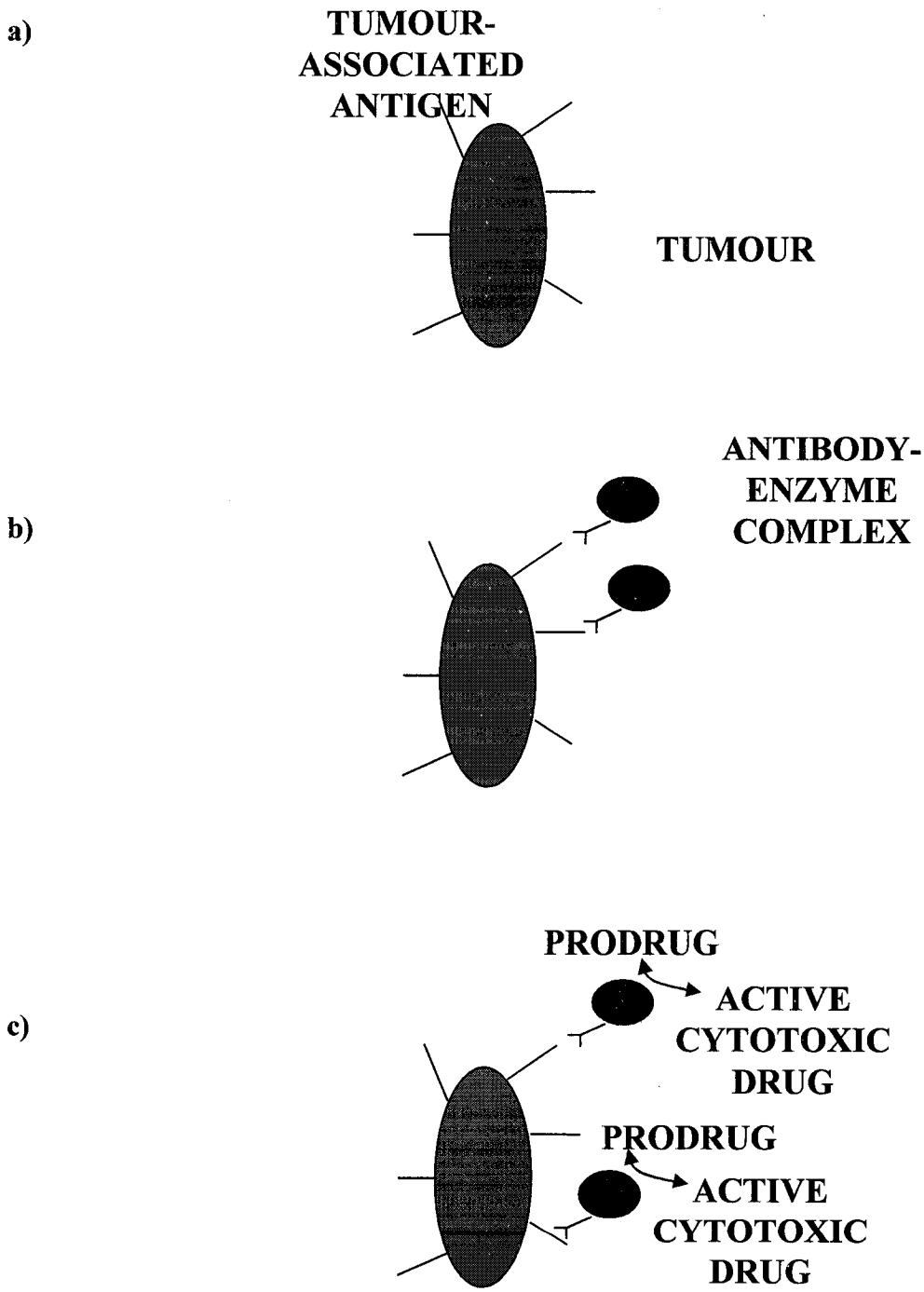


Figure 1.1. The mechanism of antibody-directed enzyme prodrug therapy (ADEPT).

active drug must possess a level of toxicity that differs greatly from that of the prodrug. Lastly, the enzyme used to convert the prodrug to its active state must be non-human, most likely of microbial origin, and non-immunogenic (Bagshawe, 1993; Springer *et. al.*, 1995). Thus, several problems have been associated with this approach, including immunogenicity to antibody-enzyme complexes. More importantly, ADEPT was not found to be efficacious *in vivo* (Bagshawe, 1993).

1.2.2. Molecular conjugation

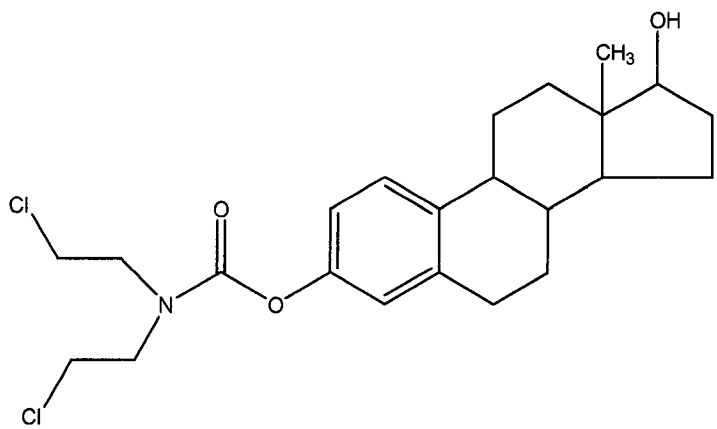
A significant body of work has accumulated to outline the differences between malignant and normal tissues (Kondapaka and Reddy, 1996; Sinha *et al.*, 1995; Sherwood *et al.*, 1999; Tsai *et al.*, 1993; Alaoui-Jamali *et al.*, 1997). As a result, several new molecular targets have been identified for cancer drugs. For example, certain growth factor receptors are overexpressed in tumour cells, and ligands to these receptors can be used to direct cancer treatment. This method is referred to as molecular conjugation, and is designed to reduce the toxicity of chemotherapy by selective drug targeting. By linking a recognition moiety, such as a receptor ligand, to a cytotoxic drug one may exploit certain characteristics that are unique to cancer cells (Guillemard and Saragovi, 2001; Langer *et al.*, 2001; Czerwinski *et al.*, 1998; Moody *et al.*, 2002). Two examples of molecular conjugation are illustrated in Figure 1.2. The first example [see (a)] is one whereby an estradiol moiety is appended to nitrogen mustard with the purpose of targeting breast tumour cells that overexpress the estrogen receptor. Unfortunately, this compound did not exhibit breast cancer selectivity *in vivo* (Petrow and Padilla, 1986; Petru *et al.*, 1988).

Another example of molecular conjugation, shown in Figure 1.2 [see (b)], is the linkage of a peripheral benzodiazepine receptor (PBR) ligand to a melphalan cross-linking agent (Kupczyk-Subotkowska *et al.*, 1997). PBR being overexpressed in brain tumours (Kupczyk-Subotkowska *et al.*, 1997), was considered a useful target for therapeutic intervention in brain tumour treatment. Unfortunately, this drug failed to induce selective targeting of PBR-overexpressing tumours. The major problem with such drug conjugates is their bulkiness that hinders binding to their target receptors.

In addition, antibodies and synthetic peptide sequences directed towards either tumour specific proteins, or proteins that are overexpressed by tumours (*e.g.* ErbB2) have been linked to cytotoxic agents and/or cytotoxins (Guillemard and Saragovi, 2001; Czerwinski *et al.*, 1998). Although some degree of tumour selectivity has been observed in *in vivo* models, the clinical efficacy of these approaches remains to be established (Moody *et al.*, 2002; Guillemard and Saragovi, 2001; Mier *et al.*, 2000).

Numerous potential anticancer targets have been identified, and compounds designed to selectively hinder tumour growth are being investigated. One such target is the epidermal growth factor receptor (EGFR), which is overexpressed by many different tumour types. The mechanism of EGFR activation, its roles in cell growth and proliferation, and the possibility of its inhibition as a chemotherapeutic option will be discussed in the following section.

a)



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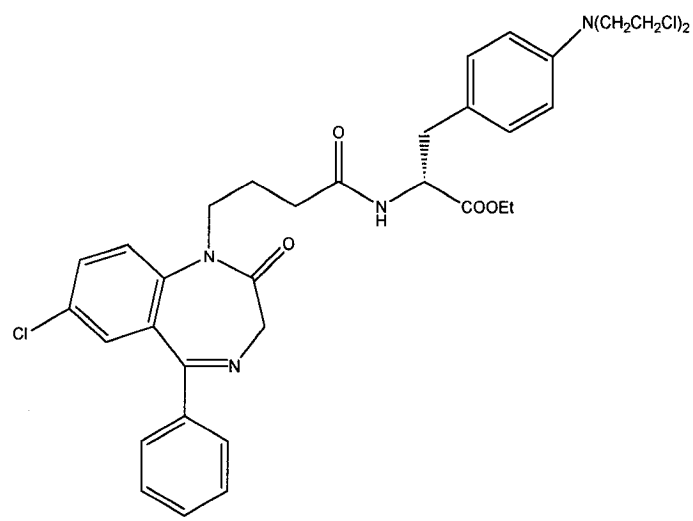


Figure 1.2. Two examples of molecular conjugation.

1.3 The Epidermal growth factor receptor (EGFR)

The Epidermal Growth Factor Receptor (EGFR) is a tyrosine kinase (TK) receptor that mediates a variety of cellular processes, such as cell proliferation and growth (Suo *et al.*, 2002; Wosikowski *et al.*, 2000). It is of particular interest for cancer researchers because of its overexpression in a large number of epidermally derived human tumours including breast, ovarian, and prostate (Wosikowski *et al.*, 1997; Suo *et al.*, 2002; Christenson *et al.*, 2001; Sherwood *et al.*, 1999; Moscatello *et al.*, 1995). Furthermore, cells may express both EGFR and its cognate ligand (*e.g.* EGF, TGF-α), leading to the formation of autocrine loops. This constitutive receptor activation often translates into aggressive tumour progression and poor patient prognosis. Thus, it is important to understand the mechanisms by which EGFR transduces signals in order to develop potential inhibitors designed to treat various EGF-dependent tumours (Baselga, 2000; Ciardello and Tortora, 2001).

1.3.1. Receptor structure and specific ligands

The erbB subfamily of receptor tyrosine kinases is comprised of four closely related receptors: erbB1 (EGFR), erbB2 (Her2), erbB3, and erbB4 (Hackel *et al.*, 1999; Harper *et al.*, 2002; Christenson *et al.*, 2001). Each receptor possesses an extracellular domain with two cysteine-rich regions (Cys), a transmembrane portion (TM), and an intracellular tyrosine kinase (TK) domain with conserved tyrosine residues (Hackel, *et al.*, 1999; McInnes and Sykes, 1997; Egeblad and Jaatela, 2000). A schematic of these domains is outlined in Figure 1.3. [see (a)]. As depicted in Figure 1.3. [see (b)], the cysteine-rich regions bind to ligands such as TGF-α or EGF and induce a conformational

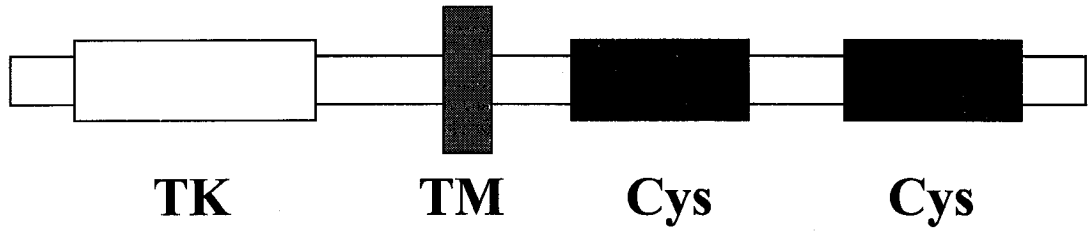
change in receptor. This change results in receptor dimerization and autophosphorylation of internal tyrosine residues and the recruitment of downstream proteins, thus propagating signals to the nucleus.

There are two major ligand families for the erbB receptors: Epidermal Growth Factor (EGF)-like ligands, and Heregulins (HRG) (Suo *et al.*, 2002; Wosikowski *et al.*, 1997; McInnes and Sykes, 1997; Egeblad and Jaatela, 2000). EGF-like ligands include EGF, Transforming Growth Factor-α (TGF-α), Betacellulin (BTC), Heparin-binding EGF (HB-EGF), Amphiregulin (AR), and Epiregulin (EPR). All members of the EGF-like ligands are able to bind directly to EGFR (erbB1), which subsequently heterodimerizes with other erbB receptors (Suo *et al.*, 2002; Wosikowski *et. al.*, 1997; McInnes and Sykes, 1997). This determines ligand specificity and increases signal diversity (Hackel *et al.*, 1999; Christenson *et al.*, 2001). ErbB4 can directly bind BTC, EPR, and HB-EGF, whereas the heregulins induce ErbB3 and ErbB4 heterodimers with other erbB receptors (Hackel *et al.*, 1999; McInnes and Sykes, 1997). Cognate ligands, dimerization partners, and TK activity of each receptor are listed in Table 1.1.

1.3.2. Downstream signaling through EGFR

Binding of EGFR to its cognate ligand induces a conformational change that results in autophosphorylation of its conserved internal tyrosine residues (Ward *et al.*, 1994; McInnes and Sykes, 1997). This recruits downstream effector proteins with Src homology-2 (SH2) domains, as depicted in Figure 1.4. SH2 domains are amino acid motifs that bind to proteins containing phosphotyrosine (pTyr) (Koch *et al.*, 1991;

a)



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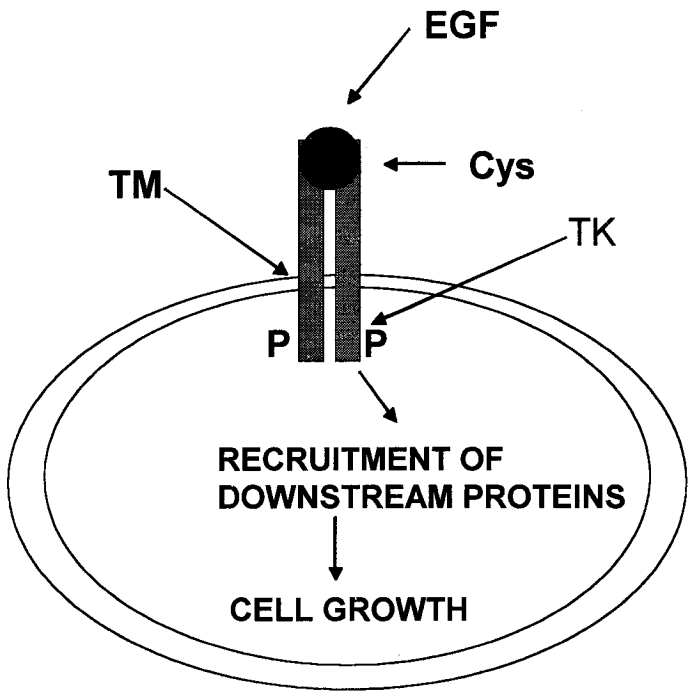


Figure 1.3. The structure of the erbB receptor subfamily (Cys=cysteine-rich regions; TM=transmembrane domain; TK=tyrosine kinase), its binding and downstream effects.

Table 1.1. The erbB subfamily of receptor tyrosine kinases.

RECEPTOR	COGNATE LIGAND(S)	PARTNER(S)	INTRINSIC TK ACTIVITY (YES/NO)	REFERENCE(S)
ErbB1	EGF, TGF-α, BTC, EGF-like ligands	ErbB2,ErbB3, ErbB4	YES	(Hackel *et al,* 1999; Harper *et al.*, 2002)
ErbB2	None	ErbB1, ErbB3, ErbB4	YES	(You *et al.*, 1998; Harari and Yarden, 2000; Alaoui-Jamali *et al.*, 1997) (Hackel *et al.*, 1999)
ErbB3	HRG, BTC, EPR, HB-EGF	ErbB1, ErbB2, ErbB4	NO	(Suo, *et al.*, 2002; Egeblad and Jaatela, 2000)
ErbB4	HRG, BTC, EPR, HB-EGF	ErbB1, ErbB2, ErbB3	YES	(Suo, *et al.*, 2002; Egeblad and Jaatela, 2000)

Pawson *et al.*, 2001; Vidal *et al.*, 2001). These domains are composed of approximately 100 amino acids and are non-catalytic. The binding of phosphotyrosine (pTyr) to an SH2 domain is largely dependent on the amino acid sequence adjacent to the pTyr at the C-terminal end (Koch *et al.*, 1991; Pawson *et al.*, 2001). In proteins possessing SH2 domains, there exists a "signature sequence" of Phe-Leu-Val-Arg-Glu-Ser (FLVRES) (Koch *et al.*, 1991; Sawyer, 1998; Pawson *et al.*, 2001). This sequence permits the formation of multiple hydrogen bonds to pTyr and essential electrostatic charges (Koch *et al,* 1991; Pawson *et al.*, 2001). SH2 domains are composed of a central antiparallel β pleated sheet flanked by an α helix on either side, and ligands containing pTyr bind to the core β sheet and interact with two binding pockets: 1) a hydrophobic binding pocket, and 2) a pocket that interacts with the third conserved pTyr residue (Sawyer, 1998; Pawson *et al.*, 2001). The presence of SH2 domains allows for temporal regulation of signal transduction pathways following activation of a growth factor receptor (Koch *et al.*, 1991; Pawson *et al.*, 2001).

Signaling through EGFR can occur through several different pathways such as Ras-Raf-MAP (mitogen-activated protein) kinase (Figure 1.4.), PLC (phospholipase C), PI_3K (phosphatidylinositol-3'–kinase), and STATs (signal transducers and activators of transcription). Many other pathways are being implicated in EGFR signaling. However, for the purpose of this thesis we will focus on the Ras-Raf-MAP kinase pathway.

1.3.3. Grb2, SH2 and SH3 domains and the recruitment of downstream signaling proteins

As depicted in Figure 1.4, following autophosphorylation, EGFR recruits Growth factor receptor binding protein-2 (Grb2) via interaction between phosphorylated tyrosine (pTyr) and the SH2 (Src-homology-2) domains of Grb2 (Harper *et al.*, 2002; McInnes and Sykes, 1997; Tari *et al.*, 1999). This protein has no catalytic function, but serves to dock other downstream effector proteins. Grb2 is a 25kDa, ubiquitously expressed protein that binds to receptor TKs and Shc adaptor protein. Grb2 accomplishes this binding by its Src-homology-3 (SH3) domain (Tari *et al*, 1999; Sawyer, 1998). An SH3 domain is a stretch of 50-70 amino acids that recognizes and binds to proline-rich sequences with a core PxxP (where x is any amino acid) (Mayer, 2002; Vidal *et al.*, 2001; Kay *et al.*, 2000). Orientation of the peptide is determined by the location of a positively charged amino acid residue relative to the PxxP core that forms a salt bridge with an acidic residue in the SH3 domain (Macias *et al.*, 2002; Kay *et al.*, 2000). There are also three shallow pockets within the SH3 domain where peptide ligands bind. Two (referred to as LP dipeptide pockets) are large enough to accommodate proline residues plus a hydrophobic residue (*i.e.* A, I, L, V, P), whereas the other pocket (pocket 1) is the specificity pocket which is bound by two loops of the SH3 domain (Macias *et al.*, 2002; Kay *et a*l, 2000; Koch *et al.*, 1991).

Interaction with SH3 domains can be either stabilized, or activated by phosphorylation. In the case of Grb2, which possesses one SH3 domain flanked on either

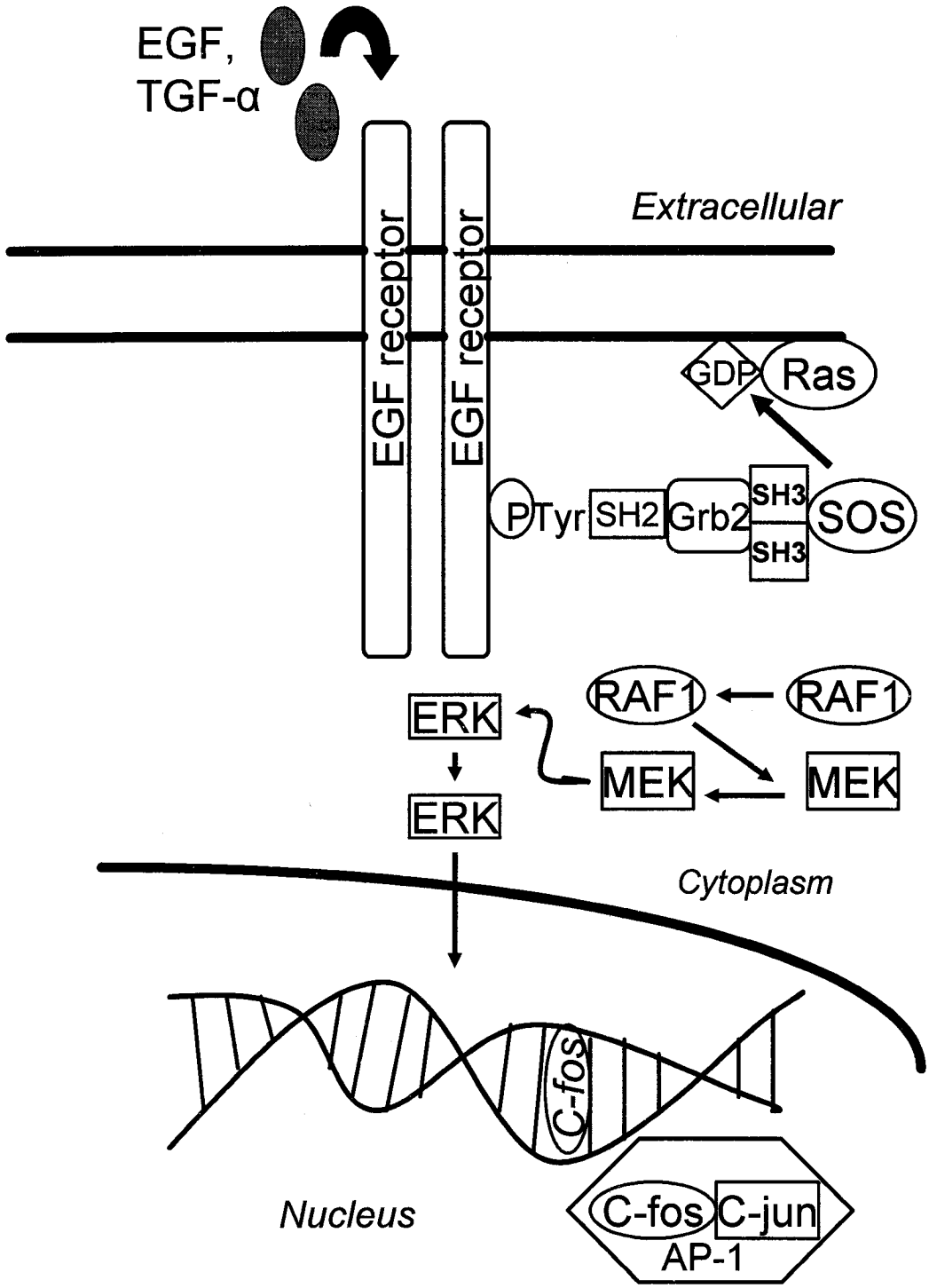


Figure 1.4. The Ras-Raf-MAP kinase signal transduction pathway mediated through EGFR.

side by an SH2 domain, interactions with the SH3 domain are stabilized by interactions between its SH2 domains and pTyr (Macias *et al.*, 2002; Mayer, 2002; Koch *et al.*, 2000; Kay *et al.*, 1999). Since proline is the only amino acid whose side chain is cyclized to its nitrogen backbone, its conformation is limited. Furthermore, multiple proline residues confer to proteins a strong preference for β sheets which orient proline in a position conducive to interaction with an SH3 domain (Macias *et al.*, 2002; Kay *et al.*, 2000). Since proline motifs are unable to bind as tightly as globular domains, due to rigidity, binding to SH3 domains can be easily modulated (Kay *et al.*, 2000; Koch *et al.*, 1991).

1.3.4. Ras GTPase

Grb2 recruits Son of Sevenless (SOS), a guanine nucleotide exchange factor (GEF), via its SH3 domain (Rowinsky *et al.*, 1999; Macias *et al*, 2002). GEFs mediate the exchange of GTP for GDP on Ras, which is the subsequent protein in this cascade (Rowinsky *et al.*, 1999; Mayer, 2002). Ras GTPase is a 21kDa protein that is mutationally active in many tumour types. It functions in gene transcription, cellular proliferation, and a number of other processes (Tari *et al.*, 1999; Rowinsky *et al.*, 1999; Nielsen *et al.*, 1999). Four alternatively spliced variants, of Ras exist, K-Ras4A, K-Ras4B, H-Ras, and N-Ras. Overexpression or mutation of Ras are highly transforming (Rowinsky *et al.*, 1999; Tsai *et al.*, 1993). The first 188 or 189 amino acids possess the highest homology, and the next 25 are varied with the relative function of the isotype. The final four amino acids (CA_1A_2X) at the C-terminus consist of cysteine plus aliphatic residues (*e.g.* V, L, I), with X being either a methionine or a serine, and are involved in subcellular localization (Rowinsky *et al.*, 1999; Nielsen *et al.*, 1999).

Ras activity is regulated by its state of activation in response to external stimuli, such as growth factors. Being a GTPase, Ras cycles between an activated GTP-bound and an inactive GDP-bound state. Wild type Ras possesses a low intrinsic GTPase activity, and thus requires GTPase accelerator protein (GAP) to enhance hydrolysis of bound GTP to GDP to inactivate Ras (Rowinsky *et al.*, 1999; Tsai *et al.*, 1993). Post-translational modifications that allow for association of Ras with the plasma membrane are also required for its activation. Farnesylation is the first step in this activation and is designed to increase the hydrophobicity of Ras by adding a 15-carbon isoprenoid group at the C-terminal end. This reaction is catalyzed by the enzyme Ras farnesyltransferase (FTase) (Rowinsky *et al.*, 1999; Gay *et al.*, 1999; Nielsen *et al.*, 1999). Following activation, Ras can act on a number of downstream pathways such as Raf-1 (serine/threonine kinase), which phosphorylates Mitogen Activated Protein (MAP) kinase.

1.3.5. The EGFR signaling pathway from Raf-1 to MAP kinase

Following recruitment to the cell membrane by an unknown mechanism of activation, Raf-1 phosphorylates two MAP kinase kinases (MEK1 and MEK2) (Harper *et al.*, 2002; McInnes and Sykes, 1997; Rowinsky *et al.*, 1999). These, in turn, phosphorylate MAP kinase (p^{44}MAPK and p^{42} MAPK), also known as Extracellular signal-related kinases 1 and 2 (ERK1 and ERK2) (Magne *et al.*, 2002; McInnes and Sykes, 1997; Mitsui *et al.*, 2001). When activated, MAP kinase is translocated to the nucleus where it phosphorylates a number of substrates (*e.g.* Elk1 nuclear transcription factor). This phosphorylation leads to activation of other kinases, transcription factors, *c-*

fos, and other downstream genes (Tari *et al.*, 1999; Mitsui *et al.*, 2001; Nelson and Fry, 2001). Activation of *c-fos* and *c-jun* results in the formation of activator protein 1 (AP-1) transcription complex. This complex induces the transcription of a number of genes involved in cell growth and differentiation, as well as cell shape and chemotaxis (Albanell *et al.*, 2001; Favoni and De Cupis, 2000).

The genes induced by AP-1 are referred to as "early response genes," since they are rapidly expressed upon binding of EGFR to its cognate ligand. Following their activation, the products of early response genes can act to induce "late response genes," such as cyclins and other proteins involved in the regulation of the cell cycle (Murray and Hunt, 1993). For example, if one was to block early response genes at the level of EGFR activation, the subsequent signaling cascade would be inhibited and the cell cycle would not progress through G1 to S phase (Moyer *et al.*, 1997; Murray and Hunt, 1993).

1.4 Implications of EGFR in the treatment of malignancies

Since EGFR activation is upstream of other signaling proteins, it was believed that the inhibition of its TK activity would translate into growth inhibition (Bos *et al.*, 1997; Ciardello and Tortora, 2001; Kallmeyer and Dobrusin, 1998). The inhibition of signaling through EGFR can be approached by targeting either the cytoplasmic TK portion, or the external receptor binding site. Since EGFR shares considerable amino acid sequence homology with a number of other receptors in the tyrosine kinase superfamily, including platelet-derived growth factor receptor (PDGFR), the design of receptor-specific inhibitors is a primary concern. For example, a class of inhibitors called quinazolines have been designed (two of which are currently in phase II and III clinical

trials) that competitively bind with high affinity to the ATP binding site of EGFR tyrosine kinase and prevent receptor autophosphorylation (Fry *et al.*, 1994; Bridges *et al.*, 1996; Herbst *et al.*, 2002). Therefore, signals transduced through the receptor are blocked. An alternative approach is the design of peptidomimetics or antibodies (*e.g.* Herceptin against erbB2) that inhibit erbB receptor function at the ligand binding site of the extracellular receptor domain (Braun *et al.*, 1984; Shawver, 1999; Fan *et al.*, 1993; Pettit *et al.*, 1997). Research is currently being performed on inhibitors designed to target several key regulators of EGFR-mediated signal transduction. Since Grb2 overexpression correlates with Grb2-SOS complex formation, peptidomimetic SH2 inhibitors have been designed to disrupt Grb2 binding to pTyr (Gay *et al.*, 1999; Tari *et al.*, 1999; Favoni and De Cupis, 2000). These phosphopeptides have demonstrated efficacy and high specificity for disruption of Ras function (Vidal *et al.*, 2001; Gay *et al.*, 1999).

Another possible therapeutic approach is the inhibition of Ras protein FTase (Rowinsky *et al.*, 1999; Nielsen *et al.*, 1999). Since mutant Ras often exhibits less GTPase than wild type, it is usually constitutively active. Using both peptidomimetics and nonpeptide inhibitors, growth inhibition of Ras-transformed fibroblasts has been demonstrated (Rowinsky *et al.*, 1999). Moreover, in pre-clinical toxicology studies these compounds have been well-tolerated and highly specific for malignant tissues (Rowinsky *et al.*, 1999; Nielsen *et al.*, 1999). Thus far, results are promising for selective chemotherapeutic approaches designed to block signaling cascades and hence inhibit the growth of cancer cells. The approach we chose to study will target the ATP binding site of the TK domain of EGFR using inhibitors of the quinazoline class.

1.5 Quinazolines

In 1994, screening efforts of EGFR TK led to the identification of 4-anilinoquinazoline heterocyclic mimics of adenine (Ward *et al.*, 1994; Rewcastle *et al.*, 1995). Since the initial discovery of these potent and selective EGFR TK inhibitors, the field of cancer chemotherapy has taken a radical turn towards signal transduction-targeted compounds (Wakeling *et al.*, 1996; Baselga, 2000).

1.5.1. Synthesis

The steps towards the synthesis of 4-anilinoquinazoline are shown in Figure 1.5.1 (Rewcastle *et al.*, 1995). First, 5-nitroanthranilonitrile **1** was refluxed for 2h in formic acid and sulfuric acid to form a heterocyclic structure **2**. Oxoquinazoline **2** was refluxed with PCl_5 for 2 h, to give chloroquinazoline **3** which was coupled with m-toluidine *in situ* in 2-propanol. The resulting 6-nitroquinazoline was hydrogenated over palladium charcoal to give 6-aminoquinazoline **4**.

1.5.2. Structure-activity relationships

When EGFR is activated by its cognate ligand, a phosphate group is transferred from ATP to its own internal tyrosine residues. This process is termed "autophosphorylation." Quinazolines are designed to interfere with this function by competitively binding to the ATP-binding site of EGFR TK. As illustrated in Figure 1.5.2. [see (a)], these compounds possess nitrogen atoms at the 1- and 3-positions of the heterocycle. As in adenine, these two nitrogens are essential for hydrogen bonding within the ATP site. They interact with methionine 769 and threonine 766 amino acid residues,

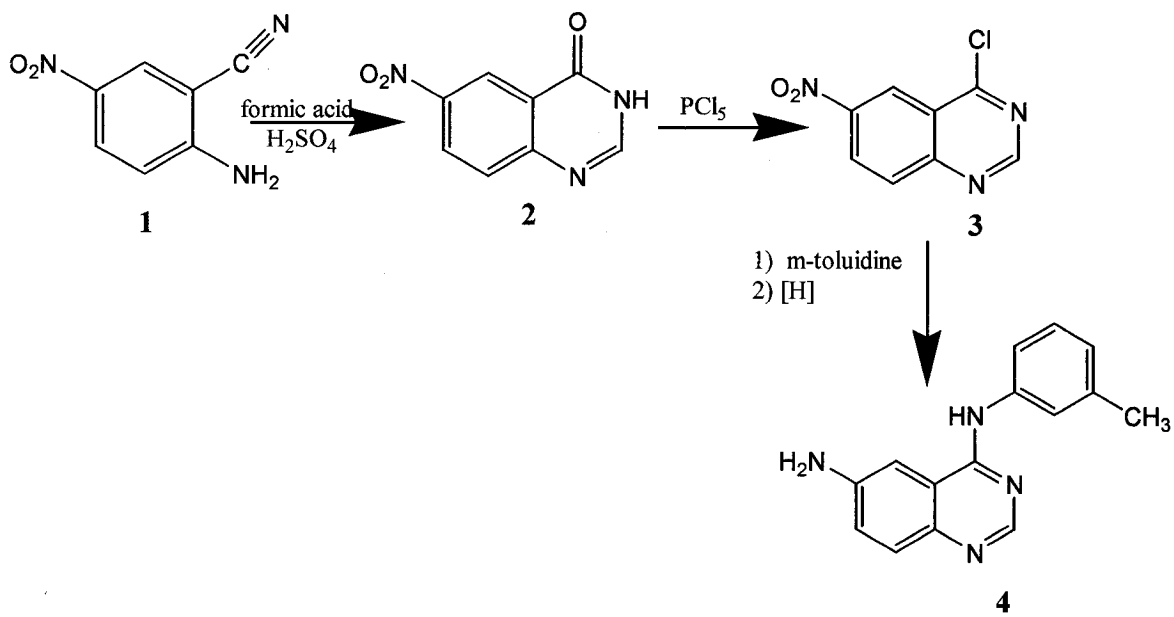


Figure 1.5.1. The synthesis of 4-anilinoquinazolines.

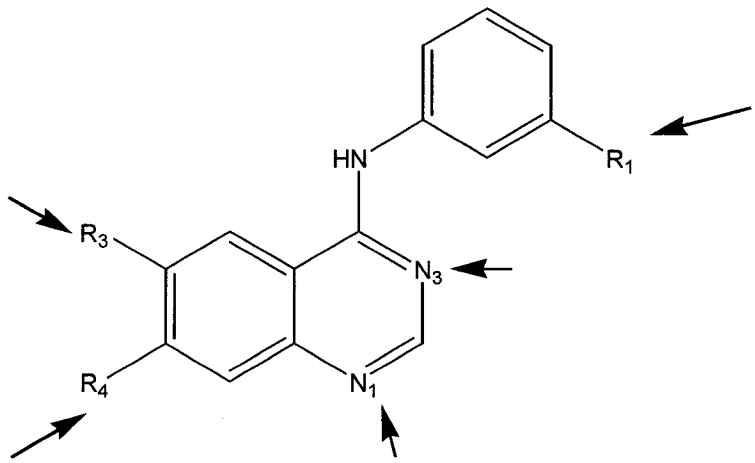
respectively (Palmer *et al.*, 1997). The binding affinity is further enhanced by the anilino group that fits into a hydrophobic pocket, an interaction that confers a major binding advantage over the adenine of ATP. Small, lipophilic moieties (especially halogens) are favoured at the 3'-position. The 6- and 7-positions are tolerant of bulky substituents. Indeed, water soluble moieties (such as aminoethylmorpholine) have been appended to these positions without significantly altering binding affinity (Vincent *et al.*, 2000; Herbst *et al.*, 2002). A docking model of quinazoline bound to the ATP binding site of EGFR (reproduced by permission from the Journal of Medicinal Chemistry) (Palmer *et al.*, 1997) is depicted in Figure 1.5.2 [see (b)].

1.5.3. Clinical applications

ZD 1839 ("Iressa"), produced by AstraZeneca, has proven the most clinically active quinazoline thus far. It is now in phase II and III clinical trials both as a monotherapy and in combination with cytotoxic drugs. It has recently been approved for use against non-small cell lung carcinoma in Japan (Ciardello *et al.*, 2000; Sirotnak *et al.*, 2000; Albanell *et al.*, 2002; Magne *et al.*, 2002; Wakeling *et al.*, 2002). Clinical trials with EGFR TK inhibitors differ from classical trials that use half of the maximum tolerated dose (MTD). Since antitumour activity depends on depletion of EGFR phosphorylation, patients are given the dose that is known to induce complete inhibition of TK *in vitro* (Sirotnak *et al.*, 2000; Ciardello *et al.*, 2000; Albanell *et al.*, 2002).

Although quinazolines have proven selective and potent inhibitors of EGFR TK activity, the reversibility of their activity remains their major drawback. These drugs require daily repeated doses in order to induce antitumour activity. Thus, quinazolines

a)



b)

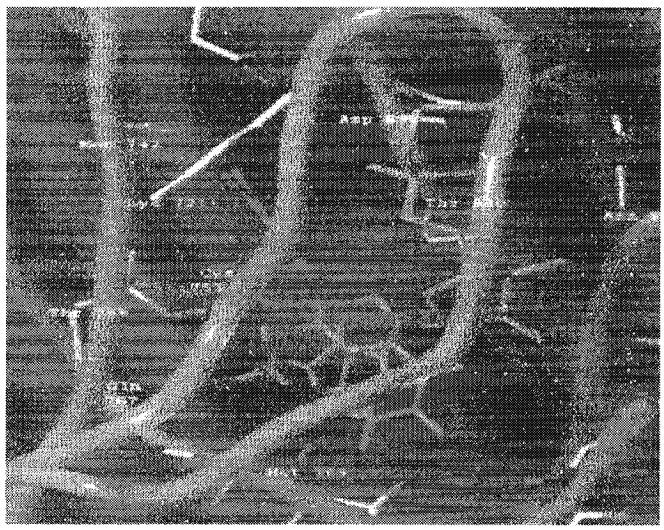
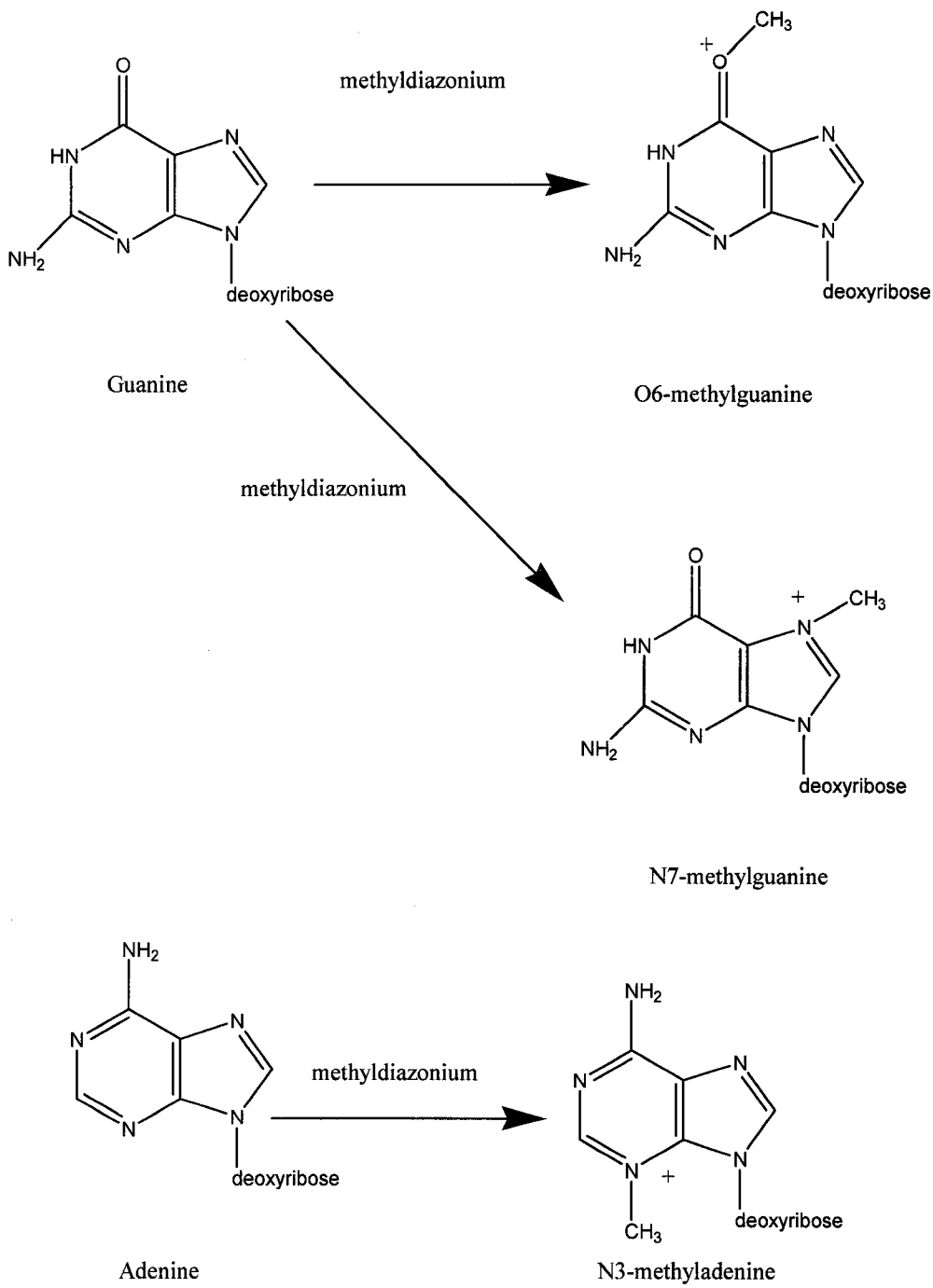


Figure 1.5.2. (a) The structure-activity relationships of 4-anilinoquinazolines. Arrows indicate positions that play a critical role in binding affinity. (b) A molecular model for their binding within the EGFR TK ATP site.

are cytostatic. To circumvent this problem, attempts have been made to render these drugs irreversible, including the addition of an acrylamide at the 6- and 7-positions of the quinazoline ring (Fry *et al.*, 1998; Vincent *et al.*, 2000). It was found that the cysteine 773 residue of the ATP binding site could add onto the double bond of the acryolyo group thereby inducing a covalent irreversible bond to the receptor with 6-substituted aminoquinazolines. One such compound (CI-1033) has been found to irreversibly inhibit EGFR TK and is now in phase I clinical trials (Fry *et al.*, 1998; Allen *et al.*, 2002).

Another factor with regards to the clinical activity of quinazolines is the level of receptor expressed by cancer cells versus normal cells. Recently, Bishop *et al.*, (2002) demonstrated that the levels of EGFR expressed by a cell line is not the only determinant of the activity of quinazoline TK inhibitors. An intact signaling cascade from EGFR activation to gene transcription is required for these compounds to be active. This is an interesting observation, because some cancer cells may express high levels of EGFR, yet be unresponsive to TK inhibitors (Baguley *et al.*, 1998; Bishop *et al.*, 2002).

The primary objective of this thesis being to use a quinazoline fused to a cytotoxic triazene tail as a probe for the Combi-targeting postulates, we will discuss herein the medicinal chemistry and pharmacology of triazenes.



1.6.1. The alkylation of DNA by monomethyltriazene.

1.6. Triazenes: classical alkylating agents used to treat cancers

Triazenes are small, degradable, molecules that have been used to treat cancer for the past 30 years (Manning *et al.*, 1985; Newlands *et al.*, 1992; Woll *et al.*, 1995). As depicted in Figure 1.6.1., under hydrolytic conditions, these compounds release an alkyldiazonium (in this case, a methyldiazonium) ion that can damage DNA by alkylating its bases at the N3 position of adenine, as well as the N7- and O6-positions of guanine (Tentori *et al.*, 1999).

1.6.1. Synthesis

a) Acyclic triazenes

As shown in Figure 1.6.2 1-aryl-3-alkyltriazenes **3**, are synthesized through the coupling of an alkylamine **2** with aromatic diazonium salts **1** (Manning *et. al.*, 1985). The resultant NNN linkage is required for antitumour activity since it generates the alkyldiazonium species that forms DNA adducts (Stevens and Newlands, 1993; Manning *et. al.*, 1997). Under aqueous conditions, these triazenes decompose by protolysis of tautomer **4** to generate aniline of type **5** plus a diazonium salt **6**. It is this diazonium salt that alkylates biological nucleophiles such as DNA bases.

Monoalkyltriazenes are extremely unstable species with $t_{1/2}$ between 5-15 minutes. 1-aryl-3,3-dialkyltriazenes were developed in order to prevent the formation of the unstable tautomer **4** that is prone to proteolysis. Unlike 1-aryl-3-alkyltriazenes, these compounds require metabolic oxidation to generate the antitumour active monoalkyltriazene (Cameron *et al.*, 1985).

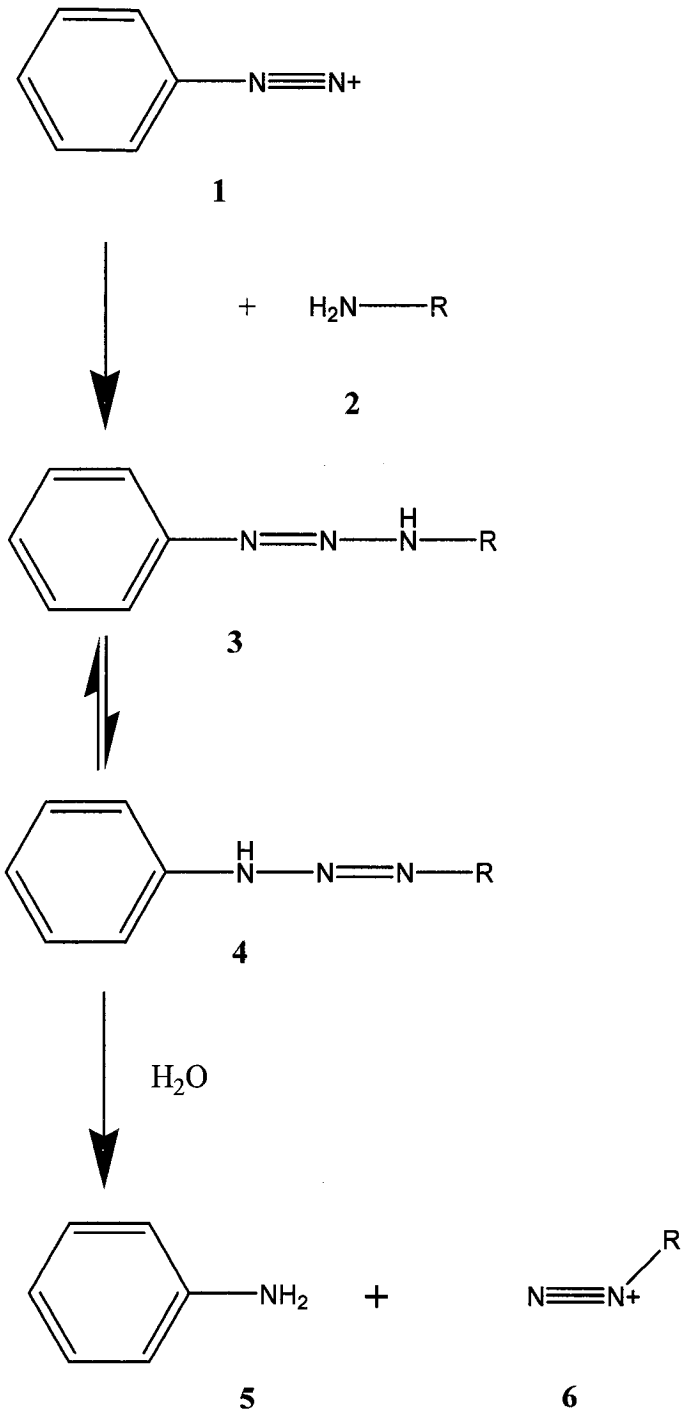
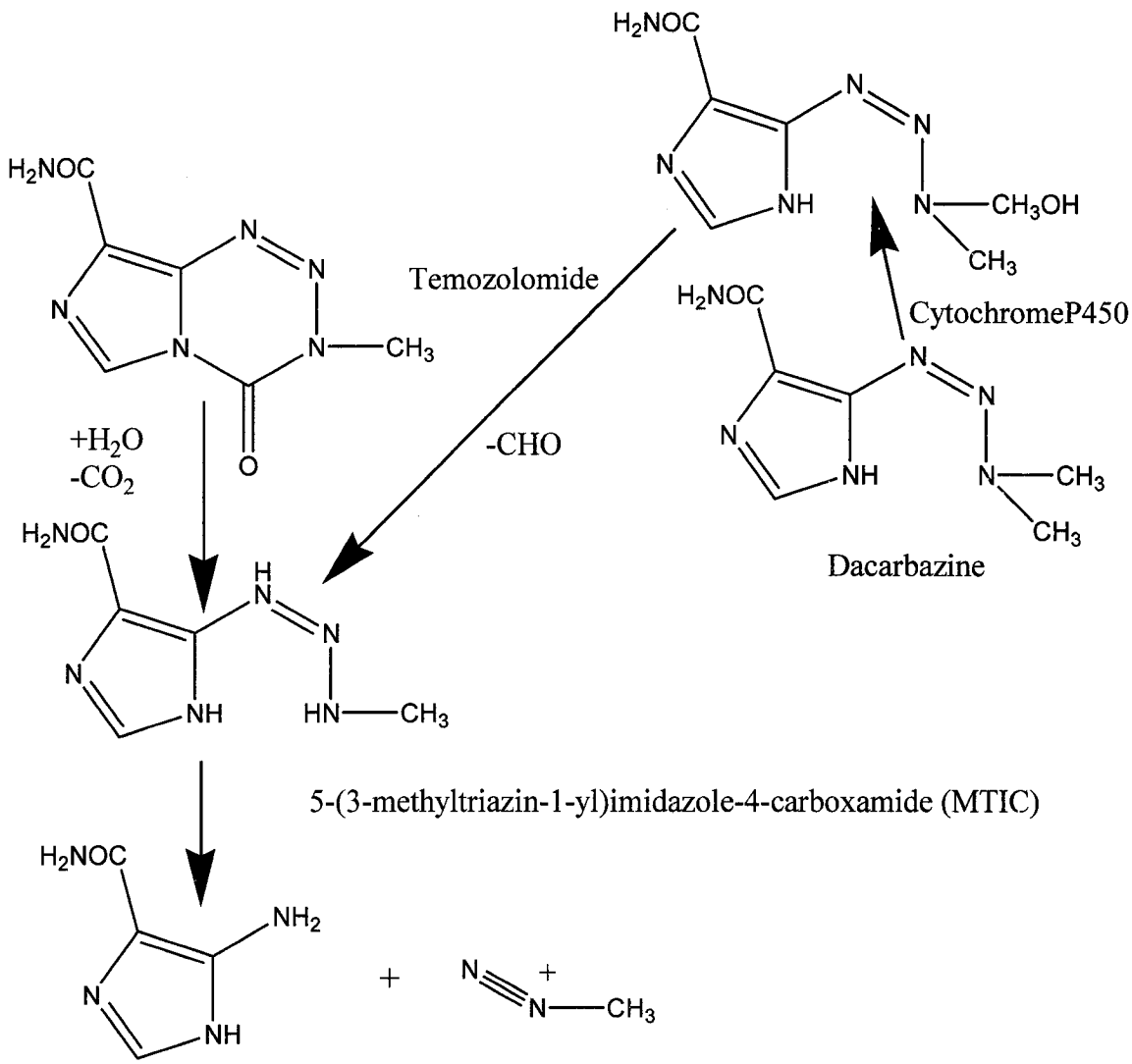


Figure 1.6.2. The synthesis and degradation of 1-aryl-3-alkyltriazene.



1.6.3. The hydrolytic degradation the cyclic triazenes temozolomide (TEM) and dacarbazine.

b) Cyclic triazenes

It is known that the metabolic activation of dimethyltriazenes, such as dacarbazine, is subject to phenotypic differences, and therefore clinical activity may vary between individuals. Temozolomide (TEM) was developed as a stable prodrug of 5-(3-methyltriazin-1-yl)imidazole-4-carboxamide (MTIC), the active metabolite of dacarbazine (Stevens *et al.*, 1987). This drug is synthesized by direct condensation of diazoimidazole zwitterions with methyl isocyanate. The degradation of cyclic triazenes dacarbazine and TEM are depicted in Figure1.6.3 (Cameron *et al.*, 1985; Iley *et al.*, 1992).

1.6.2. Structure-activity relationships of triazenes

Compounds that chloroethylate DNA are more potent than monomethylating ones since they induce cross-links. On the other hand, mono- or diethyltriazenes do not demonstrate antitumour activity, perhaps due to the quick repair of ethylated DNA. Therefore, methyl or chloroethyl groups are required for triazene activity. Thus, TEM and dacarbazine (both monomethylating agents) are the most potent clinically used triazenes (Lawley and Phillips, 1996; Newlands *et al.*, 1997).

1.6.3. Clinical applications

As discussed in section 1.6.2, TEM (and several other triazenes such as DTIC) are currently used in the clinic against a variety of tumour types (Gaya *et al.*, 2002; Newlands *et al.*, 1992). In particular, TEM is already in use against gliomas and melanomas, and is being evaluated as a treatment for Non-Hodgkin's lymphoma (Woll *et al.*, 1995; Gaya *et*

al, 2002). The major advantage to TEM is that it is orally administered, it can cross the blood-brain barrier to reach gliomas, and it is less toxic than other alkylating agents of the same class (Woll *et al.*, 1995; Newlands *et al.*, 1992; Gaya *et al.*, 2002).

One major drawback to the clinical use of TEM is the development of drug resistance. In particular, the expression of the DNA repair enzyme AGT depletes the activity of such a monomethylating triazene (Pegg, 1990; Gerson, 2002; Kokkinakis *et al.*, 2001). Furthermore, deficiencies in mismatch repair (MMR) and base excision repair (BER) contribute to clinical drug resistance and anticancer treatment problems (Bearzatto *et al.*, 2000; Aebi *et al.*, 1997; D'Atri *et al.*, 1998; Liu *et al.*, 1999). Therefore, novel agents and dosing schedules are being developed to hurdle the obstacle of resistance, and will be discussed in section 1.7.

1.7. Chemoresistance to alkyltriazenes

DNA repair is an essential process for the maintenance of genomic stability and is associated with a number of different cellular mechanisms. These mechanisms include repair enzymes such as O6-alkylguanine-DNA alkyltransferase (AGT) (Pegg, 1990; Wedge *et al.*, 1996; Gerson, 2002), mismatch repair (MMR) (Brown *et al.*, 1997; Fink *et al.*, 1998; Jiricny, 1998), and base excision repair (BER) (Liu *et al.*, 1999; Krokan *et al.*, 2000). Some of these mechanisms are also implicated in the resistance of cancer cells to alkylating agents of the triazene class. In general, triazenes most often alkylate at the N3 position of adenine (9%) and N7 or O6 of guanine (70% and 5%, respectively) (Tentori *et al.*, 1999). AGT rapidly removes O6-alkylguanine lesions (Pegg, 1990; Pegg, 2000) whereas BER can repair N3-alkyladenine and N7-alkylguanine lesions (Tentori *et al.*,

1999; Singer and Hang, 1997). Therefore these fundamental systems can induce significant resistance to anticancer chemotherapy, and various approaches are being taken towards overcoming this resistance

1.7.1. O6-alkylguanine-DNA alkyltransferase (AGT)

The primary cytotoxic DNA lesion formed by triazene compounds is O6-alkylguanine, and this lesion can be removed from DNA by the enzyme O6-alkylguanine-DNA alkyltransferase (AGT) in a single step (Pegg, 1990; Pegg, 2000). Human AGT is a 21 kDa protein that has not yet been crystallized (Daniels and Tainer, 2000; Pegg, 2000). Several homologues have been identified in a large number of species, including microorganisms and mammals, and the most homologous amino acid sequences were found around the region of the cysteine acceptor site and the helix-turn-helix DNA binding domain of the enzyme.

As shown in Figure 1.7.1, following a conformational change that allows the transfer of the O6-alkyl group to cysteine-145 in its active site, AGT repairs the DNA whilst being inactivated in the process (Pegg, 1990; Pegg, 2000). Thus, levels of AGT depend on initial cellular concentrations of the enzyme and the rate of its *de novo* synthesis (Daniels and Tainer, 2000; D'Atri *et al.*, 2000). AGT is expressed by many different species and in many different tissues, and some tumour cell lines express little to no AGT (referred to as Mer- cells) (Bearzatto *et al.*, 2000; Esteller *et al.*, 1999). This phenotype may be due to promoter hypermethylation, a common event in primary tumors that is believed to possibly be an important step in tumorigenesis (Bearzatto *et al.*, 2000; Esteller *et al.*, 1999; Lage *et al.*, 1999). In contrast, other tumour cell lines express high levels of

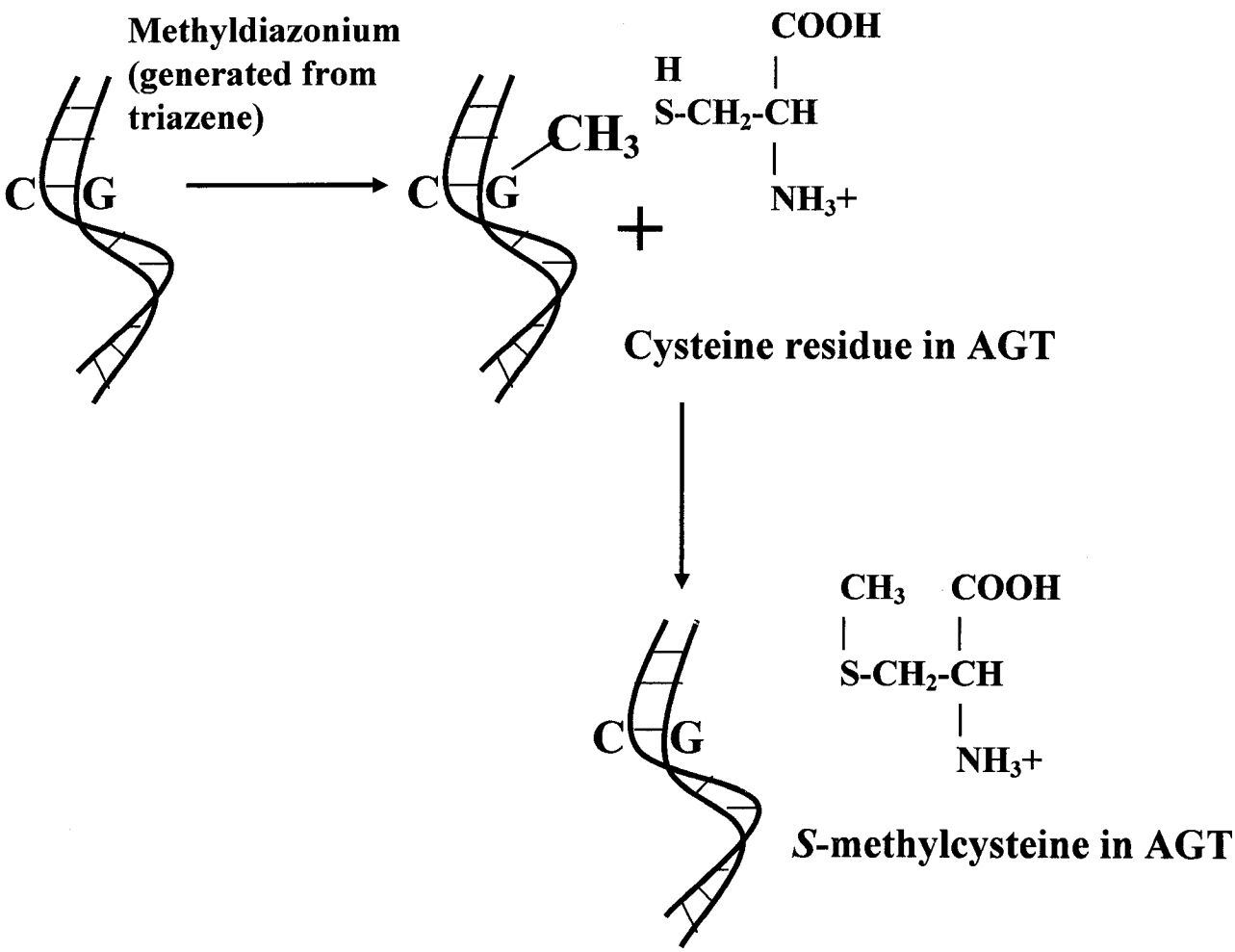
AGT, and are thus intrinsically insensitive to anticancer chemotherapeutics that rely on the O6-alkylguanine lesion for cytotoxicity (Nutt *et al.,* 2000; Kokkinakis *et al.,* 1997; Bignami *et al.*, 2000; Gerson, 2002).

Pretreatment of tumour cell lines with O6-benzylguanine (BG) depletes AGT since a cysteine residue in the enzyme active site recognizes and removes its benzyl group thus enhancing cell sensitivity to alkylating agents that form O6-alkylguanine lesions (Goodtzova *et al.*, 1997; Kreklau *et al.*, 1999). This approach has been studied in human glioma and melanoma xenograft models (Kreklau *et al.*, 1999; Wedge *et al.*, 1996), and is now undergoing clinical investigation in combination with BCNU and temozolomide (Spiro *et al.*, 1999; Kreklau *et al.*, 1999).

1.7.2. The experimental detection of DNA damage

To study the biological effects of alkyltriazenes, one must be able to detect the DNA damage incurred when cells are treated with these compounds. Several different methods have been used, including alkaline filter elution assay and single-cell microelectrophoresis (comet) assay (Lawley and Phillips, 1996).

One classic means by which to measure DNA damage is by alkaline filter elution assay. For this protocol, cells are given radiolabeled thymidine and washed before being given drug. Cells are then lysed on a polycarbonate filter through which they flow via gravity. Fractions of eluted solution are collected and analysed by liquid scintillation counting (Lawley and Phillips, 1996).



1.7.1. The mechanism of action of O6-alkylguanine DNA alkyltransferase (AGT).

A more modern means to detect DNA damage is single-cell microelectrophoresis (comet) assay (Collins *et al.*, 1997; McNamee *et al.*, 2000; Kassie *et al.*, 2000; Yang *et al.*, 1999). As shown in Figure 1.7.2, in this assay cells are exposed to DNA damaging elements, cast in agarose gels, lysed within the gel, electrophoresed, dehydrated, stained, and examined using a fluorescence microscope and detection software (Alkomet v2.1) (McNamee *et al.*, 2000). This assay has many advantages over classical methods since it is both qualitative and quantitative, and several different parameters of DNA damage can be determined (*i.e.* tail length, comet length, tail moment). Furthermore, this assay is rapid, and a large number of samples can be processed all at once. It is highly sensitive, and can be used to detect DNA damage both *in vitro* and *in vivo*. In addition, the comet assay can be used to examine both single-stranded breaks, and DNA cross-links.

1.8. The Combi-targeting concept: A novel theory for selective chemotherapy

There is a pressing need for the development of new approaches to increase the potency of current cancer therapies. As discussed earlier, "smart drugs" that target proteins overexpressed in cancer cells are showing promise both in the laboratory and in the clinic. Anticancer agents, such as the alkylating triazenes discussed in section 1.6, have traditionally been directed at DNA. Since they target rapidly dividing cells, these cytototoxic drugs are not selective for tumour cells but induce deleterious side effects due to their systemic toxicity. More importantly, acquired resistance mediated by DNA repair enzymes is a major impediment in their clinical use. Therefore, these drugs must be

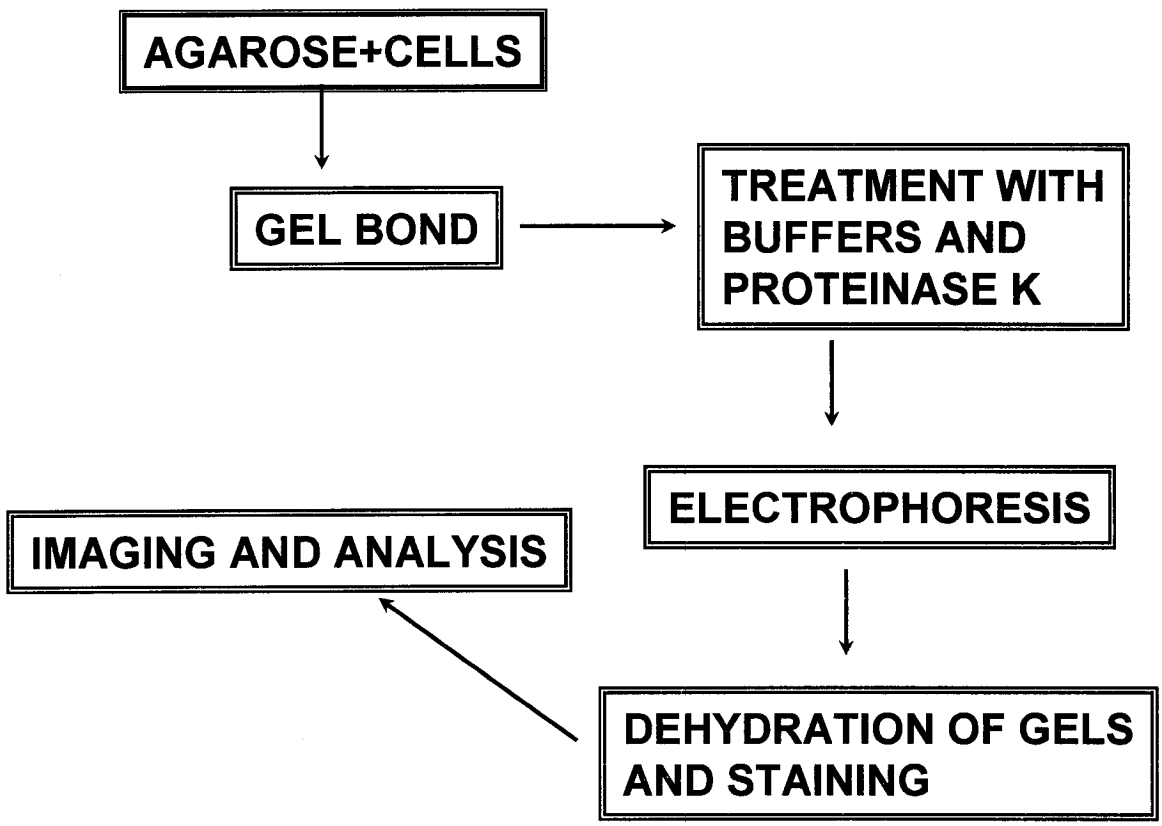


Figure 1.7.2. Single cell micro-electrophoresis (comet) assay.

rendered more selective and more potent in order for their therapeutic index to be ameliorated.

Our approach, the Combi-targeting concept, seeks to improve the activity of cytotoxic agents (in this case, alkyltriazenes) by giving them a second function. By combining a DNA-damaging moiety with a potent and selective inhibitor of a TK that is overexpressed in cancer cells, we may selectively target tumours whose progression depends on the targeted TK. As discussed in section 1.6, compounds of the quinazoline class that inhibit EGFR TK are often cytostatic agents. By combining them with DNA-damaging moieties, we have designed a concept whereby the intact "combi-molecule" can bind to EGFR TK on its own and degrade to yield another potent inhibitor plus a DNA-damaging agent.

As illustrated in Figure1.8, a triazene is linked to a TK inhibitor of EGFR to form a TZ-I. The TZ-I enters cells that are EGFR-proficient or deficient by passive diffusion. In cells expressing EGFR, the entire TZ-I may interact with the ATP binding site prior to degradation and subsequently be hydrolyzed to a DNA alkylating species (TZ) and an EGFR TK inhibitor (I). In cells that do not express EGFR, the TZ will only damage DNA. Thus, cells expressing EGFR will be doubly affected by the properties of this combi-molecule (TZ-I). Therefore, the TZ-I will not only serve to inflict DNA damage to cells but also to inhibit downstream signaling mediated by EGFR.

We decided to use the triazene class of DNA-damaging molecules to study the feasibility of the Combi-targeting approach because of their small size, and known clinical activity. This property permits the design of TZ-Is capable of retaining significant affinity for the ATP binding site of EGFR.

We combined a triazene with a quinazoline TK inhibitor of EGFR to create the first ever TZ-I, termed "**SMA41**." Its mechanism of action is described in Chapter 2, in which we confirm that: (a) SMA41 can indeed be synthesized, and (b) it is capable of degrading into a free inhibitor (termed SMA52) plus a DNA-damaging methyldiazonium species. Moreover, SMA41 is capable of inhibiting EGF-induced autophosphorylation of EGFR and inhibiting the growth of cells overexpressing EGFR despite the presence of the DNA repair enzyme AGT (Matheson *et al.*, 2001).

In Chapter 3 (Matheson *et al.*, 2003), we further studied the selective effects of SMA41 in a panel of established human tumour cell lines expressing variable levels of EGFR and AGT. We showed a correlation of the activity of SMA41with the expression of AGT, but SMA41 consistently demonstrate superior activity over that of TEM (the clinically used methyltriazene counterpart of SMA41).

Yet, the methyldiazonium released by SMA41 was too unstable to be characterized by analytical methods. Thus, we synthesized a ^{14}C-labeled analogue of SMA41. Chapter 4 describes the synthesis, radiochemical purity, and specific activity of this compound (Matheson *et. al.*, 2003 in press).

We subsequently determined how SMA41 and its subcomponent SMA52 is distributed within intact cells. As discussed in Chapter 5, we discovered that the free TK inhibitor (SMA52) generated by SMA41 fluoresces at 448.8 nm under UV (280 nm)

excitation. Therefore, we could exploit this characteristic to follow the conversion of SMA41 into SMA52 within whole cells using UV flow cytometry and UV microscopy. Furthermore, we were able to measure the intracellular half-life of SMA41.

In Chapter 6, we investigated the ability of A431 cells (expressing AGT) to repair DNA damage and cell cycle perturbations induced by SMA41. Moreover, we studied the inhibition of downstream signaling by SMA41 (*ie. c-fos* induction and MAP kinase phosphorylation), its irreversible inhibition of EGFR autophosphorylation, and its efficacy in a mouse xenograft model with A431 cells.

This thesis describes the Combi-targeting concept, from the determination of its activity and the elucidation of its mechanism of action *in vitro*, to the demonstration of its efficacy *in vivo*.

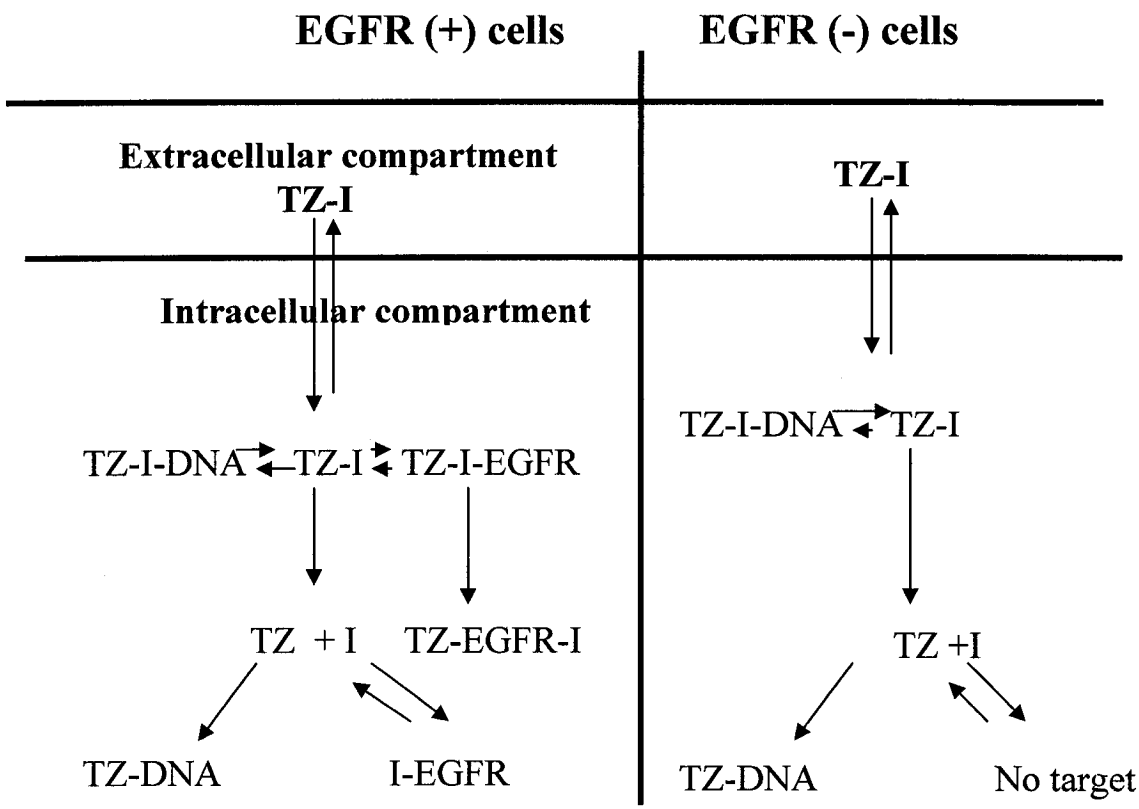


Figure 1.8. Principles of the Combi-targeting concept.

1.9. Research objectives

The primary objective of this project was to study the feasibility of the unimolecular combination of two drugs with different mechanisms of action (both DNA and EGFR targeting) against human tumour cells. This was accomplished by 1) synthesizing the first ever "combi-triazene" SMA41, 2) elucidating its mechanism of action *in vitro*, 3) studying its selectivity for cell lines expressing EGFR and its subcellular distribution, and 4) determining its effects in an *in vivo* model.

1.9.1. Project outline

For this thesis, a number of different objectives were set:

Objective 1: Synthesis of the first ever unimolecular combination of two anticancer drugs with mixed mechanisms of action involving DNA damage and EGFR inhibition.

Objective 2: Confirmation of the binary targeting properties of this molecule, and elucidation of its effects both at the cell surface and downstream to EGFR activation.

Objective 3: Demonstration of selectivity for cell lines expressing EGFR, and study of the subcellular distibution of the molecule (*i.e.* its ability to reach its intended target).

Objective 4: Demonstration of the efficacy of this molecule *in vivo*.

1.9.2. Contributions of Authors

CHAPTER 2: This paper has been published in The Journal of Pharmacology and Experimental Therapeutics 296: 832-840 (2001). My contribution was all of the experimental work, and the preparation of the manuscript. The imaging of the single-cell microelectrophoresis samples that I prepared was performed by Dr. James McNamee at Health Canada. My supervisor (Dr. Bertrand Jean-Claude) provided comments and suggestions. This research remains a cornerstone to the Combi-targeting concept, and extended to two other papers in our laboratory (Brahimi *et al.*, 2002; Qiu *et al.*, 2003- see Appendix).

CHAPTER 3: This paper has been published in Cancer Chemotherapy and Pharmacology 51:11-20 (2003). My contribution was all of the experiments and the preparation of the manuscript. Dr. James McNamee at Health Canada imaged the single-cell microelectrophoresis samples that I prepared. My supervisor assisted with suggestions and revisions.

CHAPTER 4: This manuscript is in press for the Journal of Labelled Compounds and Radiopharmaceuticals. My contribution was the experimental work, and Dr. Shadreck Mzengeza assisted in the preparation of ^{14}C-SMA41 as well as the scanning of the radio-TLC. My supervisor assisted with the HPLC analysis of the compound. I prepared the first draft of the manuscript, and my supervisor made comments and revisions.

CHAPTER 5: This manuscript is under submission to Biochemical and Biophysical Research Communications. My contribution was all of the experimental work, and preparation of the manuscript. Mme. Anne Marcil (Biotechnology Research Institute, Montreal, QC) assisted in the fluorescence microscopy, and Dr. Moulay Alaoui-Jamali (Jewish General Hospital, Montreal, QC) allowed me to perform the transduction of the SF-126 and SF-188 cell lines in his laboratory. My supervisor provided suggestions and comments about the manuscript.

CHAPTER 6: This manuscript is under submission to the Journal of the National Cancer Institute. My contribution was all of the *in vitro* experimental work (flow cytometry, Western blotting, RT-PCR, and comet assay). I performed the large-scale synthesis of SMA41 required for *in vivo* studies in the xenograft model in immunocompromised SCID mice. Although I have received training in animal handling from the McGill Animal Resources Centre, the technical work in these studies were performed by Dr. Taqui Wang (Jewish General Hospital, Montreal, QC). Furthermore, I prepared the first draft of the manuscript. My supervisor made invaluable suggestions and revisions.

1.9.3. References

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CHAPTER 2

DESIGN OF A CHIMERIC 3-METHYL-1,2,3-TRIAZENE WITH MIXED RECEPTOR TYROSINE KINASE AND DNA DAMAGING PROPERTIES: A NOVEL TUMOUR TARGETING STRATEGY

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2.1. Abstract

The mixed epidermal growth factor receptor (EGFR)-DNA targeting properties of SMA41, a 6-(3-methyl-1,2,3-triazen-1-yl)-4-anilinoquinazoline designed to release N^4-m-tolyl-quinazoline-4,6-diamine henceforth referred to as SMA52 [an inhibitor of EGFR tyrosine kinase (TK)] and methyldiazonium (a DNA methylating species) were studied in the O^6-methylguanine-DNA methyltransferase (MGMT)-proficient and high EGFR-expressing epidermoid carcinoma of the vulva cell line A431. The effects of SMA41 were compared with those of SMA52 alone, and temozolomide (TEM), a clinical prodrug of 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) which is inactive in MGMT-proficient cells. The results showed that: (a) the chimeric SMA41 could degrade in serum-containing medium ($t_{1/2}$~30 min) to generate, as predicted, the free inhibitor SMA52 as the most abundant metabolite (~81% yield); (b) in contrast to SMA52 alone, the chimeric SMA41 and TEM induced significant DNA damage in A431 cells after 30 min or 2 h drug exposures, as confirmed by alkaline single-cell gel micro-electrophoresis (comet) assay; (c) SMA41 showed 5-fold greater affinity for the ATP binding site of EGFR than independently synthesized SMA52 in an enzyme assay and blocked EGF-induced tyrosine phosphorylation and EGFR autophosphorylation in A431 cells in a dose-dependent manner; (d) these mixed targeting properties of SMA41, combined with its ability to be converted to another potent EGFR TK inhibitor (*e.g.* SMA52) by hydrolytic cleavage, translated into over 8-fold greater antiproliferative activity than TEM which showed no EGFR targeting properties (IC_{50} competitive binding >100 μM); (e) under continuous drug exposure [3-6 day sulforhodamine (SRB) and clonogenic assays], SMA41 was almost equipotent with SMA52; however, in a short 2 h drug exposure

followed by incubation in drug-free media, SMA52 showed an almost complete loss of antiproliferative activity over the whole dose range. In contrast SMA41 retained almost 100% of its activity, indicating a more sustained growth inhibitory activity. The results *in toto* suggest that the superior antiproliferative activity of SMA41 may be due to a combination of events associated with its binary EGFR TK and DNA targeting properties.

Key words: EGFR, monoalkyltriazene, temozolomide, tyrosine kinase

2.2. Introduction

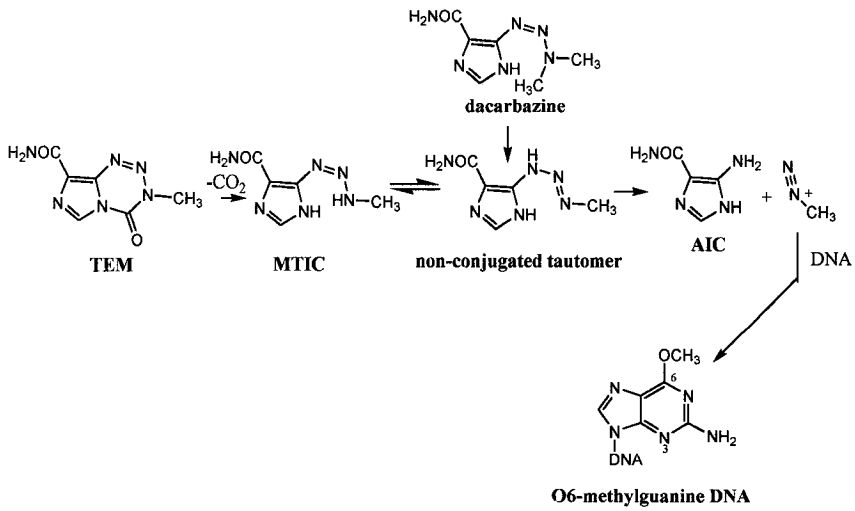
Over the past 20 years, acquired resistance mediated by DNA repair enzymes has often imposed severe limitations on the use of DNA-interactive agents and in many cases useful clinical antitumour activity could only be observed with the administration of multiple antitumour drugs of different mechanisms of action. Based upon this observation, we surmised that novel compounds with multiple intracellular targets would be more effective against resistant tumours than their classical counterparts. In this paper, we describe the first attempt to combine two major mechanisms of action (inhibition of tyrosine kinase-mediated signaling and DNA-targeting) into one single molecule (*e.g.* SMA41). The biochemical responses to the latter molecule were studied in the resistant Mer+ DNA repair-proficient human epidermoid carcinoma of the vulva cell line A431 which co-expresses high levels of EGFR TK.

The overexpression and dysfunction of TKs, directly or indirectly implicated in mitogenic signaling in tumour cells, have been extensively studied and are now considered the major functional differences between normal and tumour cells (Sinha *et al.,* 1995; Sherwood *et al.,* 1999; Kondapaka and Reddy, 1996; Alaoui-Jamali *et al.,* 1997; Tsai *et al.,* 1993). Because of their significant involvement in tumour progression, overexpressed receptor TKs have now become the modern targets for drug design and selective chemotherapeutic interventions (Carroll *et al.,* 1997; Deininger *et al.,* 1997; Levitzki and Gazit, 1995). One such target is EGFR which, in many patients, is associated with aggressive tumour progression and invasion (Xie *et al.,* 1999; Turner *et al.,* 1996; Modjtahedi and Dean, 1998; Moyer *et al.,* 1997). It has already been demonstrated that blocking signal transduction mediated by the TK activity of EGFR

translates into significant antitumour activity both *in vitro* and *in vivo,* and two novel agents are now in phase II clinical trials (Levitzki and Gazit, 1995; Ward *et al.*, 1994; Rewcastle *et al.,* 1997; Lanzi *et al.,* 1997; Moyer *et al.,* 1997; Rewcastle *et al.,* 1995; Rewcastle *et al.,* 1998). Despite being significantly less toxic than previous cytotoxic agents, in tumours where they cannot induce apoptosis, most TK inhibitors currently in clinical trial present the disadvantage of being cytostatic agents that induce reversible growth inhibitory activity (Smaill *et al.,* 1999).

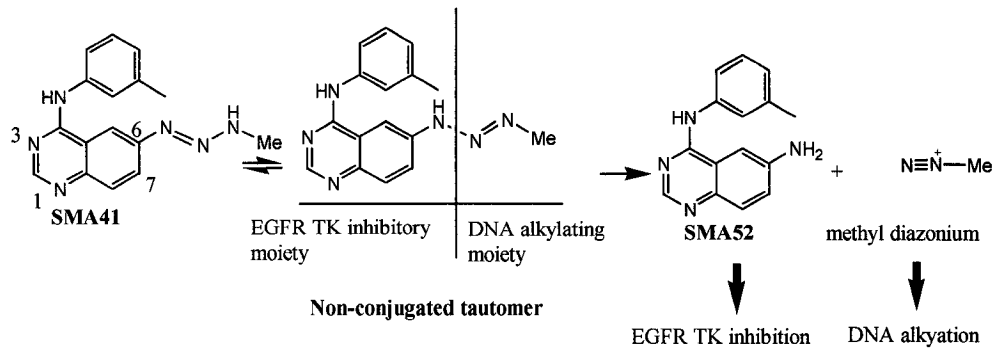
The disadvantages associated with both the classical cytotoxic agents and the modern RTK inhibitors, as well as the need for novel targets to circumvent DNA-repair-associated chemoresistance, stimulated our interest in designing molecules with mixed EGFR tyrosine kinase and DNA-targeting properties.

SMA41 exhibits two distinct structural characteristics: (a) a 1,2,3-triazene linkage, the pharmacophore of the active metabolites of dacarbazine or TEM and (b) a 4-anilinoquinazoline moiety, the pharmacophore of the potent quinazoline class of EGFR TK inhibitors which are now in clinical trial (Ching *et al.,* 1993b; Moyer *et al.,* 1997b).



Scheme 2.1

The 3-alkyl-1,2,3-triazenes such as TEM, or its metabolite MTIC, are known to heterolyze to an aromatic amine (*e.g.* AIC, Scheme 2.1) and an alkyldiazonium species (*e.g.* methyldiazonium, Scheme 2.1) under hydrolytic conditions (Cameron *et al.,* 1985; Gibson *et al.,* 1986; Baig and Stevens, 1987). Their mechanism of action is primarily based upon the generation of alkyldiazonium species that alkylate the 6- and 7-position of guanine in DNA. Substantial evidence suggests that alkylation of DNA at the O6 position of guanine is the cytotoxic lesion induced by 3-methyl or 3-(2-chloroethyl)-1,2,3-triazenes (Pegg *et al.*, 1995; Tisdale, 1987; Bodell *et al.*, 1985; Baer *et al.*, 1993). Mer+ cells expressing elevated levels of MGMT, an enzyme capable of repairing the O^6-alkylguanine lesion, show significant resistance to the action of alkylating agents like TEM or its metabolite MTIC (Tisdale, 1987; Mitchel and Dolan, 1993; Lee *et al.*, 1991; Chen *et al.*, 1993). The choice of the triazene TEM as a control drug in this study, was inspired by its proven clinical activity in the treatment of solid tumours such as gliomas and malignant melanoma and its significant inactivity in Mer+ cells.



Scheme 2.2

On the other hand, the aninilinoquinazolines are a novel class of highly receptor type specific compounds that inhibit EGFR-related signal transduction by competition at the ATP binding site (Ward *et al.,* 1994). The significant number of structure-activity-relationship (SAR) studies on 4-anilinoquinazolines and pyrido[d]pyrimidines as EGFR TK inhibitors is consistent with the compounds binding to the ATP site of EGFR (Rewcastle *et al.,* 1997; Rewcastle *et al.,* 1998; Rewcastle *et al.,* 1995). Molecular modeling suggests that the N-1 atom accepts an H-bond from Met-769, N-3 accepts an H-bond from the side chain of Thr-766 on strand 5 deep in the binding cleft, and the anilino moiety is located in an adjacent hydrophobic pocket. The model suggests that the only positions on the inhibitors where substituents can be altered without affecting binding affinity are the 6- and 7-positions which are located at the entrance of the binding cleft (Rewcastle *et al.,* 1995). Indeed a variety of compounds with bulky side chains on the 6- and 7-positions were synthesized and found to retain significant binding affinity for the EGFR ATP binding site (Rewcastle *et al.,* 1997). We therefore decided to append the alkyltriazene moiety to the 6-position of the quinazoline heterocycle. Thus, as outlined in Scheme 2.2, based upon the mechanism of hydrolytic cleavage of 1,2,3-triazenes and the SAR of quinazolines, SMA41 was designed to release (a) SMA52, a competitive inhibitor of the ATP binding site of EGFR and (b) the DNA damaging methyldiazonium species. In addition, this chimeric molecule was designed to remain small enough to be able to interact with the receptor on its own. This property was introduced with the purpose of targeting high EGFR-expressing cells. Interactions of SMA41 with the ATP binding site of EGFR would promote its intracellular retention, thereby favoring more intra- than extracellular degradation of this drug.

SMA41 was found to be able to release SMA52 in serum-containing cell culture media and to possess (a) a dual DNA damaging and a cellular phosphotyrosine inhibitory activity and (b) superior antiproliferative effects when compared with its clinical triazene counterpart TEM and its metabolite SMA52 alone. This novel strategy is designated as the "Combi-Targeting concept".

2.3. Materials and Methods

2.3.1. Drug Treatment

SMA41, and SMA52 were synthesized in our laboratories according to known procedures (Cameron *et al.,* 1985; Manning *et al.,* 1985, Rewcastle *et al.* 1995). Temozolomide was provided by Shering-Plough Inc. (Kenilworth, NJ). In all assays, drug was dissolved in DMSO and subsequently diluted in sterile RPMI-1640 media containing 10% fetal bovine serum (Life Technologies, Burlington, Canada) immediately prior to the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

2.3.2. Cell Culture

A431 cells (American Type Culture Collection, Manassas, VA) were maintained in a monolayer culture at 37°C in a humidified environment of 5% CO_2-95% air. The cultures were maintained in RPMI-1640 supplemented with fetal bovine serum (10%), penicillin (50 U/mL), and streptomycin (50 mg/mL) (Life Technologies, Burlington, Canada). Cells were maintained in logarithmic growth by harvesting with a trypsin-

EDTA solution containing 0.5 mg/mL of trypsin and 0.2 mg/mL of EDTA and replating before confluence. In all assays, the cells were plated for 24 h before drug administration.

2.3.3. Growth Inhibition Studies

Cell monolayers were exposed to different concentrations of each drug continuously for 72 h. Under short exposure, they were treated with each drug for 2 h and allowed to recover for 72 h in drug-free medium.

For the SMA52+TEM combination, the drugs were mixed at a 1:7 (SMA52/TEM) molar ratio, serially diluted and added to the monolayers for 72 h. IC_{50}'s were determined using the median effect equation as described by Chou and Talalay, 1984. The nature of drug interactions was determined using equation (1) where CI_{50} s>1, =1 or <1 indicate, antagonism, additivity, or synergism respectively (Chou *et al.*, 1984).

$$(1)\ CI_{50} = \frac{IC_{50}\text{ (TEM in combination)}}{IC_{50}\text{ (TEM alone)}} + \frac{IC_{50}\text{ (SMA52 in combination)}}{IC_{50}\text{ (SMA52 alone)}}$$

All growth inhibitory activities were evaluated using the SRB assay (Skehan *et al.*, 1990). Briefly, following drug treatment, cells were fixed using 50 μL of cold trichloroacetic acid (50%) for 60 min at 4°C, washed five times with tap water, and stained for 30 min at room temperature with SRB (0.4%) dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid, and allowed to air dry. The resulting colored residue was dissolved in 200 μL of Tris base (10 mM), and optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in

triplicate.

2.3.4. Colony forming assays.

Clonogenic assays were performed as previously described (Jean-Claude *et al.,* 1999). Briefly, cells were plated at a density of 500 cells/well and continuously exposed to each drug for 6 days. Colonies were fixed with methanol (100%) and stained with methylene blue (0.5%) after which they were counted with the SynGene GeneTools colony counting software package (Cambridge, UK). Only colonies with pixel areas of 4 or greater were counted. Data are means and SD's of two independent determinations.

2.3.5. Degradation

SMA41 (1 mg) was dissolved in DMSO (500 μL), added to RPMI with 10% fetal bovine serum (2 mL) and incubated for 24 h at 37°C. Thereafter, proteins were precipitated by addition of acetonitrile (3.5 mL) and the supernatant collected by centrifugation. The concentration of SMA52 deriving from the degradation of SMA41 was calculated using a standard curve obtained from the serial dilution of independently synthesized SMA52 incubated in serum-containing medium under identical conditions. HPLC analyses were performed on a Hewlett-Packard 1090 liquid chromatograph, using a Deltapak C4 15 μm 300x3.9 mm column (reverse phase) to characterize and quantitate the products resulting from the degradation of SMA41. The operating mode was isocratic and two solutions A (50% acetonitrile) and B (50% water) were used with a 0.5 ml/min flow rate and a 5 μL injection volume. Under these conditions, independently synthesized SMA52 and SMA41 showed retention times of 11 and 15 min respectively. For the rapid quantitation

of metabolite, a less polar acetonitrile-water (70:30) eluent was used. Under such conditions SMA52 showed a retention time of 7.49 min. For LC-MS analysis of the degradation of SMA41, the column was placed on a Spectra System P1500 HPLC coupled with a Finnigan LCQDUO mass spectrometer.

The half-life of SMA41 under physiological conditions was studied by UV spectrophotometry using an Ultrospec 2000 Pharmacia Biotech spectrophotometer. SMA41 was dissolved in a minimum volume of DMSO, diluted with RPMI medium supplemented with 10% serum and absorbances read at 340 nm in a UV cell maintained at 37 ^{o}C with a circulating water bath. The half-life was estimated by a one-phase exponential decay curve-fit method using the GraphPad software package (GraphPad Software, Inc., San Diego, CA).

2.3.6. EGFR Binding Assay

Nunc MaxiSorp 96-well plates were incubated overnight at 37 ^{o}C with 100 μL per well of 0.25mg/mL poly(L-glutamic acid-L-tyrosine, 4:1) PGT in PBS. Excess PGT was removed and the plate was washed 3 times with Tween 20 (0.1%) in PBS. The kinase reaction was performed as previously described using 15 ng/well of EGFR affinity-purified from A431 cells (Moyer *et al.*, 1997, Vincent *et al.,* 2000) (generous gift from Pfizer Inc, NJ and commercial supplies from BIOMOL, Plymouth meeting, CA). The compound was added and phosphorylation initiated by the addition of ATP. After 8 min at room temperature with constant shaking, the reaction was terminated by aspiration of the reaction mixture and rinsing the plate 4 times with wash buffer (Tween 20 (0.1%) in PBS). Phosphorylated PGT was detected following a 25 min incubation with 50 μL per

well of HRP conjugated PY54 antiphosphotyrosine antibody diluted to 0.2 μg/mL in blocking buffer (3% BSA; 0.05% Tween 20). Antibodies were removed by aspiration, and the plate washed 4 times with wash buffer. The signals were developed by the addition of 50 μL per well of TMB peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersberg, MD) and following blue color development, 50 μL of H_2SO_4 (0.09 M) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (Model 2550).

2.3.7. EGF-induced Autophosphorylation Assay

A431 cells were pre-incubated in a 6-well plate (1 x 10^6) with 0.1% serum at 37 °C overnight for 24 h after which they were exposed to a dose range of each drug for 2 h and subsequently treated with 50 ng/mL EGF for 30 min at 37 °C. Thereafter, they were washed with PBS and re-suspended in cold lysis buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% NP40, 1 mM EDTA; 5 mM NaF; 1 mM Na_3VO_4; protease inhibitor tablet (Roche Biochemicals, Laval, Canada)]. The lysates were kept on ice for 30 min and collected by centrifugation at 10 000 rpm for 20 min at 4 °C. The protein concentrations were determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (40 μg/mL) from each lysate were added to a 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). Non-specific binding on the PVDF membrane was minimized with a blocking buffer containing nonfat dry milk (3%) in PBS. The membrane was incubated with primary antibodies [either anti-phosphotyrosine antibody (UBI, Lake Placid, NY) for the detection of phosphotyrosine, or anti-EGFR (Neomarkers, Fremont, CA) for

determination of corresponding receptor levels, and anti-β-tubulin (Neomarkers, Fremont, CA) for the detection of equal loading]. Thereafter, blots were incubated with HRP-goat anti-mouse antibody (1:200 dilution; Bio-Rad Laboratories, Hercules, CA) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Band intensities were measured using the SynGene GeneTools software package (Cambridge, UK).

2.3.8. EGF-induced Total Cellular Tyrosine Phosphorylation Assay

A431 cells were pre-incubated in a 96 well plate (1 x 10^6) with 0.1% serum at 37°C overnight. The drugs were added for 15 min in serum-free media, and cells were subsequently given EGF (50 ng/mL) for 30 min at 37°C. Cells were fixed with a 1:1 mixture of methanol and acetic acid for 30 min at 4°C. Non-specific binding was blocked with BSA (1%) in PBS for 1 h at 37°C, after which 0.1 μg/mL HRP-conjugated antiphosphotyrosine antibody (UBI, Lake Placid, NY) was added in the same buffer for 1 h at room temperature. 200 μ L TMB peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersberg, MD) was added to each well and following blue colour development, H_2SO_4 (0.9 M) was administered to each well, and plates were read at 450 nm.

2.3.9. Alkaline Comet Assay for quantitation of DNA Damage

A modified alkaline comet assay technique (Singh *et al.*, 1994) was used to quantitate DNA damage induced by SMA41, SMA52, and TEM. A431 cells were exposed to drugs for 30 min or 2 h, and harvested with trypsin-EDTA. The cells were subsequently

collected by centrifugation and re-suspended in PBS. The resulting cell suspension was diluted to approximately 10^6 cells, and mixed with agarose (1%) in PBS at 37°C in a 1:10 dilution. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel casting chambers, as previously described (McNamee *et al.*, 2000), then immediately placed into a lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10mM tris-base, 1% (w/v) N-lauryl sarcosine, 10% (v/v) DMSO and 1% (v/v) Triton X-100]. After being kept on ice for 30 min, the gels were gently rinsed with distilled water and then immersed in a second lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10mM tris-base], containing 1mg/ml proteinase K for 60 min at 37 °C. Thereafter, the gels were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed into 1 M ammonium acetate for 30 min. They were further soaked in 100% ethanol for 2 h, dried overnight and subsequently stained with SYBR Gold (1/10000 dilution of stock supplied from Molecular Probes, OR) for 20 min. For evaluation of comets, DNA damage was assessed using the Tail Moment parameter (*ie.* the product of the distance between the barycentres of the head and the tail of the comet). A minimum of 50 cell comets were analyzed for each sample, using ALKOMET v3.1 software, and values are an average of tail moments for the entire cell population.

2.4. Results

2.4.1. Degradation of SMA41

SMA41 was allowed to degrade in RPMI supplemented with 10% fetal calf serum at 37°C and its half-life measured by UV spectrophotometer. It was found to be

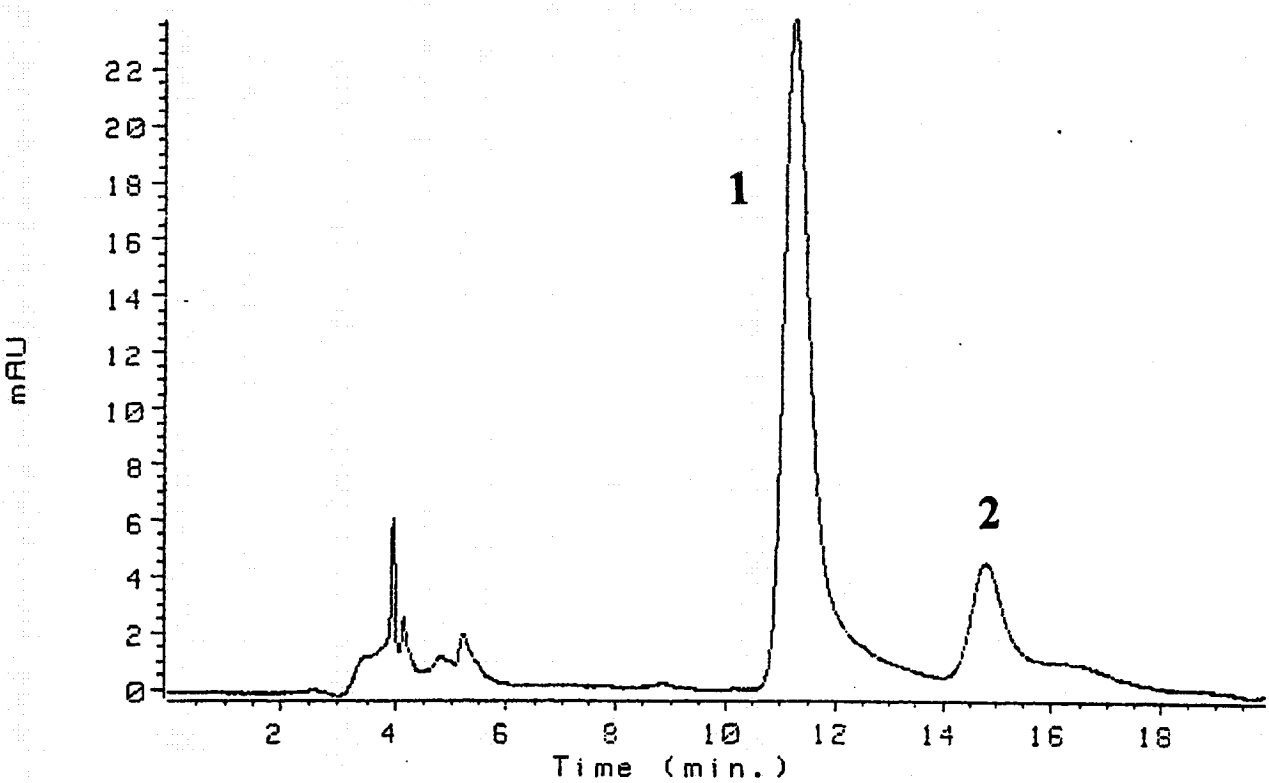


Figure 2.1. Partial degradation of SMA41 to SMA52 in RPMI medium supplemented with 10% serum at 37°C. The peak at around 11 min **(1)** corresponds to SMA52 and the one at 15 min **(2)** represents undegraded SMA41. Peaks in 2-6 min range are medium components. An almost exclusive decomposition of SMA41 into SMA52 was observed.

significantly stable with a $t_{1/2}$ of approximately 30 min in serum-containing cell culture medium at 37°C. SMA41 decomposed almost exclusively into SMA52, the structure of which was confirmed both by HPLC analysis of independently synthesized SMA52 and by LC-MS analyses (Figure 2.1) which showed a mass M+1= 251 for the chromatogram peak corresponding to its retention time. Quantification of this peak and calculations using standard curves indicated that SMA41 was converted to SMA52 in a yield of approximately 81%.

2.4.2. Anti-proliferative activity in A431 cells

The SRB assay was used to evaluate the antiproliferative activity of different compounds in the human squamous carcinoma of the vulva cell line A431 in which EGFR constitutive activity, as reflected by tyrosine phosphorylation under basal conditions, has been shown to be sensitive to antiproliferative agents targeting the EGFR *in vitro* or *in vivo* (Lanzi *et al.,* 1997). In addition this cell line expresses detectable levels of the DNA repair enzyme MGMT (Fornace *et al.* 1990). The MGMT status of our A431 cell line was also confirmed by western blotting using a commercially available anti-MGMT antibody (Pharmingen International, Toronto, Canada) (data not shown). Under 72 h continuous exposure the results, illustrated by Figure 2.2a, showed that SMA41 is 1.8-fold more potent (IC_{50}=36 μM) than its metabolite SMA52 alone (IC_{50}= 59 μM, Figure 2.2b), and 10-fold more potent than TEM (IC_{50}=366 μM, Figure 2.4) in the MGMT-proficient cell line A431. A clonogenic assay as illustrated by Figure 2.3, showed that the antiproliferative activity of SMA41 was in the same range as that of SMA52 ($IC_{50SMA41}$ 4 μM, $IC_{50SMA52}$ 3.7 μM). However, when the cells were treated for only 2 h and further

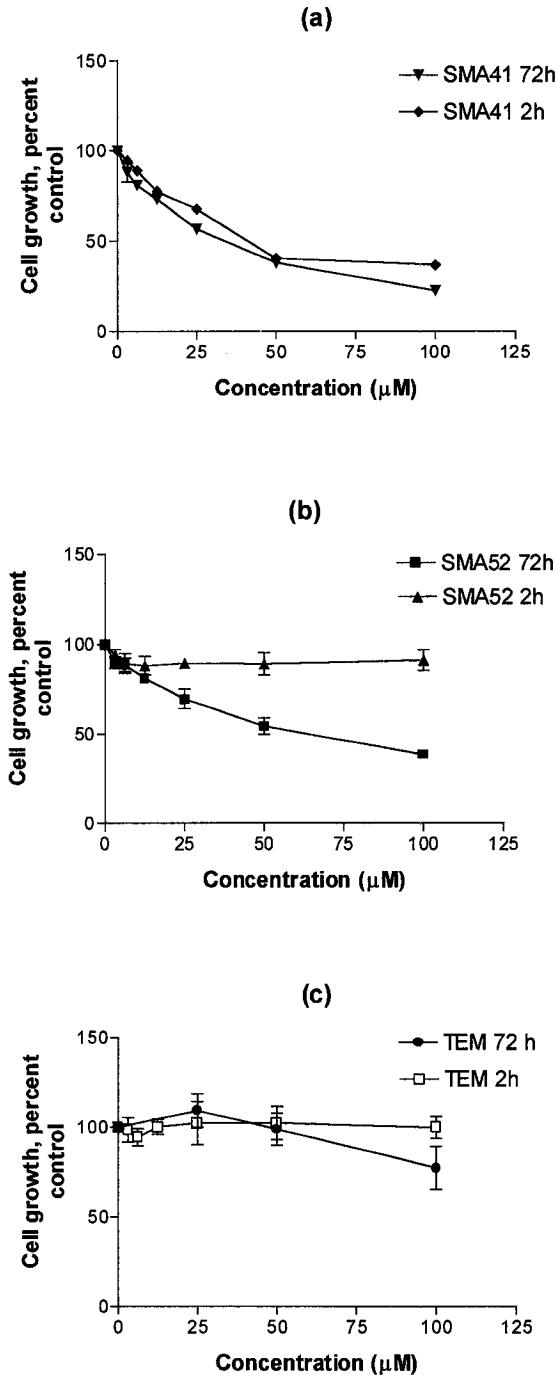


Figure 2.2. Reversibility of antiproliferative effects of (a) SMA41, (b) SMA52, and (c) temozolomide on A431 cells. Cells were exposed to each drug for either 2 h, after which they were allowed to recover for 72 h in drug-free medium, or continuously for 72 h. Cell growth was measured using the sulforhodamine B assay. Each point represents at least two independent experiments run in triplicate.

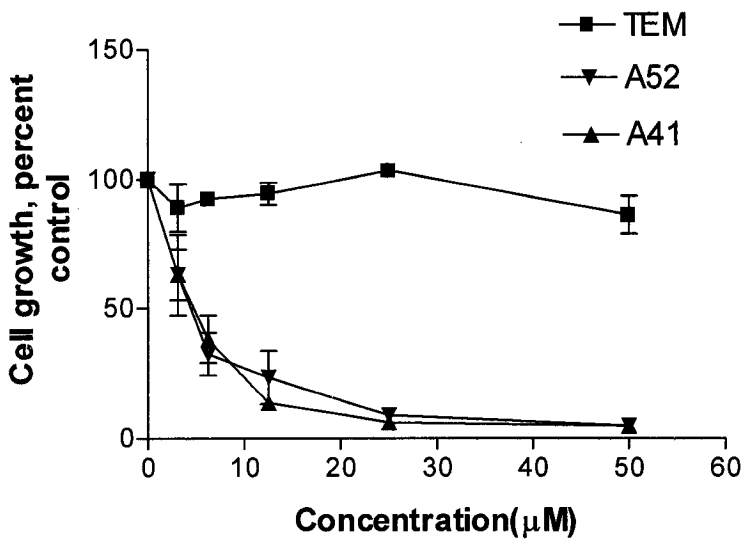


Figure 2.3. Survival of clonogenic cells for SMA41, SMA52, and temozolomide. A431 cells were plated at a density of 500 cells/well and exposed to each drug continuously for 6 days, after which colonies were detected using Syngene imaging software. Each point represents at least two independent determinations.

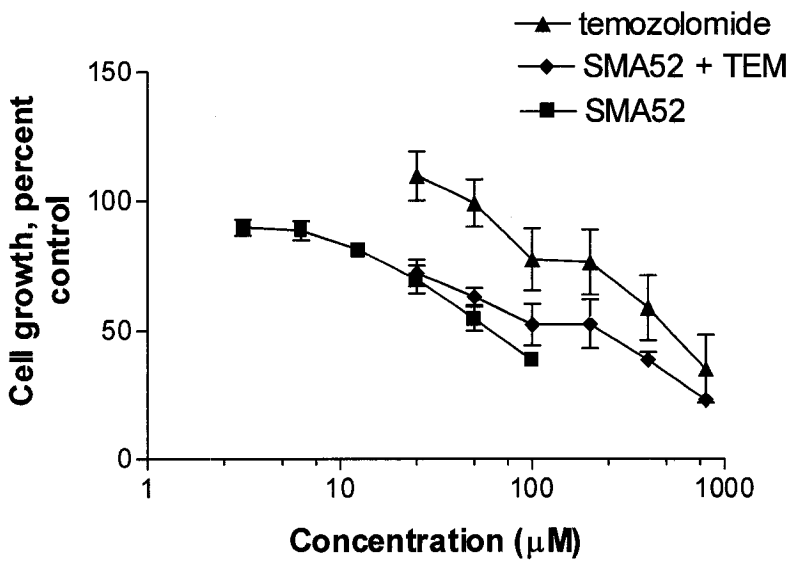


Figure 2.4. Effects of SMA52, TEM, and SMA52 + TEM on the growth of A431 cells. The cells were exposed to each drug continuously for 72 h, and cell growth measured using the sulforhodamine B assay. Each point represents at least two independent experiments run in triplicate.

incubated in drug-free medium, an almost complete loss of activity was observed for SMA52 (IC_{50}>100 μM, Figure 2.2b), indicating that it induced significantly reversible growth inhibitory activities. In contrast, SMA41 showed significant retention of activity with little change in the IC_{50} values [IC_{50} (2 h)=36 μM, IC_{50} (72 h)= ~30 μM].

In order to demonstrate the antiproliferative advantages of combining the EGFR and DNA targeting mechanisms in a single molecule, we studied the combined effect of SMA52 (independently synthesized) with that of TEM using the SRB assay (Figure 2.4). Using equation 1 to determine the nature of interactions between these two drugs, the results showed that the CI_{50} at the 50% effect for SMA52 + TEM is approximately 0.6, indicating a subadditive interaction. However, under identical conditions the antiproliferative activity of the chimeric SMA41 was 4-fold more pronounced than that of the 2-drug combination (IC_{50}, 134 μM).

2.4.3. Binary targeting properties of SMA41

The significant antiproliferative activity of SMA41 in a methyltriazene-resistant cell stimulated our interest in further dissecting its binary (EGFR and DNA) targeting properties. This was achieved by two types of assays: EGF-stimulated tyrosine phosphorylation and DNA damage.

2.4.3.1. Inhibition of EGFR TK activity

In a competitive EGFR binding assay (Figure 2.5), SMA41 (IC_{50}, 0.2 μM) showed a 5-fold stronger binding affinity than SMA52 (1.02 μM) for the ATP site of the purified

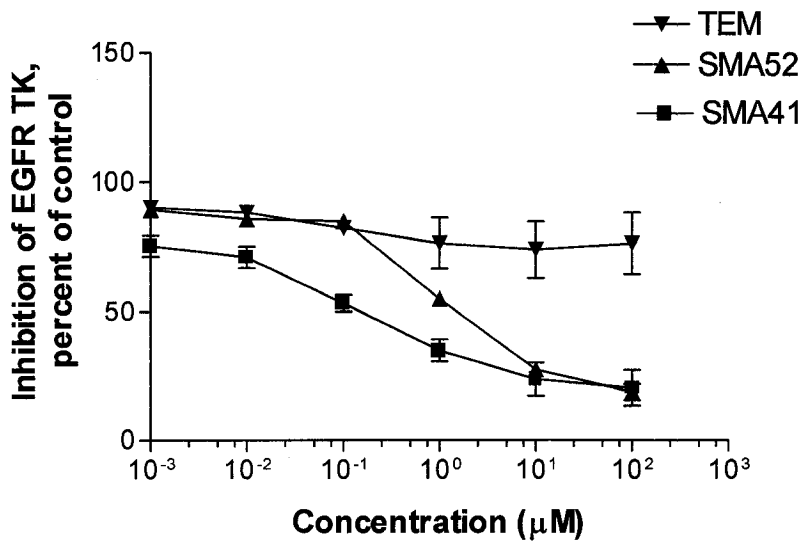


Figure 2.5. Inhibition of purified EGFR tyrosine kinase activity by SMA41, SMA52, and temozolomide. Phosphorylation of a PGT substrate by purified EGFR was measured using immunoassay with antiphosphotyrosine antibodies. Each point represents at least three independent experiments.

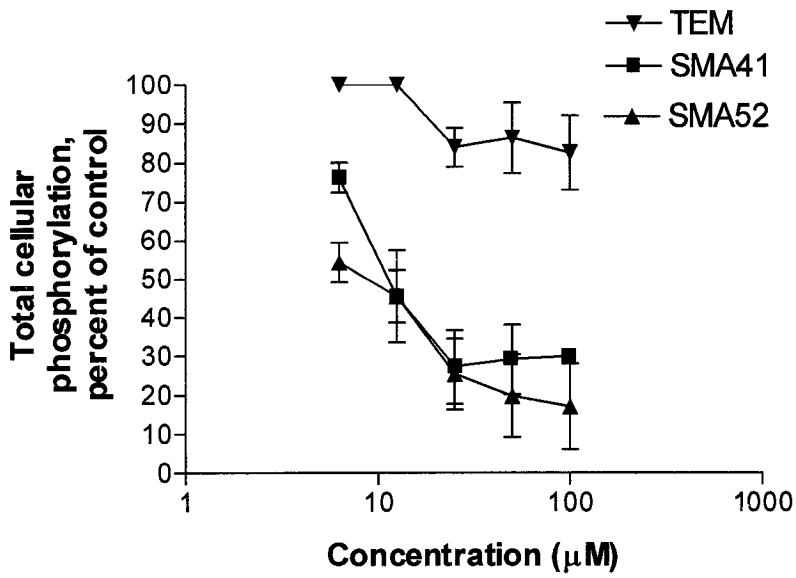
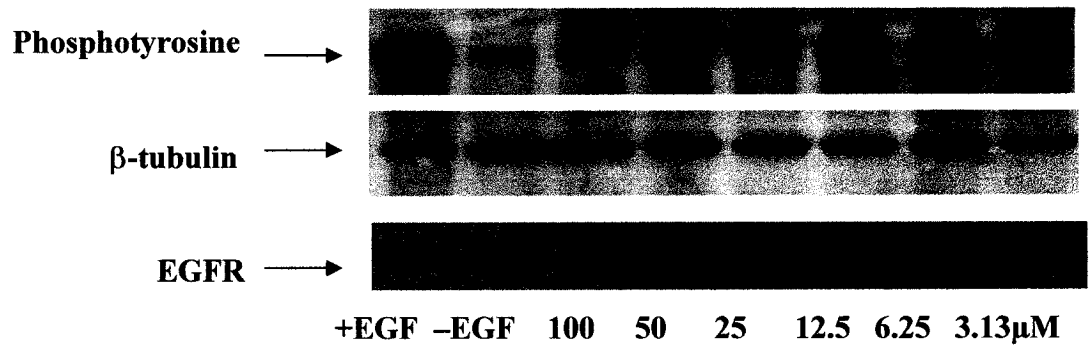


Figure 2.6. Inhibition of EGF-induced total cellular phosphorylation by SMA41, SMA52, and temozolomide. Total cellular phosphorylation was measured in A431 cells using an immunoassay with antiphosphotyrosine antibodies. Each point represents at least two independent experiments run in duplicate.

a)



b)

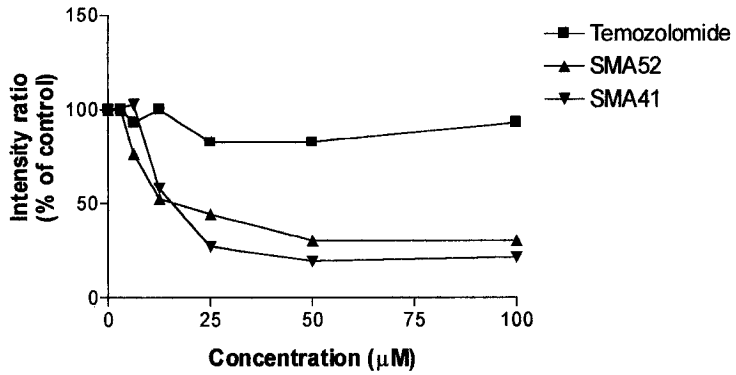


Figure 2.7. (a) Inhibition of EGF-stimulated autophosphorylation by SMA41 in A431 cells. Cells were exposed to the indicated concentrations (μM) of drug for 2 h, after which they were stimulated with 50 ng/mL EGF (*Lanes 1*, and *3-8*). A non-stimulated control is shown in *Lane 2*. Cell lysates were prepared following a 30 min exposure to EGF, and phosphotyrosine was detected by Western blotting with anti-ß tubulin used as a loading control. Membranes were stripped of antiphosphotyrosine and reprobed with anti-EGFR antibodies to detect receptor levels. (b) Comparison between the inhibition of EGF-stimulated autophosphorylation by SMA41, SMA52, and temozolomide in A431 cells. IC_{50} values were determined by sigmoidal dose-response curves obtained from band intenstities (ß-tubulin/phosphotyrosine) versus drug concentrations (μM).

receptor. TEM did not show any significant affinity for this receptor (IC_{50}>100 μM). In an ELISA-based whole cell assay, SMA41 and SMA52 showed comparable levels of inhibition of EGF-induced total cellular phosphorylation (Figure 2.6). Similarly, Western blot analysis (Figure 2.7) demonstrated that both drugs induced almost equal levels of inhibition of EGF-induced EGFR autophosphorylation [IC_{50} SMA52= 8.44 μM, IC_{50} SMA41 12.5 μM]. In contrast to SMA41 and SMA52, TEM did not exhibit any EGFR binding affinity, nor did it inhibit EGF-induced autophoshorylation in A431 cells [IC_{50}>100μM] in the specified dose ranges.

2.4.5. Quantitation of DNA damage

Using the alkaline comet assay, it was demonstrated that, in contrast to SMA52 (Figure 2.8c), both SMA41 and TEM were capable of inducing DNA damage in a dose-dependent manner. However, differences were observed in the kinetics of dose-dependent DNA damage induced by SMA41 when compared with TEM. For SMA41, the trend was to induce rapid nuclear condensation at the highest doses (25-100 μM) leading to a reduction in comet tailing. For SMA41, significant comet tail moment could only be observed in the 6-25 μM range after a short 30 min and 2 h drug exposures (Figure 2.8a). With a 2 h exposure, a decrease in tail moment was observed at concentrations above 6 μM, concurrent with observable nuclear condensation likely due to a rapid onset of apoptosis. In contrast, TEM (Figure 2.8b) exhibited a dose-dependent increase in comet tail moment under 30 min exposure with a remarkable enhancement under the longer 2 h drug exposure (Figure 2.8b). This is *prima facie* evidence that despite being two methylating agents, the mechanisms of action of SMA41 may be markedly different from

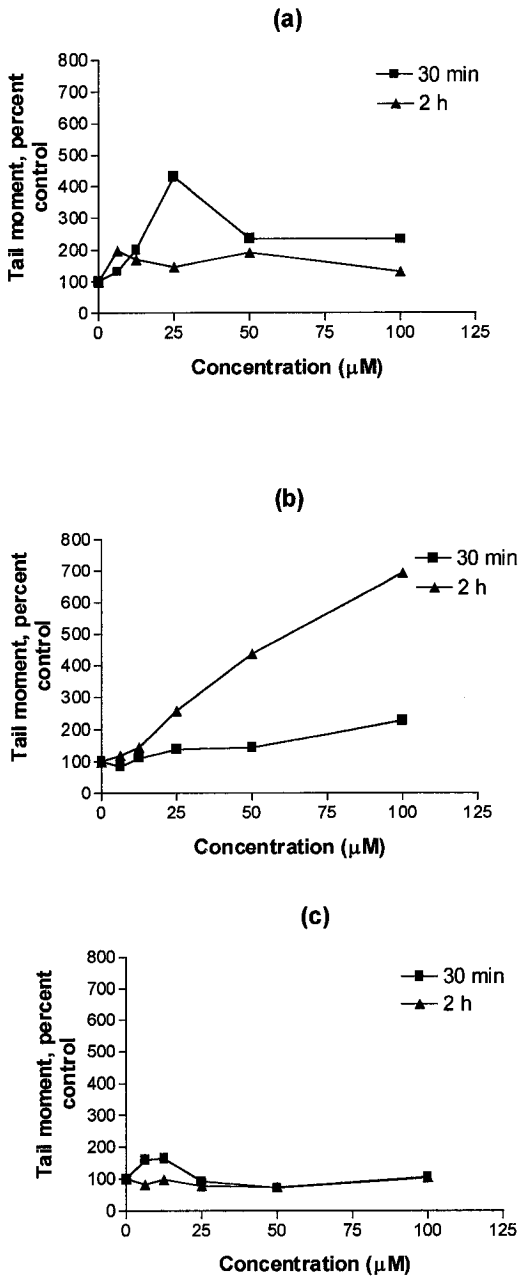


Figure 2.8. Quantitation of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in A431 cells exposed to (a) SMA41, (b) TEM, and (c) SMA52 for 30 min, or 2 h.

that of TEM.

2.5. Discussion

Overexpression of EGFR is common in a wide variety of major human solid tumours of epithelial origin such as breast, colorectal, head and neck ovarian and bladder carcinomas (Lanzi *et al.,* 1997; Modjtahedi and Dean, 1998; Yaish *et al.,* 1988). EGF binding induces receptor dimerization, autophosphorylation and activation of mitogenic signaling. The A431 cell line expresses a large number of EGF binding sites and also the high affinity EGFR ligand TGF (Lanzi *et al.,* 1997). This translates into aggressive autocrine-controlled growth *in vitro*. Blocking A431 cell proliferation has become the standard screen for anti-proliferative inhibitors of EGFR TK activity (Lanzi *et al.,* 1997; Yaish *et al.,* 1988). This cell line also expresses the alkyltriazene resistance-associated DNA repair enzyme MGMT and is as demonstrated herein (Figure 2.2c) resistant to the cyclic 1-methyl-1,2,3-triazene TEM (IC_{50}=366 μM). Therefore, it represents a good model for the determination of the pharmacological advantages of simultaneous targeting of EGFR and DNA in EGF expressing refractory tumours.

Dacarbazine and TEM, two prodrugs of monomethyltriazenes are the most active drugs in the treatment of malignant melanomas and gliomas (Hill *et al.,* 1989; Carter *et al.,* 1976; Lee *et al.,* 1992; Carter *et al.,* 1994). As outlined in Scheme 2.1, the cytotoxic monoalkyltriazene MTIC degrades under physiological conditions to generate a variety of metabolites, the critical reaction being the heterolysis of the non conjugated tautomer to generate the arylamine AIC and the alkyldiazonium species (Kolar *et al.,* 1980; Foedstad

et al., 1985; Cameron *et al.,* 1985; Manning *et al.,* 1985). It has already been shown by isotopic labeling that the latter species alkylate DNA at the 6- and 7-positions of guanine or 3-position of adenine. As we have demonstrated that SMA41 is able to generate like MTIC a free arylamine (SMA52), we expected its concomitantly generated metastable methyldiazonium to induce the same type of alkali labile DNA lesions as those associated with TEM or other classical triazenes. Indeed, in contrast to SMA52-exposed cells, significant levels of DNA damage were observed in those treated with SMA41 in the 6.25-25 μM range and the analyses were complicated by nuclear condensation at concentrations higher than its growth inhibitory IC_{50} under both 30 min and 2 h drug exposure (SRB). Since this assay involved alkaline electrophoresis of the whole cell nuclei, we believe that this fragmentation is primarily due to N7-methylguanine, a type of lesion with known alkali labile properties (Catapano *et al.,* 1987). The quantitation of this type of alkali labile DNA lesions by the classical alkaline elution assay is now well documented (Hartley *et al.,* 1986; Pera *et al.,* 1981). Single-cell microelectrophoresis (comet) assay clearly showed that, like TEM the clinical prodrug of MTIC, SMA41 possesses strong DNA damaging properties. However, the marked differences between dose-response profiles of SMA41 and TEM [DNA damage, SRB and clonogenic assays)] indicate that these two methylating agents may block cell proliferation by a different mechanism. It is noteworthy that the activity of SMA41 was approximately 8-fold greater in the clonogenic assay than in the SRB assay. This can be attributed to an increased exposure time (6 day continuous exposure).

The second target of SMA41 was first elucidated by measuring its ability to block EGFR TK phosphorylation of a PGT substrate in an ELISA assay. It should be first

remembered that the design of SMA41 was primarily based upon previously identified structure activity-relationships in the quinazoline series showing that bulky substituents are tolerated at the 6- and 7-positions. In addition, electron-donating substituents increase their binding affinity for the ATP-binding site of EGFR. Since the delocalization of electrons from N3 of the triazene chain may confer only a slight electron-donating character to SMA41, we believe that its stronger EGFR TK inhibitory activity when compared with SMA52 (which contains a stronger electron-donating group at the 6-position (Hammett constant σ_p= -0.57, σ_m=–0.09) (Hammett, 1940; Andrejus, 1988; Jean-Claude and Williams, 1998) may be due to its ability to induce methylation of nucleophilic aminoacid side chains in the ATP binding site of the receptor (*e.g* thiol function of a cysteine residue). It has recently been demonstrated that quinazolines bearing an acrylamide group at the 6-position are capable of alkylating the cysteine 773 at the TK active site (Snaill *et al.,* 1999; Jeff *et al.,* 2000), and that the 6-position is 4 Δ closer to this cysteine residue than the 7-position. The non-conjugated form of the alkyltriazene moiety having approximately the same length as the acrylamide moiety and being a strong alkylator, may undergo a similar type of alkylation in the active site of EGFR. Moreover, since an exposure time (8 min) shorter than the $t_{1/2}$ was used in the *ex vivo* tyrosine kinase assay, the observed inhibitory activity is mainly due to the binding of the intact molecule with minimal contribution of the residual SMA52 metabolite.

Having demonstrated that SMA41 is able to target isolated receptors, we investigated whether this agent could block signal transduction in A431 cells. An EGF-induced total phosphorylation assay showed that, like SMA52, SMA41 is capable of inhibiting EGF-induced total TK phosphorylation in a dose-dependent manner in A431

cells. In addition, SMA41 is capable of blocking EGF-induced autophosphorylation of the EGFR. It is important to mention that no detectable levels of tyrosine kinase inhibitory activity were observed with TEM over its whole dose range. The detection of autophosphorylation inhibitory activities for SMA41 and SMA52 is an indirect evidence of normal transport of these compounds across the cell membrane. Although it is not clear at this stage whether SMA41 is sequestered in the cells before its hydrolytic cleavage to SMA52 or whether significant extracellular cleavage occurred prior to cell penetration of these two species. However, some inference can be made in light of the macromolecular targeting results.

In contrast to the isolated enzyme assay which showed a 5-fold superior inhibitory activity for SMA41 when compared with SMA52, the whole cell phosphorylation assays exhibited similar levels of activity for these two drugs. If no DNA damage was observed, these results could indicate a total extracellular conversion of SMA41 to SMA52 prior to cell penetration. However, the significant nuclear fragmentation and the marked retention of activity observed when SMA41 was removed after 2 h (Figure 2.2a), are indirect evidence of intracellular sequestration of SMA41 since as we have demonstrated, SMA52 alone does not possess DNA damaging properties (Figure 2.8c). The loss of TK inhibitory activity of SMA41 when compared with SMA52 (from the isolated enzyme to the whole cell assays) may be due to differences in the intracellular distributions of these drugs. Nevertheless, the results *in toto* give *prima facie* evidence that SMA41 is a novel triazene with a significant EGFR tyrosine kinase inhibitory activity, a property that has never been observed before for any class of mono- or dialkyltriazenes.

Based upon known principles of medical oncology, which suggest that rapidly

proliferating cells are more sensitive to DNA damaging agents than slow-growing ones, it was feared that the cytostatic effect of EGFR TK inhibition would initially block proliferation and thereby decrease cell sensitivity to the DNA damage associated with the concomitantly generated methyldiazonium species. This would translate into a rather antagonistic effect. To test this hypothesis, we mimicked the combined effect of the two mechanisms of action by designing a 2-drug combination model involving SMA52 (an EGFR TK inhibitor) and TEM (a DNA damaging agent). The results showed a sub-additive interaction and not an antagonistic one between these two drugs. Moreover, it is noteworthy that SMA41 was more potent than the 2-drug combination. This suggests that a single molecule formulated as a masked form of these two types of agents may be more efficacious than a 2-drug combination encompassing individual monoalkyltriazenes and EGFR TK inhibitors.

Since SMA41 can both block phosphorylation induced by EGF and damage genomic DNA, its over 8-fold (SRB assay) and over 90-fold (clonogenic assay) greater potency when compared with TEM may result from the combined effects of these two distinct mechanisms of antiproliferative activities. The binary targeting may trigger signal transduction associated with the induction of apoptosis. Indeed, in contrast to SMA52 and TEM, significant nuclear condensation was observed in cells treated with SMA41 for 2 h in the 25-100 μM range. The effect of binary targeting on the expression and activity of MGMT and the mechanism of apoptosis induced by SMA41 are now being investigated in our laboratory and the results will be reported in due course.

A significant body of evidence has accumulated to suggest that overexpression of EGFR is a marker for poor prognosis in many solid tumours. Selective inhibitors of

tyrosine phosphorylation by EGFR are now considered an important class of anticancer drugs and two members of the 4-(phenylamino)quinazoline class are now in clinical trial. Despite the significant EGFR inhibitory activity of these reversible inhibitors, the high intracellular concentrations of ATP is a major barrier to sustained inhibition of EGF-stimulated signal transduction in tumour cells. More recently, this problem was addressed by Smaill *et al.* (1999) who showed that quinazolines containing acryloyl function at the 6-position could induce irreversible inhibition of EGFR by alkylating cysteine 773 of the enzyme. A recently synthesized water soluble analogue of this class has now been selected for phase I clinical trial (Jeff *et al.,* 2000). It is noteworthy that despite being irreversible inhibitor of EGFR, when apoptosis is not triggered, if the cells respond to alternative growth hormones (*e.g.* heregulin or PDGF) these compounds may still not induce a sustained growth inhibitory activity. Our novel SMA41 presents the advantage of being not only capable of blocking EGF-stimulated signal transduction on its own but also generating a DNA alkylating species that may inflict irreversible cytotoxic DNA lesions. Moreover, this compound was designed to release another intact EGFR TK inhibitory molecule (*e.g.* SMA52) that may further enhance its growth inhibitory activity. Our results showed that these combined properties conferred increased potency to a monoalkyltriazene against an MGMT-proficient tumour cell line with marked resistance to the clinical drug TEM (IC_{50}, 366 μM). Also, the current study, which was primarily designed to identify the principal targets of SMA41, has conclusively demonstrated that this one-molecule combination showed superior activity when compared with a 2-drug combination involving TEM + SMA52. Further studies are now ongoing to characterize the effects of SMA41 on growth stimulation by a wide variety of hormones including

EGF, TGF, PDGF and insulin prior to the demonstration of *in vivo* efficacy of this novel approach termed "the Combi-Targeting concept".

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2.8. Connecting Text

In the preceding chapter (Chapter 2), the Combi-targeting concept was introduced. The synthesis and mechanism of action of SMA41, the first-ever "combi-molecule" targeting both EGFR TK as well as DNA, was described in detail. Since this molecule was designed to degrade into an intact TK inhibitor plus a DNA-damaging methyldiazonium ion, we demonstrated using LCMS that this indeed occurs in serum-containing medium at 37°C with a yield of approximately 81% for the intact TK inhibitor (SMA52) and a half-life of approximately 34 minutes. Furthermore, we demonstrated that SMA41 does indeed possess binary targeting properties: it can inhibit purified EGFR TK and EGFR in whole cells, and it is capable of inducing DNA damage. These results encouraged us to continue our elucidation of the effects of SMA41, and to examine its selectivity in cell lines expressing variable levels of receptor. Moreover, we wished to determine whether the DNA repair enzyme AGT had an effect on the activity of SMA41, since its expression hampers the activity of monomethylating triazenes such as TEM. In these studies we compared the activity of TEM with that of SMA41, since their DNA-damaging properties are analogous. Where we saw little to no activity of TEM against the AGT-expressing cell line A431, SMA41 demonstrated growth inhibition. This supports the theory that a combined targeting approach is superior to that of administering a single drug against a tumour. The following chapter describes investigation into the effects of both AGT and EGFR expression on the activity of SMA41. This paper was published in the Journal of Pharmacology and Experimental Therapeutics (see Appendix).

CHAPTER 3

DIFFERENTIAL RESPONSES OF EGFR/AGT-EXPRESSING CELLS TO THE "COMBI-TRIAZENE" SMA41

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3.1.Abstract

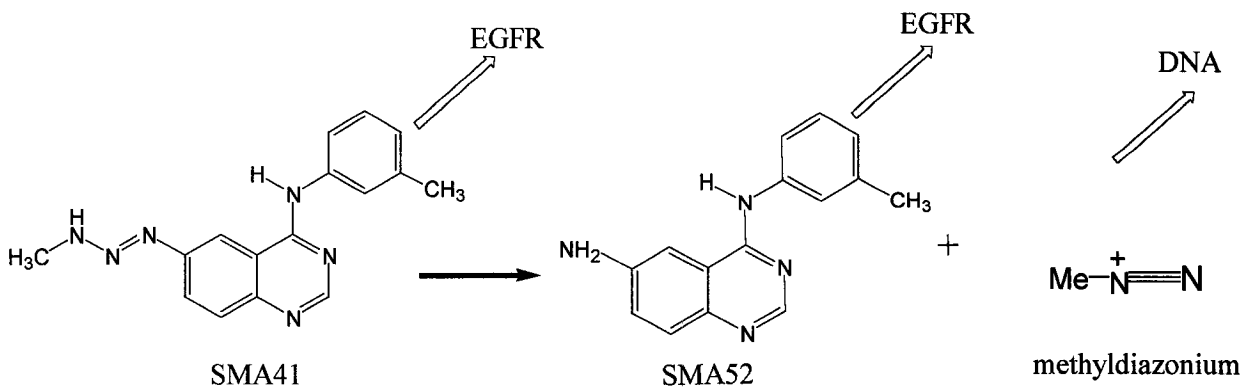
Previous studies have demonstrated enhanced potency associated with the binary [DNA/epidermal growth factor receptor (EGFR)] targeting properties of SMA41 (a chimeric 3-(alkyl)-1,2,3-triazene linked to a 4-anilinoquinazoline backbone) in the epidermal carcinoma of the vulva cell line A431. We now report on the dependence of its antiproliferative effects (*e.g.* DNA damage, cell survival) on the EGFR and the DNA repair protein O6-alkylguanine DNA alkyltransferase (AGT) contents of 12 solid tumour cell lines, two of which NIH3T3 and NIH3T3 HER14 (engineered to overexpress EGFR) being isogenic. Studies of receptor type specificity showed that SMA41: (a) preferentially inhibited the kinase activity of EGFR over those of src, insulin receptors and PKC (a serine/threonine kinase), (b) induced stronger inhibition of EGF- than platelet-derived growth factor (PDGF)- and fetal bovine serum (FBS)-stimulated growth. Despite the EGFR specificity of SMA41, there was an absence of linear correlation between the EGFR status of our solid tumour cell lines and levels of DNA damage induced by the alkylating component. Similarly, EGFR levels did not correlate with IC_{50} values for growth inhibition. The antiproliferative activities of SMA41 correlated more with the AGT status of these cells and paralleled those of the clinical triazene temozolomide (TEM). However, throughout the panel, tumour cell sensitivity to SMA41 was consistently stronger than to its closest analogue TEM. Experiments performed with the isogenic cells showed that SMA41 was capable of inducing 2-fold higher levels of DNA damage in the EGFR transfectant and delayed cell entry to G2M in both cell types. When the cells were starved and growth-stimulated with fetal bovine serum (FBS), (conditions under which both cell types were growth-stimulated), in contrast to TEM, SMA41 and its

hydrolytic metabolite SMA52 exhibited approximately 9- and 3-fold stronger inhibition of FBS-stimulated growth in the EGFR transfectant. These results suggest that in addition to its ability to induce DNA damage and cell cycle perturbations, SMA41 is capable of selectively targeting the cells with growth advantage conferred by the EGFR transfection. When compared with monoalkyltriazene prodrug TEM, its potency may be further enhanced by its ability to hydrolyze to another signal transduction inhibitor (SMA52) + a DNA alkylating agent (AA) that may further contribute to chemosensitivity. Thus, our new Combi-Targeting strategy may well represent a tandem approach to selectively block receptor tyrosine kinase (RTK)-mediated growth signaling while inducing significant levels of cytotoxic DNA lesions in refractory tumours.

Key words: signal transduction, DNA damage, cell cycle perturbation, receptor-type specificity, O6-alkylguanine DNA alkyltransferase (AGT)

3.2. Introduction

We have designed a novel strategy, termed the "Combi-Targeting Concept", to selectively target tumour cells overexpressing receptor tyrosine kinases (RTK) or growing by an autocrine stimulatory loop. This strategy seeks to synthesize chimeric molecules referred to as "combi-molecules" (C-molecules) that possess the ability to: (a) inhibit the RTK on their own, (b) generate, following hydrolytic scission under physiological conditions, another inhibitor of the same RTK and a cytotoxic DNA alkylating agent (AA) (Matheson *et. al.*, 2001). The combined effects of these three species (C-molecule, RTK inhibitor and AA) are expected to induce significant antiproliferative activity in cells expressing the RTK. Recently, we demonstrated the feasibility of this approach by synthesizing our first C-molecule, SMA41 (Scheme 1), a masked combination of SMA52 (targeted to EGFR, see arrow) and a 3-methyl-1,2,3-triazene (precursor of the DNA damaging methyldiazonium species), that showed over 10-fold greater potency than the clinical triazene TEM in the carcinoma of the vulva cell line A431 (Matheson *et. al.*, 2001). More importantly, the C-molecule showed 4-fold greater activity than the combination of equitoxic doses of TEM and the "naked" inhibitor SMA52.



Scheme 1

Since a large number of human solid tumours express high levels of EGFR, and many co-express EGFR and AGT, we thought it of interest to study the correlation between the EGFR/AGT status of established tumour cell lines and their sensitivity to the C-molecule. The latter was designed to retain significant affinity for its cognate receptor, thereby creating conditions under which cell recognition would be assisted by its affinity for the RTK and further degradation into a secondary RTK inhibitor + a DNA damaging fragment would take place in the cytosol. Since SMA41 showed (a): a 0.2 μM affinity for EGFR, (b) the ability to degrade in serum-containing media to generate 81% of SMA52 (IC_{50} EGFR inhibition: 1 μM) and (c) DNA damaging properties, we thought it of interest to study its differential effects on a panel of cell lines with varied levels of AGT and EGFR expression. Here, we report a complex relationship between EGFR/AGT status of established cells and selective targeting of EGFR in an isogenic pair of NIH 3T3 and NIH 3T3-HER14 cells (engineered to overexpress EGFR). All cells lacking AGT were remarkably sensitive to SMA41 and TEM regardless of their EGFR status. While all cells expressing AGT were resistant to TEM, the antiproliferative activities of SMA41 were consistently stronger (10-50-fold) than those of TEM whether AGT was singly or co-expressed with EGFR, confirming the superior antiproliferative properties of the C-molecule principle. More importantly, using conditions under which the sole difference between two NIH 3T3 clones is the EGFR gene, we demonstrated that, in contrast to TEM, the C-molecule selectively blocked serum-induced growth of the EGFR-transfectant.

3.3. Materials and Methods

3.3.1. Drug Treatment

SMA41, and SMA52 were synthesized in our laboratories according to known procedures (Manning *et. al.*, 1985; Cameron *et. al.*, 1985). Temozolomide was provided by Shering-Plough Inc. (Kenilworth, NJ). In all assays, the drug was dissolved in DMSO and subsequently diluted in sterile RPMI-1640 media containing 10% fetal bovine serum (Life Technologies, Burlington, Canada) immediately prior to the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

3.3.2. Cell Culture

The human tumour cell lines MDA-MB-435, MDA-MB-453, MDA-MB-468, MCF-7 (breast carcinoma), PC-3, DU-145 (prostate carcinoma), SF-126, and SF-188 (glioma) (ATCC, Manassas, VA) were maintained in RPMI-1640 supplemented with fetal bovine serum (10%), gentamycin (50 mg/mL), and HEPES (12.5 mM) (Wisent, St. Bruno, Canada). The mouse fibroblast cell line NIH 3T3, and its stable EGFR transfectant NIH 3T3/HER14 (generous gift from Dr. Moulay Aloui-Jamali), as well as MCF-10A (a normal breast cell line) were maintained in DMEM supplemented with donor calf serum (10%), gentamycin (50 mg/mL), and HEPES (12.5 mM) (Wisent, St. Bruno, Canada). All cells were maintained in a monolayer culture at 37°C in a humidified environment of 5% CO_2-95% air. Cells were maintained in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/mL of trypsin and 0.2 mg/mL of EDTA and

replating before confluence. In all assays, the cells were plated for 24 h before drug administration.

3.3.3. Growth Inhibition Studies

For non-stimulated cell growth, approximately 5000 cells were plated per well in 96 well plates. Following 24 h incubation, cell monolayers were exposed to different concentrations of drug continuously for 7 days. For growth factor- and serum-stimulated growth, approximately 5000 cells were plated in each well of a 48 well dish. After 48 h, the cells were washed 3 times with PBS and serum-free media without phenol red was added. Cells were serum-deprived for 24 h, after which growth factor (12 ng/mL EGF, 25 ng/mL TGF-α, or 50 ng/mL PDGF), or FBS (10%) were added with various concentrations of each drug. Growth inhibitory activities for both stimulated and non-stimulated growth were evaluated using the Sulforhodamine B assay (SRB) (Skehan *et. al.*, 1990). Briefly, following drug treatment, cells were fixed using 50 μL of cold trichloroacetic acid (50%) for 60 min at 4°C, washed five times with tap water, and stained for 30 min at room temperature with SRB (0.4%) dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid, and allowed to air-dry. The resulting colored residue was dissolved in 200 μL of Tris base (10 mM), and optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

3.3.4. EGFR, Src, and Insulin Receptor Binding Assays

Nunc MaxiSorp 96 well plates were incubated overnight at 37°C with 100 μL per well of 0.25 mg/mL poly(L-glutamic acid-L-tyrosine, 4:1) PGT in PBS. Excess PGT was removed and the plate was washed 3 times with Tween 20 (0.1%) in PBS. The kinase reaction was performed as previously described using 15 ng/well of EGFR affinity-purified from A431 cells (Moyer *et. al.*, 1997) (generous gift from Pfizer Inc, NJ and commercial supplies from Biomol, Plymouth meeting, PA). The compound was added and phosphorylation initiated by the addition of ATP. After 8 min at room temperature with constant shaking, the reaction was terminated by aspiration of the reaction mixture and rinsing the plate 4 times with wash buffer (0.1% Tween 20 in PBS). Phosphorylated PGT was detected following a 25 min incubation with 50 μL per well of HRP conjugated PY54 antiphosphotyrosine antibody diluted to 0.2 μg/mL in blocking buffer (3% BSA; 0.05% Tween 20). Antibodies were removed by aspiration, and the plate washed 4 times with wash buffer. The signals were developed by the addition of 50 μL per well of TMB peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersberg, MD) and following blue color development, 50μL of H_2SO_4 (0.09 M) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (Model 2550). For the Src assay, 1.2 units/well of protein were used (Biomol, Plymouth meeting, PA), and 100 mM of EDTA was added to stop the phosphorylation reaction. For the Ins-R assay, 15 ng/well of protein was used (Biomol, Plymouth meeting, PA), and to stop the reaction, EDTA (250 mM) was added. In both cases, known inhibitors were used to calibrate the assay.

3.3.5. PKC Assay

MBP coated 96 well plates (UBI, Lake Placid, NY) were reconstituted with PBS prior to the addition of drug dilution in assay dilution buffer II (UBI, Lake Placid, NY), PKC lipid activator, Mg/ATP cocktail, and purified PKC 25 ng/well (UBI, Lake Placid, NY). The reaction was allowed to occur at room temperature with constant shaking for 15min. The plate was washed 3 times with PBS, and blocking buffer was added (1% BSA in PBS) for 45min. Anti-phospho MBP antibodies (1 μg/mL in blocking buffer) (UBI, Lake Placid, NY) and goat-anti-mouse-HRP secondary antibodies (1/5000 in blocking buffer) (UBI, Lake Placid, NY) were used to detect phosphorylated substrate. The plates were washed 7 times with PBS and the signals developed by the addition of 75 μL per well of TMB peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersberg, MD). Following blue color development, 75 μL of H_2SO_4 (0.09 M) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (Model 2550).

3.3.6. Alkaline Comet Assay for quantitation of DNA Damage

A modified alkaline comet assay technique (Matheson *et. al.*, 2001; McNamee *et. al.*, 2000) was used to quantitate DNA damage induced by SMA41, SMA52, and TEM. A431 cells were exposed to drugs for 30 min and harvested with trypsin-EDTA. The cells were subsequently collected by centrifugation and re-suspended in PBS. The resulting cell suspension was diluted to approximately 10^6 cells/ml, and mixed with agarose (1%) in PBS at 37°C in a 1:10 dilution. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel casting chambers, as previously described (Matheson *et. al.*, 2001; McNamee *et. al.*, 2000) then immediately placed into a lysis

buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM tris-base, 1% (w/v) N-lauryl sarcosine, 10% (v/v) DMSO and 1% (v/v) Triton X-100]. After being kept on ice for 30 min, the gels were gently rinsed with distilled water and then immersed in a second lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base], containing 1 mg/ml proteinase K for 60 min at 37°C. Thereafter, they were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed into 1M ammonium acetate for 30 min. They were further soaked in 100% ethanol for 2 h, dried overnight and subsequently stained with SYBR Gold (1/10000 dilution of stock supplied from Molecular Probes, OR) for 20 min. For evaluation of comets, DNA damage was assessed using the Tail Moment parameter (*i.e.* the product of the distance between the barycentres of the head and the tail of the comet multiplied by the percentage DNA in the tail region). A minimum of 50 cells/comets were analyzed for each sample, using ALKOMET v3.1 software, and values are an average of tail moments for the entire cell population.

3.3.7. Flow cytometry for cell cycle analysis

Cells were grown in 6 well plates in a monolayer until confluence, washed 3 times with PBS, and grown in serum-free medium without phenol red for 24 h. Thereafter, they were harvested with Trypsin-EDTA, collected by centrifugation and washed with PBS. Vindelov's solution [0.01 M Tris base, 10 mM NaCl, 700U RNase, $7.5x10^{-5}$M propidium iodide, 0.1% NP-40] was added (400 µL) for 10 min at 37°C and the fluorescence (FL2) detected using a Becton-Dickenson FACScan. When G1 cell synchronization was

satisfactory, drugs + (FBS (10%) were added for an additional 24 h after which analyses were performed as described.

3.3.8. Western blotting

All cell lines were grown in 6 well plates (1 x 10^6) in media containing 10% serum until confluence. Thereafter, they were washed with PBS and re-suspended in cold lysis buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% NP40, 1 mM EDTA; 5 mM NaF; 1mM Na_3VO_4; protease inhibitor tablet (Roche Biochemicals, Laval, Canada)]. The lysates were kept on ice for 30 min and collected by centrifugation at 10 000 rpm for 20 min at 4°C. The protein concentrations were determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (40 μg/mL) from each lysate were added to a 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). Non-specific binding on the PVDF membrane was minimized with a blocking buffer containing nonfat dry milk (3%) in PBS. The membrane was incubated with primary antibodies [either anti-EGFR (Neomarkers, Fremont, CA) for determination of corresponding receptor levels, anti-β-tubulin (Neomarkers, Fremont, CA) for the detection of equal loading, or anti-AGT (Pharmingen, Toronto, Canada]. Thereafter, blots were incubated with HRP-goat anti-mouse antibody (1:200 dilution; UBI, Lake Placid, NY) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Band intensities were measured using the SynGene GeneTools software package (Cambridge, UK).

3.4 Results

3.4.1. Receptor-type specificity

Prior to determining whether the mixed targeting properties of SMA41 translated into selective targeting of EGFR-overexpressing cells, we thought it of interest to analyze its receptor-type specificity. This was determined using two lines of assays: (a) ELISA-based competitive binding and (b) growth factor or serum-stimulated growth of solid tumour cells. As shown in Table 3.1, SMA41 did not inhibit insulin or src tyrosine kinase activities (IC_{50}>100 μM), nor did it inhibit protein kinase C (PKC) serine/threonine kinase activity. More importantly, as shown in Figure 3.1, when the cells were starved and stimulated with various growth factors or serum, SMA41 preferentially blocked EGF- over platelet-derived growth factor (PDGF)- and serum-stimulated growth in NIH 3T3/HER-14 cells, indicating some degree of selectivity for EGFR-mediated growth.

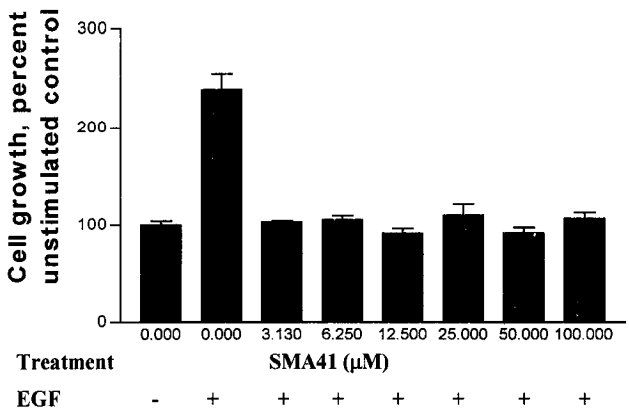
3.4.2. Correlation with EGFR-expression

Since SMA41 showed preferential affinity for the intracellular ATP binding site of EGFR, we hypothesized that this was likely to translate into selective targeting of EGF-dependent cells. More importantly, the ATP binding site being located in the cytosol, we surmised that methyldiazonium species generated by the *in situ* hydrolysis of EGFR-bound SMA41 may diffuse away from the membrane towards the nucleus, thereby inducing an EGFR-assisted elevation of the levels of DNA damage in EGFR-overexpressing cells. This was also expected to confer a direct dependence of levels of DNA damage or antiproliferative activity on the EGFR content of the cells. Growth inhibitory studies using the NCI suforhodamine B assay (Skehan *et. al.*, 1990) under a 7-

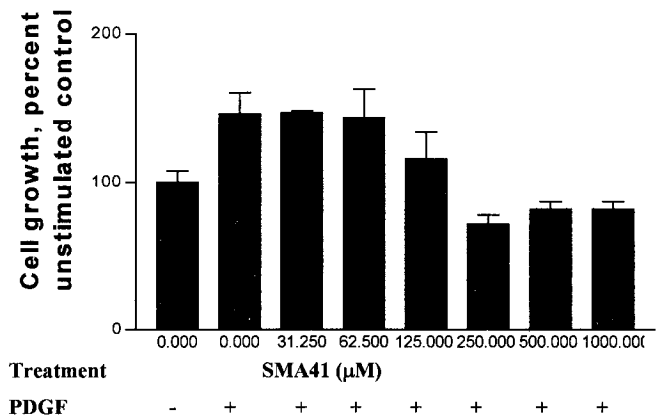
Tyrosine kinase	SMA41 IC_{50} (μM, competitive binding)	SMA52 IC_{50} (μM, competitive binding)
PKC	>100	>100
Src	>100	>100
Insulin receptor	>100	>100
EGFR(13)	0.2	1

Table 3.1. Inhibition of tyrosine and serine/threonine kinases by SMA41 and SMA52 in an ELISA-based competitive ATP binding assay. Each value was calculated by nonlinear regression analysis using Graph Pad Prism software (Graph Pad Software Inc., San Diego, CA), and represents at least two independent experiments run in duplicate.

a)



b)



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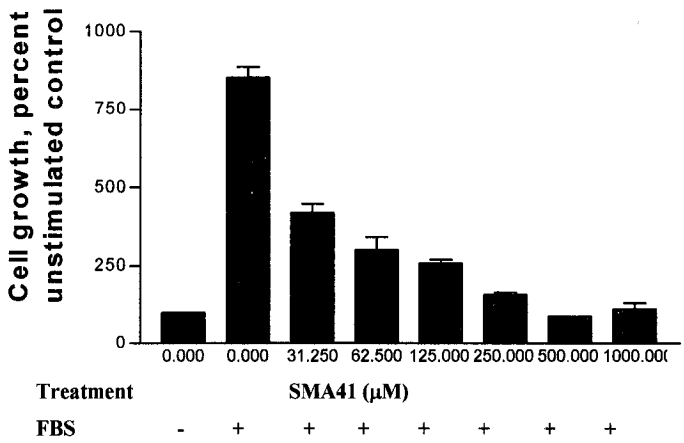


Figure 3.1. Inhibition of growth factor-stimulated cell growth by SMA41. NIH 3T3/ HER-14 cells were serum-deprived for 24 h prior to the addition of (a) EGF (12 ng/mL), (b) PDGF (50 ng/mL), or (c) serum (10%) + SMA41 at the indicated doses. Each point represents at least two experiments run in triplicate.

day continuous exposure in a panel of 10 established cell lines expressing varied levels of EGFR (Table 3.2; Figure 3.3), demonstrated little correlation between IC_{50} values and EGFR levels. Also, single-cell microelectrophoresis (comet) assays for DNA damage in all these cell lines revealed no linear correlation between levels of DNA lesions induced by TEM and EGFR status of the cells (data not shown). Similarly, no linear correlation was apparent between the IC_{50} values of the naked inhibitor SMA52 and EGFR levels.

The IC_{50} values for SMA41 varied from 1.1 to 24 μM (Table 3.2), with the lowest numbers observed for AGT-deficient cells. This differential response profile paralleled that of TEM. Linear regression analyses showed a significant correlation between IC_{50} values of SMA41 and AGT levels of the cells (Pearson coefficient $r^2=0.7$, $p<0.05$). As expected, this correlation was not significant for SMA52 ($r^2=0.2472$, $p>0.05$) (Fig.2a-b). Thus, responses to SMA41 seem to depend more on the AGT than the EGFR status of the cells. The sole SMA41-sensitive cell line with significant levels of AGT was the breast carcinoma MDA-MB-453. Since the latter line was also exquisitely sensitive to SMA52, we believe that this may be due to its expression of high levels erbB2 and erbB3 (Xinmei *et. al.*, 2000), the two closest homologues of EGFR.

It is noteworthy that despite being a monomethylating agent, the IC_{50} values of SMA41 (average IC_{50}= 16.7±3.3 μM) were more than 19-fold lower than those of TEM (average IC_{50}=318±82 μM) throughout the panel, indicating a consistently greater potency of SMA41 when compared with TEM.

Cell line	IC_{50} SMA41	IC_{50} SMA52	IC_{50} TEM	AGT (AGT/ tubulin)	EGFR (EGFR/ tubulin)
MCF- 10A	21.0	16.4	92.4	1.15	0.04
A431	21.6	8.6	604.9	0.20	2.46
PC-3	21.8	24.4	573.3	0.76	1.20
DU-145	24.3	26.2	197.8	0.81	1.64
SF-126	2.0	26.9	12.5	0	0.10
SF-188	19.8	33.8	412.6	0.36	0
MCF-7	24.1	39.6	699.6	1.29	0.51
MDA-MB-435	3.0	29.3	12.0	0.06	0.23
MDA-MB-453	1.1	8.1	451.0	0.56	1.54
MDA-MB-468	28.6	39.5	125.0	0.65	1.22

Table 3.2. AGT/EGFR levels and IC_{50} values for cell growth inhibition by SMA41, SMA52 and TEM in a panel of human tumour cell lines. Each IC_{50} value (μM) represents at least two independent experiments performed in triplicate. AGT and EGFR levels were determined by Western blotting. AGT values were normalized by subtracting band intensity ratio (AGT/tubulin) of SF-126 from those of all other cells, leaving AGT values for the latter as 0. Similarly SF188 band intensity ratio was considered the background for normalizing EGFR levels.

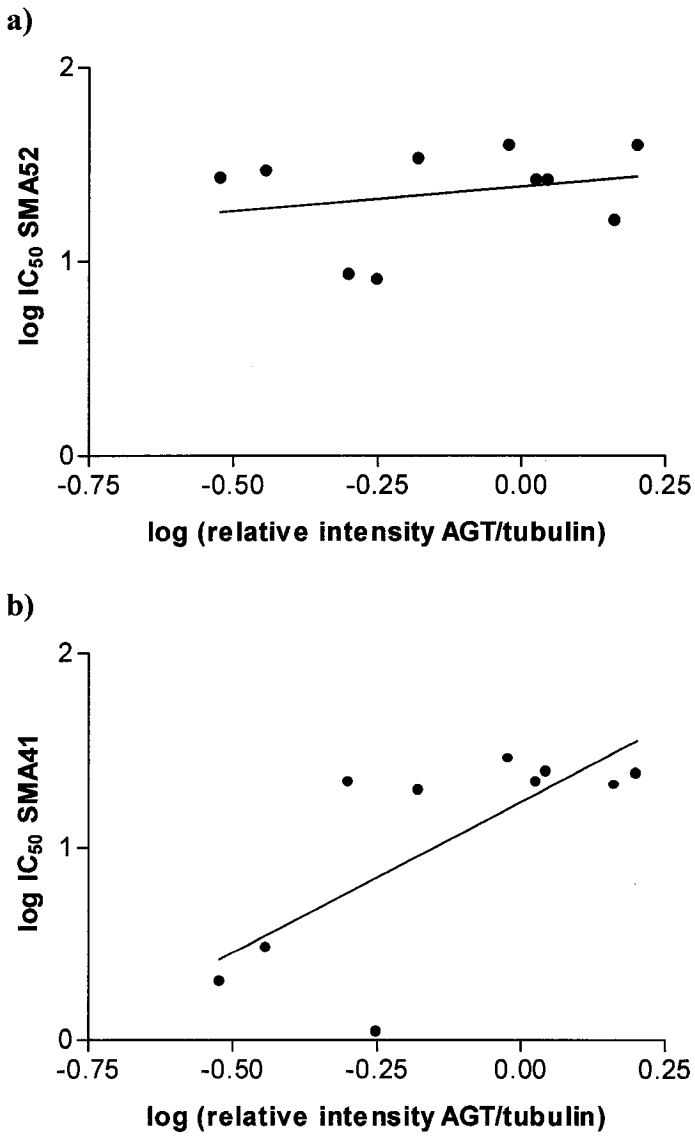


Figure 3.2. Correlation of AGT levels with IC_{50} values for (a) SMA41 and (b) SMA52). Pearson correlation coefficients were calculated as r^2=0.7241 ($p<0.05$) for SMA41, and r^2=0.2472 ($p>0.05$) for SMA52.

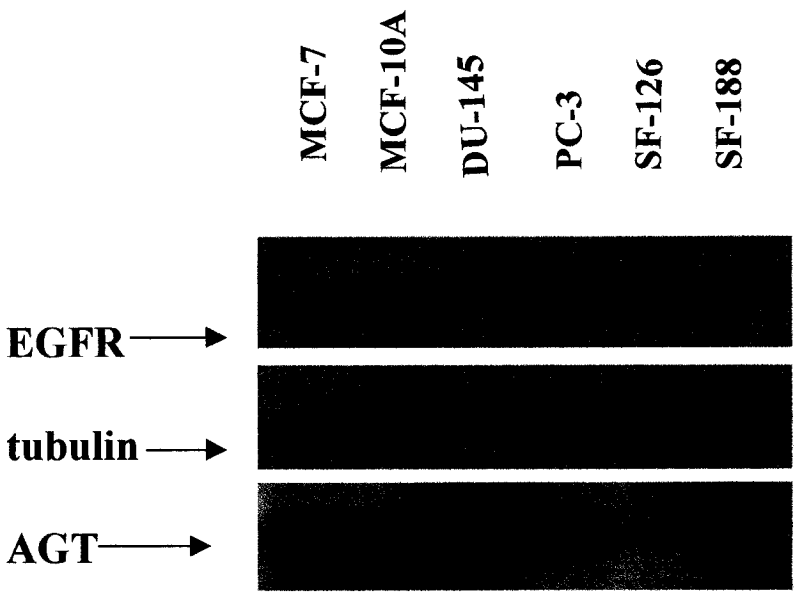


Figure 3.3. Expression of EGFR and AGT in selected cell lines from the panel. Tubulin was used as a loading control for quantitation of EGFR and AGT levels.

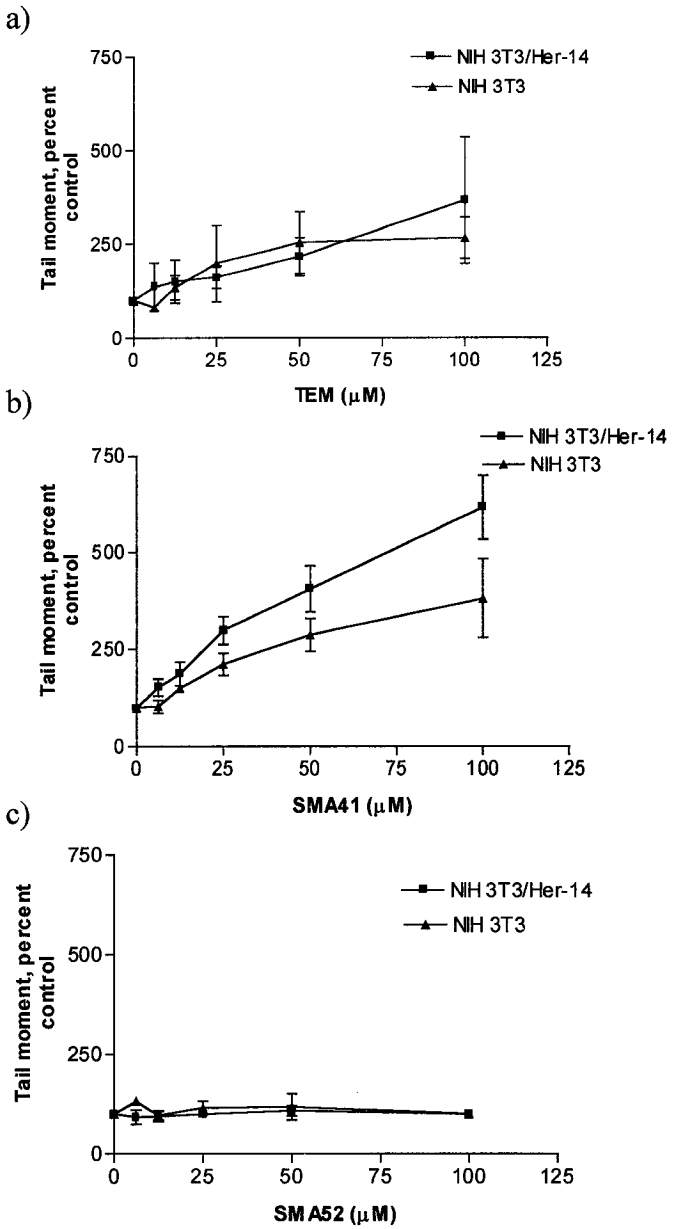
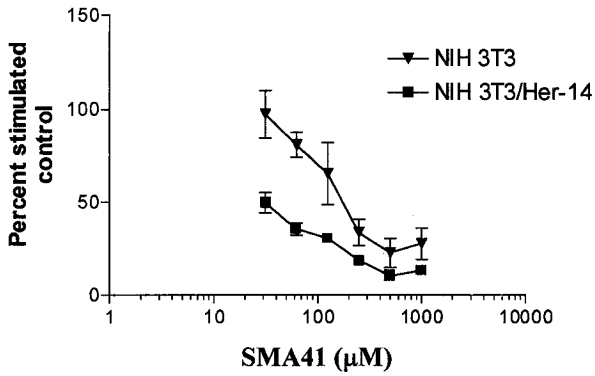
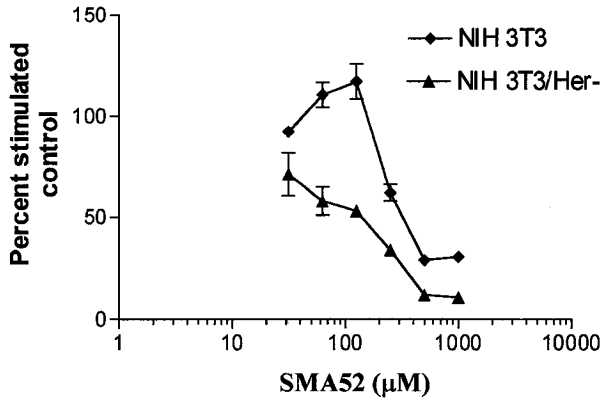


Figure 3.4. Differential DNA damage induced by (a) TEM, (b) SMA41, and (c) SMA52 in NIH 3T3 and NIH 3T3/HER14 cells as measured by the comet assay following a short 30 min exposure. Each point represents at least two independent experiments.

a)



b)



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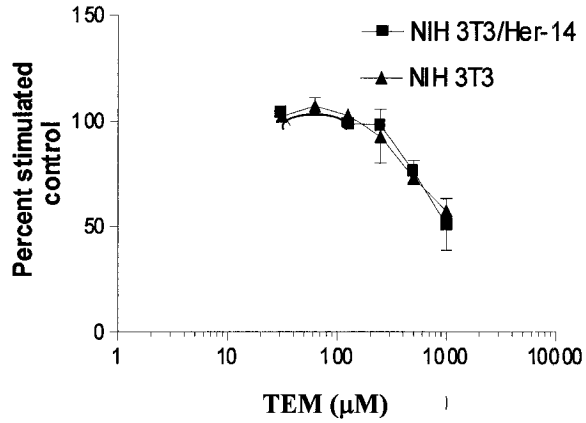

Figure 3.5. Inhibition of serum-stimulated growth of NIH 3T3 and NIH 3T3/HER14 cells by (a) SMA41, (b) SMA52, and (c) TEM. Each point represents at least two independent experiments run in triplicate.

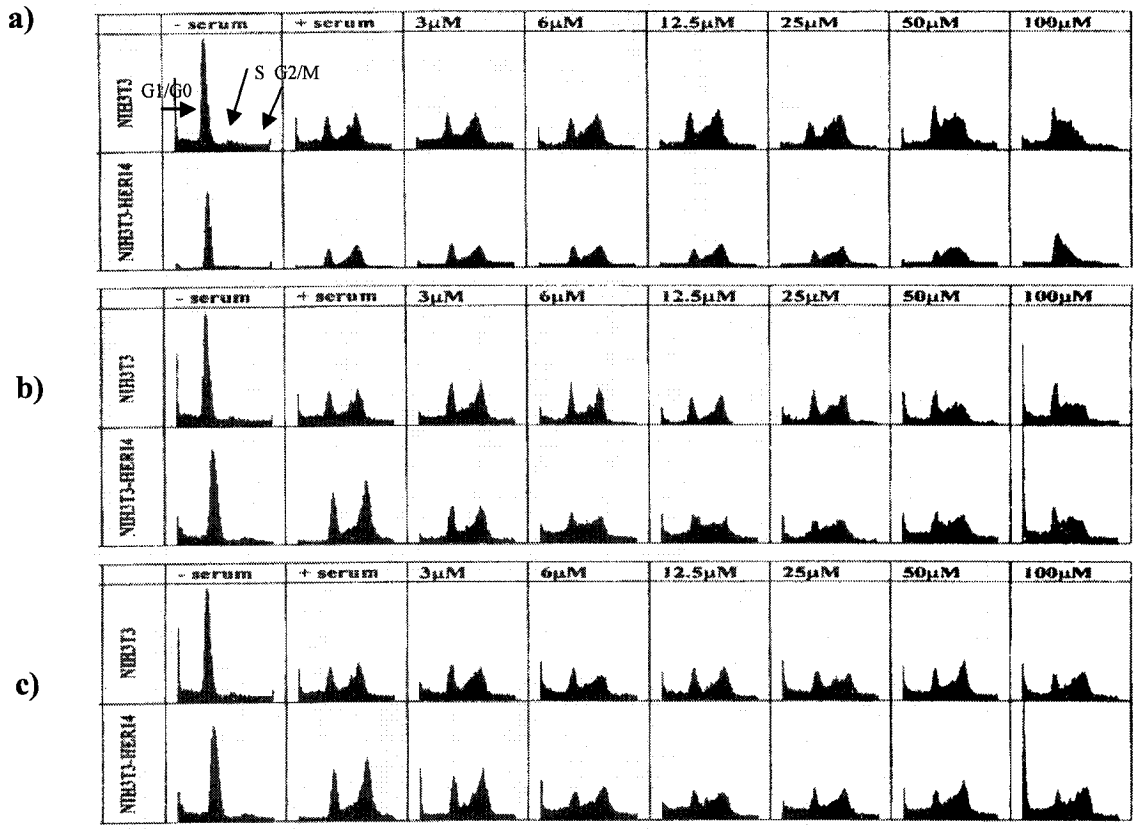

Figure 3.6. Cell cycle effects of (a) SMA41, (b) SMA52 and (c) TEM on NIH 3T3 and NIH 3T3/HER14 cells. Cells were synchronized in G1 by serum deprivation for 24 h, after which they were treated with 10% serum + drug dose range. The cell cycle phases G1/G0, S, and G2/M are labeled.

3.4.3. Differential DNA damage induced by SMA41 in the NIH 3T3 isogenic pair of cell lines

Various factors such as DNA repair status, dependence of cell growth on factors other than EGF, and unspecific binding to unidentified receptors may affect the correlation between EGFR and levels of DNA lesions or antiproliferative effects. Thus, we chose to pursue the study in an isogenic pair of cell lines [NIH 3T3 and NIH 3T3/HER14 cells (engineered to overexpress EGFR)] for which the sole difference between the two cell types was the transfected EGFR gene. Using the comet assay, we demonstrated that SMA41 induced 2-fold higher levels of DNA damage in the EGFR transfectant when compared with its parental NIH 3T3 cell line (Figure 3.4). However this did not translate into any differential effect in the SRB assay which showed a barely detectable difference in the antiproliferative activities of SMA41 in the two cell types (data not shown).

3.4.4. Growth stimulation assays with NIH 3T3/HER-14 and NIH 3T3 isogenic pair of cell lines

Having observed an absence of correlation under both poly- and isogenic conditions, we determined whether the EGFR transfection has conferred a growth advantage to these cells under conditions in which their proliferation is serum-stimulated. Further, we hypothesized that if this growth advantage is associated with EGFR-mediated signaling, abrogation of EGFR tyrosine kinase activity will selectively sensitize the EGFR-transfected cells. Thus, we designed an experiment wherein the cells were starved for 24 h and growth-stimulated with a dose range of serum. Interestingly, it was found that the EGFR transfectant grew approximately 3-fold faster than its parent non-transformed NIH

3T3 cell line, confirming that the EGFR transfection has conferred a growth advantage to these cells. With this model, we tested the effects of SMA41 and SMA52 in comparison with TEM using the serum concentration at which maximum growth-stimulation was observed (10%). The results (Figure 3.5) showed that TEM induced no differential response between the two cell types. In contrast, SMA41 selectively induced 5-fold stronger serum-stimulated growth inhibitory activity in the EGFR transfectant. Similarly the naked inhibitor SMA52 induced 3-fold stronger inhibition of serum-mediated growth in NIH 3T3/HER-14 cells.

3.4.5. EGF- and serum-induced cell cycle effects

The mechanism of this significant selectivity was further investigated by flow cytometric analyses of SMA41, SMA52 and TEM-induced cell cycle perturbation under serum-driven growth stimulation. It should be remembered that SMA41 possesses mixed signal transduction and DNA targeting properties and it is now well established that inhibition of signaling and induction of DNA damage are associated with significant cell cycle perturbations. More specifically, DNA lesions induced by alkyltriazenes of the same class as SMA41 [*e.g.* monomethyltriazenylimidazole-4-carboxamide (MMTIC) Jean-Claude *et. al.*, 1998; Erba *et. al.*, 1986) and its prodrug TEM] cause significant cell cycle arrest in S or G2M and inhibitors of EGFR arrest cell cycle in G1 (Moyer *et. al.*, 1997). Since SMA41 is a combination of these two types of mechanism, a complex cell cycle distribution was expected. Indeed, when the cells were starved by serum deprivation, a significant synchronization in G1 was observed (Figure 3.6). The addition of serum to control cells induced significant cell transition to S and G2M 24 h later. Co-addition of

SMA41 did not significantly block cell exit from G1, but rather induced a dose-dependent arrest in S phase with a noticeable accumulation of cells in G1/S at the highest 100 μM dose. At the latter concentration, a barely detectable number of cells have reached G2M 24 h after serum stimulation. More importantly, cell cycle perturbations were more pronounced in the NIH 3T3/HER-14 than in its non-transformed counterpart NIH 3T3. This was clearly observable at the highest dose wherein cell entries to G2M were delayed at earlier phases in NIH 3T3/HER14 cells [arrest in G1/S (early)] than in their parental NIH 3T3 cells (arrest in mid-S and late S) (Figure 3.6). Similarly, SMA52 delayed cell entry to G2M, however, the effects were weaker than those of SMA41. In contrast, no significant cell cycle arrest was induced by TEM. Even at the highest dose, the G2M peaks were similar to those of control cells. These results represent further evidence of the markedly distinct mechanism of action of SMA41 when compared with those of SMA52 and TEM.

3.5. Discussion

Solid tumours are often characterized by the expression of DNA repair enzymes, that confer resistance to chemotherapy, and the overexpression or dysfunction of proteins directly implicated in mitogenic signaling (Bast *et. al.*, 1998; Ilekis *et. al.*, 1997; Lamharzi *et. al.*, 1998; Nikura *et. al.*, 1997). The expression of DNA repair enzymes, such as AGT, significantly decreases the chemosensitivity of these tumours to alkylating agents of the nitrosourea and triazene classes (Baer *et. al.*, 1993; Bobola *et. al.*, 1996; Fairbairn *et. al.*, 1995; Mitchel *et. al.*, 1993; Wedge *et. al.*, 1996). On the other hand, amplification and overexpression of receptors of the erb family (*e.g.* EGFR, $p185^{neu}$) have

been detected in subpopulations of many types of human tumours and are experimentally associated with aggressive tumour progression, poor patient survival and more importantly, reduced chemosensitivity (Hengstler *et. al.*, 1999; Modjtahedi and Dean, 1998). The binding of our growth factor of interest EGF to its cognate receptor EGFR is followed by a cascade of activation events that ultimately induces the transcription of genes associated with proliferation (*e.g.* early response genes such as *c-fos* or *c-jun*) (Brader *et. al.*, 1998; Kaina *et. al.*, 1997). More recently, aberration of *c-fos* expression has been associated with greater cell sensitivity to N-methyl-N-nitrosoguanidine, a methylating agent generating the same type of lesions as SMA41 (Kaina *et. al.*, 1997). This observation lends support to approaches like the Combi-Targeting concept that seeks to combine blockade of EGF-mediated signal transduction with methylating DNA lesions in order to induce enhanced chemosensitivity and perhaps chemoselectivity in refractory tumours. The current study was designed to determine the potency of the approach in a panel of 12 cell lines with varied levels of expression of EGFR and AGT, the latter being a DNA repair enzyme that confers resistance to triazenes of the same class as our C-molecule.

We have already demonstrated that SMA41 is the precursor of two major species: SMA52 that inhibits EGFR TK (IC_{50}=1 μM), and a metastable methyldiazonium whose presence, due to its short life, could only be indirectly confirmed by the high levels of alkali labile lesions that it induced in A431 carcinoma of the vulva cells (Matheson *et. al.*, 2001). Thus, we compared the antiproliferative effects of C-molecule SMA41 with those of independently synthesized SMA52 and with the clinical methyldiazonium generator

TEM. It should be remembered that SMA41 generates 81% of SMA52 following degradation in serum-containing media (Matheson *et. al.*, 2001). In the present study, the IC_{50} values of SMA52 for cell growth inhibition were consistently in the same range in almost all the cell lines as determined by a 7 day continuous exposure SRB assay. Only values for A431 and MDA-MB-453 cells were 3-fold lower than the average (Table 3.2). Since A431 growth mechanism is driven by an aggressive autocrine growth mediated by TGF-α/EGFR (Lanzi *et. al.*, 1997), prolonged exposure and consequently sustained inhibition of EGFR TK may translate into significant antiproliferative activity. As for the MDA-MB-453 cell line which, despite its significant AGT content, was the most sensitive cells to SMA41, strong dependence of growth on erbB2 or erbB3 gene products (Xinmei *et. al.*, 2000) and unspecific binding to these receptors (the closest homologues of EGFR) may account for the superior sensitivity of these cell lines. As expected, there was no dependence of SMA52 activity on the AGT status of the cells since as previously reported, it does not alkylate DNA. In contrast, throughout the panel, the IC_{50} values of the methylating agent TEM were quite high, but significantly low in AGT null cells. This is in agreement with a great number of reports that demonstrated the direct correlation between the antiproliferative activity of TEM and the AGT status of human tumour cells (Baer *et. al.*, 1993; Fairbairn *et. al.*, 1995).

Since SMA41 is a combination of the two lines of mechanisms of action described above (*i.e.* inhibition of tyrosine kinase activity, methylation of DNA), antiproliferative activities superior to those of SMA52 and TEM alone were expected. Surprisingly, the IC_{50} values for SMA41 were in the same range as those of SMA52 in almost all cells expressing AGT except the MDA-MB-453, but 9-25-fold stronger in

AGT null cells. Co-expression of EGFR and AGT did not seem to affect the levels of antiproliferative activities induced by SMA41 in the cell panel. However, despite the absence of correlation with EGFR status, the antiproliferative activities of SMA41 were superior to those of TEM in all cells tested, regardless of their AGT status. These results suggest *in toto* that, where AGT is expressed, repair of O6-methylguanine may at least partially compromise the cytotoxic effects of the DNA damaging component of the dual mechanism of action of SMA41, leaving an antiproliferative activity that is mostly the result of the 81% conversion of the C-molecule to SMA52. The contribution of the growth inhibitory property of SMA52 may also explain the overall superior antiproliferative effects of the C-molecule SMA41 when compared with those of its clinical counterpart TEM.

The absence of correlation between the EGFR status of our cell panel and IC_{50} values for cell survival may be primarily explained by the DNA repair status of the cells on which depend the cytotoxic effects of all SMA41-induced DNA lesions. However, cell growth may be dependent on factors or hormones other than EGF that are available in the serum-containing medium. Therefore, we presumed that EGFR-selectivity studies should be analyzed in isogenic clones that differ only by their EGFR content but possess identical DNA repair status. More importantly, EGFR should confer a growth advantage to its host clone. This was in line with recent study by Bishop *et al.* (2002) who showed in the NCI panel that only EGFR-expressing cells with intact EGF-mediated mitogenic signaling pathway, were significantly sensitive to anilinoquinazolines. Thus the study was further carried out in NIH 3T3 and NIH 3T3/HER14 (engineered to overexpress EGFR).

We first demonstrated the binary targeting properties of SMA41 in these cells by analyzing levels of DNA damage and inhibition of EGF-induced growth in the EGFR-expressing transfectant (Figure 3.4). Interestingly, DNA damage appeared more enhanced in the EGFR transfectant when compared with its parental line. These results lend support to the hypothesis according to which methyldiazonium ions generated from EGFR-bound SMA41 may diffuse towards the nucleus, thereby enhancing the levels of DNA damage. Since this did not translate into EGFR-selective antiproliferative activity in continuous exposure SRB assay, we surmised that the EGFR targeting component of SMA41 was more likely to be responsible for a differential response between the two cell types. As we have demonstrated, EGF plays a role in mitogenic signaling in these cells. However, the parental NIH 3T3 cells being insensitive to EGF-induced growth, FBS (10%) was considered the most appropriate stimulation medium as it growth-stimulated both cell types and in addition, it represented a good mimic of an *in vivo* medium. SMA41 showed 9-fold selective inhibition of FBS-induced growth in the EGFR transfectant when compared with the parental cell line NIH 3T3. Similarly, its derived metabolite SMA52 induced 3-fold selectivity for NIH 3T3/HER-14. The greater selectivity of SMA41 when compared with SMA52 is in line with the order of affinity of these two drugs for EGFR, suggesting the possible dominance of the EGFR inhibitory component of SMA41 in this selective activity.

The mechanism of selectivity of SMA41 under growth stimulation can be explained in light of recent observations by He *et al.* (2001), who showed using NIH 3T3 cells that serum or PDGF activation of p21-activated kinase (PAK), a family of threonine kinases involved in cell motility and directed migration, requires EGFR. Specific

inhibitors of both PDGFR and EGFR TK block the activation of PAK. Abolition of EGFR using fibroblasts from EGFR -/- mice showed complete depletion of PAK activation, suggesting a key role for EGFR in this process. Although the implication of these types of cooperative interactions between PDGF and EGFR in cell proliferation has not yet been fully elucidated, we have clearly observed that EGFR transfection has conferred a significant growth advantage to the NIH 3T3/HER-14 transfectant over its parental line under serum stimulation. Since at concentrations of SMA41 where significant serum-induced growth were inhibited by 50% (*e.g.* 32.5 μM), no inhibition of PDGF-induced proliferation was observed, its selective growth inhibition is likely to be based on blockade of EGFR TK which at this concentration was inhibited by 100%. It could be argued that rapidly growing cells are more sensitive to cytotoxic agents than slowly growing ones. However, this can be refuted by the absence of differential cytotoxic activity produced by TEM in the two cell types. More importantly, SMA52 which showed the same levels of activity as SMA41 in basal growth assays induced only 3-fold difference between the two cell types. The preferential inhibition of serum-induced growth stimulation in the EGFR transfectant is a clear demonstration of the tumour targeting role of the signal transduction inhibitory component of our C-molecule.

In order to have insight into the effects of the detected DNA lesions induced by SMA41 in these cells, we analyzed the effects of the latter on cell cycle progression. In contrast to TEM, SMA41 significantly delayed cell cycle progression of G1-synchronized cells 24 h after growth stimulation by serum with a slightly more pronounced effect in the EGFR-transfectant NIH 3T3/HER14. Since inhibition of growth stimulation would cause a protracted arrest in G1/G0, the complex cell cycle profiles

induced by our C-molecule [*e.g* arrests in S(early) and S(middle)] when compared with SMA52 or TEM, may primarily be due to the DNA damaging component or the results of unspecific inhibition of cell cycle proteins. It is not clear at this point how the induced cell cycle delays correlate with cell death or to the overall antiproliferative activity.

In previous studies we demonstrated the feasibility of a C-molecule with mixed EGFR and DNA targeting properties. In the current study we conclusively demonstrated that the EGFR targeting component plays a significant role in blocking growth stimulation not only by EGF alone, but also by serum which contains a variety of growth factors, the most abundant of which being PDGF. We have also demonstrated that the EGFR component may assist in the selective induction of DNA damage in isogenic clones. Our studies in a heterogeneous panel of cells led to the conclusion that, while combination of a DNA damaging triazene with an EGFR inhibitory moiety confers significantly greater potency to the resulting C-molecule when compared with the classical triazenes, the DNA repair status of the cells still remains a significant barrier to optimal potency. To this end, our laboratory is now investigating other polyfunctional cytotoxic moieties that may enhance the potency of the DNA damaging component of the C-molecules. Moreover, work is undergoing to transfect the AGT null and AGT-proficient cells with EGFR in order to better outline the effects of co-expression of AGT and EGFR on their sensitivity to the C-molecules.

3.6. Acknowledgements

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3.7. References

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3.8. Connecting text

As discussed in Chapter 3, the activity of SMA41 is affected, but not suppressed, by expression of AGT in a panel of human tumour cell lines and expression of EGFR did not seem to influence the antiproliferative activity in established cell lines. However, ectopic expression of EGFR in normal cells wherein signaling is intact seemed to selectively sensitize cells to SMA41 in growth stimulation assays. Having demonstrated the receptor type specificity and the conditions under which selective tumour targeting can be achieved by the combi-molecule, we wished to subsequently study the subcellular pharmacology of the approach in order to outline the distribution and localization of the various events underlying its hydrolytic scission under physiological conditions. While SMA52 was found to be fluorescent, allowing its detection by fluorescence microscopy and flow cytometry, the methyldiazonium, because of its notorious instability could not be characterized by conventional techniques. Thus we chose to ^{14}C-label the latter species in order to trace its subcellular distribution. In this chapter, we discuss the radiosynthesis of ^{14}C labeled SMA41 performed in collaboration with Dr. S. Mzengeza, a radiochemist. The synthetic method and characterization of the compound is described in Chapter 4 that is *in press* in the Journal of Labelled Compounds and Radiopharmaceuticals (2003).

CHAPTER 4

SYNTHESIS OF 1-[4-(M-TOLYL)AMINO-6-QUINAZOLINYL]-3-[^{14}C]-METHYL TRIAZENE: A RADIOLABELED PROBE FOR THE COMBI-TARGETING CONCEPT

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4.1 Abstract

The synthesis of 1-[4-(m-tolyl)amino-6-quinazolinyl]-3-[^{14}C]-methyl triazene (SMA41) is described. This triazene was designed to be hydrolyzed under physiological conditions to N^4-m-tolyl-quinazoline-4,6-diamine (SMA52), a moderate inhibitor of the epidermal growth factor receptor (EGFR) and the DNA alkylating species [^{14}C]-methyldiazonium. A radiolabeled probe was needed to test the hypothesis that *in situ* hydrolysis of SMA41 may induce alkylation of the ATP binding site of EGFR. ^{14}C-SMA41 was obtained with a radiochemical yield of 21% and a specific activity of 54.6 mCi/mmole, as determined by HPLC quantitation and scintillation counting. Radio-TLC analyses showed 98% radiochemical purity.

Key words: ^{14}C-Labeled SMA41; EGFR tyrosine kinase inhibitor; alkyltriazene; synthesis

4.2. Introduction

Recently, we reported a novel tumor targeting strategy termed the "Combi-Targeting Concept" (Matheson *et al.*, 2001; Brahimi *et al.*, 2002; Matheson *et al.*, 2003; Qiu *et al.*, 2003). This novel approach was based on the fundamental premise that molecules designed to simultaneously damage DNA and block the tyrosine kinase activity of oncoreceptors involved in cell signaling may show superior antiproliferative effects when compared with classical single-targeted antitumor drugs. In addition, we surmised that if the molecules are designed to heterolyze to a secondary inhibitor of the same target, more sustained antitumor effects may be induced. To verify these postulates, we designed SMA41 to target both genomic DNA and EGFR, a transmembrane tyrosine kinase whose overexpression is associated with aggressive tumor progression and poor prognosis (Visakorpi *et al.*, 1992; Zelada-Hedman *et al.*, 1994; Walker and Dearing, 1999). SMA41 has been shown to: (a) block EGF-induced EGFR autophosphorylation on its own, (b) degrade to SMA52, another inhibitor of EGFR, (c) significantly damage DNA, and (d) induce 8-fold greater antiproliferative activity against EGFR-expressing tumor cells than its single-targeted DNA-damaging counterpart temozolomide (Matheson *et al.*, 2001). In order to verify whether SMA41 not only damages DNA but also alkylates the EGF receptor, we recently attempted to ^{14}C-label the methyl group of the triazene moiety. As outlined in Figure 4.1, decomposition of SMA41 (**3**) would lead to the incorporation of ^{14}C into the released methyldiazonium ion. Here we report the synthesis and analysis of the first example of radiolabeled molecular probe of the "Combi-Targeting" strategy.

4.3. Results and discussion

The synthesis of both labeled and unlabeled SMA41 proceeded as outlined in Figure 4.1. Aminoquinazoline **1**, obtained as previously described (Rewcastle *et al.*, 1995), was treated in acetonitrile with $NOBF_4$ at 0°C to provide the diazonium salt **2**. Treatment of **2** with aqueous methylamine *in situ* followed by raising the pH of the resulting mixture with potassium carbonate gave the desired quinazolinyl triazene **3** (SMA41) in good yield. Previous analysis demonstrated that in a cell culture medium, SMA41 can be converted to quinazoline diamine **4** in an 80% yield with a half-life of approximately 34 min as determined by UV analysis (Matheson *et al.*, 2001).

The synthesis of the ^{14}C-labeled SMA41 proceeded in a similar fashion. To circumvent the difficulties associated with the handling of a suspension of $NOBF_4$ pellets in acetonitrile at micro-scale, the diazotization was performed at a 100 mg scale and a fraction of this solution (1.1 mL) was injected into an ampoule containing solid $^{14}CH_3NH_3^+Cl^-$. SMA41 was obtained by subsequent injection of 100 μL K_2CO_3 (1 g/mL) into the ampoule. TLC (60/40 ethylacetate/hexane) showed a spot with R_f = 0.37, identical to that observed for an unlabeled SMA41 standard. It is important to note that due to the instability of triazenes of the same class as SMA41 under acidic conditions (Cameron *et al.*, 1985; Schmiedekamp *et al.*, 1994) no acid was used in the micro-scale radiosynthesis.

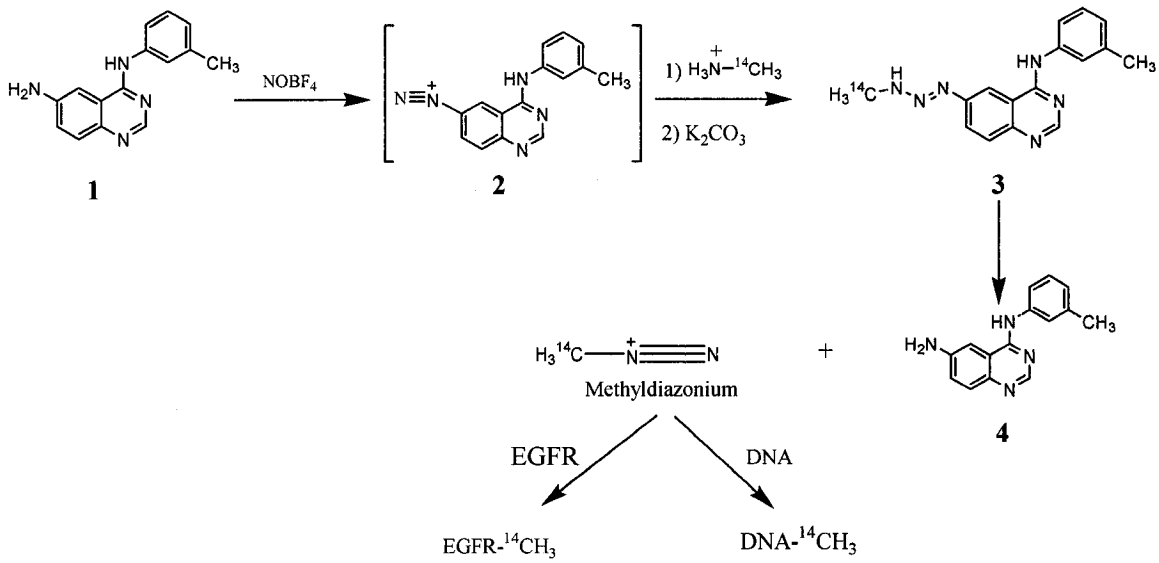

Figure 4.1. Synthesis of ^{14}C-labeled SMA41 (**3**).

As depicted in Figure 4.2, further analysis of the TLC plate with a Packard Canberra InstantImager, revealed a 98% radiochemical purity (see peak 1) with a minor impurity (2%) (see peak 2). HPLC analysis showed a peak at 9.6 min with an R_t identical to that of standard SMA41. To minimize on-column degradation, the pH of the mobile phase was adjusted to 8. A specific activity of 54.6 mCi/mmole was obtained using liquid scintillation counting of 5 μL aliquots of a ^{14}C-labeled SMA41 solution.

The level of specific radioactivity was sufficient to allow satisfactory analysis using 100 μL of a 0.1 mg/mL solution of SMA41. For example, when the EGFR overexpressing carcinoma of the vulva cell line A431 was exposed to ^{14}C-labeled SMA41 for 2 h at 37°C, the bound radioactivity was distributed through DNA, RNA and protein with counts in the 5000-18000 cpm range. Results from the macromolecular distribution studies will be reported elsewhere.

4.4. Experimental

2-Aminoquinazoline **1** was synthesized in our laboratory by known methods (Matheson *et al.*, 2001). ^{14}C-methylamine hydrochloride (56 mCi/mmol) was purchased from Amersham (Piscataway, NJ). Acetonitrile and K_2CO_3 were purchased from Fisher Scientific (Nepean, ON, Canada) and $NOBF_4$ from Sigma-Aldrich (Oakville, ON, Canada).

4.4.1. Synthesis of Unlabeled SMA41

To a solution of 6-amino-4-[(m-tolyl)amino]quinazoline (1 g, 4 mmol) in acetonitrile (50 mL) was added 5 mL of acetic acid. The mixture was kept at 0 °C for 1 h

after which a suspension of $NOBF_4$ (2 equiv.) in acetonitrile was added dropwise. A 40% aqueous methylamine solution (1 mL) was added and the resulting reddish solution neutralized by addition of saturated sodium carbonate until a two-phase mixture was formed. Ethyl acetate (200 mL) was added and the aqueous layer removed. The resulting pale brown ethyl acetate solution was dried over anhydrous potassium carbonate and evaporated under vacuum to give a brown oil which was re-dissolved in a minimum volume of the same solvent. Hexane was added by portion until a persistent pale brown precipitate was formed. The mixture was filtered and the resulting precipitate collected by filtration, and dried under vacuum to provide the monoalkyltriazene **3** (SMA41) as a pale brown powder (0.7, 60%): mp 75-80 °C (dec.); ^{1}H NMR (DMSO) δ 10.67 (br q, 1H, N*H*CH_3), 9.74 (s, 1H, NH), 8.51 (s, 2H, H2), 8.47 (s, 2H, H5), 7.9 (d, 1H, J=9, H7), 7.70 (br s d, 3H, H2', H6', H8, overlap), 7.24 (t, 1H, J= 7.5 Hz, H5'), 6.90 (d, 1H, J = 7.5 Hz, H4'), 3.07 (d, 3H, J=4, HNC*H*$_3$), 2.31 (s, 2H, ArCH_3); ^{13}C NMR 158.4, 154.1, 149.9, 148.7, 140, 138.1, 129.4, 128.879, 125.3, 124.8, 123.7, 123.4, 120.0, 116.4, 115.3, 31.3, 21.9; FABMS M+1 (I%) 293 (43), 250 (14), 234 (12).

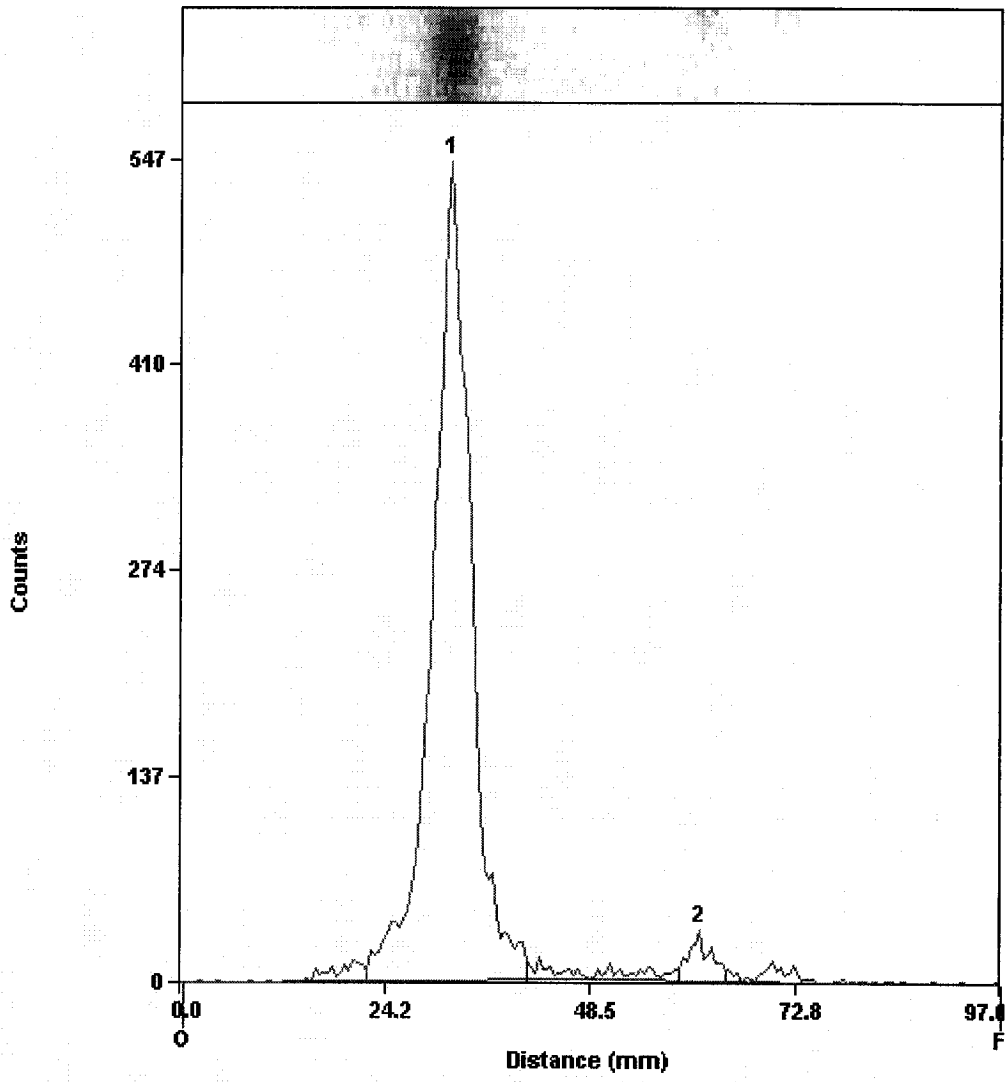

Figure 4.2. Radio-TLC of ^{14}C SMA41 eluted with a solution of 40% hexane in ethyl acetate.

4.4.2. Synthesis of ^{14}C-Labeled SMA41

Compound **1** (100 mg, 25 mmol) was dissolved in acetonitrile (33 mL) and cooled on ice to 0°C for 10 min. To this solution, a suspension of $NOBF_4$ (93.4 mg, 10.9 mmol) in acetonitrile (3 mL) was added dropwise. A fraction of the mixture (1.1 mL) was injected into previously cooled ^{14}C-methylamine hydrochloride (0.3 mg, 4.3 μmole, 250 μCi) and the mixture was made alkaline by subsequent addition of 100 μL of K_2CO_3 (1 g in 1 mL distilled water). The acetonitrile layer was removed, evaporated, and the resulting residue was redissolved in ether. This SMA41-containing solution was dried over anhydrous K_2CO_3, evaporated, and the resulting residue reconstituted in dry acetonitrile. The solution was stored at –20°C until further use. SMA41 was characterized by co-chromatography with an unlabeled sample of SMA41 on an aluminum oxide TLC plate and by HPLC. The yield was approximately 60% as estimated by HPLC quantitation. Caution! Triazenes degrade on silica gel plates and under acidic conditions.

4.4.3. Radiochemical purity

The radiochemical purity was determined by performing TLC on an aluminum oxide plate using a solution of 40% hexane in ethyl acetate. The plate was subsequently scanned in a Packard Canberra InstantImager radioanalyzer. Percent purity was calculated by determining the percentage of radioactivity in the peak corresponding to SMA41 divided by the total radioactivity of the plate using the Packard InstantImager software package.

4.4.4. Specific Radioactivity

The specific radioactivity was measured by first determining the concentration of the ^{14}C-labeled SMA41 solution. This was obtained from a calibration curve established with a series of known concentrations of unlabeled SMA41. The specific radioactivity was determined by dividing radioactivity obtained from liquid scintillation counting of 5 μL aliquots of a ^{14}C-labeled SMA41 solution by the HPLC-determined concentration.

The HPLC conditions were as follows: C4 Deltapak column (300 x 3.9 mm, 15 μm), with a mobile phase of methanol/H_2O = 7:3, pH 8, at a flow rate of 0.5 mL/min, and detection at 254 nm. The injection volume was 5 μL.

4.5. Acknowledgements

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4.7. Connecting text

Since the methyldiazonium that is released upon hydrolysis of SMA41 is an unstable and short-lived species, it cannot be characterized by HPLC methods. Its release could only be indirectly confirmed by characterizing DNA damage that it induced using single-cell microelectrophoresis (comet) assay. As described in Chapter 4, we have now a ^{14}C-labeled SMA41, the first-ever radiolabeled probe for the Combi-targeting postulates that will permit the characterization of the bound methyl group resulting from SN2 alkylation reactions with subcellular macromolecules. The successful synthesis of ^{14}C-labeled SMA41 has permitted the study of the subcellular dynamics of SMA41 in Chapter 5 and the determination of the exact DNA adducts that it induces in tumour cells (Chapter 6). In Chapter 5, the fluorescence characteristics of SMA52 and the radioactivity of ^{14}C-SMA41 have been exhaustively exploited to study the macromolecular and subcellular distribution of its released metabolites. A clear compartmentalized model is now proposed for the subcellular catabolism of SMA41.

CHAPTER 5

INTERNALIZATION OF THE BINARY EGFR/DNA-TARGETING "COMBI-TRIAZENE" SMA41

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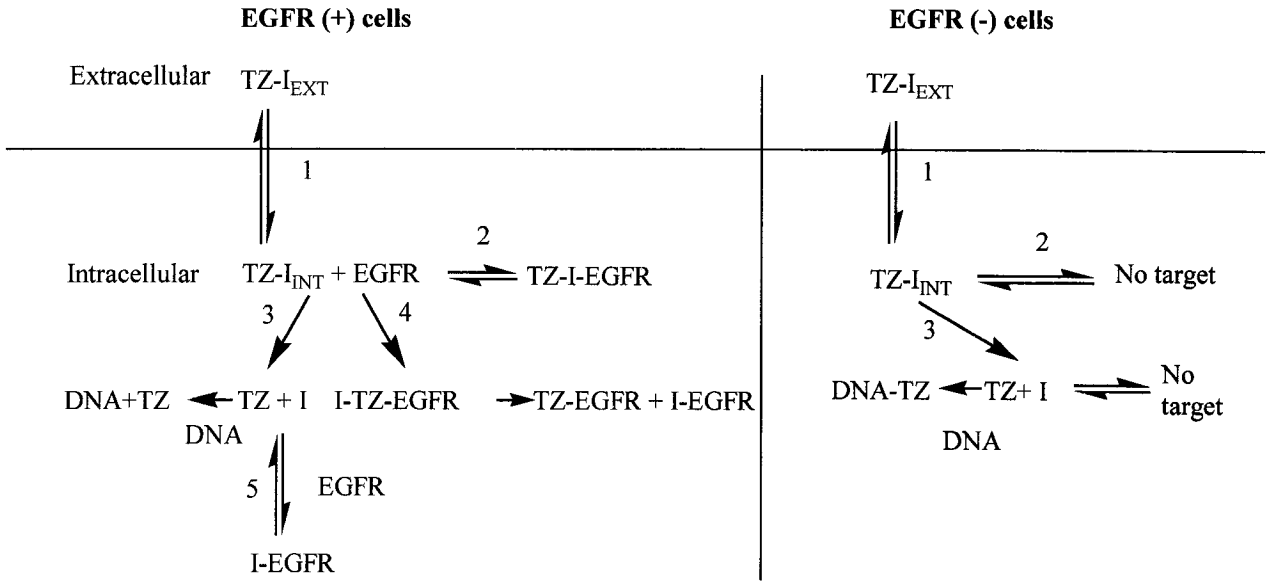
5.1. Abstract

We have designed a novel strategy that consists of designing molecules termed "combi-molecules" or TZ-I to possess multiple antitumour targets. One such molecule SMA41, the TZ-I prototype has been shown to target the epidermal growth factor receptor (EGFR) and to degrade under physiological conditions to give SMA52 (I) and methyldiazonium (TZ), the release of which could only be indirectly confirmed by the quantitation of DNA damage induced in whole cells. While the antiproliferative advantages of this strategy have now been demonstrated, the exact subcellular localization of the metabolites released from SMA41 remained elusive. Here we exploited the fluorescence properties of SMA52 to study its release from SMA41 and its subcellular distribution. Further, using ^{14}C-labelled SMA41, we determined the distribution of the methydiazonium within subcellular macromolecules. The results show that SMA41 degrades in the EGFR-overexpressing A431 carcinoma of the vulva cells to SMA52 distributed in the perinuclear region. Radioactivity associated with SMA41 ^{14}C-methyl group was distributed in DNA, RNA, and protein, the latter macromolecule being the most alkylated. Fluorescence imaging and flow cytometry in isogenic glioma cells engineered to overexpress EGFR showed approximately 2-fold higher levels of SMA52 in the SF126/EGFR cells, a cell line that expressed the highest levels of EGFR of the cell panel. Consistently higher fluorescence intensities were observed when cells were exposed to SMA41 than when directly administered SMA52, indicating that SMA41 can serve as efficient carrier of SMA52. The results suggest that SMA41 enters the cells by passive diffusion, binds to EGFR or breaks down into two species: SMA52 (I) concentrated in the perinuclear region and methyldiazonium (TZ) that can alkylate RNA, protein and nuclear DNA.

5.2. Introduction

The overexpression and dysfunction of the epidermal growth factor receptor (EGFR) is associated with aggressive tumour growth, autocrine stimulatory loops, and poor patient prognosis (Chou *et al.*, 1999; Sherwood *et al.*, 1999; Albanell *et al.*, 2001). Thus compounds have been developed that target the tyrosine kinase (TK) of EGFR (Fry *et al.*, 1994; Rewcastle *et al.*, 1996). Quinazolines are one such class of compounds that bind to the ATP binding site of EGFR TK, and have shown good tolerance and efficacy in clinical trials (Baselga *et al.*, 2000; 2001). Since these compounds block EGF-induced growth, their activity is cytostatic rather than cytotoxic, and they require long term repeated doses in order to induce tumour regression. We have already demonstrated that quinazolines lose their antiproliferative activities when cell cultures are washed free of the drugs (Matheson *et al.*, 2001). To circumvent problems associated with the reversibility of EGFR inhibitors, we recently designed a novel tumour targeting strategy that seeks to synthesize molecules termed "combi-molecules" designed to not only block EGFR TK on their own, but to release an alkylating species capable of damaging DNA thereby conferring a cytotoxic component to these inhibitors. As outlined in Scheme 5.1, a molecule termed "combi-molecule" or TZ-I (see TZ-I_{EXT}) is designed to penetrate cells by passive diffusion (path 1) and once inside the cells (see TZ-I_{INT}), the molecules can either bind to EGFR (path 2) or degrade to generate a DNA damaging agent (TZ) (paths 3 and 4) and an inhibitor of EGFR (I) (paths 3 and 5) (Matheson *et al.*, 2001; 2003). In contrast to cells that do not express EGFR, the antiproliferative activity will be solely based on the DNA damaging species (see EGFR-, path 2) and cell response will parallel that elicited by classical chemotherapeutic DNA-directed agents. As shown in Scheme

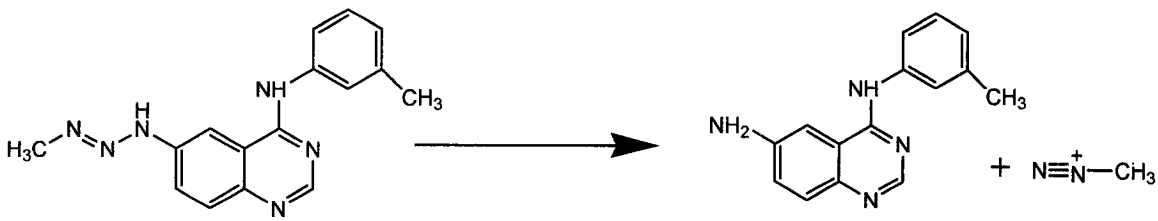
5.2, the feasibility of this type of molecules has already been demonstrated using SMA41, a quinazoline linked to an alkyltriazene (TZ-I) that has been shown to bind to EGFR on its own and release another inhibitor of EGFR (I) + a methyldiazonium (TZ) (Matheson *et al.*, 2001). The presence of TZ, because of its extremely short life, could only be indirectly confirmed by the high levels of DNA damage detected in cells exposed to SMA41.



Scheme 5.1.

Despite an extensive demonstration of the feasibility of the combi-targeting theory (Matheson *et al.*, 2001; 2003; Brahimi *et al.*, 2002), the fate of the decomposition products (*e.g.* I and TZ) in the intracellular compartment remained elusive. Fortuitously, the released TK inhibitor SMA52 (I) was found to fluoresce in the blue (448.8 nm).

Thus, its subcellular localization could be studied by UV flow cytometry and fluorescence microscopy. To trace the subcellular distribution of the methyldiazonium species, SMA41 was ^{14}C-labeled at the 3-methyl group (Matheson *et al.*, 2003 in press). The study was carried out in the EGFR-overexpressing carcinoma of the vulva cell line A431, in which previous studies were performed, and in isogenic brain tumour cells with ectopic expression of EGFR. The latter cell types were chosen on the basis of the significant potency of the lead triazenes dacarbazine and its prodrug temozolomide (TEM) in the clinic against gliomas.



TZ-I (SMA41) I (SMA52) TZ

Scheme 5.2.

5.3. Materials and methods

5.3.1. Drug treatment. The synthesis of ^{14}C-SMA41 is described elsewhere (Matheson *et al.*, 2003 in press). SMA41 and SMA52 were synthesized in our laboratories by the procedure described by Matheson *et al.*, 2001; 2003. Temozolomide was provided by Shering-Plough Inc. (Kenilworth, NJ). In all assays, drug was dissolved in DMSO and subsequently diluted in sterile RPMI-1640 media containing 10% fetal bovine serum (Life Technologies, Burlington, Canada) immediately prior to the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

5.3.2. Cell culture. The human tumour cell lines A431, SF-126, and SF-188 (human glioma) (ATCC, Manassas, VA), as well as the transduced cell lines SF-126/EGFR and SF-188/EGFR were maintained in RPMI-1640 supplemented with fetal bovine serum (10%), gentamycin (50 mg/mL), and HEPES (12.5 mM) (Wisent, St. Bruno, Canada). All cells were maintained in a monolayer culture at 37°C in a humidified environment of 5% CO_2-95% air. Cells were maintained in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/mL of trypsin and 0.2 mg/mL of EDTA (Wisent, St. Bruno, Canada) and replating before confluence. In all assays, the cells were plated at least 24 h before drug administration.

5.3.3. Emission spectra for SMA41 and SMA52. Emission-absorption spectra of SMA41 and SMA52 were taken in ethanol (70%) on a Perkin Elmer scanning spectrofluorimeter at room temperature.

5.3.4. UV flow cytometry for fluorescence in whole cells. Cells were grown in 6 well plates until confluency, and then exposed to each compound for various time points at 37°C. Following drug treatment, cells were harvested with Trypsin-EDTA, collected by centrifugation, re-suspended in PBS, centrifuged, and washed. Fluorescence intensity associated with single cells was quantitated using a Becton-Dickinson FACScan with an excitation wavelength of 250 nm.

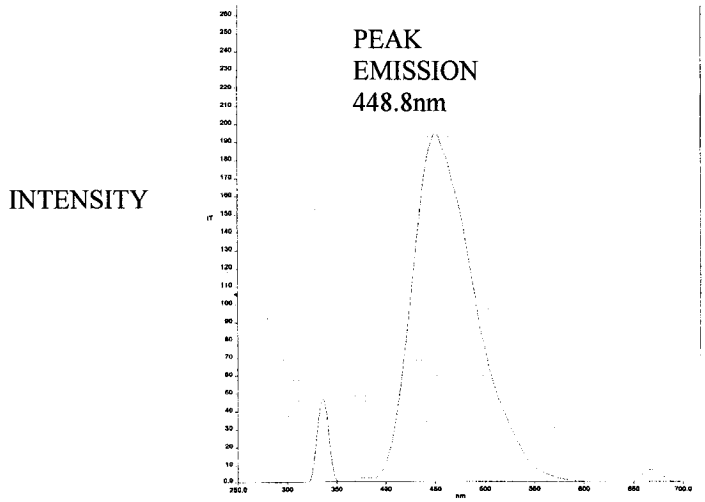
5.3.5. Transduction of cell lines. The human *EGFR* (and *Apz* or green fluorescent protein-GFP) was introduced into SF-126 and SF-188 human glioma cell lines using the

293GPG retrovirus. Stable 293GPG used in these experiments were a generous gift from Dr. Moulay Alaoui-Jamali (Jewish General Hospital, Montreal, QC). Cells (10^5) were seeded in 6 well plates for each cell line. Retrovirus (0.5 mL) and polybrene (0.5 mL; 6 μg/mL final concentration) (Sigma-Aldrich, St.Louis, MO) were administered to each well, and cells were grown at 37°C overnight (16-24 h). The media was subsequently replaced with fresh media and the cells were grown for 72h. FACS analyses were performed to detect the green fluorescence associate with GFP and indicative of positive transduction. Furthermore, western blotting was performed to confirm the presence of the *EGFR* gene in each transduced cells (*e. g* SF-126/EGFR and SF-188/EGFR).

5.3.6. UV fluorescence microscopy. Cells were plated on two well chamber slides (Nalge Nunc, Naperville IL) in complete media, and allowed to grow until confluency. Following a 2 h treatment with SMA41 or SMA52 at 37°C, media was removed and cells were washed with PBS. Subsequently, cells were fixed with formaldehyde [3.7% formaldehyde in PBS; 1 mM $MgCl_2$] for 30 min at room temperature. Thereafter, the slides were washed 3 times with PBS containing 1 mM $MgCl_2$, and cover slips were added using the SlowFade Light Antifade kit (Molecular Probes, Eugene, OR). The slides were examined with excitation at a wavelength of 340 nm.

5.3.7. Radiolabeling of isolated macromolecules by ^{14}C-SMA41. Calf liver RNA, bovine serum albumin, and calf thymus DNA (600 μg/mL) (Sigma, Oakville, ON, Canada) were exposed to 100 μM ^{14}C-SMA41 for 2 h at 37°C and pellets isolated as described by Bull and Tisdale (1987). Samples were read in a Wallac 1219 Rackbeta liquid scintillation counter with 150 μL sample plus 1 mL distilled water in 9 mL Universol scintillation cocktail (ICN, Montreal, QC, Canada).

a)



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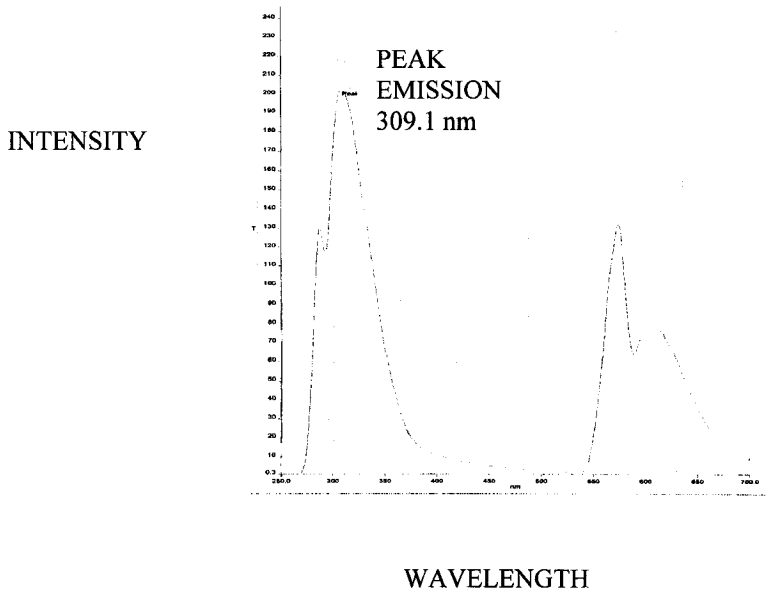


Figure 5.1. Emission spectra for SMA52 (a) and SMA41 (b). SMA52 was found to have a peak emission of 448.8 nm when excited at 280 nm. In contrast, SMA41 (b) did not exhibit this characteristic, but instead fluoresced at 309.1 nm and the 550-700 nm region.

5.3.8. Radiolabeling and isolation of macromolecules from A431 cells

A431 cells were grown in 6 well plates until confluency, and subsequently treated with 100 μCi ^{14}C-SMA41 for 2 h at 37°C. Cells were collected, lysed in perchloric acid, and the pellets were prepared as per Bull and Tisdale (1987). Radioactivity associated with DNA, RNA, and protein pellets was quantitated using liquid scintillation counting.

5.4. Results

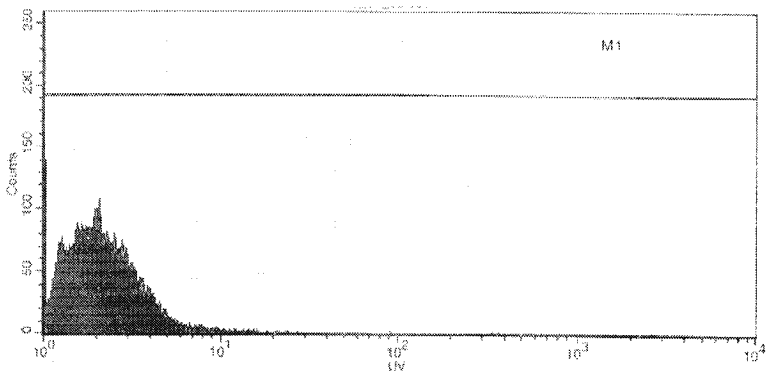
5.4.1. Emission spectra for SMA41 and SMA52

As shown in Figure 5.1, in contrast to SMA41, SMA52 fluoresces at 448.8 nm when excited at 280 nm. This indicates that light detected at 448.8 nm will have no interference with emission from SMA41. These properties have permited an unequivocal detection of SMA52 with instruments such as UV fluorescence microscope and flow cytometer with a UV excitation source.

5.4.2. Flow cytometric analysis of SMA41 and SMA52 internalization by A431 cells

The feasibility of the fluorimetric analysis of SMA52 was first tested with the high EGFR-expressing A431 cells in which binary DNA/EGFR targeting had first been demonstrated (Matheson *et al.*, 2001). Figure 5.2a shows a flow cytometry histogram of untreated A431 cells with background levels of fluorescence. When they were treated with 1mM of SMA41, a significant cohort of cells showed considerable fluorescence intensity, indicating a marked uptake of SMA52 (Figure 5.2b). A higher intensity was obtained when SMA41 was administered to cells under the same conditions. This indicates that SMA41 is converted to SMA52 (its fluorescent metabolite) within whole cells (Figure 5.2c).

a)



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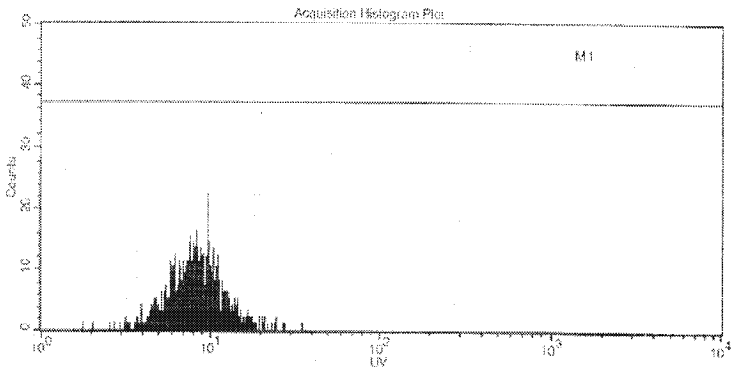

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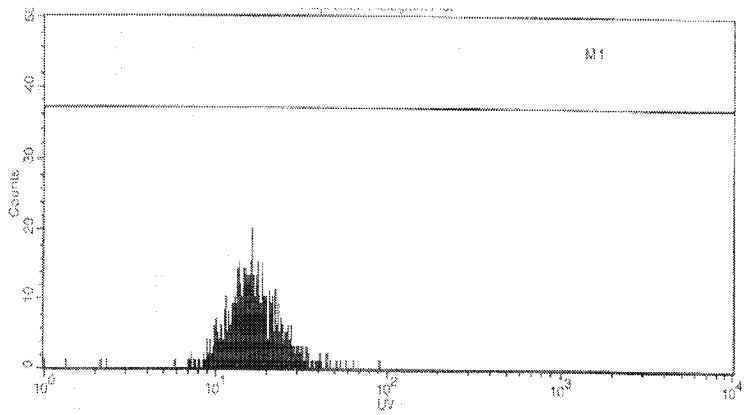


Figure 5.2. FACS histograms illustrating the internalization of SMA41 by A431 cells. When cells are (a) untreated their fluorescence is not detected upon UV excitation. If SMA52 (b) or SMA41 (c) are given for 2 h at 1 mM concentration, there is a significant increase in intensity indicating increased concentrations of SMA52 in these cells.

Having optimized the detection of SMA52, we performed the analysis on a dose range of both SMA41 and free SMA52 (Figure 5.3a). Cells treated with SMA41 showed approximately 2-fold higher fluorescence intensities than those treated with SMA52. Thus, SMA41 can serve as an excellent carrier of SMA52.

More importantly, a time course study whereby fluorescence was measured at multiple exposure times showed that SMA41 may be degraded with a half-life of 11 min, measured as the inverse of the rate of formation of SMA52 (Figure 5.3b).

5.4.3. Transduction of SF-126 and SF-188 with *EGFR*

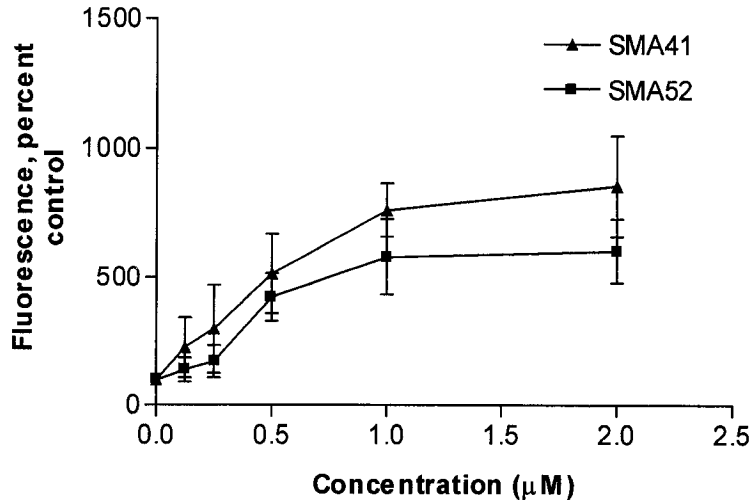
Using flow cytometry, it was confirmed that SF-126/EGFR and SF-188/EGFR indeed express *EGFR*. Since the vector used to transduce this gene also contained the gene for green fluorescent protein (GFP), FL1 analysis could confirm its presence (data not shown). Furthermore, western blot analysis for EGFR supported this data, and showed an approximately 5-fold greater expression of EGFR by SF-126/EGFR over that of SF-188/EGFR (Figure 5.4).

5.4.5. UV Flow cytometric analysis of SMA41 and SMA52 in isogenic cell lines

Single cell analysis by flow cytometry demonstrated approximately 2-fold higher levels of SMA52 in the transductant (SF-126/EGFR) cell line when compared with its non-transducted counterpart (SF-126) (Figure 5.5a). No significant difference was observed for SF-188 and SF-188/EGFR, perhaps due to low levels of EGFR expression (Figure 5.5b).

5.3.6. UV fluorescence microscopy for the internalization of SMA41 and SMA52 by isogenic cell lines

Fluorescence microscopy imaging demonstrated an increased number of cells with high



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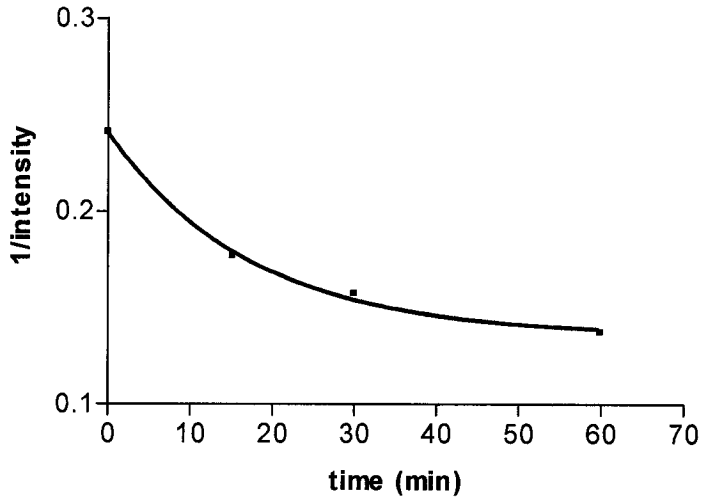


Figure 5.3. Internalization of SMA41 and SMA52 by A431 cells and the half-life of SMA41 within the cytosol. In (a), a dose range of SMA41 or SMA52 was administered to A431 for 2 h, and fluorescence was detected using flow cytometry. For (b), 1 mM of SMA41 was given for 0, 15, 30, or 60 min, and fluorescence determined using flow cytometry at each time point. Each point represents an average of two experiments.

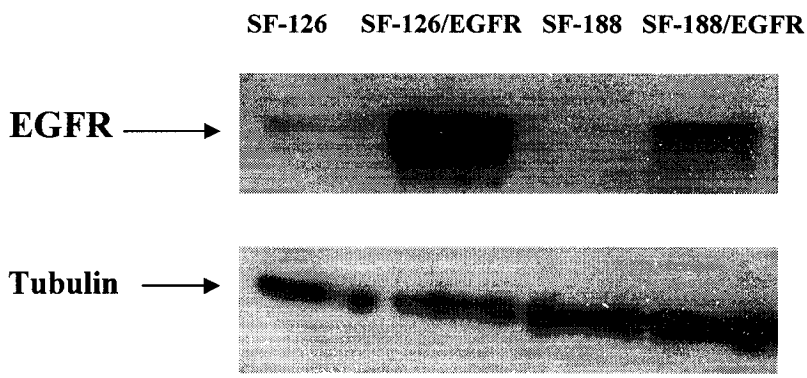


Figure 5.4. Western blotting for the detection of EGFR. Protein lysates for SF-126, SF-126/EGFR, SF-188, and SF-188/EGFR were prepared, and Western blotting was performed using anti-EGFR antibodies. Tubulin was used as a loading control.

fluorescence intensities in the SF-126 and SF-188 transductants exposed to SMA41 Figure 5.6a and b). This difference was more pronounced in the SF-126 transductants where higher expression levels of EGFR were detected. However, no significant difference was seen when cells were given SMA52 alone (Figure 5.6c and d; Figure 5.7c and d).

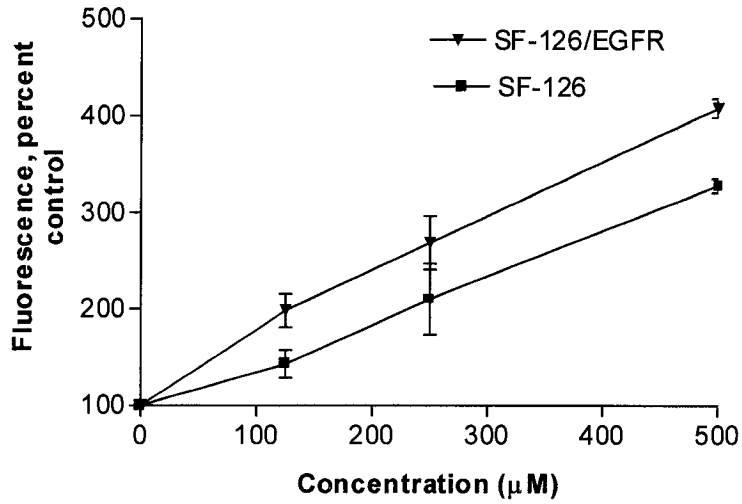
5.4.7. Distribution of radioactivity in isolated macromolecules and whole A431 cells exposed to ^{14}C-SMA41

Following 2 h exposure to radiolabeled SMA41, commercially available DNA, RNA, and protein were collected and counted using liquid scintillation. Likewise, A431 cells were exposed to ^{14}C-SMA41 for 2 h, lysed, and their DNA, RNA, and protein were isolated for liquid scintillation counting. As shown in Figure 5.8a the radioactivity is distributed in all three macromolecules, with the highest levels in the protein. A similar pattern was obtained for macromolecules isolated from whole cells (Figure 5.8b).

5.5. Discussion

The "combi-triazene" SMA41 is a chimeric and unimolecular combination of two pharmacophores associated with two major mechanisms of action: a 4-anilinoquinazoline that defines its ability to inhibit EGFR-mediated cell signaling, and a 3-methyl-1,2,3-triazene that masks its DNA alkylating methyliazonium metabolite. The hydrolytic cleavage of the alkyltriazene has been shown to lead to the formation of an aromatic amine SMA52, a reversible inhibitor of EGFR and the methyldiazonium species directed at DNA. While the binary biological effects of SMA41 (EGFR inhibition and DNA damage) have now been demonstrated, the subcellular distribution of its bioactive metabolites remained elusive.

a)



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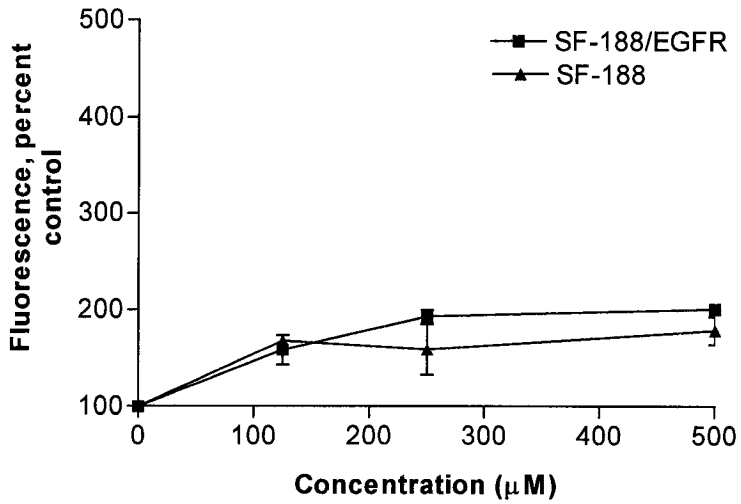
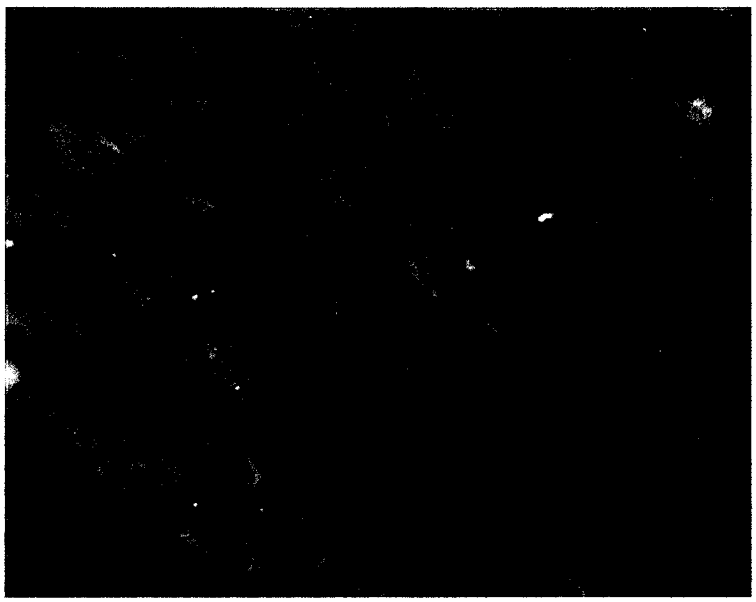


Figure 5.5. Internalization of SMA41 and SMA52 by glioma cells. Fluorescence was measured using UV flow cytometry in intact cells. Either (a) SF-126 and SF-126/EGFR cells, or (b) SF-188 and SF-188/EGFR were treated with SMA41 for 2 h. Each point represents average and standard deviation from at least two experiments

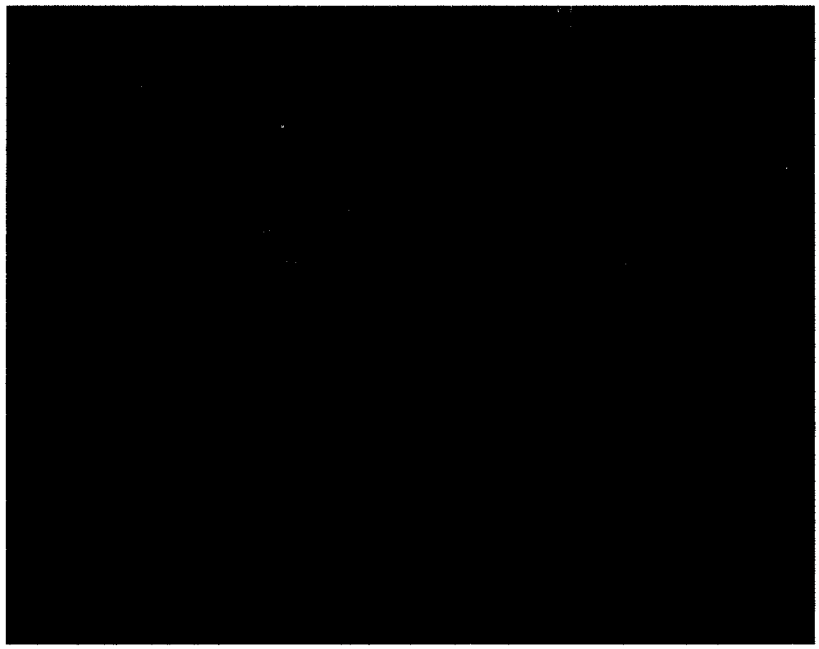
a)



b)

Figure 5.6. Fluorescence microscopy images for the internalization of SMA41 by SF-126 and SF-126/EGFR cells. For each experiment, 500 μM of SMA41 was given for 2h to either (a) SF-126 or (b) SF-126/EGFR cells (1000x magnification).

c)



d)

Figure 5.6. Fluorescence microscopy images for the internalization of SMA52 by SF-126 and SF-126/EGFR cells. For each experiment, 500 μM of SMA52 was given for 2h to either (c) SF-126 or (d) SF-126/EGFR cells (1000x magnification).

a)

b)

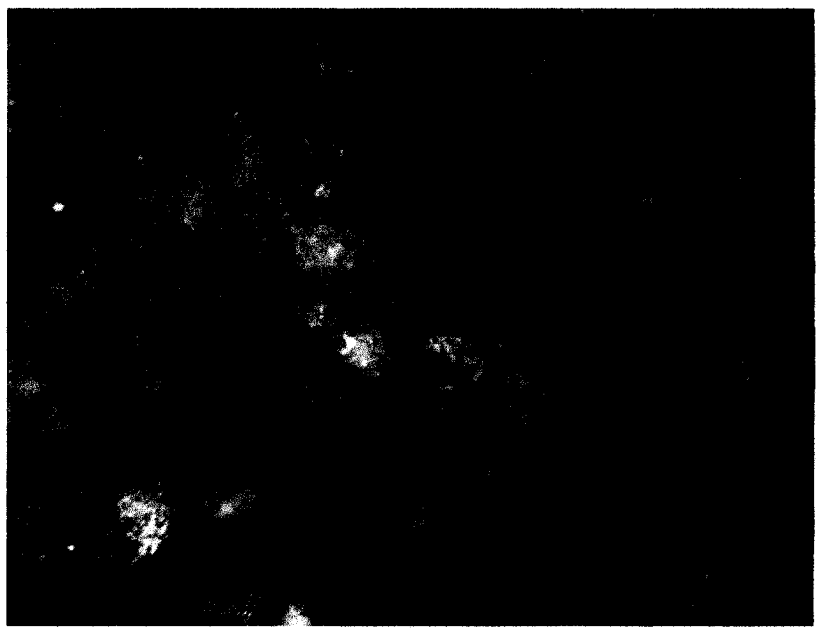


Figure 5.7. Fluorescence microscopy images for the internalization of SMA41 by SF-188 and SF-188/EGFR cells. For each experiment, 500 μM of SMA41 was given for 2h to either (a) SF-188 or (b) SF-188/EGFR cells (1000x magnification).

c)

d)

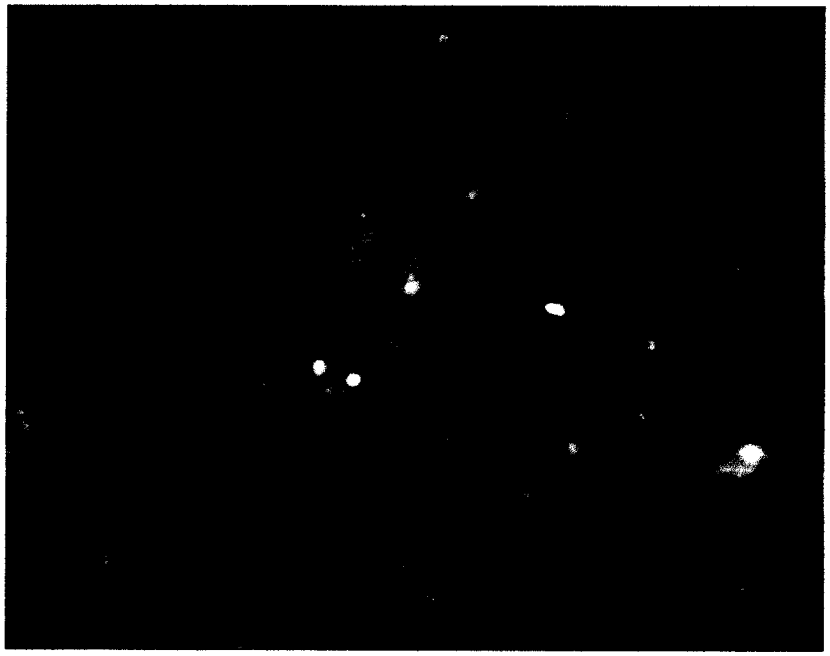


Figure 5.7. Fluorescence microscopy images for the internalization of SMA52 by SF-188 and SF-188/EGFR cells. For each experiment, 500 μM of SMA52 was given for 2h to either (c) SF-188 or (d) SF-188/EGFR cells (1000x magnification).

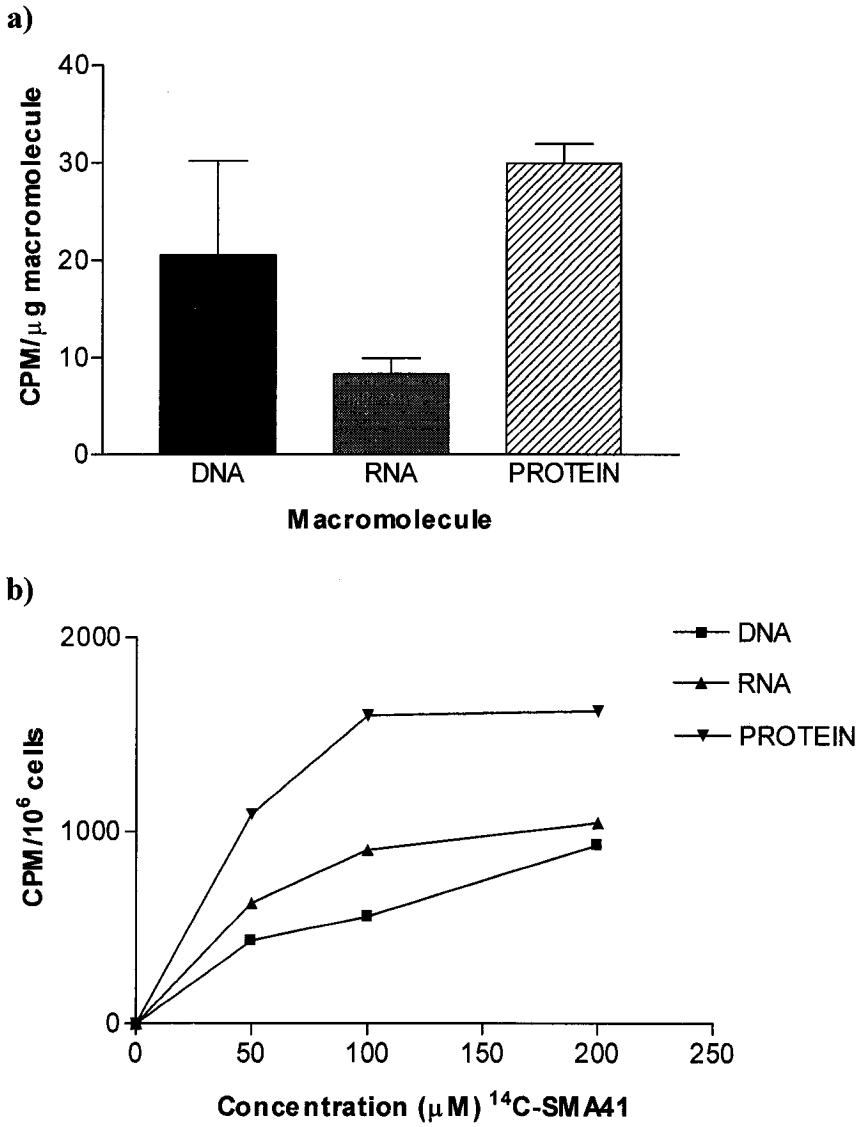


Figure 5.8. Distribution of radioactivity in macromolecules exposed to ^{14}C-SMA41. Either (a) isolated macromolecules, or (b) whole cells were exposed to ^{14}C-SMA41 for 2 h, after which DNA, RNA, and protein were isolated and radioactivity was counted.

The half-life of SMA41 is in the 34 min range, a level of stability that permits its decomposition in both the extra- and intracellular compartments. In theory, SMA52 formed in the extracellular compartment may diffuse into the cells until a $[SMA52]_{INT}=[SMA52]_{EXT}$. However intracellular decomposition of SMA41 to SMA52 may temporally increase $[SMA52]_{INT}$ and binding to specific proteins may also contribute to an increase in intracellular retention of the inhibitor. Indeed, both flow cytometry and fluorescence microscopy demonstrated elevated levels (approximately 2-fold) of SMA52 when released from SMA41. The greater lipophilicity of SMA41 when compared with SMA52 may also accelerate its diffusion into the cells and its ability to react or to be trapped in biological nucleophiles more specifically to be captured by receptors (*e.g.* EGFR) may decrease its cellular efflux thereby increasing levels of the fluorescent metabolite. It was interesting to observe that the half-life of SMA41 (measured as the inverse of the rate of formation of SMA52) in whole cells was approximately 3-fold shorter than the one measured under the same conditions in the extracellular compartment, indicating that SMA52 is rapidly released inside the cells. The fluorescence microscopic analyses demonstrated that SMA52 is mostly trapped in the perinuclear region most probably in ER/Golgi apparatus where nascent EGFR is being released. This is a striking observation as it shows a discrete organelle distribution of the released inhibitor.

The methyldiazonium being an extremely unstable and charged species is less likely to diffuse to the cells when released in the extracellular compartment. Thus the detected radioactivity in intracellular macromolecules must have been emanated from internalized SMA41. As already reported for ^{14}C-labeled temozolomide (Tisdale, 1987), the lead compound of the triazene class, the labeled methyl group was distributed through all three macromolecules with a larger distribution in proteins when compared with nucleic acids. It is now accepted that despite alkylation of all three molecules, the true cytotoxic lesions are those inflicted to DNA not those incurred by RNA or proteins.

Thus, studies in the high EGFR expressing A431 cells have confirmed the subcellular localization of the binary component of SMA41.

The facile observation of subcellular localization of SMA52, has stimulated our interest in determining whether it was influenced by the EGFR levels in the cells. Thus, two brain tumour cell lines, a tumour type for which triazenes are indicated in the clinic, were engineered to overexpress EGFR and subcellular distribution of SMA52 given directly or released from SMA41 was analyzed. Both flow cytometry and fluorescence microscopy showed a slightly more abundant distribution of SMA52 in the SF-126 transductants and this selectivity was more apparent when SMA52 was released from SMA41. While SMA52 can bind to other intracellular proteins, its preferential binding to cytosolic EGFR or related proteins may account for its increased levels in the transductants. The fact that this selectivity was more apparent in isogenic cells exposed to SMA41 may not only be explained by a more rapid cell penetration of the latter, but also by its affinity for EGFR on its own. It should be remembered that SMA41 is a 5-fold more potent inhibitor than SMA52.

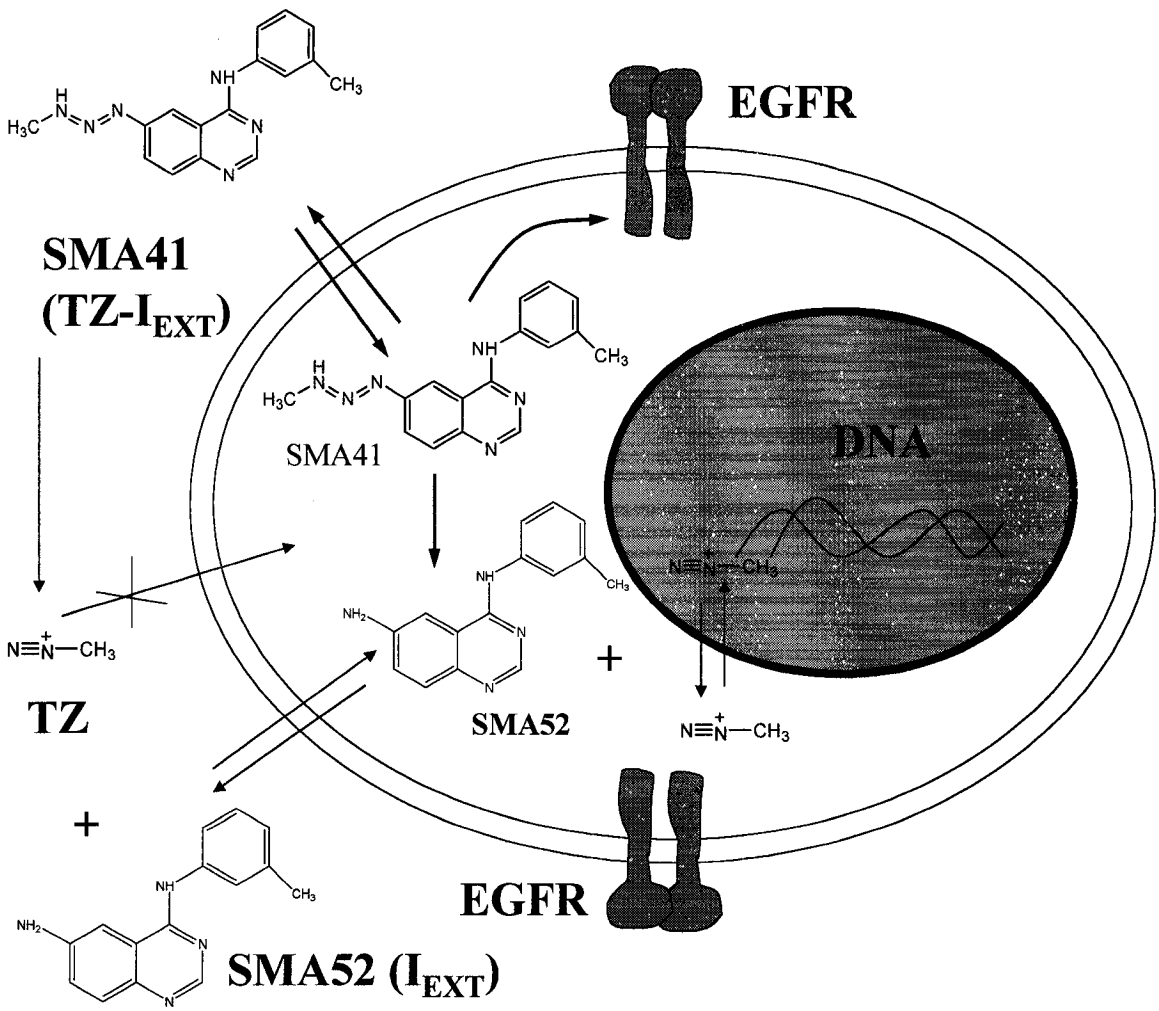
The results *in toto* have allowed us to verify our primary postulates outlined in Scheme 5.1 and to propose a model for the transport and distribution of the combi-molecule or the TZ-I. As depicted in Scheme 5.3, while the absence of fluorescence from SMA41 did not permit the tracing of its localization, we can at least propose a nuclear localization for the methyldiazonium that has been proven to bind to DNA. The model suggests: (a) an extracellular decomposition of SMA41 with permissible penetration only for SMA52 and not for the methyl diazonium (TZ), (b) a cytosolic distribution of SMA41 and SMA52 (known to bind to cytosolic domain of EGFR) as shown by ocular inspection of fluorescence microscopy imaging and (c) a nuclear localization of the methyldiazonium ion.

This study conclusively demonstrated that SMA41 is an effective carrier of SMA52, a metabolite that it can selectively deliver to high EGFR overexpressing cells.

More importantly, the results discussed herein strongly support the primary postulates of the combi-targeting theory according to which a molecule termed combi-molecule or TZ-I with affinity for EGFR, allowed to degrade to another inhibitor (I) of the same target and a DNA damaging species (TZ) that damages genomic DNA is achievable. Further studies are ongoing in our laboratory to engineer a larger panel of cells with ectopic expression of EGFR, HER2 and PDGFR and insulin in order to better assess the receptor dependence of SMA52 distribution and extent of DNA damage in epithelial cancer cells.

5.6. Acknowledgements

We thank the Cancer Research Society Inc and the Canadian Institutes for Health Research for financial support. SLM is grateful to the McGill Faculty of Medicine for a graduate scholarship. We also thank Mme. Anne Marcil at the Biotechnology Research Institute of the National Research Council for her assistance with fluorescence microscopy and Dr. Moulay Alaoui-Jamali at the Jewish General Hospital, Montreal, Canada for his assistance with the transduction.



Scheme 5.3.

5.7. References

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5.8. Connecting text

Having now demonstrated: (a) the binary targeting of the TZ-I SMA41, (b) the EGFR and DNA repair dependence of its antiproliferative activity, and (c) its subcellular dynamics, three major questions remained unanswered: the biological effects of inhibition of EGF-induced EGFR autophosphorylation, the cellular response to DNA damage and the feasibility of the combi-targeting concept *in vivo*. This final chapter uses the radiolabeled probe synthesized in Chapter 4 to damage DNA, isolate and characterize the DNA adducts by HPLC and to test the hypothesis that SMA41 may alkylate EGFR. O6-alkylguanine and N7-guanine were identified and their repair monitored. More importantly, the irreversible nature of the mechanism of EGFR by SMAA41 was demonstrated.

While the demonstration of the mechanism of action and efficacy *in vitro* of the "combi-targeting" principles were considered an important step, the clinical relevance of this novel tumour targeting strategy can only be demonstrated through human tumour xenograft models *in vivo*. Thus this ultimate chapter describes the *in vivo* efficacy of the combi-molecule SMA41 in comparison with its daughter metabolite SMA52 and the clinical DNA damaging agent TEM.

CHAPTER 6

THE COMBI-TARGETING CONCEPT: DISSECTION OF THE BINARY MECHANISM OF ACTION OF THE COMBI-TRIAZENE SMA41 *IN VITRO* AND ANTIPROLIFERATIVE EFFECTS *IN VIVO*

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6.1. Abstract

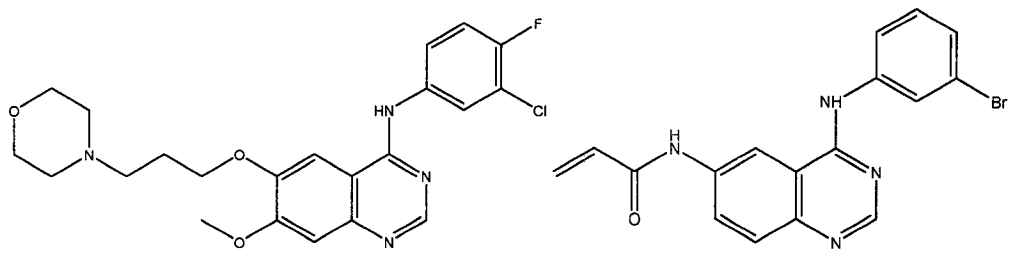
We have previously demonstrated within the framework of a novel tumour targeting strategy termed "combi-targeting" that SMA41 a unimolecular combination of an epidermal growth factor receptor (EGFR) tyrosine kinase (TK) inhibitor of the quinazoline class and a monomethyltriazene, induced sustained antitumour activity in EGFR-overexpressing A431 carcinoma of the vulva cells. SMA41 degraded to give an intact TK inhibitor (SMA52) capable of inhibiting EGF-induced EGFR autophosphorylation and a methyldiazonium species targeted to DNA. While the binary targeting potential of SMA41 has now been elucidated, the biological effects of each event (EGFR inhibition and DNA damage) and their translation into *in vivo* growth inhibition remained to be demonstrated. Using ^{14}C-SMA41 and western blotting assays, we demonstrated herein that SMA41 covalently damaged EGFR and irreversibly blocked autophosphorylation in A431 cells. Inhibition of EGF-induced autophosphorylation translated into blockade of MAP kinase activation and *c-fos* expression. DNA damage induced significant cell cycle arrest in S, G2M and the N7- or O6- alkylguanine adducts detected by radio-HPLC were found to be repaired. While O6-methylguanine lesions were rapidly repaired, N7-methylguanine adducts were still detectable even at 24 h post-treatment. These results suggest that the binary targeting properties of SMA41 are associated with a binary cascade of events in the cells that appear to culminate into significant growth retardation. Further, the growth inhibitory advantages of the "combi-targeting" approach were proven *in vivo*. At 100-200 mg/Kg, SMA41 showed approximately 2-fold superior antiproliferative activity when compared with SMA52 in A431 cells implanted in immuno-compromised SCID mice. However, the DNA damaging agent temozolomide showed superior *in vivo* efficacy, perhaps due to its greater bioavalability accociated with its significant hydrosolubility.

6.2. Introduction

The overexpression and dysregulation of tyrosine kinase (TK) is commonly observed in a large number of cancers. For example, the epidermal growth factor receptor (EGFR) is often overexpressed in breast, ovarian, and prostate tumors, and this can result in the formation of autocrine loops associated with poor patient prognosis and aggressive tumor growth (Moscatello *et al.*, 1995; Wosikowski *et al.*, 1997; Sherwood *et al.*, 1999; Suo *et al.*, 2002). Drugs have been designed to target EGFR and block the signaling cascade that is mediated by this receptor. Of these compounds, two belonging to the quinazoline class are currently in phase III clinical trials (Baselga *et al.*, 2002; Hirata *et al.*, 2002; Wakeling *et al.*, 2002; Allen *et al.*, 2002). Iressa™ **1**, a water soluble quinazoline identified as a highly effective competitive inhibitor of EGFR TK has recently been approved for the therapy of lung cancers in Japan.

The inhibitors of EGFR TK act by competitively binding to its ATP site thereby blocking subsequent activation of mitogen activated protein (MAP) kinase and the transcription of genes such as *c-fos* that are associated with cell proliferation (Mitsui *et al.*, 2001; Nelson *et al.*, 2001; Albanell *et al.*, 2001; Magne *et al.*, 2002). Due to the marked reversibility of the action of Iressa™ and related quinazolines, prolonged and repeated doses are required for the induction of sustained antitumour activity and now the combination of this drug with classical cytotoxic agents has become a logical approach to increase the potency of EGFR-directed therapy (Ciardello *et al.*, 2000; Sirotnak *et al.*, 2000). Recent studies demonstrated significant synergy between Iressa™ and the anti-metabolite 5-fluorouracil (Magne *et al.*, 2002). Similar results have been reported with

DNA damaging agents such as cisplatin, doxorubicin, and cyclophosphamide (Ciardello *et al.*, 2000; Sirotnak *et al.*, 2000).

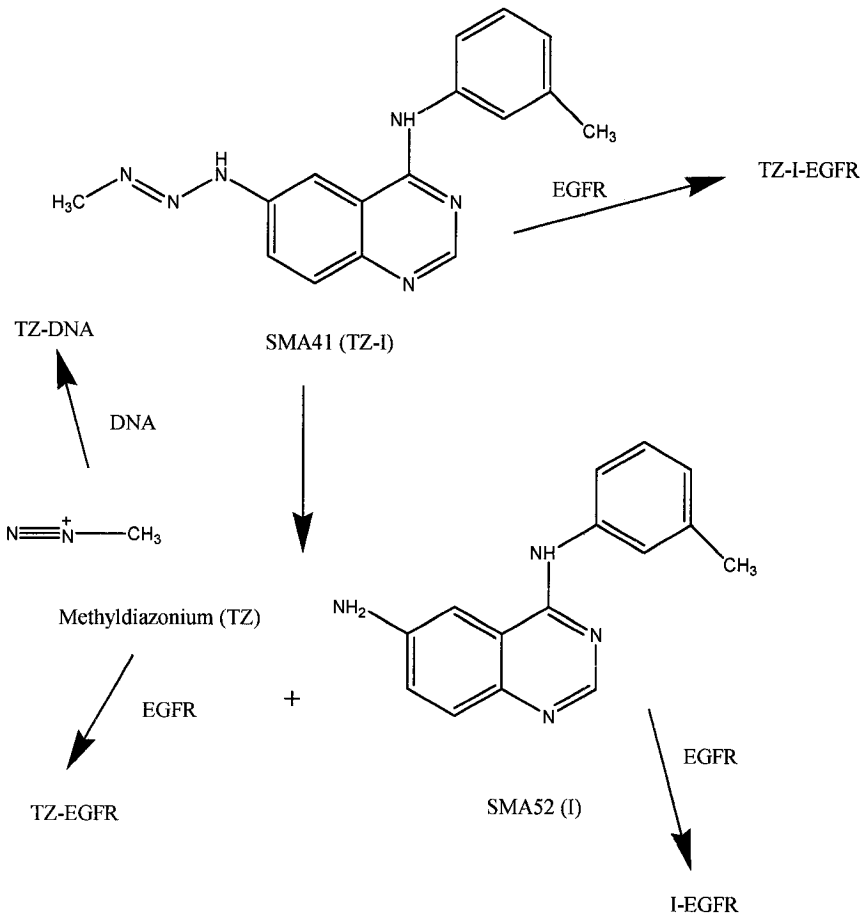


1 **2**

Another approach to overcome the reversible effects of Iressa was the development of novel compounds such as **2** containing an α,ß-unsaturated acrylamide designed to react with Cys773 of the ATP site of the receptor, thus inducing irreversible inhibition of EGFR TK (Fry *et al.*, 1998). Although these compounds were shown to be more potent than Iressa^{TM} *in vivo*, because of the heterogeneity of solid tumours, inhibition of a single target may not suffice to reduce the risk of relapse following chemotherapy.

Recently we designed a novel tumor targeting strategy termed "combi-targeting" that sought to confer a second target (*e.g.* DNA) to this type of inhibitor with the purpose of adding an alternative cytotoxic property to the cytostatic activity associated with inhibition of TK-mediated signaling (Matheson *et al.*, 2001; Brahimi *et al.*, 2002; Matheson *et al.*, 2003; Qiu *et al.*, 2003). This led to the design of small molecular systems termed "combi-molecules" capable of mimicking classical combination therapy by mixing multiple mechanisms of antitumour action. More specifically, the "combi-targeting" concept postulates that a TZ-I, designed to behave not only as an inhibitor of a tyrosine kinase but also to be hydrolyzed to another inhibitor I and a DNA-damaging

species TZ should induce more sustained antitumour activity than the reversible inhibitor alone. More importantly, if TZ-I is stable enough to penetrate the cells intact, it may not only bind to the ATP site but also react with amino acid residues of the ATP site, thereby covalently damaging and irreversibly inhibiting the receptor. Thus the TZ-I was designed to be a single molecule encompassing all currently investigated approaches to effective EGFR-based chemotherapy with small molecules: (1) inhibition of EGFR TK mediated signaling, (2) combination of EGFR TK inhibitors with cytotoxic agents, and (3) irreversible inhibition of EGFR TK activity.



Scheme 6.1.

We recently reported the synthesis of our first combi-molecule SMA41, containing a triazene tail and a quinazoline head (Matheson *et al.*, 2001; 2003). As outlined in Scheme 6.1, SMA41 (TZ-I) was found to be hydrolyzed under physiological conditions to generate the methyldiazonium ion that can damage DNA as well as an intact TK inhibitor SMA52 (I) (Matheson *et al.*, 2001; 2003). Furthermore, the molecule was shown to block EGFR autophosphorylation and to induce significant levels of DNA damage in EGFR expressing cells. Moreover, it demonstrated more than 8-fold greater antiproliferative activity than a combination of temozolomide (TEM), a clinical prodrug of the methyliazonium (TZ) plus SMA52 (I) *in vitro* (Matheson *et al.*, 2001). More recently, using a ^{14}C-labeled SMA41 and the fluorescence properties of SMA52, we demonstrated that TZ was indeed bound to DNA and I was distributed in the perinuclear region of the cells (Matheson *et al.*, 2003 in preparation). Here we analyzed the biochemical effects of each element of the dual targeting principles of the TZ-I and study the feasibility of the combi-targeting concept *in vivo*.

6.3. Materials and methods

6.3.1. Drug Treatment

SMA41, and SMA52 were synthesized in our laboratories according to known procedures (Matheson *et al.*, 2001; Matheson *et al.*, in press). Temozolomide was provided by Shering-Plough Inc. (Kenilworth, NJ). In all assays, drug was dissolved in DMSO and subsequently diluted in sterile RPMI-1640 media containing 10% fetal bovine serum (Wisent, St. Bruno, Canada) immediately prior to the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

6.3.2. Cell Culture

The human tumor cell line A431 (ATCC, Manassas, VA) was maintained in RPMI-1640 supplemented with fetal bovine serum (10%), gentamycin (50 mg/mL), and HEPES (12.5 mM) (Wisent, St. Bruno, Canada). All cells were maintained in a monolayer culture at 37°C in a humidified environment of 5% CO_2-95% air. Cells were maintained in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/mL of trypsin and 0.2 mg/mL of EDTA and replating before confluence. In all assays, the cells were plated at least 24 h before drug administration.

6.3.3. Flow cytometry for cell cycle analysis

Cells were grown in 6 well plates in a monolayer until confluency and subsequently exposed to each compound for 2 h at 37°C. Thereafter which they were allowed to recover in drug-free media for either 24 h or 48 h, harvested in Trypsin-EDTA, collected by centrifugation, and re-suspended in PBS. Samples were treated with Vindelov's solution (Vindelov, 1977) [0.01 M Tris base, 10 mM NaCl, 700U RNase, $7.5x10^{-5}$ M propidium iodide, 0.1% NP-40] for 10 min at 37°C, vortexed, and fluorescence quantitated using a Becton-Dickinson FACScan.

6.3.4. Alkaline Comet Assay for quantitation of DNA Damage

A modified alkaline comet assay technique was used to quantitate DNA damage induced by SMA41, SMA52, and TEM (Yang *et al.*, 1999; McNamee *et al.*, 2000). A431 cells were exposed to drugs for 2 h and either harvested immediately with trypsin-EDTA, or allowed to recover in drug-free media for 24 h. The cells were subsequently collected by centrifugation and re-suspended in PBS. The resulting cell suspension was diluted to approximately 10^6 cells, and mixed with agarose (1%) in PBS at 37°C in a 1:10 dilution.

The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel casting chambers, as previously described (Matheson *et al.*, 2001), then immediately placed into a lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base, 1% (w/v) N-lauryl sarcosine, 10% (v/v) DMSO and 1% (v/v) Triton X-100]. After being kept on ice for 30 min, the gels were gently rinsed with distilled water and then immersed in a second lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base], containing 1mg/ml proteinase K for 60 min at 37°C. Thereafter, the gels were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed into 1 M ammonium acetate for 30 min. They were further soaked in 100% ethanol for 2 h, dried overnight and subsequently stained with SYBR Gold (1/10000 dilution of stock supplied from Molecular Probes, OR) for 20 min. For evaluation of comets, DNA damage was assessed using the Tail Moment parameter (*ie.* the product of the distance between the barycentres of the head and the tail of the comet). A minimum of 50 cell comets were analyzed for each sample, using ALKOMET v3.1 software, and values are an average of tail moments for the entire cell population.

6.3.5. Detection of DNA adducts using HPLC

A431 cells were grown to confluency in T75 flasks, and subsequently exposed to 15.4 μCi ^{14}C-SMA41 for either 2 h or 2 h with 24 h recovery in drug-free media. DNA was isolated using the Wizard Genomic DNA Purification kit (Promega, Madison, WI) as per the manufacturer's directions. Following hydrolysis with 0.1N HCl at 80°C for 30min, samples were neutralized using 0.1N NaOH and run on a C18 HPLC column (250mm x 4.6mm) at pH3 with 10/90 methanol/acetonitrile as per Kaur and Halliwell (1996). Fractions were collected from the column every 0.5 min, and counted using liquid

scintillation in a Wallac 1219 Rackbeta counter in Universol liquid scintillation cocktail (ICN, Montreal, Canada). Standards of N7-methylguanine (Sigma, Oakville, ON, Canada), and O6-methylgaunine (Sigma, Oakville, ON, Canada) were used to calibrate the column.

6.3.6. Western blotting for irreversibility

Cells were grown in 6 well plates (1 x 10^6) in media containing 10% serum until confluency, washed three times with sterile PBS, and grown in phenol red-free RMPI without serum for 24 h. SMA41 or SMA52 was given for 90 min, washed with PBS and grown in serum-free media for 2 h, washed again and grown in serum-free media for another 4 h. Cells were subsequently stimulated with 100 ng/mL EGF for 10 min and protein lysates were prepared as previously described (Matheson *et al.*, 2001). In parallel, another set of samples was prepared without the 6 h growth in serum-free media prior to stimulation. Samples were subjected to 10% SDS-PAGE, transferred to a PVDF membrane, and probed with PY20 antiphosphotyrosine antibodies (Neomarkers, Fremont, CA) and anti-α-tubulin (Neomarkers, Fremont, CA). Proteins were detected using goat-anti-mouse-HRP conjugated antibodies and Enhanced Chemiluminescence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was subsequently stripped of antibody, and reprobed using ant-EGFR antibodies (Neomarkers, Fremont, CA) and detected using ECL. Bands were visualized using Syngene software imaging system (Cambridge, UK).

6.3.7. Immunoprecipitation of EGFR

A431 cells were grown in T150 flasks until confluency. ^{14}C-SMA41 (100 μCi) (Matheson *et al.*, in press) was given for 2 h at 37°C. Cells were collected using a cell scraper, and protein lysates were prepared as previously described (Matheson *et al.*, 2001). Anti-EGFR antibodies (Neomarkers, Fremont, CA) and protein agarose A (Invitrogen, Burlington, ON, Canada) were added to the lysates according the manufacturer's instructions. Samples were centrifuged, boiled in loading buffer, and run on 12% SDS-PAGE. Proteins were transferred to a PVDF membrane, incubated with anti-EGFR antibodies (1:100 dilution) in 3% PBS-MILK overnight at 4°C, washed, and incubated with goat anti-mouse-HRP (1:1000 dilution; Upstate Biotechnology, Lake Placid, NY) in PBS-MILK. Bands were visualized using enhanced chemiluminescence, washed, and subsequently exposed to a film for 30 days at 4°C to detect the presence of ^{14}C-SMA41.

6.3.8. Western blotting for MAP kinase

A431 cells were growth in 6 well plates until confluency, washed three times with PBS, and grown in serum-free media for 24 h. The next day, SMA41 was administered for 2 h, after which cells were stimulated with 100 ng/mL EGF for 20 min. Cells were collected, and protein lysates were prepared as previously described (Matheson *et al.*, 2001). Samples were separated on SDS-PAGE, and transferred to a PVDF membrane. The membrane was incubated with primary antibodies anti-phosphoMAP kinase (Cell Signaling research, Beverly, MA) for determination of phosphorylated MAP kinase. Thereafter, blots were incubated with HRP anti-rabbit antibody (1:1000 dilution; Cell

Signaling, Beverly, MA) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were stripped and re-probed with anti- MAP kinase (Cell signaling research, Beverly, MA) to detect corresponding protein levels, and subsequently with HRP anti-rabbit antibody (1:1000 dilution; Cell signaling research, Beverly, MA).

6.3.9. RT-PCR for the expression of *c-fos*

A431 cells were growth in 6 well plates until confluency, after which they were washed 3 times with sterile PBS and given serum- and phenol red-free medium for 24 h. Cells were then incubated with a dose range of SMA41 for 2 h, followed by 50 ng/mL of EGF for 30 min. Total RNA was isolated using the High Pure RNA isolation kit (Roche Molecular Biochemicals, Laval, QC, Canada) following the manufacturer's instructions. Quantitative analysis of *c-fos* mRNA and *G3PDH* mRNA (1 μg of RNA per sample) was performed using the Titan One Tube RT-PCR system (Roche Molecular Biochemicals, Laval, QC, Canada) following the manufacturer's instructions. The following primers were used: 5'ATGATGTTCTCGGGCTTC3' (sense) and 5'CTCCTGCCAATGCTCTGC3' (antisense) for *c-fos* and 5'CCATGGAGAAGGCTGGGG3' (sense) and 5'CAAAGTTGTCATGGATGACC3' (antisense) for *G3PDH* (Alpha DNA, Montreal, Canada). Products were run on a 1% agarose gel and stained with ethidium bromide for detection.

6.3.10. Mouse xenograft studies (A431 cells)

SCID mice were maintained as per McGill animal safety protocols. Mice were treated with $1.5x10^6$ cells suspended in 0.2 mL PBS subcutaneously injected into the flank of each mouse. Treatments began when tumors became palpable. The animals were placed into five treatment groups of 6 mice each, plus one control group given the vehicle (10% DMSO, 90% H_2O). The treatment groups were given either 100 mg/kg or 200 mg/kg SMA41, SMA52, or TEM in 0.4 mL volume (10% DMSO, 90% H_2O) every 3 days for 25 days, for a total of 7 injections. Tumor burden was measured twice a week and tumor volume was calculated using the formula TV= (tumor width)^2 x tumor length x 0.5.

6.3.11. HPLC analysis of mouse blood for metabolites

Whole blood following a 4 h injection of 400 mg/kg SMA41 was collected in sodium citrate tubes, and centrifuged to remove cells. An equal amount of acetonitrile was added to the resultant plasma, and centrifuged to prepare HPLC samples. These samples were injected onto a C_4 column (300 x 3.9mm; 15 μM) and eluted using a mixture of 70/30 methanol/water at pH8.

6.4. Results

A significant body of work has accumulated to suggest that SMA41decomposes into SMA52 (an inhibitor of EGFR TK) and a methyldiazonium species (a DNA damaging agent) under physiological conditions (Matheson *et al.* 2001; 2003). However, the exact contribution of each of these species to the mechanism of antiproliferative activity of SMA41 remained to be defined. To dissect the binary contributions of each

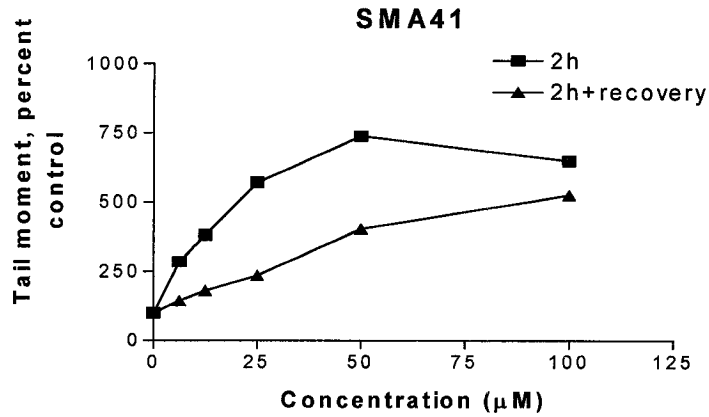
event to the mixed targeting property of SMA41, we performed two major series of assays: (a) those designed to identify the exact DNA adducts induced by the methyldiazonium ion, their repair and effects on the cell cycle, and (b) those that focused on the elucidation of the mechanisms of inhibition of EGFR and their effects on downstream signaling. Finally, we determined whether the principle of binary targeting of EGFR and DNA would be well tolerated *in vivo* and induce EGF-dependent tumour growth inhibition.

6.4.1. Characterization and repair of DNA adducts induced by the methyldiazonium (TZ)

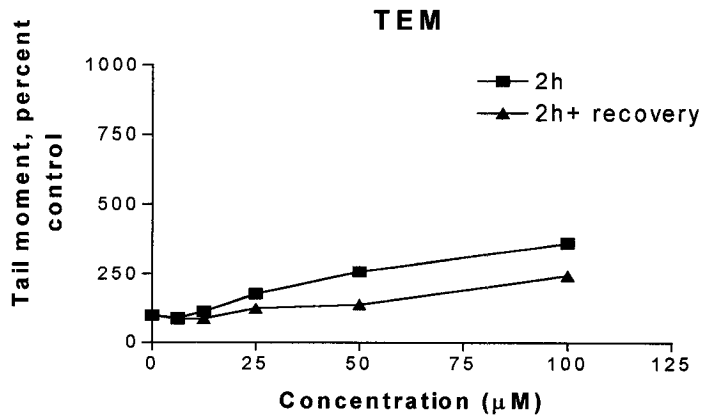
Previous studies demonstrated that SMA41 induces dose-dependent DNA damage in A431 cells (Matheson *et. al.*, 2001). Here we studied the repair of these DNA breaks by comparing DNA damage induced shortly after exposure with those observed 24 h post-treatment (Figure 6.1). DNA damage induced by both SMA41 and TEM were repaired, however their levels remained significantly high after 24 h in cells treated with SMA41. The single-cell microelectrophoresis (comet) assay being designed to detect alkali-labile lesions, we surmise that they may originate from N7-alkylguanine adducts (Bignami *et al.*, 2002). Nonetheless, the cytotoxic DNA adduct induced by alkyltriazenes of the same class as TEM is known to be O6-alkylguanine. Cells expressing O6-alkylguanine DNA alkyltransferase (AGT), like A431, are capable of repairing this adduct and known to be resistant to TEM. Thus, we decided to characterize these adducts and study their repair.

Using SMA41 ^{14}C-labeled at the 3-methyl group, DNA adducts were characterized under a drug treatment schedule similar to that of the comet DNA repair

a)



b)



c)

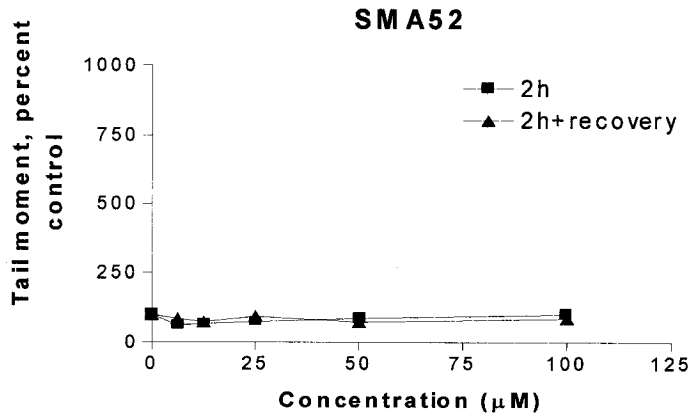


Figure 6.1. Single-cell microelectrophoresis for the detection of alkali-labile DNA lesions induced in the cell line A431. Cells were exposed to either: (a) SMA41, (b) TEM, or (c) SMA52 for 2 h or 2 h with 24 h in drug-free media.

assay. Two hours after treatment (Figure 6.2a), HPLC analysis showed a major peak at 4.6 min that corresponded to N7-methylguanine and a minor peak at 7.6 min indicating O6-methylguanine. As shown in Figure 6.2b, while the N7-methylguanine was still observed after 24 h, the O6-methylguanine peak disappeared, indicating repair of the latter lesions.

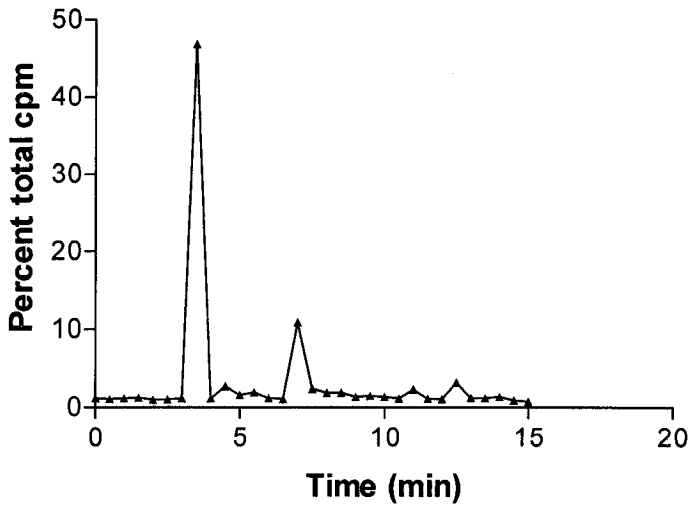
6.4.2. Effects of SMA41 on the cell cycle

In cell lines exposed to SMA41, the marked DNA repair activity observed 24 h post-treatment was accompanied by a significant dose-dependent increase in the number of cells accumulated at various phases in the cell cycle. As shown in Figure 6.3, when cells were given SMA41 for 2 h and allowed to recover for 24 h or 48 h, significant cell cycle arrest was observed in S (61% of cell population). In contrast, no significant cell cycle perturbations were observed for TEM or SMA52. Cell cycle arrests induced by SMA41 were partially reversed 24 h later.

6.4.3. Mechanisms of inhibition of EGFR

The "combi-targeting" concept postulates that the generated TZ (*e.g.* methyldiazonium) may react with the Cys773 to give a covalently modified and inactivated receptor. Thus, we hypothesized that if this reaction does indeed occur, at least a partially irreversible effect of SMA41 should be observed. Thus, autophosphorylation activity in A431 cells was measured after multiple washouts up to 8 h post-treatment. As shown in Figure 6.4, in contrast to SMA52, SMA41 retained almost 100% of its ability to block EGF-induced autophosphorylation in these cells at a dose of 25μM.

a)



b)

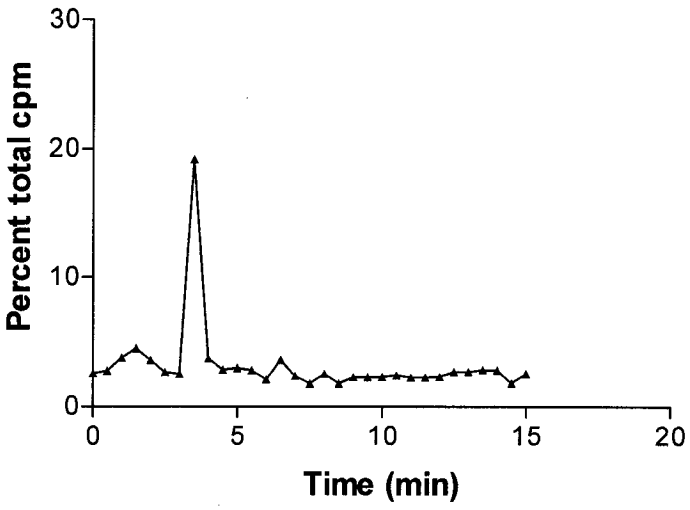


Figure 6.2. HPLC analysis of DNA adducts in cells exposed to ^{14}C-SMA41. A431 cells were treated with ^{14}C-SMA41 for either (a) 2 h, or (b) 2 h with 24 h recovery after which DNA was collected and analyze using HPLC.

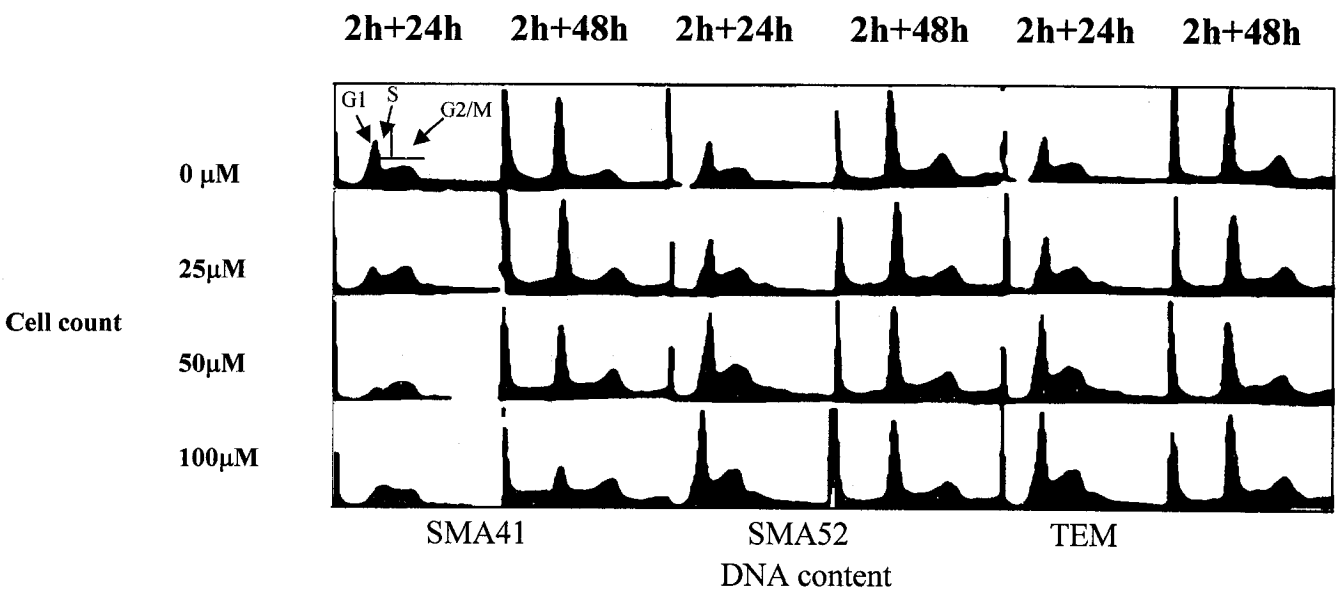


Figure 6.3. Cell cycle analysis of A431 exposed to SMA41, SMA52, or TEM. A431 cells were treated with a dose range of drug for 2 h and allowed to recover in drug-free media for either 24 h or 48 h.

In order to gain insight into the irreversible mechanism of inhibition of EGFR, the cells were exposed to ^{14}C-labeled SMA41 and immunoprecipitated by standard methods either immediately or 24 h post-treatment. As shown in Figure 6.5, significant radioactivity was observed at bands corresponding to the molecular weight of EGFR. Although this method does not allow the characterization of the alkylated site, it suggests that SMA41 may at least covalently modify the receptor.

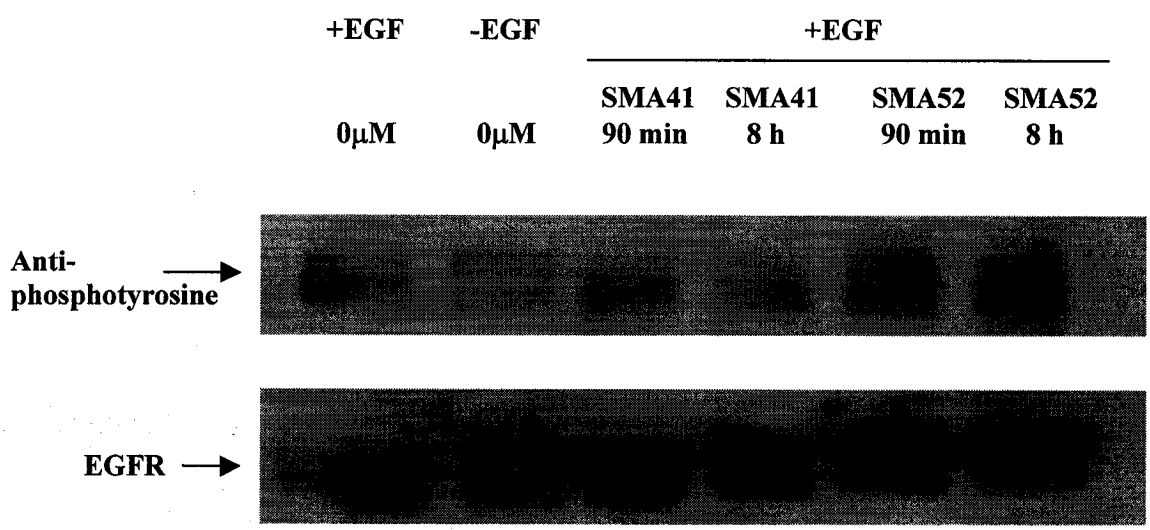
The effects of EGFR TK inhibition on downstream signaling was further investigated by analyzing MAP kinase phosphorylation and *c-fos* expression. As shown in Figure 6.6, at high doses SMA41 was capable of inhibiting MAP kinase activation and subsequent expression of *c-fos*.

6.4.4. *In vivo* studies

Having now analyzed the biological effects of DNA damage and blockade of EGFR autophosphorylation induced by the combi-molecule *in vitro*, we determined its potency in a mouse xenograft model using A431 in SCID mice. Moreover, we verified that the conversion of SMA41 to the free TK inhibitor SMA52 occurs *in vivo*.

SCID mice developed palpable tumors following subcutaneous injection with A431 cells, and SMA41, TEM, and SMA52 were administered in DMSO. Tumor burden was measured every three days, and mice were sacrificed after 25 days. As shown in Figure 6.7a and b, tumour burden was significantly delayed in mice given 100 mg/kg or 200 mg/kg SMA41 when compared with those mice given SMA52 or the vehicle only. At 21 day post-treatment, SMA41 showed approximately 2-fold greater percent growth

a)



b)

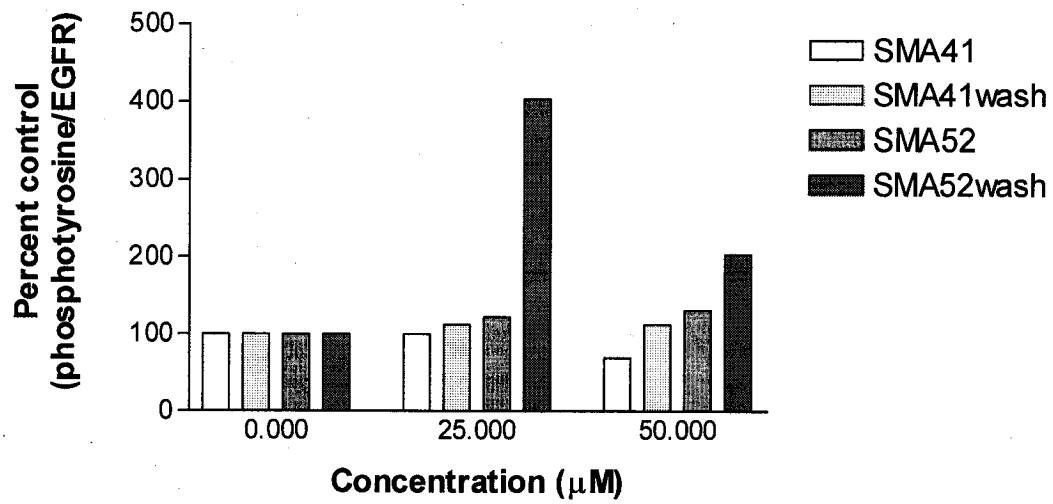


Figure 6.4. Irreversible inhibition of EGF-induced autophosphorylation of EGFR by SMA41 and SMA52 in A431 cells. Cells were treated with SMA41 for 90 min, washed free of drug for 8 h, and cell lysates were prepared for Western blotting for the detection of anti-phosphotyrosine (a); (b) shows levels of phophotyrosine/EGFR.

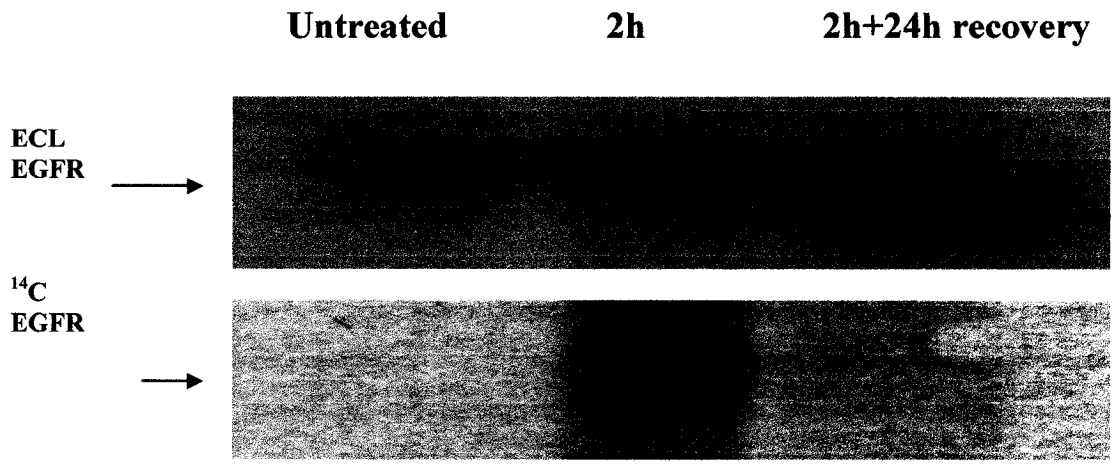


Figure 6.5. Immunoprecipitation for the detection of EGFR in A431 cells treated with ^{14}C-SMA41. Cells were treated with ^{14}C-SMA41 for 2 h or 2 h with 24 h in drug-free media, and protein lysates were prepared. EGFR was detected using both ECL and ^{14}C autoradiography.

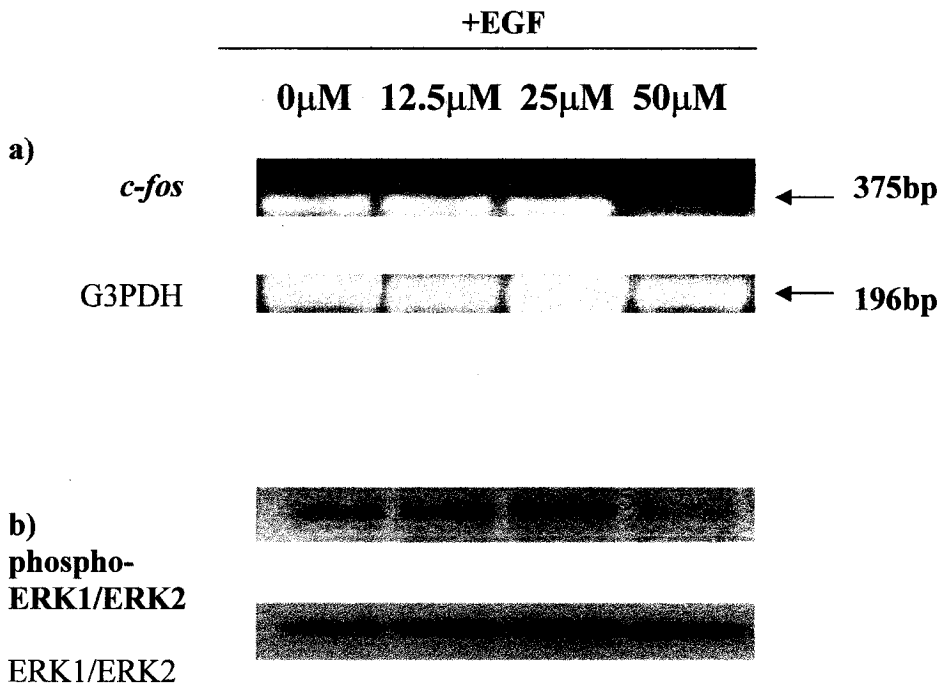


Figure 6.6. The inhibition of *c-fos* expression, and MAP kinase phosphorylation by SMA41. RT-PCR was used to determine the inhibition of *c-fos* expression following 2 h exposure to SMA41, with G3PDH used as an internal control (a). Western blotting was performed for phospho-MAP kinase following a 2 h exposure to SMA41 (b).

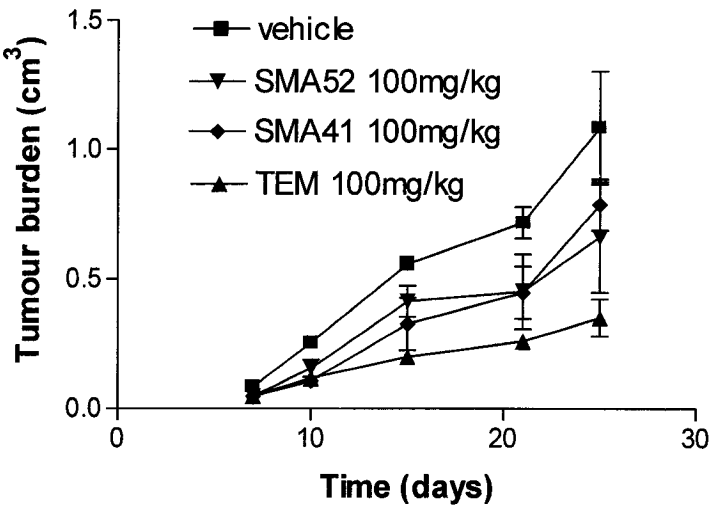
inhibition than SMA52 at 200 mg/kg. TEM showed superior activity to SMA41 due to its water solubility and bioavailability.

Mice were treated with SMA41 (200 mg/kg) for the course of the study (25 d), sacrificed, and whole blood was collected. The plasma was analyzed for metabolites, as shown in Figure 6.8. A peak was observed that corresponded to that of SMA52. Furthermore, mass spectroscopy was performed and the mass of this peak (M+1) was found to be 251.3, a molecular weight that corresponds to SMA52. Therefore, the conversion of SMA41 to SMA52 (the intact TK inhibitor) occurs not only *in vitro*, but also *in vivo* (see Appendix).

6.5. Discussion

Previous studies demonstrated the mixed EGFR/DNA targeting properties of SMA41, the first molecular probe for the combi-targeting postulates. In addition to being able to bind to the EGFR ATP binding site on its own, this combi-molecule was designed to generate another inhibitor (SMA52) and a DNA-damaging methyldiazonium species. Considerable evidence has emerged to confirm the ability of SMA41 to damage DNA in whole cells. However, the exact nature of this damage remained to be determined. Methyltriazenes of the same class as SMA41 are known to induce N7- and O6-methylguanine DNA lesions (Bignami *et al.*, 2000). Although the N7-methylguanine adducts are the most abundant (approximately 90% of total DNA adducts), the O6-methylguanine that accounts for about 10% of the total damage induced by methyltriazenes is the most cytotoxic (Kokkinakis *et al.*, 1997; Bignami *et al.*, 2000;

a)



b)

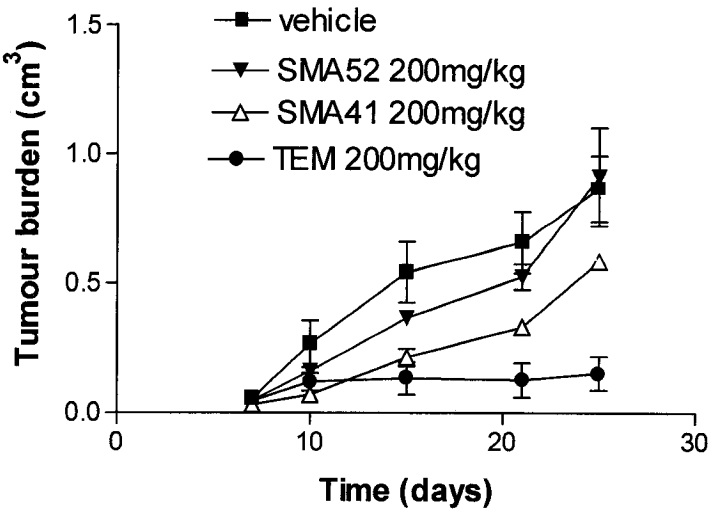


Figure 6.7. *In vivo* activity of SMA41 in A431 mouse xenografts. SCID mice were injected with A431 cells, and tumour delay was measured in mice treated with (a) 100 mg/kg, or (b) 200 mg/kg of SMA41, TEM, or SMA52.

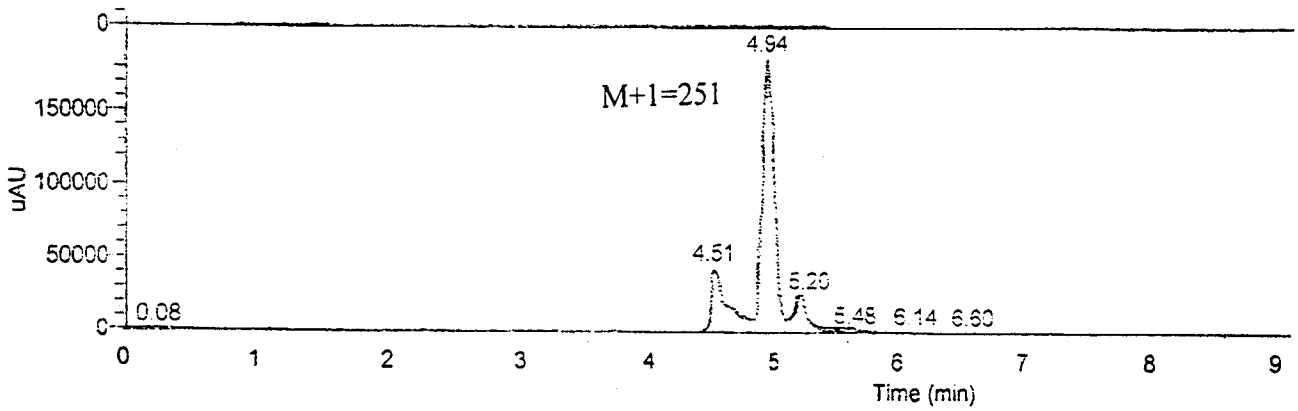


Figure 6.8. The HPLC detection of the conversion of SMA41 to SMA52 from mouse plasma. A dose of 400mg/kg of SMA41 was given for 4 h and mouse blood was collected for HPLC analysis. A peak corresponding to the control for SMA52 was identified at 4.49 min.

Kokkinakis *et al.*, 2001). Tumour cells harbouring AGT (a suicide DNA repair enzyme that captures the methyl group by transalkylation of its own cysteine residue) are resistant to methylating agents of the triazene class (Baer *et al.*, 1993; Lage *et al.*, 1999; Bignami *et al.*, 2000). In this study (and shown in Figure 6.2), the characterization of DNA adducts induced by ^{14}C-labeled SMA41 revealed the formation of both N7- and O6-alkylguanine lesions. The complete disappearance of the peak corresponding to O6-alkylguanine 24 h post-treatment indicated that this lesion is repaired, an observation that is consistent with the AGT positive status of A431. In contrast, significant levels of N7-alkylguanine adducts were apparent 24 h post-treatment although, as indicated by alkaline comet assay data, this type of alkali labile lesion is also repaired (Figure 6.1). Thus, the cytotoxic contribution of DNA damage induced by SMA41 may not depend on the O6-alkylguanine adducts, but on other types of lesions such as N7-alkylguanine or N3-adenine (the levels of which were too low to be detected by our radio-HPLC methods). More importantly in contrast to TEM, which is known to induce similar types of adducts, DNA damage by SMA41 causes significant cell cycle arrest in S (middle), S (late), and G2/M (Figure 6.3). Given that SMA41 (as demonstrated in earlier studies) decomposes within 1h into an almost stoichiometric quantity of SMA52 and a methyldiazonium species at the same proportion as TEM (Matheson *et al.*, 2001), one would expect SMA41 to induce little cell cycle perturbation. Thus, the observed arrest may not only be due to the ability of SMA41 to alkylate DNA, but also (in contrast to TEM) to its ability to interfere with other proteins in the cells, perhaps associated with EGFR signaling.

One of the premises of the combi-targeting concept was that SMA41 (TZ-I) can alkylate the receptor to form a TZ-EGFR adduct (Scheme 6.1). Covalent damage to the receptor may induce its irreversible inhibition. This was verified by studying the reversibility of inhibition of EGF-dependent receptor autophosphorylation. The results indicated that, unlike the reversible inhibitor SMA52 (I), SMA41 could induce irreversible inhibition of the receptor (Figure 6.4). The persistent reduction of phosphorylation of EGFR was not due to changes in protein content since receptor levels were constant throughout the dose range. The irreversible nature of inhibition of autophosphorylation by SMA41 lends support to the hypothesis that SMA41 may covalently modify the receptor. This was further corroborated by studies with ^{14}C-labeled SMA41 (Figure 6.5) that showed distribution of radioactivity in a band corresponding to EGFR. Although this assay could not give insight into the specifically alkylated amino acid sequence, it confirms the covalent modification of EGFR by SMA41.

The biological effects of EGF-dependent autophosphorylation were further characterized by analyzing its downstream effects. At high doses, inhibition of EGF-induced EGFR autophosphorylation by SMA41 blocked phosphorylation of MAP kinase and expression of *c-fos*. The rather high doses required for these inhibitory events suggest that blockade of autophosphorylation by this drug may also block other growth stimulation pathways. We have already demonstrated that SMA41 inhibits EGF-stimulated growth at concentrations less than 3 μM (Matheson *et al.*, 2001; 2003).

We have now demonstrated that the mixed EGFR/DNA targeting properties of the TZ-I SMA41 triggers a binary cascade of events: those associated with DNA damage,

and those related to EGF-dependent receptor autophosphorylation in A431 carcinoma cells, a cell line that expresses the DNA repair enzyme AGT and that aggressively proliferates via a TGFα/EGFR-mediated autocrine loop. While these events translate into significant antiproliferative activity *in vitro*, the demonstration of the relevance of the combi-targeting principles to the clinical therapy of EGF-dependent carcinomas required a primary *in vivo* model. It was first found that the drug, although administered as a suspension, was well tolerated with achievable doses such as 100 mg/kg and 200 mg/kg (Figure 6.7). TEM at these doses is extremely water soluble, and also known to be well tolerated. SMA52, administered as a much clearer suspension than SMA41 was also well tolerated. While once/daily doses are required for most EGFR inhibitors to induce antitumour activity, because of the cytotoxic nature of SMA41, a once every three day schedule was selected. Two major *in vitro* observations were translated *in vivo*: (a) the conversion of TZ-I to TZ + I, since we successfully detected SMA52 in plasma collected from tumour bearing animals treated with SMA41 (Figure 6.8), and (b) the *in vivo* antitumour activity of the TZ-I SMA41 was superior to that of its I (SMA52) alone. Nevertheless, the activity of TEM alone was superior to that of both drugs. This is due to the higher bioavailability of TEM which at equimolar dose may achieve significantly higher plasma concentration than SMA41 given as a suspension. High and repeated doses of TEM are known to deplete the levels of AGT, thereby increasing its cytotoxic activity (Nutt *et al.*, 2000; Kokkinakis *et al.*, 2001). Regardless, the demonstration of the superior activity of SMA41 when compared with SMA52 (a reversible inhibitor) suggests that the combi-targeting principles can be used to increase the potency of reversible inhibitors of EGFR. Furthermore, we presented herein the first evidence that, in contrast

to previous Michael acceptor-based approaches to the design of irreversible inhibitors of EGFR, increased potency is possible by conferring a different (non-EGFR) target to the TK inhibitor. Further studies are ongoing to increase the water solubility of SMA41with the purpose of enhancing its bioavailability and its efficacy.

6.6. Acknowledgements

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6.7. References

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CHAPTER 7

DISCUSSION

AND

CONTRIBUTION TO KNOWLEDGE

7.1. Discussion and contribution to knowledge

Cancer is an insidious and increasingly prevalent disease. In Canada, both men and women have a one in three lifetime chance of developing a malignant tumour (Health Canada, 2003). Moreover, conventional chemotherapy is plagued by several major drawbacks such as drug resistance and a lack of selectivity for its target. The latter translates into systemic toxicity and deleterious side effects. Cytotoxic drugs, for example nitrosoureas and triazenes, target rapidly proliferating cells (*i.e.* tumours). Unfortunately, this characteristic is not exclusive to malignant tissues and patients often experience deleterious side effects.

Over the past few decades, new approaches have been developed to render cytotoxic agents more selective for cancer cells. For example, drug conjugates that use a recognition moiety such as a receptor ligand have been linked to a cytotoxic agent in order to target tumour cells that overexpress the receptor (Petrow and Padilla, 1986; Petru *et al.*, 1988; Kupczyk-Subotkowska *et al.*, 1997; Langer *et al.*, 2001; Arencibia *et al.*, 2001). Although this appears to be a wise approach, its limitations include bulkiness that hinders binding affinity. Therefore, the quest for anticancer drug specificity has inspired the search for novel targets and new methodologies.

Through the elucidation of the biochemical differences between normal and malignant tissues, various potential molecular targets for chemotherapy have been identified. These include growth factor receptors and other downstream proteins involved in signaling cascades that mediate such diverse cellular processes as proliferation and apoptosis (Sherwood *et al.*, 1999; Chou *et al.*, 1999; Favoni and De Cupis, 2000; Wosikowski *et al.*, 2000).

Overexpression of epidermal growth factor receptor (EGFR) occurs in a large number of cancers (*e.g.* breast, ovarian, and prostate), and can lead to the formation of autocrine loops contributing to aggressive tumour progression (Sherwood *et al.*, 1999; Suo *et al.*, 2002). Therefore, EGFR has emerged as an excellent target for chemotherapy. By directing molecules to EGFR and blocking signaling through this receptor, one can selectively inhibit tumour growth in EGFR-positive cancers. In fact, tyrosine kinase (TK) inhibitors of the quinazoline class that competitively bind to the ATP-binding site of EGFR have shown tremendous efficacy and selectivity *in vitro* and *in vivo*, and are currently under investigation in various phase III clinical trials (Fry *et al.*, 1994; Fry *et al.*, 1998; Rewcastle *et al.*, 1995; Baselga *et al.*, 2002).

The most important shortcoming of EGFR TK inhibitors is that these drugs are cytostatic, and not cytotoxic. In short, they induce reversible inhibition of tumour cell growth. Therefore repeated and prolonged dosing is required for sustained antitumour activity, and these drugs are now being studied in combination with cytotoxic agents (Ciardello *et al.*, 2000; Baselga *et al.*, 2002; Wakeling *et al.*, 2002). The use of combination therapy is common since by targeting more than one element within a tumour cell, one may increase the likelihood of effective treatment (Fung and Nademanee, 2002). Furthermore, efforts have been made to render TK inhibitors irreversible by appending an acrylamide moiety to the 6-position of the quinazoline ring in order to covalently modify the Cys773 residue within the ATP binding site of the receptor (Fry *et al.*,1998; Allen *et al.*, 2002). Results have shown that this approach does indeed prolong the EGFR inhibitory activity of quinazolines (Vincent *et al.*, 2000).

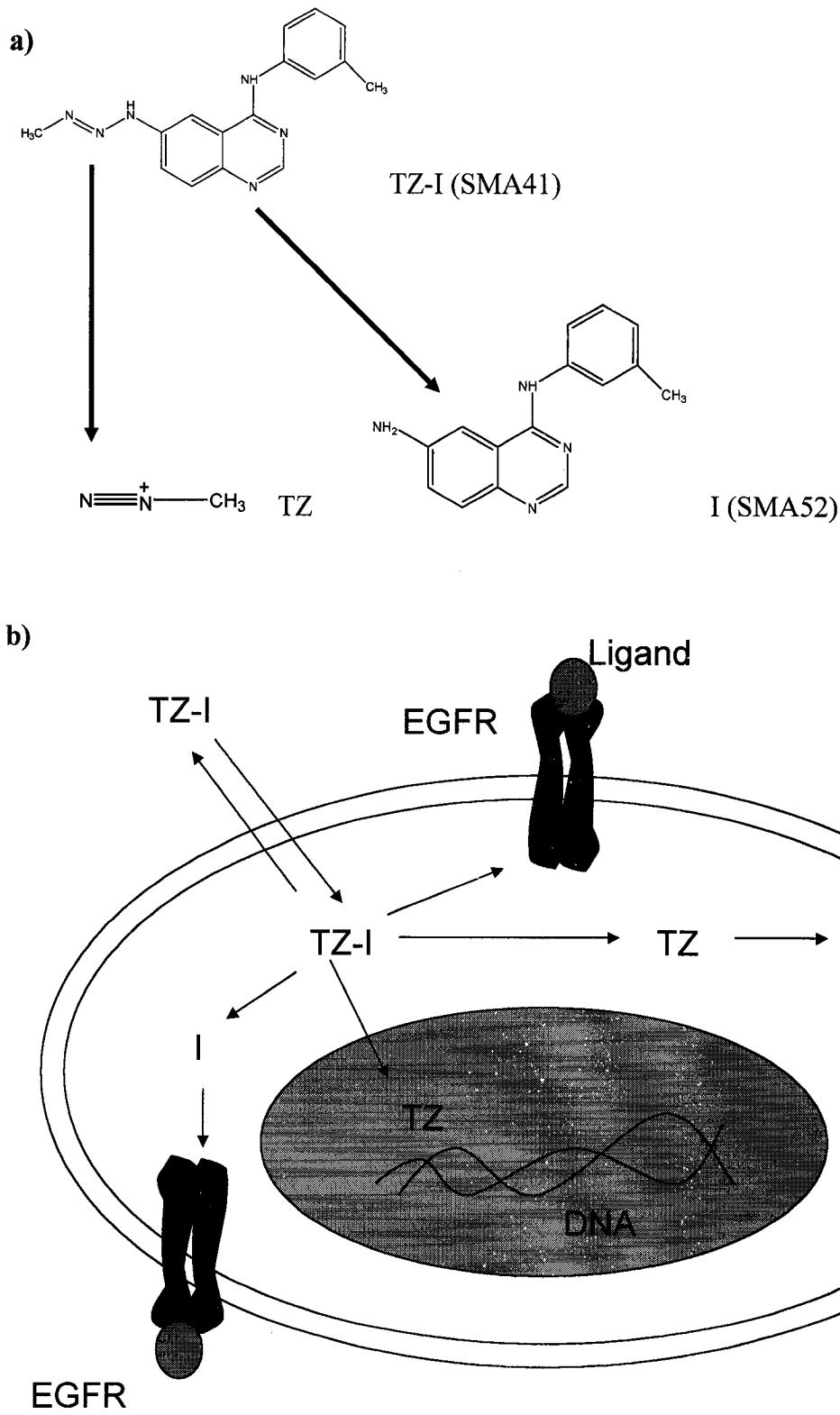

Figure 7.1. The Combi-targeting concept.

Consequently, we designed a novel tumour targeting strategy to overcome the barriers of selectivity and reversibility: The Combi-targeting concept. This theory proposes a tandem approach to treat refractory tumours. To illustrate its achievability, we synthesized the first ever unimolecular combination of a triazene DNA alkylating agent and a quinazoline TK inhibitor. As shown in Figure 7.1 [see (a)], SMA41 ("TZ-I") enters cells by passive diffusion and binds to the ATP binding site of EGFR to inhibit signaling through this receptor. Under these hydrolytic conditions, as depicted in 7.1 [see (b)], it subsequently degrades to yield the corresponding free inhibitor SMA52 ("I") plus a DNA-damaging methyldiazonium species ("TZ") that can interact with both DNA and EGFR. We have presented direct evidence of the intracellular conversion of SMA41 into SMA52 both *in vitro* and *in vivo*.

SMA41 degrades to yield SMA52 in a proportion of 81% with a half-life in serum-containing medium at 37°C of 34 min (Matheson *et al.*, 2001). This "combi-triazene" also inhibits EGF-induced receptor autophosphorylation, binds to purified EGFR in an ELISA-based assay, induces dose-dependent DNA damage, and induces sustained inhibition of cell growth in a cell line that overexpresses both EGFR and AGT (human carcinoma of the vulva, A431). Moreover, when SMA52 and temozolomide (TEM), the control methyltriazene, were used in combination and compared with SMA41, the combi-triazene showed superior antiproliferative effects. This data shows the overall viability of the Combi-targeting concept, and was published in the Journal of Pharmacology and Experimental Therapeutics (Matheson *et al.*, 2001). In addition, this work has resulted in another publication on the Combi-targeting concept (See Appendix for Qiu *et al.*, 2003).

Following the demonstration of the feasibility of our novel targeting strategy, we focused on the selectivity of SMA41 in a panel of cell lines expressing variable levels of EGFR and AGT. The results of these experiments were published in the journal Cancer Chemotherapy and Pharmacology (Matheson *et al.*, 2003). In this chapter, we demonstrated that SMA41 inhibits EGF-, but not serum- nor PDGF-, induced cell growth in a mouse fibroblast cell line transfected with *EGFR* (NIH 3T3/Her-14). In addition SMA41 binds to EGFR, but not Src kinase, insulin receptor, or protein kinase C (PKC) in an ELISA-based assay. Hence, SMA41 shows significant receptor type specificity. Despite EGFR selectivity, when IC_{50} values were determined for SMA41 in a panel of human tumour cell lines, they did not correlate with EGFR levels. However, activity appeared to be more dependent on AGT status. The same trend was observed for TEM, another monomethylating triazene. Most importantly, SMA41 showed consistently superior activity over the clinical drug TEM.

Given that there was no correlation between SMA41 IC_{50} value and EGFR status in established cell lines, we studied its selectivity in a pair of cell lines isogenic for *EGFR* (NIH 3T3 and NIH 3T3/Her-14). In the EGFR-positive, SMA41 induced: 1) higher levels of DNA damage over the EGFR-negative cell line, 2) significant cell cycle perturbations in a serum-stimulated context, and 3) approximately 9-fold greater antiproliferative activity in the EGFR-positive cell line over the EGFR-negative cell line. These results suggest that the combi-triazene may not only selectively target cells expressing EGFR via its TK inhibitor, but also enhance the activity of the individual TK inhibitor and methyltriazene with its binary targeting approach.

Having demonstrated the mechanism of action, selectivity, and internalization of the combi-molecule SMA41, we determined its downstream effects, irreversible activity, and its *in vivo* efficacy. Furthermore, we demonstrated the repair of alkali-labile DNA damage induced by both TEM and SMA41.

It had been previously hypothesized that SMA41, being an alkylating agent, may irreversibly damage the receptor. To establish whether SMA41 irreversibly interacts with EGFR, we studied autophosphorylation following a washout of cells treated with the combi-triazene or the free inhibitor, SMA52. When cells were exposed to SMA41 for 2 h, and the drug was subsequently washed off for 8 h, there was an almost 100% retention of TK inhibitory activity. In contrast, cells treated with SMA52 resumed autophosphorylation. Moreover, A431 cells treated with ^{14}C-SMA41 showed retention of radioactivity localized to the receptor.

These data suggest that there is an interaction, and covalent modification, of EGFR by the methyltriazene of SMA41. The proposed mechanism of irreversibility is shown in Figure 7.2. When SMA41 binds to the ATP-binding site of EGFR, the methyltriazene reacts with the SH-group of Cys773. Thus the methyldiazonium species may form a covalent bond with this residue *in situ* [see (a)], or be released by hydrolysis for subsequent interaction with DNA [see (b)].

Throughout this thesis, I have demonstrated that the Combi-targeting concept is indeed a valid and novel approach to tumour targeting. From the design and synthesis of the first-ever combi-molecule (SMA41), to its mechanism of action, selectivity, cellular

distribution, and *in vivo* activity, I have sought to prove the feasibility of this theory. Thus, I have demonstrated the following:

1) The synthesis of the first-ever unimolecular combination of a cytotoxic triazene (TZ) and a quinazoline EGFR TK inhibitor (I) to form "SMA41," (TZ-I)
2) The hydrolytic degradation of SMA41 (TZ-I) to give an intact TK inhibitor (I) and a DNA-damaging methyldiazonium species (TZ),
3) The receptor-type and receptor-site specificity of SMA41 (TZ-I) for EGFR,
4) The translation of the binary targeting properties of SMA41 with significant antiproliferative activity *in vitro,*
5) The distribution of both the methyldiazonium (TZ) and free TK inhibitor (I) in whole cells,
6) The good *in vivo* tolerance and efficacy of SMA41 (TZ-I) in a mouse xenograft model,
7) The conversion of SMA41 (TZ-I) into SMA52 (I) not only *in vitro*, but also *in vivo*,
8) The superior antiproliferative activity of SMA41 over the TK inhibitor SMA52 *in vivo.*

Radical advances have been made in cancer treatment throughout the past few decades. As shown in Figure 7.3, the evolution of chemotherapy has taken a major turn in the past 10 years from cytotoxic drugs to signal transduction inhibitors. Traditionally, anticancer drugs have been DNA-directed such as DNA cross-linking nitrosoureas and nitrogen mustards. These drugs are still clinically relevant, but are generally given in

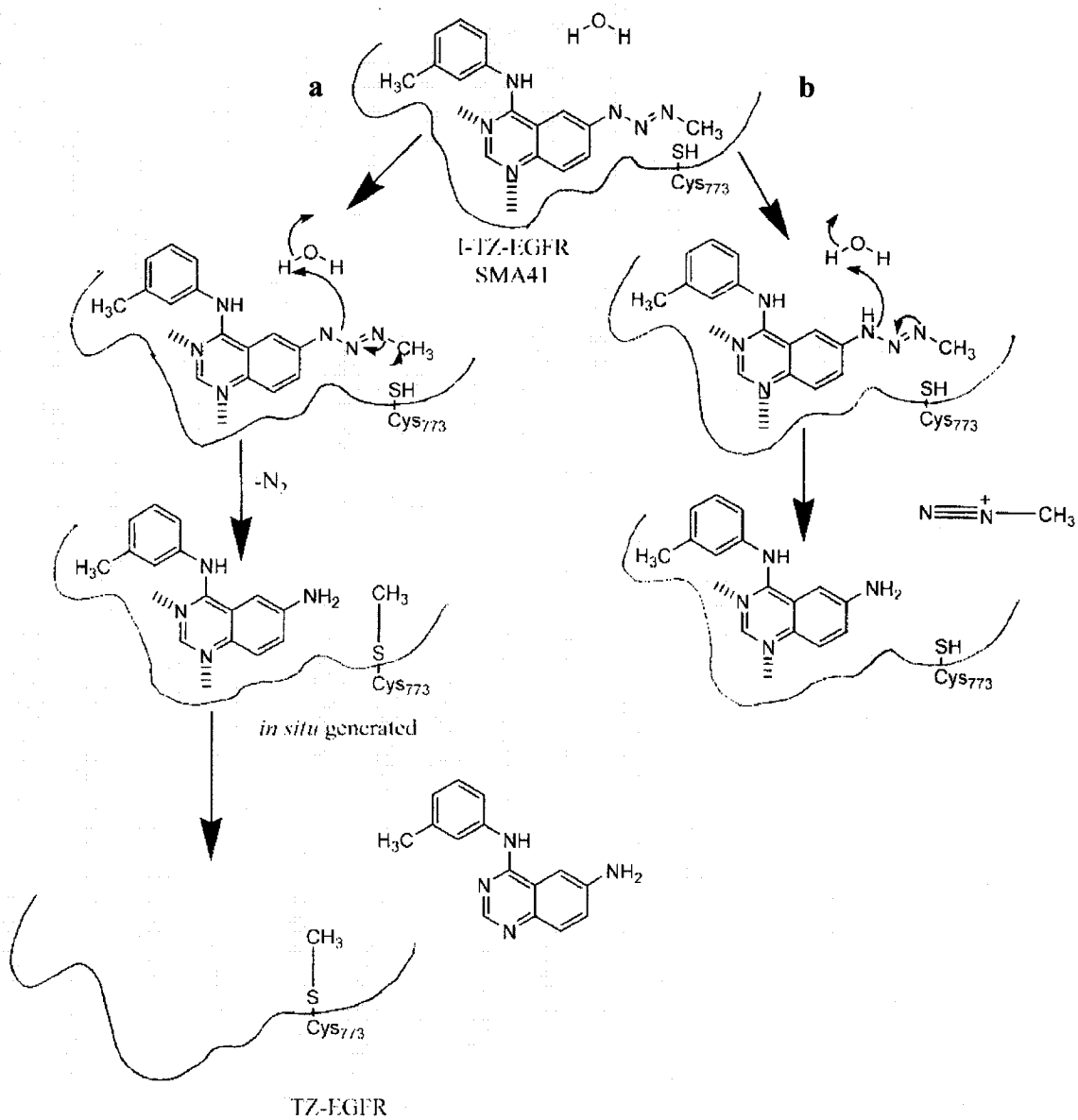

Figure 7.2. The mechanism of irreversibility of SMA41.

combination with other cytotoxic agents directed at different targets (Fung and Nademanee, 2002). Moreover, cancer cells often exhibit mechanisms of resistance to DNA-damaging drugs. For example, resistance to triazenes is mediated by the expression of O6-alkylguanine DNA alkyltransferase (AGT) (Cai *et al.*, 2000; Kokkinakis *et al.*, 2001; Gerson, 2002).

The inhibition of signal transduction pathways has become the focus of much research. Monoclonal antibodies against extracellular portions of EGFR have been developed, some of which are used in the clinic, and small molecule inhibitors of the internal EGFR TK domain have now been identified (Fan *et al.*, 1992; Fry *et al.*, 1994). TK inhibitors have shown excellent tolerability, selectivity, and efficacy in clinical trials, especially the quinazoline compound ZD1839 (Iressa) (Woodburn and Barker, 1996; Ciardello *et al.*, 2000). Since these compounds are cytostatic, and tumours regrow after cessation of treatment, an irreversible TK inhibitor (CI-1033) was synthesized and studied (Fry *et al.*, 1998; Allen *et al.*, 2002). In addition, clinical trials using ZD1839 in combination with cytotoxic drugs to increase their efficacy are currently underway (Ciardello *et al.*, 2000). Novel tumour targeting strategies are thus required to enhance the activity of TK inhibitors. Therefore, we designed and studied the Combi-targeting concept.

We sought to increase the efficacy of a quinazoline TK inhibitor by giving it an additional DNA-damaging function. Hence, cells expressing EGFR would be subject to binary targeting. The first-ever combi-triazene (SMA41) was successfully synthesized, and shown to be efficacious *in vitro* and *in vivo*.

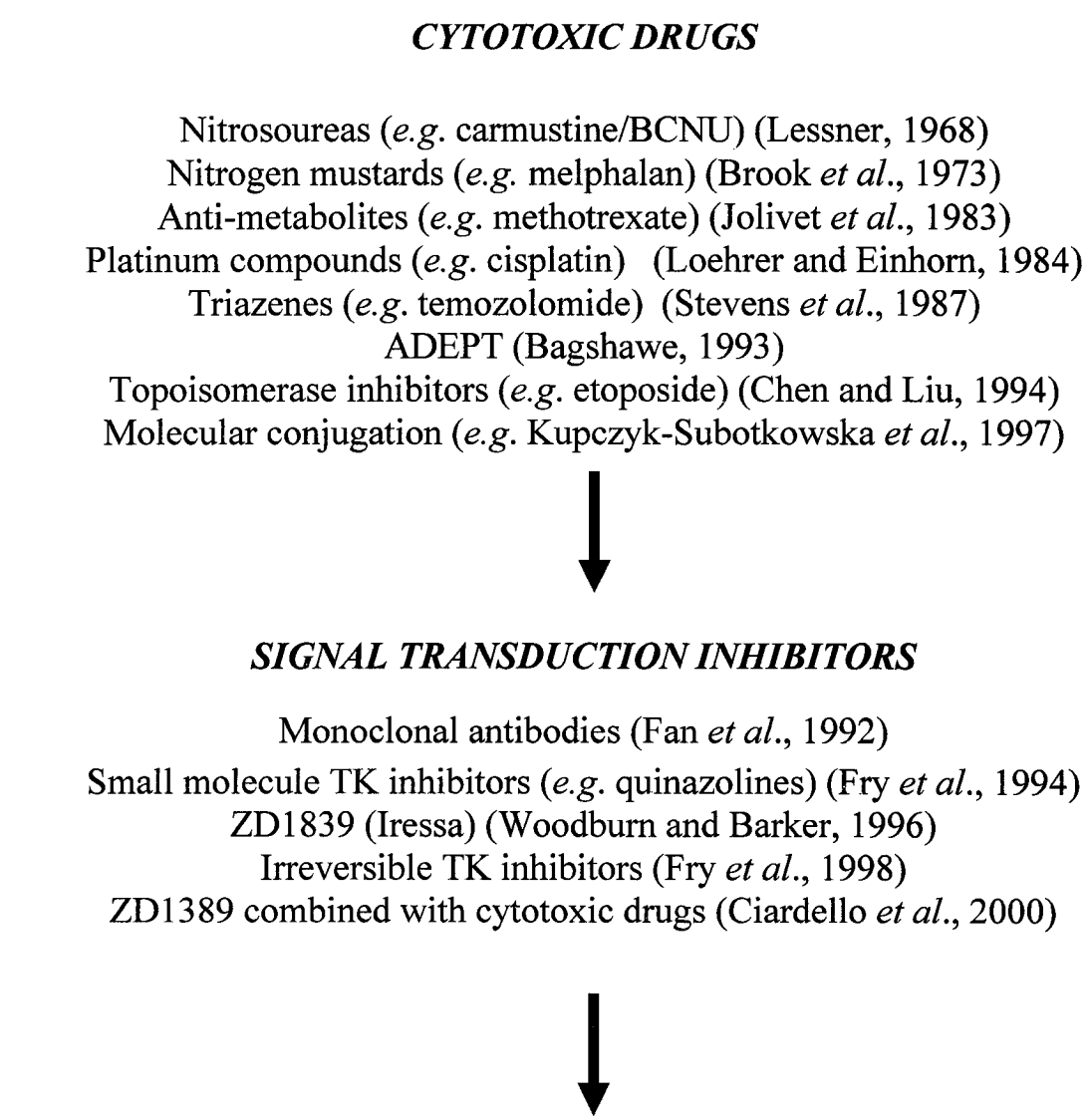

Figure 7.3. Anticancer drug therapy: past, present, and future.

The major concern about SMA41 is its limited water solubility that hinders its bioavailability. Thus, current research is focusing on the addition of solubilizing moieties (such as morpholine) to this, and other, combi-molecules to increase their *in vivo* activity.

With the dawn of the genomic era, many new targets will be identified. This shall lead the way to the development and implementation of combi-targeting to treat a variety of tumour types and not only those overexpressing EGFR. We are optimistic that the contents of this thesis have laid the foundation for the continuation of exciting and promising work on the Combi-targeting concept, and for its eventual clinical translation.

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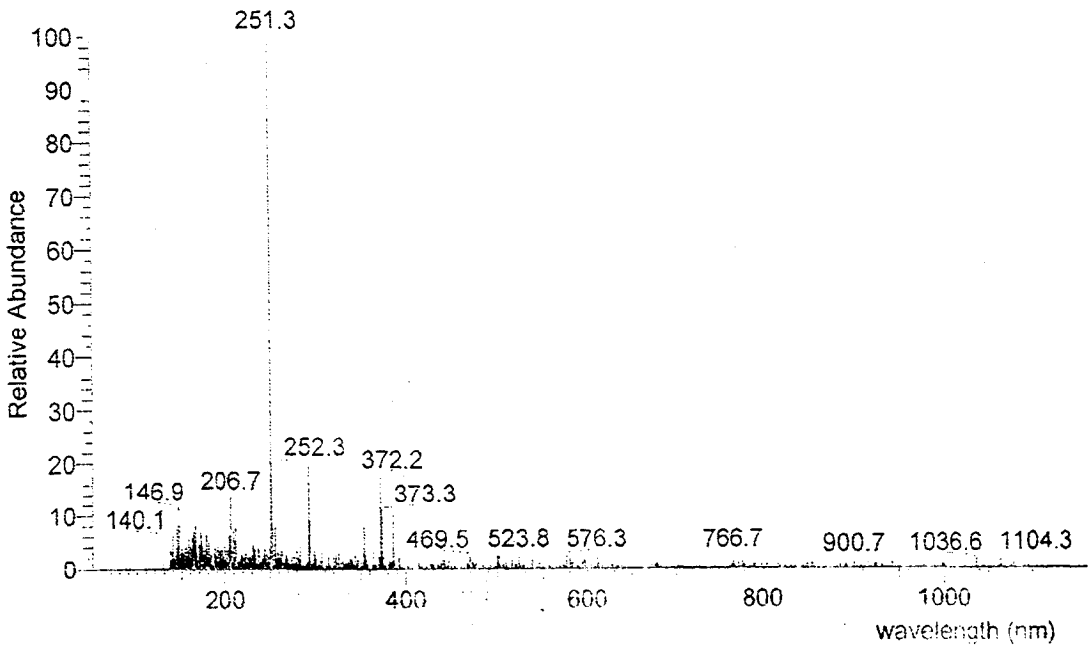
APPENDIX



Mass spectroscopy to identify the metabolites found in mouse plasma from A431 xenograft studies with SMA41 (Chapter 6).

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 4451
Investigator #: 976
Approval End Date: Nov. 30 2002
Facility Committee:

☐ Pilot ☒ New Application ☐ Renewal of Protocol #

Title (must match the title of the funding source application): Mechanism of action of target-mediated tumour selectivity of 1,2,3-triazene-containing chimeric molecules: a novel tumour targeting strategy

1. Investigator Data:

Principal Investigator: Bertrand J. Jean-Claude Office #
Department: Medicine Fax#
Address: Email:

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Bertrand J. Jean-Claude **Work #:** **Emergency #:**
Name: Stephanie Matheson **Work #:** **Emergency #:**

ACTION	✓	DATE
P.I.	✓	Dec 5'01
FACC	✓	"
RGO	✓	"
VET	✓	"
DB		

returned 4 Dec 2001

3. Funding Source:

External ☒	Internal ☐
Source (s): Cancer Research Society	**Source (s):**
Peer Reviewed: ☒ YES ☐ NO**	**Peer Reviewed:** ☐ YES ☐ NO**
Status : ☒ Awarded ☐ Pending	**Status:** ☐ Awarded ☐ Pending
Funding period: 09/2001-09/2003	**Funding period:**

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y):	01/01/02	**or ongoing** ☐
Expected Date of Completion of Animal Use (d/m/y):	01/04/02	**or ongoing** ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: **Date:** 13/9/2001

Approval Signatures:

Chair, Facility Animal Care Committee:		**Date:** 13/9/2001
University Veterinarian:		**Date:** 10/18/01
Chair, Ethics Subcommittee(as per UACC policy):		**Date:** 10/18/01
Approved Period for Animal Use	**Beginning:** Dec. 1, 2001	**Ending:** Nov. 30, 2002

☐ This protocol has been approved with the modifications noted in Section 13.

(Had Admin. approval previously)

SEP 13 2001 PM

McGill University

Royal Victoria Hosptial Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Dr. Bertrand J. Jean-Claude

ADDRESS:

TELEPHONE:

FAX NUMBER:

DEPARTMENT: Medicine

E-MAIL: |

PROJECT TITLE:

Mechanism of action and target-mediated tumour selectivity of 1,2,3-triazene-containing chimeric molecules : a novel tumour targeting strategy

2. FUNDING SOURCE: MRC/CIHR ☐ NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ Cancer Research Society

Grant No.: N/A Beginning date: 01/09/01 End date: 30/08/2003

3. Indicate if this is

☒ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date 08/01

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency: Approval End Date

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 2 3 4

Principal Investigator or course director: Dr. Bertrand J. Jean-Claude SIGNATURE date: 02/08/01 (day / month / year)

Chairperson, Biohazards Committee: Dr. Dana Baran SIGNATURE date: 28-08-01 (day / month / year)

Approved period: beginning 01 (day) 09 (month) 01 (year) ending 01 (day) 09 (month) 02 (year)

* as defined in the "McGill Laboratory Biosafety manual"

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Dr. Bertrand J. Jean-Claude	Medicine	X				
Dr. Fouad Brahimi	Medicine					X
Dr. Fabienne Dudouit	Medicine					X
Dr. Quyi Quiu	Medicine					X
Mrs. Stephanie Matheson	Medicine				X	
Mrs. Ranita Banerjee	Medicine				X	

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Bertrand J. Jean-Claude Phone No: work: home:

Name: Mrs. Stephanie Matheson Phone No: work: ɔme:

6. Briefly describe:

i) the biohazardous material involved are human tissues designated as biosafety risk group 2.

ii) the procedures involving biohazards

- Protective laboratory clothing (lab-coats) will be used
- Gloves will be used
- Working surfaces will be cleaned with 70% ethanol
- Spills will be reported to the laboratory supervisor
- Contaminated materials will be safely disposed of (autoclave or incinerated)
- No food or drink will be allowed
- Hnd washing will be reinforced

iii) the protocol for decontaminating spills

- Surfaces will be thoroughly washed with 70% ethanol
- Autoclavable materials will be sterilized

---

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

The use of a laminar flow hood for tissue culture and sterilization of work surfaces with 70% ethanol

---

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Royal Victoria Hospital	Rm 7.15	Microzone	Microzone	8017156	June 31/2001

McGill University

Royal Victoria Hospital Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Dr. Bertrand J. Jean-Claude

ADDRESS

TELEPHONE:

FAX NUMBER

DEPARTMENT: Medicine

E-MAIL:

PROJECT TITLE:

Novel unimolecular combination models for selective tumour targeting: A tandem approach to the enhancement of chemoselectivity and abrogation of chemoresistance

2. FUNDING SOURCE: MRC/CIHR ☐x NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify)

Grant No.: MOP-49440 Beginning date: 01/10/01 End date: 30/09/2004

3. Indicate if this is

☐ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency: Canadian Institute of Health Research/(Formerly Medical Res. Council (MRC) Approval End Date

☒ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 2 3 4

Principal Investigator or course director SIGNATURE date: 17/07/01 (day/month/year)

Chairperson, Biohazards Committee: Dr. Dana Baran SIGNATURE date: 20/07/01 (day/month/year)

Approved period: beginning 01/10/01 (day/month/year) ending 01/10/02 (day/month/year)

* as defined in the "McGill Laboratory Biosafety manual"

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Dr. Bertrand J. Jean-Claude	Medicine	X				
Dr. Fouad Brahimi	Medicine					X
Dr. Fabienne Dudouit	Medicine					X
Dr. Quyi Quiu	Medicine					X
Mrs. Stephanie Matheson	Medicine				X	
Mrs. Ranita Banerjee	Medicine				X	

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Bertrand J. Jean-Claude Phone No: work: home:

Name: Mrs. Sthephanie Matheson Phone No: work: home:

6. Briefly describe:

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- Hnd washing will be reinforced

iii) the protocol for decontaminating spills

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7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

The use of a laminar flow hood for tissue culture and sterilization of work surfaces with 70% ethanol

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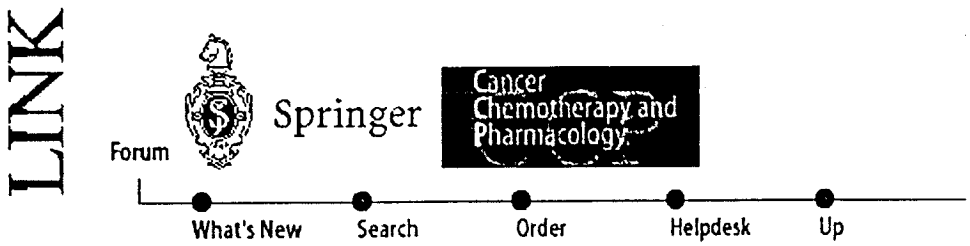
10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Royal Victoria Hospital	Rm 7.15	Microzone	Microzone	8017156	June 31/2001



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Cancer Chemotherapy and Pharmacology

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Title of article: DIFFERENTIAL RESPONSES OF EGFR/AGT-EXPRESSING CELLS TO THE "CENBI-TRIAZENE" SMA 41

Author(s): S.L. MATHESEN, J.P. MCNAMEE, R.J. JEAN-CLAUDE

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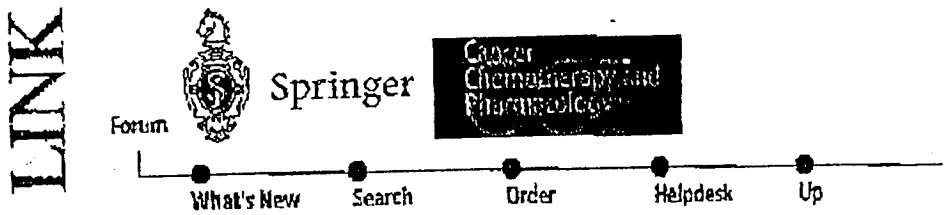

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Title of article: Differential responses of EGFR/AGT-expressing cells to the "Combi-triazene" SM41.

Author(s): S.L. Mathews, J.P. McNamee, & B.J. Tess-Claude

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Title of article: DIFFERENTIAL RESPONSES OF EGFR/ABT-EXPRESSING CELLS TO THE "COMBI-TRIAZENE" SMA41

Author(s): S.L. MATHESON, J.P. ~~MCNAMEE~~, B.J. JEAN-CLAUDE

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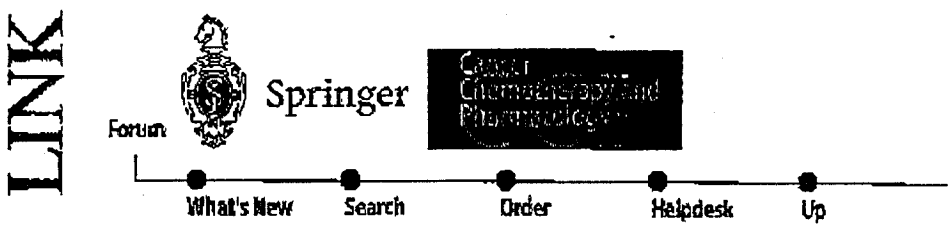

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Title of article: Differential responses of EGFR/AGT-expressing cells to the "Combi-triazene" SM41.

Author(s): S.A. Matheson, J.P. McNamee, & B.J. Jean-Claude

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Design of a Chimeric 3-Methyl-1,2,3-triazene with Mixed Receptor Tyrosine Kinase and DNA Damaging Properties: A Novel Tumor Targeting Strategy

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ABSTRACT

The mixed epidermal growth factor receptor (EGFR)-DNA targeting properties of SMA41, a 6-(3-methyl-1,2,3-triazen-1-yl)-4-anilinoquinazoline designed to release N^4-*m*-tolyl-quinazoline-4,6-diamine henceforth referred to as SMA52 [an inhibitor of EGFR tyrosine kinase (TK)] and methyldiazonium (a DNA methylating species) were studied in the O^6-methylguanine-DNA methyltransferase (MGMT)-proficient and high EGFR-expressing epidermoid carcinoma of the vulva cell line A431. The effects of SMA41 were compared with those of SMA52 alone, and temozolomide (TEM), a clinical prodrug of 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) that is inactive in MGMT-proficient cells. The results showed that 1) the chimeric SMA41 could degrade in serum-containing medium ($t_{1/2}$ of ~30 min) to generate, as predicted, the free inhibitor SMA52 as the most abundant metabolite (~81% yield); 2) in contrast to SMA52 alone, the chimeric SMA41 and TEM induced significant DNA damage in A431 cells after 30-min or 2-h drug exposures, as confirmed by alkaline single-cell gel microelectrophoresis (comet) assay; 3) SMA41 showed 5-fold greater affinity for the ATP binding site of EGFR than independently synthesized SMA52 in an enzyme assay and blocked EGF-induced tyrosine phosphorylation and EGFR autophosphorylation in A431 cells in a dose-dependent manner; 4) these mixed targeting properties of SMA41, combined with its ability to be converted to another potent EGFR TK inhibitor (e.g., SMA52) by hydrolytic cleavage, translated into over 8-fold greater antiproliferative activity than TEM, which showed no EGFR targeting properties (IC_{50} competitive binding >100 μM); 5) under continuous drug exposure (3–6-day sulforhodamine and clonogenic assays), SMA41 was almost equipotent with SMA52; however, in a short 2-h drug exposure followed by incubation in drug-free media, SMA52 showed an almost complete loss of antiproliferative activity over the whole dose range. In contrast, SMA41 retained almost 100% of its activity, indicating a more sustained growth inhibitory activity. The results in toto suggest that the superior antiproliferative activity of SMA41 may be due to a combination of events associated with its binary EGFR TK and DNA targeting properties.

Over the past 20 years, acquired resistance mediated by DNA repair enzymes has often imposed severe limitations on the use of DNA-interactive agents and in many cases useful clinical antitumor activity could only be observed with the administration of multiple antitumor drugs of different mechanisms of action. Based upon this observation, we surmised that novel compounds with multiple intracellular targets would be more effective against resistant tumors than their classical counterparts. In this article, we describe the first attempt to combine two major mechanisms of action (inhibition of tyrosine kinase-mediated signaling and DNA targeting) into one single molecule (e.g., SMA41). The biochemical responses to the latter molecule were studied in the resistant Mer+ DNA repair-proficient human epidermoid carcinoma of the vulva cell line A431, which coexpresses high levels of EGFR TK.

The overexpression and dysfunction of TKs, directly or indirectly implicated in mitogenic signaling in tumor cells, have been extensively studied and are now considered the major functional differences between normal and tumor cells (Tsai et al., 1993; Sinha et al., 1995; Kondapaka and Reddy, 1996; Alaoui-Jamali et al., 1997; Sherwood et al., 1999). Because of their significant involvement in tumor progression, overexpressed receptor TKs have now become the modern targets for drug design and selective chemotherapeutic interventions (Levitzki and Gazit, 1995; Carroll et al., 1997;

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ABBREVIATIONS: EGFR, epidermal growth factor receptor; TK, tyrosine kinase; RTK, receptor tyrosine kinase; TEM, temozolomide; MTIC, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; DMSO, dimethyl sulfoxide; SRB, sulforhodamine B; HPLC, high pressure liquid chromatography; PGT, poly(L-glutamic acid-L-tyrosine, 4:1); PBS, phosphate-buffered saline; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; EGF, epidermal growth factor; MGMT, O^6-methylguanine DNA methyl transferase.

Deininger et al., 1997). One such target is EGFR, which, in many patients, is associated with aggressive tumor progression and invasion (Turner et al., 1996; Moyer et al., 1997; Modjtahedi and Dean, 1998; Xie et al., 1999). It has already been demonstrated that blocking signal transduction mediated by the TK activity of EGFR translates into significant antitumor activity both in vitro and in vivo, and two novel agents are now in phase II clinical trials (Ward et al., 1994; Levitzki and Gazit, 1995; Rewcastle et al., 1995, 1997, 1998; Lanzi et al., 1997; Moyer et al., 1997). Despite being significantly less toxic than previous cytotoxic agents, in tumors where they cannot induce apoptosis, most TK inhibitors currently in clinical trial present the disadvantage of being cytostatic agents that induce reversible growth inhibitory activity (Smaill et al., 1999).

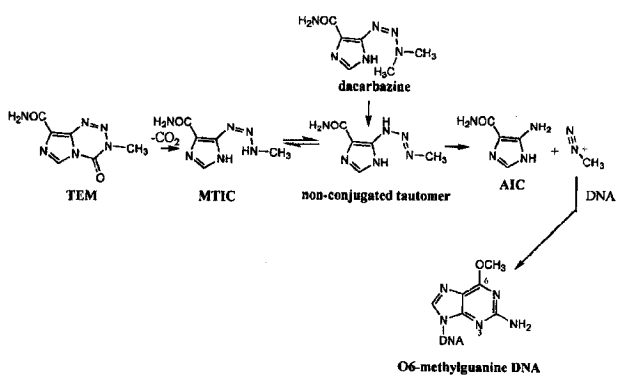
The disadvantages associated with both the classical cytotoxic agents and the modern RTK inhibitors, as well as the need for novel targets to circumvent DNA repair-associated chemoresistance, stimulated our interest in designing molecules with mixed EGFR tyrosine kinase and DNA targeting properties.

SMA41 exhibits two distinct structural characteristics: 1) a 1,2,3-triazene linkage, the pharmacophore of the active metabolites of dacarbazine or TEM; and 2) a 4-anilinoquinazoline moiety, the pharmacophore of the potent quinazoline class of EGFR TK inhibitors, which are now in clinical trial (Ching et al., 1993; Moyer et al., 1997).

The 3-alkyl-1,2,3-triazenes such as TEM, or its metabolite MTIC, are known to heterolyze to an aromatic amine (e.g., 5-aminoimidazole-4-carboxamide, Scheme 1) and an alkyldiazonium species (e.g., methyldiazonium, Scheme 1) under hydrolytic conditions (Cameron et al., 1985; Gibson et al., 1986; Baig and Stevens, 1987). Their mechanism of action is primarily based upon the generation of alkyldiazonium species that alkylate the 6- and 7-position of guanine in DNA. Substantial evidence suggests that alkylation of DNA at the O6 position of guanine is the cytotoxic lesion induced by 3-methyl or 3-(2-chloroethyl)-1,2,3-triazenes (Bodell et al., 1985; Tisdale, 1987; Baer et al., 1993; Pegg et al., 1995). Mer+ cells expressing elevated levels of MGMT, an enzyme capable of repairing the O^6-alkylguanine lesion, show significant resistance to the action of alkylating agents such as TEM or its metabolite MTIC (Tisdale, 1987; Lee et al., 1991; Chen et al., 1993; Mitchel and Dolan, 1993). The choice of the triazene TEM as a control drug in this study was inspired by its proven clinical activity in the treatment of solid tumors such as gliomas and malignant melanoma and its significant inactivity in Mer+ cells.

On the other hand, the aninilinoquinazolines are a novel class of highly receptor type-specific compounds that inhibit EGFR-related signal transduction by competition at the ATP binding site (Ward et al., 1994). The significant number of structure-activity relationship studies on 4-anilinoquinazolines and pyrido[d]pyrimidines as EGFR TK inhibitors is consistent with the compounds binding to the ATP site of EGFR (Rewcastle et al., 1995, 1997, 1998). Molecular modeling suggests that the N-1 atom (see SMA41, Scheme 2) accepts an H-bond from Met-769, N-3 accepts an H-bond from the side chain of Thr-766 on strand 5 deep in the binding cleft, and the anilino moiety is located in an adjacent hydrophobic pocket. The model suggests that the only positions on the inhibitors where substituents can be altered without affecting binding affinity are the 6- and 7-positions, which are located at the entrance of the binding cleft (Rewcastle et al., 1995). Indeed a variety of compounds with bulky side chains on the 6- and 7-positions were synthesized and found to retain significant binding affinity for the EGFR ATP binding site (Rewcastle et al., 1997). We therefore decided to append the alkyltriazene moiety to the 6-position of the quinazoline heterocycle. Thus, as outlined in Scheme 2, based upon the mechanism of hydrolytic cleavage of 1,2,3-triazenes and the structure-activity relationship of quinazolines, SMA41 was designed to release 1) SMA52, a competitive inhibitor of the ATP binding site of EGFR, and 2) the DNA damaging methyldiazonium species. In addition, this chimeric molecule was designed to remain small enough to be able to interact with the receptor on its own. This property was introduced with the purpose of targeting high EGFR-expressing cells. Interactions of SMA41 with the ATP binding site of EGFR would promote its intracellular retention, thereby favoring more intra- than extracellular degradation of this drug.

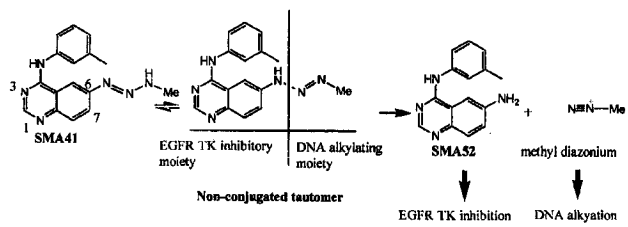
SMA41 was found to be able to release SMA52 in serum-containing cell culture media and to possess 1) a dual DNA damaging and a cellular phosphotyrosine inhibitory activity, and 2) superior antiproliferative effects compared with its clinical triazene counterpart TEM and its metabolite SMA52 alone. This novel strategy is designated as the "combi-targeting concept".



Scheme 1.

Materials and Methods

Drug Treatment. SMA41 and SMA52 were synthesized in our laboratories according to known procedures (Cameron et al., 1985; Manning et al., 1985; Rewcastle et al., 1995). Temozolomide was provided by Schering-Plough Inc. (Kenilworth, NJ). In all assays, drug was dissolved in DMSO and subsequently diluted in sterile RPMI-1640 media containing 10% fetal bovine serum (Life Technologies, Burlington, Canada) immediately before the treatment of cell



Scheme 2.

cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

Cell Culture. A431 cells (American Type Culture Collection, Manassas, VA) were maintained in a monolayer culture at 37°C in a humidified environment of 5% CO_2, 95% air. The cultures were maintained in RPMI-1640 supplemented with fetal bovine serum (10%), penicillin (50 U/ml), and streptomycin (50 mg/ml) (Life Technologies). Cells were maintained in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/m trypsin and 0.2 mg/ml EDTA and replating before confluence. In all assays, the cells were plated for 24 h before drug administration.

Growth Inhibition Studies. Cell monolayers were exposed to different concentrations of each drug continuously for 72 h. Under short exposure, they were treated with each drug for 2 h and allowed to recover for 72 h in drug-free medium.

For the SMA52 + TEM combination, the drugs were mixed at a 1:7 (SMA52/TEM) molar ratio, serially diluted, and added to the monolayers for 72 h. IC_{50} values were determined using the median effect equation as described by Chou and Talalay (1984). The nature of drug interactions was determined using eq. 1 where CI_{50} values >1, =1, or <1 indicate antagonism, additivity, or synergism, respectively (Chou and Talalay, 1984).

$$CI_{50} = \frac{IC_{50}\text{ (TEM in combination)}}{IC_{50}\text{ (TEM alone)}} + \frac{IC_{50}\text{ (SMA52 in combination)}}{IC_{50}\text{ (SMA52 alone)}}$$

All growth inhibitory activities were evaluated using the SRB assay (Skehan et al., 1990). Briefly, following drug treatment, cells were fixed using 50 μl of cold trichloroacetic acid (50%) for 60 min at 4°C, washed five times with tap water, and stained for 30 min at room temperature with SRB (0.4%) dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid and allowed to air dry. The resulting colored residue was dissolved in 200 μl of Tris base (10 mM), and optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

Colony-Forming Assays. Clonogenic assays were performed as previously described (Jean-Claude et al., 1999). Briefly, cells were plated at a density of 500 cells/well and continuously exposed to each drug for 6 days. Colonies were fixed with methanol (100%) and stained with methylene blue (0.5%) after which they were counted with the SynGene GeneTools colony-counting software package (Cambridge, UK). Only colonies with pixel areas of four or greater were counted. Data are means and standard deviations of two independent determinations.

Degradation. SMA41 (1 mg) was dissolved in DMSO (500 μl), added to RPMI-1640 with 10% fetal bovine serum (2 ml), and incubated for 24 h at 37°C. Thereafter, proteins were precipitated by addition of acetonitrile (3.5 ml) and the supernatant collected by centrifugation. The concentration of SMA52 deriving from the degradation of SMA41 was calculated using a standard curve obtained from the serial dilution of independently synthesized SMA52 incubated in serum-containing medium under identical conditions. HPLC analyses were performed on a Hewlett Packard 1090 liquid chromatograph, using a Deltapak C4 15-μm 300 × 3.9-mm column (reverse phase) to characterize and quantitate the products resulting from the degradation of SMA41. The operating mode was isocratic and two solutions A (50% acetonitrile) and B (50% water) were used with a 0.5-ml/min flow rate and a-5 μl injection volume. Under these conditions, independently synthesized SMA52 and SMA41 showed retention times of 11 and 15 min, respectively. For the rapid quantitation of metabolite, a less polar acetonitrile/water (70:30) eluent was used. Under such conditions SMA52 showed a retention time of 7.49 min. For liquid chromatography-mass spectrometry analysis of the degradation of SMA41, the column was placed on a Spectra System P1500 HPLC coupled with a Finnigan LCQDUO mass spectrometer.

The half-life of SMA41 under physiological conditions was studied by UV-spectrophotometry using an Ultrospec 2000 Pharmacia Biotech spectrophotometer. SMA41 was dissolved in a minimum volume of DMSO, diluted with RPMI-1640 medium supplemented with 10% serum, and absorbances read at 340 nm in a UV cell maintained at 37°C with a circulating water bath. The half-life was estimated by a one-phase exponential decay curve-fit method using the GraphPad software package (GraphPad Software, Inc., San Diego, CA).

EGFR Binding Assay. Nunc MaxiSorp 96-well plates were incubated overnight at 37°C with 100 μl/well of 0.25 mg/ml poly(L-glutamic acid-L-tyrosine, 4:1) PGT in PBS. Excess PGT was removed and the plate was washed three times with Tween 20 (0.1%) in PBS. The kinase reaction was performed as previously described using 15 ng/well EGFR affinity-purified from A431 cells (Moyer et al., 1997; Vincent et al., 2000) (generous gift from Pfizer Inc., Groton, CT, and commercial supplies from BIOMOL, Plymouth Meeting, PA). The compound was added and phosphorylation initiated by the addition of ATP. After 8 min at room temperature with constant shaking, the reaction was terminated by aspiration of the reaction mixture and rinsing the plate four times with wash buffer [Tween 20 (0.1%) in PBS]. Phosphorylated PGT was detected following a 25-min incubation with 50 μl/well of HRP-conjugated PY54 anti-phosphotyrosine antibody diluted to 0.2 μg/ml in blocking buffer (3% bovine serum albumin; 0.05% Tween 20). Antibodies were removed by aspiration, and the plate washed four times with wash buffer. The signals were developed by the addition of 50 μl/well of 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersberg, MD) and following blue color development, 50 μl of H_2SO_4 (0.09 M) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550).

EGF-Induced Autophosphorylation Assay. A431 cells were preincubated in a six-well plate (1×10^6) with 0.1% serum at 37°C overnight for 24 h after which they were exposed to a dose range of each drug for 2 h and subsequently treated with 50 ng/ml EGF for 30 min at 37°C. Thereafter, they were washed with PBS and resuspended in cold lysis buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% Nonidet P-40, 1 mM EDTA; 5 mM NaF; 1 mM Na_3VO_4; protease inhibitor tablet (Roche Biochemicals, Laval, Canada)]. The lysates were kept on ice for 30 min and collected by centrifugation at 10,000 rpm for 20 min at 4°C. The protein concentrations were determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (40 μg/ml) from each lysate were added to a 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding on the polyvinylidene difluoride membrane was minimized with a blocking buffer containing nonfat dry milk (3%) in PBS. The membrane was incubated with primary antibodies [either anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) for the detection of phosphotyrosine, or anti-EGFR (Neomarkers, Fremont, CA) for determination of corresponding receptor levels, and anti-β-tubulin (Neomarkers) for the detection of equal loading]. Thereafter, blots were incubated with HRP-goat anti-mouse antibody (1:200 dilution; Bio-Rad Laboratories) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Band intensities were measured using the SynGene GeneTools software package.

EGF-Induced Total Cellular Tyrosine Phosphorylation Assay. A431 cells were preincubated in a 96-well plate (1×10^6) with 0.1% serum at 37°C overnight. The drugs were added for 15 min in serum-free media, and cells were subsequently given EGF (50 ng/ml) for 30 min at 37°C. Cells were fixed with a 1:1 mixture of methanol and acetic acid for 30 min at 4°C. Nonspecific binding was blocked with bovine serum albumin (1%) in PBS for 1 h at 37°C, after which 0.1 μg/ml HRP-conjugated anti-phosphotyrosine antibody (Upstate Biotechnology) was added in the same buffer for 1 h at room temperature. 3,3′,5,5′-Tetramethylbenzidine peroxidase (200 μl) substrate (Kierkegaard and Perry Laboratories) was added to each well

and following blue color development, H_2SO_4 (0.9 M) was administered to each well, and plates were read at 450 nm.

Alkaline Comet Assay for Quantitation of DNA Damage. A modified alkaline comet assay technique was used to quantitate DNA damage induced by SMA41, SMA52, and TEM. A431 cells were exposed to drugs for 30 min or 2 h, and harvested with trypsin-EDTA. The cells were subsequently collected by centrifugation and resuspended in PBS. The resulting cell suspension was diluted to approximately 10^6 cells, and mixed with agarose (1%) in PBS at 37°C in a 1:10 dilution. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel casting chambers, as previously described (McNamee et al., 2000), and then immediately placed into a lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base, 1% (w/v) *N*-lauryl sarcosine, 10% (v/v) DMSO, and 1% (v/v) Triton X-100]. After being kept on ice for 30 min, the gels were gently rinsed with distilled water and then immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base), containing 1 mg/ml proteinase K for 60 min at 37°C. Thereafter, the gels were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed into 1 M ammonium acetate for 30 min. They were further soaked in 100% ethanol for 2 h, dried overnight, and subsequently stained with SYBR Gold (1/10,000 dilution of stock supplied from Molecular Probes, Eugene, OR) for 20 min. For evaluation of comets, DNA damage was assessed using the Tail Moment parameter (i.e., the product of the distance between the barycenters of the head and the tail of the comet). A minimum of 50 cell comets was analyzed for each sample, using ALKOMET version 3.1 software, and values are an average of tail moments for the entire cell population.

Results

Degradation of SMA41

SMA41 was allowed to degrade in RPMI-1640 supplemented with 10% fetal calf serum at 37°C and its half-life measured by UV-spectrophotometry. It was found to be significantly stable with a $t_{1/2}$ of approximately 30 min in serum-containing cell culture medium at 37°C. SMA41 decomposed almost exclusively into SMA52, the structure of which was confirmed both by HPLC analysis of independently synthesized SMA52 and by liquid chromatography-mass spectrometry analyses (Fig. 1), which showed a mass M + 1 = 251 for the chromatogram peak corresponding to its retention time. Quantification of this peak and calculations using standard curves indicated that SMA41 was converted to SMA52 in a yield of approximately 81%.

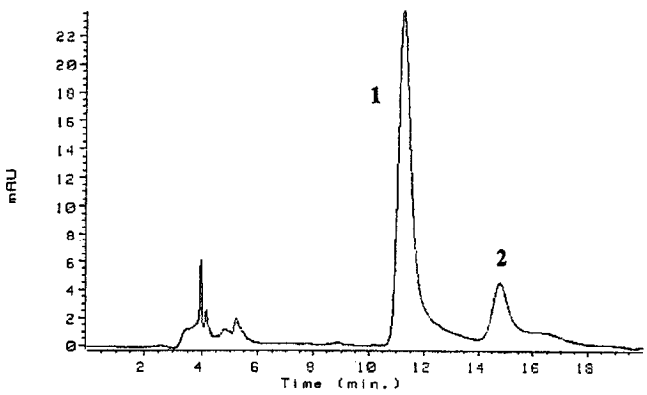


Fig. 1. Partial degradation of SMA41 to SMA52 in RPMI-1640 medium supplemented with 10% serum at 37°C. The peak at around 11 min (1) corresponds to SMA52 and the one at 15 min (2) represents undegraded SMA41. Peaks in 2- to 6-min range are medium components. An almost exclusive decomposition of SMA41 into SMA52 was observed.

Antiproliferative Activity in A431 Cells

The SRB assay was used to evaluate the antiproliferative activity of different compounds in the human squamous carcinoma of the vulva cell line A431 in which EGFR constitutive activity, as reflected by tyrosine phosphorylation under basal conditions, has been shown to be sensitive to antiproliferative agents targeting the EGFR in vitro or in vivo (Lanzi et al., 1997). In addition, this cell line expresses detectable levels of the DNA repair enzyme MGMT (Fornace et al. 1990). The MGMT status of our A431 cell line was also confirmed by Western blotting using a commercially available anti-MGMT antibody (Pharmingen International, Toronto, Canada) (data not shown). Under 72-h continuous exposure the results, illustrated by Fig. 2a, showed that SMA41 is 1.8-fold more potent (IC_{50} = 36 μM) than its metabolite SMA52 alone (IC_{50} = 59 μM, Fig. 2b), and 10-fold more potent than TEM (IC_{50} = 366 μM, Fig. 4) in the MGMT-proficient cell line A431. A clonogenic assay as illustrated by Fig. 3 showed that the antiproliferative activity of SMA41 was in the same range as that of SMA52 (IC_{50} SMA41 4 μM, IC_{50} SMA52 3.7 μM). However, when the cells were treated for only 2 h and further incubated in drug-free medium, an almost complete loss of activity was observed for SMA52 (IC_{50} > 100 μM, Fig. 2b), indicating that it induced significantly reversible growth inhibitory activities. In contrast, SMA41 showed significant retention of activity with little change in the IC_{50} values [IC_{50} (2 h) = 36 μM, IC_{50} (72 h) = ~30 μM].

To demonstrate the antiproliferative advantages of combining the EGFR and DNA targeting mechanisms in a single molecule, we studied the combined effect of SMA52 (independently synthesized) with that of TEM using the SRB assay (Fig. 4). Using eq. 1 to determine the nature of interactions between these two drugs, the results showed that the CI_{50} at the 50% effect for SMA52 + TEM is approximately 0.6, indicating a subadditive interaction. However, under identical conditions the antiproliferative activity of the chimeric SMA41 was 4-fold more pronounced than that of the two-drug combination (IC_{50} = 134 μM).

Binary Targeting Properties of SMA41

The significant antiproliferative activity of SMA41 in a methyltriazene-resistant cell stimulated our interest in further dissecting its binary (EGFR and DNA) targeting properties. This was achieved by two types of assays: EGF-stimulated tyrosine phosphorylation and DNA damage.

Inhibition of EGFR TK Activity. In a competitive EGFR binding assay (Fig. 5), SMA41 (IC_{50} = 0.2 μM) showed a 5-fold stronger binding affinity than SMA52 (1.02 μM) for the ATP site of the purified receptor. TEM did not show any significant affinity for this receptor (IC_{50} > 100 μM). In an ELISA-based whole cell assay, SMA41 and SMA52 showed comparable levels of inhibition of EGF-induced total cellular phosphorylation (Fig. 6). Similarly, Western blot analysis (Fig. 7) demonstrated that both drugs induced almost equal levels of inhibition of EGF-induced EGFR autophosphorylation (IC_{50} SMA52 = 8.44 μM, IC_{50} SMA41 12.5 μM). In contrast to SMA41 and SMA52, TEM did not exhibit any EGFR binding affinity, nor did it inhibit EGF-induced auto-

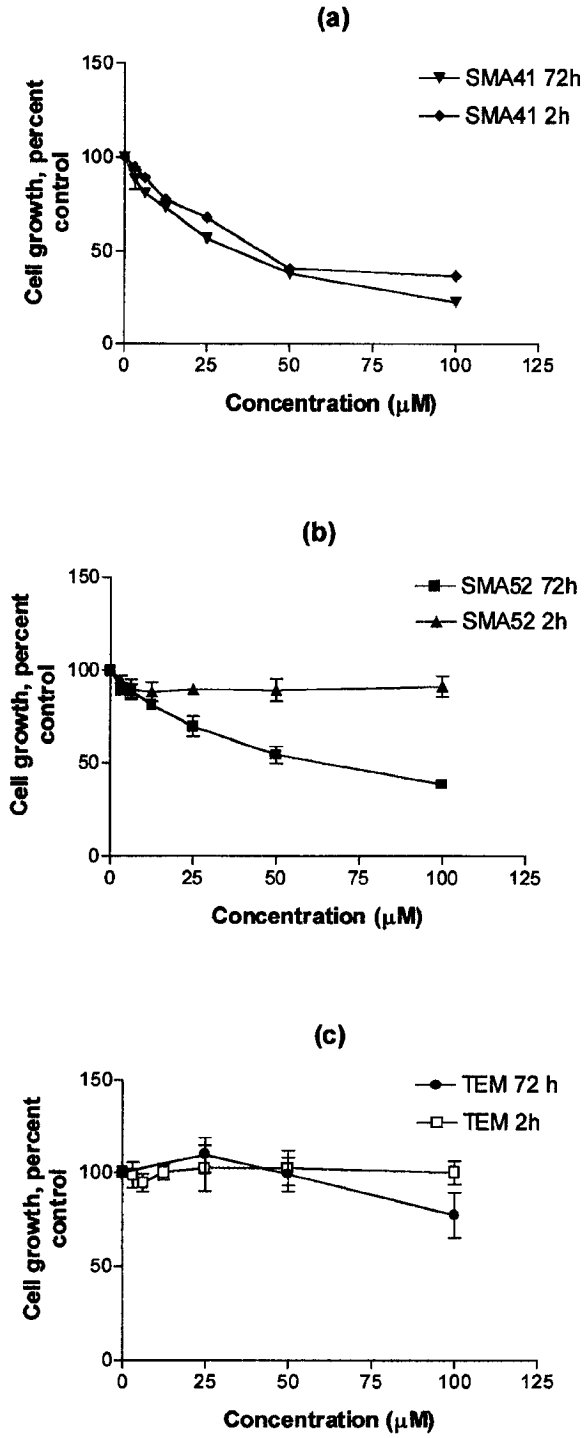


Fig. 2. Reversibility of antiproliferative effects of SMA41 (a), SMA52 (b), and temozolomide (c) on A431 cells. Cells were exposed to each drug for either 2 h, after which they were allowed to recover for 72 h in drug-free medium, or continuously for 72 h. Cell growth was measured using the sulforhodamine B assay. Each point represents at least two independent experiments run in triplicate.

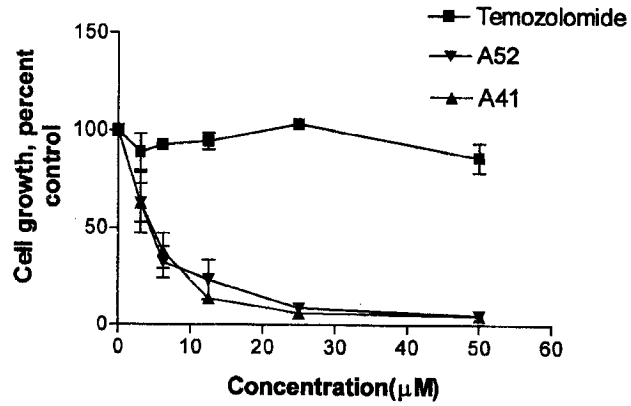


Fig. 3. Survival of clonogenic cells for SMA41, SMA52, and temozolomide. A431 cells were plated at a density of 500 cells/well and exposed to each drug continuously for 6 days, after which colonies were detected using Syngene imaging software. Each point represents at least two independent determinations.

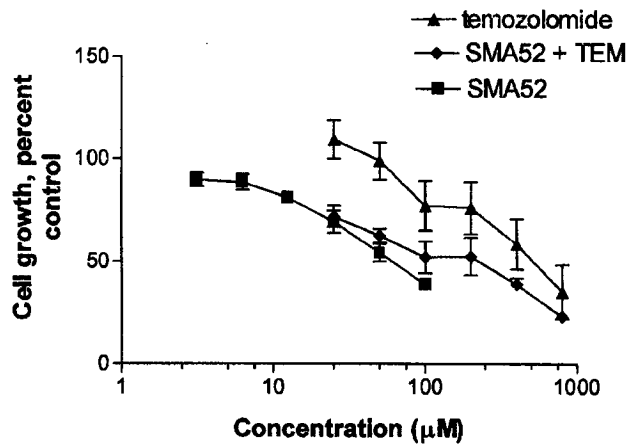


Fig. 4. Effects of SMA52, TEM, and SMA52 + TEM on the growth of A431 cells. The cells were exposed to each drug continuously for 72 h, and cell growth measured using the sulforhodamine B assay. Each point represents at least two independent experiments run in triplicate.

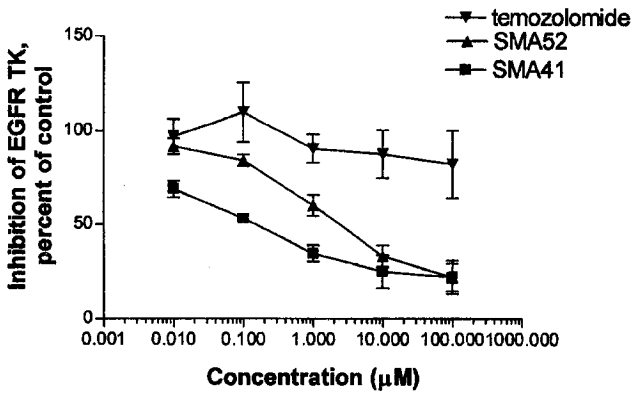


Fig. 5. Inhibition of purified EGFR tyrosine kinase activity by SMA41, SMA52, and temozolomide. Phosphorylation of a PGT substrate by purified EGFR was measured using immunoassay with anti-phosphotyrosine antibodies. Each point represents at least three independent experiments.

phoshorylation in A431 cells (IC_{50} > 100 μM) in the specified dose ranges.

Quantitation of DNA Damage. Using the alkaline comet assay, it was demonstrated that, in contrast to SMA52 (Fig. 8c), both SMA41 and TEM were capable of inducing DNA damage in a dose-dependent manner. However, differences were observed in the kinetics of dose-dependent DNA damage induced by SMA41 compared with TEM. For SMA41, the trend was to induce rapid nuclear condensation at the high-

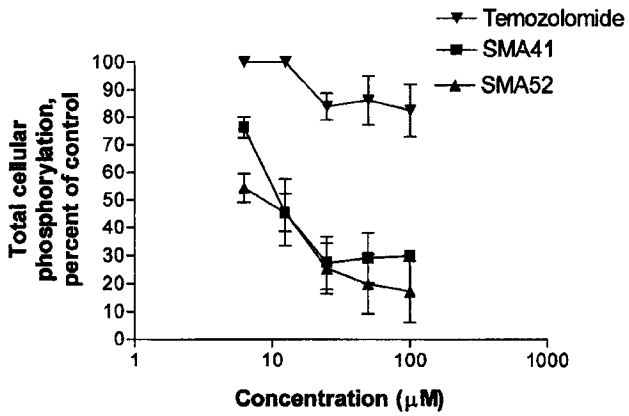


Fig. 6. Inhibition of EGF-induced total cellular phosphorylation by SMA41, SMA52, and temozolomide. Total cellular phosphorylation was measured in A431 cells using an immunoassay with anti-phosphotyrosine antibodies. Each point represents at least two independent experiments run in duplicate.

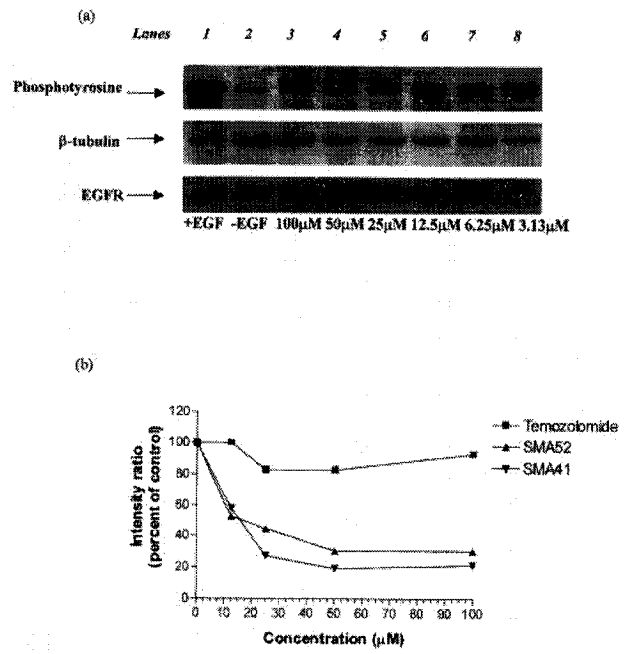


Fig. 7. a, inhibition of EGF-stimulated autophosphorylation by SMA41 in A431 cells. Cells were exposed to the indicated concentrations (μM) of drug for 2 h, after which they were stimulated with 50 ng/ml EGF (lanes 1 and 3–8). A nonstimulated control is shown in lane 2. Cell lysates were prepared following a 30-min exposure to EGF, and phosphotyrosine was detected by Western blotting with anti-β-tubulin used as a loading control. Membranes were stripped of anti-phosphotyrosine and reprobed with anti-EGFR antibodies to detect receptor levels. b, comparison between the inhibition of EGF-stimulated autophosphorylation by SMA41, SMA52, and temozolomide in A431 cells. The film was scanned and the bands were analyzed and quantified using Syngene GeneTools software. Values are percentage of control of phosphotyrosine/β-tubulin.

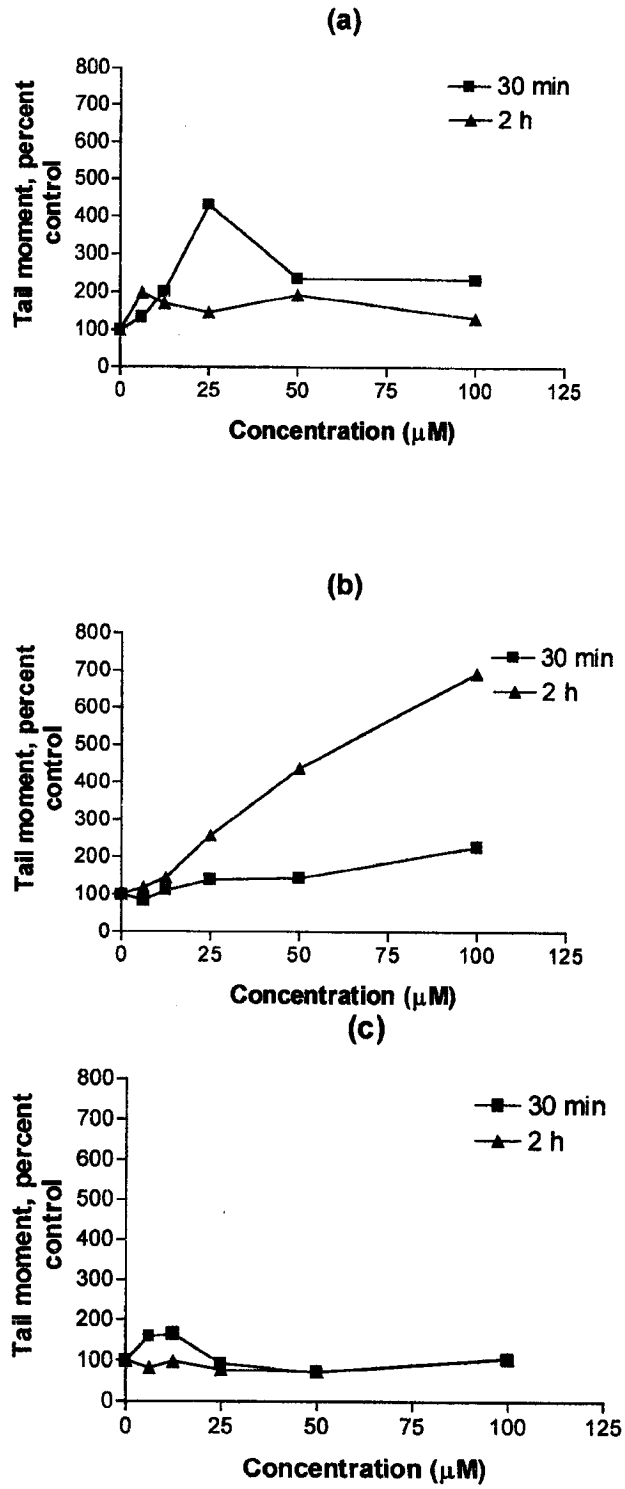


Fig. 8. Quantitation of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in A431 cells exposed to SMA41 (a), TEM (b), and SMA52 (c) for 30 min or 2 h.

est doses (25–100 μM), leading to a reduction in comet tailing. For SMA41, significant comet tail moment could only be observed in the 6 to 25 μM range after a short 30-min and 2-h drug exposures (Fig. 8a). With a 2-h exposure, a decrease in tail moment was observed at concentrations above 6 μM, concurrent with observable nuclear condensation likely due to a rapid onset of apoptosis. In contrast, TEM (Fig. 8b) exhibited a dose-dependent increase in comet tail moment under 30-min exposure with a remarkable enhancement under the longer 2-h drug exposure (Fig. 8b). This is prima facie evidence that despite being two methylating agents, the mechanisms of action of SMA41 may be markedly different from that of TEM.

Discussion

Overexpression of EGFR is common in a wide variety of major human solid tumors of epithelial origin such as breast, colorectal, and head and neck ovarian and bladder carcinomas (Yaish et al., 1988; Lanzi et al., 1997; Modjtahedi and Dean, 1998). EGF binding induces receptor dimerization, autophosphorylation, and activation of mitogenic signaling. The A431 cell line expresses a large number of EGF binding sites and also the high-affinity EGFR ligand transforming growth factor TGFα (Lanzi et al., 1997). This translates into aggressive autocrine-controlled growth in vitro. Blocking A431 cell proliferation has become the standard screen for antiproliferative inhibitors of EGFR TK activity (Yaish et al., 1988; Lanzi et al., 1997). This cell line also expresses the alkyltriazene resistance-associated DNA repair enzyme MGMT and is as demonstrated herein (Fig. 2c) resistant to the cyclic 1-methyl-1,2,3-triazene TEM (IC_{50} = 366 μM). Therefore, it represents a good model for the determination of the pharmacological advantages of simultaneous targeting of EGFR and DNA in EGF-expressing refractory tumors.

Dacarbazine and TEM, two prodrugs of monomethyltriazenes, are the most active drugs in the treatment of malignant melanomas and gliomas (Carter et al., 1976; Hill et al., 1989; Lee et al., 1992; Carter et al., 1994). As outlined in Scheme 1, the cytotoxic monoalkyltriazene MTIC degrades under physiological conditions to generate a variety of metabolites, the critical reaction being the heterolysis of the nonconjugated tautomer to generate the arylamine 5-aminoimidazole-4-carboxamide and the alkyldiazonium species (Kolar et al., 1980; Cameron et al., 1985; Foedstad et al., 1985; Manning et al., 1985). It has already been shown by isotopic labeling that the latter species alkylate DNA at the 6- and 7-positions of guanine or 3-position of adenine. Because we have demonstrated that SMA41 is able to generate, like MTIC, a free arylamine (SMA52), we expected its concomitantly generated metastable methyldiazonium to induce the same type of alkali-labile DNA lesions as those associated with TEM or other classical triazenes. Indeed, in contrast to SMA52-exposed cells, significant levels of DNA damage were observed in those treated with SMA41 in the 6.25 to 25 μM range and the analyses were complicated by nuclear condensation at concentrations higher than its growth inhibitory IC_{50} under both 30-min and 2-h drug exposure (SRB). Since this assay involved alkaline electrophoresis of the whole cell nuclei, we believe that this fragmentation is primarily due to N7-methylguanine, a type of lesion with known alkali-labile properties (Catapano et al., 1987). The quantitation of this type of alkali-labile DNA lesion by the classical alkaline elution assay is now well documented (Pera et al., 1981; Hartley et al., 1986). Single-cell microelectrophoresis (comet) assay clearly showed that, like TEM, the clinical prodrug of MTIC, SMA41 possesses strong DNA damaging properties. However, the marked differences between dose-response profiles of SMA41 and TEM (DNA damage, SRB, and clonogenic assays) indicate that these two methylating agents may block cell proliferation by a different mechanism. It is noteworthy that the activity of SMA41 was approximately 8-fold greater in the clonogenic assay than in the SRB assay. This can be attributed to an increased exposure time (6-day continuous exposure).

The second target of SMA41 was first elucidated by measuring its ability to block EGFR TK phosphorylation of a PGT substrate in an ELISA assay. It should be first remembered that the design of SMA41 was primarily based upon previously identified structure activity-relationships in the quinazoline series, showing that bulky substituents are tolerated at the 6- and 7-positions. In addition, electron-donating substituents increase their binding affinity for the ATP binding site of EGFR. Since the delocalization of electrons from N3 of the triazene chain may confer only a slight electron-donating character to SMA41, we believe that its stronger EGFR TK inhibitory activity compared with SMA52 [which contains a stronger electron-donating group at the 6-position (Hammett constant $\sigma_p = -0.57$, $\sigma_m = -0.09$) (Hammett, 1960; Andrejus, 1988; Jean-Claude and Williams, 1998)] may be due to its ability to induce methylation of nucleophilic amino acid side chains in the ATP binding site of the receptor (e.g., thiol function of a cysteine residue). It has recently been demonstrated that quinazolines bearing an acrylamide group at the 6-position are capable of alkylating the cysteine 773 at the TK active site (Smaill et al., 1999; Jeff et al., 2000), and that the 6-position is 4 Å closer to this cysteine residue than the 7-position. The nonconjugated form of the alkyltriazene moiety, having approximately the same length as the acrylamide moiety and being a strong alkylator, may undergo a similar type of alkylation in the active site of EGFR. Moreover, since an exposure time (8 min) shorter than the $t_{1/2}$ was used in the ex vivo tyrosine kinase assay, the observed inhibitory activity is mainly due to the binding of the intact molecule with minimal contribution of the residual SMA52 metabolite.

Having demonstrated that SMA41 is able to target isolated receptors, we investigated whether this agent could block signal transduction in A431 cells. An EGF-induced total phosphorylation assay showed that, like SMA52, SMA41 is capable of inhibiting EGF-induced total TK phosphorylation in a dose-dependent manner in A431 cells. In addition, SMA41 is capable of blocking EGF-induced autophosphorylation of the EGFR. It is important to mention that no detectable levels of tyrosine kinase inhibitory activity were observed with TEM over its whole dose range. The detection of autophosphorylation inhibitory activities for SMA41 and SMA52 is an indirect evidence of normal transport of these compounds across the cell membrane. Although it is not clear at this stage whether SMA41 is sequestered in the cells before its hydrolytic cleavage to SMA52 or whether significant extracellular cleavage occurred before cell penetration of these two species. However, some inference can be made in light of the macromolecular targeting results.

In contrast to the isolated enzyme assay that showed a 5-fold superior inhibitory activity for SMA41 compared with SMA52, the whole cell phosphorylation assays exhibited similar levels of activity for these two drugs. If no DNA damage was observed, these results could indicate a total extracellular conversion of SMA41 to SMA52 before cell penetration. However, the significant nuclear fragmentation and the marked retention of activity observed when SMA41 was removed after 2 h (Fig. 2a) are indirect evidences of intracellular sequestration of SMA41 since as we have demonstrated, SMA52 alone does not possess DNA damaging properties (Fig. 8c). The loss of TK inhibitory activity of SMA41 compared with SMA52 (from the isolated enzyme to the whole cell assays) may be due to differences in the intracellular distributions of these drugs. Nevertheless, the re-

sults in toto give prima facie evidence that SMA41 is a novel triazene with a significant EGFR tyrosine kinase inhibitory activity, a property that has never been observed before for any class of mono- or dialkyltriazenes.

Based upon known principles of medical oncology, which suggest that rapidly proliferating cells are more sensitive to DNA damaging agents than slow-growing ones, it was feared that the cytostatic effect of EGFR TK inhibition would initially block proliferation and thereby decrease cell sensitivity to the DNA damage associated with the concomitantly generated methyldiazonium species. This would translate into a rather antagonistic effect. To test this hypothesis, we mimicked the combined effect of the two mechanisms of action by designing a two-drug combination model involving SMA52 (an EGFR TK inhibitor) and TEM (a DNA damaging agent). The results showed a subadditive interaction and not an antagonistic one between these two drugs. Moreover, it is noteworthy that SMA41 was more potent than the two-drug combination. This suggests that a single molecule formulated as a masked form of these two types of agents may be more efficacious than a two-drug combination encompassing individual monoalkyltriazenes and EGFR TK inhibitors.

Since SMA41 can both block phosphorylation induced by EGF and damage genomic DNA, its over 8-fold (SRB assay) and over 90-fold (clonogenic assay) greater potency compared with TEM may result from the combined effects of these two distinct mechanisms of antiproliferative activities. The binary targeting may trigger signal transduction associated with the induction of apoptosis. Indeed, in contrast to SMA52 and TEM, significant nuclear condensation was observed in cells treated with SMA41 for 2 h in the 25 to 100 μM range. The effect of binary targeting on the expression and activity of MGMT and the mechanism of apoptosis induced by SMA41 are now being investigated in our laboratory and the results will be reported in due course.

A significant body of evidence has accumulated to suggest that overexpression of EGFR is a marker for poor prognosis in many solid tumors. Selective inhibitors of tyrosine phosphorylation by EGFR are now considered an important class of anticancer drugs and two members of the 4-(phenylamino) quinazoline class are now in clinical trial. Despite the significant EGFR inhibitory activity of these reversible inhibitors, the high intracellular concentrations of ATP is a major barrier to sustained inhibition of EGF-stimulated signal transduction in tumor cells. More recently, this problem was addressed by Smaill et al. (1999) who showed that quinazolines containing acryloyl function at the 6-position could induce irreversible inhibition of EGFR by alkylating cysteine 773 of the enzyme. A recently synthesized water-soluble analog of this class has now been selected for phase I clinical trial (Jeff et al., 2000). It is noteworthy that despite being irreversible inhibitor of EGFR, when apoptosis is not triggered, if the cells respond to alternative growth hormones (e.g., heregulin or platelet-derived growth factor) these compounds may still not induce a sustained growth inhibitory activity. Our novel SMA41 presents the advantage of being not only capable of blocking EGF-stimulated signal transduction on its own but also generating a DNA alkylating species that may inflict irreversible cytotoxic DNA lesions. Moreover, this compound was designed to release another intact EGFR TK inhibitory molecule (e.g., SMA52) that may further enhance its growth inhibitory activity. Our results showed that these combined properties conferred increased potency to a monoalkyltriazene against an MGMT-proficient tumor cell line with marked resistance to the clinical drug TEM (IC_{50} = 366 μM). Also, the current study, which was primarily designed to identify the principal targets of SMA41, has conclusively demonstrated that this one-molecule combination showed superior activity compared with a two-drug combination involving TEM + SMA52. Further studies are now ongoing to characterize the effects of SMA41 on growth stimulation by a wide variety of hormones, including EGF, transforming growth factor, platelet-derived growth factor, and insulin before the demonstration of in vivo efficacy of this novel approach termed the combi-targeting concept.

Acknowledgments

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ORIGINAL ARTICLE

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Differential responses of EGFR-/AGT-expressing cells to the "combi-triazene" SMA41

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Abstract *Purpose*: Previous studies have demonstrated enhanced potency associated with the binary [DNA/epidermal growth factor receptor (EGFR)] targeting properties of SMA41 (a chimeric 3-(alkyl)-1,2,3-triazene linked to a 4-anilinoquinazoline backbone) in the A431 (epidermal carcinoma of the vulva) cell line. We now report on the dependence of its antiproliferative effects (e.g. DNA damage, cell survival) on the EGFR and the DNA repair protein O6-alkylguanine DNA alkyltransferase (AGT) contents of 12 solid tumor cell lines, two of which, NIH3T3 and NIH3T3 HER14 (engineered to overexpress EGFR), were isogenic. *Methods*: Receptor type specificity was determined using ELISA for competitive binding, as well as growth factor-stimulation assays. DNA damage was studied using single-cell microelectrophoresis (comet) assays, and levels of EGFR were determined by Western blotting. The effects of SMA41 on the cell cycle of NIH3T3 cells were investigated using univariate flow cytometry. *Results*: Studies of receptor type specificity showed that SMA41: (a) preferentially inhibited the kinase activity of EGFR over those of Src, insulin receptor and protein kinase C (PKC, a serine/threonine kinase), (b) induced stronger inhibition of growth stimulated with EGF than of growth stimulated with platelet-derived growth factor (PDGF) or fetal bovine serum (FBS). Despite the EGFR specificity of SMA41, there was an absence of a linear correlation between the EGFR status of our solid tumor cell lines and levels of DNA damage induced by the alkylating component. Similarly, EGFR levels did not correlate with IC_{50} values. The antiproliferative activities of SMA41 correlated more with the AGT status of these cells and paralleled those of the clinical triazene temozolomide (TEM). However, throughout the panel, tumor cell sensitivity to SMA41 was consistently stronger than to its closest analogue TEM. Experiments performed with the isogenic cells showed that SMA41 was capable of inducing twofold higher levels of DNA damage in the EGFR transfectant and delayed cell entry to G_2/M in both cell types. When the cells were starved and growth-stimulated with FBS (conditions under which both cell types were growth-stimulated), in contrast to TEM, SMA41 and its hydrolytic metabolite SMA52 exhibited approximately nine- and threefold stronger inhibition of growth of the EGFR transfectant. *Conclusions*: These results suggest that, in addition to its ability to induce DNA damage and cell cycle perturbations, SMA41 is capable of selectively targeting the cells with a growth advantage conferred by EGFR transfection. When compared with the monoalkyltriazene prodrug TEM, its potency may be further enhanced by its ability to hydrolyze to another signal transduction inhibitor (SMA52) plus a DNA alkylating agent that may further contribute to chemosensitivity. Thus, our new "combi-targeting" strategy may well represent a tandem approach to selectively blocking receptor tyrosine kinase-mediated growth signaling while inducing significant levels of cytotoxic DNA lesions in refractory tumors.

Keywords Signal transduction · DNA damage · Cell cycle perturbation · Receptor-type specificity · O6-alkylguanine DNA alkyltransferase (AGT)

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Introduction

We have designed a novel strategy, termed the "combi-targeting" concept, to selectively target tumor cells

overexpressing receptor tyrosine kinases (RTK) or growing by an autocrine stimulatory loop. This strategy seeks to synthesize chimeric molecules referred to as "combi-molecules" (C-molecules) that possess the ability to: (a) inhibit RTK on their own, (b) generate, following hydrolytic scission under physiological conditions, another inhibitor of the same RTK and a cytotoxic DNA alkylating agent [1]. The combined effects of these three species (C-molecule, RTK inhibitor and alkylating agent) would be expected to induce significant antiproliferative activity in cells expressing RTK. Recently, we have demonstrated the feasibility of this approach by synthesizing our first C-molecule, SMA41 (Scheme 1), a masked combination of SMA52 (targeted to EGFR, see arrow) and a 3-methyl-1,2,3-triazene (precursor of the DNA-damaging methyldiazonium species), that shows over tenfold greater potency than the clinical triazene temozolomide (TEM) in the carcinoma of the vulva cell line A431 [1]. More importantly, the C-molecule shows fourfold greater activity than the combination of equitoxic doses of TEM and the "naked" inhibitor SMA52.

Since a large number of human solid tumors express high levels of EGFR, and many coexpress EGFR and O6-alkylguanine DNA alkyltransferase (AGT), we thought it of interest to study the correlation between the EGFR/AGT status of established tumor cell lines and their sensitivity to the C-molecule. The latter was designed to retain significant affinity for its cognate receptor, thereby creating conditions under which cell recognition would be assisted by its affinity for RTK, and further degradation into a secondary RTK inhibitor plus a DNA-damaging fragment would take place in the cytosol. Since SMA41 showed (a) a 0.2 μM affinity for EGFR, (b) the ability to degrade in serum-containing medium to generate 81% of SMA52 (IC_{50} EGFR inhibition 1 μM), and (c) DNA-damaging properties, we thought it of interest to study its differential effects on a panel of cell lines with various levels of AGT and EGFR expression.

Here, we report a complex relationship between the EGFR/AGT status of established cells and selective targeting of EGFR in an isogenic pair of NIH 3T3 and NIH 3T3-HER14 cells (engineered to overexpress EGFR). All cells lacking AGT were remarkably sensitive to SMA41 and TEM regardless of their EGFR status. While all cells expressing AGT were resistant to TEM, the antiproliferative activities of SMA41 were consistently stronger (10- to 50-fold) than those of TEM whether AGT was expressed singly or coexpressed with EGFR, confirming the superior antiproliferative properties of the C-molecule principle. More importantly, using conditions under which the sole difference between two NIH 3T3 clones was the EGFR gene, we demonstrated that, in contrast to TEM, the C-molecule selectively blocked serum-induced growth of the EGFR transfectant.

Materials and methods

Drug treatment

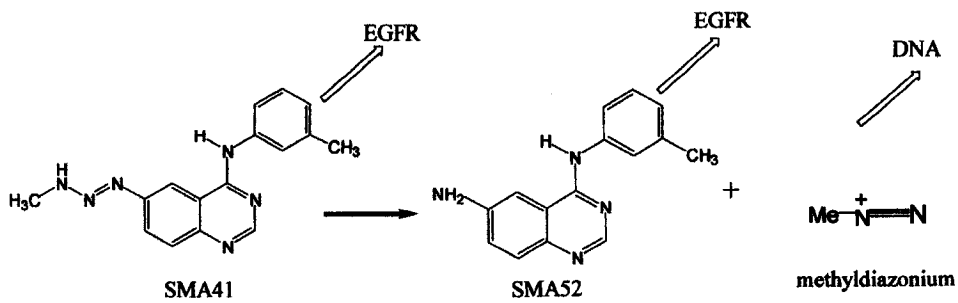
SMA41 and SMA52 were synthesized in our laboratories according to known procedures [2, 3]. TEM was provided by Schering-Plough (Kenilworth, N.J.). In all assays, the drug was dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in sterile RPMI-1640 medium containing 10% fetal bovine serum (FBS; Life Technologies, Burlington, Canada) immediately prior to the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% v/v.

Cell culture

The human tumor cell lines MDA-MB-435, MDA-MB-453, MDA-MB-468, MCF-7 (breast carcinoma), PC-3, DU-145 (prostate carcinoma), SF-126, and SF-188 (glioma) (ATCC, Manassas, Va.) were maintained in RPMI-1640 supplemented with FBS (10%), gentamicin (50 mg/ml), and HEPES (12.5 mM) (Wisent, St. Bruno, Canada). The mouse fibroblast cell line NIH 3T3, and its stable EGFR transfectant NIH 3T3/HER14 (generous gift from Dr. Moulay Aloui-Jamali), as well as MCF-10A (a normal breast cell line) were maintained in DMEM supplemented with donor calf serum (10%), gentamicin (50 mg/ml), and HEPES (12.5 mM) (Wisent). All cells were maintained in monolayer culture at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were maintained in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/ml of trypsin and 0.2 mg/ml of EDTA and replating before confluence. In all assays, the cells were plated for 24 h before drug administration.

Growth inhibition studies

For nonstimulated cell growth, approximately 5000 cells were plated per well in 96-well plates. Following a 24-h incubation, cell monolayers were exposed to different concentrations of drug continuously for 7 days. For growth factor- and serum-stimulated growth, approximately 5000 cells were plated in each well of a 48-well dish. After 48 h, the cells were washed three times with phosphate-buffered saline (PBS), and serum-free medium without



Scheme 1 C-molecule SMA41 and its degradation into a secondary RTK inhibitor (SMA52) plus a DNA-damaging fragment (methyldiazonium)

phenol red was added. Cells were serum-deprived for 24 h, after which growth factor (12 ng/ml EGF, 25 ng/ml TGF-α, or 50 ng/ml PDGF) or FBS (10%) were added with various concentrations of each drug. Growth inhibitory activities for both stimulated and nonstimulated growth were evaluated using the sulforhodamine B (SRB) assay [4]. Briefly, following drug treatment, cells were fixed using 50 µl cold trichloroacetic acid (50%) for 60 min at 4°C, washed five times with tap water, and stained for 30 min at room temperature with SRB (0.4%) dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid, and allowed to air-dry. The resulting colored residue was dissolved in 200 µl Tris-base (10 m*M*), and the optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

EGFR, Src, and insulin receptor binding assays

Nunc MaxiSorp 96-well plates were incubated overnight at 37°C with 100 µl per well of 0.25 mg/ml poly(L-glutamic acid-L-tyrosine) (4:1, PGT) in PBS. Excess PGT was removed and the plate was washed three times with Tween 20 (0.1%) in PBS. The kinase reaction was performed as previously described using 15 ng/well of EGFR affinity-purified from A431 cells [5] (generous gift from Pfizer, N.J., and commercial supplies from Biomol, Plymouth Meeting, Pa.). The compound was added and phosphorylation initiated by the addition of ATP. After 8 min at room temperature with constant shaking, the reaction was terminated by aspiration of the reaction mixture and rinsing the plate four times with wash buffer (0.1% Tween 20 in PBS). Phosphorylated PGT was detected following a 25-min incubation with 50 µl per well of PY54 anti-phosphotyrosine antibody conjugated with horseradish peroxidase (HRP) diluted to 0.2 µg/ml in blocking buffer comprising 3% bovine serum albumin (BSA) and 0.05% Tween 20. Antibodies were removed by aspiration, and the plate washed four times with wash buffer. The signals were developed by the addition of 50 µl per well of tetramethylbenzidine (TMB) peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersburg, Md.) and, following blue color development, 50 µl H_2SO_4 (0.09 *M*) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550). For the Src assay, 1.2 U/well of protein were used (Biomol), and 100 m*M* of EDTA was added to stop the phosphorylation reaction. For the Ins-R assay, 15 ng/well of protein was used (Biomol), and to stop the reaction, EDTA (250 m*M*) was added. In both cases, known inhibitors were used to calibrate the assay.

Protein kinase C assay

Myelin basic protein (MBP)-coated 96-well plates (UBI, Lake Placid, N.Y.) were reconstituted with PBS prior to the addition of drug diluted in assay dilution buffer II (UBI), protein kinase C (PKC) lipid activator, Mg/ATP cocktail, and purified PKC 25 ng/well (UBI). The reaction was allowed to occur at room temperature with constant shaking for 15 min. The plate was washed three times with PBS, and blocking buffer was added (1% BSA in PBS) for 45 min. Anti-phospho-MBP antibodies (1 µg/ml in blocking buffer; UBI) and goat anti-mouse HRP secondary antibodies (1:5000 in blocking buffer; UBI) were used to detect phosphorylated substrate. The plates were washed seven times with PBS and the signals developed by the addition of 75 µl per well of TMB peroxidase substrate. Following blue color development, 75 µl H_2SO_4 (0.09 *M*) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550).

Alkaline comet assay for quantitation of DNA damage

A modified alkaline comet assay technique [1, 6] was used to quantitate DNA damage induced by SMA41, SMA52, and TEM. Cells were exposed to drugs for 30 min and harvested with trypsin-EDTA. The cells were subsequently collected by centrifugation and resuspended in PBS. The resulting cell suspension was diluted to approximately 10^6 cells/ml, and mixed with agarose (1%) in PBS at 37°C at a dilution of 1:10. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel-casting chambers, as previously described [1, 6], then immediately placed into a lysis buffer (2.5 *M* NaCl, 0.1 *M* tetrasodium EDTA, 10 m*M* Tris-base, 1% w/v *N*-lauryl sarcosine, 10% v/v DMSO and 1% v/v Triton X-100).

After being kept on ice for 30 min, the gels were gently rinsed with distilled water and then immersed in a second lysis buffer (2.5 *M* NaCl, 0.1 *M* tetrasodium EDTA, 10 m*M* Tris-base) containing 1 mg/ml proteinase K for 60 min at 37°C. Thereafter, they were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed into 1 *M* ammonium acetate for 30 min. They were further soaked in 100% ethanol for 2 h, dried overnight and subsequently stained with SYBR Gold (1:10,000 dilution of stock supplied by Molecular Probes, Eugene, Ore.) for 20 min. For evaluation of comets, DNA damage was assessed using the tail moment parameter (i.e. the product of the distance between the barycenters of the head and the tail of the comet multiplied by the percentage DNA in the tail region). A minimum of 50 cells/comet were analyzed for each sample, using ALKOMET v3.1 software, and the values presented are average tail moments for the entire cell population.

Flow cytometry for cell cycle analysis

Cells were grown in six-well plates in a monolayer until confluence, washed three times with PBS, and grown in serum-free medium without phenol red for 24 h. Thereafter, they were harvested with trypsin-EDTA, collected by centrifugation and washed with PBS. Vindelov's solution (0.01 *M* Tris-base, 10 m*M* NaCl, 700 U RNAse, 7.5×10^{-5} *M* propidium iodide, 0.1% NP-40) was added (400 µl) for 10 min at 37°C and the fluorescence (FL2) detected using a Becton-Dickinson FACScan. When G_1 cell synchronization was satisfactory, drugs plus FBS (10%) were added for an additional 24 h, after which analyses were performed as described.

Western blotting

NIH 3T3/HER14 cells were preincubated in a six-well plate (1×10^6 cells/well) with 0.1% serum at 37°C overnight for 24 h, after which they were exposed to a dose range of each drug for 2 h and subsequently treated with 50 ng/ml EGF for 30 min at 37°C. All other cell lines were grown in six-well plates (1×10^6 cells/well) in medium containing 10% serum until confluence. Thereafter, they were washed with PBS and resuspended in cold lysis buffer (50 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 1% NP40, 1 mM EDTA, 5 m*M* NaF, 1 m*M* Na_3VO_4, protease inhibitor tablet; Roche Biochemicals, Laval, Canada). The lysates were kept on ice for 30 min and collected by centrifugation at 10,000 rpm for 20 min at 4°C. The protein concentrations were determined against a standardized control using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). Equal amounts of protein (40 µg/ml) from each lysate were added to a 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, Mass.). Nonspecific binding on the PVDF membrane was minimized with a blocking buffer containing 3% nonfat dried milk in PBS. The membrane was incubated with the following primary antibodies: anti-EGFR (Neomarkers, Fremont, Calif.) for determination of corresponding receptor levels, anti-β-tubulin (Neomarkers) for the detection of equal loading, or anti-AGT (Pharmingen, Toronto, Canada). Thereafter, blots were incubated with HRP-goat anti-mouse antibody (1:200 dilution; UBI) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK). Band intensities were measured using the SynGene GeneTools software package (Cambridge, UK).

Results

Receptor type specificity

Prior to determining whether the mixed targeting properties of SMA41 translated into selective targeting of EGFR-overexpressing cells, we thought it of interest to analyze its receptor type specificity. This was determined using two lines of assays: (1) ELISA-based competitive binding and (2) growth factor- or serum-stimulated growth of solid tumor cells. As shown in Table 1, SMA41 did not inhibit insulin or Src tyrosine kinase activities ($IC_{50} > 100$ μM), nor did it inhibit PKC serine/threonine kinase activity. More importantly, as shown in Fig. 1, when the cells were starved and stimulated with various growth factors or serum, SMA41 preferentially blocked growth of NIH 3T3/HER14 cells stimulated with EGF over those stimulated with platelet-derived growth factor (PDGF) or serum, indicating some degree of selectivity for EGFR-mediated growth.

Correlation with EGFR expression

Since SMA41 showed preferential affinity for the intracellular ATP binding site of EGFR, we hypothesized that this was likely to translate into selective targeting of EGF-dependent cells. More importantly, the ATP binding site being located in the cytosol, we surmised that methyldiazonium species generated by the in situ hydrolysis of EGFR-bound SMA41 may diffuse away from the membrane towards the nucleus, thereby inducing an EGFR-assisted elevation of the levels of DNA damage in EGFR-overexpressing cells. This was also expected to confer a direct dependence of levels of DNA damage or antiproliferative activity on the EGFR content of the cells. Growth inhibitory studies using the NCI SRB assay [4] under a 7-day continuous exposure in a panel of ten established cell lines expressing various levels of EGFR (Table 2; Fig. 2), demonstrated little correlation between IC_{50} values and EGFR levels. Also, single-cell microelectrophoresis (comet) assays for DNA damage in all these cell lines revealed no linear correlation between levels of DNA lesions induced by TEM and EGFR status of the cells (data not shown). Similarly, no linear correlation was apparent between the IC_{50} values of the naked inhibitor SMA52 and EGFR levels.

The IC_{50} values for SMA41 varied from 1.1 to 24 μM (Table 2), with the lowest value observed for AGT-deficient cells. This differential response profile paralleled that for TEM. Linear regression analyses showed a significant correlation between the IC_{50} values of SMA41 and the AGT levels of the cells (Pearson

Table 1 Inhibition of tyrosine and serine/threonine kinases by SMA41 and SMA52 in an ELISA-based competitive ATP binding assay. Each value was calculated by nonlinear regression analysis using GraphPad Prism software (GraphPad Software, San Diego, Calif.), and represents the results of at least two independent experiments run in duplicate

Tyrosine kinase	IC_{50}, competitive binding (μM)	
	SMA41	SMA52
PKC	>100	>100
Src	>100	>100
Insulin receptor	>100	>100
EGFR	0.1	1

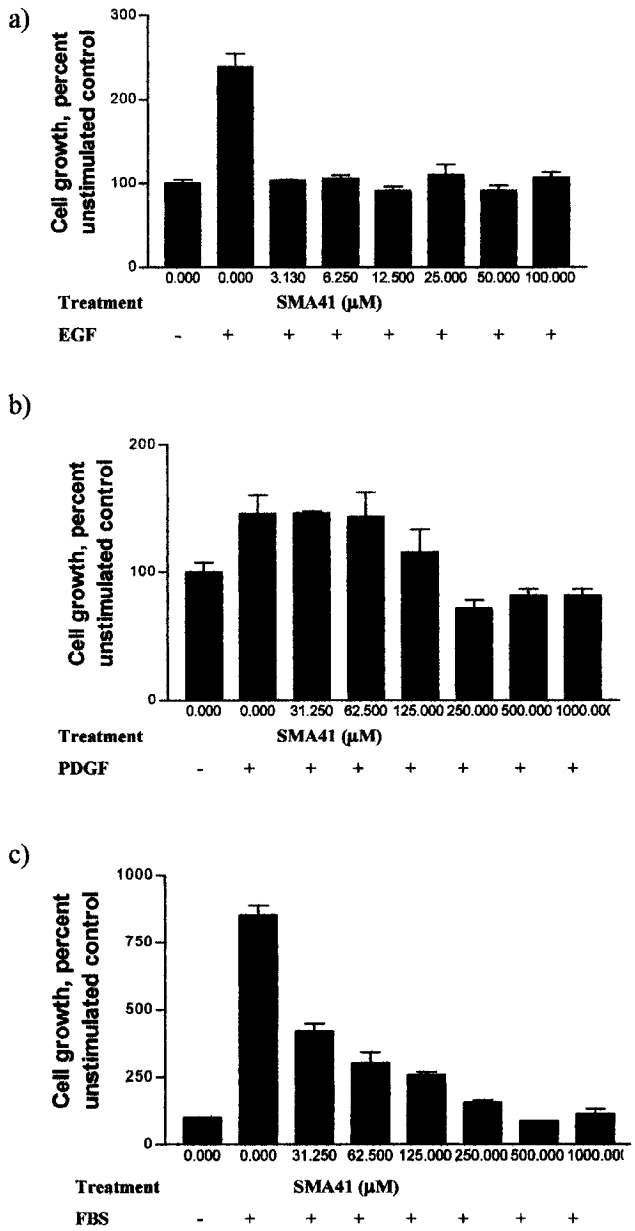


Fig. 1a–c Inhibition of growth factor-stimulated cell growth by SMA41. NIH 3T3/HER14 cells were serum-deprived for 24 h prior to the addition of (**a**) EGF (12 ng/ml), (**b**) PDGF (50 ng/ml), or (**c**) serum (10%) plus SMA41 at the indicated doses. Each point represents the average results from at least two independent experiments run in triplicate

Table 2 AGT/EGFR levels and IC_{50} values for cell growth inhibition by SMA41, SMA52 and TEM in a panel of human tumor cell lines. Each IC_{50} value (μM) represents the results from at least two independent experiments performed in triplicate. AGT and EGFR levels were determined by Western blotting. AGT values were normalized by subtracting the band intensity ratio (AGT/tubulin) of SF-126 from those of all other cells, leaving AGT values for the latter as 0. Similarly, the SF-188 band intensity ratio was considered the background for normalizing EGFR levels

Cell line	IC_{50}			AGT	EGFR
	SMA41	SMA52	TEM	(AGT/tubulin)	(EGFR/tubulin)
MCF-10A	21.0	16.4	92.4	1.15	0.04
A431	21.6	8.6	604.9	0.20	2.46
PC-3	21.8	24.4	573.3	0.76	1.20
DU-145	24.3	26.2	197.8	0.81	1.64
SF-126	2.0	26.9	12.5	0.0	0.10
SF-188	19.8	33.8	412.6	0.36	0.0
MCF-7	24.1	39.6	699.6	1.29	0.51
MDA-MB-435	3.0	29.3	12.0	0.06	0.23
MDA-MB-453	1.1	8.1	451.0	0.56	1.54
MDA-MB-468	28.6	39.5	125.0	0.65	1.22

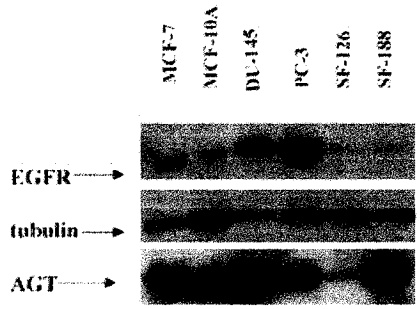


Fig. 2 Expression of EGFR and AGT in selected cell lines from the panel. Tubulin was used as a loading control for quantitation of EGFR and AGT levels

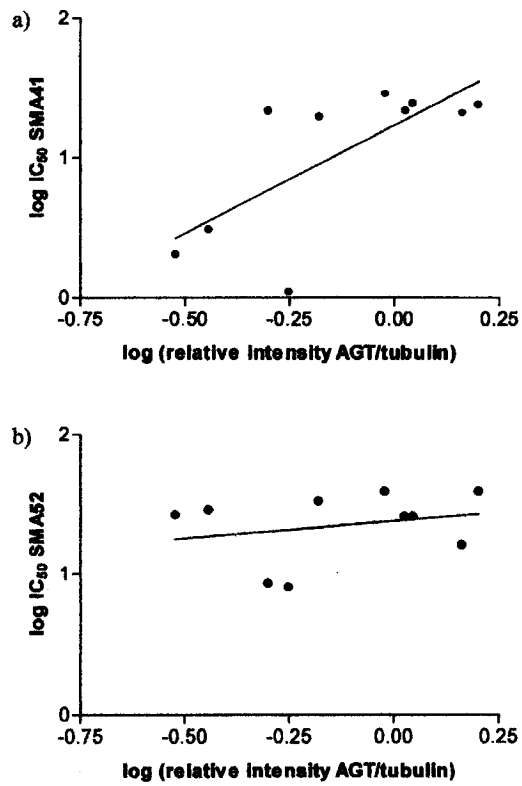


Fig. 3a, b Correlation of AGT levels with IC_{50} values for (**a**) SMA41 and (**b**) SMA52. Pearson correlation coefficients were calculated as $r^2 = 0.7241$ ($P < 0.05$) for SMA41, and $r^2 = 0.2472$ ($P > 0.05$) for SMA52

coefficient $r^2 = 0.7$, $P < 0.05$). As expected, this correlation was not significant for SMA52 ($r^2 = 0.2472$, $P > 0.05$; Fig. 3). Thus, responses to SMA41 seem to depend more on the AGT than the EGFR status of the cells. The sole SMA41-sensitive cell line with significant levels of AGT was the breast carcinoma MDA-MB-453. Since the latter line was also exquisitely sensitive to SMA52, we believe that this may have been due to its expression of high levels erb2 and erb3 [7], the two closest homologues of EGFR.

It is noteworthy that despite being a monomethylating agent, the IC_{50} values of SMA41 (average 16.7 ± 3.3 μM) were more than 19-fold lower than those of TEM (average 318 ± 82 μM) throughout the panel, indicating a consistently greater potency of SMA41 when compared with TEM.

Differential DNA damage induced by SMA41 in the NIH 3T3 isogenic pair of cell lines

Various factors, such as DNA repair status, dependence of cell growth on factors other than EGF, and nonspecific binding to unidentified receptors, may affect the correlation between EGFR and levels of DNA lesions or antiproliferative effects. Thus, we chose to pursue the study in an isogenic pair of cell lines (NIH 3T3 and NIH 3T3/HER14 cells, engineered to overexpress EGFR), the sole difference between which was the transfected EGFR gene. Using the comet assay, we demonstrated that SMA41 induced twofold higher levels of DNA damage in the EGFR transfectant when compared with its parental NIH 3T3 cell line (Fig. 4). However, this did not

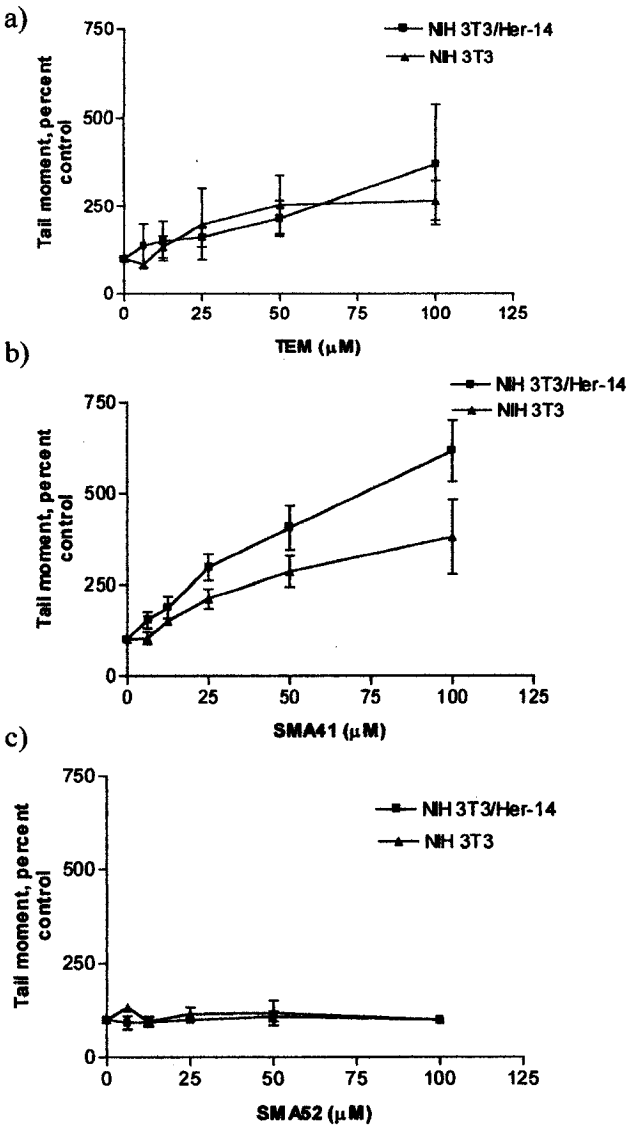


Fig. 4a–c Differential DNA damage induced by (a) TEM, (b) SMA41, and (c) SMA52 in NIH 3T3 and NIH 3T3/HER14 cells as measured by the comet assay following a short 30-min exposure. Each point represents the average results from at least two independent experiments

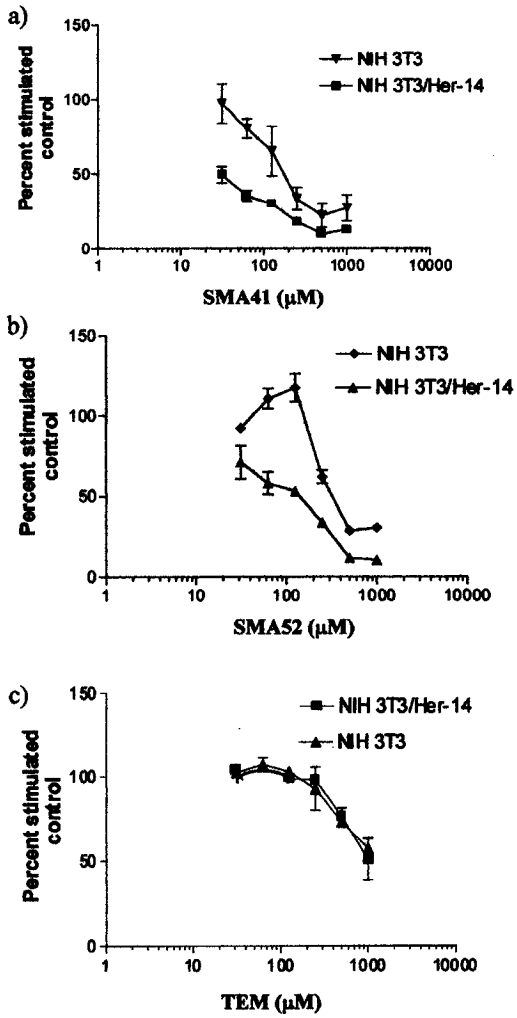


Fig. 5a–c Inhibition of serum-stimulated growth of NIH 3T3 and NIH 3T3/HER14 cells by (a) SMA41, (b) SMA52, and (c) TEM. Each point represents the average results from at least two independent experiments run in triplicate

translate into any differential effect in the SRB assay, which showed a barely detectable difference in the antiproliferative activities of SMA41 in the two cell types (data not shown).

Growth stimulation assays with NIH 3T3/HER14 and NIH 3T3 isogenic pair of cell lines

Having observed an absence of correlation under both poly- and isogenic conditions, we sought to determine whether the EGFR transfection had conferred a growth advantage on these cells under conditions in which their proliferation was serum-stimulated. Further, we hypothesized that if this growth advantage were associated with EGFR-mediated signaling, abrogation of EGFR tyrosine kinase activity would selectively sensitize the EGFR-transfected cells. Thus, we designed an experiment in which the cells were starved for 24 h and growth-stimulated with a dose range of serum. Interestingly, it was found that the EGFR transfectant grew approximately threefold faster than its parent non-transformed NIH 3T3 cell line, confirming that the EGFR transfection had conferred a growth advantage on these cells. With this model, we tested the effects of SMA41 and SMA52 in comparison with that of TEM using the serum concentration at which maximum growth stimulation was observed (10%). The results (Fig. 5) showed that TEM induced no differential response between the two cell types. In contrast, SMA41 selectively induced fivefold stronger serum-stimulated growth inhibitory activity in the EGFR transfectant. Similarly, the naked inhibitor SMA52 induced threefold

stronger inhibition of serum-mediated growth in NIH 3T3/HER14 cells.

EGF- and serum-induced cell cycle effects

The mechanism of this significant selectivity was further investigated by flow cytometric analyses of SMA41-, SMA52- and TEM-induced cell cycle perturbation under serum-driven growth stimulation. It should be remembered that SMA41 possesses mixed signal transduction and DNA tar geting properties, and it is now well established that inhibition of signaling and induction of DNA damage are associated with significant cell cycle perturbations. More specifically, DNA lesions induced by alkyltriazenes of the same class as SMA41 (e.g. monomethyltriazenylimidazole-4-carboxamide, MMTIC) [8, 9], and its prodrug TEM) cause significant cell cycle arrest in S or G_2/M and inhibitors of EGFR arrest the cell cycle in G_1 [5]. Since SMA41 is a combination of these two types of mechanism, a complex cell cycle distribution was expected.

Indeed, when the cells were starved by serum deprivation, a significant synchronization in G_1 was observed (Fig. 6). The addition of serum to control cells induced significant cell transition to S and G_2/M 24 h later. Coaddition of SMA41 did not significantly block cell exit from G_1, but rather induced a dose-dependent arrest in S phase with a noticeable accumulation of cells in G_1/S at the highest dose of 100 μM. At the latter concentration, a barely detectable number of cells had reached G_2/M 24 h after serum stimulation. More importantly, cell cycle perturbations were more pronounced in the NIH 3T3/HER14 than in its nontransformed counterpart NIH 3T3. This was clearly observable at the highest dose at which cell entry to G_2/M was delayed at earlier phases in NIH 3T3/HER14 cells (arrest in G_1/S, early) than in their parental NIH 3T3 cells (arrest in mid-S and late S; Fig. 6). Similarly, SMA52 delayed cell entry to G_2/M, but the effects were weaker than those of SMA41. In contrast, no significant cell cycle arrest was induced by TEM. Even at the highest dose, the G_2/M peaks were similar to those of control cells. These results provide further evidence of the markedly distinct mechanism of action of SMA41 when compared with those of SMA52 and TEM.

Discussion

Solid tumors are often characterized by the expression of DNA repair enzymes, that confer resistance to chemotherapy, and the overexpression or dysfunction of proteins directly implicated in mitogenic signaling [10, 11, 12, 13]. The expression of DNA repair enzymes, such as AGT, significantly decreases the chemosensitivity of these tumors to alkylating agents of the nitrosourea and triazene classes [14, 15, 16, 17, 18]. On the other hand, amplification and overexpression of receptors of the erb family (e.g. EGFR, $p185^{neu}$) have been detected in subpopulations of many types of human tumors and are experimentally associated with aggressive tumor progression, poor patient survival and more importantly, reduced chemosensitivity [19, 20]. The binding of our growth factor of interest, EGF, to its cognate receptor EGFR is followed by a cascade of activation events that ultimately induces the transcription of genes associated with proliferation (e.g. early response genes such as c-*fos*

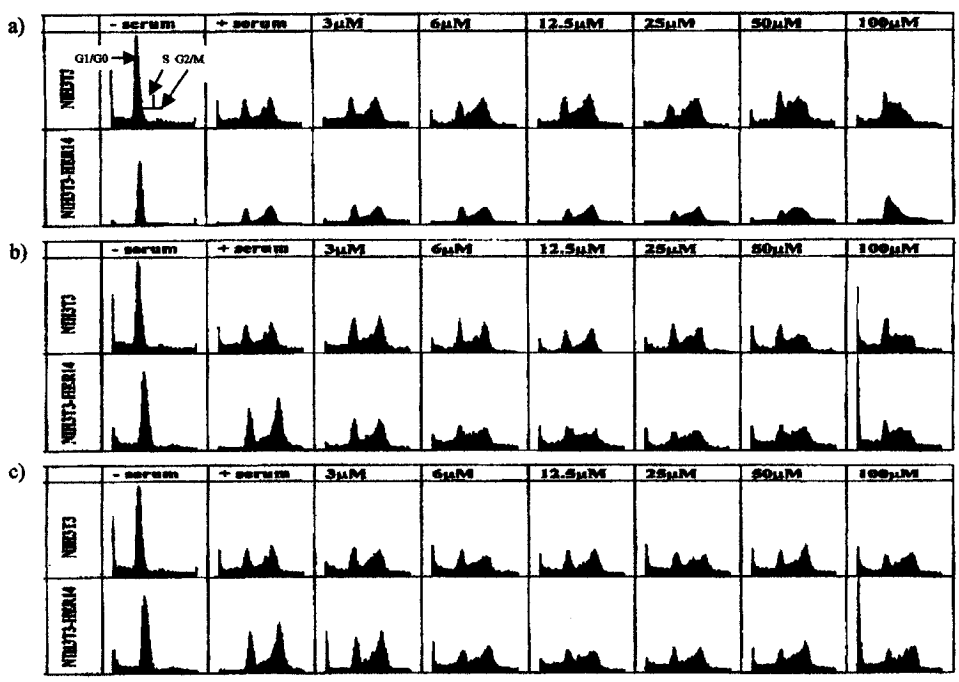


Fig. 6a–c Cell cycle effects of (**a**) SMA41, (**b**) SMA52 and (**c**) TEM on NIH 3T3 and NIH 3T3/HER14 cells. Cells were synchronized in G_1 by serum deprivation for 24 h, after which they were treated with 10% serum plus a range of drug doses. The cell cycle phases G_1/G_0, S, and G_2/M are labeled

and c-*jun*) [21, 22]. More recently, aberration of c-*fos* expression has been associated with greater cell sensitivity to *N*-methyl-*N*-nitrosoguanidine, a methylating agent generating the same type of lesions as SMA41 [22]. This observation lends support to approaches like the combi-targeting concept that seeks to combine blockade of EGF-mediated signal transduction with methylating DNA lesions in order to induce enhanced chemosensitivity and perhaps chemoselectivity in refractory tumors. The current study was designed to determine the potency of the approach in a panel of 12 cell lines with various levels of expression of EGFR and AGT, the latter being a DNA repair enzyme that confers resistance to triazenes of the same class as our C-molecule.

We have already demonstrated that SMA41 is the precursor of two major species: SMA52 that inhibits EGFR tyrosine kinase (TK) (IC_{50} 1 μM), and a metastable methyldiazonium whose presence, due to its short life, could only be indirectly confirmed by the high levels of alkali-labile lesions that it induced in A431 cells [1]. Thus, we compared the antiproliferative effects of C-molecule SMA41 with those of independently synthesized SMA52 and with the clinical methyldiazonium generator TEM. It should be remembered that SMA41 generates 81% of SMA52 following degradation in serum-containing medium [1]. In the present study, the IC_{50} values of SMA52 for cell growth inhibition were consistently in the same range in almost all the cell lines as determined by a 7-day continuous exposure SRB assay. Only values for A431 and MDA-MB-453 cells were threefold lower than the average (Table 2). Since A431 growth is driven by an aggressive autocrine growth mediated by TGF-α/EGFR [23], prolonged exposure and consequently sustained inhibition of EGFR TK may translate into significant antiproliferative activity. As for MDA-MB-453 cells which, despite their significant AGT content, were the cells most sensitive to SMA41, strong dependence of growth on erb2 or erb3 gene products [7] and nonspecific binding to these receptors (the closest homologues of EGFR) may account for the superior sensitivity of these cell lines. As expected, there was no dependence of SMA52 activity on the AGT status of the cells since, as previously reported, it does not alkylate DNA. In contrast, throughout the panel, the IC_{50} values of the methylating agent TEM were quite high, but significantly low in AGT-null cells. This is in agreement with a great number of reports of a direct correlation between the antiproliferative activity of TEM and the AGT status of human tumor cells [14, 16].

Since SMA41 is a combination of the two lines of mechanisms of action described above (i.e. inhibition of TK activity, methylation of DNA), antiproliferative activities superior to those of SMA52 and TEM alone were expected. Surprisingly, the IC_{50} values for SMA41 were in the same range as those of SMA52 in almost all cells expressing AGT except the MDA-MB-453, but were 9- to 25-fold stronger in AGT-null cells. Coexpression of EGFR and AGT did not seem to affect the levels of antiproliferative activities induced by SMA41 in the cell panel. However, despite the absence of correlation with EGFR status, the antiproliferative activities of SMA41 were superior to those of TEM in all cells tested, regardless of their AGT status. These results suggest overall that, where AGT is expressed, repair of O6-methylguanine may at least partially compromise the cytotoxic effects of the DNA-damaging component of the dual mechanism of action of SMA41, leaving an antiproliferative activity that is mostly the result of the 81% conversion of the C-molecule to SMA52. The contribution of the growth-inhibitory property of SMA52 may also explain the overall superior antiproliferative effects of the C-molecule SMA41 when compared with those of its clinical counterpart TEM.

The absence of correlation between the EGFR status of our cell panel and IC_{50} values for cell survival may be primarily explained by the DNA repair status of the cells on which depend the cytotoxic effects of all SMA41-induced DNA lesions. However, cell growth may be dependent on factors or hormones other than EGF that are available in the serum-containing medium. Therefore, we presumed that EGFR selectivity should be analyzed in isogenic clones that differ only in their EGFR content but possess identical DNA repair status. More importantly, EGFR should confer a growth advantage to its host clone. This is in line with recent study by Bishop et al. [24] who showed in the NCI panel that only EGFR-expressing cells with an intact EGF-mediated mitogenic signaling pathway were significantly sensitive to anilinoquinazolines. Thus the study was further carried out in NIH 3T3 and NIH 3T3/HER14 (engineered to overexpress EGFR).

We first demonstrated the binary targeting properties of SMA41 in these cells by analyzing levels of DNA damage and inhibition of EGF-induced growth in the EGFR-expressing transfectant (Fig. 4). Interestingly, DNA damage appeared more enhanced in the EGFR transfectant when compared with its parental line. These results lend support to the hypothesis that methyldiazonium ions generated from EGFR-bound SMA41 may diffuse towards the nucleus, thereby enhancing the levels of DNA damage. Since this did not translate into EGFR-selective antiproliferative activity in the continuous exposure SRB assay, we surmise that the EGFR-targeting component of SMA41 is more likely to have been responsible for the differential response between the two cell types. As we have demonstrated, EGF plays a role in mitogenic signaling in these cells. However, for the parental NIH 3T3 cells, being insensitive to EGF-induced growth, FBS (10%) was considered the most appropriate stimulation medium as it stimulated the growth of both cell types and in addition represented a good mimic of an in vivo medium. SMA41 showed ninefold selective inhibition of FBS-induced growth in the EGFR transfectant when compared with the parental cell line NIH 3T3. Similarly, its derived metabolite SMA52 induced threefold selectivity for NIH 3T3/HER14. The greater selectivity of SMA41 when compared

with SMA52 is in line with the order of affinity of these two drugs for EGFR, suggesting the possible dominance of the EGFR-inhibitory component of SMA41 in this selective activity.

The mechanism of selectivity of SMA41 under growth stimulation can be explained in light of recent observations by He et al. [25], who showed using NIH 3T3 cells that serum or PDGF activation of p21-activated kinase (PAK), a family of threonine kinases involved in cell motility and directed migration, requires EGFR. Specific inhibitors of both PDGFR and EGFR TK block the activation of PAK. Abolition of EGFR using fibroblasts from $EGFR^{-/-}$ mice shows complete depletion of PAK activation, suggesting a key role for EGFR in this process. Although the implication of these types of cooperative interactions between PDGF and EGFR in cell proliferation has not yet been fully elucidated, we clearly observed that EGFR transfection conferred a significant growth advantage on the NIH 3T3/HER14 transfectant over its parental line under serum stimulation. Since at concentrations of SMA41 at which significant serum-induced growth was inhibited by 50% (e.g. 32.5 μM), no inhibition of PDGF-induced proliferation was observed, its selective growth inhibition is likely to be based on blockade of EGFR TK which at this concentration was inhibited by 100%.

It could be argued that rapidly growing cells are more sensitive to cytotoxic agents than slowly growing ones. However, this can be refuted by the absence of differential cytotoxic activity produced by TEM in the two cell types. More importantly, SMA52 which showed the same levels of activity as SMA41 in basal growth assays induced only a threefold difference between the two cell types. The preferential inhibition of serum-induced growth stimulation in the EGFR transfectant is a clear demonstration of the tumor targeting role of the signal transduction inhibitory component of our C-molecule.

In order to gain an insight into the effects of the detected DNA lesions induced by SMA41 in these cells, we analyzed the effects of the latter on cell cycle progression. In contrast to TEM, SMA41 significantly delayed cell cycle progression of G_1-synchronized cells 24 h after growth stimulation by serum with a slightly more pronounced effect in the EGFR transfectant NIH 3T3/HER14. Since inhibition of growth stimulation would cause a protracted arrest in G_1/G_0, the complex cell cycle profiles induced by our C-molecule [e.g. arrests in S(early) and S(middle)] when compared with SMA52 or TEM may primarily have been due to the DNA-damaging component or the results of nonspecific inhibition of cell cycle proteins. It is not clear at this point how the induced cell cycle delays correlate with cell death or with the overall antiproliferative activity.

In previous studies we have demonstrated the feasibility of a C-molecule with mixed EGFR- and DNA-targeting properties. In the current study we conclusively demonstrated that the EGFR-targeting component plays a significant role in blocking growth stimulation not only by EGF alone, but also by serum which contains a variety of growth factors, the most abundant of which is PDGF. We have also demonstrated that the EGFR component may assist in the selective induction of DNA damage in isogenic clones. Our studies in a heterogeneous panel of cells led to the conclusion that, while combining a DNA-damaging triazene with an EGFR-inhibitory moiety confers significantly greater potency to the resulting C-molecule when compared with the classical triazenes, the DNA repair status of the cells still remains a significant barrier to optimal potency. To this end, other polyfunctional cytotoxic moieties that may enhance the potency of the DNA-damaging component of the C-molecules are now being investigated in our laboratory. Moreover, work is underway to transfect the AGT-null and AGT-proficient cells with EGFR in order to better outline the effects of coexpression of AGT and EGFR on their sensitivity to C-molecules.

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ORIGINAL ARTICLE

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The combi-targeting concept: a novel 3,3-disubstituted nitrosourea with EGFR tyrosine kinase inhibitory properties

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Abstract *Purpose*: To study the dual mechanism of action of FD137, a 3,3-disubstituted nitrosourea designed to block signaling mediated by the epidermal growth factor receptor (EGFR) on its own and to be hydrolyzed to an inhibitor of EGFR plus a DNA-damaging species. *Materials and methods*: HPLC was used to determine the half-life ($t_{1/2}$) of FD137 and to characterize its derived metabolite FD110. The dual mechanisms of DNA damaging and EGFR tyrosine kinase (TK) targeting were ascertained by the comet assay for DNA damage and by inmunodetection of phosphotyrosine in an ELISA and a whole-cell assay for EGFR-mediated signaling. The antiproliferative effects of the different drugs and their combinations were determined by the sulforhodamine B (SRB) assay. *Results*: In contrast to BCNU, FD137 significantly blocked EGF-induced EGFR autophosphorylation (IC_{50} 4 μM) in the human solid tumor cell line A431. DNA damage induced by FD137 could only be observed after 24 h exposure, but the level of DNA damage remained 3.6-fold lower than that induced by BCNU. This difference was rationalized by the 160-fold greater stability of FD137 when compared with BCNU in serum-containing medium. Further, degradation of FD137 was accompanied by the slow release of FD110, an extremely potent inhibitor of EGFR TK [IC_{50} (EGFR autophosphorylation) < 0.3 μM]. The complex properties of FD137 translated into a 55-fold greater antiproliferative activity than BCNU against the EGFR-overexpressing A431 cells that coexpresses the O^6-alkylguanine transferase (AGT). Depletion of AGT in these cells by the use of O^6-benzylguanine (O^6-BG) enhanced their sensitivity to BCNU by 8-fold, but only by 3-fold to FD137. *Conclusions*: The results overall suggest that the superior antiproliferative activity of FD137 when compared with BCNU may be associated with its ability to behave as a combination of many species with different mechanisms of action. However, the enhancement of its potency by O^6-BG suggests that its antiproliferative effect was at least partially mitigated by AGT and perhaps it may be largely dominated by its signal transduction inhibitory component.

Keywords Nitrosourea · Signal transduction · Tyrosine kinase · EGFR · BCNU

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Introduction

The nitrosoureas are among the most potent alkylating agents used in the clinic in the therapy of lymphoproliferative diseases, glioma, small-cell lung carcinoma, melanoma and gastrointestinal cancer. However, despite their broad spectrum of activity, nitrosoureas are inactive against tumors expressing the O^6-alkylguanine transferase (AGT) enzyme [1, 2], a DNA-repair protein capable of repairing the O^6-alkylguanine DNA lesion. This limitation stems from the very mechanism of action of these drugs that are believed to kill tumor cells by generating, upon hydrolysis, a chloroethyldiazonium species responsible for the alkylation of guanine at the O^6-position [3]. The resulting O^6-(2-chloroethyl)guanine adduct alkylates a base (e.g. the 7-position of guanine or the cytosine amino group) in the complementary DNA strand, thereby inducing a DNA crosslink that is believed to be the lethal lesion induced by bifunctional nitrosoureas [1, 2, 4]. Tumor cell resistance to chloroethylnitrosoureas is imputed to the AGT-mediated repair of the primary O^6-alkylguanine lesion, which

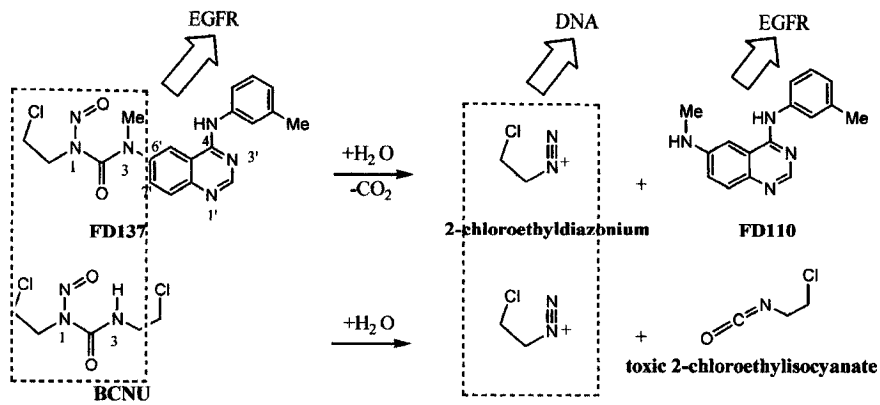
prevents the formation of the lethal DNA crosslink. This mechanism is corroborated by the use of O^6-benzylguanine (O^6-BG), an irreversible inhibitor of AGT that significantly sensitizes tumor cells to the action of 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (BCNU) (Scheme 1) in a number of animal models [5, 6]. However, recent observations of acquired resistance to repeated doses of BCNU plus O^6-BG in tumor cells [7, 8, 9] and the significant sensitivity of bone marrow cells to the latter combination [10] cast doubt on its ultimate clinical efficacy. Chemoprotection of the bone marrow using stem cells transfected with an O^6-BG-insensitive mutant form of AGT is an active field of research [8, 11]. Thus, the development of strategies designed to enhance the clinical efficacy of bifunctional nitrosoureas remains a burgeoning field.

Our laboratory is currently studying a novel approach termed "combi-targeting" that consists of imprinting into the structure of cytotoxic drugs, new molecular features allowing them to interfere with mitogenic signal transduction, while retaining their ability to damage DNA [12, 13, 14]. It is expected that the hydrolytic cleavage of these molecules termed "combi-molecules" (C-molecules) will generate other bioactive derivatives, the antiproliferative effects of which may add to or synergize with each other. We designed FD137 (Scheme 1) that contains a 1-chloroethyl-1-nitrosourea function and a 3-methyl-3-(4′-anilinoquinazolin-6′-yl) moiety. Each of the substituents around the ureido function is designed to play a specific role following the degradation of the C-molecule: (a) the 1-chloroethyl-1-nitrosourea moiety is responsible for the postdegradative generation of the DNA-damaging 2-chloroethyldiazonium species, (b) the methyl group is designed to prevent the formation of a toxic isocyanate following cleavage of the ureido function, and (c) the 4′-anilinoquinazoline moiety contains all the structural requirements for competitively blocking of ATP binding to the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR).

EGFR is a transmembrane glycoprotein with an external domain that binds activating ligands such as EGF and tumor growth factor α (TGF-α). It contains an intracellular TK domain, that upon activation (by binding of EGF or TGF-α) phosphorylates both the receptor itself and a variety of effector proteins that ultimately triggers DNA synthesis and proliferation of EGFR-proficient cells [15, 16]. Overexpression of EGFR and often coexpression of cognate ligands (e.g. TGF-α, amphiregulin) that leads to autocrine growth, are associated with aggressive tumor proliferation and invasiveness. Agents like the anilinoquinazolines that are capable of blocking EGFR-mediated signaling have already been shown to induce significant antitumor activity in vivo [17, 18]. In this study, we chose to combine our nitrosourea moiety with the pharmacophores of anilinoquinazolines because of their known tolerance of substituents at the 6′- and 7′-positions (see Scheme 1) which in their binding mode to EGFR are located at the entrance of the binding cleft. Indeed, a variety of compounds with bulky side chains on the 6′- and 7′-positions have been synthesized and found to retain significant affinity for the EGFR ATP binding site [17, 19, 20, 21].

Most of the known quinazolines are reversible inhibitors of EGFR that require repeated dosing schedules for sustained inhibition in vivo and the irreversible ones are specifically directed at EGFR or its closest homologue $p185^{neu}$ [17, 18]. Thus, these agents target a single mechanism of cell growth, leaving all alternative growth pathways unaffected. Recently, synergistic combinations of EGFR TK inhibitors with other cytotoxic drugs have been evoked as an alternative to the lack of sustained growth inhibition induced by EGFR TK inhibitors when used as single agents [22]. This is consistent with our strategy that not only seeks to combine these two mechanisms in a single molecule (C-molecule), but also to target the latter to EGFR.

As shown in Scheme 1, the C-molecule (FD137) is designed to target EGFR on its own and to further decompose into the cytotoxic 2-chloroethyldiazonium species directed at DNA plus another less bulky inhibitor (e.g. FD110) capable of inducing stronger EGFR TK inhibitory activity than its parent C-molecule. Conversely, its classical counterpart BCNU does not possess an intracellular target on its own and is merely designed to generate the DNA-damaging chloroethyldiazonium plus a toxic isocyanate upon hydrolysis (see Scheme 1). In the study reported here, we



Scheme 1 C-molecule FD137 degradation to DNA-damaging 2-chloroethyldiazonium, the less bulky inhibitor FD110 and the toxic 2-chloroethylisocyanate

demonstrated that the C-molecule FD137, a nitrosourea tethered to a 4-anilinoquinazoline was capable of: (a) generating a less bulky, more potent EGFR inhibitor FD110 upon hydrolysis, (b) blocking EGFR TK activity and EGF-induced EGFR autophosphorylation in whole cells, and (c) inducing DNA damage perhaps through the generation of a chloroethyldiazonium species. The levels of DNA damage induced by FD137 were about 3-fold less than those induced by BCNU. However, FD137 was 55-fold more potent than BCNU against the A431 cell line that coexpresses EGFR and AGT.

Materials and methods

Drug treatment

FD137 and FD110 were synthesized in our laboratory according to known procedures [19, 20]. O^6-BG was a generous gift from Dr. Robert Moschel (National Cancer Institute, USA). BCNU was purchased from Sigma Chemical Company (Mississauga, Canada). In all experiments, drugs were dissolved in DMSO and subsequently diluted in sterile RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Life Technologies, Burlington, Canada) prior to addition to the cell culture medium. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

Cell culture

A431 cells (American Type Culture Collection, Manassas, Va.) and SF126 cells (generous gift from Mrs. Dolores Dougherty, Brain Tumor Research Center, University of California, Calif.) were maintained in RPMI-1640 supplemented with FBS (10%) and ciprofloxacin 10 μg/ml (Mediatech, Herndon, Va.). The cultures were kept in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA and replating before confluence. All cell lines used repeatedly tested negative for mycoplasma contamination.

HPLC determinations of FD137 degradation to FD110

A volume of 25 μl of FD137 (24 m*M*) was added to RPMI-1640 supplemented with 10% FBS (1 ml) and incubated for various periods (6–168 h) at 37°C. Proteins were precipitated by addition of acetonitrile (1.75 ml) and the supernatant collected by centrifugation. HPLC analyses were performed with a Hewlett Packard 1090 liquid chromatograph, using a Waters C4 15-μm 300×3.9-mm column (reverse phase) to characterize and quantify the products resulting from the degradation of FD137. The concentration of FD110 deriving from the degradation of FD137 was calculated using a standard curve obtained from the serial dilution of independently synthesized FD110. The operating mode was isocratic and two solutions, A (53% acetonitrile) and B (47% water) were used with a flow rate of 0.5 ml/min and an injection volume of 10 μl. Under these conditions, the retention times of independently synthesized FD110 and FD137 were 7.6 and 11.9 min, respectively.

Combination studies

For the combination of FD110 and BCNU, the drugs were mixed at their IC_{50} ratio (FD110/BCNU, 1:125), serially diluted and added to the monolayers for 96 h. IC_{50} values were determined using the median effect equation as described by Perez et al. [23]. The results of drug interactions were determined using Eq. 1 where CI_{50} values >1, $=1$, and <1 indicate antagonism, additivity and synergism, respectively. Cell growth-inhibitory activities were evaluated using the sulforhodamine B (SRB) assay [24].

$$CI_{50} = \frac{IC_{50}(\text{FD110 in combination})}{IC_{50}(\text{FD110 alone})} + \frac{IC_{50}(\text{BCNU in combination})}{IC_{50}(\text{BCNU alone})}$$

Enzyme assay

The EGFR kinase assay was similar to the one described previously [13]. Nunc Maxisorp 96-well plates were incubated overnight at 37°C with poly(L-glutamic acid/L-tyrosine, 4:1; PGT) in phosphate-buffered saline (PBS). The kinase reaction was performed using 4.5 ng/well EGFR affinity-purified from A431 cells. Following drug addition, phosphorylation was initiated by the addition of ATP. A typical assay was performed at 0.001–100 μ*M*. The reaction was terminated by aspiration of the reaction mixture and phosphorylated substrate was detected with anti-phosphotyrosine antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, Calif.). The signals were developed by the addition of 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersburg, Md.) and H_2SO_4 (0.09 *M*) was added to stop the reaction. The plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550).

Autophosphorylation assay

A431 cells (1×10^6) were preincubated in a six-well plate with 10% serum at 37°C overnight for 48 h and starved for 24 h, after which they were exposed to a dose range of each drug for 2 h and subsequently treated with 50 ng/ml EGF for 20 min at 37°C. Cells were washed with PBS and resuspended in cold lysis buffer. The lysates were kept on ice for 30 min and collected by centrifugation at 10,000 rpm for 20 min at 4°C. The concentrations of protein were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). Equal amounts of protein (80 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). Nonspecific binding on the membrane was minimized with a blocking buffer containing nonfat dried milk (3%) in PBS Tween. Thereafter, the primary antibodies were incubated with membranes, either anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, N.Y.) for the detection of phosphotyrosine, or anti-EGFR (Neomarkers, Fremont, Calif.) for the determination of corresponding receptor levels. Blots were incubated with HRP-goat anti-mouse antibody (BioRad Laboratories) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK). Band intensities were measured using the SynGene GeneTools software package.

Alkaline comet assay for quantification of DNA damage

The alkaline comet assay technique was used to quantify DNA damage induced by FD137, FD110 and BCNU as previously described [12, 25]. Briefly, A431 cells were exposed to drugs for 2 or 24 h, harvested with trypsin-EDTA, subsequently collected by centrifugation and resuspended in PBS. Cell suspensions were diluted to approximately 10^6 cells, and mixed with agarose (1%) in PBS at 37°C at a dilution of 1:10. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel-casting chambers, as previously described [25], and then immediately placed into a lysis buffer (2.5 *M* NaCl, 0.1 *M* tetrasodium EDTA, 10 m*M* Tris-base, 1% w/v *N*-lauryl sarcosine, 10% v/v DMSO, and 1% v/v Triton X-100, pH 10.0). After being kept on ice for 30 min, the gels were gently rinsed with distilled water and immersed in a second lysis buffer (2.5 *M* NaCl, 0.1 *M* tetrasodium EDTA,

10 m*M* Tris-base) containing 1 mg/ml proteinase K for 60 min at 37°C. The gels were then rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed in 1 *M* ammonium acetate for 30 min. They were then soaked in 100% ethanol for 2 h, dried overnight, and stained with SYBR Gold (1:10,000 dilution of stock supplied by Molecular Probes, Eugene, Ore.) for 20 min.

Comets were visualized at ×330 magnification and DNA damage was quantitated using the tail moment parameter (i.e. the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet). A minimum of 50 cells/comet were analyzed for each sample, using ALKOMET version 3.1 image analysis software. For the determination of interstrand crosslink levels, the tail moments of irradiated control cells (4000 rad) were compared with those of irradiated drug-treated cells and the percentage decrease in tail moment was calculated as described by Hartley et al. [26] according to the formula:

$$\%\ \text{decrease in tail moment} = \left(1 - \left\{\frac{\text{Tm(treat} - \text{irr)} - \text{Tm(unirr)}}{\text{Tm(irr)} - \text{Tm(unirr)}}\right\}\right) \times 100$$

where Tm(treat-irr) is the tail moment of the drug-treated irradiated sample, Tm(unirr) is the tail moment of the untreated unirradiated control, and Tm(irr) is the tail moment of the untreated irradiated control.

Results

Degradation of FD137

The decomposition of FD137 was analyzed by monitoring the variations of the area under the HPLC chromatogram peaks corresponding to FD137 and FD110, as a function of time. As expected, an inverse relationship was observed between the disappearance of FD137 and the appearance of FD110. From these results, a $t_{1/2}$ of 41 h was calculated using a one-phase exponential decay curve fit (Fig. 1). This $t_{1/2}$ was 160-fold slower than that reported for BCNU, a nitrosourea known to be extremely unstable in serum-containing medium ($t_{1/2}$ 15 min) [3, 27, 28]. The retention time of FD110 was ascertained by HPLC analysis of an independently synthesized standard. After complete degradation (72 h), the overall yield of FD110 was approximately 80%.

Binary targeting properties of FD137

Inhibition of substrate phosphorylation. The EGFR TK-inhibitory activity of FD137 was demonstrated through an ELISA assay whereby the drug was incubated with the isolated full-length EGFR for a short 8-min period and through an EGF-induced autophosphorylation assay in A431 cells involving a 2-h exposure. As shown in Fig. 1, the kinetics of degradation ($t_{1/2}$ 41 h) indicated that within these short exposure periods (8 and 120 min), the entire structure remained intact in the medium. Thus, in this time-frame the analysis of EGFR TK-inhibitory activity of FD137 was not complicated by that of the concomittant generation of FD110. In the ELISA assay, FD137 blocked PGT substrate phosphorylation in a dose-dependent manner (IC_{50} 1 μ*M*) but its activity was 20-fold lower than that of FD110 (IC_{50} 0.05 μ*M*; Fig. 2), indicating that it is a prodrug of a more potent inhibitor of EGFR TK. As expected, the EGFR TK-inhibitory activity of BCNU was negligible.

Inhibition of EGF-induced autophosphorylation. In further analysis, the cells were starved for 24 h and stimulated with EGF in the presence of the different drugs for 2 h. Inhibition of autophosphorylation of EGFR was analyzed by Western blotting. The observed order of potency from this whole cell assay paralleled that of the ELISA. In contrast to BCNU, FD137 blocked EGFR autophosphorylation in a dose-dependent manner (IC_{50} 4 μ*M*; Fig. 3a, b), but its potency was more than 14-fold less than that of FD110 which induced an almost 100% inhibition of EGFR TK at concentrations as low as 0.3 μ*M* (Fig. 3b). This further confirms that FD137 is a stable carrier of a more potent inhibitor.

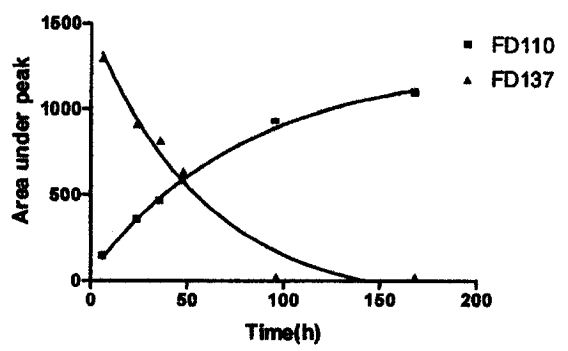


Fig. 1 Conversion of FD137 (*triangles*) to FD110 (*squares*) in RPMI supplemented with 10% FBS at 37°C. Protein precipitation and HPLC determination were performed as described in Materials and methods

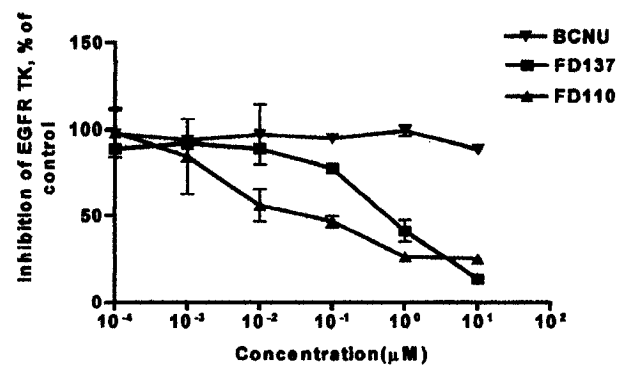


Fig. 2 Competitive binding to EGFR by BCNU (*down triangles*), FD137 (*squares*) and FD110 (*up triangle*). PGT substrate phosphorylation was detected using an anti-phosphotyrosine antibody. Each point represents at least two experiments run in triplicate

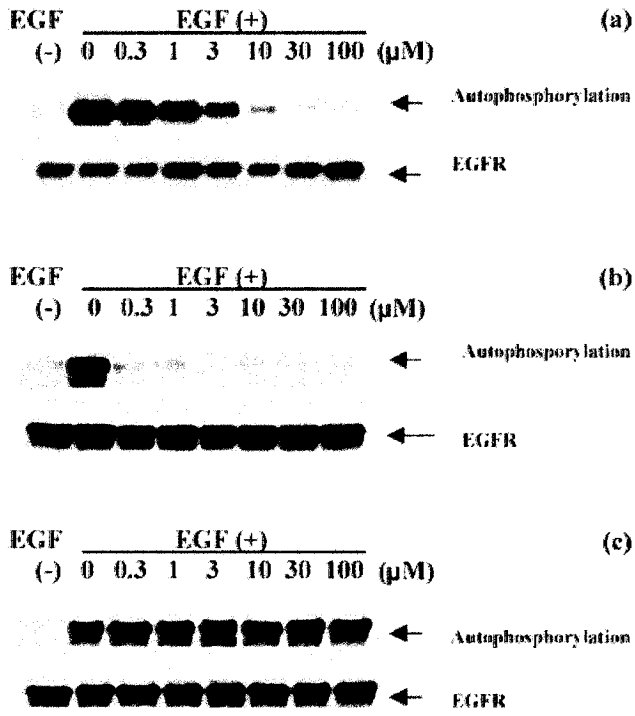


Fig. 3a–c Inhibition of EGF-stimulated EGFR autophosphorylation by FD137 (**a**), FD110 (**b**) and BCNU (**c**) in the A431 cell line. Cells were starved for 24 h and 50 ng/ml EGF (20 min) was added with different concentrations of compounds for 2 h. Western blotting was performed with an anti-phosphotyrosine antibody (diluted 1:1000). The same PVDF membrane was stripped and EGFR detected with anti-EGFR antibody

DNA-damaging property of FD137

While the released FD110 was directed at EGFR, the concomitantly generated chloroethyldiazonium (see Scheme 1) was designed to damage DNA. It is noteworthy that the latter species, due to its short life, was not detectable by our available methods. Thus, we surmised that induction of DNA damage by FD137 would be an indirect evidence of the formation of the latter. We used the single-cell microelectrophoresis (comet assay) to characterize DNA strand breaks from A431 cells following exposure to all three drugs. This assay permits the microscopic observation and quantitation of DNA damage from whole cell nuclei [29]. When we used the 2-h drug exposure time normally allowed for analysis of DNA damage by nitrosoureas [27, 30], BCNU induced a dose-dependent increase in strand breaks (Fig. 4a). In contrast, levels of DNA damage inflicted by FD137 were similar to those of FD110 which, as expected, induced negligible levels of DNA lesions. However, when the cells were exposed to drug for 24 h (Fig. 4b), in contrast to BCNU which exhibited a saturation curve, FD137 induced a dose-dependent linear increase in DNA strand breaks with no appearance of a plateau at concentrations as high as 100 μ*M*.

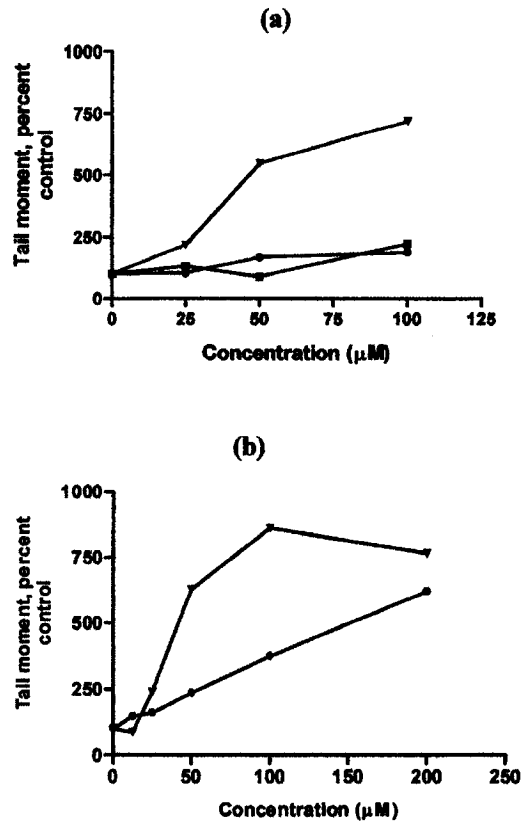


Fig. 4a, b Alkaline comet assay for quantitation of DNA damage. The levels of DNA damage were measured as tail moments in A431 cells exposed (**a**) to FD137 (*circles*), FD110 (*squares*), BCNU (*triangles*) for 2 h and (**b**) to FD137 (*circles*) or BCNU (*triangles*) for 24 h. Tail moment parameters (formula shown in Materials and methods) were measured with ALKOMET image analysis software for at least 50 cells per dose

Calculations using the slope of the linear part of these curves showed that BCNU was a 3.6-fold stronger DNA-damaging agent than FD137, a result that is in line with the order of stability of these two nitrosoureas. Likewise, a crosslink assay involving comparison between tail moments of γ-irradiated drug-treated and control samples (4000 rad) showed that at 25–50 μ*M*, BCNU induced 6–20% decrease in tail moment, indicating detectable levels of crosslinks at these doses. In contrast, in cells treated with FD137, the same levels of crosslinks could only be observed at twice higher concentrations (50–100 μ*M*; Fig. 5). This further confirms that BCNU is a much stronger DNA-damaging agent than FD137.

Cell growth inhibition of FD137

Comparative effects of the three agents. To demonstrate whether the binary properties of FD137 translate into enhanced antiproliferative activities when compared with its nitrosourea counterpart BCNU, we evaluated the effects of each drug on cell proliferation using a

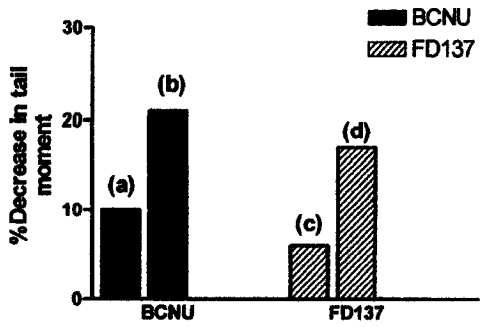


Fig. 5 Comparison between levels of DNA interstrand crosslinks induced by BCNU (*a* 25 μ*M*, *b* 50 μ*M*) and FD137 (*c* 50 μ*M*, *d* 100 μ*M*) as measured in terms of the percentage decrease in tail moments of γ-irradiated control and treated cells

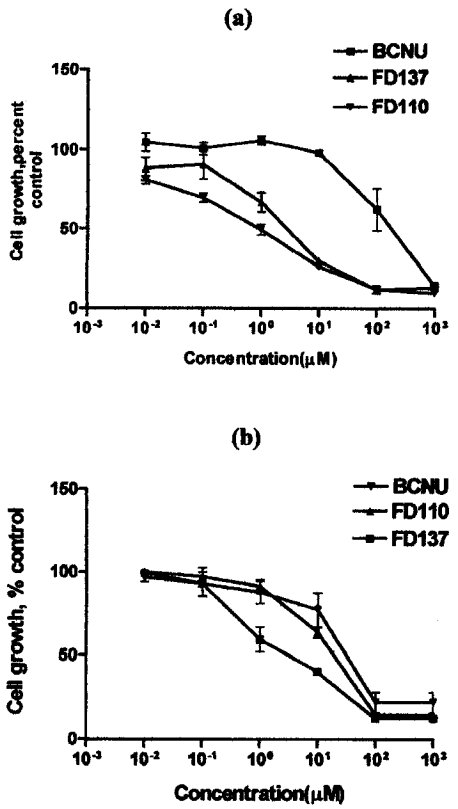


Fig. 6a, b Comparison between the antiproliferative activity of BCNU, FD137 and FD110 (**a**) in the AGT/EGFR-proficient A431 cell line and (**b**) in the AGT/EGFR-deficient SF126 glioma cell line, as determined by the SRB assay. Each point represents at least three independent experiments run in triplicate

4-day SRB assay in the A431 cells that overexpress EGFR and coexpress AGT. More importantly, it has already been demonstrated that antiproliferative activities of quinazolines strongly correlate with their ability to block EGF autophosphorylation in this cell line [31, 32]. Our results showed a 2.3-fold superior potency of FD110 (IC_{50} 1.11 μ*M*) when compared with FD137 (IC_{50} 2.57 μ*M*; Fig. 6a). However, the latter was more than 50-fold more potent than its nitrosourea counterpart BCNU. Interestingly when these drugs were tested in the glioma cell line SF126 that is deprived of both AGT and EGFR, a different order of potency was observed. FD137 (IC_{50} 1.7 μ*M*) was approximately 8-.fold more potent than FD110 (IC_{50} 13 μ*M*; Fig. 6b). Interestingly, the latter was almost equipotent with BCNU (IC_{50} 17 μ*M*) in this AGT/EGFR-deficient glioma cell line.

Effects of AGT depletion in A431 cells. From the DNA damage studies, it appeared that in contrast to BCNU, FD137 could induce detectable DNA damage at concentrations 20–50 times higher than its IC_{50} (25–100 μ*M*). Under the conditions of the comet assay, the type of lesions observed may probably be the alkali labile *N*7-alkylpurines. Despite their abundance in cells treated with *N*-alkyl-*N*-nitrosoureas, these lesions are far less cytotoxic than the O^6-alkylguanine adducts, as proven by the significant sensitization of AGT-proficient cells to BCNU when the activity of AGT is depleted [33]. Thus, we surmised that if the chloroethyldiazonium ion was indeed released by FD137 or that DNA alkylation at O^6-guanine was induced, its activity should be enhanced by coadministration of O^6-BG. Indeed depletion of AGT activity by its inhibitor O^6-BG sensitized the cells to BCNU by 8-fold (Fig. 7c) and by 3-fold to FD137 (Fig. 7a). As expected, O^6-BG had no effect on cell sensitivity to FD110 that does not possess DNA-damaging properties (Fig. 7b). This indicates that the cytotoxic effects of the O^6-alkylguanine DNA adducts were indeed induced by FD137, but may have been at least partly mitigated by AGT.

Comparison with classical two-drug combinations. FD137 being a unimolecular combination of two antiproliferative properties combining inhibition of the EGFR pathway with DNA damage, we thought it of interest to compare the potency of this form of combination with that of two different drugs representing the two antiproliferative mechanisms under study. We surmised that the closest mimic was a combination of FD110 (the metabolite resulting from FD137) with BCNU (a prodrug of the 2-chloroethyldiazonium species; Fig. 8a). From IC_{30} to IC_{80}, we only observed near-additive to subadditive interactions (Fig. 8b). As an example, the CI_{50} at the 50% effect for FD110 plus BCNU was 0.85, indicating a subadditive interaction. More importantly, FD137 was 23-fold more potent than the latter combination against the A431 cells (Fig. 8a).

Discussion

The combi-targeting concept postulates that a molecule (C-molecule) possessing receptor affinity on its own and that is able to further degrade into another inhibitor of the same target and DNA-damaging species should induce significantly sustained antiproliferative activity in cells expressing these receptors. The results presented here demonstrated that FD137 was indeed capable of

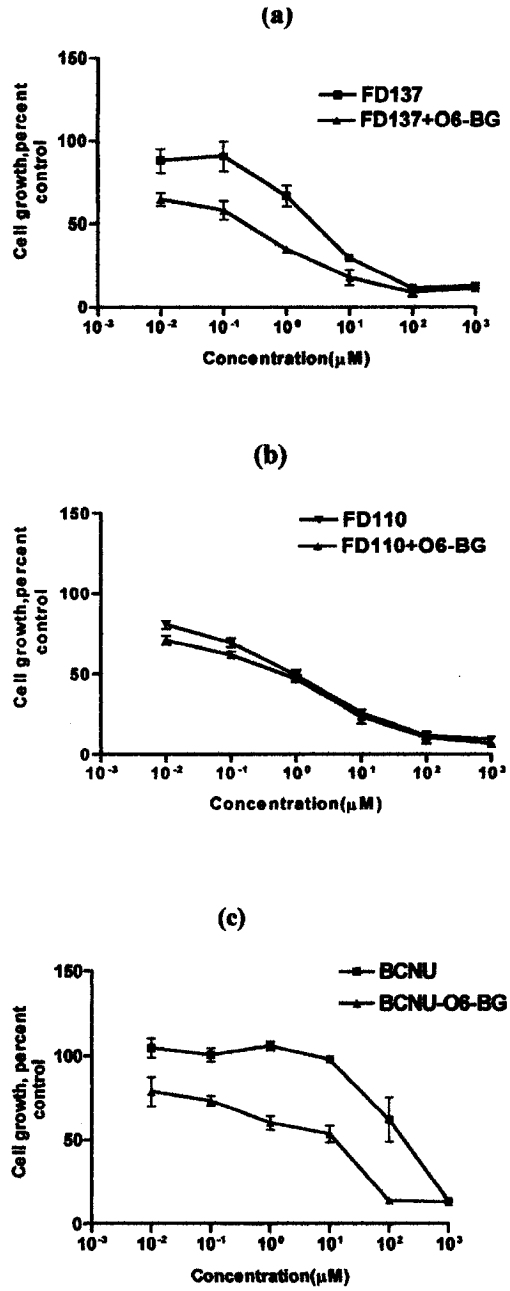


Fig. 7a–c Effect of O^6-BG (AGT inhibitor) on (a) FD137-, (b) FD110- and (c) BCNU-induced cell growth inhibition in A431 cells treated with the different drugs and their corresponding combinations with O^6-BG for 96 h. Cell growth control percentages were determined by the SRB assay. Each data point represents an average of triplicate samples from two separate experiments

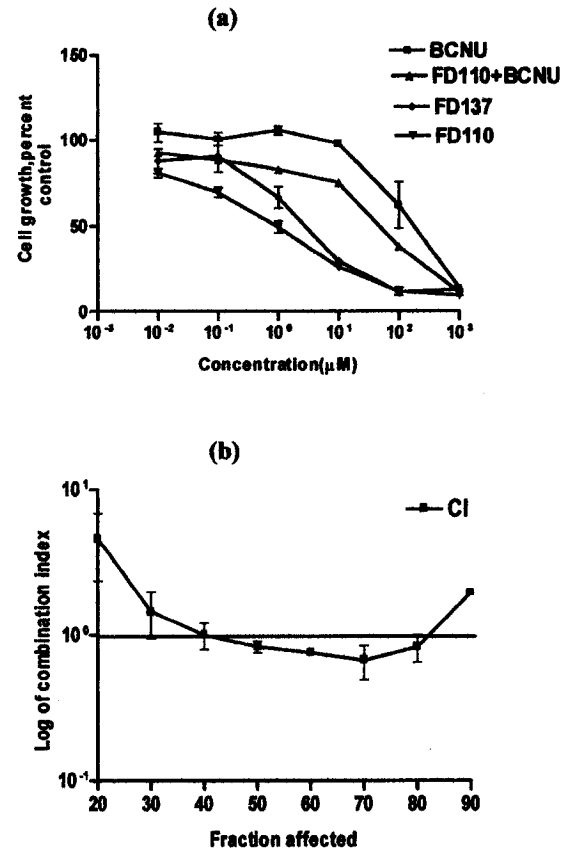


Fig. 8 a Comparison of the antiproliferative effects of FD110 plus BCNU (*up triangles*) with those of FD110 (*down triangles*) and BCNU (*squares*) and FD137 (*diamonds*) alone. FD110 and BCNU were mixed at their IC_{50} ratio (1:125) and each point represents a combination of two independent experiments. b Log of combination index calculated from the dose-response curves of a as a function of the effects expressed as a percentage. Combination index values < 1, $= 1$, and > 1 indicate synergism, additivity and antagonism, respectively

slowly degrading to FD110 with a $t_{1/2}$ of nearly 41 h in serum-containing medium at 37°C, a rate of degradation markedly slower than that of BCNU ($t_{1/2}$ 15 min in serum). The superior stability of FD137 is primarily due to the bulkiness of the 3,3-disubstituted nitrosourea function wherein both the 3-methyl and the 4′-anilino-quinazolin-6′-yl groups significantly hinder the carbonyl of the ureido moiety, thereby decreasing the rate of hydrolysis of the latter. More importantly, the stability of this molecule is superior to that of SarCNU, an experimental 3,3-disubstituted nitrosourea that is known to exhibit a $t_{1/2}$ of 2.69 h in serum-containing medium [34]. The 3-methyl group hindering the urea moiety was included in the structure design to prevent the formation of a toxic isocyanate function following hydrolysis of the urea moiety. It is now known that the chloroethylisocyanate species derived from the hydrolysis of BCNU is responsible for its carbamoylating power and perhaps may increase its in vivo toxicity [35].

The stability of FD137 was in agreement with the barely detectable levels of DNA damage observed following a 2-h drug exposure. In contrast, BCNU ($t_{1/2}$ about 15 min in serum) [3, 28, 29, 36] induced significant DNA damage within the same exposure period. Perhaps, during this short period, the concentration of the generated chloroethyldiazonium species was insufficient

to induce detectable levels of DNA damage. Likewise, the slow release of the chloroethyldiazonium species accounts for the 3-fold lower levels of DNA damage induced by FD137 when compared with BCNU, even after a 24-h continuous exposure. Indeed, our kinetic analysis suggests that only 29% of the C-molecule was converted into FD110 by 24 h. Complete degradation of FD137 was observed only after 72 h in the culture medium, an exposure time considered too long for quantitation of DNA damage due to complications introduced by DNA repair and cell loss.

It is noteworthy that the slow rate of degradation of the C-molecule created conditions under which the cells were exposed to high but decreasing concentrations of intact FD137 during the first 48 h and increasingly high doses of FD110 during the subsequent 48 h. With the assumption that the release of each mole of FD110 is associated with one equivalent of the metastable chloroethyldiazonium, the DNA lesions may have been slowly but continuously inflicted in the presence of FD110 and FD137 gradients, forming then a three-drug-like combination over a long period. We clearly characterized the target of each of the constitutive elements of FD137 in the human carcinoma of the vulva cell line A431 that overexpresses EGFR and proliferates by a TGF-α-mediated autocrine induction [31]. More importantly, the observation that FD110 was a more than 20-fold stronger inhibitor of EGFR TK than its parent FD137, confirms the postulate according to which the C-molecule is designed to block EGF-induced EGFR autophosphorylation on its own and to further degrade to a smaller molecule with significantly higher affinity for the ATP binding site of EGFR. Thus, FD137 represents a complex model of a three-drug combination wherein decreasing and increasing concentrations of two agents directed at the same target but possessing different levels of affinity are generated over a long period. In addition, the decreasing concentration of the parent agent (the C-molecule) may be accompanied by the slow release of a DNA-alkylating species.

Having demonstrated the events proposed in Scheme 1, we characterized their effects on antiproliferative activities in the A431 cells using the SRB assay. The advantages of the unimolecular combinations were first determined by comparison with BCNU and FD110 alone. FD137 was clearly more potent than BCNU but less potent than FD110. It is interesting to note that despite being a precursor of FD110, FD137 was 2.3-fold less potent than the latter. This can be rationalized in light of its kinetics of degradation that showed a plateau concentration for FD110 only 72 h after treatment. In contrast, when administered alone, the cells were exposed for 96 h to a steady concentration of FD110, a 20-fold more potent EGFR inhibitor than FD137. More importantly, the order of potency of these drugs in the SRB assay paralleled that of their EGFR TK-inhibitory IC_{50} values (BCNU > FD137 > FD110). Since it is well established that the potency of compounds of the anilinoquinazoline class against A431 cells increases with increasing EGFR TK-inhibitory activity [31, 32], these results may also indicate that the antiproliferative activity of FD137 may be largely dominated by its effect on EGFR-mediated signaling. Thus, what is the contribution of the chloroethyldiazonium-induced DNA lesions to the overall antiproliferative activity?

Our comet analysis demonstrated that, although at supratoxic concentrations, FD137 was capable of inducing DNA crosslinks. Assuming that the FD137-derived chloroethyldiazonium species induced the primary cytotoxic O^6-alkylguanine DNA adduct, the use of an inhibitor to deplete the activity of the DNA repair enzyme AGT should potentiate its action. It is now well established that inhibition of AGT significantly sensitizes cells to BCNU and other related bifunctional nitrosoureas [33, 36, 37]. We found that exposure of the cells to nontoxic 25 μM O^6-BG dramatically sensitized them to the effect of the C-molecule. Indeed coadministration of O^6-BG decreased FD137 IC_{50} to a slightly lower value than that of FD110 which is a 20-fold stronger EGFR TK inhibitor. This is an indirect evidence of the generation of the chloroethyldiazonium and that its effect is somewhat mitigated by AGT. More importantly, these results suggest that blockade of EGFR-mediated signaling may not downregulate or affect the activity of AGT in the cells.

Further evidence of absence of crosstalk between AGT levels and EGFR-mediated signaling was provided by the absence of a synergistic interaction between an isoeffective combination of BCNU and FD110. The two effects were found to be mostly additive. Interestingly, the C-molecule, a single agent encompassing the two antiproliferative mechanisms of these two drugs was 23-fold more effective than their individual combinations. Perhaps the ability of the stable and lipophilic C-molecule to penetrate the cells prior to releasing the DNA damaging species plus the EGFR TK inhibitors may account for its superior antiproliferative activity when compared with the classical two-drug combination of the two agents BCNU (unstable) plus FD110 (a polar free amine).

The results overall suggest that the growth-inhibitory activity of the C-molecule in the A431 cells was chiefly achieved through inhibition of EGFR and any contribution from the DNA-damaging component should originate from cytotoxic lesions other than O6-alkylguanine (e.g. N7-alkylguanine, N3-alkyladenine). Thus, the AGT-dependence of FD137 antiproliferative activity suggests that its mechanism of cell killing may be similar to that of BCNU or SarCNU, two nitrosoureas bearing the *N*-(2-chloroethyl)-*N*-nitrosourea moiety.

FD137 is the first nitrosourea possessing TK-inhibitory properties ever reported. While our model clearly demonstrated its ability to damage DNA, the impact of the generated lesions appeared at least partially mitigated by the presence of AGT in these cells. However, this stable nitrosourea could induce 55-fold stronger antiproliferative activity than BCNU in AGT-expressing cells perhaps due to an alternative mechanism by which

it can block EGF- or TGFα-mediated mitogenic signaling. Hence, this is the first generation of "smart" nitrosoureas that may primarily behave like multidrug combinations in EGF-dependent tumor cells and like classical nitrosoureas in the absence of EGFR and AGT. In order to enhance the potency of the approach, our laboratory is currently optimizing the EGFR TK affinity of FD137 and improving its water solubility prior to the demonstration of its activity in vivo.

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Inhibition of Epidermal Growth Factor Receptor-Mediated Signaling by "Combi-Triazene" BJ2000, a New Probe for Combi-Targeting Postulates

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ABSTRACT

The Combi-Targeting concept postulates that a molecule termed combi-molecule (C-molecule) with binary epidermal growth factor receptor (EGFR) targeting/DNA-damaging properties and with the ability to be hydrolyzed to another EGFR inhibitor should induce sustained antiproliferative activity in cells overexpressing EGFR. Because we postulate that the EGFR affinity of the C-molecule and that of its hydrolytic metabolites are critical parameters for sustained potency against EGFR-overexpressing cells, we synthesized BJ2000 (IC_{50} = 0.1 μM, competitive binding at ATP site), a novel C-molecule that can decompose into a 6-amino-4-anilinoquinazoline FD105 (IC_{50} = 0.2 μM). Studies using the EGFR-overexpressing A431 cells revealed that BJ2000 could damage DNA and block epidermal growth factor-stimulated EGFR autophosphorylation by a partially irreversible mechanism. Blockade of EGFR autophosphorylation subsequently induced inhibition of mitogen-activated protein kinase activation and c-fos gene expression. Enzyme-linked immunosorbent assay and growth factor-mediated stimulation of proliferation assays in the EGFR-expressing NIH3T3HER14 demonstrated the preferential EGFR-targeting properties of BJ2000, and more importantly suggest that blockade of EGFR phosphorylation by this drug translate into significant growth inhibitory effects. These properties culminated into irreversible antiproliferative effects as confirmed by a sulforhodamine B assay. Five days after a 2-h treatment, BJ2000 retained significant antiproliferative effect in A431 cells, whereas its reversible metabolite FD105 almost completely lost its activity. This result in toto lend support to the Combi-Targeting concept according to which a molecular conjugate kept small enough to interact with EGFR and designed to degrade into another inhibitor of the same target plus a DNA-damaging species may induce sustained growth inhibitory effect in EGFR-overexpressing cells.

The ErbB family, particularly EGFR and ErbB2, are overexpressed in various tumors and have been correlated with an adverse prognosis for cancer patients (Prigent and Lemoine, 1992). One major limitation of current EGFR tyrosine kinase (TK) inhibitors is the high intracellular concentration of ATP that represents a major barrier to sustained inhibition of EGF-stimulated signal transduction in tumor cells. Where they cannot induce apoptosis, EGFR TK inhibitors are cytostatic agents that induce reversible antitumor effects. Recently, irreversible inhibitors of the EGFR family have been synthesized that showed greater potency than their reversible predecessors (Fry, 1998, 1999; Smaill et al., 2000). Unfortunately, irreversible inhibition of EGFR may not suffice to induce sustained antitumor activity if the cells possess alternative growth mechanisms, and combination with cytotoxic drugs to potentiate the action of the EGFR TK inhibition is now being considered a useful alternative (Ciardiello et al., 2000).

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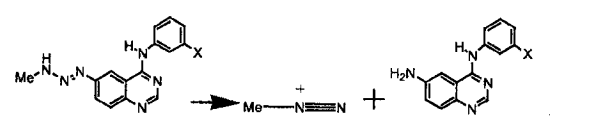
ABBREVIATIONS: EGFR, epidermal growth factor receptor; TK, tyrosine kinase; EGF, epidermal growth factor; C-molecule, combi-molecule; TEM, temozolomide; MTIC, monoalkyltriazene 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HPLC, high-pressure liquid chromatography; PGT, poly(L-glutamic acid-L-tyrosine, 4:1); PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; Erk2, extracellular signal-regulated kinase 2; RT-PCR, reverse transcription-polymerase chain reaction; TGFα, transforming growth factor-α; SRB, sulforhodamine B; AGT, O^6-alkylguanine DNA *transferase*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

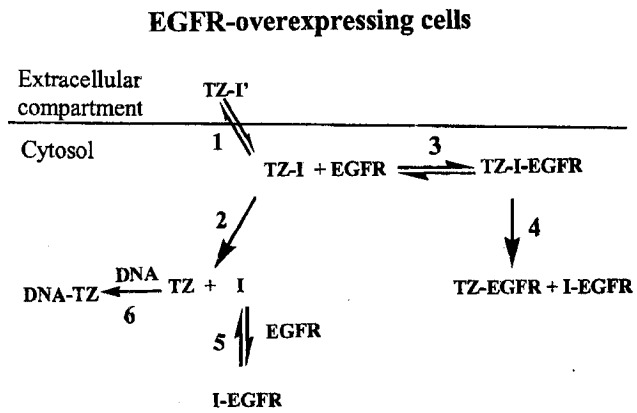
Our novel Combi-Targeting strategy seeks to combine the signal transduction inhibitory mechanisms of receptor TK inhibitors with the cytotoxic effects of DNA-damaging fragments into one single agent termed C-molecule. The latter is designed to 1) inhibit the receptor TK on its own and 2) be converted into another inhibitor of the same receptor TK plus a DNA-damaging species upon hydrolysis. This principle simply leads to a receptor-affinity TZ-*I* (Scheme 1) capable of generating a cytotoxic molecule (TZ) plus another inhibitor, *I*.

As depicted in Scheme 2, in EGFR-overexpressing cells, we predict that the extracellular TZ-*I'* (TZ representing the cytotoxic element and *I* or *I'*, the EGFR inhibitory components) can diffuse through the membrane and in the cytosol (see TZ-*I*) either directly bind to the EGFR ATP-binding site to provide the TZ-*I*-EGFR complex (path 3) or degrade into a cytotoxic TZ molecule plus an EGFR TK inhibitor *I* (path 2). Although the TZ will exert cytotoxic activity by damaging DNA (see DNA-TZ), the generated inhibitor *I* is designed to inhibit EGFR-induced growth (path 5). The combined cytostatic and cytotoxic effects may lead to enhanced antiproliferative activity of the TZ-*I* in EGFR-overexpressing cells. More importantly, the TZ-*I* may directly alkylate the EGFR as outlined in path 4 wherein an inactivated (covalently modified) receptor (TZ-EGFR) may be formed, leaving an irreversibly inhibited receptor. If *I* loses affinity for the damaged receptor, we surmise that it will be released and subsequently bind to undamaged EGFR molecules (see I-EGFR).

Recently, we reported preliminary evidence of the feasibility of a mixed EGFR/DNA-directed molecule termed SMA41 that contained a 3-methyl-1,2,3-triazene moiety appended to position 6 of a 4-anilinoquinazoline moiety (Matheson et al., 2001). It was shown to possess mixed EGFR/DNA-targeting properties on its own (IC_{50} competitive binding = 0.2 μM) and to degrade into another inhibitor SMA52 (IC_{50} competitive binding = 1 μM). The choice of the 3-alkyl-1,2,3-triazene as a DNA-alkylating moiety was inspired by its small size and its ability to heterolyze to an aromatic amine and an alkyldiazonium species that kill cells by alkylating DNA at position 6 of guanine (Cameron et al., 1985; Baig and Stevens, 1987). In comparison with temozolomide (TEM), a cyclic prodrug of the monoalkyltriazene 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) (Stevens et al., 1984, 1987), we demonstrated that the potency of the C-molecule was superior to that of a classical combination of SMA52 and TEM at equitoxic doses. Although SMA41 possessed significant EGFR TK inhibitory activity, the stable molecule that it released (SMA52) was a weak EGFR TK inhibitor (IC_{50} = 1 μM). Thus, we surmised that because the degradation of the TZ-*I* will lead to a stable inhibitor *I* that will remain longer in the cell medium, the potency of the latter may play a critical role in the overall antiproliferative activity. Therefore, we sought for a TZ-*I* capable of generating an *I* with stronger



Scheme 1. A receptor-affinity TZ-*I* capable of generating a cytotoxic molecule (TZ) plus another inhibitor, *I*.



Scheme 2. The target-mediated selectivity component of our approach is based on the strong affinity of the C-molecule for the cytosolic domain of EGFR that may influence the equilibria TZ-*I'* (extracellular compartment)/TZ-*I* (cytosolic) (path 1) and TZ-*I* (cytosolic)/TZ-*I*-EGFR.

affinity than SMA52. Herein, we replaced the anilino methyl group by a less bulky chloro substituent to produce BJ2000, a C-molecule with 2-fold stronger affinity than SMA41 and capable of generating an *I* with 5-fold stronger affinity than SMA52. In this article, we demonstrated that the TZ-*I* BJ2000 was able to release the *I* termed FD105 (yield 87%) in cell culture medium supplemented with serum. This novel model exhibited 1) a mixed DNA-damaging and EGFR phosphotyrosine inhibitory activity, confirming path 1, 2, and 3; 2) a preferential inhibition of EGF-induced growth over PDGF and serum; and 3) a potent inhibition of EGFR-induced gene expression. More importantly, we demonstrated that BJ2000 induced irreversible inhibition of autophosphorylation, suggesting a pathway involving covalent binding of the alkylating product with EGFR (path 4) and partially irreversible inhibition of cell growth. This TZ-*I* or C-molecule has proven an invaluable tool for testing our Combi-Targeting postulates.

Materials and Methods

Drug Treatment

BJ2000 and FD105 were synthesized in our laboratories according to known procedures (Cameron et al., 1985; Manning et al., 1985; Rewcastle et al., 1995). Temozolomide was provided by Schering Plough (Kenilworth, NJ). In all assays, drug was dissolved in DMSO and subsequently diluted in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Wisent Inc., St-Bruno, QC, Canada) or in Dulbecco's modified Eagle's medium containing 10% bovine calf serum (Invitrogen, Burlington, ON, Canada) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

Cell Culture

The cell line used in this study, the human epidermoid carcinoma of the vulva A431, was obtained from the American Type Culture Collection (Manassas, VA). The mouse fibroblasts NIH3T3 and NIH3T3HER14 (NIH3T3 cells stably transfected with EGFR gene) were generous gifts from Dr. Moulay Aloui-Jamali (Montreal Jewish General Hospital, Montreal, Canada). The A431 cell line was maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics as described previously (Matheson et al., 2001). NIH3T3 and NIH3T3HER14 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. All cells were maintained in an atmosphere of 5% CO_2.

Degradation

The half-life of BJ2000 under physiological conditions was studied by UV-spectrophotometer. BJ2000 was dissolved in a minimum volume of DMSO, diluted with RPMI 1640 medium supplemented with 10% FBS, and absorbances read at 340 nm in a UV cell maintained at 37°C with a circulating water bath. The half-life was estimated by a one-phase exponential decay curve-fit method using the GraphPad software package (GraphPad Software, San Diego, CA).

The study of the conversion of BJ2000 into FD105 by HPLC was performed by adding BJ2000 (625 μM) to RPMI 1640 medium with 10% FBS (2 ml) and incubating it for different periods at 37°C. Thereafter, proteins were precipitated by addition of acetonitrile (3.5 ml) and the supernatant collected by centrifugation. The concentration of FD105 resulting from the degradation of BJ2000 was calculated using a standard curve obtained from the serial dilution of independently synthesized FD105 incubated in serum-containing medium under identical conditions. HPLC analyses were performed on a 1090 liquid chromatograph (Hewlett Packard, Palo Alto, CA) using a C_4 reverse phase column (15 μm, 300 × 3.9 mm; Waters, Milford, MA) to characterize and quantitate the products resulting from the degradation of BJ2000. The operating mode was isocratic and two solutions A (53% acetonitrile) and B (47% water) were used with a 0.5-ml/min flow rate and 10-μl injection volume. The peaks were detected at 254 nm. Under these conditions, independently synthesized FD105 and BJ2000 showed retention times around 10.5 and 15.2 min, respectively.

Kinase Assays

EGFR Kinase. This assay is similar to the one described previously (Matheson et al., 2001). Maxisorp 96-well plates (Nalge Nunc International, Naperville, IL) were incubated overnight at 37°C with 100 μl/well of 25 ng/ml PGT in PBS. Excess PGT was removed and the plate was washed three times with wash buffer (0.1% Tween 20 in PBS). The kinase reaction was performed by using 4.5 ng/well EGFR affinity-purified from A431 cells (Moyer et al., 1997; Vincent et al., 2000). The compound was added and phosphorylation initiated by the addition of ATP (20 μM). After 8 min at room temperature with constant shaking, the reaction was terminated by aspiration mixture and by rinsing the plate four times with wash buffer. Phosphorylated PGT was detected after a 25-min incubation with 50 μl/well of horseradish peroxidase-conjugated PY20 anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 0.2 mg/ml in blocking buffer (3% bovine serum albumin and 0.05% Tween 20 in PBS). Antibody was removed by aspiration and the plate washed four times with wash buffer. The signals were developed by the addition of 50 μl/well of 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and after blue color development, 50 μl of H_2SO_4 (0.09 M) was added per well, and plates were read at 450 nm using an ELISA reader (model 2550; Bio-Rad, Hercules, CA).

c-src and Insulin Kinases. Conditions for the c-src and insulin kinase assays were the same as those for the EGFR kinase assay, except for the addition of 1 mM manganese chloride to the assay buffer and a final ATP concentration of 100 μM. The reaction was terminated by the addition of 50 μl of 250 mM EDTA before aspiration. For experiments comparing inhibition of EGFR with c-src-kinase or insulin receptor, Baculovirus-expressed human c-src (1.2 units/well; Upstate Biotechnology, Lake Placid, NY) or baculovirus-expressed cytoplasmic domain of the insulin receptor β subunit (15 ng/ml; BIOMOL Research Laboratories, Plymouth Meeting, PA) was substituted for the EGFR. As positive controls, PP1 (BIOMOL Research Laboratories), a selective inhibitor of c-src (IC_{50} = 170 nM), and HNPMA-$(AM)_3$ (BIOMOL Research Laboratories), an inhibitor of insulin receptor (IC_{50} = 100 μM), were used.

Autophosphorylation Assay

Inhibition of receptor autophosphorylation in viable cells was determined by anti-phosphotyrosine Western blots. Cells were grown to confluence in six-well plates, washed twice with PBS, and exposed to serum-free medium for 18 h. They were subsequently treated with the compounds for 90 min and then with EGF (100 ng/ml) for 10 min (A431 cells), or with PDGF (100 ng/ml) for 10 min (NIH3T3 cells). Thereafter, they were washed with PBS and resuspended in cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, and protease inhibitor tablet; Roche Applied Science, Laval, QC, Canada). The lysates were kept on ice for 30 min and collected by centrifugation at 10,000 rpm for 20 min at 4°C. Protein concentrations were determined against a standardized control using a protein assay kit (Bio-Rad). Equal amounts of protein from each cell lysate were added to an 8% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding on the membrane was minimized with a blocking buffer containing 3% nonfat dry milk in PBS. The membrane was blotted for 1 h with anti-phosphotyrosine antibody PY20 (NeoMarkers, Fremont, CA) or anti-EGFR antibodies (NeoMarkers) and anti-β-tubulin antibodies (NeoMarkers) for the detection of equal loading. It was subsequently incubated with horseradish peroxidase-goat anti-mouse antibody (Bio-Rad) and the bands visualized with an enhanced chemiluminescence system (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Band intensities were measured using the GeneTools software package (SynGene, Cambridge, UK).

For the study of inhibition of mitogen-activated protein kinase (MAPK) activation by BJ2000, protein lysates were obtained as described above and Western blot was performed as reported by Tari and Lopez-Berestein (2000). The membrane was incubated with anti-phosphorylated MAPK (Erk2) antibodies or antibodies specific for Erk2 (Cell Signaling Technology Inc., Beverly, MA).

Reverse EGFR Autophosphorylation

This assay was performed as described by Fry et al. (1998). A431 cells were grown to confluence in six-well plates and then incubated in serum-free medium for 18 h. Duplicate sets of cells were then treated with 30 μM of each compound for 90 min. One set of cells was then stimulated with EGF (100 ng/ml) for 10 min, and extracts were made as described under the Western blotting procedure described above. The other set of cells was washed free of the compound with warmed serum-free media and incubated for 2 h. Thereafter, the cells were washed, incubated for another 2 h, washed again, and then incubated for a further 4 h. This set of cells was then stimulated with EGF, and extracts were prepared as for the first set.

RT-PCR for c-fos Expression

A431 cells were grown to confluence in six-well plates and then incubated in serum-free medium for 18 h. Cells were exposed to the indicated concentrations of drug before stimulation with EGF (50 ng/ml) for 30 min. Total RNA was isolated using the High Pure RNA Isolation kit (Roche Applied Science, Mannheim, Germany), following the manufacturer's instructions. Quantitative analysis of c-fos mRNA and GAPDH mRNA (2 μg of RNA for each sample) was preformed by Titan One Tube RT-PCR kit (Roche Applied Science), following the manufacturer's instructions and using the following primers: 5′ATGATGTTCTCGGGCTTC3′ (sense) and 5′CTCCTGCCAATGCTCTGC3′ (antisense) for c-fos, and 5′CCATGGAGAAGGCTGGGG3′(sense) and 5′CAAAGTTGTCATGGATGACC3′ (antisense) for GAPDH.

In Vitro Growth Inhibition Assay

To study the effect of our compounds on growth factor-stimulated proliferation, cells were grown to 70% of confluence in 48-well plates and washed twice with PBS after which they were exposed to serum-

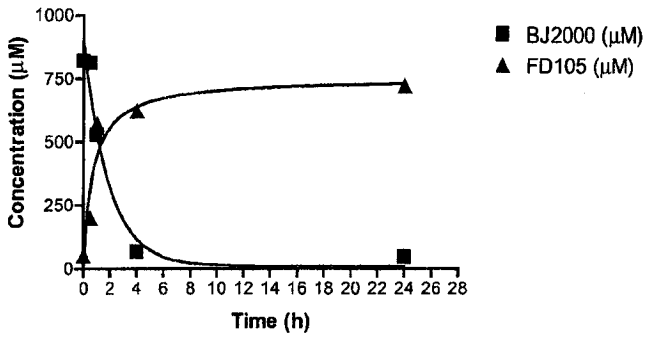


Fig. 1. Degradation of BJ2000 to FD105 in RPMI 1640 medium supplemented with 10% FBS at 37°C. Experiments were carried out by reverse phase HPLC analysis: BJ2000 (625 μM) was added to RPMI 1640 medium with 10% FBS (2 ml) and incubated for different periods at 37°C. Thereafter, proteins were precipitated by addition of acetonitrile (3.5 ml) and the supernatant collected by centrifugation. The concentration of FD105 resulting from the degradation of BJ2000 was calculated using a standard curve obtained from the serial dilution of independently synthesized FD105 incubated in serum-containing medium under identical conditions.

free medium for 18 h. Cells were exposed to each drug and growth factors (EGF, TGFα, PDGF, or serum) for 72 h and cell growth measured using the sulforhodamine B (SRB) assay (Skehan et al., 1990). Briefly, after drug treatment, cells were fixed using 50 μl of cold trichloroacetic acid (50%) for 60 min at 4°C, washed five times with tap water, and stained for 30 min at room temperature with SRB (0.4%) dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid and allowed to air dry. The resulting colored residue was dissolved in 200 μl of Tris base (10 mM) and optical density read for each well at 540 nm using a microplate reader (model 2550; Bio-Rad). Each point represents the average of at least two independent experiments run in triplicate.

To study the reversibility of the antiproliferative effects of our compounds, cells were grown to 70% confluence in 96-well plates and subsequently washed twice with PBS after which they were exposed to serum-free medium for 18 h. Under continuous exposure, cells were exposed to different concentrations of each drug for 120 h. Under short exposure, they were exposed to each drug for 2 h, after which they were allowed to recover for 120 h in drug-free medium. Growth inhibitory activities were evaluated using the SRB assay as described above. Each point represents the average of at least two independent experiments run in triplicate.

Alkaline Comet Assay for Quantitation of DNA Damage

The alkaline comet assay was performed as described previously (Matheson et al., 2001). The cells were exposed to drugs (BJ2000, FD105, or TEM) for 30 min, harvested with trypsin-EDTA, subsequently collected by centrifugation, and resuspended in PBS. Cell suspensions were diluted to approximately 10^6 cells and mixed with agarose (1%) in PBS at 37°C in a 1:10 dilution. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, ON, Canada) using gel casting chambers as described previously (McNamee et al., 2000) and then immediately placed into a lysis buffer [2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base, 1% (w/v) *N*-lauryl sarcosine, 10% (v/v) DMSO, and 1% (v/v) Triton X-100, pH 10.0]. After being kept on ice for 30 min, the gels were gently rinsed with distilled water and immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetrasodium EDTA, and 10 mM Tris-base) containing 1 mg/ml proteinase K for 60 min at 37°C. Thereafter, the gels were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed in 1 M ammonium acetate for 30 min. Thereafter, they were soaked in 100% ethanol for 2 h, dried overnight, and stained with SYBR Gold (1/10, 000 dilution of stock supplied from Molecular Probes, Eugene, OR) for 20 min. Comets were visualized at 330× magnification and DNA damage was quantitated using the Tail Moment parameter (i.e., the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet). A minimum of 50 cell comets was analyzed for each sample, using ALKOMET, version 3.1, image analysis software.

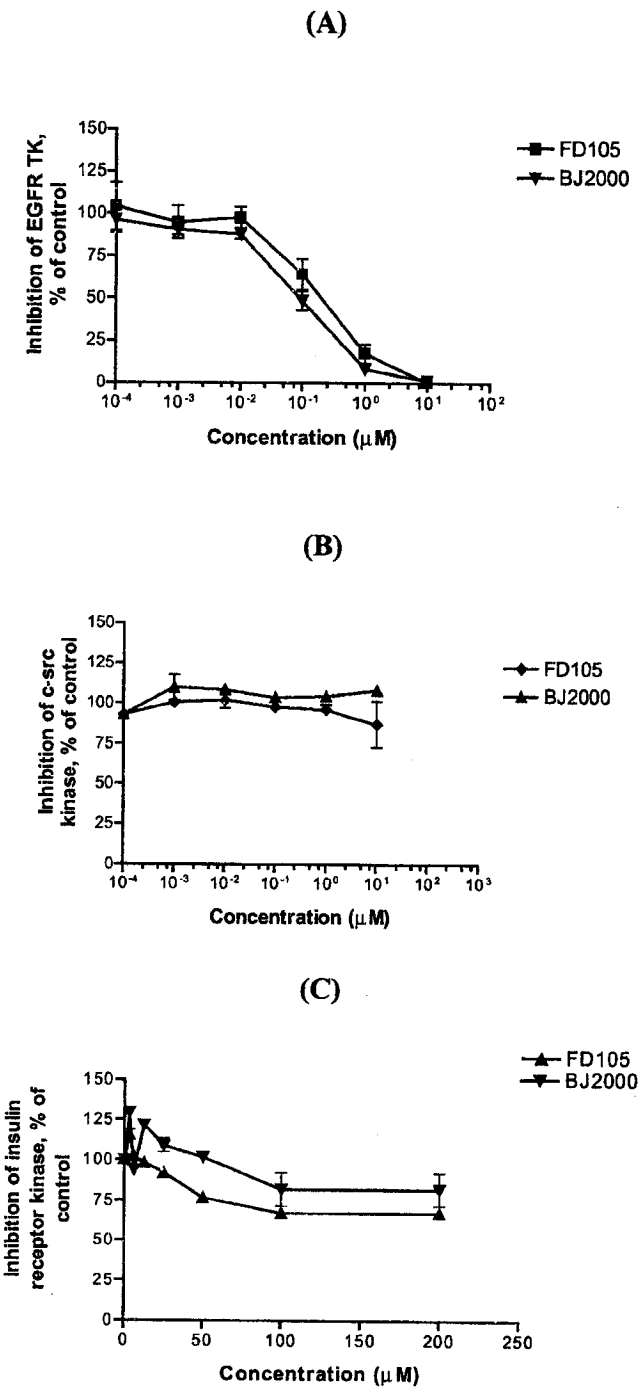


Fig. 2. Selectivity of BJ2000 and FD105 for EGFR TK (A) versus c-src kinase (B) and insulin receptor kinase (C). Inhibition of purified kinases was measured as described under *Materials and Methods*. Each point represents at least two independent experiments.

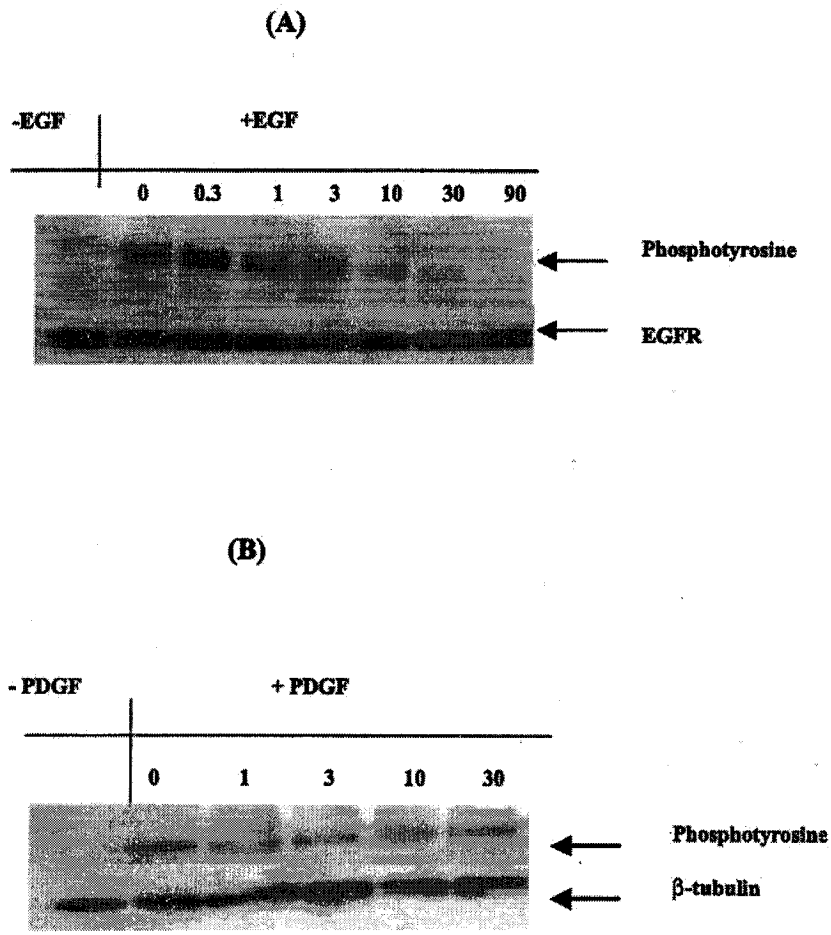


Fig. 3. Selective inhibition of EGFR and PDGFR autophosphorylation in intact cells by BJ2000. Serum starved A431 cells (A) or NIH3T3 cells (B) were preincubated for 90 min with the indicated concentrations of BJ2000 before stimulation with EGF (A) for 10 min or PDGF (B) for 10 min. Equal amount of cell lysates was analyzed by Western blotting using anti-phosphotyrosine antibodies. Membranes were stripped of anti-phosphotyrosine and reprobed with anti-EGFR or anti-β-tubulin antibodies as a loading control. Band intensities were measured using the GeneTools software package (SynGene).

Results

Degradation of BJ2000

The half-life of BJ2000 measured by UV-spectrophotometry at 340 nm was 34 min in RPMI 1640 medium supplemented with 10% FBS at 37°C; however, we noted that absorbance at this wavelength reached early saturation at high absorbance units (e.g., 0.6). Therefore, the study was performed with HPLC analysis whereby the decreasing and increasing peaks associated with the disappearance of BJ2000 and appearance of FD105, could be clearly monitored. As expected, an inverse relationship was observed between the areas of the peaks associated with these two species with observed half-life of 75 min for BJ2000. Calculations based upon area/concentration standard curve [Area = 2.796x (concentration) − 38.65; R^2 = 0.99] indicated that BJ2000 was converted into FD105 in an 87% yield after a 24-h incubation in RPMI 1640 medium supplemented with 10% FBS at 37°C (Fig. 1).

Inhibition of EGFR TK Activity

In a competitive EGFR binding assay, BJ2000 (IC_{50} = 0.1 μM) showed 2-fold greater binding affinity than its metabolite FD105 (IC_{50} = 0.2 μM) for the ATP site of purified EGFR. Therefore, both the drug and its corresponding prodrugs showed significant affinity for EGFR. As previously reported (Matheson et al., 2001), TEM did not show any effect on the tyrosine kinase activity of this receptor (IC_{50} > 100 μM) (Fig. 2A). Furthermore, the EGFR selectivity of these two agents was tested by comparing their EGFR inhibitory activities with those of other TKs such as c-src and the insulin receptor. In ELISA assays, BJ2000 and its metabolite FD105 did not block c-src TK activity nor did it exert any effect on insulin receptor TK in the 1 to 100 μM range, indicating the selectivity of these agents for EGFR (Fig. 2, B and C).

Inhibition of EGFR Autophosphorylation

Western blot analysis demonstrated that BJ2000 blocked EGF-induced EGFR autophosphorylation in A431 cells in a dose-dependent manner with an IC_{50} value of ~6 μM without affecting the levels of EGFR (Fig. 3A). At concentrations as high as 30 μM, it had no effect on PDGF-induced PDGFR autophosphorylation in NIH3T3 cells (Fig. 3B). These results represent further evidence of BJ2000 selectivity for EGFR.

Mechanism of EGFR Inhibition

Unlike FD105, BJ2000 is a reactive molecule capable of alkylating nucleophiles. Thus, we surmised that it might inflict some covalent damage to the ATP site of EGFR, thereby inducing irreversible inhibition. To test this hypothesis, we used the reversibility assay described by Smaill et al. (2000) and Fry et al. (1998) according to which the cells are

treated with drug for 90 min and the culture medium repeatedly removed and replaced three times after treatment, after which EGFR autophosphorylation is measured. As expected, BJ2000 and FD105 at 30 μM completely suppressed EGF-dependent EGFR autophosphorylation in A431 cells immediately after drug exposure. However, at 8 h post-treatment in drug-free medium (after repeated washouts), only 40% of the EGFR autophosphorylation activity was restored in cells treated with BJ2000, indicating that the latter is capable of inducing partially irreversible inhibition of EGFR autophosphorylation. In contrast, 96% of EGFR autophosphorylation activity was restored in cells treated with FD105 at the same dose. As expected, TEM did not show any inhibitory activity immediately after the 90-min treatment, nor did it induce any effect at 8 h post-treatment (Fig. 4).

Antiproliferative Activity of BJ2000

Inhibition of Growth Factor-Stimulated Proliferation. SRB assays demonstrated that like FD105 (Fig. 5A), BJ2000 (Fig. 5B) was capable of selectively blocking EGF or TGFα-induced proliferation in NIH3T3 cells stably transfected with the EGFR gene (NIH3T3HER14) (100% growth inhibition at 1 μM). This C-molecule and its metabolite were approximately 30-fold less effective in inhibiting PDGF-stimulated growth (Fig. 5, A and B) (100% inhibition at around 30 μM). Similarly, they exhibited a lesser effect on serum-stimulated growth in NIH3T3HER14 cells (100% growth inhibition at concentrations >30 μM) (Fig. 5, A and B). These EGFR-selective effects are in agreement with those observed from ELISA and whole cell autophosphorylation assays.

Reversibility of Growth Inhibitory Activity. The A431 cells express TGFα and overexpress its cognate receptor EGFR, leading to an aggressive autocrine cell growth. These cells have been shown to be sensitive to antiproliferative agents targeting the EGFR both in vitro or in vivo (Lanzi et al., 1997). Moreover, they also express the DNA repair enzyme O^6-alkylguanine transferase (AGT) known to be responsible for resistance to monoalkyltriazenes of the same class as BJ2000 (Mitchel and Dolan, 1993). Thus, this cell line was found an appropriate model for testing the sustainability of the antiproliferative effects of our different agents. Under 120-h continuous exposure, the results obtained from SRB assay as illustrated by Fig. 6A, showed that BJ2000 was ~2-fold more potent (IC_{50} = 15 μM) than its metabolite FD105 alone (IC_{50} = 47 μM; Fig. 6B) in the AGT-proficient cell line A431. In contrast, TEM at concentrations as high as 200 μM did not show any significant antiproliferative activity in these cells (Fig. 6C). More importantly, in a short exposure assay (2 h) followed by 120-h recovery, an almost complete loss of activity was observed for FD105 in the A431 cell line (IC_{50} > = 100 μM; Fig. 6B), indicating that it induced significantly reversible growth inhibitory activity. In contrast, BJ2000 showed significant retention of activity (IC_{50} = 38 μM; Fig. 6A), indicating a more sustained effect of the latter.

Inhibition of EGFR-Mediated Signaling

To determine whether blockade of EGFR autophosphorylation translates into inhibition of downstream signaling, we analyzed the effect of the C-molecule on EGF-induced phosphorylation of MAPK (Erk2) and c-fos expression in A431 cells. The results showed that BJ2000 induced complete inhibition of Erk2 phosphorylation at concentrations as low as 1 μM without affecting the levels of Erk2 (Fig. 7). Similarly, RT-PCR analysis showed that BJ2000 induced nearly 100% inhibition of EGF-mediated c-fos gene expression at low concentrations (0.3–3 μM) (Fig. 8), indicating that inhibition of EGFR phosphorylation by the C-molecule is accompanied by a significant blockade of EGFR-dependent downstream signaling.

Quantitation of DNA Damage

Using the alkaline comet assay, we demonstrated that, in contrast to FD105 (Fig. 9) and like TEM (Matheson et al., 2001), BJ2000 induced a dose-dependent DNA damage in both A431 and NIH3T3HER14 cells after a 30-min drug exposure (Fig. 9), implying indirect evidence of the formation of metastable methyldiazonium species.

Discussion

Agents targeting EGFR and its closest homolog $p185^{neu}$, the erbB2 gene product, present two major advantages: 1) they induce target-selective antitumor activities and 2) they exhibit good toxicity profiles. However, where they cannot induce apoptosis, they are cytostatic agents that induce reversible antitumor effects. For sustained antitumor activity, combinations with other cytotoxic drugs (e.g., cisplatin, doxorobucin, and taxans) have proven a useful alternative (Ciardiello et al., 2000). However, the lack of selectivity of the latter agents may negatively alter the overall toxicity profiles of these regimens. We now propose a novel approach to this problem that seeks to combine EGFR TK inhibitors with the pharmacophores of known cytotoxic DNA-damaging properties into single chimeric molecules targeted to EGFR. As

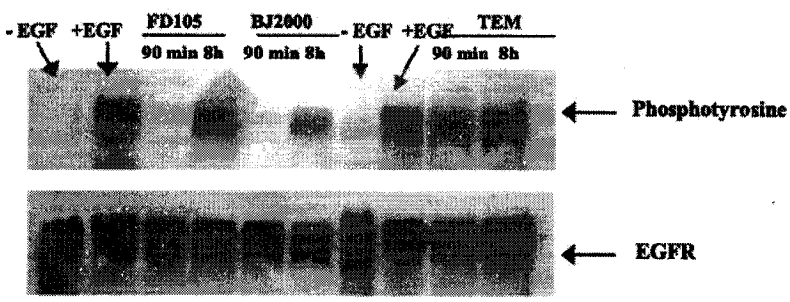


Fig. 4. Reversal of EGFR autophosphorylation in the presence of BJ2000, FD105, or TEM in A431 cells. Duplicate sets of cells were treated with 30 μM of designated compound to be tested as a reversible EGFR inhibitor for 90 min. One set of cells was then stimulated with EGF for 10 min, and extracts were made as described under the Western blotting procedure. The other set of cells was washed free of the compound with warmed serum-free media, incubated for 2 h, washed again, incubated for another 2 h, and incubated for a further 4 h after a subsequent wash. This set of cells was then stimulated with EGF, and extracts were made similar to the first set. The phosphotyrosine and EGFR were detected as described under *Materials and Methods*.

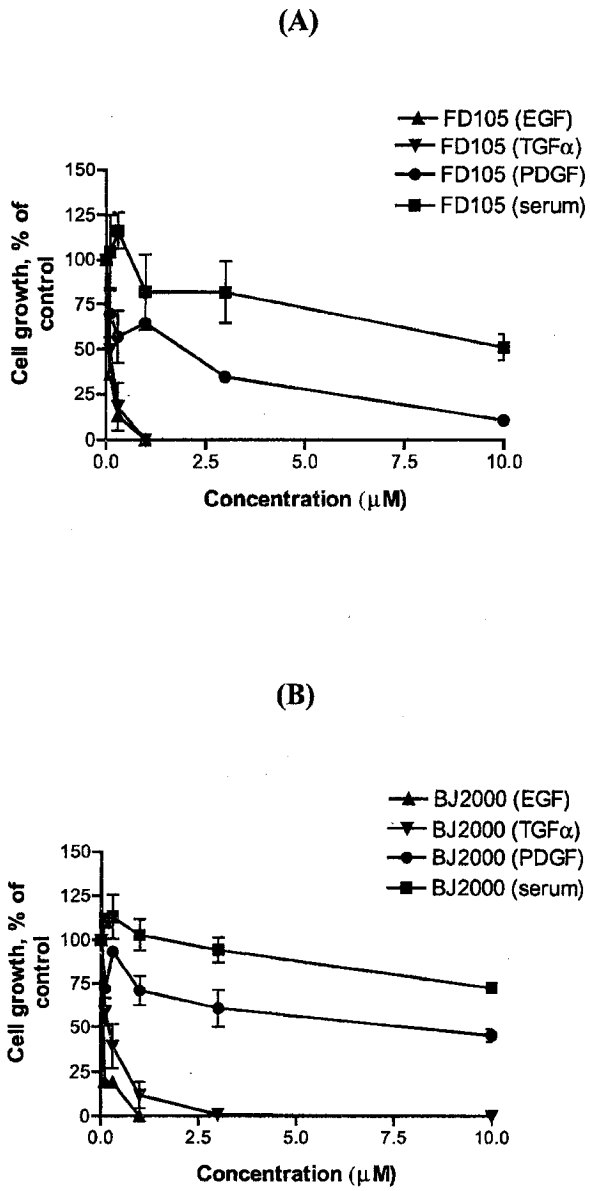


Fig. 5. Effect of FD105 and BJ2000 on growth factor-stimulated proliferation in NIH3T3HER14 cells. A, FD105 + EGF (TGFα, PDGF, or serum). B, BJ2000 + EGF (TGFα, PDGF, or serum). Cells were exposed to each drug and growth factors (EGF, TGFα, PDGF, or serum) for 72 h. Cell growth was measured using SRB assay. Each point represents at least two independent experiments run in triplicate.

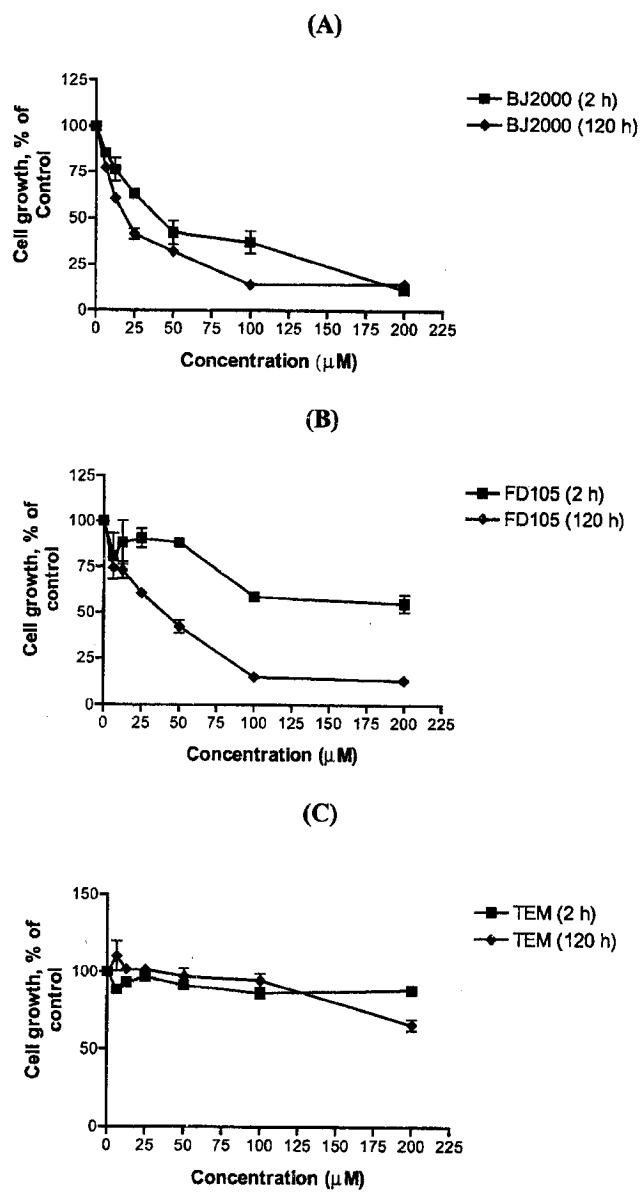


Fig. 6. Reversibility of antiproliferative effect of BJ2000 (A), FD105 (B), and TEM (C) in A431 cells. Cells were exposed to each drug for 2 h, after which they were allowed to recover for 120 h in drug-free medium or continuously for 120 h. Cell growth was measured using SRB assay. Each point represents at least two independent experiments run in triplicate.

outlined in Scheme 2, the target-mediated selectivity component of our approach is based on the strong affinity of the C-molecule for the cytosolic domain of EGFR that may influence the equilibria TZ-*I'* (extracellular compartment)/TZ-*I* (cytosolic) (path 1) and TZ-*I* (cytosolic)/TZ-*I*-EGFR (Scheme 2). Both bound and unbound fractions of TZ-*I* will eventually degrade to generate *I* that may further block EGFR TK (path 5). Fractions of unbound TZ-*I* may diffuse through the nucleus where the generated methyldiazonium species (TZ) may alkylate and damage DNA (path 6). Bound fractions may react with amino acid residues of the active site of the receptor, thereby irreversibly inhibiting it. More importantly, unreacted fractions of the in situ generated TZ may diffuse away from the receptor. If the in situ-generated *I* loses affinity for the damaged receptor, it may bind to other nondamaged receptor molecules, leading to a more sustained EGFR TK inhibition (path 4). Using BJ2000 as a probe, the work discussed herein gave prima facie support to our postulates.

BJ2000 was designed to possess two major components: an EGFR inhibitory component imprinted into the quinazoline moiety and a DNA-damaging methyldiazonium species masked by the appended 3-methyl-1,2,3-triazene moiety. The effects of the EGFR component were demonstrated by both enzyme and whole cell assays, whereby the ability of the C-molecule to block substrate (ELISA) and EGFR autophosphorylation (Western blotting) was clearly shown. Further-

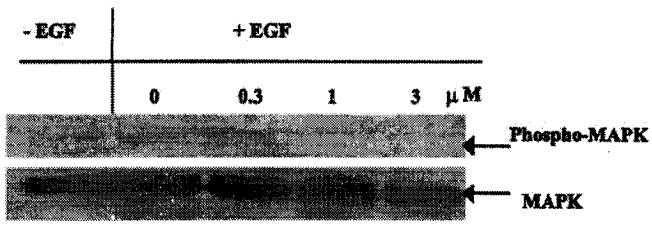


Fig. 7. Effect of BJ2000 on MAPK (Erk2) activation in A431 cells. Serum-starved cells were preincubated for 90 min with the indicated concentrations of BJ2000 before stimulation with EGF for 10 min. Protein lysates were obtained, and Western blot was performed as described by Tari and Lopez-Berestein (2000).

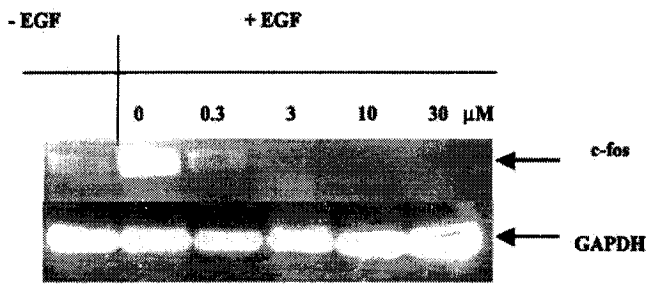


Fig. 8. Effect of BJ2000 on c-fos gene expression in A431 cells. Serum-starved cells were preincubated for 90 min with the indicated concentrations of BJ2000 before stimulation with EGF for 30 min. Quantitative analysis of c-fos and GAPDH was preformed by RT-PCR, as described under *Materials and Methods*.

more, BJ2000 exhibited selectivity for EGFR in both ELISA and growth factor-stimulated proliferation assays. The reversibility assay showed an only 40% recovery of the autophosphorylation activity, 8 h after cell exposure to BJ2000, despite multiple washouts. In contrast, almost complete recovery of initial EGFR autophosphorylation activity was observed in cells treated with FD105 at the same dose (Fig. 4). It is not clear at this point whether the partial inactivation of the EGFR TK occurred through alkylation of amino acid residues located at the active site. Nevertheless, Smaill et al. (2000) and Fry et al. (1998) demonstrated that acryloyl moieties attached to position 6 of quinazoline reacted with cysteine 773 of EGFR, leaving an irreversibly inhibited receptor. The triazene chain of our C-molecule being appended to the same position and possessing approximately the same length as the acryloyl moiety is likely to undergo a similar type of interaction.

As for the DNA-damaging component, we compared our activities with those of the clinical drug TEM, a cyclic triazene that generates an open chain monoalkyltriazene of the same type as BJ2000 (Baig and Stevens, 1987). Like TEM, BJ2000 induced significant DNA damage in A431 cells after a 30-min drug exposure (Fig. 9). This represents an indirect confirmation of the formation of the DNA-damaging methyldiazonium species (TZ) postulated by path 6. The complete confirmation of the latter path that predicts the hydrolytic conversion of TZ-*I* into TZ + *I* was provided by HPLC detection of *I* (FD105) and the kinetic analysis. The results clearly indicated an inverse relationship between the degradation of BJ2000 ($t_{1/2}$ = 75 min) and the formation of FD105 in an 87% yield after complete degradation (Fig. 1, 4–24 h). Thus far, the results in toto lend support to almost all the postulates depicted in Scheme 2 (paths 1–6), according to which a TZ-*I* (or C-molecule) can bind to EGFR (paths 1–3) or degrade to another high-affinity inhibitor *I* (paths 2 and 5) plus a reactive species that can damage DNA (path 6) and perhaps the receptor (path 4).

The principle by which the multiple properties of BJ2000 should culminate into sustained growth inhibition was demonstrated in comparison with its derived inhibitor FD105. In contrast to the latter, BJ2000 antiproliferative activity in A431 cells was partially retained as long as 5 days after a 2-h drug exposure. The complete loss of activity of FD105 and the marked inactivity of TEM, a known DNA-damaging agent, in the A431 cells, suggest that the sustained antiproliferative activity of our C-molecule may result from an interactive effect between its DNA-damaging component and its signal transduction inhibitory properties. This assumption may be further corroborated by our demonstration of the ability of the C-molecule to block MAPK phosphorylation and c-fos gene expression at low concentrations (1–3 μM). Because MAPK is a critical signal transduction protein known to induce c-fos gene expression and to mediate the mitogenic effects of EGF-activated signaling (Davis, 1993, 1995), blockade of EGFR autophosphorylation by our C-molecule and by its derived inhibitor *I* may translate into inhibition of downstream signaling associated with expression of genes required to rescue the cells. Indeed, Kaina et al. (1997) demonstrated that c-fos-deficient cells exhibited a more severe mutagen-induced block to DNA replication and were compromised in the abolition of replication blockage. Thus, the mechanism by which blockade of EGF-induced signal transduction by the TK inhibitory element down-regulates DNA repair enzymes (e.g., AGT and DNA glycosylases) or other critical proteins can be evoked to rationalize the EGFR-mediated sustained inhibition of proliferation induced by our

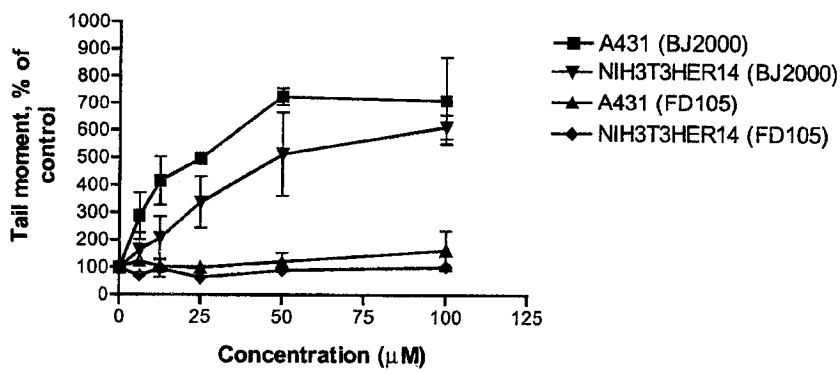


Fig. 9. Quantitation of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in A431 and NIH3T3HER14 cells exposed to BJ2000 and FD105 for 30 min. Each point represents at least two independent experiments.

C-molecule. We are now verifying this hypothesis by analyzing the regulatory DNA repair genes induced in response to cell exposure to our C-molecules.

In summary, the testing of our Combi-Targeting postulates has led to the development of a potent molecule capable of behaving as a masked form of multiple effects: 1) direct inhibition of EGFR autophosphorylation, 2) inhibition of the same target by its derived inhibitor *I*, 3) induction of DNA damage and perhaps covalent damage of EGFR, 4) sustained growth inhibitory activity, and 5) inhibition of EGFR-mediated MAPK activation and c-fos gene expression. Thus, our work may contribute to the development of novel binary tumor targeting strategies with the prospect of circumventing the adverse effects associated with the lack of selectivity and potency of classical chemotherapy.

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