A Novel Small Molecule-Based Multi-Targeting Approach for the Selective Therapy of Epidermal Growth Factor Receptor (EGFR)- or Her2-Expressing Carcinomas

by

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Abstract

The chemotherapy of solid tumours is hampered by two major obstacles: 1) the lack of selectivity of the current drugs and 2) the reduced sensitivity to tumour drugs. Recent advances in cancer biology have led to the identification of novel molecular targets responsible for aggressive proliferation, drug resistance and invasiveness. Targeting one such factor, epidermal growth factor receptor (EGFR), has proven an effective strategy to block the progression of solid tumours. However, the potency of the current clinical drugs directed at EGFR is mitigated by their reversible cytostatic activity and reduced cytotoxicity. Here we describe the design and synthesis of a new class of agents termed "cascade release combi-molecule" (I-TZ), "programmed" to hammer the receptor by releasing multiple bioactive metabolites (I-TZ', I-TZ") in a step-wise fashion and a reactive DNA damaging species (TZ), with the purpose of promoting sustained inhibition of EGFR tyrosine kinase (TK) and drug-induced cytotoxicity. This thesis describes the synthesis of one such molecule, RB24 (I-TZ), and demonstrates that it was capable of being hydrolyzed to its various sub-components with an overall half-life of 42 min under physiological conditions. The I-TZ induced sustained inhibition of the EGFR TK and strong antiproliferative activity in human solid tumour cells. It also inflicted higher levels of DNA damage to cancer cells transfected with the EGFR or HER2 gene. This selectivity was based on a novel mechanism of targeting described as a bystander effect, whereby following distribution of the I-TZ in the perinuclear region, the alkylating species (TZ) was subsequently released towards the nucleus. The I-TZ was significantly more potent than its clinical counterpart, Temodal™. Further investigation of the mechanism underlying its superior potency show that blockade of EGFR-

mediated signaling led to down-regulation of: 1) MAPK mitogenic signaling, 2) the BER protein, XRCC1 and 3) anti-apoptotic signaling mediated by Bad. Further down-regulation of XRCC1, with a MEK1 inhibitor, led to the discovery of a significant synergistic antiproliferative effect and a novel formula to further enhance the potency of the I-TZ in refractory tumours. Furthermore, the *in vivo* activity of the cascade release principle was proven in a prostate cancer xenograft study using a more water-soluble analogue of RB24. Thus, we have developed a novel model for the therapy of growth factor receptor-expressing tumours from the design and synthesis of the probe drugs, to the elucidation of their mechanism of action and the demonstration of the *in vivo* efficacy of the proposed strategy.

Résumé

La chimiothérapie des tumeurs solides se heurte à deux obstacles majeures: 1) un manque de sélectivité et 2) une chimiorésistance contre les médicaments anticancéreux. Les récentes découvertes en oncogénèse ont permis d'identifier de nouvelles cibles moléculaires responsables de la prolifération non contrôlée, de la chimiorésistance et de la formation des tumeurs métastasiques. L'inhibition du récepteur de facteur de croissance (EGFR) s'est avérée être une stratégie efficace pour ralentir la progression des tumeurs solides. Cependant, l'efficacité des médicaments actuels ciblant ce récepteur est compromise par une activité cytostatique réversible et un manque de cytotoxicité.

Dans ce travail, nous présentons la conception et la synthèse d'une nouvelle classe d'agents anticancéreux, dénommée 'combi-molécules à décomposition en chaîne' (I-TZ). Ces molécules sont conçues pour se fragmenter progressivement en multiples métabolites bioactifs (I-TZ', I-TZ'') aboutissant, au final, à la libération d'une espèce alkylante (TZ). Cela a pour effet d'induire l'inhibition soutenue de l'activité tyrosine kinase du récepteur et de promouvoir la cytotoxicité. Ce manuscrit traite de la synthèse et de l'hydrolyse dans les conditions physiologiques d'une molécule type RB24 (I-TZ), caractérisée par un temps de demi-vie global de 42 min. De ce fait, elle possède une activité antiproliférative envers des tumeurs solides grâce, d'une part, à l'inhibition soutenue du récepteur de facteur de croissance et, d'autre part, à l'endommagement de l'ADN. Nous avons noté que les cellules tumorales transfectées par des gènes codant pour l'EGFR ou HER2 montrent des lésions de l'ADN plus importantes. Ce résultat peut être expliqué par la concentration des molécules dans la région péri-nucléaire

rapprochant ainsi l'entité alkylante (TZ) du noyau. Le composé RB24 a montré une activité supérieure à celle du médicament de référence Temodal^{TM}. L'étude approfondie du mécanisme d'action a démontré que l'inhibition du récepteur de facteur de croissance conduit à la suppression: 1) du signal mitogène transmis via MAP Kinase, 2) de l'expression des protéines BER, XRCC1 et 3) du signal anti-apoptotique régulé par Bad. La co-administration d'un inhibiteur de MEK1 capable de bloquer l'expression de XRCC1 a permis de découvrir une étonnante synergie antiproliférative, et d'établir une nouvelle formule pour l'optimisation de l'effet de I-TZ envers les tumeurs réfractaires. De plus, nous avons également démontré l'efficacité *in vivo* d'un analogue plus hydrosoluble de RB24, dans un modèle de xénogreffes de la prostate.

En conclusion, nous avons réalisé un cycle complet de découverte d'une nouvelle méthode de traitement des tumeurs exprimant le récepteur de facteur de croissance: de la conception et la synthèse des molécules sondes, l'élucidation de leur mécanisme d'action, à la démonstration d'une efficacité de cette stratégie *in vivo*.

Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.

-Louis Pasteur (1822-1895)

This thesis is dedicated to my uncle Shambhu Banerjee, whose life was cut short at such a young age, while bravely battling with cancer.

You will forever remain in our hearts.

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Finally, I would like to thank my parents, Rabindra and Reba, and my sister, Rupa, for all their love and encouragement.

Foreword

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

As an alternative to the traditional thesis format, the thesis 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	O^6-alkylguanine transferase
BER	base excision repair
BG	benzylguanine
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
FAK	focal adhesion kinase
HPLC	high pressure liquid chromatography
HRG	heregulin
HRP	horseradish peroxidase
I	inhibitor
JNK	c-jun-N-terminal kinase
MAPK	mitogen-activated protein kinase
MMR	mismatch repair
MTIC	5-(3-methyltriazen-1-yl)imidazole-4-carboxamide
PBS	phosphate-buffer saline
PGT	poly (L-glutamic acid-L-tyrosine, 4:1)
RTK	receptor tyrosine kinase
SAPK	stress activated protein kinase
SRB	sulforhodamine B assay
SSB	single strand break

TEM	temozolomide, Temodal™
TK	tyrosine kinase
TZ	methyldiazonium

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CHAPTER 1

INTRODUCTION

1.1. Preface

Cancer originates from the development of numerous mutations that lead to uncontrolled cellular proliferation. Certain mutations lead to abnormal or aberrant expression of signaling proteins and transcription factors. Despite the importance of the aforementioned proteins in cancer progression, traditional chemotherapeutic treatment regimens are primarily directed at damaging DNA or blocking proteins required for DNA replication. The lack of tumour selectivity and toxicity associated with these regimens are considered major deterrents for their use as therapeutic agents. Recently, therapies targeting the receptor tyrosine kinases (RTK) or cytoplasmic RTKs, that control the activation of numerous signaling cascades (e.g. cell cycle progression, anti-apoptotic signaling, cellular proliferation) have proven highly efficacious. These cellular receptors include the insulin-like growth factor receptor (IGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptors (EGFR), as well as its closely related homologue, c-ErbB2 (HER2). All these receptors are implicated in tumour progression, aggressiveness and invasiveness of various carcinomas. Since the purpose of this thesis was to develop a novel approach for tandem targeting of EGFR and DNA, a brief review of current knowledge acquired on this class of receptors is presented herein.

1.2 EGF Receptor and its signaling pathways

1.2.1. EGFR family

The epidermal growth factor receptor subfamily consists of EGFR (ErbB1), HER2 (ErbB2/neu) an orphan receptor, HER3 (ErbB3) lacking kinase function and HER4

(ErbB4) (Hackel *et al.*, 1999; Christensen *et al.*, 2001). HER2 is the preferential dimerization partner for all the members of the ErbB receptor family (Christensen *et al.*, 2001; Roskoski, 2004). The HER3 receptor lacks a tyrosine kinase domain and therefore, can only activate downstream signaling cascades when it dimerizes with another ErbB receptor (Roskoski, 2004). High levels or overexpression of EGFR and HER2 have been correlated with aggressiveness and poor prognosis, making EGFR a promising target for effective chemotherapy of tumours that express this class of RTK (Alaoui-Jamali *et al.*, 1997; Harari and Yarden, 2000). Similarly, the EGFRvIII is a truncated form of EGFR caused by an in-frame deletion of exons 2-7. The resulting protein lacks 267 amino acids (aa 6-273) in the extracellular domain resulting in a 145 kDa truncated structure, the activation of which is ligand-independent. Thus, EGFRvIII is constitutively activated (Alaoui-Jamali *et al.*, 1997; Harari and Yarden, 2000). It has been shown that cells expressing EGFRvIII have a greatly enhanced capacity for unregulated growth due to the ability of the EGFRvIII to promote tumour cell survival and proliferation. This aberrant receptor is expressed in many different types of cancer, including breast carcinomas, non-small cell lung carcinomas and glioblastoma multiforme (Okamoto *et al.*, 2003; Rae *et al.*, 2004; Heimberger *et al.*, 2005).

1.2.2. EGFR structure and activation

EGFR is a 170-kDa glycoprotein that contains three functional domains: a transmembrane domain, a carboxy terminal and an extracellular domain (ectodomain) to which its cognate ligand can bind (Roskoski, 2004; Normano *et al.*, 2006). The extracelluar domain consists of the following four subdomains: L1 and L2, the ligand

binding domains, CR1 and CR2, the cysteine rich regions that participate in receptor dimerization (Fig. 1.1) (Burgess *et al.*, 2003). The 11 different ligands determined for the ErbB class of receptors are EGF, transforming growth factor (TGF)-α, heparin-binding (HB)-EGF, betacellulin, amphiregulin, epiregulin, epigen and the neuregulins 1-4 which are also known as heregulins (HRG) (Hackel *et al.*, 1999; Normano *et al.*, 2006).

In the absence of ligand, EGFR is found on the cell surface in both monomer and dimer form. Following ligand binding to the L2 region, which is situated in the extracellullar domain of EGFR, the receptor undergoes receptor dimerization (Normano *et al.*, 2006). Once the receptor is ligand-bound (e.g. EGF, TGF-α), a loop from the CR1 domain of one receptor, known as the 'dimerisation loop', interacts with the CR1 loop in the partner (Roskoski, 2004; Normano *et al.*, 2006). Mutations or deletions in the dimerization loop region have been shown to completely block EGFR activation (Burgess *et al.*, 2003). The transmembrane domain has also been found to play an important role in receptor association through either van der Waals interactions or hydrogen bonding (Lemmon *et al.*, 1997). In order to promote receptor dimerization, it is believed that a significant portion of EGFR molecules are distributed in clusters throughout the membrane (Maione *et al.*, 1999; Jorissen *et al.*, 2003). This proximal distribution permits rapid interaction between receptors upon ligand binding. The C-terminal consists of several important residues that are required for both initiation of signaling cascades and receptor endocytosis (Jorissen *et al.*, 2003). Once activated, the ATP binding site for EGFR, which is located between the N- and C-terminal domains, catalyses the transfer of

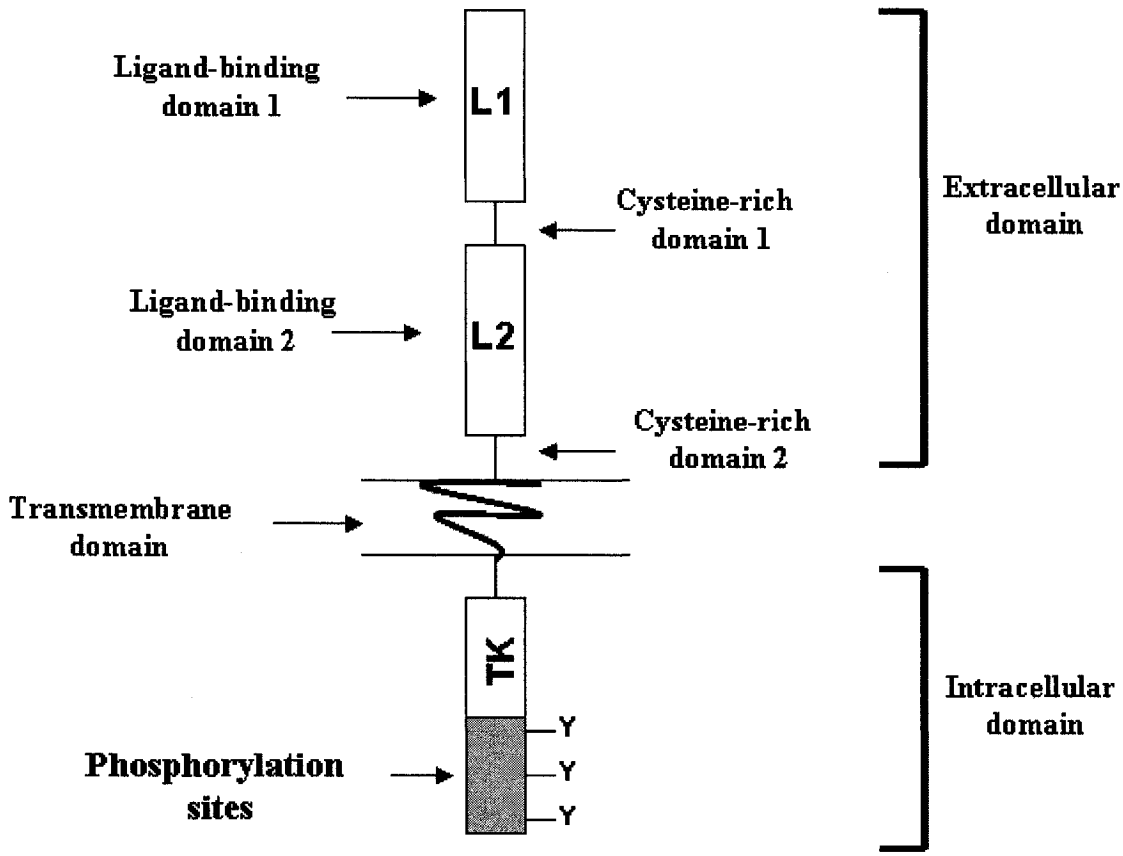


Figure 1.1. Structure of the ErbB class of receptors.

the gamma-phosphate of bound ATP to tyrosine residues found on both intracellular targets and the C-terminal domain of the EGFR TK (Bishayee *et al.*, 1999). There are five tyrosine residues in the C-terminal domain that are considered important substrates for EGFR signal transduction (Y1068, Y1148, Y1173, Y992, Y1086) (Bishayee *et al.*, 1999). These tyrosine residues can either be autophosphorylated (a process by which the receptor phosphorylates its own amino acid residues or residues of a partnering receptor) or transphosphorylated by other kinases in the cell and function as docking sites for various adaptor proteins or signaling proteins (Koch *et al.*, 1991; Normano *et al.*, 2006).

The strength and duration of the signal from EGFR depends upon how quickly the activated protein is internalized and recycled. The naturally occurring EGFR mutant known as EGFR vIII (version III) is constitutively activated and, therefore, not internalized (Pedersen *et al.*, 2001). Cells that express this mutant have been shown to harbour elevated levels of phosphotidylinositol-3-kinase (PI3K) and c-jun-N-terminal kinase (JNK) activation, as well as upregulation of the antiapoptotic protein Bcl-xL, a member of the Bcl-2 class of proteins (Nagane *et al.*, 1996; Antonyak *et al.*, 1998; Pedersen *et al.*, 2001).

1.2.3. Recruitment of adaptor proteins and activation of signal transduction

Numerous pathways are initialized following receptor dimerization and autophosphorylation, and two such pathways, the Ras/Raf/MAPK and the PI3K/Akt are the most extensively studied. The phosphorylated tyrosine residues found in the C-terminal of activated EGFR can recruit various adaptor proteins, such as Grb2 which is

constitutively bound to the guanine nucleotide exchange factor, Sos, through its two SH3 (*src* homology-3) domains (Koch *et al.*, 1991; Mayer, 2001; Vidal *et al.*, 2001). The latter domain interacts with proline-rich sequences. Following EGFR activation, Grb2 interacts with the phosphorylated residues on EGFR through its SH2 (*src* homology-2) domain or indirectly through another adaptor protein Shc (Fig. 1.2.1) (McInnes and Sykes, 1997). The latter can subsequently recruit Grb2 or other signaling proteins. Once Grb2 is relocated to the cell surface via receptor interaction, it can interact with Sos and the membrane-bound Ras, a serine-threonine kinase, that facilitates the exchange of GDP-bound Ras for a GTP-bound state (Macias *et al.*, 2002). As depicted in figure 1.2, activation of Ras triggers the Ras/Raf/MAPK signaling cascade and this ultimately culminates into the expression of early response genes such as c-fos and other genes that control proliferation (Tari *et al.*, 1999; Rowinsky *et al.*, 1999).

1.2.4. The Ras/Raf/MAPK signaling pathway

The Raf/MEK/ERK cascade generally controls cellular growth and differentiation (Rowinsky et al., 1999). When EGFR is activated upon EGF-binding, adaptor proteins, such as Grb2, are recruited to assist in the activation of Ras (Alessi *et al.*, 1994; Sebolt-Leopold, 2000; Chang *et al.*, 2003). GTP-bound Ras activates Raf, which can subsequently phosphorylate MEK1/2 on Ser 217 and Ser 221 residues. Further, phosphorylation of ERK 1 and 2 on threonine (e.g. T185, T202) and tyrosine (e.g. Y204, Y187) residues by MEK1/2 lead to the activation of various cytoplasmic targets proteins, including the intracellular domain of EGFR and the p90 ribosomal S6 kinase (RSK) (Alessi *et al.*, 1994; Chang *et al.*, 2003).

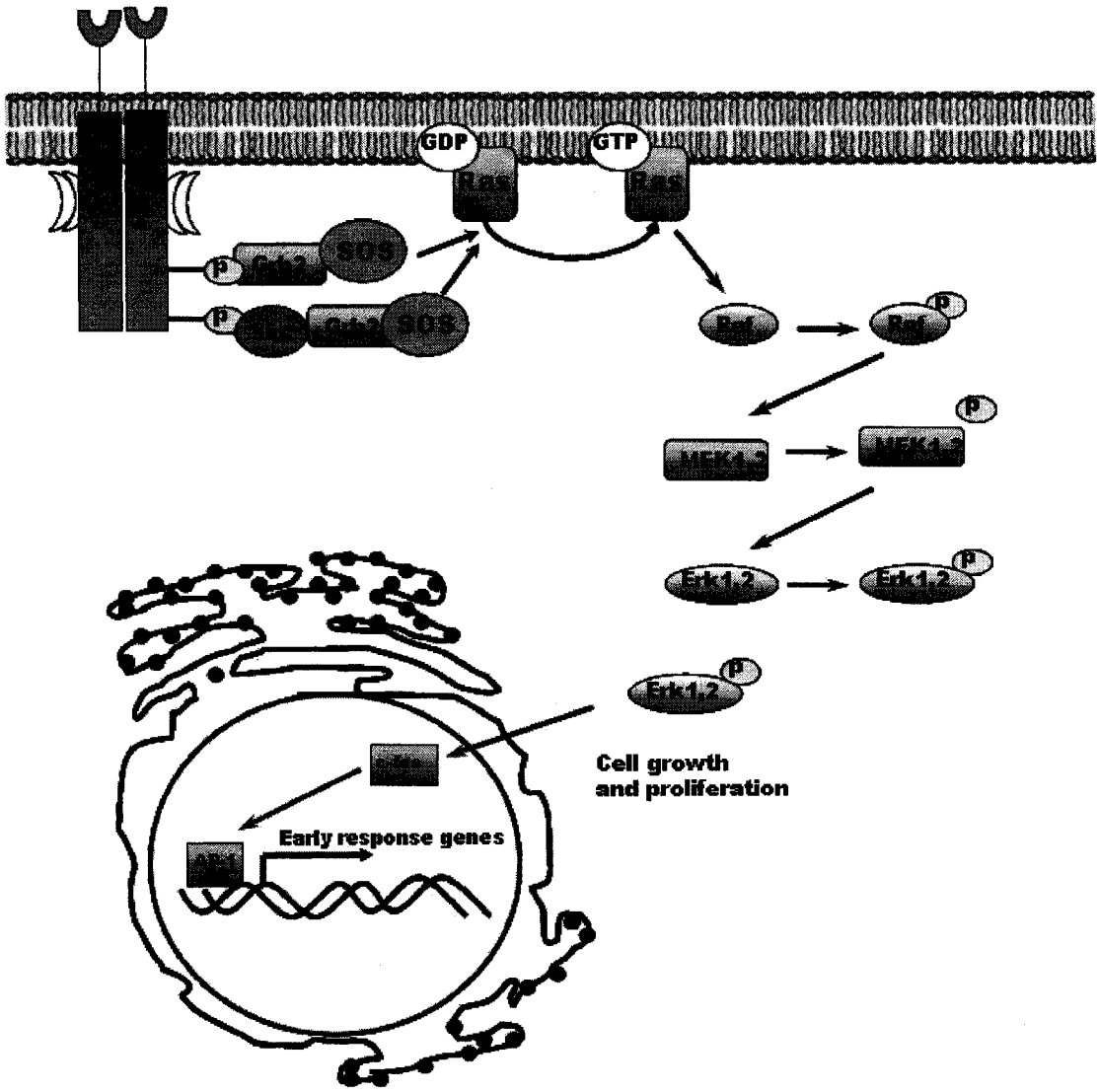


Figure 1.2.1. Activation of the Ras-Raf-MAPK signaling cascade following EGFR activation.

The ERK kinases translocate into the nucleus, where they can phosphorylate numerous transcription factors that initiate cell cycle progression from the G0 (quiescent phase)/G1 (GAP1) phase to the S (Synthesis) phase of the cell cycle (Murray and Hunt, 1993).

Growth factor induced signaling cascades, such as the Ras/Raf/MAPK pathway, culminate into the transcription of both "early-response" and "delayed-response genes" (Murray and Hunt, 1993). Early-response genes encode transcription factors such as c-fos and c-jun, that heterodimerize to form the AP-1 transcription factor complex and subsequently stimulate the transcription of "delayed response" genes required for cell cycle progression (e.g. cyclin D1) (Murray and Hunt, 1993). Accumulation of cyclin D1 following growth factor stimulation results in the formation of cyclin D1/Cdk4,6 complexes (Cheng *et al.*, 1998) that phosphorylate the retinoblastoma protein (pRb). pRb is a tumour suppressor that exerts its growth-suppressing effects by trapping various proteins, including the E2F family of transcription factors (see Fig. 1.2.2) (Murray and Hunt, 1993; Zhu, 2005). Unbound (free) E2F transcription factors induce the expression of genes that regulate the G_1-S phase transition that precedes DNA replication. Following initial phosphorylation by the cyclin D1/CDK4 complex, pRb releases E2F, permitting transcription and accumulation of a second cyclin, cyclin E (Boonstra, 2003). The cyclin E forms a complex with cdk2 that further phosphorylates pRb. Hyperphosphorylation of pRb leads to the full activation of the E2F transcription factors (Fig. 1.2.2) (Murray and Hunt, 1993).

When growth factors are removed from cell culture medium, transcription of G_1-phase cdks and cyclins is halted (Moyer *et al.*, 1998). As a result, the cells cannot enter the S

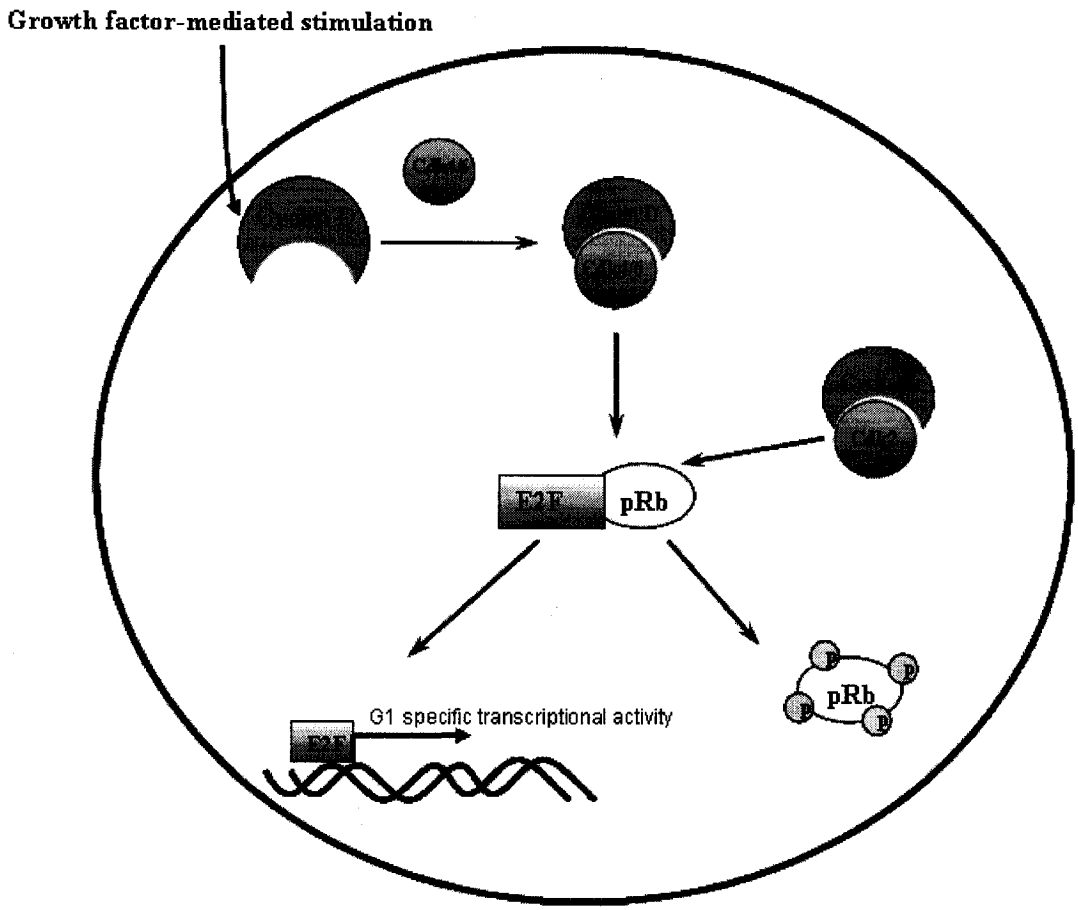


Figure 1.2.2. Cell cycle progression from the G1 to the S phase mediated by pRb phosphorylation.

phase and remain in G1. Inversely, when growth factors (e.g. EGF) are added, the activation of RTKs such as EGFR, allow the cell to progress through the cell cycle and proliferate and is referred to as growth-stimulation. This is a procedure used in this thesis to study the inhibition of EGFR. Briefly, the cells are placed in serum-free media for 24 h to deprive them of growth factors and subsequently exposed to either EGF or TGF-α. Phosphorylated tyrosine residues found on the activated EGF receptor following addition of growth factors are then detected using an anti-phosphotyrosine antibody.

1.2.5. The PI3K/Akt pathway and anti-apoptotic signaling

Cancer cells are endowed with the unique property to not only escape controlled growth and proliferation, but also to evade apoptosis, or "programmed cell death". Apoptosis is a process that entails several morphological changes such as chromatin condensation, DNA fragmentation and plasma membrane blebbing (or budding) (Vermeulen *et al.*, 2005). The apoptotic process can be activated by several factors such as the activation of death receptors (e.g. Tumour Necrosis Factor Receptor), DNA damage in the absence of DNA repair or exposure of cells to cytotoxic drugs and irradiation (Fumarola and Guidotti, 2004; de Thonel and Eriksson, 2005).

Extrinsic apoptotic signaling is mediated by the activation of "death receptors" which are cell surface receptors that include the tumor necrosis factor receptor (TNFR) gene superfamily. This family encompasses TNFR-1, Fas, and the TRAIL receptors (de

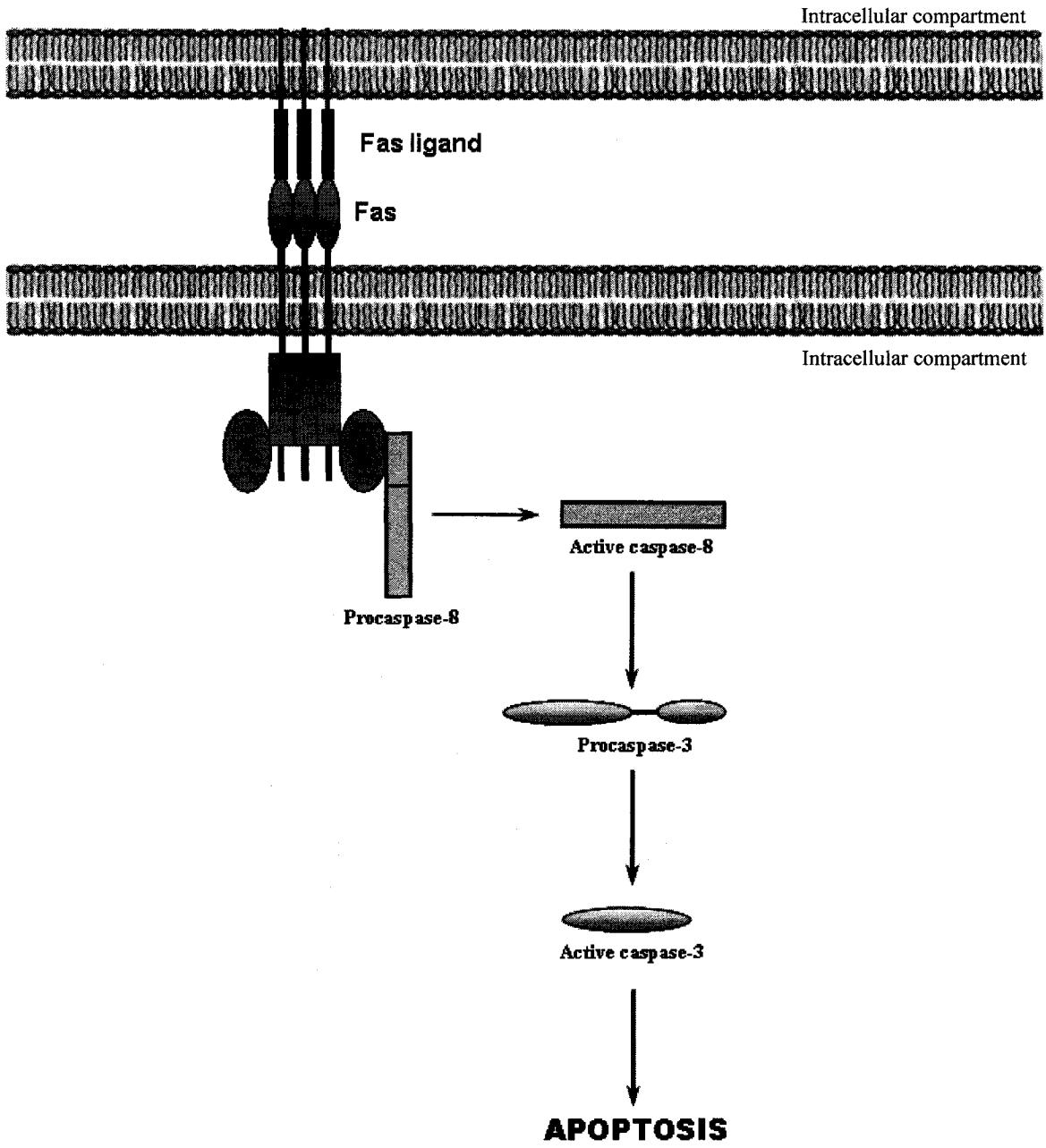


Figure 1.3.1. Extrinsic apoptotic signaling mediated by the death receptor, Fas.

Thonel and Eriksson, 2005; Hail *et al.*, 2006). As depicted in Fig. 1.3.1, activation of Fas recruits adaptor proteins such as Fas-associating protein with death domain (FADD), which subsequently activate proteins termed caspases (Houston and O'Connell, 2004; de Thonel and Eriksson, 2005). Caspases are cysteine proteases that are originally synthesized as inactive zymogens called procaspases. In order to be activated, the latter proteins require cleavage at their N-terminus between the large and small subunit. This scission forms an active heterotetramer consisting of two small and two large subunits (Thornberry and Lazebnik, 1998; Debatin, 2004). Activation of death receptors (e.g. Fas) initiates the apoptotic process via cleavage of procaspase-8 that belongs to the initiator class of caspases. The latter includes procaspase-2, -9 and -10 (Thornberry and Lazebnik, 1998; Ho and Hawkins, 2005). Caspase-8 can subsequently induce a cascade of effector caspases (e.g. procaspase-3, -6, -7) that cleave critical substrates such as DNA repair proteins (e.g. PARP), focal adhesion kinase (FAK) and the nuclear lamina (Thornberry and Lazebnik, 1998).

In some cells, apoptotic signals can also be mediated via intrinsic (intracellular) signaling pathways that culminate into a mitochondria-dependent apoptotic process (de Thonel and Eriksson, 2005). Mitochondrial permeability is generally regulated via members belonging to the Bcl-2 family of proteins that consists of both pro-survival (e.g. Bcl-2, Bcl-XL) and pro-apoptotic (e.g. Bax, Bak, Bid) members (Heiser *et al.*, 2004; de Thonel and Eriksson, 2005; Armstrang, 2006). Protein-protein interactions between anti-apoptotic and pro-apoptotic Bcl-2 members prevent the oligomerization of Bax, an event required for mitochondrial pore formation (see Fig. 1.3.2). Activation of Bax allows for

the release of mitochondrial proteins, such as cytochrome c, into the cyctoplasm (de Thonel and Eriksson, 2005; Armstrang, 2006). Once cytochrome c is released, it can form a complex with the cytoplasmic protein Apaf-1, termed the apoptosome. This apoptosome can then bind and cleave procaspase-9, releasing its activated form and subsequently initiating the caspase cascade through caspase-3 (Thornberry and Lazebnik, 1998; Heiser *et al.*, 2004).

Intrinsic apoptotic signaling is not only regulated by downstream signaling cascades induced by death receptors, but can also be triggered by growth factor receptors, such as EGFR. One of the major pathways by which EGFR can regulate anti-apoptotic signaling is by the phosphatidylinositol-3-kinase (PI3K)/ Akt pathway (Fig. 1.3.2) (Grant *et al.*, 2002; Martelli *et al.*, 2006). Since the central theme of this thesis is EGFR targeting, we will focus herein on the activation of this pathway by growth factor stimulation. PI3K is composed of a catalytic p110 domain and a p85 regulatory subunit that can directly interact with activated members of the ErbB family through an SH2 domain (Grant et *al.*, 2002; Kim *et al.*, 2005). As shown in Fig. 1.3.2., PI3K catalyses the phosphorylation of the 3' position of phophatidylinositols (PtdIns), that bind to their downstream target, the serine/threonine kinase, Akt/PKB (Datta *et al.*, 1999). This kinase is extremely important in the cellular response to anti-tumour drugs. Therefore, a brief description of its properties is provided below.

The Akt family is composed of three members: 1)Akt1/PKBα, 2) Akt2/PKBβ and 3) Akt3/PKBγ (Songyang *et al.*, 1997; Datta *et al.*, 1999). This class of serine/threonine

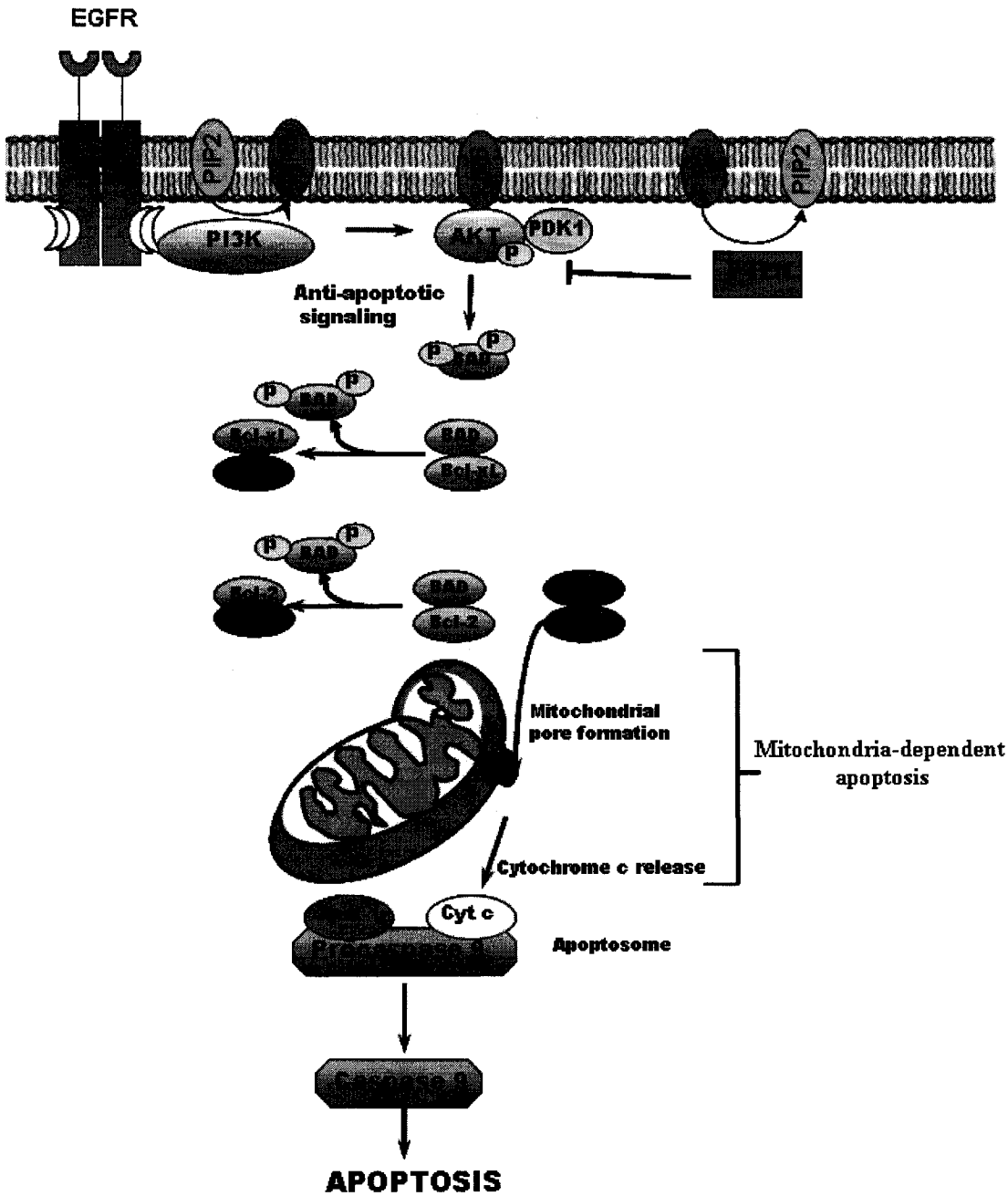


Figure 1.3.2. Activation of the PI3K/Akt pathway by EGFR.

kinase is activated by various stimuli in a PI3K–dependent manner, including the activation of EGFR following EGF binding (Fig. 1.3.2). PI3K converts phosphoinositide, PtdIns-3,4-P2 (PIP2) into PtdIns3,4,5-P3 (PIP3) that can bind directly to the pleckstrin homology (PH) domain of Akt, a process that mediates membrane translocation and a conformational change (Datta *et al.*, 1999). This interaction permits the phosphorylation of the activation loop of Akt at Thr 308 by the phosphoinositide-dependent protein kinase 1 (PDK1) (Grant *et al.*, 2002; Martelli *et al.*, 2006). Anti-apoptotic properties of the PI3K/Akt signaling cascade are mediated by the ability of Akt to negatively regulate the pro-apoptotic protein Bad by phosphorylating the Ser136 residue, inhibiting Bad from interacting and sequestering Bcl-2 and Bcl-xL (Martelli *et al.*, 2006). Bcl-2 and Bcl-xL exert their anti-apoptotic activity by binding to Bax, thereby preventing mitochondrial pore formation (Thornberry and Lazebnik, 1998; Martelli *et al.*, 2006).

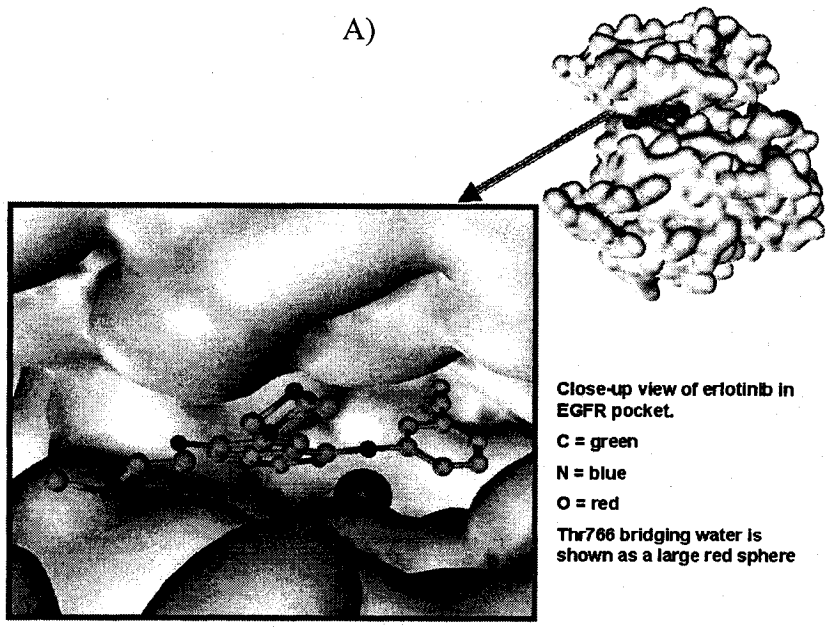
While activation of the PI3K/Akt plays an important role in anti-apoptotic signaling, its deactivation is also considered an important regulatory function in apoptotic signaling and is carried out by the dual-specificity protein and lipid phosphatase, PTEN (Pommery and Henichart, 2005; Cully *et al.*, 2006). One of the central roles for PTEN is to remove the D3 phosphate group of PIP3 thereby depleting levels of PIP3 and inactivating Akt (see Fig. 1.3.2) (Pommery and Henichart, 2005; Cully *et al.*, 2006). Once cells mutate or delete PTEN, the regulatory function of this protein is lost and this allows for the constitutive activation of the anti-apoptotic protein, Akt (Cully *et al.*, 2006; Dong, 2006). Mutations or deletions of PTEN are found in numerous cancers, including glioblastomas and prostate carcinomas. These cells have demonstrated chemoresistance to various

agents, such as cisplatin and 5-fluorouracil (Oki *et al.*, 2005). A number of studies have also demonstrated that tumour cells lacking PTEN activity are rendered insensitive to clinical EGFR targeted agents (Bianco *et al.*, 2003; She *et al.*, 2003; Kokubo *et al.*, 2005).

1.3. Small molecule approach: Tyrosine kinase inhibitors

Tyrosine kinase inhibitors (TKIs) bind to the ATP-binding site of the receptor TK and block its ability to phosphorylate tyrosine residues located in its own kinase domain or on downstream effectors (Fry *et al.*, 1994; Bridges *et al.*, 1996). By inhibiting receptor activation, the TKI can be used to control unregulated cell growth and proliferation. One class of TKI currently being used in the clinic is the quinazoline, the structures of which straddle that of adenine in ATP (Fry *et al.*, 1994; Levitzki, 1999). As depicted in Figure 1.4.1., the structures of all the EGFR TK inhibitors exemplified therein retain the aminopyrimidine backbone of adenine. Once bound to the receptor, quinazolines can stabilize the receptor and create inactive EGFR dimers (Arteaga *et al.*, 1997). Examination of structure activity relationship (SAR) studies of quinazoline derivatives led to the determination of three critical regions required for quinazoline binding to the ATP binding pocket of the receptor. As exemplified in Figure 1.4.1., the binding of the clinical EGFR TKI, erlotinib, in the ATP binding pocket is primarily sustained by 2 nitrogen (N) atoms and a hydrophobic substituent on the anilino ring: 1) the N1-position of the agent interacts with Met769 through hydrogen bonding, 2) the N3 position is bridged by a water molecule present within the binding pocket, to H-bond with Thr766 and 3) a hydrophobic group is favoured at the 3' position of the anilino ring (Assefa *et*

A)



B)

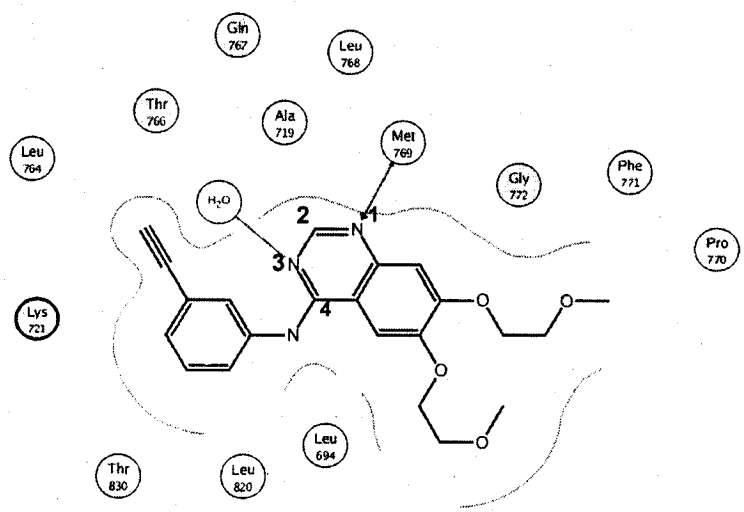


Figure 1.4.1. Structure activity relationship quinazolines. A) 3-D view of erlotinib in the ATP-binding pocket of EGFR and B) critical regions for enhanced affinity for the quinazoline in the ATP-binding pocket of EGFR.

al., 2003). Initial modeling studies of the binding of reversible anilinoquinazoline and anilinopyridopyrimidine inhibitors suggested the 6- and 7-positions point out of the ATP binding pocket, at the entrance of the binding cleft. Therefore, both positions can tolerate steric bulk without losing binding affinity (Smaill *et al.*, 1999; Smaill *et al.*, 2004; Palmer *et al.*, 1997; Vincent *et al.*, 2000; Herbst *et al.*, 2002). As an example, Smaill *et al.* (1999) appended bulky acrylamido side chains at the 6- and 7-position and the resulting molecule did not lose EGFR TK inhibitory activity. It was the purpose of the current work to modify the 6-position of the purine ring for the design of receptor affine "combi-molecules."

1.3.1. Clinical activity

Gefitinib (Iressa™, ZD1839) (see Fig. 1.4.2a) is a specific, orally active synthetic quinazoline known to block EGFR-activated induction of downstream signaling cascades (Herbst *et al.*, 2002). Although studies have demonstrated the ability of gefitinib to induce apoptosis in various tumour cell lines, the inhibitory activity of the TKI is generally cytostatic. In corroboration, clinical trials have shown that the drug must be given in a once daily schedule to induce sustained inhibition of EGFR TK activity (Blackledge and Averbuch, 2004). Results from two clinical studies, Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL)-1 and IDEAL-2, demonstrated that gefitinib was well tolerated and generated overall response rates ranging from 10%–20% in patients with non-small cell lung carcinoma (NSCLC) given 250 and 500 mg/day (El-Rayes and LoRusso, 2004). A recent study has also demonstrated that inhibitory activity induced by gefitinib translated into significant clinical antitumour activity in a particular

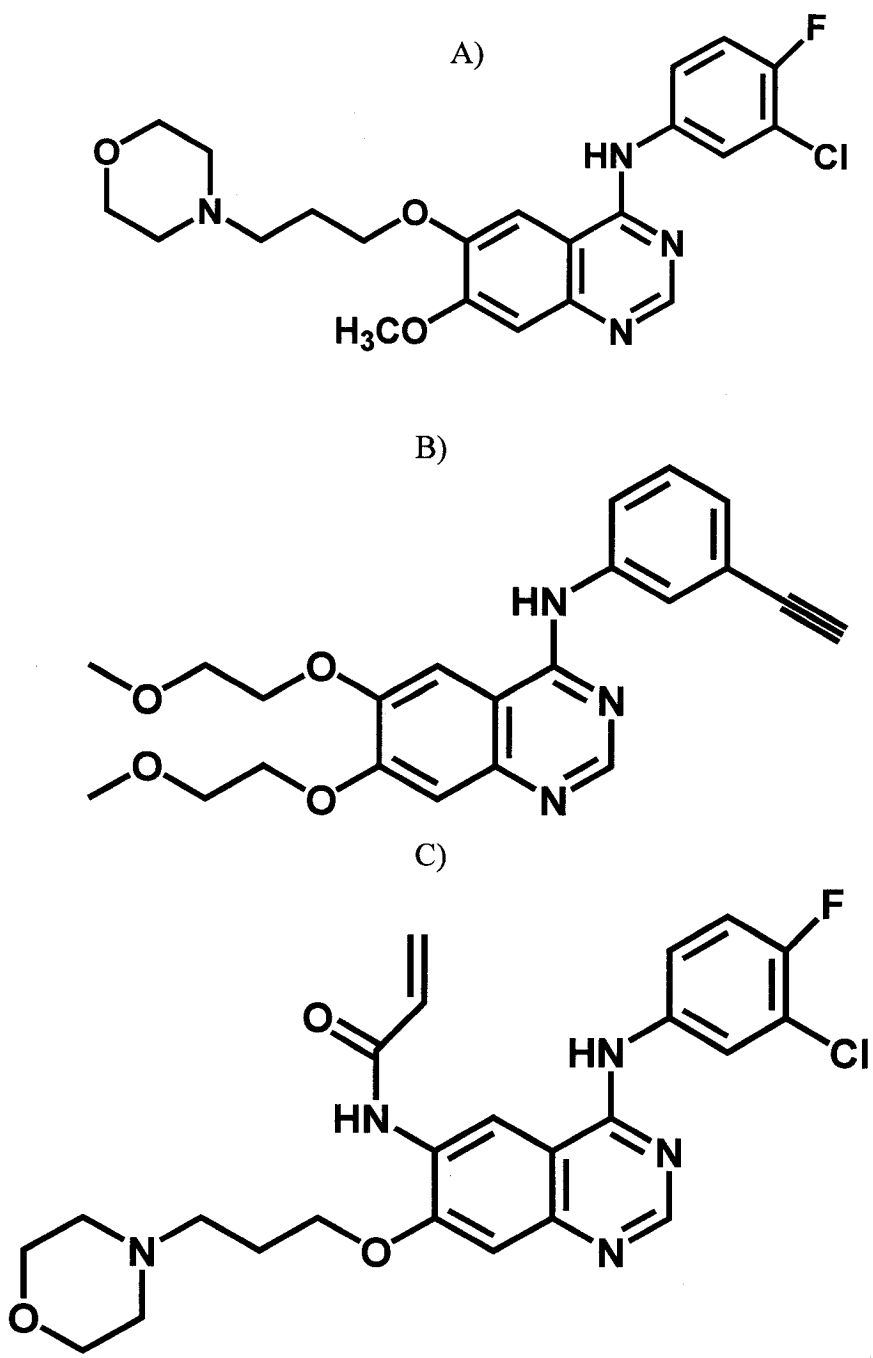


Figure 1.4.2. Molecular structure of ErbB receptor tyrosine kinase inhibitors, A) gefitinib, B) erlotinib and C) CI-1033.

subset of NSCLC patients whose tumours harboured mutations in the intracellular region of EGFR. These mutations influence the rate at which the receptor was internalized following ligand-binding, thereby affecting the duration of the signal generated by the activated receptor (Paez *et al.*, 2004; Hsieh *et al.*, 2006). Similarly, mutations surrounding the ATP-binding site of the EGFR TK domain were detected in tumours from NSCLC patients with no prior gefitinib therapy that conferred enhanced sensitivity to gefitinib inhibition (Lynch *et al.*, 2004)

Another quinazoline derivative directed towards EGFR that has currently entered clinical trials is the orally active compound, erlotinib (Tarceva, OSI-774) (Fig. 1.4.2b). Erlotinib is currently being used as a second-line or third-line drug in the therapy of advanced NSCLC, in patients who fail on platinum-based treatment regimens (Kumar and Wakelee, 2006). Phase III clinical trials of erlotinib in NSCLC patients following first or second line chemotherapy, demonstrated the ability of erlotinib to generate prolonged survival and an 8.9% response rate (Maione *et al.*, 2006).

Although clinical EGFR TK inhibitors like gefitinib and erlotinib are well tolerated agents that have demonstrated antitumour activity in numerous cancers, such as breast, ovarian and lung cancer, the potency and efficacy of these drugs are limited by their virtually reversible EGFR inhibitory activity (Lynch and Kim, 2005; Kwak *et al.*, 2005). Patients have been known to relapse after an initial response to these EGFR inhibitors due to mutations, such as the T790M mutation, that causes steric hindrance in the ATP-binding pocket of EGFR, blocking the ability of the TKI to bind to the receptor (Thomas

et al., 2005).

Several inhibitors of EGFR have already demonstrated significant inhibitory activity. However, the potency of reversible TKIs are hampered by high intracellular concentrations of ATP. In order to ameliorate the activity of quinazolines, a class of EGFR inhibitors was designed that target and irreversibly inactivate the EGFR TK. The first class of irreversible inhibitors were designed to posses an acrylamide side-chain on the 6- or 7- position of the molecule that covalently modify the Cysteine 773 residue, located within the ATP binding pocket, upon binding (Bridges, 1999; Smaill *et al.*, 2004). These irreversible inhibitors demonstrated *in vivo* antitumour activity superior to their reversible counterparts (Denny, 2002). CI-1033 (Fig. 1.4.2c) is an orally administered irreversible inhibitor of all the members of the ErbB family that exhibited antitumour activity in preclinical studies against various cancers such as breast and ovarian carcinomas (Allen *et al.*, 2003). Phase II clinical studies for CI-1033 demonstrated approximately one third of patients achieving stable disease in numerous tumours, such as ovarian cancer (Campos *et al.*, 2005). Although thrombocytopenia was observed in patients at high doses, CI-1033 demonstrated substantial activity in dose regimens that ranged from once daily to once weekly (Calvo *et al.*, 2004).

In summary, studies have demonstrated that targeted therapies that block activation of EGFR are promising. However, cross-talk between a multitude of intracellular pathways can diminish the potency of EGFR-targeted therapies. These observations support the argument that inhibition of the receptor alone does not generate enhanced and sustained therapeutic response. In order to augment the clinical potency of these targeted agents,

combination studies with other traditional chemotherapeutic agents have emerged as a promising avenue (Gasparini *et al.*, 2005; Shimoyama *et al.*, 2006; Reckamp *et al.*, 2006). Within the same line of thinking, the purpose of this thesis was to introduce the concept of targeting EGFR TK with single drugs that not only possess the ability to bind to the ATP pocket, but also to damage DNA. The class of DNA damaging agent used belong to the triazene class and we summarize herein previous results obtained on their structure, mechanism of action and clinical efficacy.

1.4. 3-Methyltriazenes

Aromatic triazenes are simple molecules often containing an N=N-N linkage that upon hydrolysis release an arylamine and a methyldiazonium (Vaughan *et al.*, 1984; Manning *et al.*, 1985). Methylating agents are the most common class of alkylators used in the therapy of solid tumors and leukemias (Newlands *et al.*, 1992). One such agent, Temodal® (TEM), is an orally available methylating agent used in the therapy of malignant melanomas and gliomas (Stevens *et al.*, 1987; Nagasubramanian and Dolan, 2003). As depicted in Figure 1.5.1, hydrolytic cleavage of TEM gives rise to the 5-aminoimidazole-6-carboxamide (AIC) and DNA damaging methyldiazonium species. This species reacts with several nucleophilic sites in DNA to give rise to various DNA adducts including N7-methylguanine (N^7meG), N3-methyladenine (N^3meA) and O6-methylguanine (O^6meG) (Fig. 1.5.2) (Tentori *et al.*, 1999). The most prevalent lesion induced by 3-methyltriazenes is the N^7meG (~70% of all lesions) followed by N^3meA (~10%) and O^6meG (~5%) (Tentori *et al.*, 1999). Although the O^6meG is the least abundant adduct formed upon treatment with methylating agents, it is by far the most

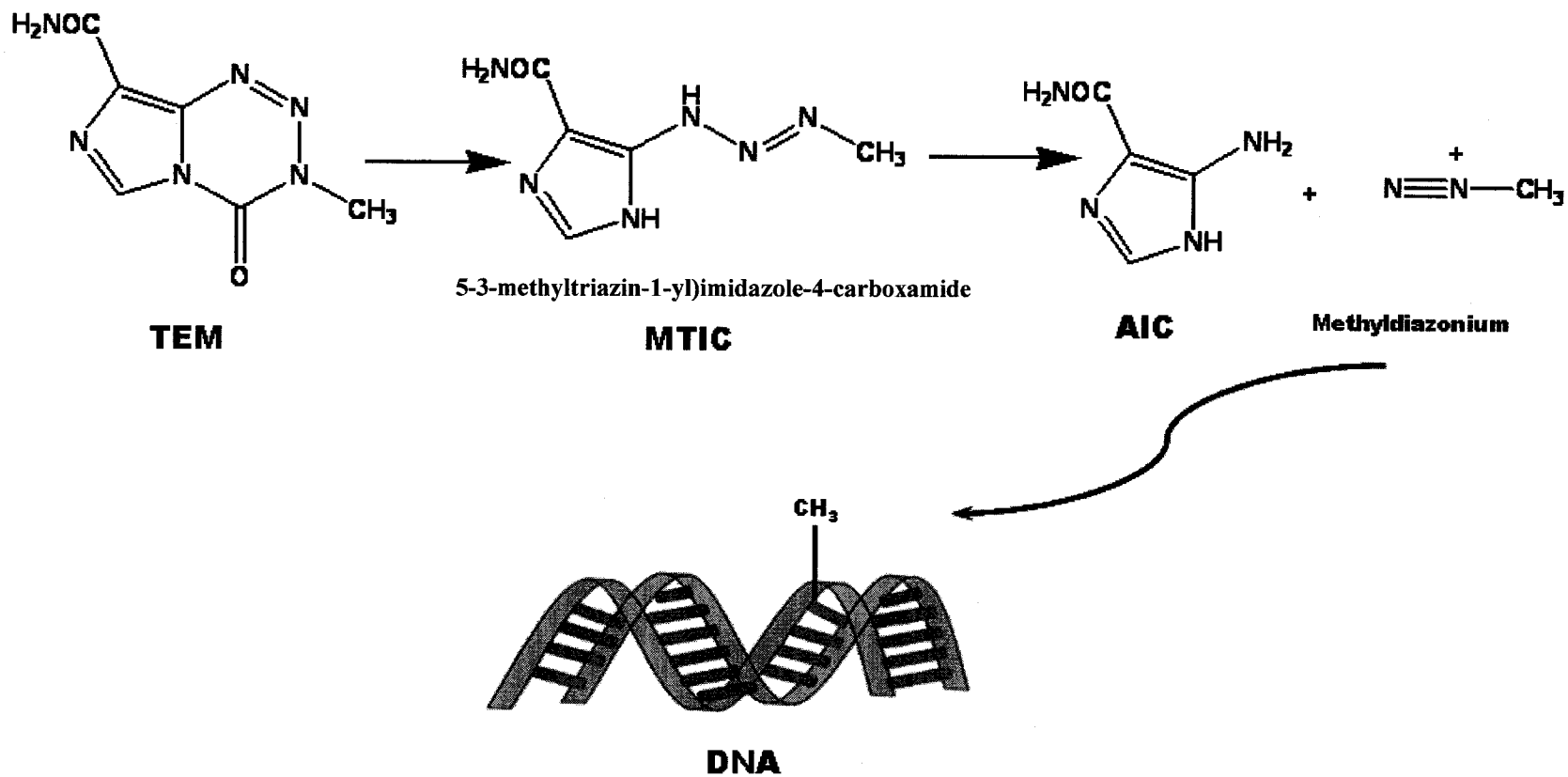


Figure 1.5.1. The hydrolytic decomposition of the clinical triazene, Temozolomide (TEM).

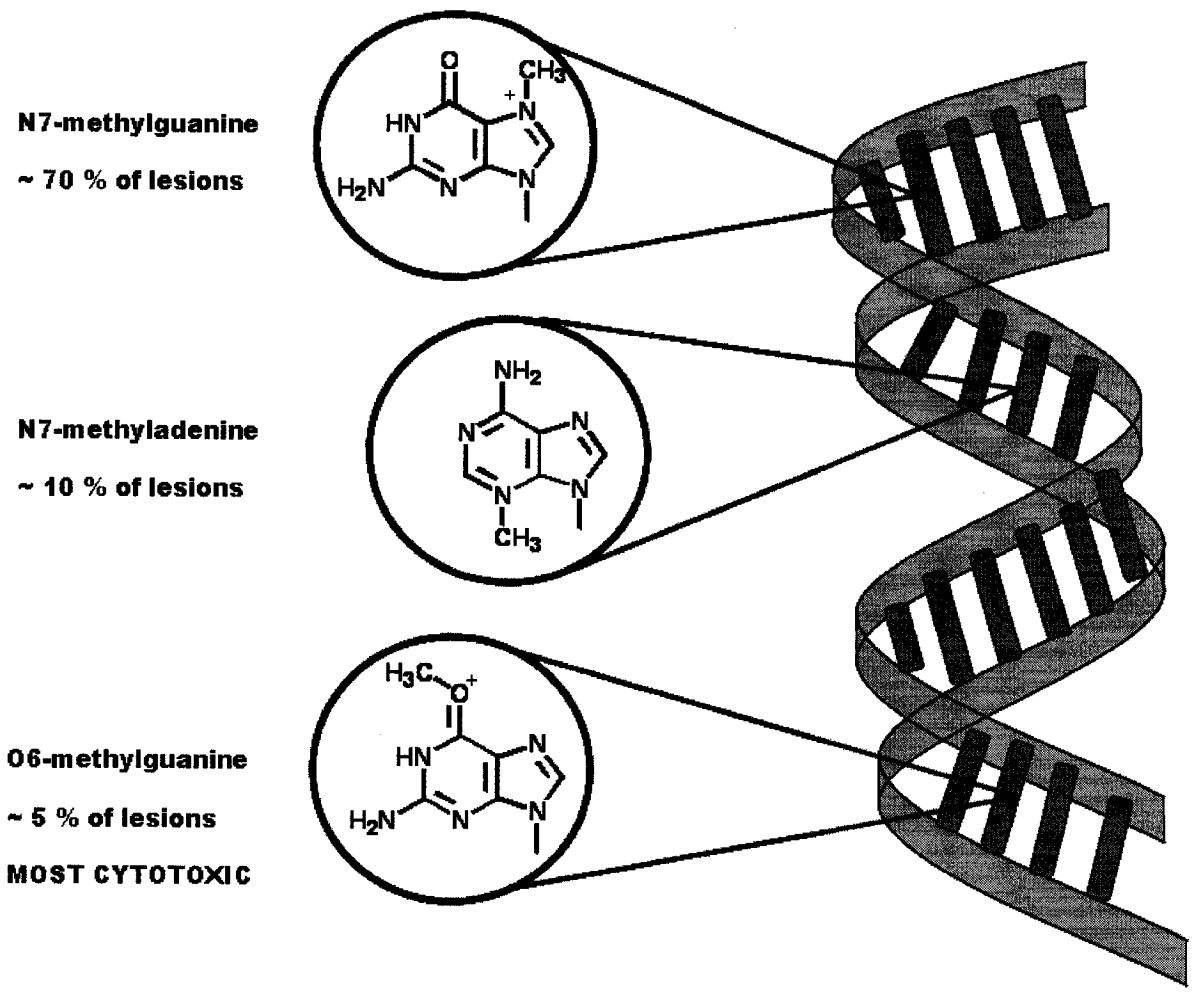
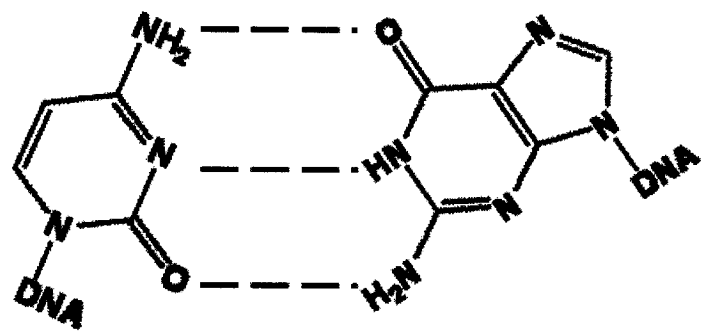
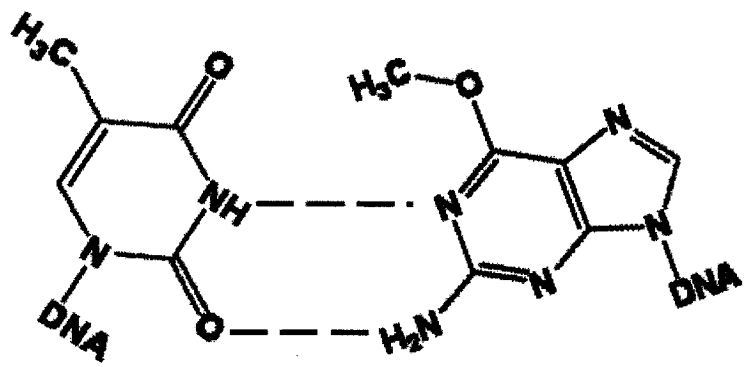


Figure 1.5.2. Formation of key DNA adducts following exposure to methylating agents of the triazene class.



Cytosine Guanine



Thymine O6-methylguanine

Figure 1.5.3. Base pairing observed between guanine and cytosine and O^6-methylguanine with thymine.

cytotoxic lesion induced by these drugs (Middleton and Margison, 2003). As shown in Figure 1.5.3, methylation at the O6-position of guanine (G) disrupts hydrogen bonding of G with cytosine (C) and if the methyl is not removed, it will mispair with thymine (T) in the following round of DNA replication (Middleton and Margison, 2003). The presence of O^6-alkyguanine transferase (AGT), a DNA repair enzyme that can remove the methyl group from the O6-position of G, confers resistance to tumour cells exposed to methylating agents such as TEM (Pegg and Byers, 1992). Further details on the mechanism of action of AGT in chemoresistance will be presented in the DNA repair section 1.5.2.

1.5. Cellular response to triazenes

Cellular responses to DNA damaging agents play an important role in regulating the response of tumours to chemotherapy and radiation. As previously mentioned, the three most common DNA lesions induced by methylating agents such as TEM and dacarbazine, are the O^6meG, the N^7meG and the N^3meA adducts (Tentori *et al.*, 1999). N^7meG and N^3meA lesions, which are the most prevalent adducts observed, are repaired efficiently and handled by the repair mechanism termed Base Excision Repair (BER).

1.5.1. Base excision repair (BER)

Despite its abundance, N^7meG adducts cause very little distortion of the DNA double helix. However, they are readily recognized by a class of enzymes termed damage-specific DNA glycosylases (Krokan *et al.*, 1997, Fromme *et al.*, 2004). As depicted in Figure 1.6.1, DNA glycosylases are generally small, monomeric proteins that cleave the N–C1′ glycosydic bond between the base and the deoxyribose ring. This cleavage leads

to the release of the damaged base, thereby generating a gap termed abasic site (Krokan *et al.*, 1997). Abasic sites can also be formed by simple non-enzymatic hydrolysis of the glycosidic bond (Fig. 1.6.2).

The human DNA glycosylase that repairs alkylation damage is known as the 3-methyladenine DNA glycosylase or alkyladenine glycosylase (AAG) (Scharer and Jiricny, 2001). The AAG protein removes N^3meA, N^7meG and various forms of DNA adducts induced by alkylating agents used in chemotherapy (Krokan *et al.*, 1997; Scharer and Jiricny, 2001). Although N^7meG and N^3meA do not cause distortion in the DNA structure, binding of AAG to DNA causes nucleotide unstacking from the DNA helix and exposure of the alkylated base (Krokan *et al.*, 1997; Scharer and Jiricny, 2001). The active site of AAG has high affinity for the electron-deficient methylated base and a water molecule within the site is positioned to attack the flipped-out nucleotide, allowing the displacement of the damaged base from DNA (Krokan *et al.*, 1997; Hollis *et al.*, 2001).

Following base removal, mammalian cells have two pathways that replace the formation of the abasic site: 1) short-patch repair or 2) the minor long-patch repair pathway.

1.5.1.1. Short-patch repair

The short patch repair pathway involves single nucleotide gap replacement. As shown in Figure 1.6.1, following removal of the damaged base by the DNA glycosylase, 5'AP endonuclease creates a single nucleotide gap (Scharer and Jiricny, 2001) that is subsequently filled by the DNA polymerase β. The latter attaches a single nucleotide to

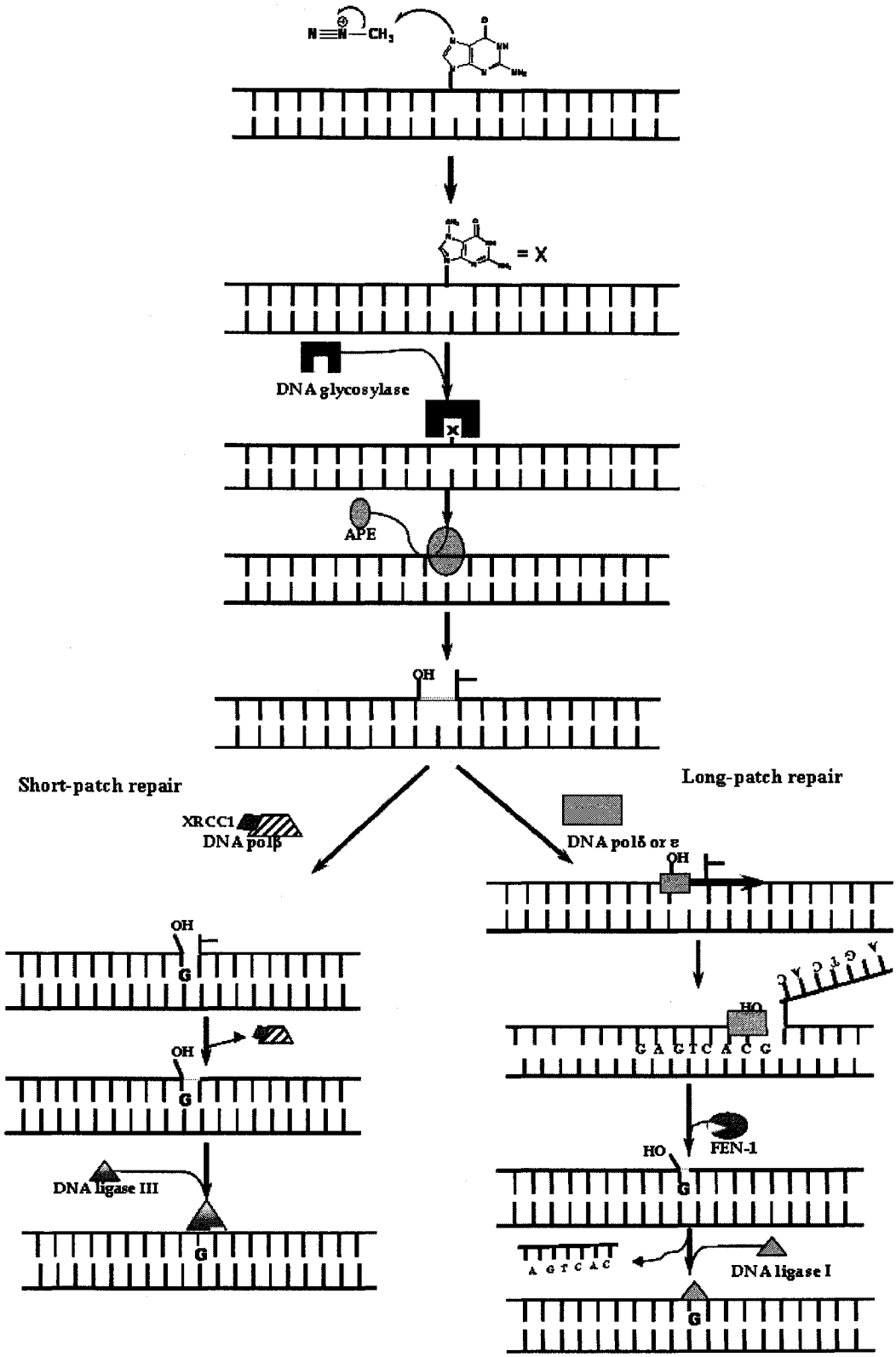


Figure 1.6.1. Repair of N^7meG and N^3meA lesions by the BER pathway.

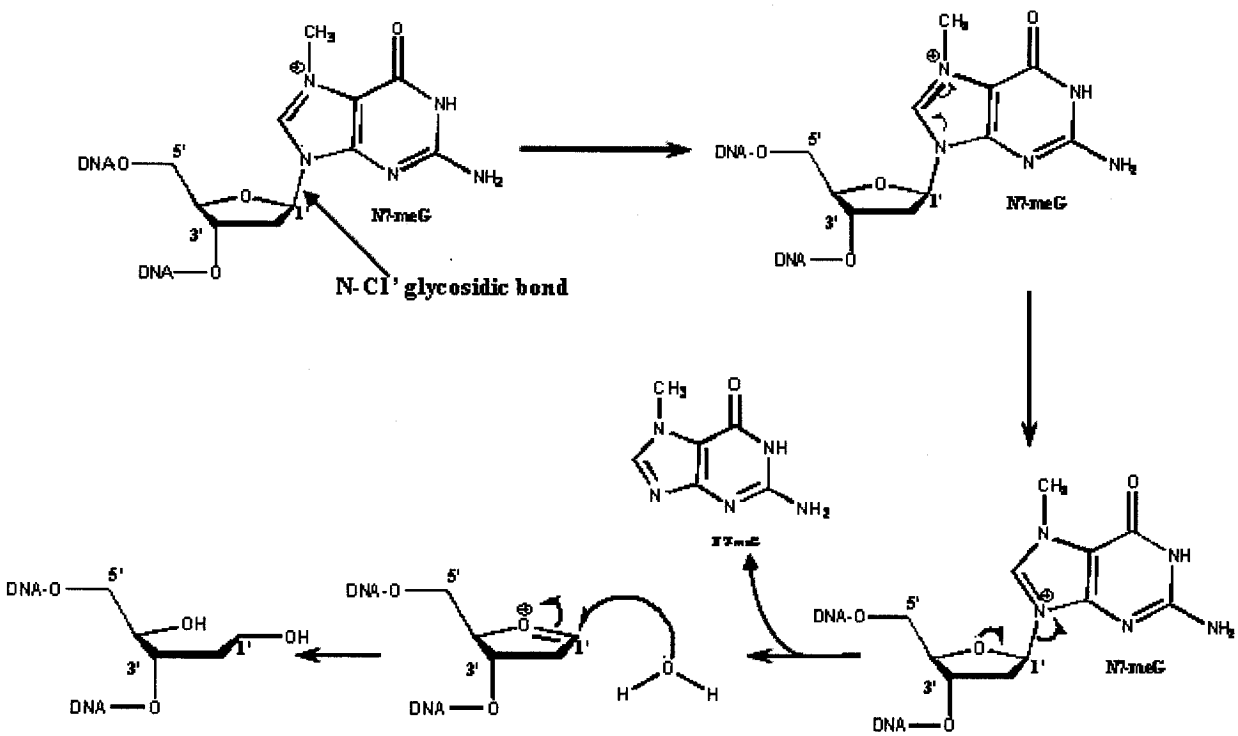


Figure 1.6.2. Spontaneous hydrolysis following methylation at the N7-position of guanine.

the newly generated 3'OH and dislodges the baseless sugar-phosphate backbone. The scaffold protein, X-ray cross-complementation protein 1 (XRCC1) interacts with DNA polymerase β through its N-terminal domain, whereas the C-terminal region of XRCC1 interacts with DNA ligase III (Krokan *et al.*, 1997). Through interactions with XRCC1, DNA ligase III is brought to the site of the nick in the DNA backbone where it connects the broken strand by catalysing a covalent phosphodiester bond formation (Krokan *et al.*, 1997). Substantial evidence indicates an important role for XRCC1 in single strand break repair (SSBR) and BER. Mutant Chinese hamster ovary (CHO) cells with no functional XRCC1 protein are hypersensitive to a broad range of DNA damage induced by methylating agents, reactive oxygen species or ionizing radiation (Zdzienicka *et al.*, 1992). This is probably due to a greater number of unrepaired SSBs encountered by the replication fork.

1.5.1.2. Long-patch repair

In the alternative long patch repair pathway, gap filling of several nucleotides (2-10 nucleotides) is performed. As depicted in Figure 1.6.1, the AP endonuclease performs the 5'-incision followed by DNA synthesis assisted by DNA polymerase δ or ε in conjunction with the proliferating cell nuclear antigen (PCNA) (Scharer and Jiricny, 2001; Gary *et al.*, 1999). The flap produced by the displaced DNA strand is removed by the flap endonuclease/exonuclease termed FEN-1 and the nick is sealed by DNA ligase I (Scharer and Jiricny, 2001).

The protective effect of BER in tumour cells has been demonstrated in numerous studies

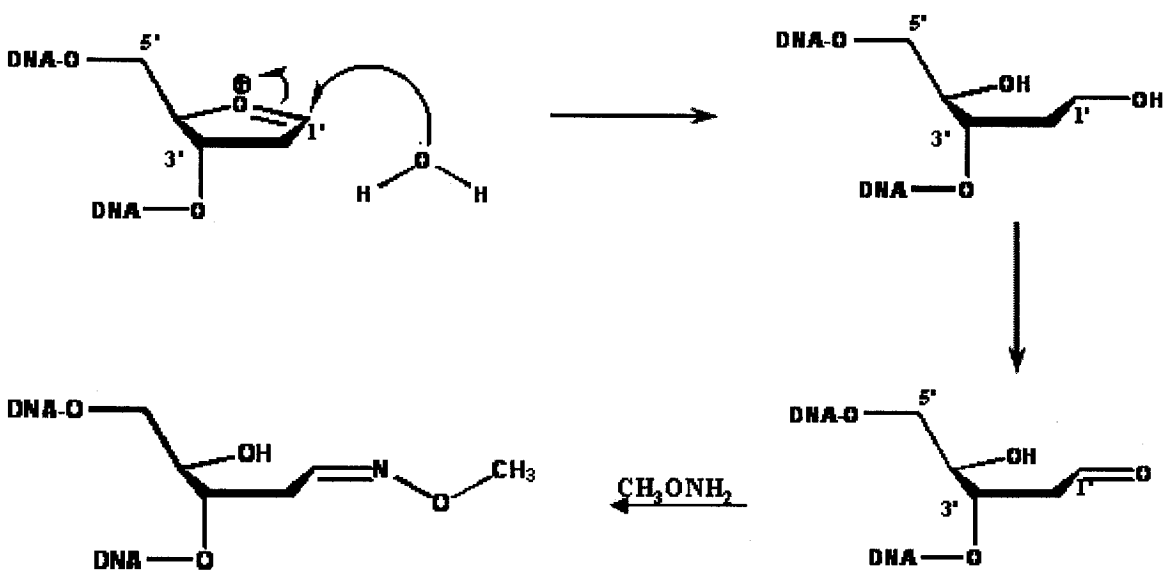


Figure 1.6.3. Methoxylamine reaction with DNA.

using inhibitors of proteins involved in BER such as methoxylamine (MX). Methoxylamine, by reacting with the aldehyde group formed at AP sites on the sugar-phosphate backbone of DNA, can generate a DNA-MX adduct that interrupts the BER, leaving high levels of unrepaired single strand breaks in the DNA (Liu and Gerson, 2004) (Fig. 1.6.3). Studies performed by Liu *et al.* (1999) demonstrated that this translated into MX potentiation of cytotoxicity induced by TEM in cells regardless of mismatch repair (MMR) status.

Although N^7meG is produced at a significantly higher frequency (>70% of all lesions) than the O^6meG lesion (approximately 5%), studies have demonstrated that it is the O^6meG adduct that plays a major role in the cytotoxicity of triazenes (Tentori *et al.*, 1999). As previously mentioned, tumours that express the DNA repair enzyme O6-alkylguanine transferase (AGT) are resistant to the methylating class of chemotherapeutic agents.

1.5.2. O6- alkylguanine transferase (AGT) repair mechanism

1.5.2.1. Structure

The O^6meG residues in DNA are the primary cytotoxic lesions induced by antitumour agents such as TEM and dacarbazine (Tentori *et al.*, 1999). Conversely, the most critical factor in the resistance of tumours to methylating agents of the triazene class is the expression of the 21kDa DNA repair enzyme, O^6-alkylguanine transferase (AGT), that binds to the lesion and removes the methyl group (Pegg and Byers, 1992; Middleton and Margison, 2003).

The overall structure of AGT is generally conserved. However, the C-terminal domain is well conserved, with two overlapping and tight turns stabilized by an asparagine (the Asn-hinge), and a helix-turn-helix (HTH) DNA-binding motif (Daniels and Tainer, 2000). Crystallographic studies by Daniels and Tainer (2000) demonstrated that AGT binds through the minor groove via a DNA binding sequence that anchors deep into the minor groove.

1.5.2.2. Function

As depicted in Figure 1.7.1, following binding of the O^6-alkylguanine substrate, AGT transfers the alkyl group to its active-site cysteine (Cys145) in an irreversible and stoichiometric reaction (Pegg and Byers, 1992; Daniels and Tainer, 2000). The presence of a water molecule in the active site forms two hydrogen bonds, one with the SH group of Cys145 in the active site and another with the His146 located within a hydrophobic region. Upon activation, the histidine N abstracts the H proton leading to a highly nucleophilic thiolate that undergoes an SN_2 attack on the methyl group. This gives rise to an S-methylated enzyme that is ubiquitinated and degraded by proteosomes. Due to this mechanism, AGT is commonly referred to as a "suicide" enzyme.

1.5.2.3. Strategies to down-regulate AGT activity

There is a great deal of variablility in the expression of AGT between cells, tissues and individuals (Lee *et al.*, 1996; Wani *et al.*, 1997). Resistance against DNA-alkylating agents based on increased AGT production is observed during chemotherapy

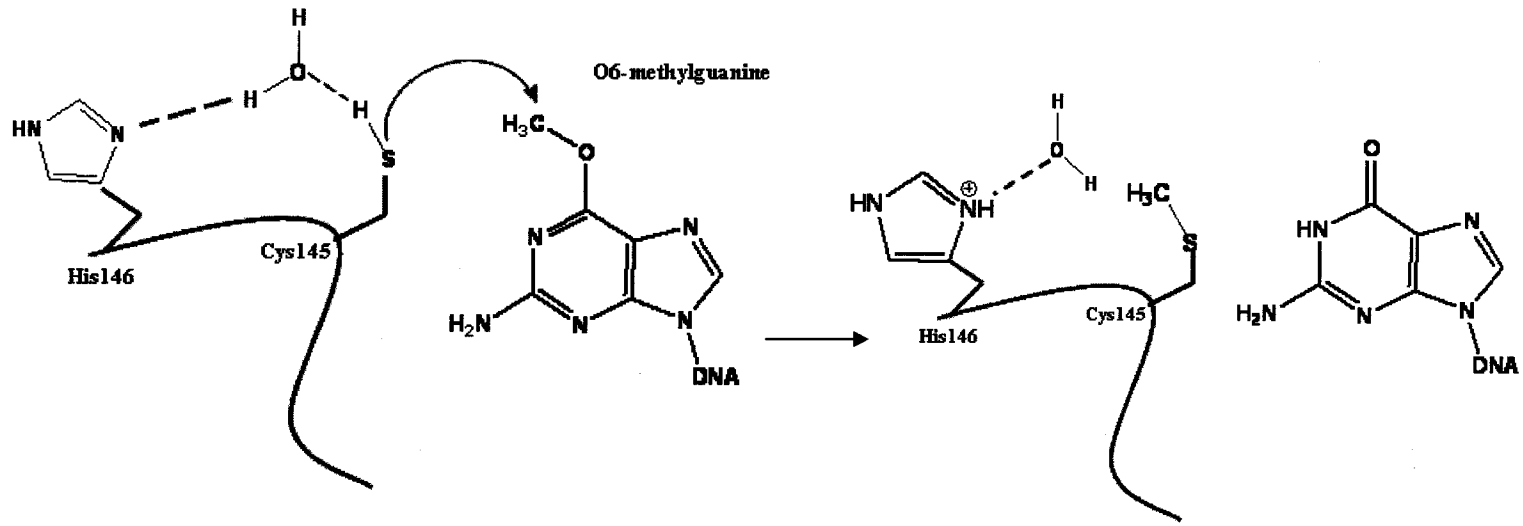


Figure 1.7.1. The stoichiometric reaction involving the removal of a methyl group from the O6-position of guanine by O^6-alkylguanine transferase (AGT).

with alkylators. There have been numerous strategies on improving sensitivity to DNA alkylating agents by depleting AGT levels.

One approach to deplete the levels of AGT was to alter the dosing schedule of the methylating agent, TEM. Patients resistant to TEM were given a bolus dose to deplete levels of the DNA repair enzyme and a second dose was administered when AGT levels were substantially depleted. Although this approach resulted in significant AGT depletion, its efficacy was compromised by increased myelotoxicity in clinical trial (Brandes *et al.*, 2001).

1.5.2.4. AGT inhibitors

Depletion of AGT levels in cells that are resistant to methylating agents by exposure to free O^6-alkylguanine derivatives has been extensively studied. The first AGT inhibitor synthesized was free O^6-methylguanine. However, due to the poor AGT affinity and limited cellular uptake of the latter agent, other inhibitors were developed (Dolan *et al.*, 1985). Studies on a series of O^6-alkylgunaine analogs by Pegg *et al.* (1998) demonstrated that O^6-benzylguanine (O^6-BG, see Fig. 1.7.2) to be the favoured substrate over O^6meG adducts. The two guanine derivatives currently being studied in clinical trials are O^6-BG and O^6-4-bromothenylguanine (O^6-BTG), an AGT inhibitor found to be 10 times more potent than O^6-BG (Kaina *et al.*, 2004).

Mutational screens were used to isolate the key regions in the binding pocket of the enzyme that would render AGT resistant to O^6-BG. The results showed that specific

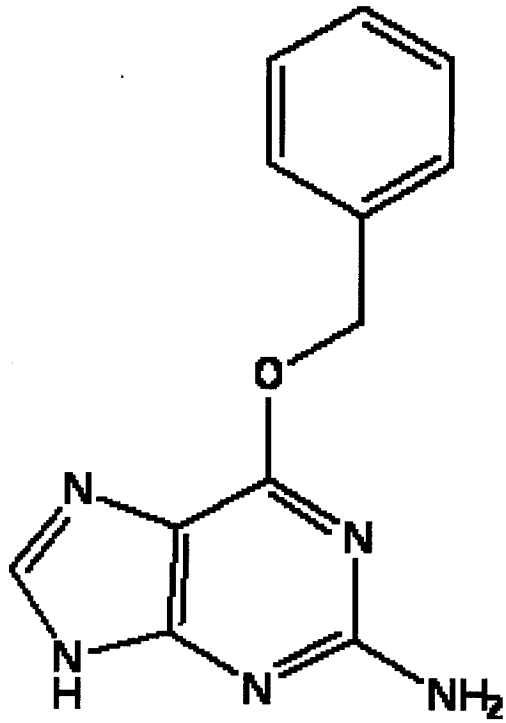


Figure 1.7.2. Molecular structure of the AGT inhibitor, O^6-benzylguanine (O^6-BG).

point mutations of Pro138, Pro140 and Thr158 were the most effective in producing O^6-BG resistance without hindering AGT activity (Xu-Welliver *et al.* 1998).

1.5.2.5. Clinical response

Results from preclinical studies in human tumour xenografts demonstrated that combining clinical alkylating agents, TEM or carmustine (BCNU), with O^6-BG sensitized cells to the alkylators (Kokkinakis *et al.*, 2001; Kokkinakis *et al.*, 2003). BCNU belongs to the class of bi-functional nitrosoureas used in the therapy of many solid tumours including melanomas and gliomas (Ewend *et al.*, 2005). As shown with methylating agents of the triazene class, BCNU is capable of inducing cytotoxic O^6-alkyl lesions that are readily repaired by AGT (Chen *et al.*, 1993). Although the 2-drug combination sensitized cells, pronounced blockade by O^6-BG in cells with basal levels of the enzyme induced myelotoxicity.

Phase I clinical trial with patients with malignant glioma treated with a combination of O^6-BG + the clinical alkylator BCNU demonstrated O^6-BG to be well-tolerated and capable of depleting AGT activity. However, partial or complete responses were not observed in any of the patients treated with the BCNU/O^6-BG combination. It is believed that this is due to the MTD of the two drug combination being far below the therapeutic dose of BCNU alone. As expected, patients treated with the combination experienced severe myelotoxicity at higher doses (Schilsky *et al.*, 2000; Quinn *et al.*, 2002).

Although AGT was proven an effective target to sensitize cells to methylating agents *in*

vitro, clinical outcomes of therapies involving depletion of AGT levels are hindered by the following obstacles: 1) the possibility of patients developing single point mutations that produce O^6-BG resistant forms of AGT, thereby generating tumours that remain resistant to the alkylator, and 2) enhanced myelotoxicity when used in combination with DNA alkylators. Therefore, there is an urgent need to develop other drugs that can enhance the activity of methylating agents in resistant tumours that do not significantly enhance the toxicity associated with DNA damaging agents. It is the purpose of this thesis to develop alternative strategies to sensitize cells resistant to methylating agents by synthesizing unimolecular agents that not only damage DNA, but also target various intracellular proteins such as EGFR, thereby potentiating the cytotoxic activity of the DNA methylating moiety.

1.5.3. Mismatch Repair (MMR)

In the absence of AGT, upon formation of O^6meG, a mispairing occurs with thymine (T) during DNA replication and generates a G:T mismatch (Fig. 1.5.3) (Tentori *et al.*, 1995; Peponni *et al.*, 2003) . The presence of G:T mismatches activates the DNA mismatch repair (MMR) system that consists of at least five homologs of the bacterial protein MutS (hMSH) and at least four homologs of the bacterial MutL protein (hMLH) (Stojic *et al.*, 2004a). The central elements of the system are the multimeric MutS/MutL complexes that are endowed with the mechanism to recognize base-base mismatches (e.g. G-T) and insertions-deletion loops (Stojic *et al.*, 2004a). One of the proposed mechanisms or models for the recognition of base mismatches by MMR is the thermodynamic

destabilization of the DNA double helix by the O^6meG-T mispair (Isaacs and Speilmann, 2004).

As depicted in Figure 1.8, the hMSH2/6 heterodimer (MutSα) binds to the base-base mismatch through an amino-terminal mismatch-recognition domain and exchanges ADP for ATP (Lamers *et al.*, 2000; Stojic *et al.*, 2004a). A conformational change occurs upon ATP binding and the complex acts as a sliding clamp (Iaccarino *et al.*, 2000; Stojic *et al.*, 2004a). The lateral movement allows the complex to differentiate between the template and newly synthesized strand by the presence of 3'-hydroxy terminals. The DNA:MutS:ATP complex then recruits the MutL complex (a heterodimer composed of hMLH1 and hPMS2) and the exonuclease termed Exo1 (Stojic *et al.*, 2004a). Exo1 excises the newly synthesized DNA fragement containing the mismatch and the resulting single-stranded DNA is stabilised by replication protein A (RPA) (Stojic *et al.*, 2004a; Guo *et al.*, 2006). The latter protein also facilitates DNA resynthesis by permitting DNA polymerases to perform DNA synthesis (Stojic *et al.*, 2004a). Interestingly, as shown in Figure 1.8, the newly synthesized DNA strand will still contain the mispaired T and the entire process will be triggered by the MutSα complex. This series of "futile" cycles of DNA repair initiate multiple pathways ultimately leading to cell cycle arrest or apoptosis (Tentori *et al.*, 2002; Stojic *et al.*, 2004a; Stojic *et al.*, 2004b). Cells that lack a functional MMR system do not recognize the G:T mispair, thereby by-passing MMR-induced DNA strand breaks. It has been reported that MMR-deficient cells are relatively resistant to methylating agents, whereas cells with a functioning MMR system enter either G2/M arrest or apoptosis (Hawn *et al.*, 1995). Recently, Tentori *et al.* (2002) demonstrated that

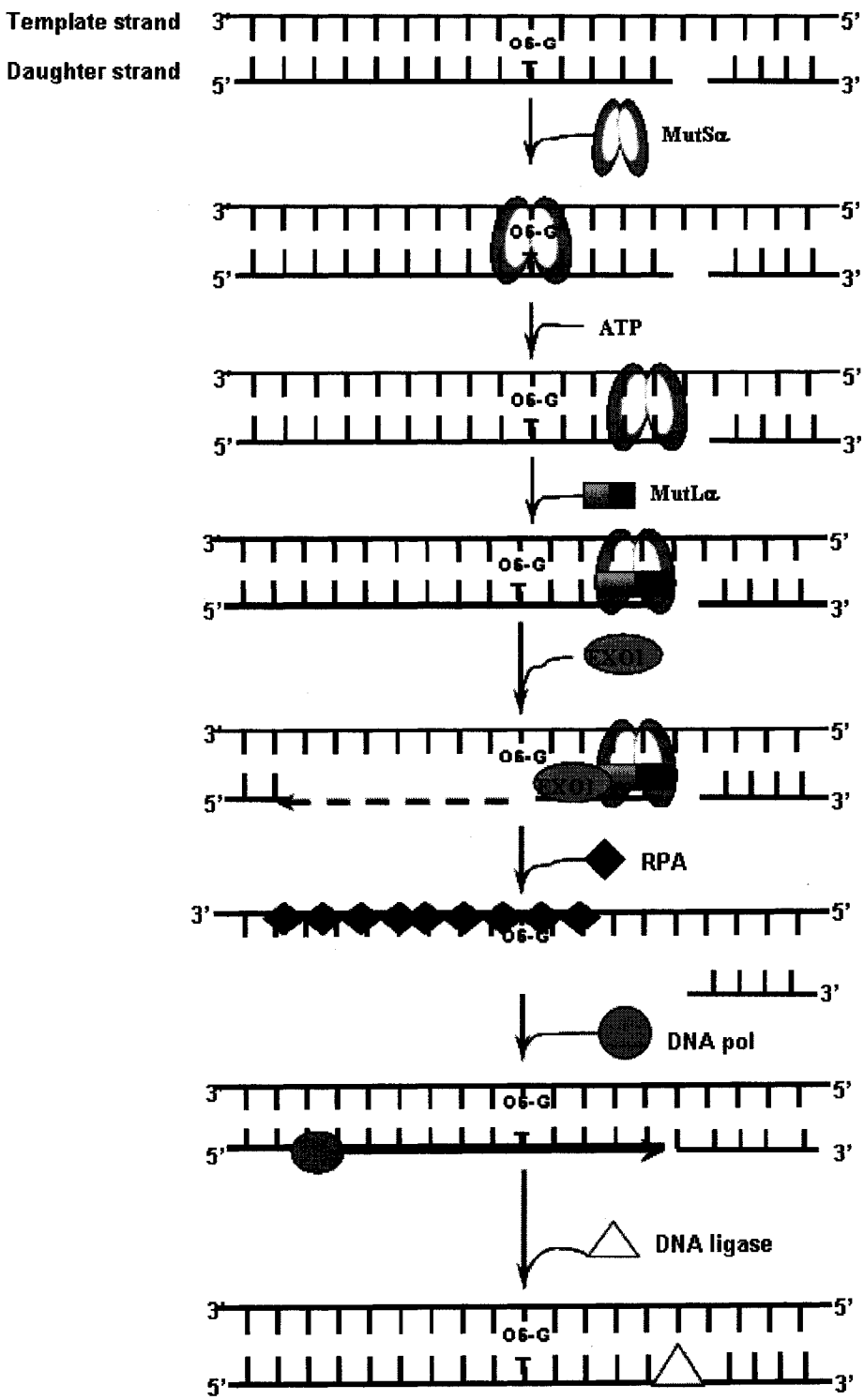


Figure 1.8. Repair of G:T base mismatches mediated through the human mismatch repair pathway (MMR).

AGT-null glioma cells with MMR-deficiency were resistant to TEM treatment. Studies carried out by D'Atri *et al.* (1998) showed that TEM induced apoptosis and G2/M cell cycle arrest in MMR-proficient cells and that these responses were absent in MMR-deficient cells.

1.5.4. Activation of DNA damage checkpoints

As previously mentioned, the proliferation of dividing cells is characterized by several transitions in the cell cycle known as checkpoints (Hoglund, 2006; O'Brien and Brown, 2006). At these checkpoints, genomic integrity is probed by two major players, the PI3K-related kinases ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) (Hiom, 2005). ATM and ATR are phosphorylated upon DNA damage through yet an unknown mechanism.

While cellular response to methylating agents is highly dependent on DNA repair processes such as BER and MMR, it has been shown to be influenced by the G2/M checkpoint (D'Atri *et al.*, 1998). The G2/M cell cycle checkpoint is an important control measure that inhibits cell cycle progression and the ability of the cell to enter mitosis (Stark and Taylor, 2006). As illustrated in Figure 1.9, the activation of ATR and ATM is followed by phosphorylation of the two checkpoint kinases, Chk1 and Chk2, as well as the tumour suppressor, p53 (Niida *et al.*, 2006). The dual specificity phosphatase Cdc25C is phosphorylated upon Chk1 and Chk2 activation, inhibiting its ability to release the cyclin-dependent kinase, cdc2, from cyclinB1 complex, thereby generating a blockade between the G2 to M transition (Niida *et al.*, 2006; Stark and Taylor, 2006).

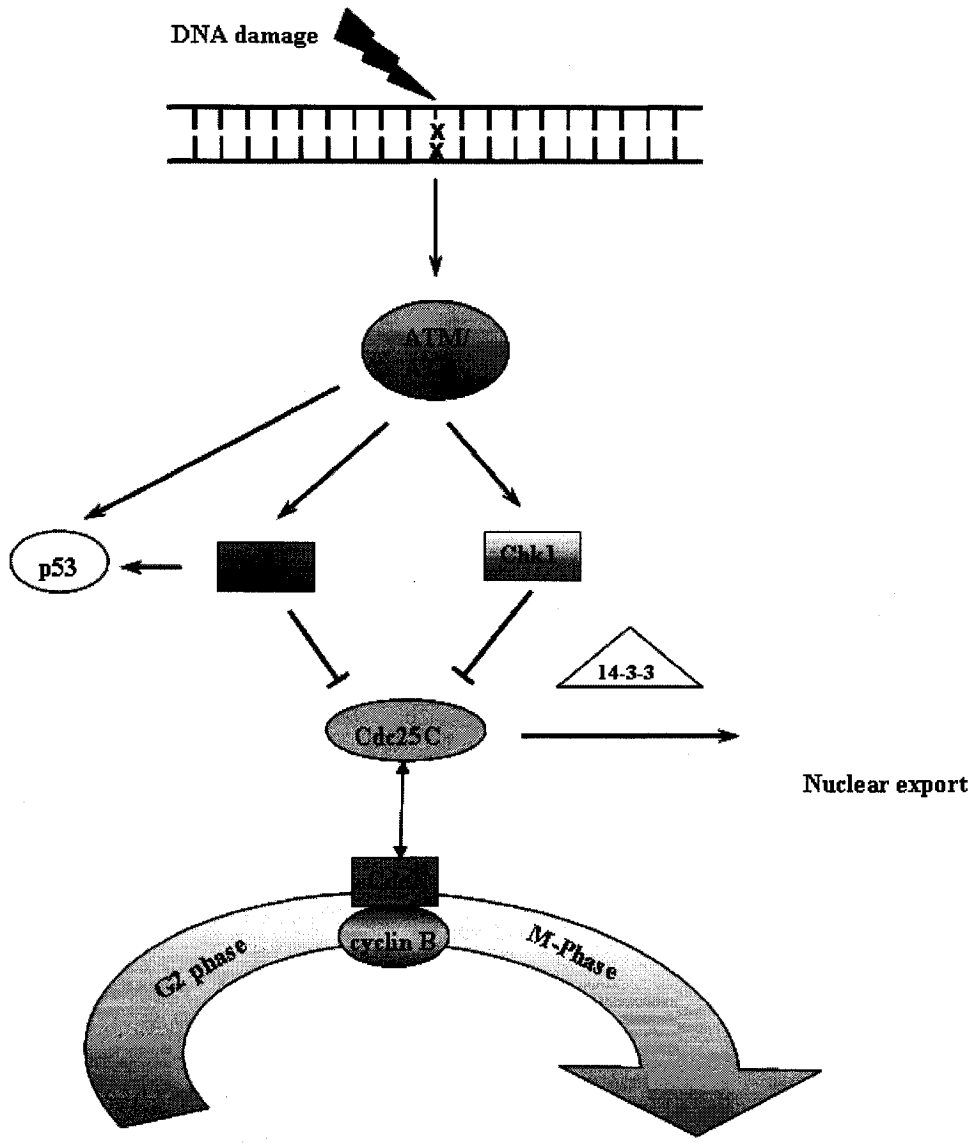


Figure 1.9. Activation of the G2/M checkpoint following DNA damage.

Studies with the methylating agent, TEM, showed that this drug that can induce N^7meG, N^3meA and O^6meG lesions in cells, blocks the cell cycle in G2/M (D'Atri *et al.*, 1998; Stojic *et al.*, 2004b). However, Hirose *et al.* (2001) demonstrated that MMR-proficient cells, in which Chk1 is deactivated by the inhibitor, UCN-01, were unable to arrest in G2/M, thereby enhancing the cytotoxic activity of TEM. The results indicated that Chk1 is critical for the cytoprotective role of G2/M arrest in these cells.

1.5.5. The stress response pathway (MAPK family)

In addition to cell cycle perturbation, in response to DNA damage, other signaling cascades known as stress response pathways, are activated to promote cell survival or cell death (Herr and Debatin, 2001; Fang and Richardson, 2005). The stress-activated protein kinases (SAPK) are a family of serine/threonine kinases that include c-jun N-terminal kinase (JNK) and p38 MAP kinase (MAPK14) (Herr and Debatin, 2001; Fang and Richardson, 2005). The SAPKs are activated predominantly by cellular stress signals, but have also been shown to be activated by mitogens (Dent *et al.*, 2003). These cellular responses are mediated by the induction of various signaling cascades and the transcriptional activity of the AP-1 complex, a dimer consisting of various combinations of the transcription factors, c-jun, c-fos and ATF-2 (Herr and Debatin, 2001).

The JNK family of kinases are activated following phosphorylation by the upstream kinase JNKK/MKK4 and/or MKK7 (see Fig. 1.10). JNK-mediated phosphorylation can either increase the stability or transcriptional activity of AP-1 components, c-jun and ATF-2, thereby promoting cell survival (Vasilevskaya and O'Dwyer, 2003). Among the

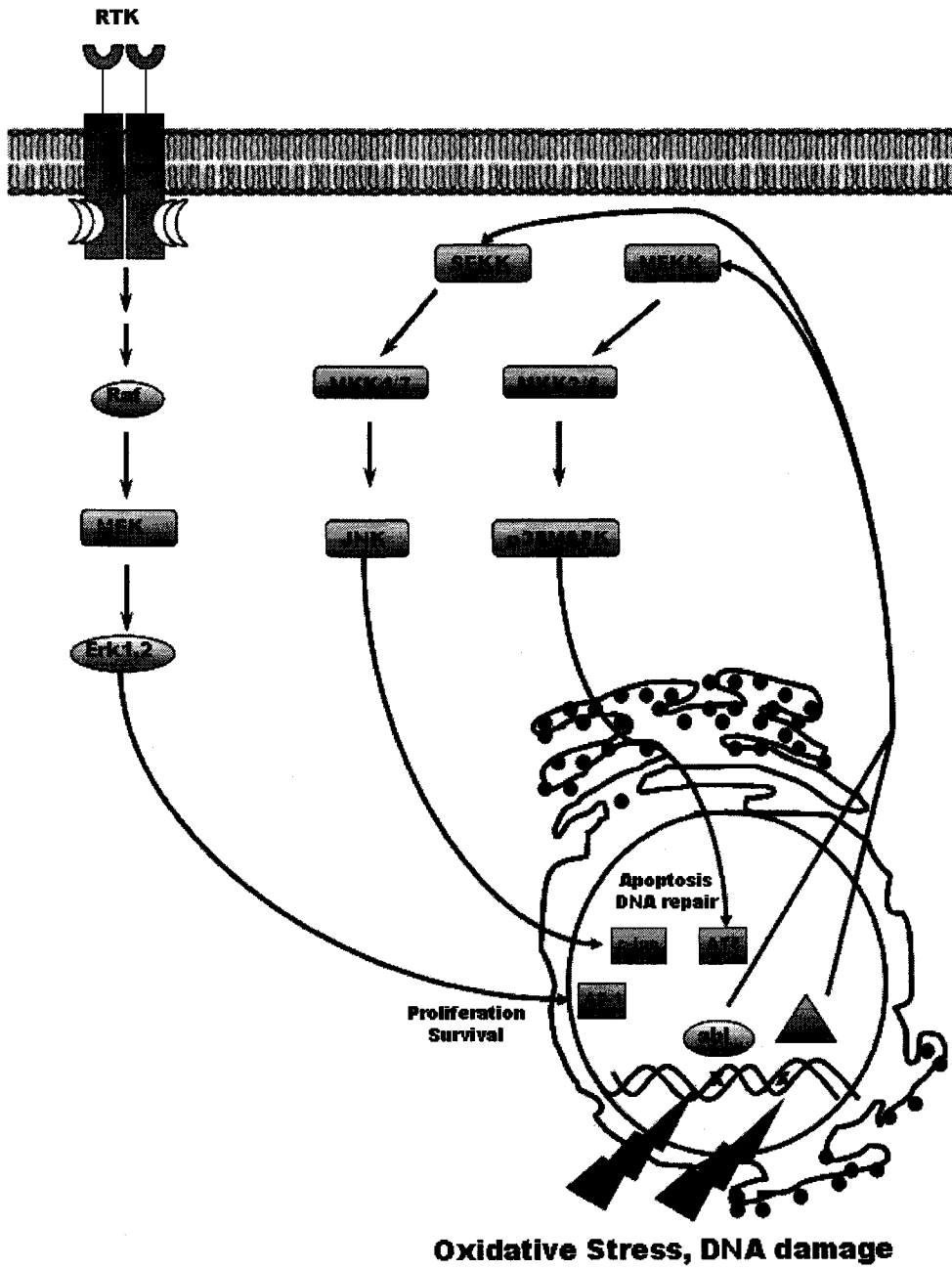


Figure 1.10. Initiation of the stress response pathway by DNA damage.

pro-apoptotic targets of c-jun is the promoter for the death inducing ligand TNF-α, which contains binding site for AP-1 (Mikami *et al.*, 2006). Another method by which JNK can regulate apoptosis is by phosphorylating members of the anti-apoptotic Bcl-2 class of proteins (e.g. Bcl2, Bcl-xL), thereby promoting mitochondrial-dependent apoptosis (Yamamoto *et al.*, 1999; Xiao *et al.*, 2004; Zu *et al.*, 2005). Studies by Tournier *et al.* (2000), revealed defective cellular responses in JNK null primary fibroblasts following exposure to UV irradiation and the methylating agent, methyl methane-sulfonate (MMS). The impaired activation of mitochondrial-dependent apoptosis was caused by defective initiation of the effector caspases (e.g. caspase-3).

As shown in Figure 1.10, the p38 family of SAPKs are regulated by the upstream kinases, MKK3 and/or MKK6, in response to external stresses (Ono and Han, 2000). Like JNK, p38MAPK can also target a variety of transcription factors including, p53 and ATF-2 (Ono and Han, 2000; Hayakawa *et al.*, 2003). Similar to JNK activation, studies have also shown p38 to play a role in apoptosis. Interestingly, activation of p38MAPK has been linked to cell cycle arrest at various checkpoints (e.g. G1/S, G2/M) following cell exposure to external stress signals (Ono and Han, 2000; Zhu and Zhang, 2003; Kurosu *et al.*, 2005). One such pathway, known for the response to the methylating agent, N-methyl-N'-nitro-nitrosoguanidine (MNNG), involves activation of ATM/ATR kinases and the subsequent phosphorylation of p38MAPK following DNA damage that resulted in G1/S arrest (Zhu and Zhang, 2003). Studies by Hirose *et al.* (2003) also demonstrated a role for p38MAPK in G2/M arrest following exposure to the clinical agent, TEM, in MMR-proficient cells. The results showed that activation of the G2/M checkpoint

initiated following TEM treatment was in parallel with p38MAPK phosphorylation and could be attenuated by the p38 inhibitors, SB203580 and SB202590. This p38-mediated G2/M arrest was also suggested to be critical in mediating chemoresistance since inhibition of the latter protein in MMR-proficient cells resulted in increased sensitivity to the methylating agent (Hirose *et al.*, 2003).

Although a significant body of work has accumulated to demonstrate the pro-apoptotic role of the stress activated kinases, JNK and p38. Recent studies indicate a pro-survival role for SAPKs during cancer progression (Dent *et al.*, 2003; Uzgare *et al.*, 2003; Maroni *et al.*, 2004). Results obtained by Harper *et al.* (2002) demonstrate that activation of both p38 and JNK in the prostate carcinoma cell line, DU145, produced a distinct growth advantage that was mitigated when the cells were exposed to an EGFR TKI. This drug downregulated both p38 and JNK activation. Other studies suggest a role for p38MAPK activation in the development of breast cancer. Breast epithelial cells stimulated with EGF induce EGFR-mediated activation of both ERK and p38 via Ras, and are associated with cellular proliferation and invasive growth (Kim *et al.*, 2003).

1.6. The Combi-Targeting Concept

Traditional chemotherapeutic approaches to cancer treatment are primarily based on multi-drug combinations. The therapeutic activities of these regimens are frequently hampered by severe toxicity and the lack of chemosensitivity of solid tumours at the advanced stages. To circumvent these problems, drugs with more targeted effects are urgently needed. Furthermore, due to tumour target heterogeneity in advanced diseases, combinations of single agents with pleiotropic effects would be more effective than

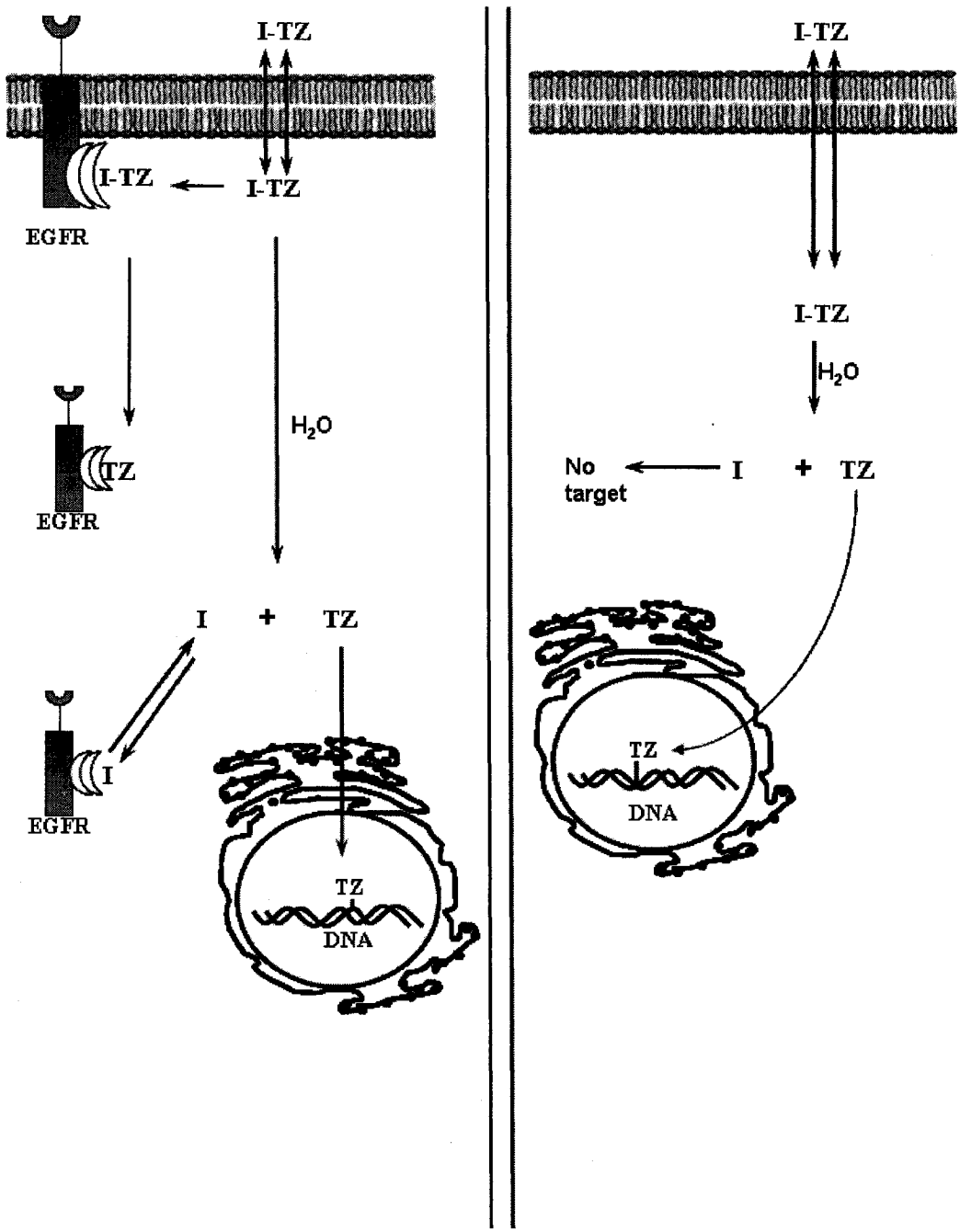


Figure 1.11. Postulates of the combi-targeting concept.

single targeted agents. It is within this context that our laboratory recently developed a novel approach termed "combi-targeting." The main thrust of this novel targeting concept is to synthesize drugs known as "combi-molecules," that are designed to generate a variety of fragments with multiple antitumour targets under hydrolytic conditions (Matheson *et al.*, 2001). As illustrated in Figure 1.11, the combi-molecule represented by I-TZ, wherein I represents the TK recognition moiety and TZ the DNA damaging tail, was designed to penetrate the cell by passive diffusion and bind to the ATP binding pocket to give rise to an EGFR-I-TZ complex or to be converted to another EGFR TK inhibitory molecule + a DNA damaging species that diffuses towards the nucleus. It was also assumed that the combi-molecule I-TZ could degrade within the active site and react with a Cys773 residue of the receptor, thereby inducing irreversible inhibitory activity. In contrast, in cells that do not express the EGFR, the I-TZ will not have any target and will simply behave as a classical DNA damaging agent. Thus, we surmised that the I-TZ, due to its combination of effects, would be more potent in the receptor expressing cells than the null ones.

Previous studies by Matheson *et al.* (2001; 2003a; 2004a; 2004b) have provided significant data to propose a model that confirmed the primary postulates of the combi-targeting concept. Radiolabeled combi-molecule, SMA41 (I-TZ, see Fig. 1.12), was used to study the subcellular distribution of the DNA alkylating species and was found to react with all three macromolecules including protein, RNA and DNA (Matheson *et al.*, 2003b; Matheson *et al.*, 2004a; Matheson *et al.*, 2004b). However, fluorescence microscopy showed that the inhibitor I (SMA52) was specifically localized in the

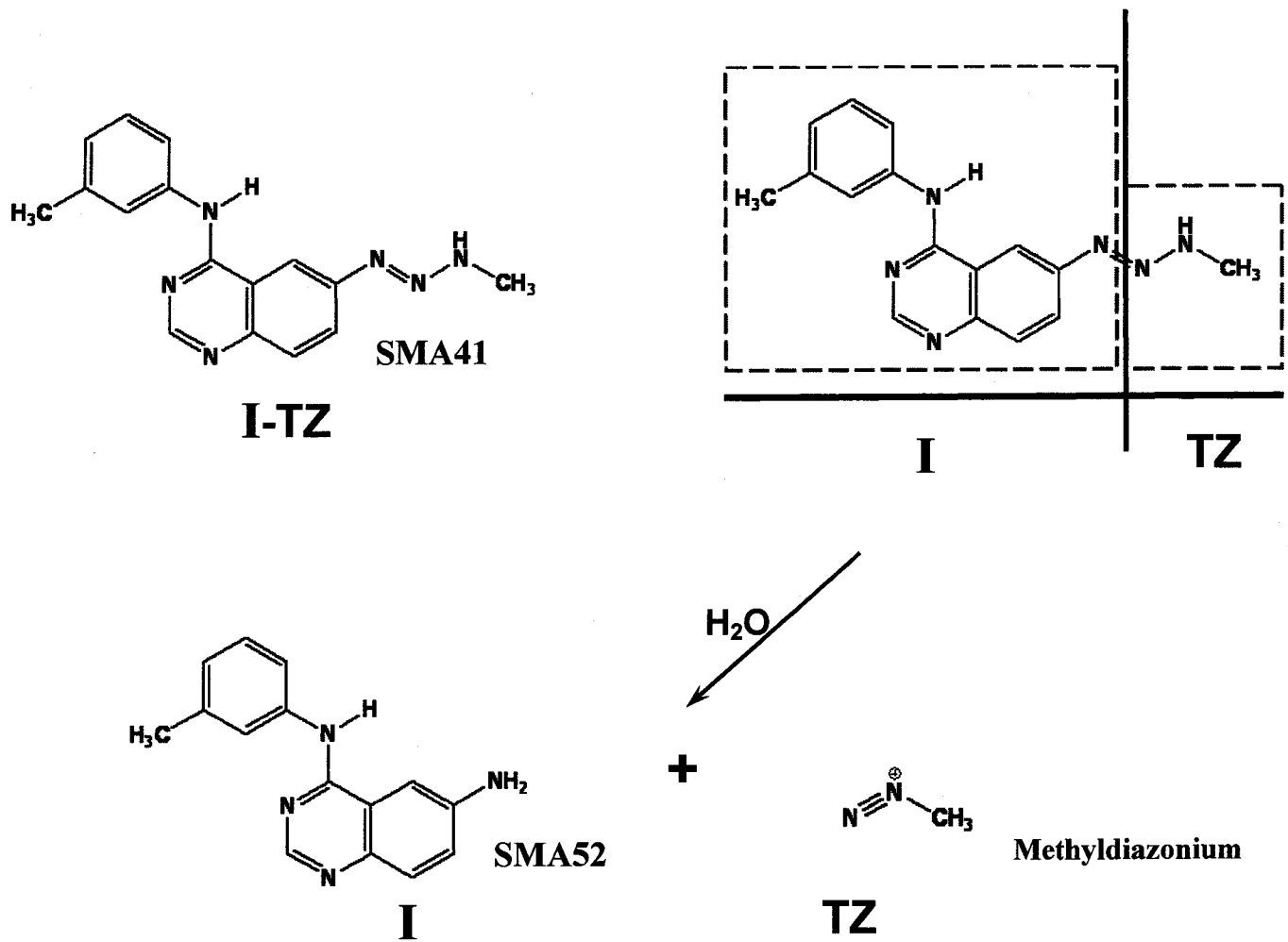


Figure 1.12. Structure of SMA41, the first prototype synthesized for the combi-targeting concept.

perinuclear region (Matheson *et al.*, 2004a). Moreover, the I-TZ was found to be internalized at a faster rate than its corresponding I administered alone. More importantly, flow cytometric analysis demonstrated that the half-life of SMA41 was considerably shorter ($t_{1/2}$=15min) inside the cell than in exogenous medium ($t_{1/2}$=40min) (Matheson *et al.*, 2004a). Thus, the proposed model was the following: a rapid diffusion of I-TZ into the cell, followed by its rapid intracellular decomposition into an inhibitor I, concentrated in the perinuclear region, and a methyldiazonium species TZ, that reacted with all three major macromolecules in the cell (e.g. RNA, DNA, protein) (Matheson *et al.*, 2004a). As it will be discussed later, new discoveries in the context of this thesis have urged a revision of the model.

A significant body of work has accumulated to demonstrate that the combi-molecules are not only agents programmed to release multiple fragments, but also to block intracellular signaling pathways. Brahimi *et al.* (2002), demonstrated that BJ2000, an I-TZ of the same class as SMA41, was capable of blocking EGF-induced EGFR phosphorylation in a partially irreversible fashion in the carcinoma of the vulva cell line, A431. This inhibitory activity translated into a dose-dependent blockade of MAPK activation and c-fos transcription. Using a different type of combi-molecule of the nitrosourea class (FD137), Qiu *et al.* (2003; 2004) obtained similar results showing that the I-TZ was capable inducing a tandem blockade of EGFR phosphorylation and DNA damage in prostate cancer cells. These events also translated into blockade of the early response gene, c-fos. In all cases, the combined EGFR/DNA targeting properties of the combi-molecules translated into superior antiproliferative activity when compared with a

classical two-drug combination. Other types of combi-molecules designed to block another tyrosine kinase, bcr-abl, were shown to inhibit TK phosphorylation in cells and to damage DNA in a similar manner (Rachid *et al.*, 2003b; Katsoulas *et al.*, 2005).

It is important to note that combi-molecules tested thus far demonstrated significant antiproliferative activity in cells that are resistant to their parent alkylating agents. For instance, previously synthesized combi-molecules (e.g. BJ2000, SMA41, FD137) were all more potent than the classical clinical agents TEM and BCNU in cells that express AGT (Brahimi *et al.*, 2002; Matheson *et al.*, 2003a; Qiu *et al.*, 2003; Qiu *et al.*, 2004).

The two major objectives of the combi-targeting principle being to exert increased potency in advanced refractory tumours and to achieve target selectivity, receptor specificity studies were performed to confirm the selectivity of the combi-molecules for EGFR or Her2. These studies were performed according to two major strategies: 1) using selective inhibition of growth factor stimulation and 2) analyzing effects of the combi-molecules on isogenic pairs of cells transfected with the EGFR or HER2 genes. Brahimi *et al.* (2002) demonstrated that in contrast to TEM, the I-TZ BJ2000 preferentially blocked EGF- or TGF-α-induced growth stimulation over PDGF- or serum-stimulation of cells. In the same study, it was shown that the combi-molecule preferentially inhibited EGFR TK over src kinase and IGFR. On the other hand, studies by Rachid *et al.* (2003a), using an extended series of combi-molecules of the triazene class demonstrated that the I-TZs selectively block NIH3T3 cells transfected with neu (ErbB2) and interestingly the levels of antiproliferative activity linearly correlated with their IC_{50} values for

competitive binding of EGFR TK, indicating that these compounds may target both EGFR and the ErbB2 receptor. More importantly, Rachid *et al.* (Rachid *et al.*, 2003a) showed that compounds designed to solely block EGFR TK or to damage DNA were significantly less potent than the combi-targeted agents.

The translation of the *in vitro* activity of the combi-molecules into *in vivo* potency was extensively studied in xenograft models. The primary purpose of these studies was to investigate whether the binary EGFR/DNA targeting properties of these molecules was a toxic event and to demonstrate their potency in refractory tumours (i.e. expressing AGT) known to be resistant to methylating agents. While Brahimi *et al.* (Brahimi *et al.*, 2005) showed that ZRBA1 was more potent than SMA41 in MDA-MB-468 (AGT+) breast cancer model, toxic side effects were observed. In addition, comparisons of combi-molecules with their corresponding classical combination were hampered by the difference in plasma concentrations achieved at equi-doses. The major obstacle encountered in the *in vivo* translation with combi-molecule was the reduced water solubility of quinazoline-based molecules. Matheson *et al.* (2004b) showed that SMA41 was well tolerated. However, significant antiproliferative activity for SMA41 could only be observed at a dose as high as 200mg/kg in an aqueous suspension. Thus, demonstration of *in vivo* potency of combi-molecules with water-soluble probes remains to be achieved.

Knowledge on combi-molecules prior to this thesis can be summarized by a) demonstration of the binary EGFR/DNA targeting properties of the combi-molecule, b)

demonstration of selective antiproliferative activity and c) evidence for the superior activity of combi-molecules over classical two-drug combinations *in vitro*. However, strategies to induce sustained potency of the combi-molecules and to elucidate cross-talk between various signaling pathways awakened by mixed EGFR/DNA targeting activities remain elusive. Further, *in vivo* demonstration of activity with a well-tolerated, water-soluble combi-molecular probe still remained to be achieved.

In light of progress on the combi-molecular strategy, my work has brought the following innovations summarized herein as the plan of this thesis: a) Chapter 2: design and synthesis of a novel combi-molecule termed "cascade release" combi-molecule "programmed" to hammer the EGFR TK with several inhibitors released in a stepwise fashion + a DNA damaging species, with the purpose of inducing sustained inhibition of phosphorylation, b) Chapter 3: analysis of the sustained antiproliferative mechanisms by the complex cascade release combi-molecule and demonstration of its ability to behave as a targeted precursor of multiple inhibitors and an alkylating species in the resistant AGT-expressing A431 carcinoma of the vulva cell line, c) Chapter 4: elucidation of the selective mechanism of target-mediated DNA damage by the cascade release combi-molecule and description of a novel model for the combi-targeting postulate, d) Chapter 5: analysis and discovery of a novel cross-talk mechanism between the stress response and EGFR-MAPK pathways in response to the multi-targeting effect of the cascade release combi-molecule and, e) Chapter 6: *in vivo* translation of the efficacy of the cascade release combi-molecule in a prostate cancer xenograft model.

1.7. Summary

A detailed description of all the intracellular mechanisms discussed in this thesis was described. These various aspects are presented in detail since the thesis deals with molecules that are not only cell signaling inhibitors but also DNA damaging agents that trigger complex stress response pathways. Thus, in summary we described:

1. The EGF receptor family and its signaling pathways
2. The small molecule inhibitors that target the ATP-binding site of EGFR TK
3. The 3-methyltriazenes and their mechanism of action
4. The DNA repair pathways induced by the triazene class of agents (e.g. BER, AGT, MMR)
5. The stress response pathways in response to methylating agents
6. Previous knowledge acquired from the "combi-targeting" concept

1.8. Research objectives

The objective of this thesis was to synthesize a novel class of combi-molecule termed "cascade release" combi-molecule capable of inducing sustained inhibition of EGFR TK while damaging DNA throughout its degradation cascade and to elucidate its mechanism of action in EGFR- or Her2-expressing cells.

1.8.1 Project outline

The following research objectives were pursued in order to realize the goals of this thesis:

Objective 1: to synthesize a novel combi-molecule capable of degrading into multiple inhibitors of EGFR TK + a methyldiazonium species and confirm its binary EGFR/DNA targeting properties

Objective 2: to characterize the sustained and irreversible antiproliferative activity of the complex combi-molecule

Objective 3: to elucidate which intracellular pathways can be targeted to enhance the potency of the combi-molecule, inducing synergistic antiproliferative activity

Objective 4: to demonstrate the *in vivo* potency of a new generation of water-soluble complex combi-molecules

1.8.2. Contribution of Authors

CHAPTER 2: This paper has been published in the Journal of Medicinal Chemistry 46(25):5546-51 (2003). My contribution was the synthesis of the novel combi-molecules with the exception of ZR08, which was synthesized by Dr. Zakaria Rachid. All of the *in vitro* analyses were performed by myself, and preparation of the first draft of the manuscript. While I performed the single-cell microelectrophoresis, the imaging and quantitation of the samples was done by Dr. James P. McNamee at Health Canada. My supervisor (Dr. Bertrand J. Jean-Claude) made comments and revisions.

CHAPTER 3: This paper has been published in the British Journal of Cancer 91(6):1066-73 (2004). My contribution was the majority of the experimental work and preparation of the first draft of the manuscript. Dr. Zakaria Rachid synthesized the combi-molecule, ZR08. Dr. Qiyu Qiu provided one set of RT-PCR data and I performed the following RT-PCR experiments. Dr. Ana Tari at the M.D. Anderson Cancer Center in Houston provided the Western blot for Erk1,2 activation on the samples I prepared. In addition, I ran the single-cell microelectrophoresis and the imaging of the samples was performed by Dr. James McNamee at Health Canada. My supervisor assisted in the revision of the manuscript.

CHAPTER 4: This manuscript will be submitted to Oncogene. My contribution was all of the experimental work and preparation of the manuscript. The imaging of the single

cell microelectrophoresis samples was performed by Dr. James McNamee. Dr. Jean-Claude made suggestions and the necessary revisions to the manuscript.

CHAPTER 5: This manuscript is under submission to Cancer Research. I performed the majority of the *in vitro* experiments and the first draft the manuscript. Dr. Qiu performed the antiproliferative SRB assay for one of the control compounds (TEM). All the imaging of the single-cell microelectrophoresis samples that I prepared were performed by Dr. James McNamee. My supervisor provided suggestions and comments on the manuscript.

CHAPTER 6: This manuscript will be submitted to the Journal of Experimental Pharmacology and Therapeutics. I carried out all the *in vitro* experiments and the imaging of the single cell microeletrophoresis was prepared by Dr. James McNamee. I also synthesized the water-soluble combi-molecule required for the *in vivo* xenograft studies. Although I received training for animal handling from the McGill Animal Resources Centre, all the technical work was carried out by the Centre. I also prepared the first draft of the manuscript that was further revised by my supervisor, Dr. Jean-Claude.

1.8.3. References

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CHAPTER 2

SYNTHESIS OF A PRODRUG DESIGNED TO RELEASE MULTIPLE INHIBITORS OF THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) TYROSINE KINASE AND AN ALKYLATING AGENT: A NOVEL TUMOUR TARGETING CONCEPT

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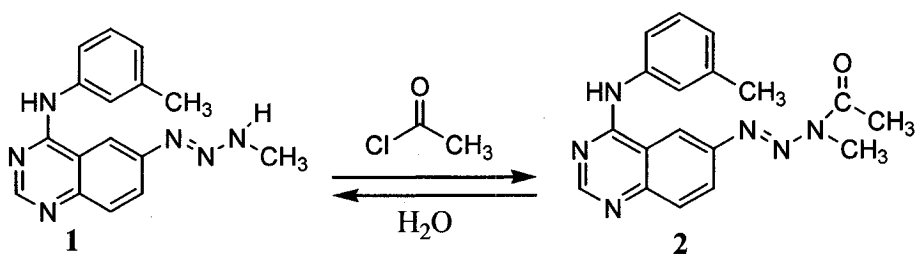
2.1. Abstract

The synthesis of a novel acetoxymethyltriazene designed to be a prodrug of multiple inhibitors of the epidermal growth factor receptor (EGFR) and a methyldiazonium species is described. Studies with each of the expected metabolites demonstrated significant EGFR tyrosine kinase inhibitory activities and the released methyldiazonium was trapped with p-nitrobenzylpyridine. Their ability to damage genomic DNA in whole cells was demonstrated by using the single-cell microelectrophoresis (comet) assay. The results suggest that this approach may well represent a novel drug combination strategy involving single molecules masking multiple bioactive agents.

2.2. Introduction

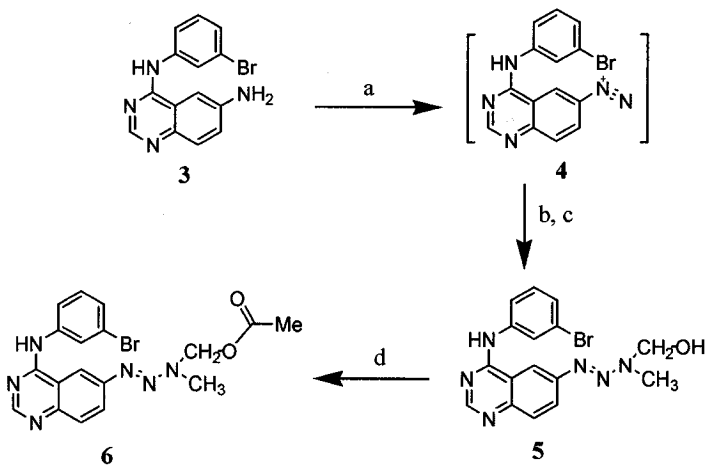
The altered protein expression and activity of receptor tyrosine kinases (TK) are implicated in the progression of various types of cancers. One such dysfunction is the overexpression of the epidermal growth factor receptor (EGFR) and its closest homologues HER2 (erbB2) that correlates with aggressive tumor progression and poor prognosis (Modjtahedi and Dean, 1994). Recently, we developed a novel strategy that seeks to combine DNA damaging properties and EGFR TK inhibitory activities into single molecules termed "combi-molecules" designed to kill EGFR-expressing tumour cells (Matheson *et al.*, 2001; Brahimi *et al.*, 2002; Qiu *et al.*, 2003; Matheson *et al.*, 2003a). In order to enhance the EGFR inhibitory potency and stability of these compounds, we designed a novel strategy termed "cascade release" (CR) that seeks to mask the combi-molecule into a prodrug "programmed" to release the antitumour species by hydrolytic activation. Since these molecules henceforth referred to as "cascade release molecules" (CRM) are also designed to retain significant EGFR affinity on their own, this principle leads to conditions under which three generations of inhibitors can be derived from the parent CRM.



Scheme 2.1

To test the CRM principle, we initially designed structure **2** (Scheme 2.1) with the hope that it would not only retain affinity for EGFR but also hydrolyze to generate monoalkyltriazene **1** which, as previously demonstrated, heterolyzes into the

corresponding 6-amino-4-anilinoquinazoline (an inhibitor of EGFR) + a methyldiazonium species (Matheson *et al.*, 2001; Brahimi *et al.*, 2002). Thus, CRM **2** was designed to be the prodrug of two known inhibitors of EGFR. Its synthesis proceeded according to Scheme 2.1. Structure **1**, obtained as described earlier was treated with acetyl chloride in methylene chloride at 0°C to give **2** in an 18% yield (Matheson *et al.*, 2003b). Studies of the hydrolysis of **2** under physiological conditions (i.e. incubation in serum containing media at 37°C) demonstrated little degradation after 24 h. Further exposure for an additional 24 h led to minor degradation. Therefore, it was concluded that this molecule was not a suitable probe for the demonstration of the CRM principle.



Scheme 2.2. Synthesis of 6-(3-Acetoxymethyl-3-methyl-triazenyl)-4-(3'-bromophenyl-amino)quinazoline. a, $NOBF_4$; b, $MeNH_2/H_2CO/H^+$; c, K_2CO_3; d, $(CH_3CO)_2O$ in pyridine.

We subsequently designed structure **6** (Scheme 2.2), which would not only offer the opportunity to further mask the CRM, but also to degrade according to known kinetics and mechanisms (Manning *et al.*, 1985; Cameron *et al.*, 1985). It has already been demonstrated that acetoxymethyltriazenes rapidly degrade to the corresponding hydroxymethyl triazene, which in turn is converted to a monoalkyltriazene of type **1**

(Matheson *et al.*, 2003b). Further, heterolysis of triazene is known to generate an aromatic amine and an alkylating agent (Cameron *et al.*, 1985). Thus, we surmised that these mechanisms of degradation might lend themselves to the demonstration of the CRM principle.

2.3. Experimental Section

2.3.1. Chemistry

^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Varian 400 or 300 spectrometer. Mass spectroscopy was performed by the McGill University Mass spectroscopy Center. ESI and APCI spectra were performed on a Finnigan LC QDUO spectrometer. Data are reported as m/z (intensity relative to base peak = 100). Melting points were determined in open capillary tubes on a Meltemp melting point apparatus and were uncorrected.

2.3.2. Synthesis of 6-(3-Acetyl-3-methyl-triazenyl)-4-(*m*-toluidyl)quinazoline (2)

To a solution of 6-(3-methyltriazenyl) -4 anilinoquinazoline **1** (100 mg, 0.400 mmol) in methylene chloride (5 mL) was added triethylamine (0.1 mL). The mixture was stirred for 15min at 0°C and acetyl chloride (0.05 mL, 0.920 mmol) was added dropwise. Thereafter, the solution was evaporated under vacuum and the resulting brown residue purified on a silica gel column (40% ethyl acetate in hexane) to give **2** as a brown powder (18 mg, 16%) : mp 188°C; FABS *m/z* 335.2 (MH^+), 235 (M-3-acetyl-3-methyltriazene) *m/z* 335.162034 (MH^+), $C_{18}H_{18}N_6O_1$ requires 335.16190; ^{1}H NMR (300MHz, DMSO-*d6*) δ 8.84 (s, 1H, NH), 8.58 (s, 1H), 8.14 (d, 1H, J=2.1Hz), 8.12 (d,

1H, J=2.1Hz), 7.83 (d, 1H, J=8.7Hz), 7.68 (s, 1H), 7.66 (s, 1H), 7.26 (t, 1H, J=7.8Hz), 6.95 (d, 1H, J=8.1Hz), 3.39 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 2.34 (s, 3H, CH_3); ^{13}C NMR (75MHz, DMSO-*d*6) δ 173.3, 158.6, 155.7, 150.7, 146.6, 139.5, 138.2, 130.0, 128.9, 125.3, 124.5, 123.6, 120.6, 120.4, 116.0, 28.4, 22.8, 22.0.

2.3.3. Synthesis of 6-(3-Hydroxymethyl-3-methyl-triazenyl)-4-(3'-bromophenyl-amino)quinazoline (5)

To a solution of 4-(3'-bromophenyl-amino)-6-aminoquinazoline **3** (100 mg, 0.318 mmol) in acetonitrile (20 mL) kept at 0°C on an ice bath, nitrosonium tetrafluoroborate (74.3 mg, 0.637 mmol) was added dropwise. The solution was stirred for 20 min and 0.9 mL of a mixture of methylamine 40% (0.075 mL, 0.954 mmol), formaldehyde 37% (0.75 mL, 9.54 mmol), concentrated HCl (0.1 mL) was added all at once. The diazonium solution was subsequently alkalinized with potassium carbonate (400 mg, 2.86 mmol) and the precipitate was filtered to give a white solid contaminated with excess potassium carbonate. The precipitate was therefore re-suspended in THF and the resulting solution filtered. The organic solvent was further evaporated to give **4** as a pure brown solid (90 mg, 73%) : mp 150°C; FABS *m/z* 387 (MH^+ with ^{79}Br), 389 (MH^+ with ^{81}Br), 359 (M-N_2) *m/z* 387.056895 (MH^+), $C_{16}H_{15}N_6O_1Br$ requires 387.05670; ^1H NMR (400MHz, DMSO-*d*6) δ 9.91 (s, 1H, NH), 8.57 (s, 1H), 8.50 (s, 1H), 8.25 (s, 1H), 7.99 (d, 1H, J=8.8Hz), 7.92 (d, 1H, J=7.6Hz), 7.76 (d, 1H, J=8.8Hz), 7.34-7.25 (m, 2H), 6.37 (s, 1H, OH), 5.17 (s, 2H, CH_2), 3.19 (s, 3H, CH_3); ^{13}C NMR (75MHz, DMSO-*d*6) δ 164.3, 158.0, 154.0, 148.9, 149.8, 130.9, 129.5, 126.3, 125.2, 124.7, 121.8, 121.2, 116.4, 116.1, 78.8, 34.0.

2.3.4. Synthesis of 6-(3-Acetoxymethyl-3-methyl-triazenyl)-4-(3'-bromophenyl-amino)quinazoline (6)

6-(3-Hydroxymethyl-3-methyl-triazenyl)-4-(3'-bromophenyl-amino)quinazoline **5** (100 mg, 0.258 mmol) was dissolved in pyridine (2 mL) after which acetic anhydride (0.055 mL, 0.516 mmol) was slowly added. The solution was further kept on ice for 1.5 h and the pyridine evaporated as an azeotrope with toluene to give **6** as a pure brown residue (105 mg, 95%) : mp 140°C; FABS *m/z* 429 (MH^+ with ^{79}Br), 431 (MH^+ with ^{81}Br), 298 [M-(3-acetoxymethyl-3-methyltriazene)], 401 ($M-N_2$) *m/z* 429.067460 (MH^+), $C_{18}H_{17}N_6O_2Br$ requires 429.06728; ^1H NMR (400MHz, DMSO-*d6*) δ 10.02 (s, 1H, NH), 8.61 (s, 2H), 8.25 (s, 1H), 7.99 (d, 1H, J=8.4Hz), 7.93 (d, 1H, J=7.6Hz), 7.79 (d, 1H, J=9.2Hz), 7.35-7.26 (m, 2H), 5.86 (s, 2H, CH_2), 3.26 (s, 3H, CH_3), 2.07 (s, 3H, CH_3); ^{13}C NMR (100MHz, DMSO-*d6*) δ 170.8, 158.1, 154.4 149.5, 147.7, 141.8, 130.9, 129.7, 126.5, 125.1, 124.8, 121.8, 121.3, 117.3, 116.2, 79.4, 35.5, 21.6.

2.3.5. Drug Treatment

Compounds **1**, **2**, **3**, **5**, **6** and **8** were synthesized in our laboratories according to known procedures. Temozolomide (TEM) was provided by Shering-Plough Inc. (Kenilworth, NJ, USA). In all assays, drugs were dissolved in DMSO and subsequently diluted in RPMI-1640 containing 10% fetal bovine serum (FBS) (Wisent Inc. St-Bruno, Canada) or in DMEM containing 10% bovine calf serum (GIBCO BRL, Burlington, Canada) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

2.3.6. Purity of Compounds

Purity analyses were performed by high performance liquid chromatography (HPLC) using a Spectrasystem (Thermoquest) and a Waters C4 15-μm 300x3.9-mm column (reverse phase). The operating mode was isocratic and two different systems of solvents were used: the first one was 100% acetonitrile with a 0.35mL/min flow rate at 254nm; and the second system was 80% isopropanol and 20% dioxane with a 0.5 mL/min flow rate at 254 nm.

2.3.7. Cell Culture

The cell lines used in this study, the mouse fibroblasts NIH3T3, NIH3T3/HER14 (NIH3T3 cells stably transfected with the EGFR gene) and NIH3T3/neu (NIH3T3 cells stably transfected with HER2) were generous gifts from Dr. Moulay Aloui-Jamali of the Montreal Jewish General Hospital. NIH3T3, NIH3T3/HER14 and NIH3T3/neu cells were maintained in DMEM supplemented with 10% FBS and antibiotics. All cells were maintained in an atmosphere of 5% CO_2.

2.3.8. Degradation

The half-lives of **6**, **5** and **8** under physiological conditions were studied by a UV-spectrophotometer. The compounds were dissolved in a minimum volume of DMSO, diluted with RPMI-1640 supplemented with 10% FBS, and absorbances were read at 240 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, USA).

The study of the conversion of the compounds (**6**, **5** and **8**) to their corresponding free inhibitor (**3**) was performed by spectrofluorometer as the latter amine is fluorescent (absorption 290 nm, emission 450 nm). Briefly, 125 μM of the drug conjugates were added to RPMI-1640 with 10% FBS and incubated for 4 h at 37°C in a microplate spectrofluorometer (SpectraMax Gemini flourescence reader, Molecular Device, CA). The data are acquired and analyzed by SoftMax®Pro (Molecular Device, CA).

HPLC analysis was performed to study the degradation of compounds **2, 5**, and **6**. The compounds were dissolved in a minimum volume of DMSO, diluted with RPMI-1640 supplemented with 10% FBS, using a Spectrasystem (Thermoquest) and a Waters C4 15-μm 300x3.9-mm column (reverse phase). The operating mode was isocratic using a 60% acetonitrile and 40 % water solvent system with a 0.5 mL/min flow rate at 254 nm.

2.3.9. EGFR Kinase Assay

Nunc Maxisorp 96-well plates 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 (Tween 20 (0.1%) in PBS). The kinase reaction was performed by using 4.5 ng/well EGFR affinity-purified from A431 cells. The compounds were added and phosphorylation was 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 following a 25 min incubation with 50 μL/well of HRP-conjugated PY20 anti-phosphotyrosine antibody (Santa Cruz Biotechnology, CA) diluted to 0.2 mg/mL in

blocking buffer (3% bovine serum albumin; 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, Gaithersberg, MD, USA) 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).

2.3.10. *In vitro* Growth Inhibition Assay

To study the effect of our compounds on serum-stimulated proliferation, cells were grown to 70 % of confluence in 96-well plates and washed twice with PBS after which they were exposed to serum-free media for 24 h. Cells were exposed to each drug + serum for 72 h and cell growth measured using the sulforhodamine B (SRB) assay. 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 read for each well at 492 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

2.3.11. Alkylating Activity Assay

For each time point, 10 μl of compound (20 μM) were added to a 2% p-nitrobenzylpyridine (NBP): ethyleneglycol (100 μL) mixed with Tris pH 7.5 (27%

ethanol) (70 μL) and incubated at 37°C. The color was developed with a solution of acetone:triethylamine (50/50, v/v). Optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550).

2.3.12. Comet Assay for Quantitative Analysis of DNA Damage

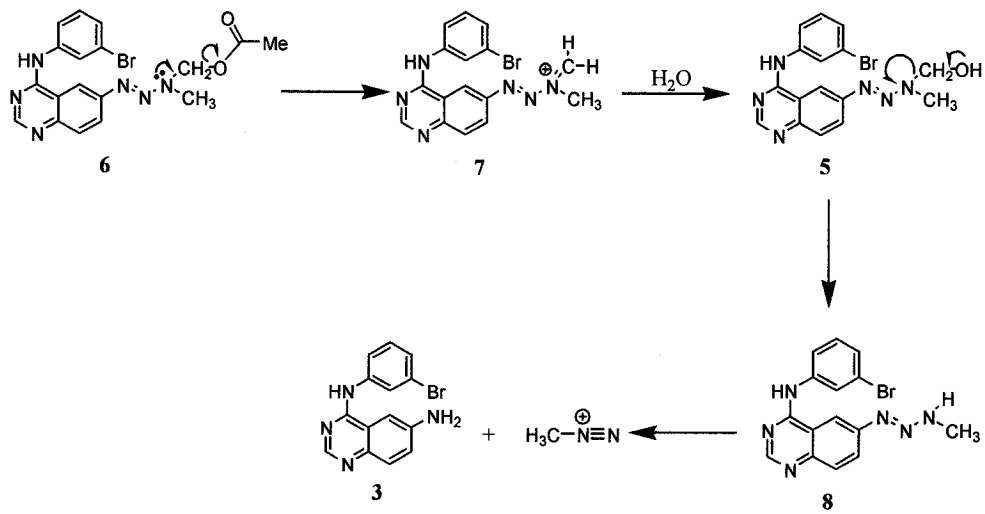
A modified alkaline comet assay technique was used to quantitate DNA damage induced by **6** and **5** in MDA-MB-435 cells. The cells were exposed to drugs for 24 h and harvested with trypsin-EDTA. They were subsequently collected by centrifugation and re-suspended in PBS. The resulting cell suspensions were diluted to $3x10^5$ cell/mL, and mixed with 0.75% agarose in PBS at 37°C in a 1:9 dilution. The gels were cast on Gelband strips (Mandel Scientific, Guelph, ON, Canada) using gel casting chambers, as previously described, then immediately placed into a lysis buffer (2.5 M NaCl, 0.1 M tetrasodium 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 immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetrasodium 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 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.

2.4. Results and Discussion

2.4.1. Chemistry

The synthesis of **6** proceeded according to Scheme 2.2. Briefly, compound **3** was treated in acetonitrile with $NOBF_4$ to generate intermediate **4**, which was treated *in situ* with a 10:1 mixture of formaldehyde/methylamine. Alkalinization with aqueous K_2CO_3 led to precipitation of compound **5**, in high yield. However, this compound co-precipitated with solid K_2CO_3, which was removed by re-suspension in THF followed by collection and evaporation of the filtrate. Hydroxymethyltriazene **5**, was obtained in a pure state (yield: 70%) without requirement for further purification. Treatment of **5** with acetic anhydride in pyridine gave **6** as a pure solid. We have already reported the synthesis of **8** (Rachid *et al.*, 2003).



Scheme 2.3.

The stability of **6** and that of each of its putative metabolites were studied by allowing them to degrade in both phosphate buffered saline (PBS) and serum-containing media using UV spectrophotometric analysis. Compound **6** was much less stable than **2**, with a half-life of 42 min in serum-containing media (Table 2.1). Interestingly, its putative metabolite **5** (Scheme 2.3) showed a half-life of 40 min suggesting that conversion of **6** to **5** may be a rapid event. Indeed, it has already been reported that acetoxymethyltriazenes are rapidly converted to the corresponding hydroxymethyltriazene in PBS (Hemens *et al.*, 1984; Hemens and Vaughan, 1986; Reid *et al.*, 1999).

Attempts to monitor the formation of **5** by HPLC failed due to its instability under the separation conditions. However, when **6** was allowed to degrade overnight under physiological conditions, we could successfully detect amine **3** (Schemes 2.2 and 2.3) as the sole product, suggesting that the putative intermediary metabolites may have been formed according to the mechanism outlined in Scheme 2.3. This mechanism has already been proposed for similar triazenes (Hemens *et al.*, 1984; Hemens and Vaughan, 1986). Addition of water to iminium ion **7** may generate the methylol **5** which may self-deformylate to give the monoalkyltriazene **8.** To circumvent problems associated with on-column degradation of **6** and that of its metabolite **5**, we designed a fluorescence detection assay in order to monitor the formation of **3**, the ultimately generated stable metabolite. Absorption-emission spectra of the latter showed absorption at 290 nm and emission at 450 nm. Thus we could unambiguously analyze the kinetics of formation of **3** by spectrofluorimetry. Interestingly, the rate of conversion of **5** to **3** was slower than that of **8** to **3**. These data support a stepwise degradation of the CRM in the sequence

proposed in Scheme 2.3. More importantly, the symmetrically inversed relationship between the degradation of the diverse metabolites and the formation of **3** suggest a near stoichiometric conversion of **6** to **3** (Fig. 2.1). A similar result was reported for triazenoquinazolines of types **1** and **8** (Brahimi *et al.*, 2002).

2.4.2. Biology

Our objective being to generate multiple inhibitors from a parent one, we tested the EGFR TK inhibitory activity of each of the putative metabolites alone in a short 8-min exposure ELISA. The assay was based on the inhibition of phosphorylation of poly(L-glutamic acid-L-tyrosine, 4:1) (PGT) by EGFR tyrosine kinase. Structure-activity relationships (SAR) in the quinazoline series have already demonstrated tolerance of bulky substituents at the 6- and 7-positions (Rewcastle *et al.*, 1995; Rewcastle *et al.*, 1997; Rewcastle *et al.*, 1998). Indeed the triazene appendage to the 6-position of this series did not significantly alter the EGFR binding affinities when compared with **3**. As an example, the IC_{50} values for **3** and **8** were in the same range [e.g. **8** (IC_{50}= 0.07 μM); **3** (IC_{50}= 0.04 μM)] (Table 2.1). More importantly, previous studies have demonstrated the strong dependence of the SAR of quinazolines on the electronic character of the substituents at the 6-position (Rewcastle *et al.*, 1995; Rewcastle *et al.*, 1998). Electron-donating groups at the 6-position enhance activity, whereas electron-withdrawing ones are deleterious to affinity. In this study, it was found that acylation of **1** decreased the affinity of the resulting compound **2** by threefold (Table 2.1). This may be due to the decrease in the electron-donating character of the triazene, that may deplete the partial charges on the nitrogens in the quinazoline ring. Indeed, when acylation was

Table 2.1. EGFR TK inhibition, alkylating activity, and half-lives of compounds **1–3**, **5–6** and **8**.

Compounds	Inhibition of EGFR IC_{50}, μM[a]	Alkylating Activity ($10^{-3}min^{-1}$)[b]	Half-life (RPMI) ($t_{1/2}$),min.[c]	Half-life (PBS) ($t_{1/2}$),min.[d]
1	0.2	9.1	35	69
2	0.578	1.5	>200	>200
3	0.04	0.05	N/A	N/A
5	0.11	6.6	40	70
6	0.13	5.8	42	84
8	0.07	8.9	38	68
TEM	N/A	11.3	N/A	N/A

[a]EGFR TK values are means of two experiments, alkylating activity and half-lives values were obtained from single experiments. [b]Measured as a rate of alkylation of NBP at pH 7.5. [c,d]Measured by UV absorbance at 290nm.

Table 2.2. Inhibition of serum-stimulated growth in NIH3T3, NIH3T3/HER14 and NIH3T3/neu cell lines by compounds **1–3, 5–6, 8, TEM, AG1478** and combination of **TEM + AG1478.**

Compounds	NIH3T3 IC_{50}, μM[a]	NIH3T3/HER14 IC_{50}, μM[a]	NIH3T3/neu IC_{50}, μM[a]
1	125 ± 1.7	28.9 ± 5.5	16.5 ± 2.3
2	200 ± 6.3	149 ± 14.2	60.6 ± 6.9
3	14.5 ± 1.9	10.4 ± 0.5	0.42 ± 0.03
5	12.8 ± 0.45	2.1 ± 0.3	0.09 ± 0.03
6	15.4 ± 0.55	3.4 ± 1.2	0.21 ± 0.05
8	15 .5 ± 1.9	8.8 ± 0.6	0.09 ± 0.03
TEM	214.0 ± 11.1	207.2 ± 12.7	210.0 ± 16
AG1478	28.6 ± 3.6	9.8 ± 0.3	4.0 ± 0.2
TEM + AG1478	39.4 ± 5.4	6.2 ± 0.5	1.1 ± 0.06

[a]*Values are means of IC_{50} values and SEMs from three experiments.*

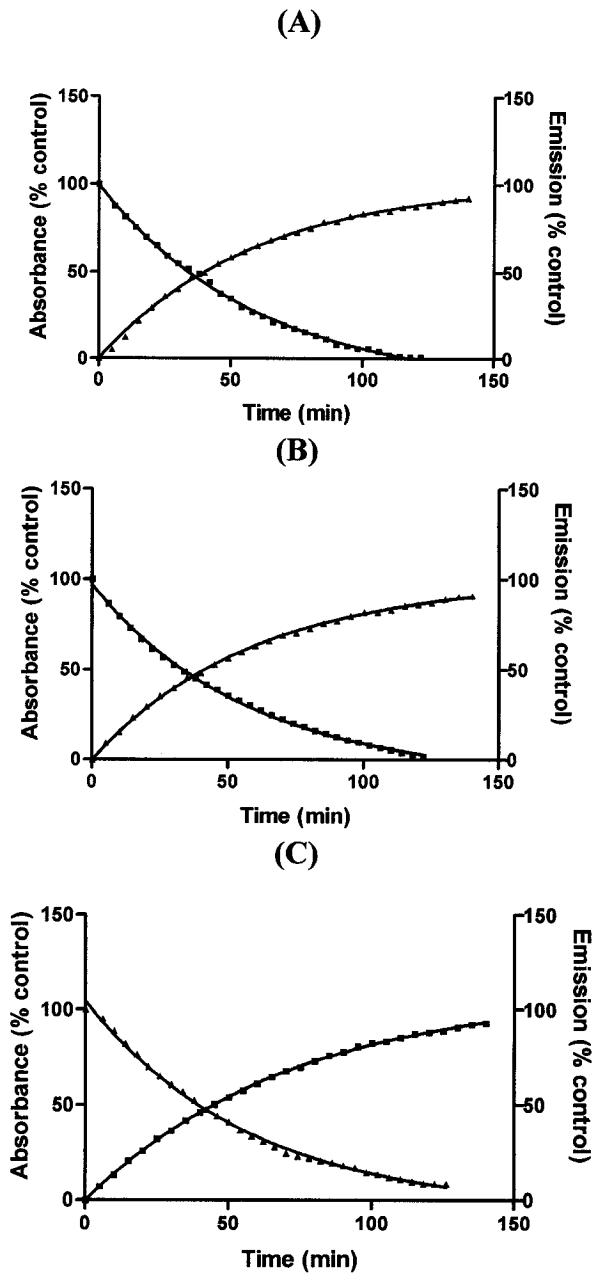


Figure 2.1. Formation (■) of **3** during the degradation (▲) of a) **6**, b) **5** and c) **8** in RPMI supplemented with FBS 10 % at 37°C. Degradation experiments were carried out by UV spectrophotometric analysis. The analyses were performed at 25μM. Drugs were added to RPMI-1640 with 10% FBS (2 ml) and incubated at 37°C.

performed in a manner that did not affect the p-extended system of 1,2,3-triazene (as in **6**), there was little change in binding affinity when compared with the parental monomethyl triazene **8**. A significant decrease in the electron density at N1 was already demonstrated by ^{15}N spectroscopy when N3 in cyclic and acyclic triazenes are acylated (Wilman, 1990; Jean-Claude and Williams, 1998).

Previous studies demonstrated that serum stimulation of isogenic cells transfected with EGFR or the HER2 gene product represent good models for the determination of tumour selectivity of combi-molecules since they offer the advantage of growth-stimulating the two cell lines with a common growth factor (Matheson *et al.*, 2003a). When this model was used, all compounds possessing EGFR TK inhibitory activities selectively blocked serum-stimulated growth of NIH 3T3 cells transfected with EGFR (NIH3T3/HER14) or HER2 (NIH3T3/neu), indicating translation of the EGFR TK inhibition into blockade of growth factor-mediated proliferation (Table 2.2). A typical NIH3T3/NIH3T3neu differential growth response profile is shown in Figure 2.2. Moreover, it is noteworthy that the cascade release molecule **6** is significantly more potent than its metabolite **8** ($p<0.001$) in the EGFR transfected cell line, NIH3T3/Her14. However, the order of potency of these two molecules was inverted in the NIH3T3/neu cell line, indicating that perhaps **6** is less selective for HER2/neu than **8** and therefore may not serve as an efficiently targeted prodrug of **8** in these cells.

In order to further demonstrate the efficacy of the cascade combi-molecular approach, the effects of **6** were compared with those of classical combination of Temozolomide

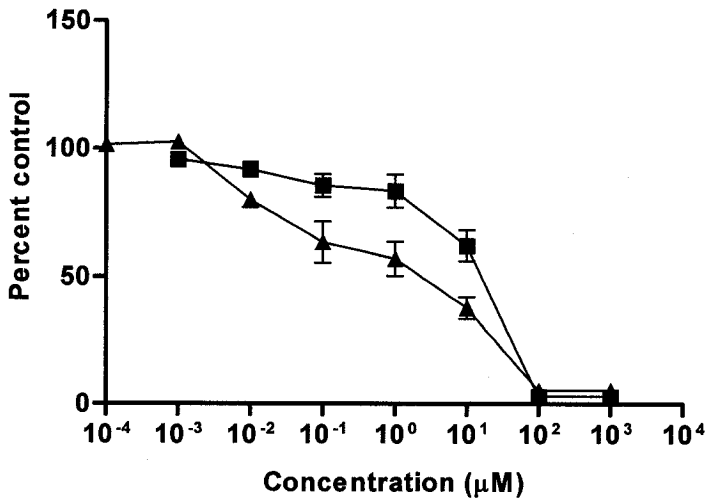
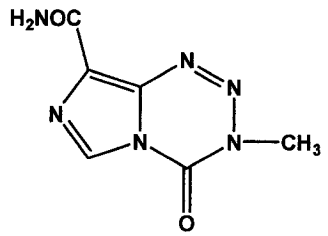
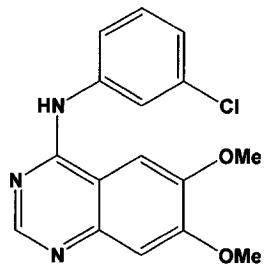


Figure 2.2. Effect of **6** on serum stimulated-proliferation in NIH3T3 (■) and NIH3T3/neu (▲) cells. Cells were starved for 24 h and exposed to each drug + serum for 72 h and growth inhibition was measured using SRB assay. Each point represents at least two independent experiments run in triplicate.

(TEM) and AG1478, a known *in vivo* active EGFR-TK inhibitor (Nagane *et al.*, 2001). The combinations were performed at IC_{50}(TEM)/IC50(AG1478) molar ratios.



TEM



AG1478

Structures of the clinical agents Temozolomide and AG1478

The results showed that **6** was 2.5- and 5-fold more potent than equi-effective doses of TEM+AG1478 in NIH3T3/Her14 and NIH3T3/neu cells respectively. Superior activity (e.g 10-fold) when compared with the TEM+AG478 combinations was observed for **8** only in the NIH3T3/neu cells, which is in agreement with its strong activity in the latter cell line. These results support the significant antiproliferative potency of our combi-targeting and combi-molecular CRM approaches.

The hydrolysis of our CRM is expected to generate multiple inhibitors of EGFR and at the final stage of degradation the methyldiazonium species. Since the latter is known to be the cytotoxic DNA alkylating metabolite of triazenes, we studied the alkylating potency of the different agents using the 4-(p-nitrobenzyl)pyridine (NBP) test (Nelis *et al.*, 1982; Brahimi *et al.*, 2002; Jean-Claude *et al.*, 2002; Qiu *et al.*, 2003; Matheson *et al.*, 2003a). In this colorimetric assay, the alkylating species is trapped by NBP and a

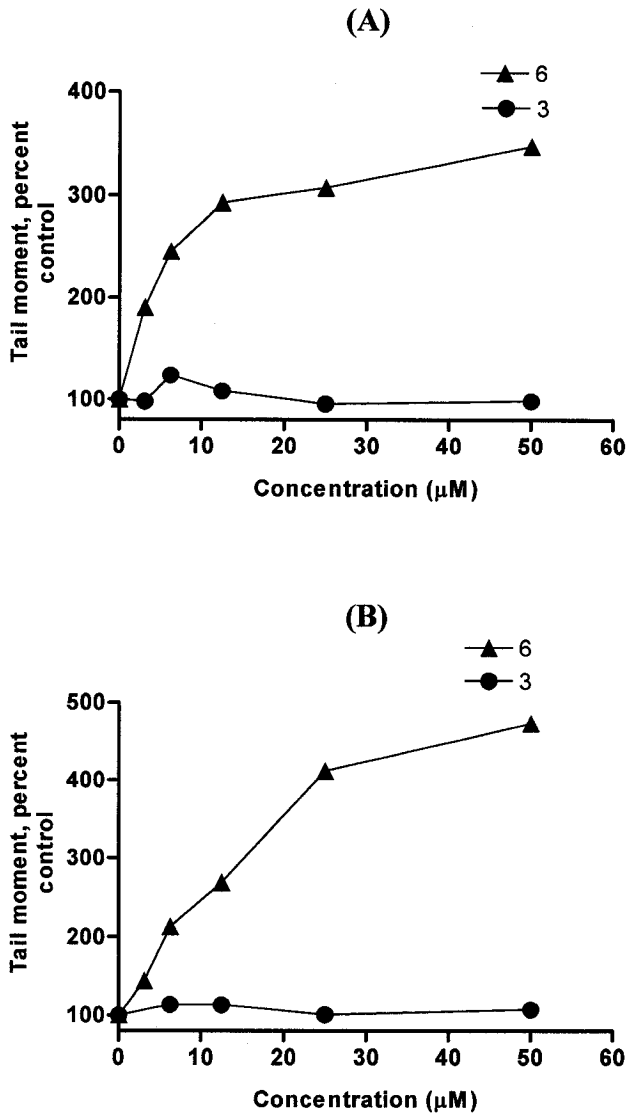


Figure 2.3. Quantitation of DNA damage using the alkaline comet assay. DNA damage induced by RB24 and RB10 in a) NIH 3T3 and b) NIH 3T3/neu cell lines. Tail moment was used as a parameter for the detection of DNA damage in NIH 3T3 and NIH 3T3/neu cells exposed to RB24 or RB10 for 30 min.

purple color is developed following addition of base. As a control, we compared the alkylating capacity of our compounds with that of a clinical DNA methylating cyclic triazene, Temozolomide (TEM). The NBP assay indicated that the parent compound **6** had a slower rate of alkylation when compared with the putative metabolite **5**, **8** and TEM (Table 2.1). As expected, the naked inhibitor **3** demonstrated no alkylating activity and the stable acylated compound **2** was found to have the slowest rate of alkylation in the entire triazene series.

The translation of the alkylating activity of **5** and **6** into DNA damage in human tumour cells was tested using the single cell microelectrophoresis (comet) assay in mouse fibroblast isogenic cell lines NIH 3T3 and NIH3T3/neu. In contrast to the naked inhibitor **3,** the acetoxymethyltriazene **6** was found to induce a dose-dependent increase in DNA strand breaks in both NIH 3T3 and its transfected counterpart, NIH 3T3/neu (Figure 2.3) confirming the binary targeting properties of the CRM.

This study conclusively demonstrates the feasibility of a molecule that may degrade in a stepwise mechanism into intermediary structures which when tested independently showed EGFR TK inhibitory activities. Although it was not possible to monitor the kinetics of formation of all the degradation products (e.g. the methylol intermediate **5**, or the monoalkyltriazene **8**), their ultimate conversion to the stable metabolite **3** suggests that they may have been formed according to the mechanism outlined in Scheme 2.3. Further, in addition to the ability of the CRMs to release inhibitors of EGFR, their alkylating activity and their DNA damaging potential suggest the formation of the putative methyldiazonium species. Thus, the combi-targeting and CRM principles mimic

the effects of complex multi-drug combinations involving modern inhibitors of EGFR TK and a classical DNA damaging agent. More importantly, the results presented herein suggest that these effects may be selectively targeted to cells expressing the HER2 or EGFR oncogenes.

2. 5. Acknowledgements

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2.7. Connecting text

In the preceding chapter (Chapter 2), a detailed synthesis of the first ever "cascade release" combi-molecule, RB24 (Structure **6**), was described. This novel class of combi-molecule is a masked form of monomethyltriazene that degrades to release several daughter inhibitors of EGFR TK and at the last step of the cascade, a methyldiazonium species. Independent synthesis of each of the putative degradation products of RB24 [e.g. RB14 (Structure **5**), ZR08 (Structure **8**), RB10 (Structure **3**)] permitted the determination of their individual *in vitro* activity. Since all of them retained significant EGFR TK inhibitory activity, we surmised that RB24 would not be deactivated through degradation. These results stimulated us to examine whether this property would translate into sustained EGFR TK inhibition. Furthermore, we sought to determine whether this sustained EGFR inhibitory activity + DNA damage would translate into irreversible antiproliferative activity. Thus, the next chapter investigates the reversible mechanism of action of RB24 and its effect on downstream signalling cascades. This paper was published in the British Journal of Cancer 91:1066-73 (2004) (see Appendix).

CHAPTER 3

SUSTAINED ANTIPROLIFERATIVE MECHANISMS BY RB24, A TARGETED PRECURSOR OF MULTIPLE INHIBITORS OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AND A DNA ALKYLATING AGENT IN THE A431 EPIDERMAL CARCINOMA OF THE VULVA CELL LINE

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3.1. Abstract

Recent results on clinical trials of epidermal growth factor receptor (EGFR) inhibitors showed a rather moderate potency for the reversible inhibitors, a debility that might be due to their lack of sustained antiproliferative activity in human tumours. With the purpose of enhancing the potency of EGFR-based therapies, we designed a novel strategy termed "Cascade-release targeting" that seeks to develop molecules capable of degrading to multiple tyrosine kinase inhibitors and highly reactive electrophiles, in a stepwise fashion. Here we report on the first prototype of this model, RB24, a masked methyltriazene, that in addition to being an inhibitor on its own, was designed to degrade to RB14, ZR08, RB10 + a DNA alkylating methyldiazonium species. The cascade degradation of RB24 requires the generation of two reactive electrophiles: a) an iminium ion and b) a methyldiazonium ion. Thus, we surmise that these species could alkylate the active site of EGFR, thereby irreversibly blocking its action and that DNA damage could be induced by the methyldiazonium. Using the EGFR-overexpressing human epidermoid carcinoma of the vulva cell line, A431, we demonstrate herein that: a) RB24 and its derived species (e.g. RB14, ZR08), irreversibly inhibit EGFR autophosphorylation, b) RB24 induced significant levels of DNA strand breaks, c) its sustained inhibition of EGFR was associated with blockade of MAPK activation and c-fos gene expression, d) it induced irreversible cell growth inhibition with a 100-fold greater potency than Temodal™, a clinical methyltriazene. The pronounced growth inhibitory potency of RB24 was attributed to its ability to simultaneously damage DNA and to irreversibly block EGFR TK activity.

Keywords: EGFR, triazene, DNA, quinazoline

3.2. Introduction

Overexpression of the epidermal growth factor receptor (EGFR) family and its cognate ligand has been correlated with aggressiveness and poor prognosis in various tumours such as breast, ovarian and prostate cancer (Modjtahedi *et al.*, 1994; Xie *et al.*, 1995; Turner *et al.*, 1996; Meden *et al.*, 1997; Chen *et al.*, 2000). The implication of these receptors in cancer progression has garnered significant attention and agents capable of blocking disordered growth signaling mediated by these proteins are now in a significant number of clinical trials against many cancers. One such agent, Iressa®, a trademark of the AstraZeneca group of Companies exhibits a broad spectrum of anti-tumour activity against many human solid tumour xenografts of various origins including breast, lung, colorectal and head and neck (Ciardiello *et al.*, 2001a; Moulder *et al.*, 2001;Heimberger *et al.*, 2002; Magné *et al.*, 2002; Ranson *et al.*, 2002). However, despite the significant activity of this compound in pre-clinical models, in Phase II clinical trial, it only induced a response rate of approximately 10% in a cohort of patients with non-small cell lung cancer (NSCLC) (Magné *et al.*, 2002; Dancey *et al.*, 2003). Several possible explanations have been put forth to explain the failure to demonstrate a benefit: inadequate dosing, reduced drug delivery to tumour, lack of sustained potency and failure to select patients on the basis of having tumours in which EGFR presents a growth advantage (Dancey *et al.*, 2003).

The lack of sustainability of the antitumour action of reversible inhibitors has stimulated the design of new irreversible inhibitors of EGFR tyrosine kinase (TK). One such compound, PD183805, bearing a 6-acrylamido group designed to alkylate cysteine 773 in the active site of the ATP binding pocket irreversibly blocks EGFR TK and is now in

Phase I development in patients with head and neck, breast and non-small cell lung carcinoma (Ciardiello *et al.*, 2001b). Other approaches to enhance the potency of EGFR inhibitor-based therapy include combinations of inhibitors with several cytotoxic agents including taxol, cytoxan and adriamycin (Ciardiello *et al.*, 1999; Ciardiello *et al.*, 2001a; Magné *et al.*, 2002; Dancey *et al.*, 2003). Within the same line of idea, with the purpose of developing more potent and targeted therapies, we developed a novel strategy termed "Combi-targeting" that seeks to synthesize single molecules capable of both blocking EGFR tyrosine kinase (TK) and inducing cytotoxicity by damaging DNA (Matheson *et al.*, 2001; Brahimi *et al.*, 2002; Matheson *et al.*, 2003b; Matheson *et al.*, 2003c; Qiu *et al.*, 2003a; Qiu *et al.*, 2003b; Rachid *et al.*, 2003). Two such compounds, SMA41 (Matheson *et al.*, 2003a; Matheson *et al.*, 2003b) and BJ2000 (Brahimi *et al.*, 2002), the first models designed to demonstrate the feasibility of this principle showed significant DNA damage ability and irreversible blockade of EGFR autophosphorylation in A431 cells. More importantly, these agents induced a more sustained antiproliferative activity when compared with a reversible inhibitor of EGFR (Matheson *et al.*, 2001; Brahimi *et al.*, 2002; Matheson *et al.*, 2003a). In addition, these molecules, also termed "Combi-molecules," selectively induced antiproliferative activity against EGFR-transfectants in isogenic models (Brahimi *et al.*, 2002; Matheson *et al.*, 2003b).

In order to further augment the potency of the Combi-targeting approach, we recently designed RB24 (an acetoxymethyltriazene) to generate three inhibitors of EGFR and at the final stage of degradation a methyldiazonium species capable of damaging DNA (Banerjee *et al.*, 2003). This masked cluster of molecules was expected to produce more sustained anti-tumour effects with the prospect of inducing activities similar or

superior to that of classical combinations involving a reversible EGFR inhibitor + a cytotoxic drug. Here we study the mechanism of action of RB24, the first prototype of this strategy termed "cascade release", and demonstrated the sustainability of its antitumour activity in A431 cells which overexpress EGFR and its cognate ligand, TGF-α (Lanzi *et al.*, 1997). The ability of this cell line to aggressively proliferate by a TGF-α mediated autocrine induction has made it an ideal model for studying the mechanism of action of EGFR TK inhibitors. In addition, these cells express O6-alkylguanine transferase (O6-AGT), a DNA repair enzyme that by repairing the O6-alkylguanine DNA adduct confers significant resistance to AGT+ cells (Lee *et al.*, 1991; Mitchel *et al.*, 1993; Pegg *et al.*, 1995; Cai *et al.*, 2000; Yingna *et al.*, 2000; Matheson *et al.*, 2003b).

3.3. Materials and Methods

3.3.1. Drug Treatment

RB24, RB14, ZR08 and RB10 were reported elsewhere (Banerjee *et al.*, 2003). Temodal™ was provided by Shering-Plough Inc. (Kenilworth, NJ, USA). In all assays, drug was dissolved in DMSO and subsequently diluted in RPMI-1640 containing 10% fetal bovine serum (FBS) (Wisent Inc. St-Bruno, Canada) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

3.3.2. 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, USA). The A431 cell line was maintained in RPMI-1640 supplemented with 10% FBS and antibiotics as described previously (Matheson *et al.*, 2001). All cells were maintained in an atmosphere of 5% CO_2.

3.3.3. *In vitro* Cytokinetic Growth Inhibition Assay

To study the irreversible effects of our compounds on cell proliferation, 200 cells/well were plated in 96-well plates with serum-containing media. Cells were exposed to each drug + serum for either 2h, 8h, 12h, 24h or 48h. After each time point the drug was removed and cells were washed 2x with PBS and allowed to recover with fresh serum-containing media for a total of 96 hrs. Cell growth was measured using the sulforhodamine B (SRB) assay. Briefly, following drug treatment and recovery, 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 492 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. Autophosphorylation Assay

A431 cells (1×10^6) were preincubated in a six-well plate with 10% serum at 37°C for 48 h and starved 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 15 min at 37°C. Cells were washed with PBS and resuspended in cold lysis buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% Nonidet P-40, 1mM 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 concentrations of protein were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were added to a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Non-specific binding on the membrane was minimized with a blocking buffer containing nonfat dry milk (5%) in PBST. Thereafter, the membranes were 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. Blots were incubated with HRP-goat anti-mouse antibody (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.

To study the effects of RB24 on the activation of extraceullular signals regulated kinases 1,2 (Erk1,2), protein lysates were obtained as described above and Western blot was performed as previously reported (Tari *et al.*, 2000). The membrane was incubated with anti-phosphorylated Erk1,2 antibodies or antibodies specific for Erk1,2 (Cell Signaling, Beverly, MA, USA).

3.3.5. Reverse EGFR Autophosphorylation

This assay was performed as previously described (Fry et al., 1998). A431 cells were grown to confluence in 6-well plates and then incubated in serum-free medium for 24 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 (50 ng/ml) for 15 min and extracts were made as described under the Western blotting procedure 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.

3.3.6. RT-PCR for c-fos Expression

A431 cells were grown to confluence in 6-well plates and then incubated in serum-free medium for 24 h. Cells were exposed to the indicated concentrations of drug prior to stimulation with EGF (50 ng/ml) for 30 min. Total RNA was isolated using the High Pure RNA Isolation Kit of Roche Molecular Bochemicals (Germany), following the manufacturer's instructions. Quantitative analysis of c-fos mRNA and G3PDH mRNA (2 μg of RNA for each sample) was preformed by Titan One Tube RT-PCR Kit (Roche Molecular Bochemicals), following the manufacturer's instructions and using the following primers: 5'ATGATGTTCTCGGGCTTC3' (sense), 5'CTCCTGCCAATGCT CTGC3' (antisense) for c-fos and 5'CCATGGAGAAGGCTGGGG3'(sense), 5'CAAA GTTGTCATGGATGACC3' (antisense) for G3PDH.

3.3.7. Alkaline Comet Assay for Quantitation of DNA Damage

The alkaline comet assay was performed as previously described (Matheson *et al.*, 2001). The cells were exposed to drugs (RB24, RB14 or RB10) 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, 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, 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 mM Tris-base) containing 1mg/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 20 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 330x 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.

3.4. Results

3.4.1. Inhibition of EGFR autophosphorylation

Western blot analysis demonstrated that RB24 blocked EGF-induced EGFR autophosphorylation in A431 cells in a dose-dependent manner with an $IC_{50} \approx 2$ μM without affecting the levels of EGFR (Figure 3.1). RB24 is a degradable molecule capable of generating an extremely reactive iminium ion intermediate that may alkylate the receptor and irreversibly inhibit EGFR activity. To test this hypothesis, we used the reversibility assay previously described (Fry *et al.*, 1998; Smaill *et al.*, 2000) according to which the cells were treated with drug for 90 min and the culture medium repeatedly removed and replaced 3 times after treatment, after which EGFR autophosphorylation was measured. As expected, RB24 at 30 μM completely suppressed EGF-dependent EGFR autophosphorylation in A431 cells immediately after drug exposure (Figure 3.2a). However, at 8 h post-treatment following repeated washouts in drug-free medium, EGFR autophosphorylation activity was completely inhibited in cells treated with RB24, indicating that the latter is capable of inducing irreversible inhibition of EGFR autophosphorylation. Similarly, irreversible inhibition was observed in cells exposed to the daughter molecules, RB14 and ZR08, however with a 20% recovery of the total activity (Figure 3.2b). In contrast, the naked inhibitor, RB10 was shown to have completely reversible inhibition of EGFR autophosphorylation (Figure 3.2a).

3.4.2. Inhibition of EGFR-mediated signaling

Antiproliferative activity induced by RB24 requires the translation of inhibition of EGFR autophosphorylation into inhibition of downstream signaling. To determine

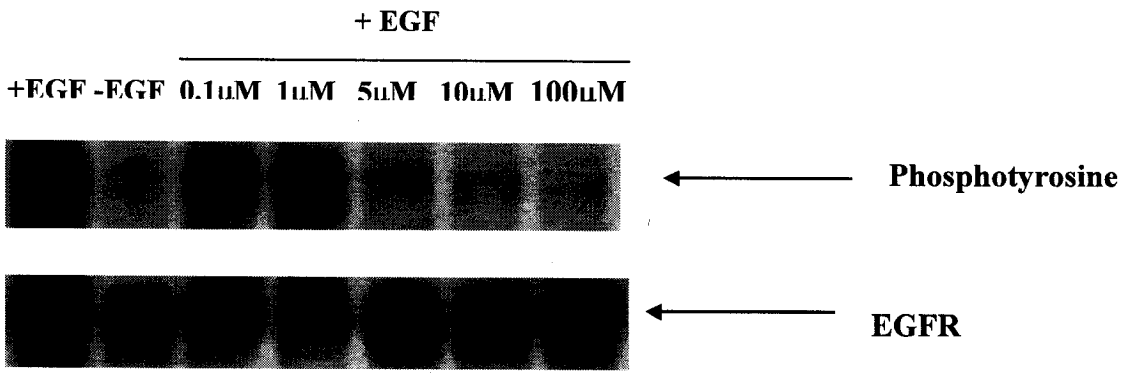


Figure 3.1. Selective inhibition of EGFR autophosphorylation in intact cells by RB24. Serum starved A431 cells were pre-incubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF for 15 min. Equal amount of cell lysates was analyzed by western blotting using anti-phosphotyrosine antibodies. Membranes were stripped of anti-phosphotyrosine and reprobed with anti-EGFR antibodies as a loading control. Band intensities were measured using the SynGene GeneTools software package.

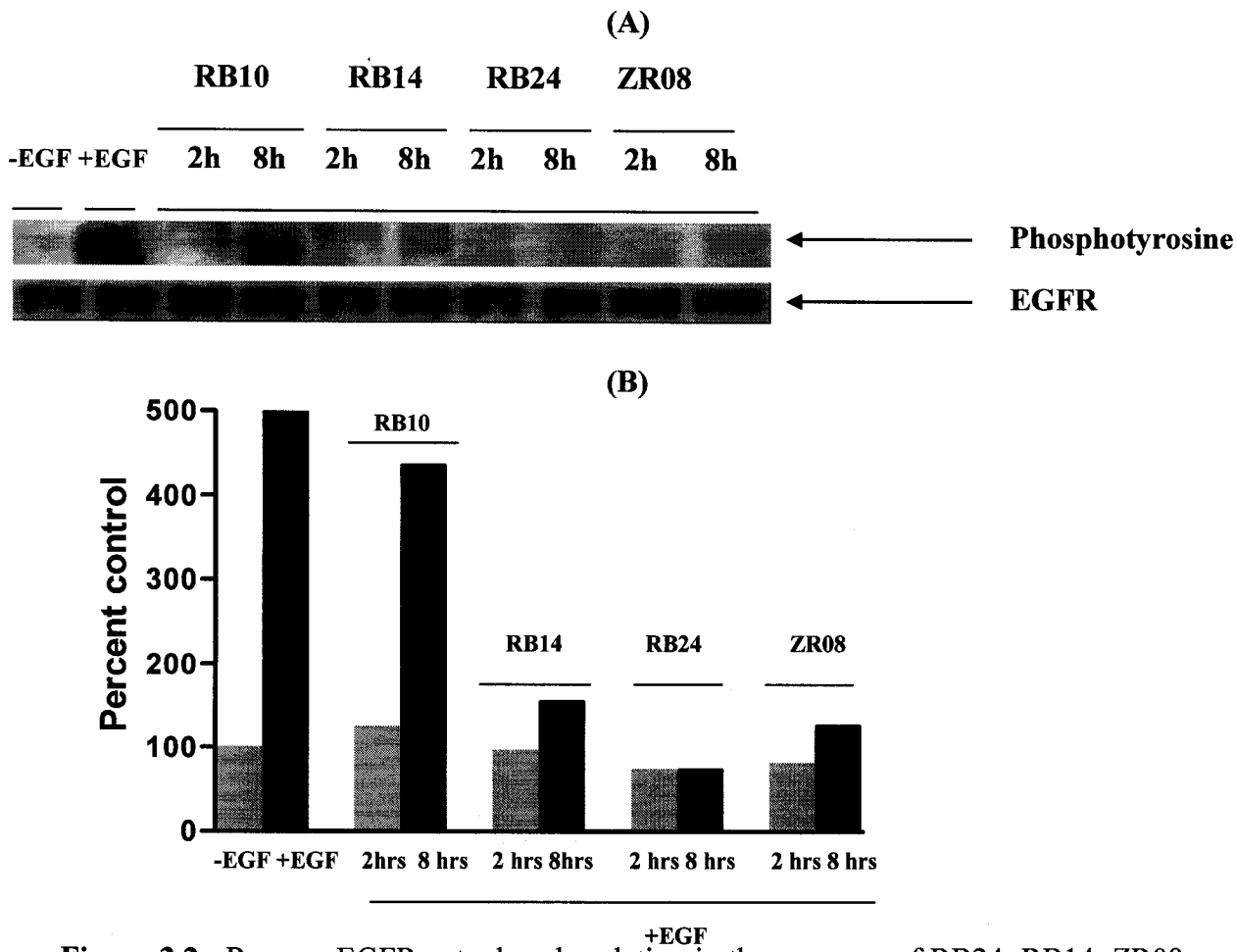


Figure 3.2. Reverse EGFR autophosphorylation in the presence of RB24, RB14, ZR08 and RB10 in A431 cells. (A) Duplicate sets of cells were treated with 30 μM of designated compound to be tested as a reversible EGFR inhibitor for 90 min. One set of cells were then stimulated with EGF for 15 min, and extracts were made as described under the Western blotting procedure above. The other set of cells were washed free of the compound with serum-free media, incubated for 2 h, and further washed twice and incubated for 4 h. This set of cells was then stimulated with EGF, and extracts were made similar to the first set. (B) Comparison between the inhibition of autophosphorylation activity induced by RB24, RB14, ZR08 and RB10. The film was scanned and band intensities were quantified using Syngene GeneTools software. Values are percentage of control of phosphotyrosine/EGFR.

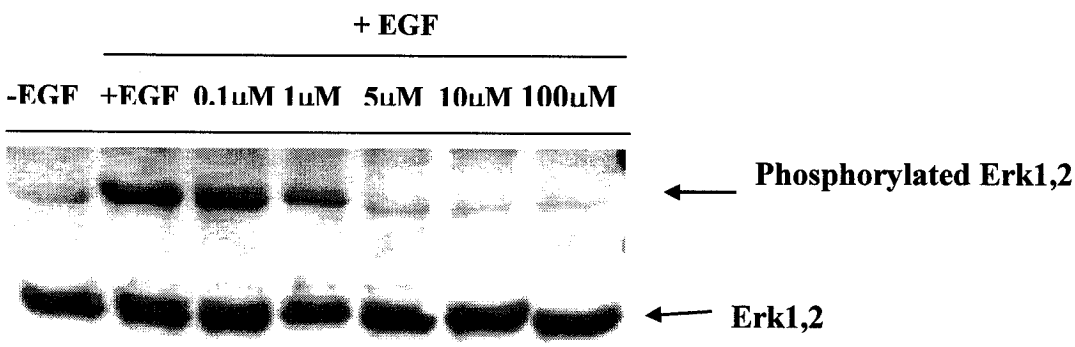


Figure 3.3. Effect of RB24 on Erk1,2 activation in A431 cells. Serum-starved cells were preincubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF for 15 min. Protein lysates were obtained and Western blot was performed as described by Tari and Lopez-Berestein (2000).

whether blockade of EGFR autophosphorylation translates into inhibition of downstream signaling, we analyzed the effect of the parent compound, RB24, on EGF-induced phosphorylation of Erk1,2 and c-fos expression in A431 cells. The results showed that RB24 induced complete inhibition of Erk1,2 phosphorylation at concentrations as low as 5 μM without affecting the levels of Erk1,2 (Figure 3.3). Similarly, RT-PCR analysis showed that RB24 induced nearly 100% inhibition of EGF-mediated c-fos gene expression at low concentrations (1μM) (Figure 3.4), indicating that inhibition of EGFR phosphorylation by RB24 is accompanied by a significant blockade of EGFR-dependent downstream signaling.

3.4.3. Quantitation of DNA damage

Using the alkaline comet assay, we demonstrated that like TEM (Matheson *et al.*, 2001), RB24 and RB14 induced a dose-dependent DNA damage in A431 cells after a 30 min drug exposure (Figure 3.5). Interestingly, RB24 and RB14 induced identical levels of DNA damage, which is in agreement with the fact that the former is a prodrug of the latter. As expected, the reversible inhibitor RB10, did not demonstrate any DNA damaging activity.

3.4.4. Irreversible growth inhibitory activity

SRB assays demonstrated that RB24 and RB14 retained significant antiproliferative activity after a short 2 h exposure and a 4 day recovery (Figure 3.6a,b). In contrast, the free inhibitor, RB10, lost at least 95% of its activity under the same conditions (Figure 3.6d). The monoalkyltriazene, ZR08, showed partially reversible activity with an 85% retention of activity following 2 h drug exposure (Figure 3.6c). In contrast to the

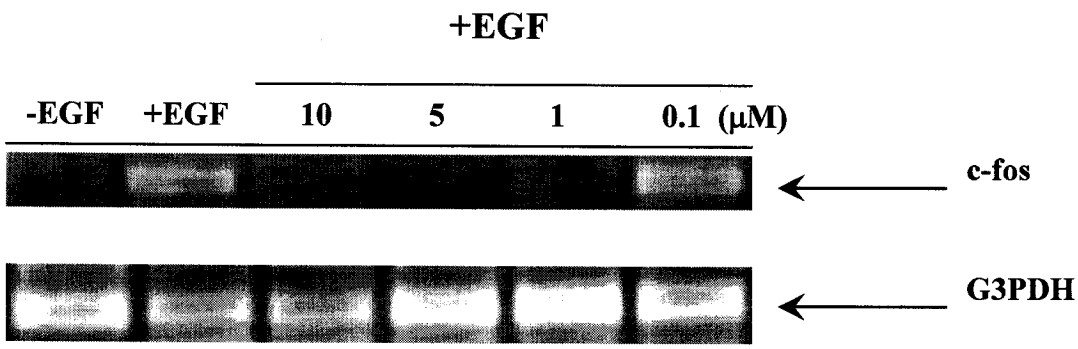


Figure 3.4. Effect of RB24 on c-fos gene expression in A431 cells. Serum-starved cells were preincubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF for 30 min. Quantitative analysis of c-fos and GAPDH was preformed by RT-PCR as described in "Materials and Methods".

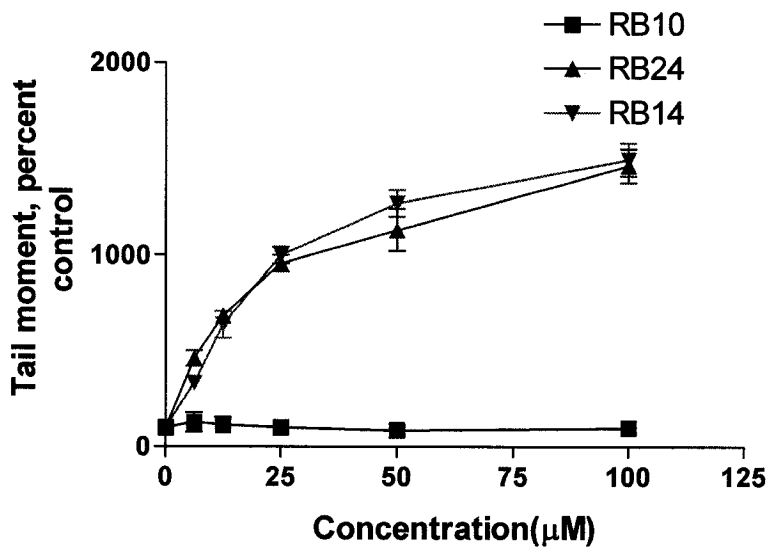


Figure 3.5. Quantitation of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in A431 cells exposed to RB24, RB10 and RB14 for 30 min. Each point represents at least two independent experiments.

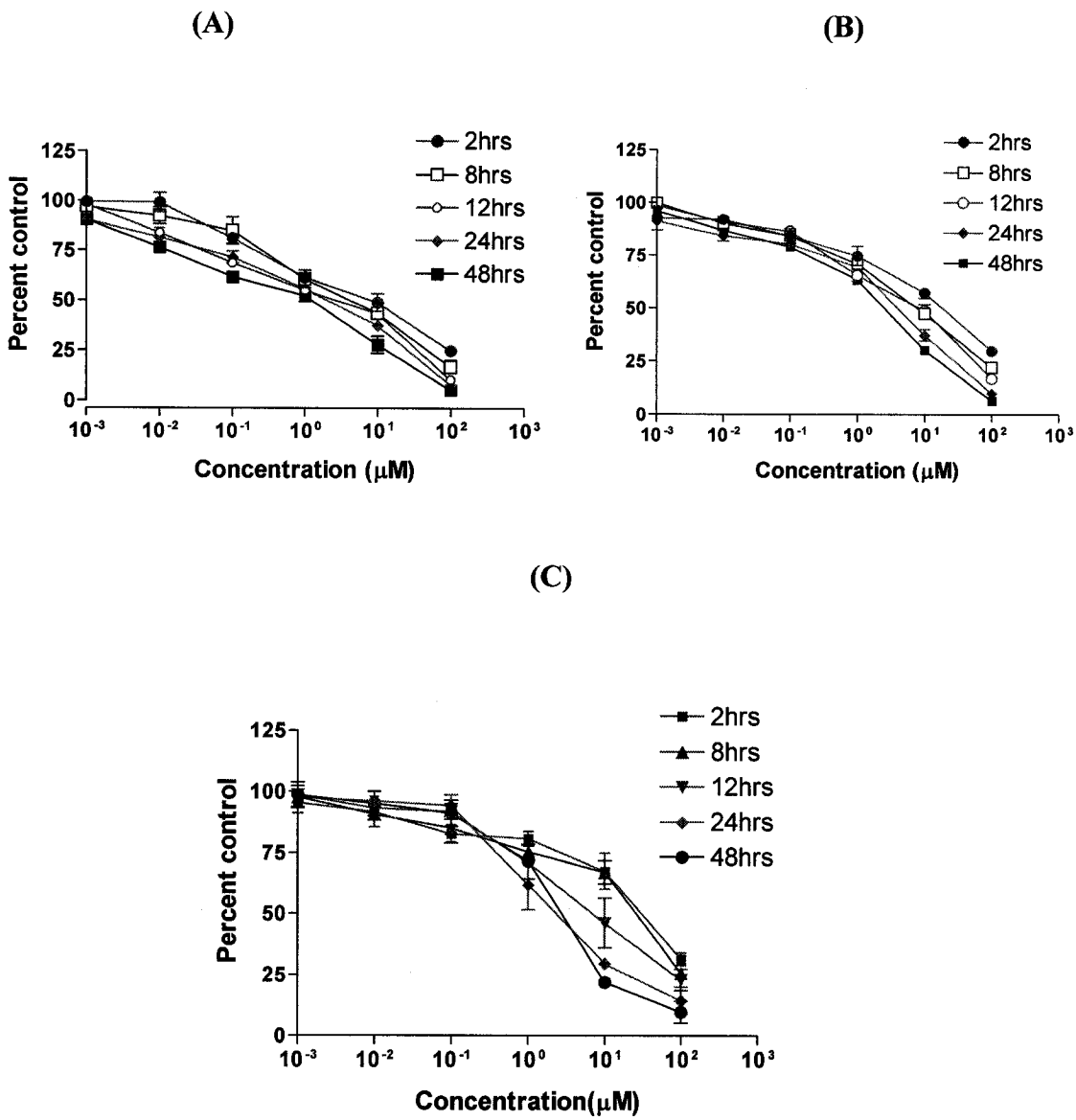
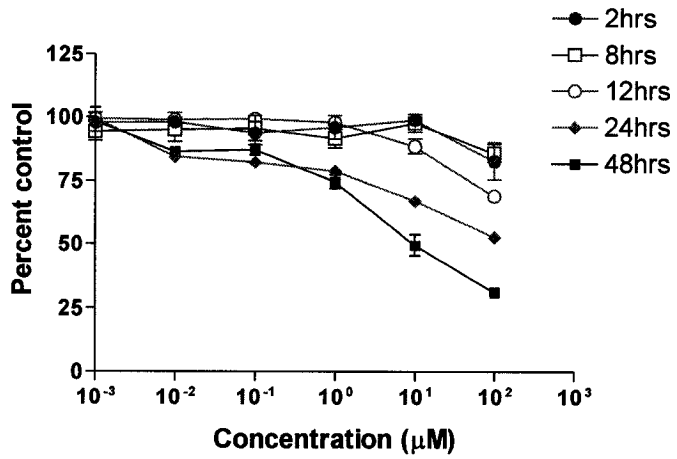


Figure 3.6. Irreversible growth inhibition for RB24, RB14, ZR08, RB10 and TEM in A431 cells. Cells were exposed to A) RB24, B) RB14 or C) ZR08 for 2, 8, 12, 24 or 48 h following recovery for a total of 96 h. Cell growth was measured using SRB assay. Each point represents at least two independent experiments run in triplicate.

(D)



(E)

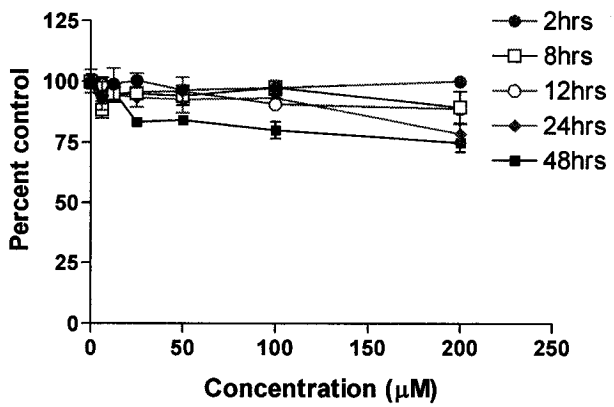


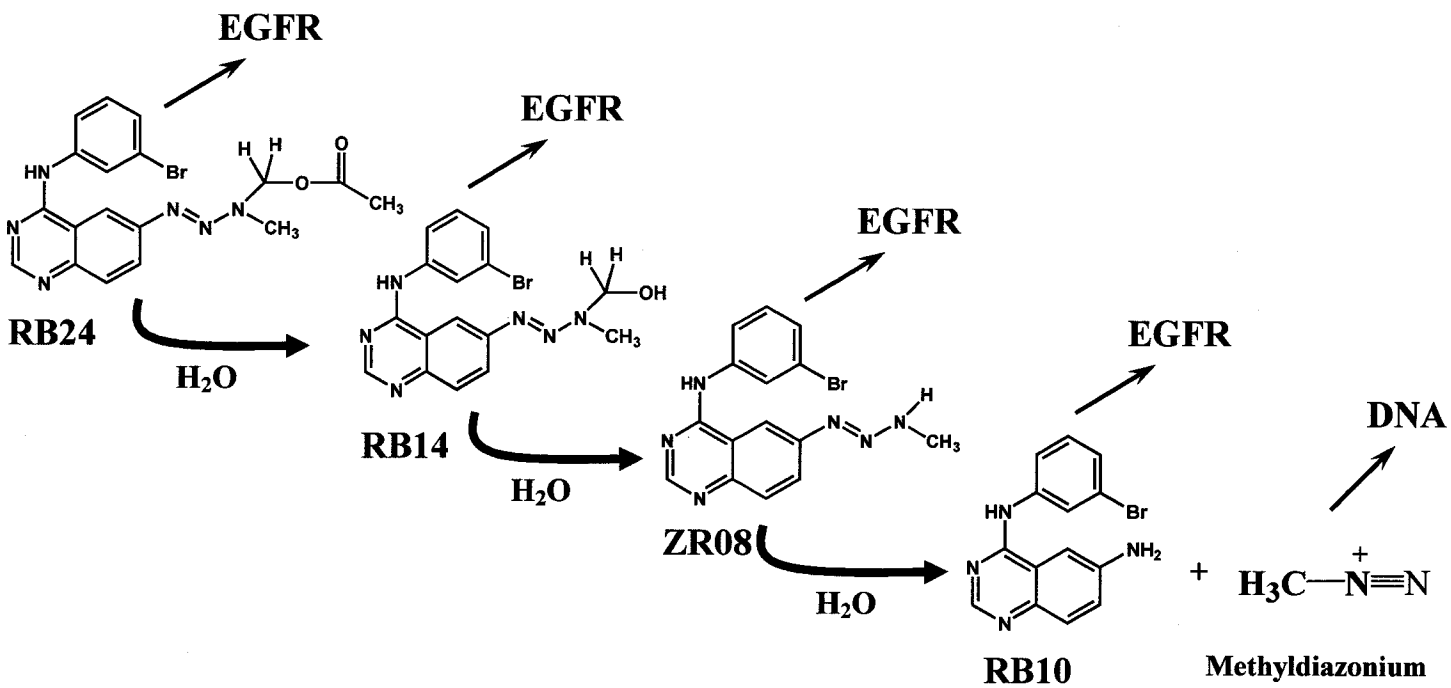
Figure 3.6. Irreversible growth inhibition for RB24, RB14, ZR08, RB10 and TEM in A431 cells. Cells were exposed to D) RB10 or E) TEM for 2, 8, 12, 24 or 48 h following recovery for a total of 96 h. Cell growth was measured using SRB assay. Each point represents at least two independent experiments run in triplicate.

antiproliferative effects of RB24, increasing exposure time was associated with significant increase in potency for all other molecules of the degradation cascade (RB14, ZR08, RB10). Despite being a potent alkylating agent, the clinical triazene, Temodal™, demonstrated no significant activity at any of the exposure times in the A431 cells (Figure 3.6e). In summary, the strength of the retention of potency was in the following order: RB24>RB14>ZR08>>RB10.

3.5. Discussion

3.5.1. Mechanisms of EGFR TK inhibition

The design of our combi-molecule, RB24, was based on the premise that acetoxymethyltriazenes are known to be hydrolyzed to a hydroxymethyltriazene intermediate that rapidly degrades into the corresponding monoalkyltriazene (Hemens *et al.*, 1984; Cameron *et al.*, 1985; Hemens *et al.*, 1986). The latter further heterolyzes to an aromatic amine + a DNA damaging species. In a recent publication (Banerjee *et al.*, 2003), we demonstrated that indeed RB24 was capable of generating the ultimate amino compound RB10, in a symmetrically inversed relationship. As depicted in Scheme 3.1, decomposition through the intermediates, RB14 and ZR08, is the sole mechanism by which RB24 can be converted to RB10. Thus, the significant EGFR inhibitory potency of the putative intermediates RB14, ZR08 and RB10, indicates that RB24 would maintain its TK inhibitory potency throughout the multistep degradation cascade. More importantly, RB24 and its two progenitors (RB14, ZR08) induced irreversible inhibition of EGFR TK when tested alone, a protracted inhibition that may be rationalized in light of recent data on the mechanism of irreversible acrylamide-based inhibitors. It is now known that 4-anilinoquinazolines bearing a 6-acrylamido group react with the cysteine



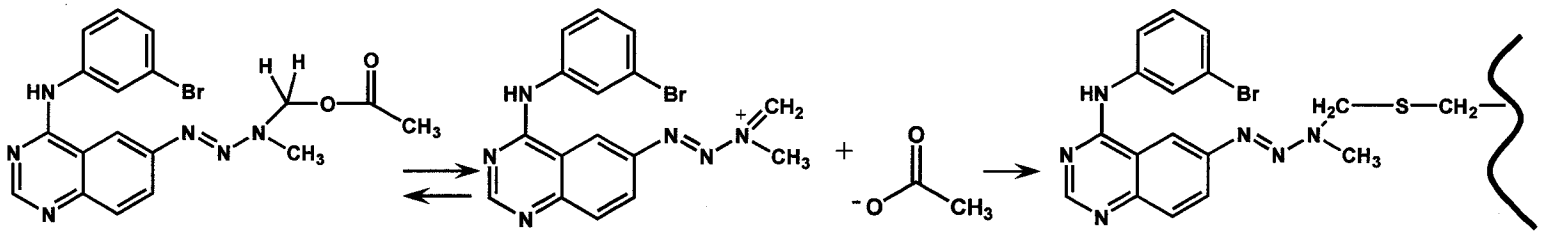
Scheme 3.1. Degradation cascade for RB24 under hydrolytic conditions.

773 of EGFR, thereby irreversibly blocking the receptor (Fry *et al.*, 1998; Smaill *et al.*, 1999; Smaill *et al.*, 2000). Based upon the mechanism of decomposition of RB24 (Banerjee *et al.*, 2003), we did not expect a direct reaction between the cysteine thiol group and the acetoxy moiety. Perhaps, if the iminium ion is formed in the active site of EGFR, it may react with the cysteine as depicted in Scheme 3.2. Addition of thiols group to the iminium ions of acetoxymethyltriazene have already been reported by Iley *et al.* (1991) who developed this type of triazenes as lyase inhibitors. Moreover, the ultimate triazene metabolite of RB24, ZR08, may also directly alkylate the cysteine residue. Quinazolinotriazenes of the same class, BJ2000 and SMA41, have already been demonstrated to induce irreversible inhibition (Brahimi *et al.*, 2002; Matheson *et al.*, 2003a; Matheson *et al.*, 2003d).

Although it was not the focus of this study to identify the site of alkylation of the receptor, the irreversible nature of TK inhibition by RB24, indicates that it may have inflicted covalent damage at a site of EGFR that is critical for its TK activity. In corroboration, RB10 that does not possess a reactive triazene tail induced reversible EGFR TK inhibitory activity.

3.5.2. DNA damage

The iminium ion is a stable cation that may not be reactive enough to account for the strong DNA damaging potential of RB24. Despite the significant body of results that confirm its formation in the mechanism of hydrolysis of acetoxymethyltriazenes (Hemens *et al.*, 1984; Hemens *et al.*, 1986; Iley, 1987; Vaughan *et al.*, 1988; Merrin *et al.*, 1992), its implication in the DNA damaging properties of the latter class of



Scheme 3.2

compounds is yet to be demonstrated. In contrast, it is now common knowledge that the methyldiazonium is capable of inducing significantly high levels of DNA alkylation particularly at N7 and O6 positions of guanine, thereby inducing DNA damage and lethal mutations in tumour cells (Bodell *et al.*, 1985; Tisdale, 1987; Baer *et al.*, 1993). The promutagenic O6-alkylguanine adduct is considered to be the primary cytotoxic lesion induced by triazenes. Cells like A431 that express high levels of AGT, a DNA repair enzyme that repairs the O6-alkylguanine adduct, are known to be resistant to alkyltriazenes. In corroboration, the A431 cells used in the study were markedly insensitive to Temodal™, a cyclic triazene known to release the methyldiazonium species upon hydrolysis (Cameron *et al.*, 1985; Gibson *et al.*, 1986; Baig *et al.*, 1987).

3.5.3. Irreversible growth inhibition

The ability of RB24 to degrade into several species with significant EGFR inhibitory activities and to damage DNA, may perhaps be responsible for its sustained potency in A431 cells. More importantly, while its antiproliferative effect was maintained even 4 days after a 2-h drug exposure, under the same conditions, ZR08 induced a partially reversible growth inhibitory activity. We have already reported similar results in A431 cells for analogous quinazolinotriazenes (e.g SMA41 and BJ2000). Thus, the marked irreversibility of the action of RB24 may be partially imputed to additive antiproliferative contribution of species generated during the degradation steps that precede the formation of ZR08. This antiproliferative contribution may result from their ability to block EGFR-mediated growth signaling, since as previously mentioned, the transient iminium ion species generated during these degradation steps may not damage DNA. The partially reversible growth inhibitory potency of ZR08 (third step of the

cascade) may perhaps be due to the dependence of its antiproliferative activity on the ultimately released reversible inhibitor RB10, the concentrations of which might have been drastically depleted by multiple washouts. This indicates that the ultimate antiproliferative effect of ZR08 is mediated by a combination of DNA damage inflicted by the methyldiazonium species and EGFR-TK inhibitory activity induced by RB10.

A synergistic interaction between the strong EGFR TK inhibitory potency and the marked DNA damaging potential of RB24 may also partly account for its remarkable potency against the A431 cells. Indeed, blockade of EGFR in these cells was accompanied by potent inactivation of Erk1,2 kinase and inhibition of c-fos gene expression. We now know, based upon previous experiments, that blockade of EGFR-mediated signaling may not affect the levels or function of the AGT enzymes. However, Yacoub *et al.* (2003) recently demonstrated that EGF up-regulates the DNA repair genes XRCC1 and ERCC1 in prostate cell lines through Erk1,2 signaling. Thus, blockade of EGFR-mediated signaling may downregulate these DNA repair enzymes that are involved in the repair of DNA strand breaks, thereby exacerbating the cytotoxic effect of the latter lesions.

In conclusion, we have demonstrated that a molecule engineered to possess complex EGFR inhibitory properties and capable of further releasing DNA damaging species could induce significantly more sustained antiproliferative activity than a reversible inhibitor of the same class (e.g. RB10). Moreover, its multiple properties have conferred antiproliferative activity in a cell line in which a classical methylating agent of the same class does not show any detectable activity. Thus this model may well represent a new

strategy to ameliorate the chemotherapy of EGF-dependent refractory tumours. Further studies are ongoing to demonstrate the potency of this novel drug *in vivo*.

3.6. Acknowledgements

We thank the Canadian Institutes of Health Research for financial support.

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3.8. Connecting text

As described in Chapter 3, we have now demonstrated the irreversible mechanism of EGFR TK inhibition of the cascade release combi-molecule, RB24. The latter agent was also capable of blocking EGFR-mediated activation of: 1) the MAPKs, Erk1,2 and 2) transcription of the early-response gene, c-fos. Furthermore, the potent blockade of EGFR TK activation and downstream signaling cascades translated into sustained antiproliferative activity for RB24 in an EGFR-overexpressing cell line. Having demonstrated the pronounced growth inhibitory activity of RB24, we wished to determine whether the EGFR recognition moiety of the combi-molecule could selectively target the entire molecule and assist in the selective delivery of DNA damage to EGFR or Her2-expressing cells. Therefore, in Chapter 4 we will examine the target selectivity of RB24 in an isogenic panel of breast cell lines transfected with the EGFR or Her2 gene. It should be noted that breast cancers are characterized by overexpression of the EGFR or Her2 gene and this is a negative prognostic factor.

CHAPTER 4

THE COMBI-TARGETING CONCEPT: SELECTIVE TARGETING OF THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)- AND HER2-EXPRESSING BREAST CANCER CELLS BY THE COMPLEX COMBI-MOLECULE RB24 (NSC 741279)

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4.1. Abstract

Overexpression of the EGFR and/or Her2 oncogene in breast cancer is associated with resistance to drug induced apoptosis and aggressive proliferation. Within the context of a new strategy termed "combi-targeting", we designed a novel molecule (RB24, NSC 74129) to block EGFR and Her2 and to further degrade upon hydrolysis to another inhibitor termed RB10 + a cytotoxic methyldiazonium species. Here we show that RB24, could block the growth of established cell lines (MDA-MB-468, MDA-MB-453, MDA-MB-435) and selectively induce apoptosis in the EGFR and ErbB2-transfectants in an MDA-MB-435 isogenic panel of cell lines. Experiments designed to elucidate the targeting mechanism of RB24 revealed that: (a) it was capable of releasing 2-3-fold higher levels of the free inhibitor, RB10, in the transfectants than in their wild-type counterpart, (b) the released RB10 was concentrated in the perinuclear region of the cells, and (c) the selective concentration of RB10 in ErbB transfectants was concomitant with elevated levels of DNA damage in the latter cells. However, selective induction of DNA damage by RB24 was abolished by co-incubation of cells with exogenous RB10. Conversely, co-incubation with a non-fluorescent inhibitor JDA41 diffused the fluorescence from the perinuclear region, indicating that the entire combi-molecule may bind to its cognate site prior to degradation. These targeting events are accompanied by a dose-dependent depletion of EGFR and ErbB2 phosphorylation, down-regulation of the anti-apoptotic protein Bad and concomitant activation of JNK: three sets of cooperative events that account for selectively high levels of apoptosis induced in the transfectants. The results *in toto* suggest the following targeting model: the combi-molecule penetrate the cells and blocks the tyrosine kinase activity of EGFR/ErbB2 and significantly binds to perinuclear located proteins from which they degrade to generate RB10 and the DNA

damaging methyldiazonium that may diffuse away from its cognate site towards the nucleus.

Key words: EGFR, Her2, perinuclear localization, DNA damaging agent, JNK

4.2. Introduction

Members of the Epidermal Growth Factor (EGF) family of receptors, including EGFR, Her2 (ErbB2) and EGFRvIII (truncated EGFR) are overexpressed in 20-40% of breast cancers (Tang *et al.*, 2000; Koenders *et al.*, 1991) The Her2 receptor, the closest EGFR homologue, is overexpressed in 20-25% of overall breast cancers, 60-70% of ductal carcinoma *in situ* (DCIS) and is often associated with highly aggressive tumour phenotypes and poor disease-free survival (Slamon *et al.*, 1989; Arteaga, 2001). The average survival of breast cancer patients with Her2-positive tumors is 2-3 years when compared with 6-7 years of Her2-negative tumors (Slamon *et al.*, 1987; Borg *et al.*, 1990). More importantly, this dysfunction is also associated with resistance to endocrine therapy and chemotherapy (Slamon *et al.*, 1987). The significant implication of the ErbB family members in tumour progression has made them important targets for breast cancer therapy. Indeed, blockade of EGFR or Her2 has proven highly effective in breast xenograft models and in the clinic (Ciardello *et al.*, 1999; Moulder *et al.*, 2001). Although gefitinib that targets the EGFR tyrosine kinase (TK) has shown moderate activity in breast cancer models, Herceptin, a monoclonal antibody directed towards Her2, has demonstrated significant activity in advanced breast carcinomas (Albain *et al.*, 2001; von Minckwitz *et al.*, 2005; Tokunaga *et al.*, 2006). Its effectiveness is such that it is now being evaluated for adjuvant breast cancer therapy. However, despite its significant potency against Her2-positive breast tumours, its efficacy in patients who express EGFRvIII with loss of PTEN function remain to be demonstrated. As a corollary to the great potency of Herceptin, strategies are now being developed to enhance its potency in breast tumours using combinations with cytotoxic DNA damaging drugs (Ciardello *et al.* 2006; Gasparini *et al.*, 2006). Here we describe a

similar strategy based on the use of a small molecule approach to target Her2 TK-mediated signaling pathways while damaging DNA. This strategy, termed "combi-targeting", seeks to design a single molecule targeted to the TK function of Her2 and EGFR and programmed to be a latent DNA damaging agent (Matheson *et al.*, 2001; Brahimi *et al.*, 2002; Matheson *et al.*, 2003a; Banerjee *et al.*, 2003; Matheson *et al.*, 2003b; Rachid *et al.*, 2003; Qiu *et al.*, 2003; Matheson *et al.*, 2004a; Matheson *et al.*, 2004b; Banerjee *et al.*, 2004; Brahimi *et al.*, 2004; Qiu *et al.*, 2004).

Activation of EGFR and Her2 leads to the recruitment of multiple adaptor proteins such as Grb2, followed by activation of the Ras-Raf-MAPK pathway that culminates into the expression of genes that regulate cellular growth and proliferation (McCubrey *et al.*, 2006). More importantly, activation of the latter receptors prevent apoptosis through induction of phosphorylation of the Phosphatidylinositol 3-kinase (PI3K) and its downstream target Akt, a sequence of events that leads to the inactivation of the pro-apoptotic protein, Bad, a member of the Bcl-2 family of proteins (McCubrey *et al.*, 2006; Stauffer *et al.*, 2005). On the other hand, cell exposure to cytotoxic agents is often associated with induction of apoptotic signaling through stress activated protein kinases such as c-jun N-terminal kinase (JNK) that is known to interact with members of the Bcl-2 family (Ohtsuka *et al.*, 2003; Huang *et al.*, 2006). Based upon these mechanisms we surmise that molecules capable of inducing DNA damage while blocking receptor-mediated signaling should induce significant levels of apoptosis and cell kill in breast cancer cells expressing these receptors. More importantly, the molecules being designed to penetrate the cell intact can bind to the ATP binding site of the TK domain prior to releasing their DNA damaging fragments, thereby targeting them to cells that

overexpress these receptors. Here we study the mechanism of action of one such molecule, RB24, which possesses the following properties: a) it has a half-life comparable with that of a clinical methylating agent, Temodal™ (TEM) ($t_{1/2}$=45 min in serum), b) it blocks EGFR TK activity both in enzyme and whole cell phosphorylation assays, and c) it has been shown to induce significant levels of DNA damage in tumour cells. This study sought to demonstrate its ability to selectively induce antiproliferative activity in breast cancer cells that express the target receptor and analyzes the mechanisms by which it selectively targets EGFR- or Her2-transfected cells.

4.3. Materials and Methods

4.3.1. Drug Treatment

RB24, RB10 and JDA41 were synthesized in our laboratory according to known procedures (Banerjee *et al.*, 2003). Temozolomide was provided by Shering-Plough Inc. (Kenilworth, NJ, USA). Gefitinib (ZD1839) was provided by AstraZeneca. In all assays, drug was dissolved in DMSO and subsequently diluted in DMEM containing 10% fetal bovine serum (FBS) (Wisent Inc. St-Bruno, Canada) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

4.3.2. Cell Culture

The cell lines used in this study, the human breast carcinoma MDA-MB-435, MDA-MB-435/EGFR (MDA-MB-435 cells stably transfected with the EGFR gene) and MDAMB435/ErbB2 (MDA-MB-435 cells stably transfected with the HER2 gene) were generous gifts from Dr. Moulay Aloui-Jamali of the Montreal Jewish General Hospital.

The human breast cancer cell lines, MDA-MB-468 and MDA-MB-453, were obtained from the American Type Culture Collection (Manassas, VA). All MDA-MB-435 cells (transfected and non-transfected) were maintained in DMEM supplemented with 10% FBS. MDA-MB-468 and MDA-MB-453 cells were maintained in RPMI-1640 supplemented with 10% FBS and antibiotics as described previously (Matheson *et al.*, 2001). All cells were maintained in an atmosphere of 5% CO_2.

4.3.3. Growth Inhibition Assay

For non-stimulated cell growth inhibition, approximately 10 x 10^3 cells/well were plated in 96-well plates. After a 24 h incubation at 37^0C in a humidified environment of 5% CO_2, cell monolayers were exposed to different concentrations of each drug continuously for 6 days and all growth inhibitory activities were evaluated using the sulforhodamine B (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 4^0C, washed four 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.

4.3.4. Autophosphorylation Assay

4.3.4.1. EGFR or ErbB2 activation

MDA-MB-435/EGFR or MDA-MB-435/ErbB2 cells (1x10^6) were plated in six-

well plates with 10% serum at 37°C for 24 h and starved overnight for 24 h, after which they were exposed to a range of drug for 2 h and subsequently treated for 15 min with 50 ng/ml EGF or 50ng/ml of HRGα, respectively. Cells were washed with PBS and resuspended in cold lysis buffer [50 mM Tris-HCL PH 7.5; 150 mM NaCl; 1% Nonidet P-40, 1mM EDTA; 5 mM NaF; 1mM Na3VO4; 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 concentrations of protein were determined using the Bio-Rad protein assay Kit (Bio-Rad laboratories, Hercules, CA). Equal amounts of protein were added to an 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Non-specific binding on the membranes were minimized with a blocking buffer containing nonfat dry milk (5%) in PBST. Thereafter, the membranes were incubated with primary anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) for the detection of phosphotyrosine. Membranes were stripped and re-probed with anti-EGFR (Neomarkers, Fremont, CA) or anti-ErbB2 (Upstate Biotechnology, Lake Placid, NY) for determination of corresponding receptor levels. Blots were incubated with HRP-goat anti-mouse antibody (1:1000 dilution; Cell signaling research, Beverly, MA) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Matheson *et al.*, 2001).

4.3.4.2. Bad activation

Inhibition of Bad activation was determined by exposing serum-starved MDA-

MB-435/EGFR or MDA-MB-435/ErbB2 cells for 2 h with the indicated concentrations of RB24 or TEM prior to stimulation with EGF (50ng/ml) or HRGα (50ng/ml), respectively. Equal amounts of cell lysates were analyzed by Western blot using anti-phosphoBad (Ser136) antibodies (Cell Signaling Tech., Danvers, MA). Membranes were stripped of antibody and reprobed with anti-Bad antibodies (Cell Signaling Tech., Danvers, MA).

4.3.4.3. JNK activation

Activation of JNK was determined by exposing MDA-MB-435/ErbB2 cells for 4 h with the indicated concentrations of RB24 or TEM. Equal amounts of cell lysates were analyzed by Western blot using anti-phosphoJNK (Thr183/Tyr185) and anti-JNK antibodies (Cell Signaling Tech., Danvers, MA).

4.3.5. UV Flow Cytometric Analysis of Intracellular Fluorescence

MDA-MB-435, MDA-MB-435/EGFR and MDA-MB-435/ErbB2 cells were seeded in DMEM with 10% FBS for 24 h and grown in six-well plates (1×10^6/well). Cells were then treated with varying concentrations of RB24 and 10% FBS at 37°C for 2 h. Thereafter, cells were harvested with trypsin-EDTA, subsequently collected by centrifugation and resuspended in PBS. Fluorescence levels were measured using the BD LSR flow cytometer (Becton Dickinson, Oakville, ON, Canada).

4.3.6. Fluorescence Microscopy Imaging for Intracellular Release of the Amine

MDA-MB-435, MDA-MB-435/EGFR and MDA-MB-435/ErbB2 cells were seeded in

DMEM with 10% FBS for 24 h and grown in six-well plates (1×10^6/well). Cells were then treated with varying concentrations of RB24 alone or in combination with the non-fluorescent EGFR TK inhibitor, JDA41 (40μM) and 10% FBS at 37°C for 2 h and media was replaced for drug-free media in each well. Thereafter, plates were analyzed using a Zeiss LSM 510 confocal microscope and cells were excited at 405nm and emission was at 250nm.

4.3.7. Alkaline Comet Assay for Quantitation of DNA Damage

The alkaline comet assay was performed as previously described (Matheson *et al.*, 2001). The cells were exposed to drugs (RB24, RB10, TEM or RB24+RB10) for 2 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 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, 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 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 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 330x 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 were analyzed for each sample, using ALKOMET version 3.1 image analysis software. Statistical significance was determined using the one-tailed Student t-test.

4.3.8. Annexin V Binding

Cells were grown in 6-well plates until confluence and then incubated with the compounds for 24 h. The cells were then harvested, washed twice with PBS, and centrifuged. Cells (10^5) were treated with annexin V-FITC and propidium iodide (PI) using the apoptosis Detection Kit (BD Bioscience Pharmingen, San Jose, CA) and the supplier's protocol. Annexin V-FITC and PI binding were analyzed by flow cytometry. Data were collected using logarithmic amplification of both the FL1 (FITC) and FL2 (PI) channels. Quadrant analysis of co-ordinate dot plots was performed with CellQuest software. Unstained cells were used to adjust the photomultiplier voltage and for compensation setting adjustment in order to eliminate spectral overlap between the FL1 and FL2 signals. Statistical significance was determined using the Student t-test.

4.4. Results

4.4.1. Antiproliferative activity of RB24, RB10, gefinitib and TEM

The combi-molecule, RB24, is designed to hydrolyze into various derivatives that inhibit EGFR TK activation, the most stable metabolite being RB10. As previously

demonstrated, at the last stage of the decomposition pathway, concomitantly with the release of RB10, a methyldiazonium is formed that damages DNA (Banerjee *et al.*, 2003). In a similar fashion, TEM is also hydrolyzed in a stepwise manner to release the latter species that methylates DNA. However, its transient metabolites, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) and hydroxymetabolite, do not have any biological target and we have already demonstrated that TEM is deprived of EGFR inhibitory activity (Matheson *et al.*, 2001). Therefore, TEM represents an appropriate comparison for our combi-molecule as it can only damage DNA but not block EGFR activation. On the other hand, gefitinib, a clinical EGFR TK inhibitor that does not have any DNA damaging potential was used as an appropriate mechanistic control for this study. Thus, we first examined the antiproliferative activities of all these compounds, including the expected degradation product for RB24, RB10, in a panel of breast cancer cells and focused the mechanistic studies in a subset of isogenic breast cancer cells, MDA-MB-435, and its ErbB transfectants, MDA-MB-435/EGFR, MDA-MB-435/ErbB2. The results showed that RB24 is selectively more potent in breast cancer cells overexpressing EGFR or Her2, with IC_{50} values ranging from 0.8μM-2.5μM and 7μM for the wild-type (Table 4.1). Evidence of this selectivity was strengthened by the response of the isogenic cell line panel to the various drugs. Indeed, RB10 and gefitinib selectively targeted the EGFR and Her2 transfectants with IC_{50} values ranging from 9-21μM (Table 4.1). Interestingly, RB24 was 8-13 fold more potent against the transfectants than its EGFR inhibitory counterparts RB10 and gefitinib. Inversely, TEM selectivity targeted the non-transfected wild-type breast cancer cells, MDA-MB-435 (IC_{50}=18μM) and was found to be inactive in the ErbB

Table 4.1. Antiproliferative effects of RB24, RB10, gefitinib and TEM in the isogenic breast carcinoma panel, MDA-MB-435, MDA-MB-435/EGFR and MDA-MB-435/ErbB2.

Compounds	MDA-MB-435 (IC_{50}, μM)*	MDA-MB-435/EGFR (IC_{50}, μM)*	Fold selectivity for EGFR	MDA-MB-435/ErbB2 (IC_{50}, μM)*	Fold-selectivity for ErbB2
RB24	7 ± 2.4	1 ± 0.4	7	0.8 ± 0.2	8.8
RB10	41 ± 2.1	9 ± 1.4	4.5	14 + 1.3	3
TEM	18 ± 1.3	>50	-	> 100	-
Gefitinib	48 ± 1.9	11 ± 2.1	4.4	21 ± 1.8	2.4

** Values are means of IC_{50} values and SEMs from three experiments.*

Table 4.2. Antiproliferative effects of RB24, RB10, gefitinib and TEM in ErbB-negative cell line, MDA-MB-435, and the ErbB-expressing cell lines, MDA-MB-468 and MDA-MB-453.

Compounds	MDA-MB-435 (IC_{50}, μM)*	MDA-MB-468 (IC_{50}, μM)*	Fold selectivity	MDA-MB-453 (IC_{50}, μM)*	Fold-selectivity
RB24	7 ± 2.4	2.5 ± 0.2	2.8	1.3 ± 0.5	4.1
RB10	41 ± 2.1	22.2 ± 1.6	1.8	18.3 ± 1.1	2.2
TEM	18 ± 1.3	>100	-	>100	-
Gefitinib	48 ± 1.9	18.4 ± 1.8	2.6	ND	-

** Values are means of IC_{50} values and SEMs from three experiments.*

transfectants (IC_{50}>50μM). It was more than 50-fold less potent than RB24 (Table 4.1).

4.4.2. Mechanism of target selectivity of the combi-molecule

The combi-molecules being designed to target both EGFR and DNA, various factors such as DNA repair enzymes and non-specific binding, may affect their potency in established cell lines. Therefore, we chose to pursue our studies on the mechanism of target selectivity of the combi-molecule in the isogenic cell line panel, MDA-MB-435, MDA-MB-435/EGFR and MDA-MB-435/ErbB2 that do not express AGT, a DNA repair protein known to mitigate cellular response to the DNA lesions induced by TEM or the combi-molecules (Fornace *et al.*, 1990; Pegg *et al.*, 1995; Matheson *et al.*, 2003b). First, comet assay analysis was performed to determine the extent of DNA damage in the isogenic cell panel. The results showed that RB24 induced significantly higher levels of DNA damage in the ErbB transfectants (EGFR, ErbB2) than in the wild-type ($p<0.05$) (Fig. 4.1b). By contrast, TEM did not exhibit any selectivity for the transfectants, with equal levels of DNA damage in all three cell lines (Fig. 4.1c). More importantly, RB24 was a 3.1 and 1.9-fold more potent DNA damaging agent than TEM in MDA-MB-435/EGFR and MDA-MB-435/ErbB2, respectively.

The ability of our compound to induce superior levels of DNA damage than TEM in the cells and further, to selectively damage cells expressing EGFR and ErbB2, stimulated our interest in determining the mechanism underlying this selectivity. Having found that the released inhibitor RB10 was fluorescent, we undertook a fluorescence microscopy

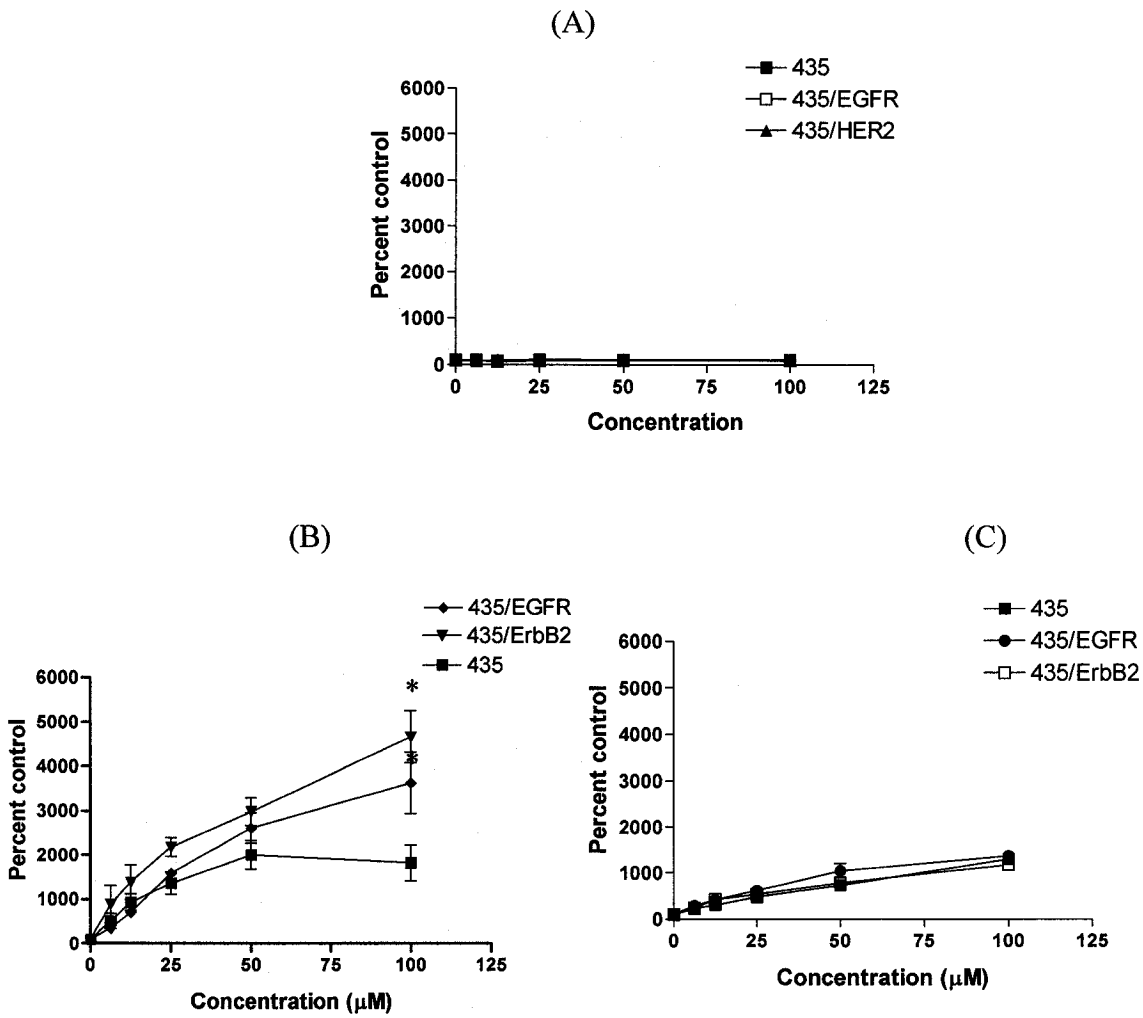

Figure 4.1. Effects of RB24, RB10 or TEM on levels of DNA damage in the isogenic MDA-MB-435 cell line panel. MDA-MB-435, MDA-MB-435/EGFR and MDA-MB-435/ErbB2 cells were preincubated for 2 h with A) RB10 B) RB24 or C) TEM and levels of DNA damage were quantitated using the alkaline comet assay. Tail moment (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) was used as a parameter for the detection of DNA damage in cells exposed to RB24, RB10 or TEM. * p-value < 0.05. *Points*, mean percentage of untreated cells (n=3); *bars*, SD.

analysis to observe its subcellular localization following exposure to RB24. The results showed that intracellular levels of RB10 released by RB24 degradation were concentrated in the perinuclear region and interestingly, the fluorescence intensity was higher in the ErbB transfectants then in the wildtype (Fig. 4.2). This result was further confirmed by UV flow cytometric quantitation that showed significant difference (2-3 fold) between the wildtype and the transfectants (Fig. 4.3). This is in agreement with the previous results showing higher levels of DNA damage in the transfectants. Thus, these results lead to the hypothesis that the enhanced DNA damage inflicted by RB24 may be due to a bystander effect, whereby RB24 anchors in its cognate sites in the perinuclear region from which the methyldiazonium is released (Fig. 4.4). To test this hypothesis, we designed a competitive experiment whereby RB10 was given in combination with RB24 to prevent the interactions of the latter with its binding sites.

In theory, if binding of RB24 to its cognate sites around the nucleus played a bystander effect, we would expect competitive binding of RB10 to deplete the levels of DNA damage induced by RB24. Indeed, as depicted in Figure 4.5, combined treatment with RB10 + RB24 significantly depleted the levels of DNA damage induced by RB24 in all MDA-MB-435 cells. More importantly, the difference was significantly enhanced in the cells expressing Her2 or EGFR, indicating that the latter receptors significantly contribute to the enhanced DNA damage observed with RB24. It should also be noted here that the levels of DNA damage induced by the combination of RB10 + RB24 were similar to that of TEM and more interestingly, selectivity for the ErbB transfectants was completely abolished (Fig. 4.5a). This is further corroborated by the depletion

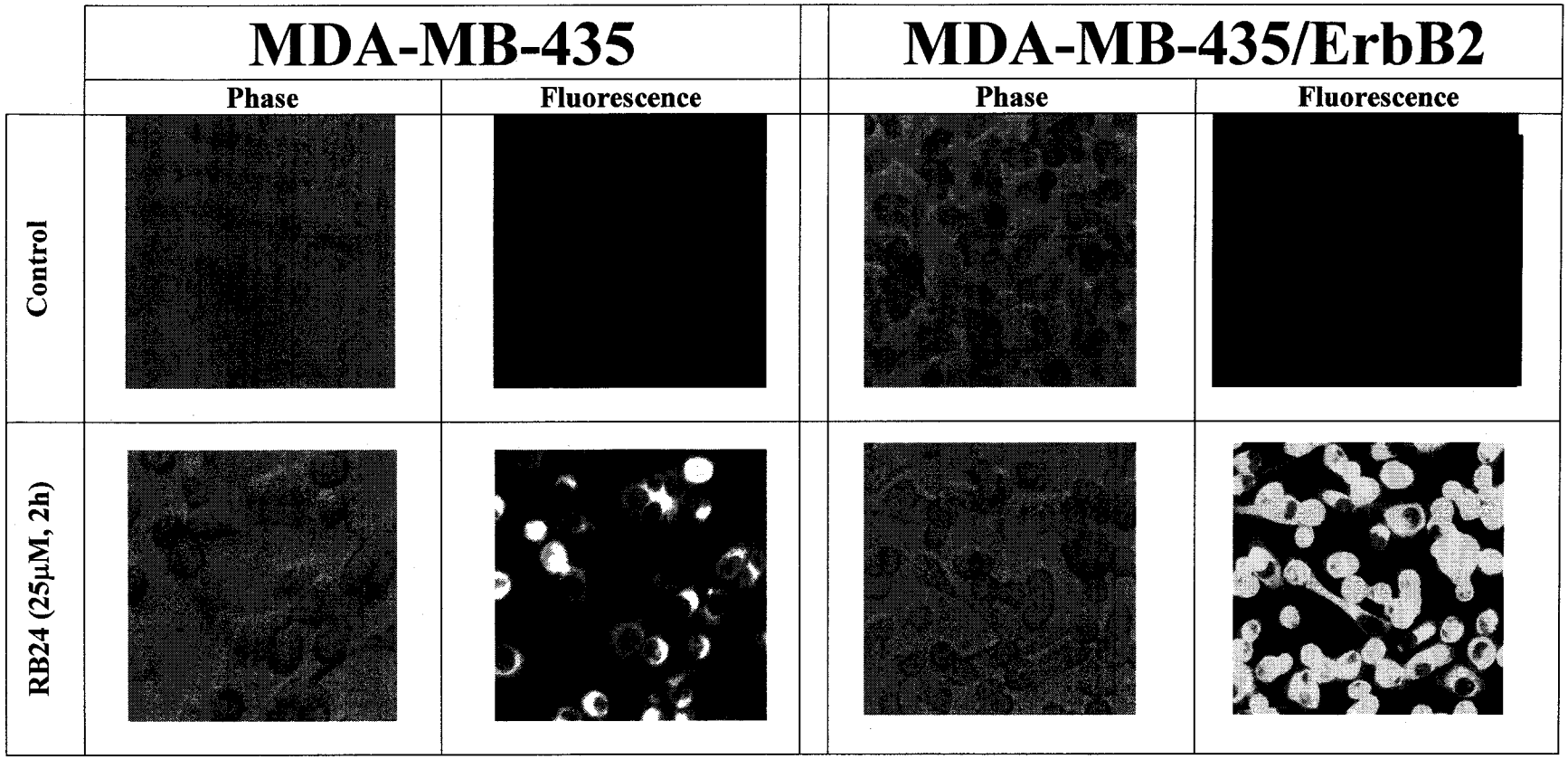

Figure 4.2. Internalization of RB24 in MDA-MB-435 and MDA-MB-435/ErbB2 cells. Localization of the compound was determined by fluorescence microscopy. RB24 (25 μM) was given for 2 h prior to observation (400x magnification).

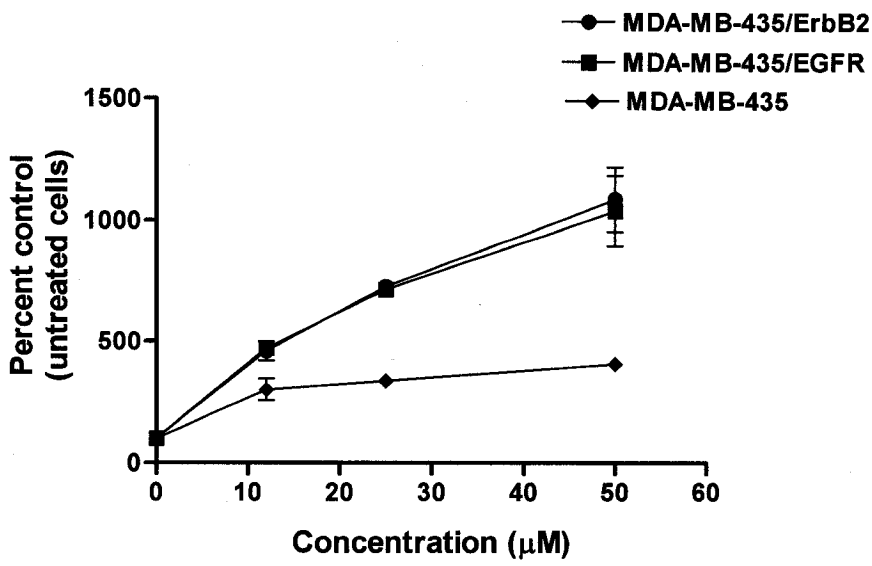

Figure 4.3. UV flow cytometric analysis of intracellular levels of RB10 released by RB24 in the isogenic MDA-MB-435 cell line panel. MDA-MB-435, MDA-MB-435/EGFR and MDA-MB-435/ErbB2 cells were treated with a dose range of RB24 for 2 h prior to analysis with the BD LSR flow cytometer. *Columns*, mean percentage of untreated cells (n=2); *bars*, SD.

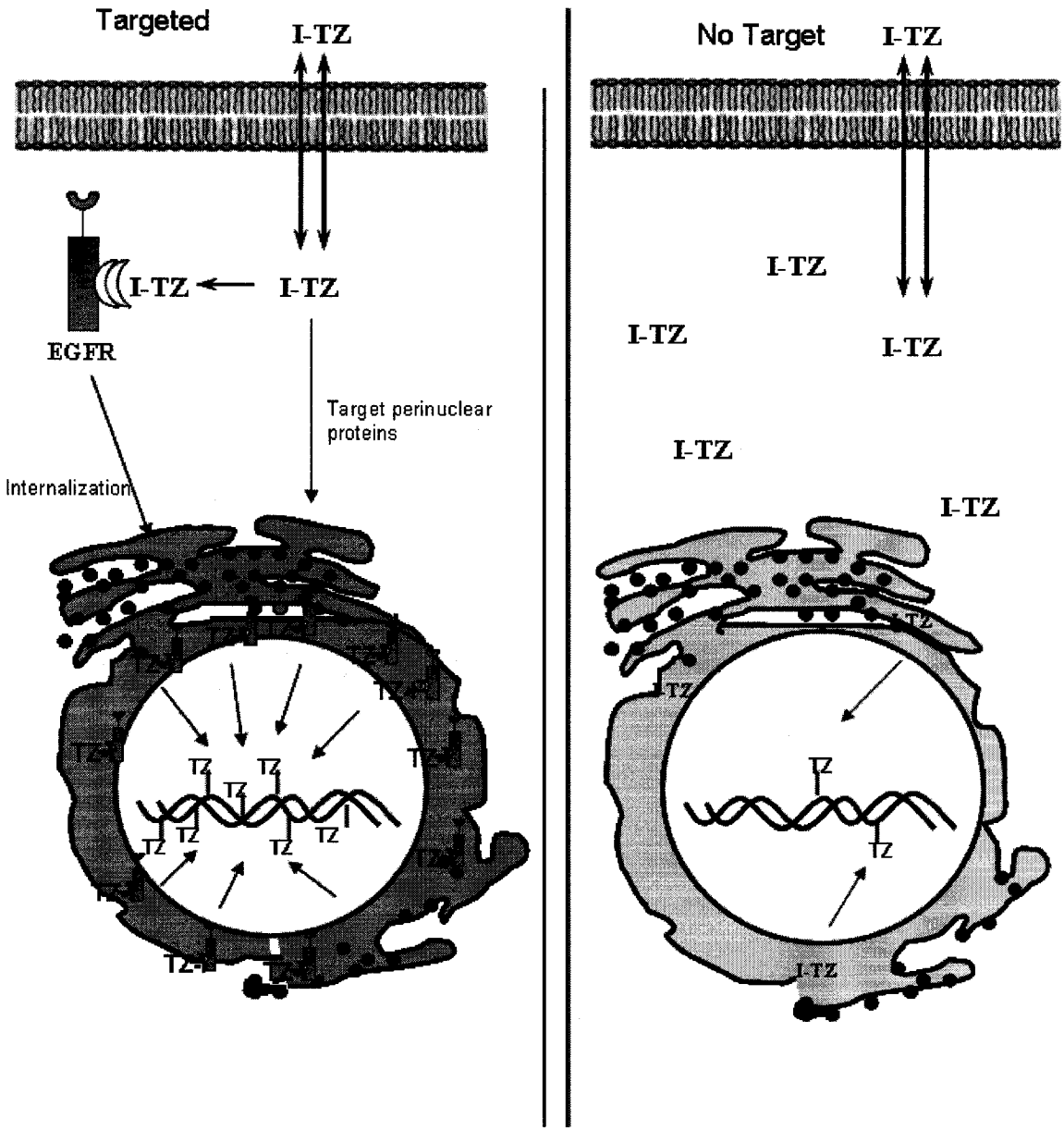

Fig. 4.4. Schematic representation of the intracellular distribution of the combi-molecule RB24 (I-TZ) in ErbB-expressing or ErbB-null cells.

and delocalization of the fluorescence associated with RB24 upon co-incubation with JDA41 (a non fluorescent inhibitor of EGFR with IC_{50} competitive binding within the same range as RB24) (Fig. 4.5b).

4.4.3. Mechanism of selective cell-killing

While it was obvious that RB24 could induce substantially higher levels of DNA damage than TEM in these cells, the translation of the latter effect into cell killing remained to be elucidated. Thus, the levels of JNK phosphorylation, a signaling protein known to be activated by DNA damage and to be involved in promoting apoptosis, were analyzed (Huang *et al.*, 1999, Ohtsuka *et al.*, 2003). The results showed that JNK was activated at concentrations of RB24 that induced significant DNA damage (25-100μM) (Fig. 4.6a). Conversely, at the same concentrations, TEM did not induce significant DNA damage and no JNK activation was apparent. To further ascertain the correlation between DNA damage and JNK activation, we repeated the analysis of TEM at doses at which equi-levels of DNA damage were induced (e.g. 200, 400μM) (Fig. 4.6a,b). Interestingly, like in RB24 treated cells, JNK activation was detected for TEM at its DNA damaging doses. Further, the pro-apoptotic role of JNK in these cells was corroborated by analysis of levels of apoptosis induced in the presence of its potent inhibitor SP600125. Indeed, Annexin V flow cytometric analysis showed that SP600125 reduced levels of apoptosis in cells treated with RB24 and TEM by *ca* 33-35% (Fig. 4.6b).

(A)

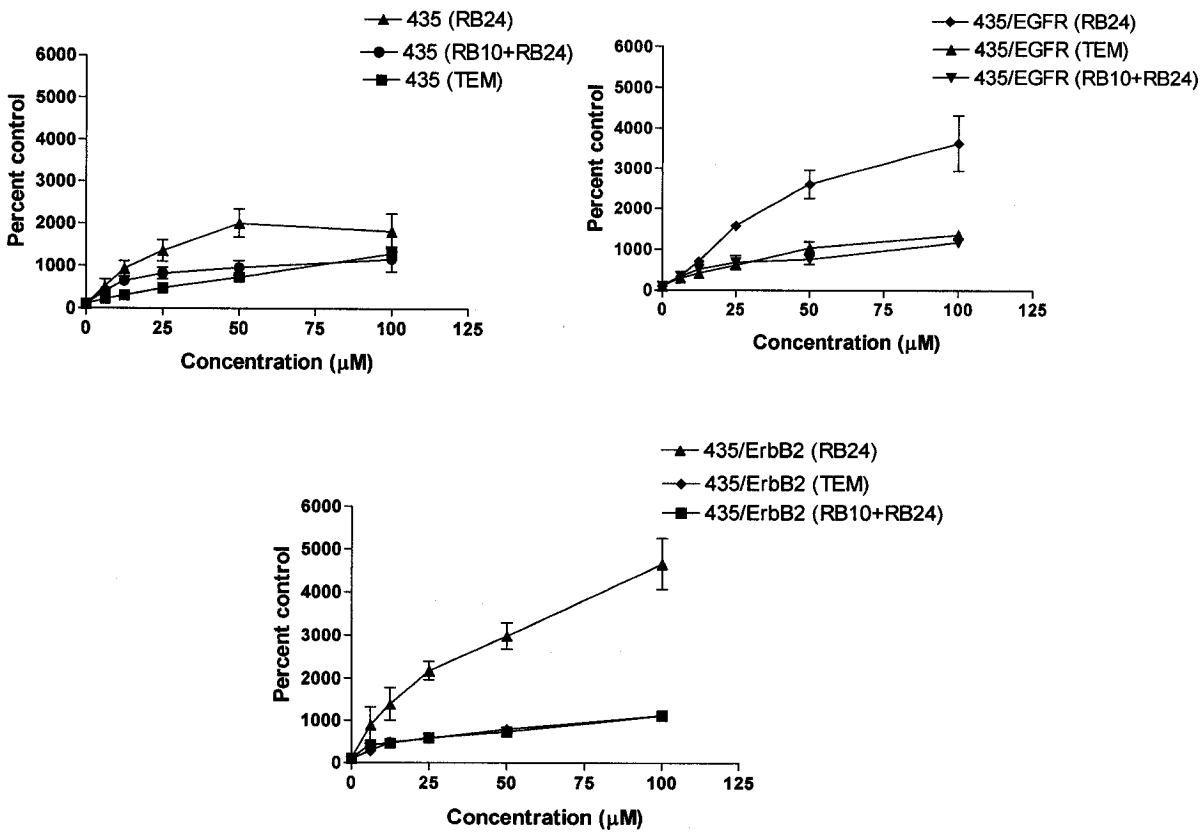

Figure 4.5. Displacement of RB24 with RB10 and levels of DNA strand breaks induced in MDA-MB-435, MDA-MB-435/EGFR or MDA-MB-435/ErbB2 cells. A) MDA-MB-435, MDA-MB-435/EGFR and MDA-MB-435/ErbB2 cells were preincubated for 2 h with a dose range of RB24 in combination with RB10 (40μM) and levels of DNA damage were quantitated using the alkaline comet assay. Tail moment (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) was used as a parameter for the detection of DNA damage in cells exposed to RB24, RB10 or TEM. *Points*, mean percentage of untreated cells (n=2); *bars*, SD.

(B)

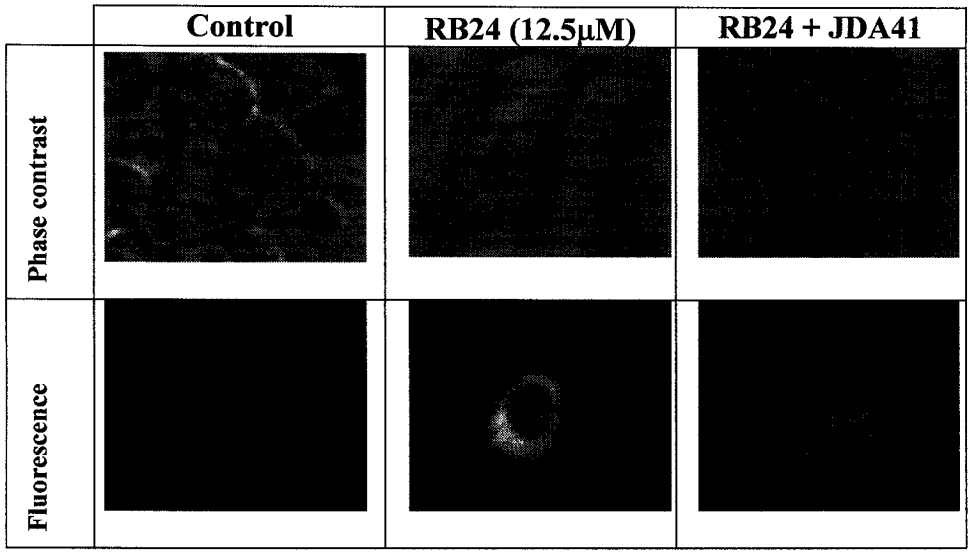

Figure 4.5. Displacement of RB24 with RB10 and levels of DNA strand breaks induced in MDA-MB-435, MDA-MB-435/EGFR or MDA-MB-435/ErbB2 cells. B) Intracellular localization of RB24 in MDA-MB-435/ErbB2 cells exposed to either RB24 alone (25μM) or in combination with the non-fluorescent EGFR inhibitor, JDA41 (40μM) for 2 h (400x magnification).

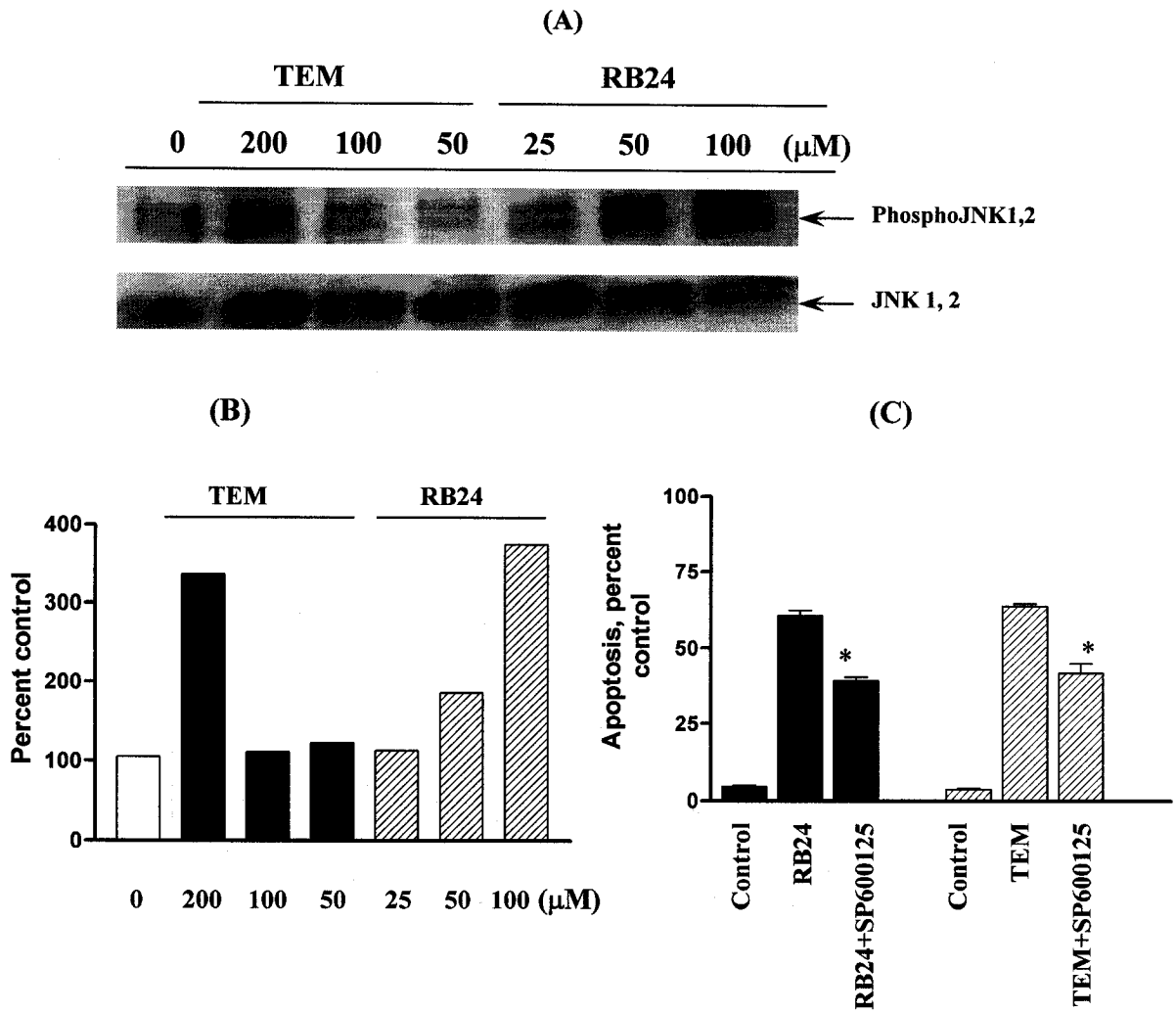

Figure 4.6. Effects of RB24, RB10 or TEM on activation of the stress activated protein kinases, JNK1, 2 and apoptosis. A) JNK activation in MDA-MB-435/ErbB2 cells exposed to a dose range of RB24 or TEM for 4 h. B) Quantitation of phosphorylated JNK levels in MDA-MB-435/ErbB2 cells exposed to RB24 or TEM. Equal amount of cell lysate was analyzed by Western blot and probed for anti-phosphoJNK or anti-JNK antibodies. C) Annexin V analysis of MDA-MB-435/ErbB2 cells treated with dose range of RB24 or TEM alone or in combination of the JNK inhibitor, SP600125. Levels of apoptotic cells were determined using Annexin V-FITC staining. * p-value < 0.05. *Columns*, mean percentage of Annexin-V-negative cells (n=4); *bars*, SD.

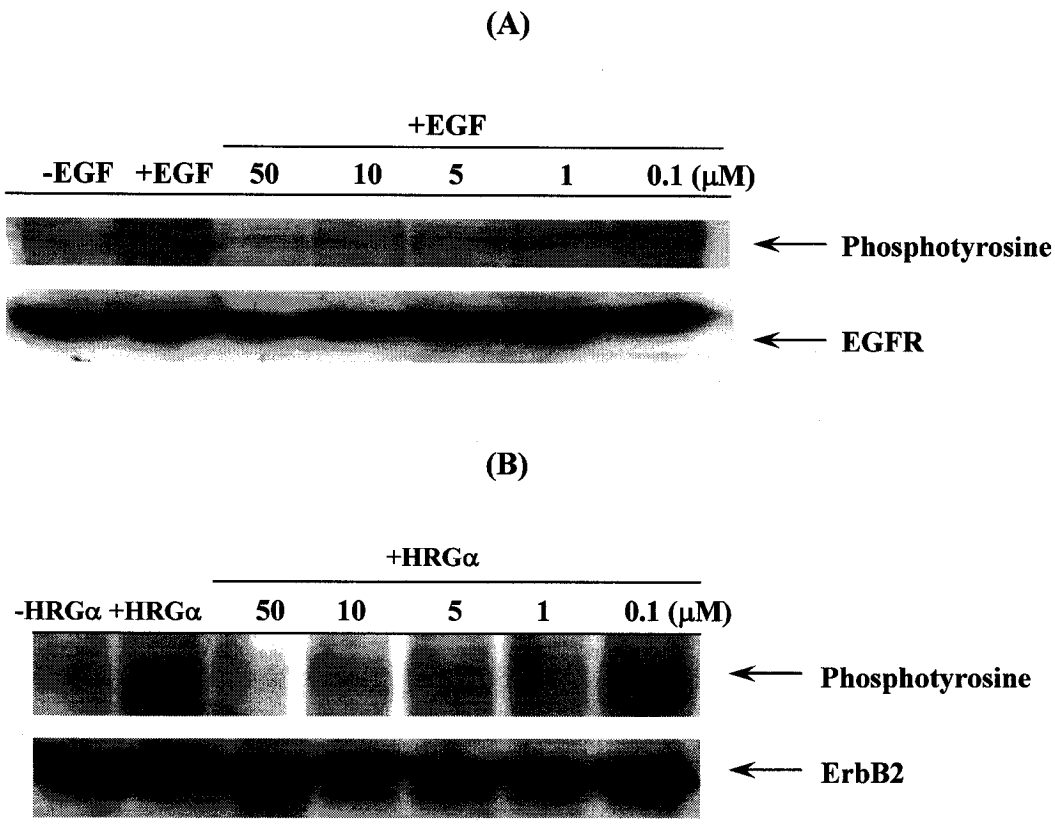

Figure 4.7. Selective inhibition of ErbB receptor activation in both MDA-MB-435/EGFR and MDA-MB-435/ErbB2 cells. Serum-starved A) MDA-MB-435/EGFR or B) MDA-MB-435/ErbB2 cells exposed to the indicated concentrations of RB24 for 2 h and subsequently stimulated with EGF (25ng/ml) or HRGα (50ng/ml), respectively. Equal amount of cell lysate was analyzed by Western blotting using anti-phosphotyrosine, anti-EGFR and anti-ErbB2 antibodies. Band intensities were measured using the SynGene GeneTools software package.

It is now known that inactivation of apoptosis is well regulated by EGFR- or ErbB2-mediated signaling and that JNK activation promotes apoptosis in response to DNA damage. Therefore, we surmise that the lack of sensitivity of the ErbB transfectant cells to TEM may be mediated by EGFR or ErbB2. Thus, we analyzed the activation of these signaling pathways in response to RB24 and TEM. Activation of EGFR (Fig. 4.7a) and ErbB2 (Fig. 4.7b) correlated with Bad phosphorylation in the transfectants. However, treatment with RB24 led to a dose-dependent inhibition of both EGFR/ErbB2 and Bad phosphorylation in these cells, indicating an alleviation of the anti-apoptotic effects of ErbB receptors (Fig. 4.7a,b, Fig. 4.8a,b). In contrast, TEM was incapable of depleting levels of Bad phosphorylation at a concentration as high as 50μM, which is in agreement with its inability to induce neither JNK nor to block EGFR TK mediated signaling (Fig. 4.7c,d).

We further determined whether the ability of the combi-molecule to selectivity block signaling and damage DNA would translate into ErbB selective apoptosis. Interestingly, RB24 induced approximately 2-fold higher level of apoptosis in the ErbB transfectants than in the wild-type (58-73% 24 h after treatment) (Fig. 4.9). Inversely, TEM was only capable of inducing significant levels of apoptosis in the wild-type cells (Fig. 4.9). RB10, an inhibitor of EGFR, induced moderate levels of apoptosis in the ErbB-expressing cells (25-30%) (Fig. 4.9c).

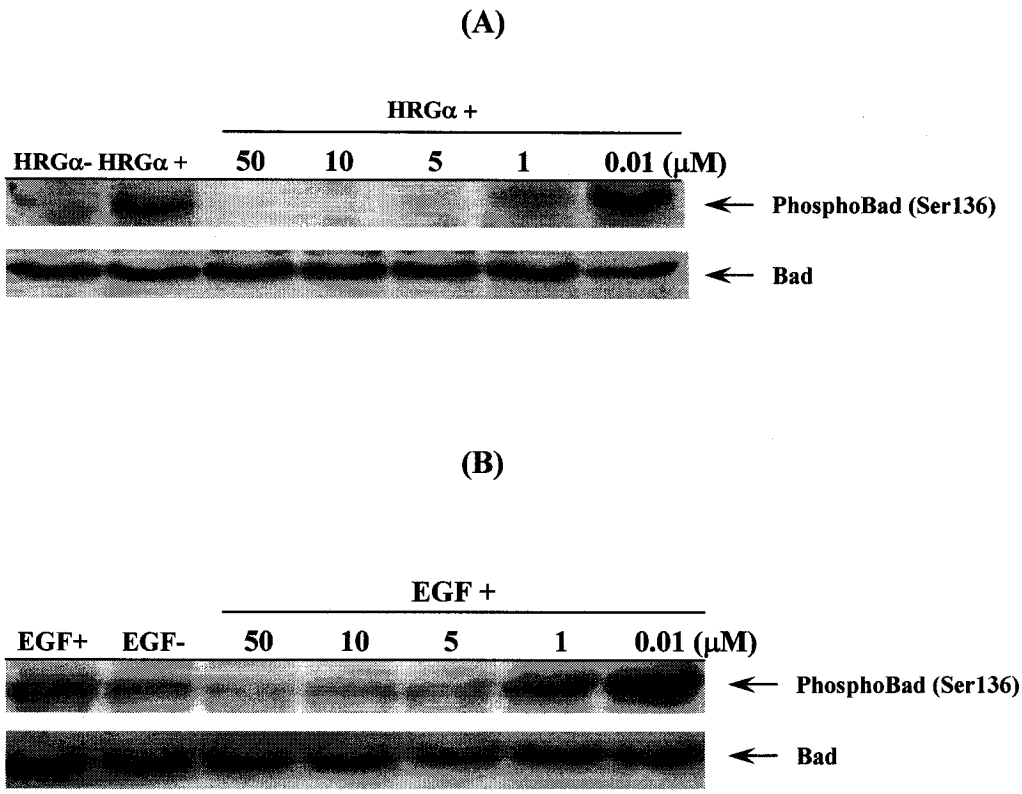

Figure 4.8. Assessment of inhibitory activity on Bad activation by RB24. Levels of Bad phosphorylation at Ser136 were examined in serum-starved A) MDA-MB-435/ErbB2 or B) MDA-MB-435/EGFR cells that were preincubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF (25ng/ml) or HGRα (50ng/ml), respectively.

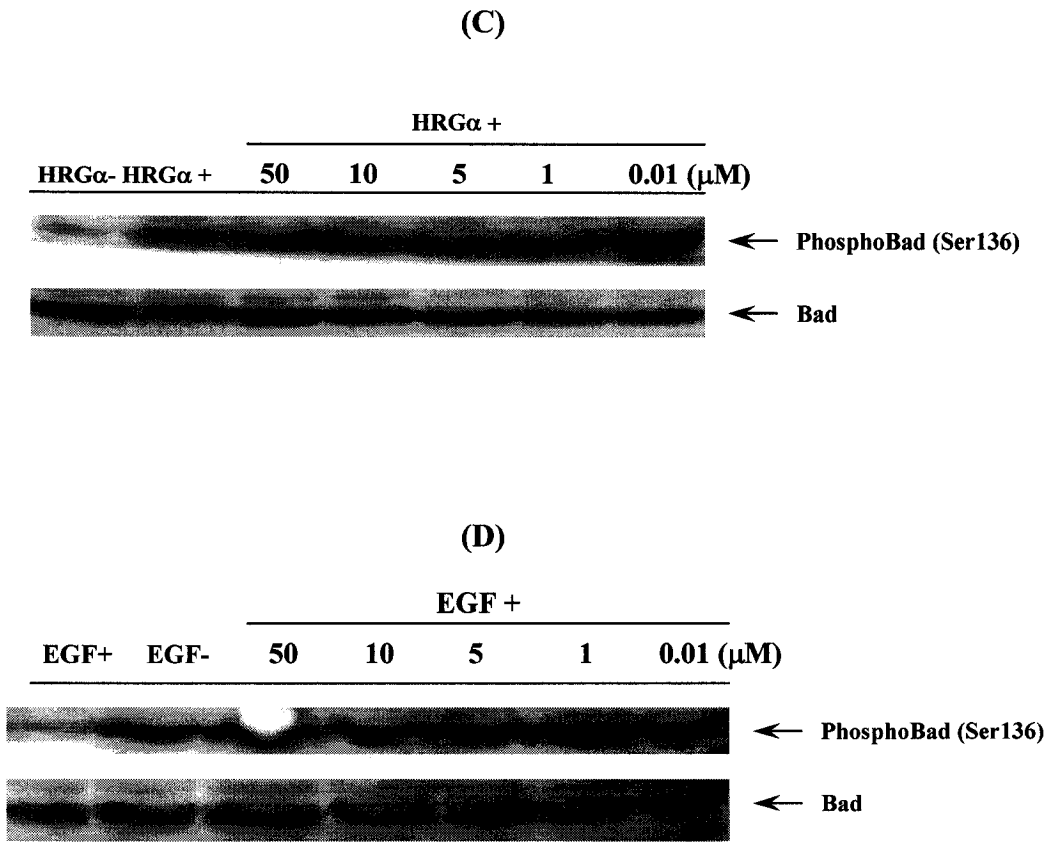

Figure 4.8. Assessment of inhibitory activity on Bad activation by TEM. Serum-starved C) MDA-MB-435/ErbB2 or D)MDA-MB-435/EGFR cells were exposed to a dose range of TEM for 2h prior to EGF (25ng/ml) or HRGα (50ng/ml) stimulation, respectively. Equal amounts of cell lysates were analyzed by Western blot using anti-phosphoBad (Ser 136) or anti-Bad antibodies.

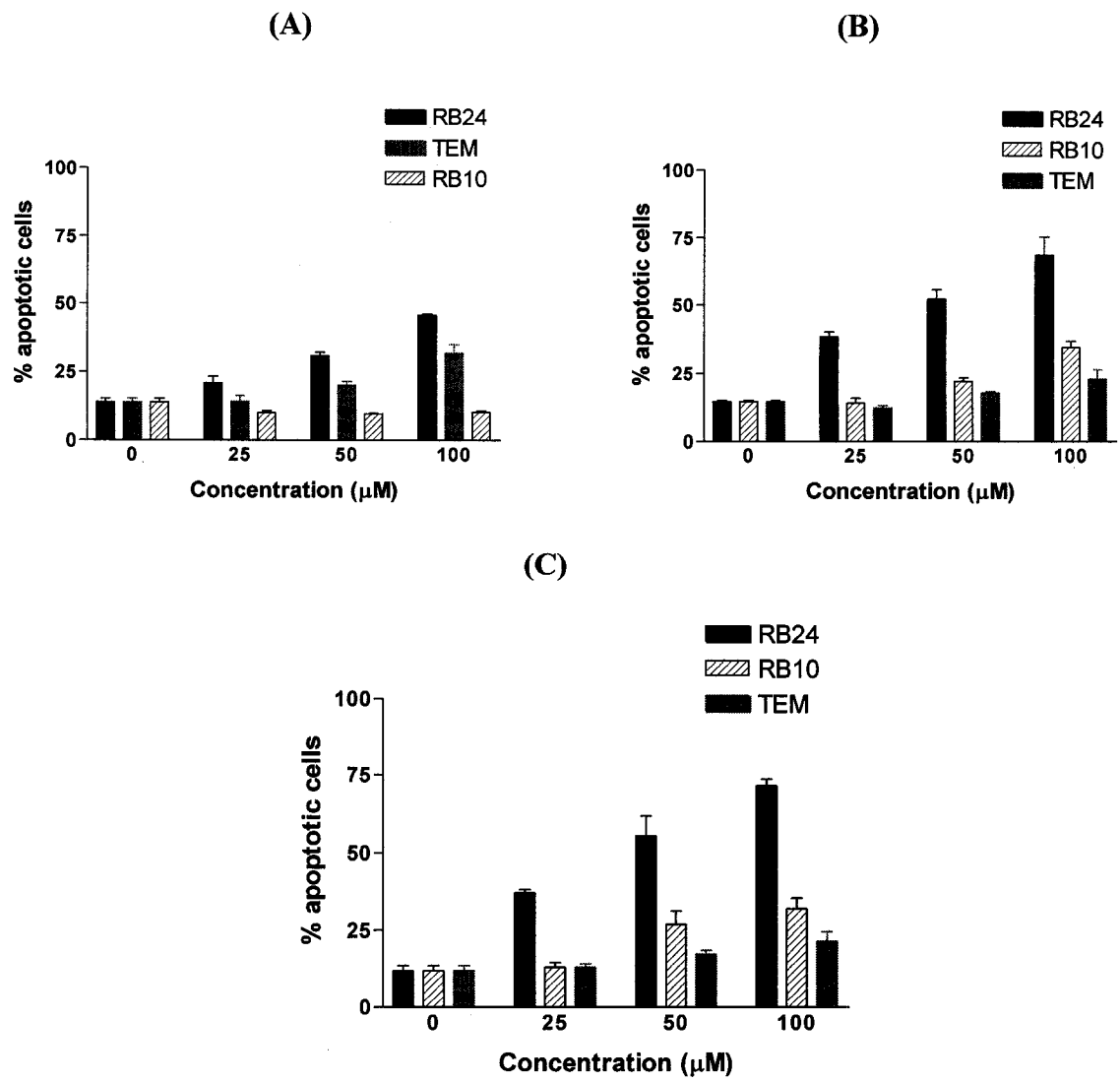

Figure 4.9. Assessment of apoptotic effects induced by RB24, RB10 and TEM. A) MDA-MB-435, B) MDA-MB-435/EGFR and C) MDA-MB-435/ErbB2 cells were treated with a dose range of RB24, TEM or RB10 for 24 h. Levels of apoptotic cells were determined using Annexin V-FITC staining. *Columns*, mean percentage of Annexin-V-negative cells (n=3); *bars*, SD.

4.5. Discussion

Alkylating agents are among the most potent cytotoxic drugs used in the clinic for the therapy of many solid tumours including breast and lung carcinomas (Evans, 2005; Trudeau *et al.*, 2005). However the potency of these compounds is often mitigated by resistance associated with the expression of DNA repair proteins (Garcia-Campelo *et al.*, 2005). Furthermore, their lack of selectivity for tumour tissues has become a major deterrent in their use for the therapy of solid tumours. This study was designed to analyze the selective potency of a novel class of combi-molecules carrying a methylating species. In this model, EGFR and ErbB2 transfection significantly reduced cell sensitivity to TEM, which is not targeted to the aforementioned receptors. Inversely, EGFR and ErbB2 transfection enhanced the potency of RB24, a methylating triazene of the same class as TEM. Thus, we hypothesize that selective and enhanced potency of RB24 against the transfectants is based upon mechanisms related to receptor targeting and cell signaling.

The first significant observation of this study was the differential cytotoxicity and DNA damage induction produced by the combi-molecule in the isogenic panel. In contrast to TEM, levels of DNA damage induced by RB24 appeared to correlate with receptor levels. Correlation of DNA damage with levels of receptors suggests that their mechanism of intracellular distribution may differ from that of TEM. Based upon our results, it appears that the combi-molecule may enter the cells by passive diffusion as previously described for other combi-molecules (Matheson *et al.*, 2001; Banerjee *et al.*, 2003; Qiu *et al.*, 2003) and bind to its cognate sites (e.g. nascent or internalized EGFR proteins or related proteins) located in the ER (Murthy *et al.*, 1986; Offterdinger *et al.*,

2004; Khan *et al.*, 2006). The loss of selective delivery of DNA alkylation upon competitive binding with other EGFR inhibitors indicates that the entire combi-molecule may bind to the perinuclear region prior to degradation and release of the methyldiazonium ion. This model is further supported by: a) the low levels of DNA damage induced by RB24 in the wild-type cells line when compared with the transfected counterparts and b) the lower levels of DNA damage induced by TEM, a non-EGFR targeting molecule (Matheson *et al.*, 2001), in all the cell lines. It is also important to mention herein that RB24 induced slightly higher levels of DNA damage than TEM in wild-type cell line which may be due to unspecific binding.

In order to elucidate the contribution of the high levels of DNA damage to cell killing, we examined various pathways related to the receptor and in response to DNA alkylation. On one hand, it is known that EGFR and ErbB2 exert anti-apoptotic effects through the PI3K/Akt signaling cascade, that ultimately leads to the inhibition of mitochondrial pore formation via Bax oligomerization (McCubrey *et al.*, 2006). On the other hand, pro-apoptotic effects in response to DNA damage is mediated by the stress activated protein kinase, JNK (Huang *et al.*, 1999, Ohtsuka *et al.*, 2003). Thus, we analyzed key elements of these pathways (e.g. Bad and JNK activation) in the transfectants. Bad being downstream of EGFR or Her2 activation, its observed inactivation by the combi-molecule is an evidence that inhibiting the receptors alleviates their anti-apoptotic effects. Activation of JNK in response to DNA damage is pro-apoptotic, therefore receptor inactivation and induction of DNA lesions are two cooperative events that can contribute to enhanced apoptosis. More importantly, the fact that activation of JNK was only seen at doses that correspond to significant levels of

DNA damage by both RB24 and TEM provides a direct link between levels of DNA damage and apoptosis in these cells. Indeed, substantially higher levels of apoptosis were observed in the transfectants when compared with their wild-type counterpart.

In summary, the results presented herein can allow us to propose a model for selective targeting and cell killing of breast cancer cells expressing ErbB receptors by the combi-molecular concept. As depicted in Figure 4.4, binding of a fraction of the combi-molecules to the TK domain of the receptors at the membrane induces blockade of downstream signaling including the anti-apoptotic PI3K/Akt pathway. Following internalization, binding of a large fraction of the combi-molecules to EGFR in the perinuclear region plays a bystander effect that localizes the alkylating species to the nucleus. The elevated levels of DNA damage induced by the combi-molecule, enhances apoptosis through activation of JNK and other alternative pathways associated with the anti-apoptotic effect of EGFR/Her2 receptor-mediated signaling.

4.6. Acknowledgements

We would like to thank the Canadian Breast Cancer Research Alliance/Canadian Institutes for Health Research (CBCRA/CIHR) for financial support.

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4.8. Connecting text

In Chapter 4, we investigated the mechanism underlying the selective potency of the cascade release combi-molecule, RB24, in an isogenic panel of breast cancer cell lines. We demonstrated therein, that the selectivity of RB24 is based upon its ability to induce enhanced DNA damage in cells transfected with EGFR or Her2. More importantly, enhanced DNA damage correlated with activation of the stress activated protein kinase, JNK, and blockade of EGFR with down-regulation of Bad-mediated anti-apoptotic signaling. This stimulated our interest in identifying cross-talk between signaling pathways that would converge to synergistic cell killing by the combi-molecule. To this end, we chose a cellular model wherein growth factor receptor activation correlated with expression of DNA repair proteins. The DU145 prostate carcinoma cells have been shown to express increased levels of the BER protein, XRCC1, upon EGFR activation and this cell line is also known to exhibit robust resistance to methylating agents of the same class as RB24. Thus, the following chapter is a detailed analysis of the cell signaling pathways and cross-talk in response to the binary stress (e.g. DNA damage, blockade of EGFR) inflicted to the cells by the combi-molecule, RB24.

CHAPTER 5

THE COMBI-TARGETING CONCEPT: MECHANISM OF ACTION OF THE PLEIOTROPIC COMBI-MOLECULE RB24 (NSC 741279) AND DISCOVERY OF A NOVEL CELL SIGNALING-BASED COMBINATION PRINCIPLE

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5.1. Abstract

RB24 (NSC 741279), a 3-methyltriazene termed "combi-molecule" designed to possess mixed epidermal growth factor receptor (EGFR) targeting and DNA methylating properties showed over 100-fold greater antiproliferative activity than Temodal® (TEM), 4-fold greater potency than gefitinib and 5-fold stronger activity than an equi-effective combination of gefitinib+TEM against the O^6-alkylguanine transferase (AGT)-proficient DU145 cell line that co-expresses EGFR. Investigation of the mechanisms underlying the unique potency of RB24 revealed that cell exposure to TEM was accompanied by activation of p38MAPK and concomitant elevation of the levels of X-ray repair cross-complementing (XRCC1) protein. Levels of phospho-p38MAPK and XRCC1 were increased by 2-fold in EGF-stimulated cells. In contrast, EGF-stimulation did not alter the status of these proteins in RB24-treated cells and this translated into a 2-fold lower level of XRCC1 when compared with those exposed to TEM + EGF. These effects correlated with significantly delayed DNA repair activity in combi-molecule-treated cells when compared with TEM-exposed ones. Further analysis demonstrated that in contrast to TEM, RB24 could block Bad phosphorylation at serine 136 in a dose-dependent manner and induced significantly higher levels of apoptosis than the latter molecule. Tandem depletion of XRCC1 and Bad activation through alternative pathways using the MEK1 inhibitor, PD98059, led to substantial levels of apoptosis in RB24-treated cells. The results *in toto* indicate that the superior activity of the combi-molecule may be attributed to its ability to down-regulate DNA repair proteins such as XRCC1 and to alleviate anti-apoptotic signaling through blockade of EGFR-mediated signaling while inflicting high levels of DNA lesions to the cells.

5.2. Introduction

Binding of growth factors to their cognate receptor is associated with the activation of signaling cascades that ultimately translate into genes associated with cell proliferation (Modjtahedi and Dean, 1994; Moyer *et al.*, 1997; Ciardello and De Vita, 2005). Tumours in which certain growth factors, such as the epidermal growth factor receptor (EGFR) or its closest homologue Her2 are overexpressed, show aggressive growth and reduced sensitivity to chemotherapeutic drugs (Slamon, 1989; Tanner *et al.*, 1996; Yen *et al.*, 1999; Hengstler *et al.*, 1999). Recent strategies directed at blocking pathways activated by these receptors led to inhibition of tumour growth *in vivo* and gefitinib (ZD1839, Iressa™) is the first inhibitor of EGFR tyrosine kinase (TK) that exhibited antitumour activity in the clinic (Magne *et al.*, 2002; Ranson *et al.*, 2002; Sgambato *et al.*, 2004; Bellezza *et al.*, 2006). However, despite the significant attention given to EGFR-mediated signaling, little emphasis was placed on developing agents that target other pathways that could synergize with down-regulation of genes associated with depletion of EGFR autophosphorylation. Recently, within the framework of a novel tumour targeting strategy termed "Combi-Targeting", our laboratory has developed a novel class of pleiotropic inhibitors capable of not only blocking EGFR-mediated signaling but also inducing DNA damage, a type of insult known to subvert multiple signaling pathways in the cell, including apoptosis, stress response and cell cycle perturbation (Matheson *et al.*, 2001; Brahimi *et al.*, 2002; Matheson *et al.*, 2003a; Banerjee *et al.*, 2003; Matheson *et al.*, 2003b; Rachid *et al.*, 2003; Qiu *et al.*, 2003; Matheson *et al.*, 2004a; Matheson *et al.*, 2004b; Banerjee *et al.*, 2004; Qiu *et al.*, 2004). As outlined in Figure 5.1, the combi-targeting principle is based on the premise that a combi-molecule I-TZ (wherein TZ represents the alkylating function and I the EGFR

recognition moiety) will penetrate the cell by passive diffusion and bind to the ATP-binding site of EGFR to form the TZ-I-EGFR complex. I-TZ being designed to be hydrolyzed under physiological conditions can break down to generate the TZ (an alkylating species) that damages DNA and I, a free inhibitor that can further block EGFR-mediated signaling. Since resistance to DNA damaging agents is often mediated by DNA repair enzymes, we surmised that down-regulation of DNA repair proteins through blockade of EGFR phosphorylation by I-TZ and I, would enhance cell-killing, thereby inducing more potent antitumour activity than currently available inhibitors of EGFR alone or individual DNA damaging agents.

We have already demonstrated that the combi-molecule RB24 was a unique combi-molecule with ability to block EGF-induced signaling on its own and also to be degraded into multiple species with EGFR TK inhibitory properties + a DNA alkylating methyldiazonium species (Fig. 5.1) (Banerjee *et al.*, 2003; Banerjee *et al.*, 2004). RB24 belongs to the same class of methylating agents as the clinical drug Temodal® (TEM), which is known to induce three types of DNA adducts: N7-methylguanine (N^7-MeG), N3-methyladenine (N^3-MeA) and O6-methylguanine (O^6-MeG). While the N^7-MeG and N^3-MeA account for 85% of the total adducts and are handled by base excision repair (BER) enzymes, the O^6-MeG adducts are repaired by a specific enzyme termed O^6-alkylguanine transferase (AGT) (Gibson *et al.*, 1986; Tisdale, 1987; Fornace *et al.*, 1990; Baer *et al.*, 1993; Mitchel and Dolan, 1993; Pegg *et al.*, 1995). The O^6-MeG lesions are believed to be the most cytotoxic adducts, as evidenced by the marked resistance of cells that express AGT. Strategies designed to enhance the potency of the

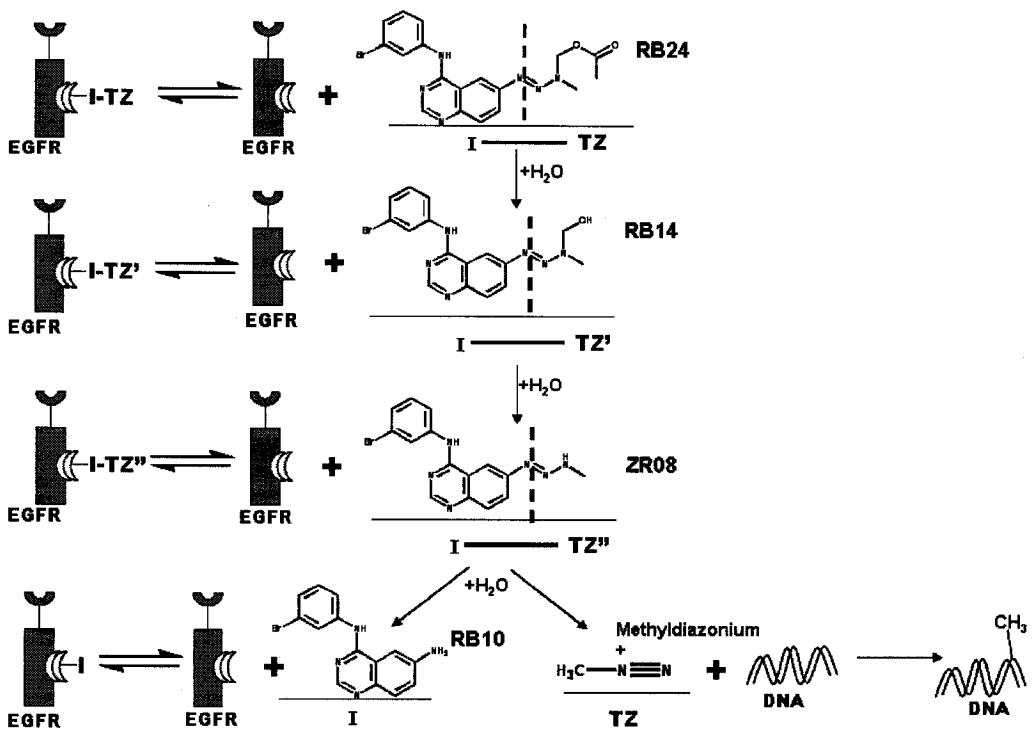

Figure 5.1. Stepwise release of the metabolites generated during the degradation cascade of the combi-molecule, RB24. The parent combi-molecule (I-TZ) hydrolyzes to release a series of daughter inhibitors (I-TZ', I-TZ") and at the final stage of degradation, a free inhibitor (I) + a DNA methylating species (TZ).

latter agents in resistant tumours are primarily based on the development of DNA repair inhibitors. O6-benzylguanine (O6-BG), an inhibitor of AGT, has been shown to sensitize cells to TEM (Cai *et al.*, 2000; Quinn *et al.*, 2005). Similarly, methoxylamine or poly-ADP-ribose polymerase (PARP) inhibitors have been used to inhibit base excision repair in resistant cells (Horton *et al.*, 2000; Liu *et al.*, 2002; Miknyoczki *et al.*, 2003; Tentori *et al.*, 2003; Liu and Gerson, 2004). Despite the marked importance of BER in chemoresistance, studies designed to develop molecules capable of indirectly disrupting it through down-regulation of small proteins such as XRCC1, a member of the BER complex, are scant. Here, we study the signaling pathways that contribute to the potency of one such molecule, RB24, a combi-molecule designed to not only down-regulate DNA repair through EGFR inhibition but also to methylate DNA.

In this study, we have chosen the androgen-independent prostate cancer cell line DU145 as a model for the following reasons: (1) it expresses AGT that repairs the cytotoxic O^6-MeG lesion, (2) EGF-stimulation in these cells significantly up-regulates nucleotide excision repair protein, ERCC1, and BER protein XRCC1 and (3) EGF-stimulation is accompanied by activation of anti-apoptotic signaling that leads to Akt phosphorylation of Bad (Yacoub *et al.*, 2003; Maroni *et al.*, 2004; Fan *et al.*, 2004; Hagan *et al.*, 2004). Thus, the DU145 cells accumulate almost all the critical signaling disorders required to confer a robust resistance to methylating agents. In this study, we demonstrate that in contrast to the clinical methylating agent, TEM, these cells are exquisitely sensitive to the combi-molecule and investigate alternative cell signaling pathways that can be hammered to further sensitize them to RB24. We also demonstrate that blockade of

MEK1 phosphorylation exquisitely sensitizes cells to RB24, a novel type of mixed EGFR-DNA targeting molecule, but not to TEM.

5.3. Materials and Methods

5.3.1. Drug Treatment

RB24 and RB10 were synthesized in our laboratory according to known procedures (Banerjee *et al.*,2003; Rachid *et al.*,2003). Temodal® (TEM) was provided by Schering-Plough Inc. (Kenilworth, NJ, USA). Gefitinib was provided by AstraZeneca. SB203580 and PD98059 were purchased from Biomol (Plymouth Meeting, PA). U0126 was purchased from Cell Signaling Technology (Danvers, MA). In all assays, drug was dissolved in DMSO and subsequently diluted in RPMI-1640 containing 10% fetal bovine serum (FBS) (Wisent Inc., St-Bruno, QC, Canada) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

5.3.2. Cell Culture

The cell line used in this study was the human prostate carcinoma, DU145, which was obtained from the American Type Culture Collection (Manassas, VA). DU145 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, and antibiotics as described previously (Matheson *et al.*, 2001). Cells were maintained at 37°C in a humidified environment of 5% CO_2, 95% air. The cell cultures were maintained in logarithmic growth by harvesting with a trypsin-EDTA solution (0.5mg/ml trypsin and 0.2 mg/ml EDTA) and replating before confluence.

5.3.3. Growth Inhibition Assay

Growth inhibition studies were carried out with approximately 5 x 10^3 cells/well plated in 96-well plates. After a 24 h incubation at 37°C in a humidified environment of 5% CO_2, cell monolayers were exposed to different concentrations of each drug continuously for 6 days. All growth inhibitory activities were evaluated using the sulforhodamine B (SRB) assay (Skehan *et al.*,1990). The drug combinations were performed at IC_{50} molar ratios. Briefly, following drug treatment, cells were fixed using 50 µl of cold trichloroacetic acid (50%) for 60 min at 4°C, washed four 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 492 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

Antiproliferative effects that were generated by RB24 or TEM in combination with various inhibitory agents (e.g. SB203580, PD98059, O6-BG, U0126, gefitinib) were assessed by determining the combination index (CI). CI values were calculated using the following median effect equation (Perez *et al.*, 1993) where CI_{50} values >1, =1, and <1 demonstrated antagonistic, additive or synergistic activity, respectively:

$$CI_{50} = \frac{IC_{50}\text{ (Compound A in combination)}}{IC_{50}\text{ (Compound A alone)}} + \frac{IC_{50}\text{ (Compound B in combination)}}{IC_{50}\text{ (Compound B alone)}}$$

5.3.4. EGF-induced or Activated Proteins

5.3.4.1. XRCC1 levels

DU145 cells ($1x10^6$) were plated in six-well plates with 10% serum at 37°C for 24 h and starved overnight for 18 h, after which one set was exposed to 25μM of RB24 and 100μM of TEM for 2 h and subsequently treated with 20 ng/ml EGF for 5 h at 37°C. The other set was treated with 25μM of RB24 and 100μM of TEM for 2 h and subsequently replaced with drug- and serum-free media for 5 h at 37°C. Cells were washed with PBS and resuspended in cold lysis buffer [50 mM Tris-HCL PH 7.5; 150 mM NaCl; 1% Nonidet P-40, 1mM EDTA; 5 mM NaF; 1mM Na3VO4; protease inhibitor tablet (Roche Biochemicals, 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. The concentrations of protein were determined using the Bio-Rad protein assay Kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were added to an 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Non-specific binding on the membranes were minimized with overnight incubation at 4°C with blocking buffer containing nonfat dry milk (5%) in PBST. Thereafter, the membranes were incubated with primary anti-XRCC1 antibodies (Neomarkers, Fremont, CA) for 1 h for the detection of XRCC1 levels. Membranes were stripped and re-probed with anti-actin and anti-tubulin (Neomarkers, Fremont, CA) for determination of corresponding receptor levels. Blots were incubated with HRP-goat anti-mouse antibody (1:1000 dilution; Cell Signaling Research, Beverly, MA) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Matheson *et al.*, 2001).

5.3.4.2. p38MAPK and JNK activation

DU145 cells ($1x10^6$) were plated in six-well plates with 10% serum at 37°C for 24 h and starved overnight for 18 h, after which cells were exposed to 25μM of RB24 and 100μM of TEM for 2 h and subsequently treated with or without 20 ng/ml EGF for 5 h at 37°C. Cells were washed with PBS and lyzed by the aforementioned method. Equal amounts of protein were loaded into 10%SDS-PAGE and western blotting was performed as described. Antigens were detected with primary anti-phosphop38MAPK antibody or anti-phosphoJNK (Neomarkers, Fremont, CA) for 24 h for the detection of activated p38MAPK or JNK levels. Membranes were stripped and re-probed with anti-p38MAPK or anti-JNK (Neomarkers, Fremont, CA) for determination of corresponding enzyme levels. Blots were incubated with HRP-goat anti-mouse antibody (1:1000 dilution; Cell Signaling Research, Beverly, MA, USA) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

5.3.4.3. EGFR and Bad activation

Inhibition of EGFR and Bad activation were determined by exposing serum-starved cells for 2 h with the indicated concentrations of RB24 or TEM prior to stimulation with EGF (50ng/ml and 25ng/ml, respectively). Another set of serum-starved cells were treated with 10μM of RB24 alone or in combination with 30μM of U0126 for 6 h. Equal amounts of cell lysates were analyzed by Western blot using anti-phosphotyrosine (Upstate USA, Charlottesville, VA), anti-phosphoBad (Ser136, Ser112) antibodies (Cell Signaling Tech., Danvers, MA). Membranes were stripped of antibody and reprobed with anti-EGFR

or anti-Bad antibodies (Cell Signaling Tech., Danvers, MA).

5.3.5. Annexin V Binding

Cells were grown in 6-well plates until confluence and then incubated with the compounds for 24 h. The cells were then harvested, washed twice with PBS, and centrifuged. Cells (10^5) were treated with annexin V-FITC and propidium iodide (PI) using the apoptosis Detection Kit (BD Bioscience Pharmingen, San Jose, CA) and the supplier's protocol. Annexin V-FITC and PI binding were analyzed by flow cytometry. Data were collected using logarithmic amplification of both the FL1 (FITC) and FL2 (PI) channels. Quadrant analysis of co-ordinate dot plots was performed with CellQuest software. Unstained cells were used to adjust the photomultiplier voltage and for compensation setting adjustment in order to eliminate spectral overlap between the FL1 and FL2 signals. Statistical significance was determined using the Student t-test (n=4).

5.3.6. Alkaline Comet Assay for Quantitation of DNA Damage

The alkaline comet assay was performed as previously described (Matheson *et al.*, 2001). The cells were exposed to a dose range of RB24 for 2h, harvested with trypsin-EDTA, subsequently collected by centrifugation and resuspended in PBS. In DNA repair kinetics studies, cells were treated with RB24 or TEM for 2 h and allowed to recover for 2, 4, 18, 24, and 48h. 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 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, 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 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 room temperature, 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 330x 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 were analyzed for each sample, using ALKOMET version 3.1 image analysis software.

5.4. Results

5.4.1. Inhibition of EGFR activation by RB24

We first tested the ability of RB24 to inhibit EGF-induced EGFR phosphorylation in the DU145 cells. The results showed that RB24 could robustly block EGF-induced EGFR phosphorylation, as evidenced by 100% inhibition observed at a dose as low as 1 μM without affecting the levels of the latter receptor (Fig. 5.2).

5.4.2. Antiproliferative potency of RB24

As mentioned earlier, the DU145 cells possess characteristics (e.g. AGT expression) that confer high resistance to DNA methylating agents (Tisdale *et al.*, 1987; Fornace *et al.*, 1990; Yacoub *et al.*, 2003). Thus, its potency was compared with that of the clinically active methylating agent TEM in an SRB assay. RB24, despite possessing a similar methylating mechanism, was over 100-fold more potent than the clinical triazene TEM in a growth inhibitory assay (Table 5.1). Being a dual acting molecule designed to degrade ($t_{1/2}$=42 min) to the potent EGFR inhibitor, RB10, we also sought to determine whether RB24 owes its remarkable potency solely to the effects of the latter molecule. The results showed that RB24 was over 3-fold more potent than the free inhibitor RB10 against the DU145 cells (Table 5.1). This enhanced potency when compared with its daughter inhibitor (IC_{50}competitive binding = 40nM) indicates that perhaps the DNA lesions that it induced also contributed to its pronounced antiproliferative activity. It is noteworthy that RB24 was over 4-fold more potent than gefitinib (Table 5.1)

5.4.3. DNA damage and activation of the MAPK pathway

DU145 cells being AGT+ and virtually insensitive to TEM exposure, O6-methylguanine adducts were excluded as possible cytotoxic DNA lesions induced by RB24. Thus, we surmised that cytotoxic DNA damage may perhaps be induced through N-alkylated guanine or adenine bases, the repair of which is handled by BER and that perhaps blockade of EGFR-mediated signaling by RB24 may down-regulate XRCC1, a key player of BER. To this end, we analyzed signaling associated with cell response to DNA damage, chiefly through the MAPK pathway. Prior to the latter analysis, we first confirmed the presence of DNA damage in cells exposed to a dose range of RB24 using

Table 5.1. Antiproliferative effects of RB24 and TEM alone or in combination with cell signaling inhibitors in the prostate carcinoma cell line, DU145 .

Compounds	Antiproliferative activity (IC_{50}, μM)*	Combination Index
RB24	12 ± 2.6	-
RB10	38 ± 2.8	-
Gefitinib	49 ± 3.5	-
RB24+O6-BG	7 ± 1.4	0.34
RB24+ SB203580	30 ± 1.7	0.98
RB24 + PD98059	0.7 ± 0.03	0.03
RB24 + U0126	0.6 ± 0.04	0.03
TEM	486 ± 11.6	-
TEM+O6-BG	55 ± 1.1	0.31
TEM + SB203580	417 ± 9.6	1.51
TEM+PD98059	353 ± 5.8	1.38
TEM + U0126	316 ± 7.2	1.25
TEM + gefitinib	59 ± 6.7	0.23
TEM + gefitinib + U0126	25 ± 2.3	0.16
RB24 + gefitinib + U0126	18 ± 0.21	0.1
RB24 + gefitinib + U0126	2.5 ± 0.53	0.1

** Values are means of IC_{50} values and SEMs from three experiments.*

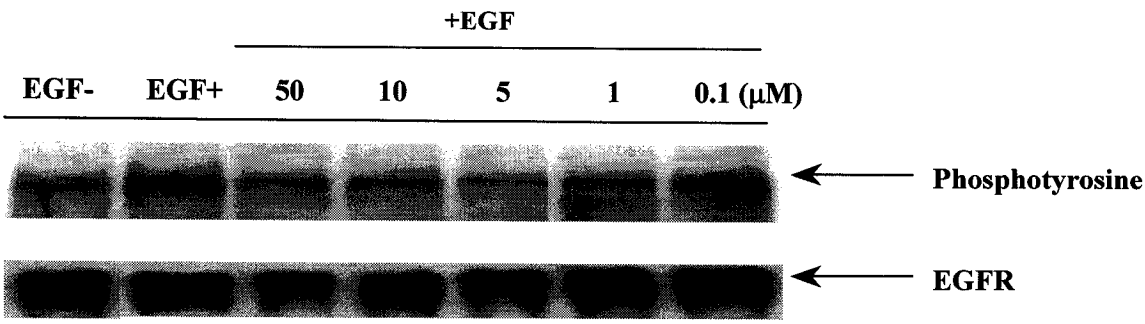

Figure 5.2. Inhibition of EGFR autophosphorylation in intact cells by RB24. Serum-starved DU145 cells were pre-incubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF (50ng/ml) for 15 min. Equal amount of cell lysate was analyzed by Western blotting using anti-phosphotyrosine and anti-EGFR antibodies. Band intensities were measured using the SynGene GeneTools software package.

the comet assay. Marked levels of DNA strand breaks were observed at concentration as low as 6 μM following a 2 h drug exposure and more importantly, levels of DNA damage by RB24 were approximately 3-fold higher than those by TEM (Fig. 5.3A). Further comet analysis at 2-48 h post treatment showed sustained levels of DNA damage in cells treated with RB24 (Fig. 5.3b). In contrast, at a dose of TEM that induced identical levels of DNA lesions, combined tail moment in TEM-treated cells returned to control levels as early as 18h-post-treatment, indicating rapid DNA repair (Fig. 5.3B). Thus, we analyzed the levels of the base excision repair protein XRCC1 triggered by the presence of DNA lesions upon exposure to: (a) RB24, (b) TEM and (c) RB24+EGF in comparison with TEM+EGF.

The results showed that serum-starved cells stimulated with EGF produced elevated levels of the XRCC1 protein. These levels were 2-fold higher in cells exposed to TEM + EGF (Fig. 5.3C). In the absence of EGF stimulation, TEM induced significant levels of XRCC1, presumably through a stress response pathway. Similarly, induction of XRCC1 was also observed with RB24 in the absence of EGF stimulation. However, in contrast to TEM, RB24 blocked induction of XRCC1 through EGF-stimulation leading to a 2-fold lower level of the latter protein in cells exposed to RB24 when compared with those exposed to TEM (Fig. 5.3C). The free inhibitor RB10 that does not damage DNA did not trigger expression of XRCC1. However, it completely suppressed its expression through EGF stimulation, suggesting that the residual levels of XRCC1 observed following treatment with RB24 is associated with its DNA damaging

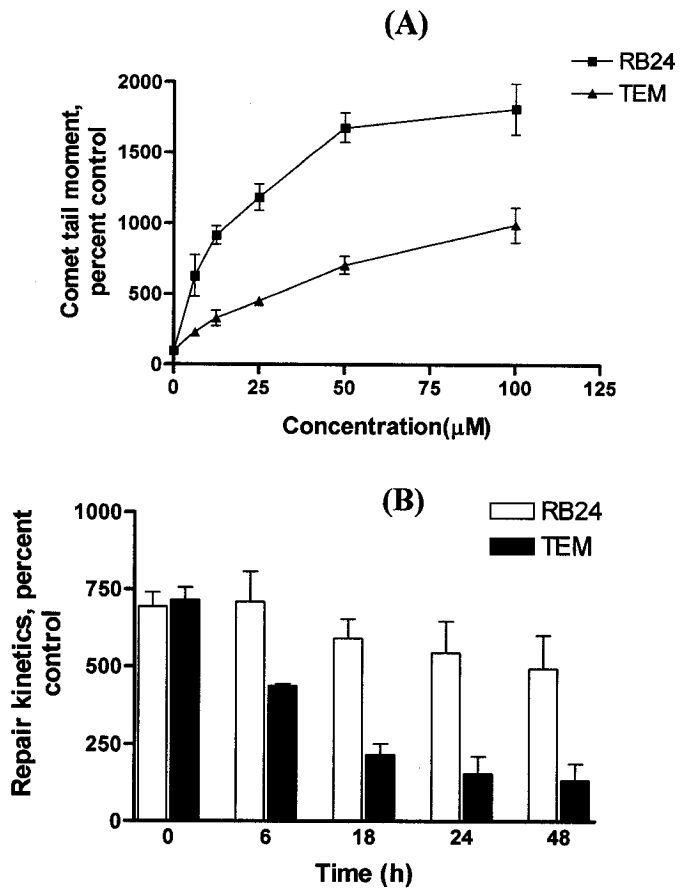

Figure 5.3. Effects of RB24 or TEM on levels of DNA damage, XRCC1 and activation of stress response in the DU145 cell line. A, DU145 cells were treated with RB24 (■) or TEM (▲) for 2 h and levels of DNA damage were quantitated using the alkaline comet assay. Tail moment (i.e., the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet) was used as a parameter for the detection of DNA damage in cells exposed to RB24 or TEM. *Points*, mean percentage of untreated cells (n=3); *bars*, SD. B, comet assay analysis of DU145 cells pretreated with RB24 (*white columns*) or TEM (*black columns*) for 2 h followed by washout and replacement of drug-containing media with fresh media. Cells were allowed to recover for multiple timepoints. C*olumns,* mean of three experiments; *bars*, SD.

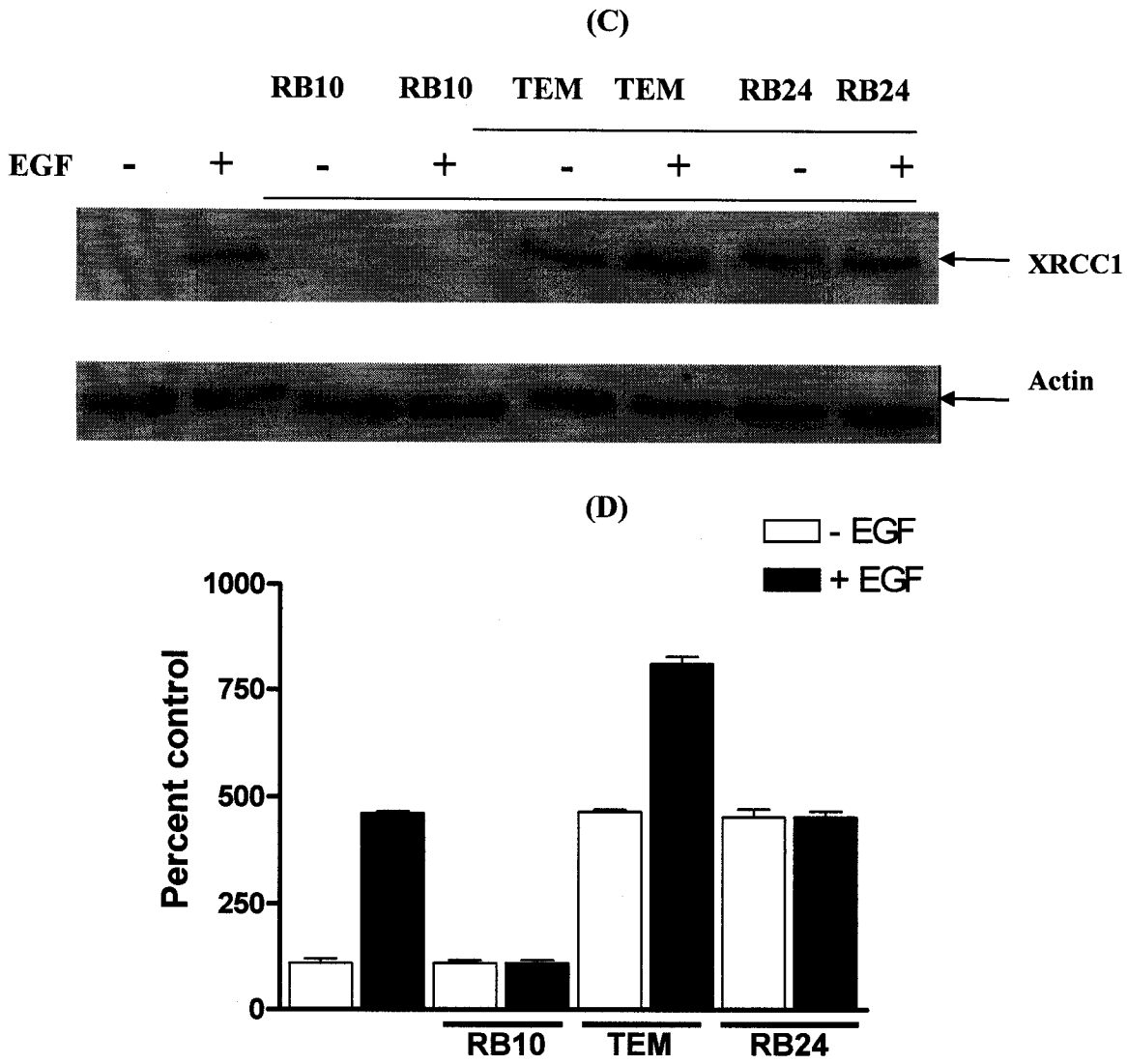

Figure 5.3. Effects of RB24 or TEM on levels of DNA damage, XRCC1 and activation of stress response in the DU145 cell line. C, serum-starved DU145 cells were preincubated for 2 h with RB24, RB10 or TEM following treatment with or without EGF (20ng/ml) for 5 h. Cell lysates were isolated and analyzed by Western blot for indicated proteins. Quantitation of XRCC1 protein levels were measured using the SynGene GeneTools software package as. *Columns*, ratio of XRCC1/actin and mean of three experiments; *bars*, SD.

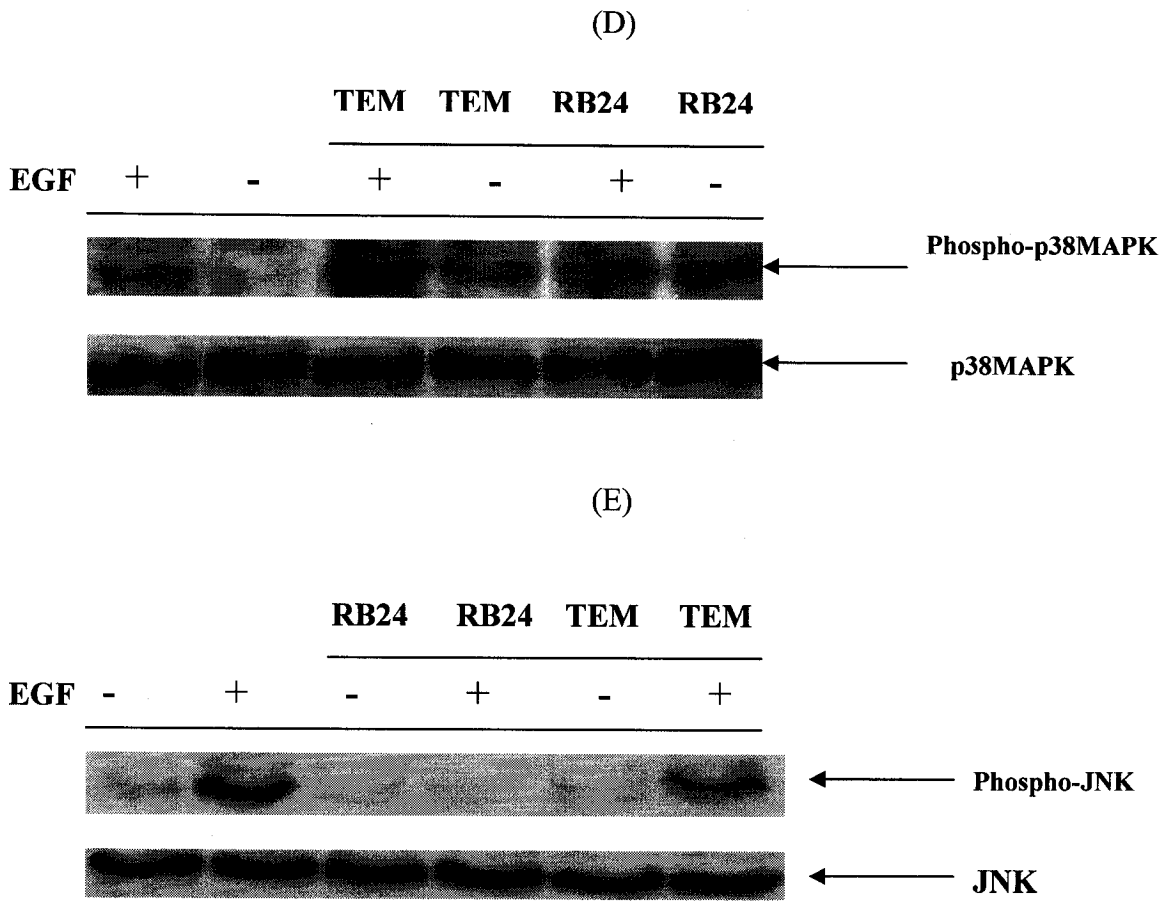

Figure 5.3. Effects of RB24 or TEM on levels of DNA damage, XRCC1 and activation of stress response in the DU145 cell line. D, E p38MAPK and JNK activation of serum-starved DU145 cells exposed for 2 h to RB24 or TEM. Following compound exposure, cells were treated with or without EGF (20ng/ml) for 3 h. Equal amount of cell lysate was analyzed by Western blot and probed for anti-phosphop38MAPK, anti-phosphoJNK, anti-JNK or anti-p38MAPK antibodies.

component. Thus, we further characterized the path leading to XRCC1 expression in the absence of EGF stimulation.

Exposure to DNA damage of the types induced by methylating agents results in the activation of complex signaling pathways including the ATM and MAPK cascades (Mabuchi *et al.*, 2002; Uzgare *et al.*, 2003; Hirose *et al.*, 2003; Stojic *et al.*, 2004). Based upon studies demonstrating the involvement of p38MAPK and JNK activation following DNA damage induced in cells treated with alkylating agents (Uzgare *et al.*, 2003; Hirose *et al.*, 2003; Stojic *et al.*, 2004), we investigated the phosphorylation status of the latter proteins both under EGF-induced mitogenic signaling and under direct exposure to DNA damage. The results showed markedly elevated levels of phospho-p38MAPK both in cells exposed to TEM or RB24 in the absence of EGF. However, in the presence of EGF, the activation of p38MAPK followed the same trend as that observed for EGF-induced XRCC1 expression in response to DNA methylation. Under EGF-stimulation, RB24 induced approximately 2-fold less activation of p38MAPK than TEM (Fig. 5.3D). Direct cell exposure to TEM or RB24 did not activate JNK, indicating that XRCC1 expression is not induced through the JNK pathway (Fig. 5.3E). Thus, under EGF stimulation and exposure to DNA damage, the DU145 cells are protected by a tandem induction of XRCC1 expression both through EGFR-mediated mitogenic signaling and through DNA damage-induced stress response, culminating into a robust resistance to DNA lesions.

5.4.4. Inhibition of anti-apoptotic signaling by the combi-molecule

Following EGFR activation, numerous signaling cascades are induced that regulate not

only cellular proliferation and growth, but can also promote cell survival. One of the key anti-apoptotic signaling pathways that are regulated by EGFR, is the PI3K/Akt pathway that ultimately lead to Bad activation (Mitsiades *et al.*, 2004). Phosphorylation of Bad by Akt inhibits binding to Bcl-xL, leading to a blockade of the apoptotic pathway (Mabuchi *et al.*, 2002). Thus, we examined the ability of our combi-molecule, RB24, to inhibit phosphorylation of Bad via inhibition of EGFR activation, a process that could alleviate the apoptotic blockade. Western blot analyses showed that RB24 inhibited phosphorylation of Bad at Ser136, the target of Akt, in a dose-dependent manner (Fig. 5.4A). It is also known that activation of Bad through phosphorylation at a different serine residue, Ser112, is regulated by the MAPK family. Therefore, we studied the ability of RB24 to inhibit phosphorylation of Bad through the MAPK cascade. The results showed that RB24 was capable of inhibiting Bad phosphorylation at Ser112 after a pulse 2 h exposure. However, when DU145 cells were treated with RB24 alone over a 6 h period, we observed partial recovery of Bad (Fig. 5.4B). In contrast, DU145 cells exposed to a dose-range (3-100μM) of the methylating agent TEM, resulted in no inhibition of Bad phosphorylation on both the aforementioned serine residues (data no shown).

To determine whether the pleiotropic effects of RB24 (i.e. DNA damage, EGFR blockade, inhibition of Bad phosphorylation) would culminate into enhanced apoptosis, we examined annexin V binding to DU145 cells previously treated with RB24 or TEM. In contrast to TEM, RB24 induced significant levels of apoptosis ($p<0.01$) after a 24 h drug exposure (Fig. 5.4C).

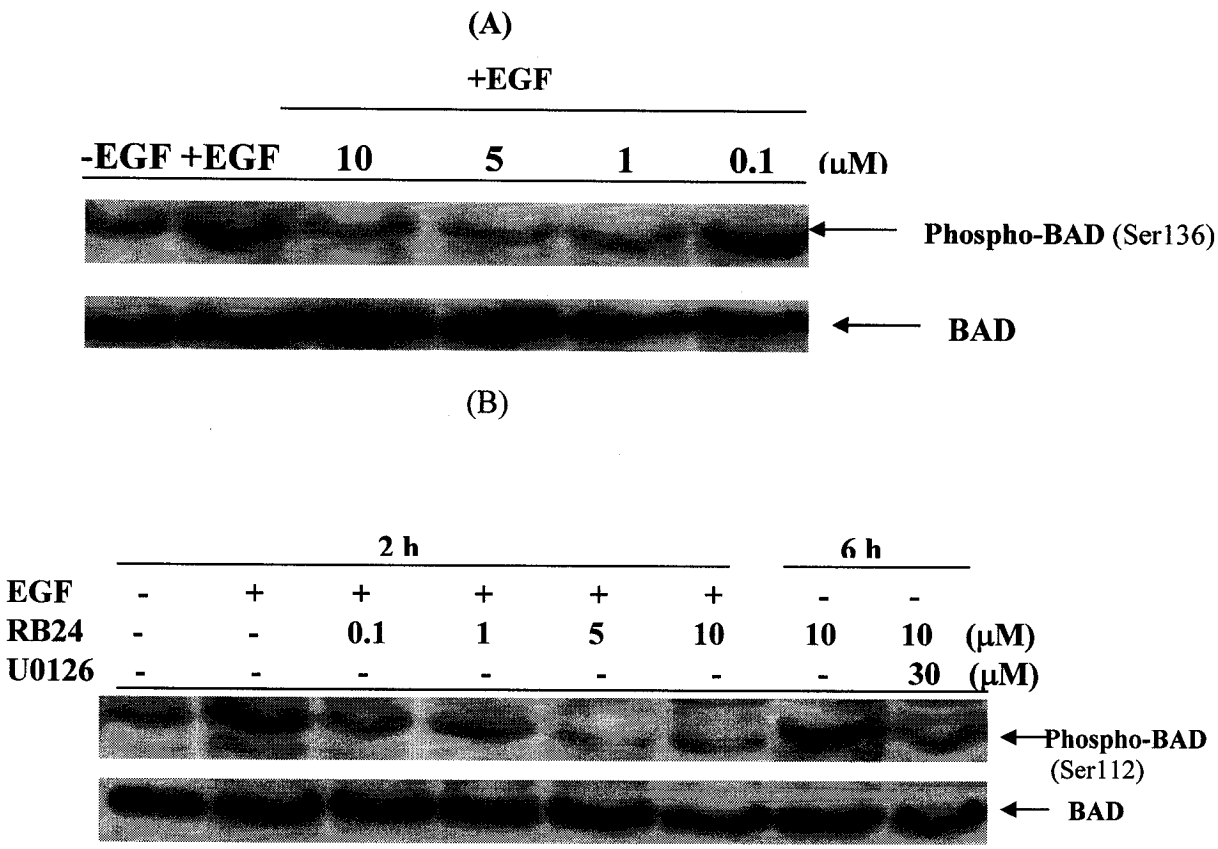

Figure 5.4. Assessment of the apoptotic effects of RB24 alone or in combination with cell signaling inhibitors in DU145 cells. A, levels of Bad phosphorylation at Ser136 were examined in serum-starved cells that were preincubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF (25ng/ml) for 15 min. In B, serum-starved cells were exposed to RB24 for 2h prior to EGF (25/mg/ml) stimulation (15 min) or treated with RB24 for 6h at the indicated doses alone or in combination with U0126. Equal amounts of cell lysates were analyzed by Western blot using anti-phosphoBAD (Ser 136), anti-phosphoBAD (Ser 112), or anti-BAD antibodies.

(C)

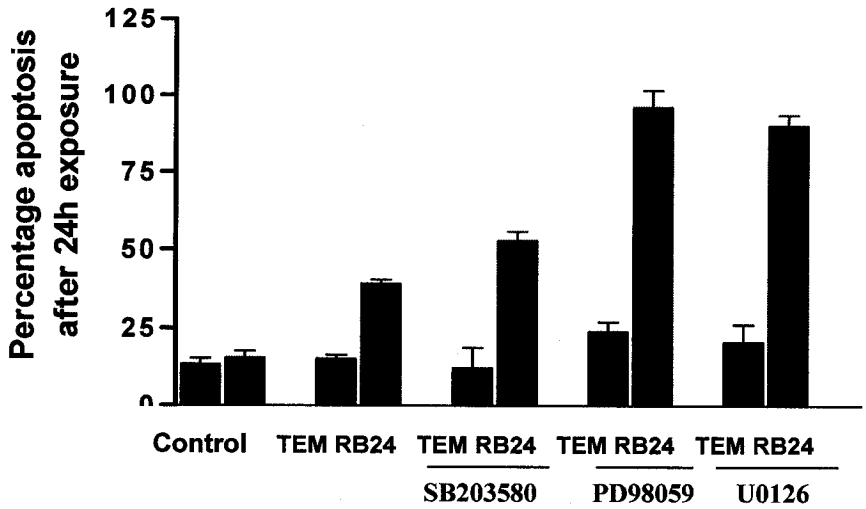

Figure 5.4. Assessment of the apoptotic effects of RB24 or TEM alone or in combination with cell signaling inhibitors in DU145 cells. C, DU145 cells were treated with 25 μM RB24 or TEM alone or in combination with SB203580, PD98059, U0126 for 48 h. Levels of apoptotic cells were determined using Annexin V-FITC staining. *Columns*, mean percentage of Annexin-V-negative cells (n=4); *bars*, SD.

5.4.5. Enhancing the potency of the combi-molecule

In addition to being a masked form of a two-drug combination, the combi-molecule is a single molecule. Therefore, it can be combined with other antitumour agents. Studies on the multiple signaling pathways indicated that blockade of EGFR-mediated signaling did not suffice to completely suppress XRCC1 expression in these cells. Thus, p38MAPK, the activation of which does not require EGFR phosphorylation, was pointed as a logical target to enhance the potency of the DNA damaging component of RB24. The results showed that blockade of p38MAPK with SB203580 at an equi-effective dose induced an additive effect (Table 5.1).

Having found that inhibition of MEK1-related phosphorylation of Bad was restored rapidly and with current knowledge of its significant involvement in cross-talk between mitogenic, apoptotic and stress signaling, MEK1 appeared as a critical target for enhancement of the potency of the combi-molecule. The results showed that while the combination of a p38MAPK inhibitor with RB24 yielded minimal inhibition of XRCC1 expression, inhibition of its upstream MEK kinase with PD98059 induced significant depletion of the latter protein in the cells and significant depletion of Bad phosphorylation when combined with U0126 (Fig. 5.4B, 5.5A). This translated into substantial levels of apoptosis (Fig. 5.4C) and a unique combination synergy (CI_{50}=0.03) in the SRB antiproliferative assay (Table 5.1, Fig 5.5B). In contrast, the potency of the triazene TEM could only be moderately enhanced by the MEK inhibitors, PD98059 and U0126 (Table 5.1, Fig. 5.5C).

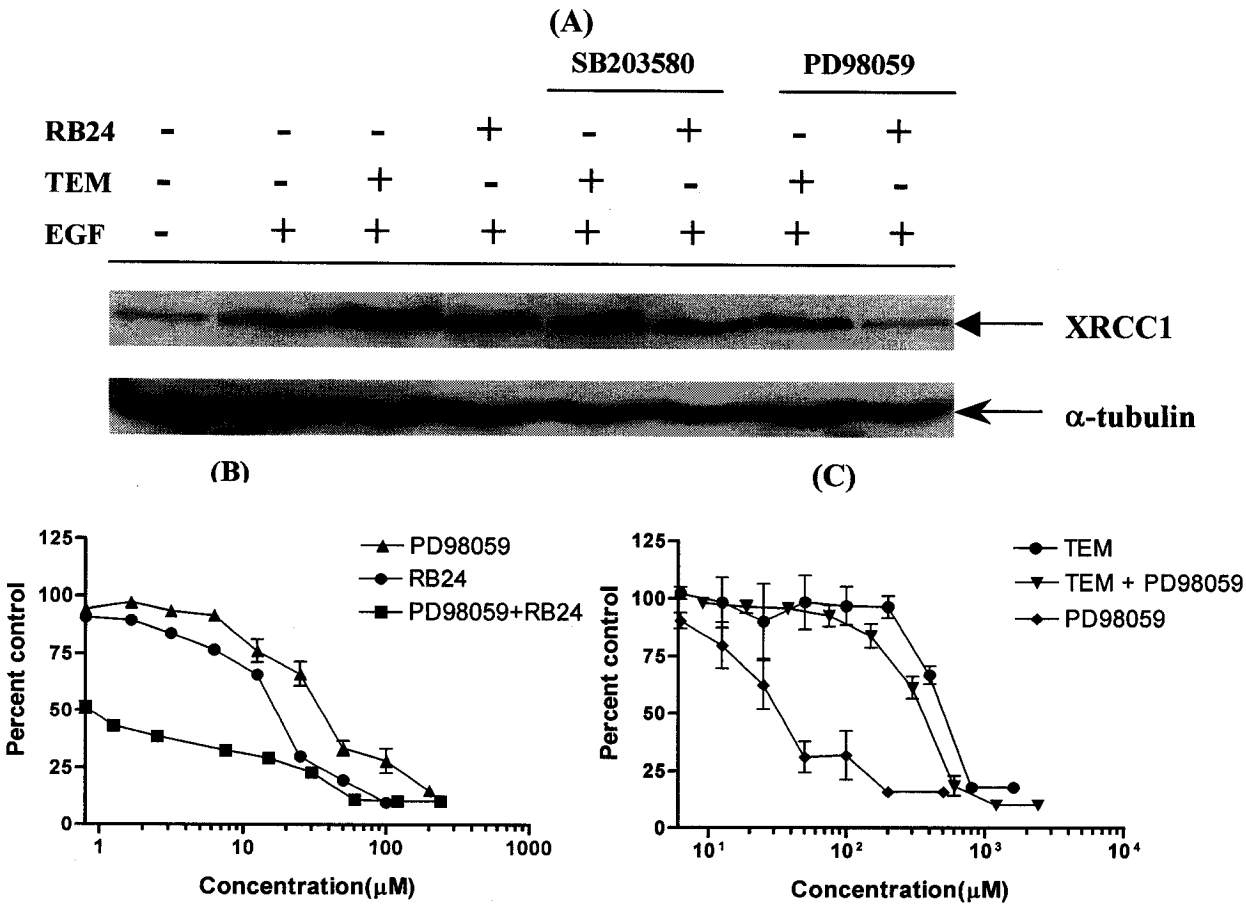

Figure 5.5. Characterization of the enhancement in growth inhibitory activity of RB24 or TEM when in combined with SB203580 or PD98059. A, serum-starved DU145 cells were exposed to RB24 or TEM alone or in combination with either SB203580 or PD98059 for 2h followed by a 5 h exposure to EGF (20ng/ml). Levels of the BER protein, XRCC1, were analyzed by Western blot using anti-XRCC1 or anti-α-tubulin antibodies. B, antiproliferative activity studies were carried in DU145 cells exposed to RB24 (●) or the MEK1 inhibitor, PD98059 (▲), alone or in combination (■) for a continuous 6 days. C, cell growth inhibition studies in DU145 cells treated for 6 days continuously with TEM (●) or PD98059 (♦), alone or in combination (▼). Antiproliferative activity was determined using the SRB assay. *Points*, mean of three experiments run in triplicate; *bars*, SD.

5.4.6. Deconvolution of the potency of the combi-molecule and discovery of a novel combination formula

Since RB24 possesses both EGFR inhibitory and DNA damaging properties, we sought to determine whether a triple combination of TEM (clinical DNA damaging)+gefitinib (clinical EGFR inhibitor) +U0126 (potent MEK1 inhibitor) would prove synergistic or more efficacious than the 2-drug combination RB24+MEK1 inhibitor. Although the combination of TEM+gefitinib+U0126 did result in a synergistic activity, that of RB24+U0126 was more than 20-fold more potent than the latter formula at equi-effective doses (Table 5.1). When using RB24 in a triple drug combination with either TEM+U0126 or gefitinib+U0126, the results indicated that both triple combinations were synergistic, but still remained approximately 30- or 4-fold less advantageous than the two-drug combination of RB24 + U0126 (or PD59068) at equi-effective doses, respectively (Table 5.1).

5.5. Discussion

The potency of DNA alkylating agents is significantly limited by the expression of DNA repair enzymes that process the DNA adducts in resistant tumour cells (Pegg *et al.*, 1995; Miknyoczki *et al.*, 2003; Yacoub *et al.*, 2003). To circumvent this problem, numerous investigations have been directed at sensitizing cells using DNA repair inhibitors (Cai *et al.*, 2000; Horton *et al.*, 2000; Miknyoczki *et al.*, 2003). Despite the significant advances on the molecular biology of stress response, strategies designed to sensitize the cells using relevant signal transduction pathways are scant. As an example, the potency of TEM is enhanced by the use of O6-benzylguanine, a direct inhibitor of AGT (Cai *et al.*, 2000; Quinn *et al.*, 2005), or by inhibitors of PARP (Miknyoczki *et al.*,

2003; Tentori *et al.*, 2003). More recent studies have used methoxylamine to indirectly deplete BER and sensitize cells to TEM (Liu and Gerson, 2004). Here we exploited the relationship between DNA repair and EGFR-mediated signaling to design a single molecule capable of inducing a tandem damage of DNA and blocking of signal transduction. Indeed, the results presented herein showed that RB24 could block XRCC1 expression through the depletion of EGFR phosphorylation while being capable of damaging DNA. Unfortunately, being a DNA damaging agent, it also induced XRCC1 presumably through activation of the stress response pathway. The ability of RB24 to down-regulate XRCC1 through EGFR inhibition led to an overall two-fold lower level of EGF-stimulated XRCC1 in DU145 cells when compared with TEM-treated ones. Indeed, cell exposure to TEM induced elevation of XRCC1 through both the stress response pathway and EGF stimulation, leading to an additive augmentation of the latter DNA repair protein. The implication of the stress response pathway in the induction of XRCC1 in the absence of EGF-stimulation was ascertained by the marked upregulation of phospho-p38MAPK in these cells when RB24 or TEM were directly administered to the cells alone. Thus, this study led to the discovery that additive induction of XRCC1, through EGFR activation and alternative pathways, may be at least partly responsible for resistance to DNA methylating agents. TEM, being a simple alkylating agent, was incapable of blocking the elevation of XRCC1 levels associated with EGFR TK activation. In contrast, by strongly blocking EGFR, the combi-molecule was able to deplete the base excision repair protein and more importantly, we demonstrated that this was associated with a remarkable delay in DNA strand break repair in these cells. It is important to note herein that taking into account the ability of the combi-molecules to induce 3-fold higher levels DNA damage than TEM, the analysis was performed at

concentrations that inflict equi-level of DNA strand breaks by these two agents. Therefore the observed delay is truly dependent upon biological factors and not to levels of DNA damage. Thus, the complex combi-molecule RB24 is the first example of a DNA damaging molecule capable of both inducing DNA lesions while capable of preventing the repair of the latter damage.

Furthermore, despite the fact that DU145 cells are protected by anti-apoptotic mechanisms driven by EGFR-mediated activation of the Akt-Bad cascade as well as overexpression of Bcl-2 (Eisenmann *et al.*, 2003; Mitsiades *et al.*, 2004; Xiao and Singh, 2006), the combi-molecule was capable of inducing marked levels of apoptosis in these cells. This again can be rationalized in light of its ability to block EGFR activation as confirmed by the significant inhibition of Bad phosphorylation at serine 136, which is a target of Akt (Mabuchi *et al.*, 2002). It is now well known that Bad phosphorylation prevents its heterodimerization with Bcl-xL, thereby allowing it to sequester Bax and subsequently interrupt mitochondrial pore formation. In contrast to the combi-molecule, TEM, that was deprived of anti-signaling properties, could only induce a barely detectable level of apoptosis in these cells. This is in agreement with the constant activation of Bad observed in TEM-exposed cells even at supralethal doses.

Increase in levels of XRCC1 produced through the activation of the stress response pathway was not completely abolished by the combi-molecule. Thus, we surmised that strategies designed to enhance the potency of RB24 should be directed at blocking key players of the stress response pathway that may be activated upon induction of DNA damage. It is now known that DNA lesions trigger activation of ATM or c-abl, which in

turn lead to further activation of members of the MAPK pathway (Stojic *et al.*, 2004). Thereafter, the repair of certain types of DNA lesions can be mediated either through JNK activation (e.g. cisplatin adducts) or through p38MAPK phosphorylation (e.g. methylating agents) which is then followed by induction of early response genes (Uzgare *et al.*, 2003; Hirose *et al.*, 2003; Stojic *et al.*, 2004). Since JNK was not activated upon exposure to RB24 and TEM, response to DNA damage induced by the latter drugs may be mediated primarily through p38MAPK activation. Indeed blockade of p38MAPK enhanced the potency of RB24 producing an additive effect which may be associated with partial depletion of XRCC1.

MEK1 is known to be involved in cross-talk between various cascades including growth factor stimulation and antiapoptotic signaling (Yacoub *et al.*, 2003; Maroni *et al.*, 2004; Stojic *et al.*, 2004). Further, previous studies by Yacoub *et al.* (2003) demonstrated that blockade of MEK1 by PD98059 led to significant depletion of XRCC1 in γ-irradiated DU145 cells. Similarly, marked reduction of XRCC1 levels was seen when PD98059 or U0126 were co-administered with RB24 or TEM. However, in RB24-treated cells, levels of apoptosis induced by the latter treatment were remarkably higher than any treatment that depleted XRCC1 in this study. This unique synergy can be explained by the blockade of reactivation of Bad by MEK1 signaling. Indeed inhibition of MEK1 through EGFR was reversible, being recovered as early as 6 h after treatment. It is noteworthy that in the cell growth inhibitory assay, the potency of RB24+PD98059 or U0126 combinations were superior to that of classical combinations involving RB24+O6-BG, TEM+O6-BG or 3-drug combination principles

TEM+gefitinib+PD98059 or U0126, at equi-effective concentrations. This new discovery demonstrates that while the combi-molecule principle can produce superior antiproliferative activity with a single molecule in lieu of two, it can also produce a similar effect with two- instead of three-way combinations. It is now known that the reduction of the number of drugs in a combination may decrease the risk of additive toxicity (Powis, 1991).

This study conclusively demonstrates that the combi-molecular strategy can be applied to significantly modify the biological activity of classical pharmacophores. Here, it has offered the opportunity to study two DNA methylating agents with the same DNA damage pharmacophore (an alkyltriazene function) but with substantially different antiproliferative activity profiles: one of them being virtually inactive and the other exquisitely potent against a resistant prostate tumour cell line. We also demonstrated that this difference is imputed to the additional growth factor receptor TK inhibitory property imprinted in the molecule that induced a tandem depletion of DNA repair protein and inactivation of anti-apoptotic signaling while high level cytocidal DNA lesions were being inflicted. Further, we outlined herein that in addition to being a combi-molecule, RB24 can exert additive and synergistic effects in combination with cell signaling inhibitors that disrupt alternative pathways in the cells. This novel strategy sets premise for exploring new avenues in the development of novel types of alkylating agents for the therapy of growth factor receptor-expressing refractory tumours.

5.6. Acknowledgements

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5. 8. Connecting text

In the preceding chapter, we demonstrated that the binary targeting properties of the cascade release combi-molecule could induce significant antiproliferative activity through a co-operative and multi-targeting mechanism in a cell line known to express various survival characteristics (e.g. autocrine expression of EGF and EGFR, expression of AGT, overexpression of Bcl-2). In the following chapter, we will determine whether this translates into anti-tumour activity *in vivo* using the more water-soluble 3'-chloro analogue of RB24, RB107. We will also demonstrate that *in vivo*, RB24 can fragment into the predicted sub-components of the cascade.

CHAPTER 6

DEGRADATION, CYTOKINETICS AND PHARMACOANALYSIS OF ACETOXYMETHYLTRIAZENES TERMED CASCADE RELEASE COMBI-MOLECULES DESIGNED TO INHIBIT THE EPIDERMAL GROWTH FACTOR RECEPTOR WHILE DAMAGING DNA

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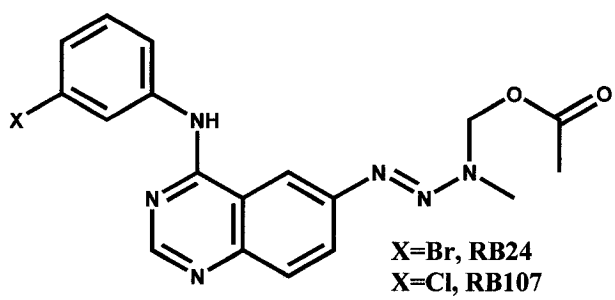
6.1. Abstract

Within the context of a new strategy termed "Combi-Targeting", one class of molecule containing an acetoxymethyltriazene was designed to degrade into multiple inhibitors of EGFR TK + a methyldiazonium species. Although sustained antiproliferative activity was induced *in vitro* by the lead combi-molecule, RB24, also known as a "cascade release" combi-molecule, the characterization of its *in vivo* degradation as well as its antitumour activity in mice models remain to be demonstrated. Here we report the first evidence for the formation of the diverse metabolites *in vivo* following i.v. injection of RB24, using LC-MS analysis. The potency of the cascade release approach *in vivo* was demonstrated with a more water-soluble analogue of RB24, RB107, in alkylating-agent-resistant DU145 human prostate carcinoma xenograft model. *In vitro*, RB107 was shown to induce the same cell response profile as RB24, with significant EGFR/DNA targeting potential and almost identical effect on cell cycle progression. They induced significant cell cycle arrest in G1 at low doses and mild G2/M arrest at higher doses. RB107 was tolerated at doses as high as 200 mg/kg and induced significant antitumour activity at 15 days post-treatment (100 mg/kg, i.p. injection, once every other day). Its activity was superior to that of the clinical EGFR TK inhibitor, gefitinib, and the triazene, Temodal™ (TEM) administered alone at equi-doses (100 mg/kg). The combi-molecule also showed superior potency when compared with classical combination of TEM+gefitinib at equi-doses (50 mg/kg). The results *in toto* indicate that the cascade release approach leads to the stepwise release of multiple bioactive species in plasma and translates into significant antiproliferative activity *in vivo*.

6.2. Introduction

The epidermal growth factor receptor (EGFR) is often overexpressed in breast, ovarian and prostate carcinomas (Hu and Xing, 2003; Shelton *et al.*, 2005; Baszley and Gullick, 2005). Aberrant expression of EGFR is associated with tumour invasiveness, enhanced anti-apoptotic signaling and reduced drug sensitivity (Modjtahedi and Dean, 1994; Baszley and Gullick, 2005). In search for new strategies to sensitize EGFR-expressing tumour cells to cancer therapeutics, we recently developed a strategy termed "combi-targeting"(Matheson *et al.*, 2001; Brahimi *et al.,* 2002; Qiu *et al.*, 2003; Matheson *et al.*, 2003; Rachid *et al.*, 2003; Banerjee *et al.*, 2003; Qiu *et al.*, 2004; Banerjee *et al.*, 2004, Matheson *et al.*, 2004a; Matheson *et al.*, 2004b). This strategy was based on the premise that blockade of EGFR would down-regulate anti-apoptotic signaling, as well as DNA repair enzymes responsible for tumour cell resistance to cytotoxic DNA alkylating agents. Thus, we designed a novel class of pleiotropic drugs containing a quinazoline moiety to block the tyrosine kinase (TK) activity of EGFR + a triazene chain "programmed" to release a DNA damaging species upon hydrolysis. This new class of molecule termed "combi-molecule" has now been shown to induce significant antiproliferative activity in EGFR-expressing cells that are resistant to Temodal® (TEM), a clinical DNA damaging agent of the same class (Matheson *et al.*, 2001; Qiu *et al.*, 2003; Matheson *et al.*, 2003; Banerjee *et al.*, 2003; Banerjee *et al.*, 2004, Matheson *et al.*, 2004a). Combi-molecules of the triazene class are particularly useful probes for the combi-targeting concept due to their small size and their reasonable rate of hydrolysis under physiological conditions. SMA41, the first combi-molecule or combi-triazene used to prove the feasibility of this concept, induced DNA damage and blocked EGFR autophosphorylation in the human TEM-resistant, A431 carcinoma of the vulva cell line

(Matheson *et al.*, 2001). It also induced significant antiproliferative activity *in vivo* at high doses (Matheson *et al.*, 2004b). However, the rather moderate EGFR TK inhibitory of its released inhibitor SMA52 (IC_{50}=1μM) and its poor water solubility precluded its further development. To circumvent these problems, we designed a new class of molecule with two major features: a) replacement of the methyl by a less bulky chloro- or bromo-group to enhance interaction with the hydrophobic pocket and b) appendage of an acetoxymethyl to the N3 position of the triazene chain to promote water solubility. These novel compounds termed "cascade release" combi-molecules, that owe their names to their ability to be hydrolyzed into multiple inhibitors of EGFR TK, were designed to generate more potent monoalkyltriazene analogues of SMA41 (e.g. BJ2000, IC_{50}=0.1μM; ZR08, IC_{50}=0.039μM) (Brahimi *et al.*, 2002; Rachid *et al.*, 2003; Banerjee *et al.*, 2003). The ability of the metabolites generated during the degradation cascade to block EGFR was proven by independent synthesis of each of the degradation products and their testing in an ELISA for isolated EGFR and whole cell assay involving EGF-induced EGFR autophosphorylation. Because of the rapid conversion of the entire combi-molecule into the final steps of the cascade, the formation of each of the transient metabolites *in vivo* remained hypothetical. Thus, in this study we sought to analyze the



Scheme 6.1

cascade using LC-MS analysis of plasma samples collected immediately or 5 min following i.v. injection. RB24, the most studied cascade release combi-molecule (Banerjee *et al.*, 2003; Banerjee *et al.*, 2004; Banerjee *et al.*, 2006), was used for analysis of the cascade *in vivo* and its chloro-analogue, RB107, being more water-soluble, was used for efficacy studies (see Scheme 6.1). We show herein that its cellular response profiles parallel that of RB24 and examine its antiproliferative potency in a DU145 prostate cancer xenograft model.

6.3. Materials and Methods

6.3.1. Drug Treatment

RB24, RB107, RB105, BJ2000, RB10 and FD105 were synthesized in our laboratory according to known procedures (Banerjee *et al.*, 2003; Rachid *et al.*, 2003). Temodal® (TEM) was provided by Schering-Plough Inc. (Kenilworth, NJ, USA). Gefitinib was provided by AstraZeneca. In all assays, drug was dissolved in DMSO and subsequently diluted in RPMI-1640 containing 10% fetal bovine serum (FBS) (Wisent Inc., St-Bruno, QC, Canada) immediately before the treatment of cell cultures and the concentration of DMSO never exceeded 0.2% (v/v).

6.3.2. Cell Culture

The cell line used in this study was the human prostate carcinoma, DU145, obtained from the American Type Culture Collection (Manassas, VA). The cell line was maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics as described previously (Matheson *et al.*, 2001; Banerjee *et al.*, 2004). Cells were maintained in a monolayer at 37 °C in a humidified environment of 5% CO_2, 95% air.

The cultures were maintained in logarithmic growth by harvesting with a trypsin-EDTA (0.5 mg/mL trypsin and 0.2 mg/mL EDTA) and replating before confluence.

6.3.3. EGFR Kinase Enzyme Assay

The EGFR kinase assay is similar to the one previously described (Matheson *et al.*, 2001; Banerjee *et al.*, 2003). MaxiSorp 96-well plates (Nalge Nunc international, Naperville, IL) were incubated overnight at 37°C with 100 µL/well of 0.25 ng/mL poly [L-glutamic acid –L-tyrosine, 4:1] (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 using 4.5 ng/well EGFR affinity-purified from A431 cells (Matheson *et al.*, 2001; Banerjee *et al.*, 2003). The compound was added and phosphorylation initiated by the addition of ATP (20 µM). After 8 min at room temperature, the reaction was terminated by aspiration of the reaction mixture and 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 µg/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 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 to stop the reaction. The plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550; Bio-Rad, Hercules, CA).

6.3.4. *In vitro* Growth Inhibition Assay

To study the effect of our compounds on cellular proliferation, cells were grown in 96-well plates and exposed to a dose range of each drug for 6 days. Cell growth was measured using the sulforhodamine B (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 read for each well at 492 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

6.3.5. Autophosphorylation Assay

DU145 cells ($1x10^6$) were preincubated in a six-well plate with 10% serum at 37°C for 24 h and starved overnight for 18 h, after which they were exposed to a dose range of RB107 for 2 h and subsequently treated with 50 ng/mL EGF for 15 min at 37°C. Cells 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, 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. The concentrations of protein were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were added to a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene

difluoride membrane (Millipore, Bedford, MA). Non-specific binding on the membranes were minimized with a blocking buffer containing nonfat dry milk (5%) in PBST. Thereafter, the membranes were incubated with primary anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) for the detection of phosphotyrosine. Membranes were stripped and re-probed with anti-EGFR (Neomarkers, Fremont, CA) for determination of corresponding receptor levels. Blots were incubated with HRP-goat anti-mouse antibody (1:1000 dilution; Cell Signaling Research, Beverly, MA) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Matheson *et al.*, 2001).

6.3.6. Alkaline Comet Assay

The alkaline comet assay technique was used to quantitate DNA strand breaks induced by RB107 and TEM in DU145 cells. The cells were exposed to drugs for 2 h and harvested with trypsin-EDTA. They were subsequently collected by centrifugation and re-suspended in PBS. The resulting cell suspensions were diluted to $3x10^5$ cell/mL, and mixed with 0.75% agarose in PBS at 37°C in a 1:9 dilution. The gels were cast on Gelband strips (Mandel Scientific, Guelph, ON, Canada) using gel casting chambers, as previously described, then immediately placed into a lysis buffer (2.5 M NaCl, 0.1 M tetrasodium 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 immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetrasodium 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 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, 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 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 (McNamee *et al.*, 2000)

6.3.7. Flow Cytometric Analysis of Cell Cycle Progression

DU145 cells ($1x10^6$) were preincubated in six-well plates with 10% serum at 37°C for 24 h. Thereafter, cells were treated with the indicated dose range of RB24, RB107, RB10, FD105 and TEM for 24 h. The cells were harvested with trypsin-EDTA, centrifuged and re-suspended in PBS. Vindelov's solution [0.01M Tris-base, 10mM NaCl, 700 U RNase, $7.5x10^{-5}$ M propidium iodide, 0.1% NP-40] was added (400µl) for 10min at 37°C, and the fluorescence (FL2) detected DNA content was measured using a Becton Dickinson Flow Cytometer.

6.3.8. Pharmacoanalysis

The drug, RB24, was administered i.v. to CD-1 male mice (3/group/time point) (Charles River, Saint-Constant, QC, Canada) at 100 mg/kg dose in 0.4 mL of a Tween 80 (6%)/ethanol (10%) saline solution. Blood samples were collected in heparinized tubes

either immediately or 5 min post-injection administration and centrifuged for 8 min at 3000 rpm and plasma separated with a micropipette and stored at – 80 °C. Plasma aliquots (50μL) were mixed with acetonitrile (100 μL) to precipitate the proteins and the mixture centrifuged for 5 min at 6000 rpm. Samples were analyzed by LC-MS using a Discovery C18 (15cmx3mm, 5mm) column at pH=9 to identify the peaks corresponding to the expected metabolites.

6.3.9. Mouse Xenograft Studies (DU145)

CD-1 nude male mice (River, Saint-Constant, QC, Canada) were maintained as per McGill animal safety protocols. Dose finding was performed with 2 CD-1 nude mice/group and the maximum tolerated dose defined as dose that does not induce more than 15% weight loss over a period of at least 14 days. For xenograft studies, mice were treated with $1x10^6$ cells suspended in 0.2 mL PBS injected subcutaneously into the flank of each mouse. Treatments began when tumors became palpable. The animals were placed into treatment groups of 6 mice each and one control group that received the vehicle for RB107(Tween 80 (6%)/ethanol (10%) in saline), TEM (10% DMSO in saline) and gefitinib (10% Tween 80 in saline). The treatment groups were given RB107 (e.g. 100mg/kg), TEM (100 mg/kg) or gefitinib (100 mg/kg) in 0.4 mL volume i.p. every other day. Tumor burdens were measured before each injection and tumor volume was calculated using the formula $TV = [(\text{tumor width} + \text{tumor length})/4]^3 \times 4/3 \times 3.14159$. Statistical significance was determined using a two-tailed Student t-test (n=6).

6.4. Results

6.4.1. *In vitro* analyses

6.4.1.1. *Binary EGFR/DNA targeting properties*

The dual EGFR/DNA targeting properties of RB107 were characterized by Western blotting of EGF-stimulated DU145 cells and the single-cell microelectrophoresis (comet) assay. Using an ELISA, we first demonstrated that RB107 was capable of blocking activation of isolated EGFR in a dose-dependent manner with an IC_{50} of 0.28μM (Table 6.1). Likewise, Western blotting results showed a dose-dependent blockade of EGF-induced EGFR phosphorylation (IC_{50}=4μM) (Fig.6.1a).

On the other hand, comet analysis following a 2 hour drug treatment, a time period that allows maximal intracellular decomposition of RB107, demonstrated that the drug induced significant levels of DNA damage (Fig. 6.2b). More importantly, these levels were approximately 1.5-fold higher than that inflicted by the clinical drug, Temodal™ (TEM) (Fig.6.2b).

6.4.1.2. *Antiproliferative activity*

Having demonstrated the binary targeting properties of RB107, we examined its antiproliferative activity in the DU145 cell line that has already been shown to be sensitive to EGFR inhibitors *in vitro* (Vicentini *et al.*, 2003; Sgambato *et al.*, 2004). More importantly, DU145 cells also express the DNA repair enzyme O^6-alkylguanine transferase (AGT) that is known to confer resistance to methylating agents of the same

Table 6.1. Inhibition of EGFR TK activation and antiproliferative activity in DU145 cells by RB24, RB107, RB105, BJ2000, FD105, TEM and gefitinib.

Compound	IC_{50} competitive binding (μM)	Antiproliferative activity (IC_{50}, μM)
RB24	**0.12**[a]	**12 ± 2.6**[d]
RB107	**0.28**	**24 ± 1.3**
RB105	**0.23**	**21 ± 1.1**
BJ2000	**0.1**	**26 ± 1.3**
FD105	**0.2**[b]	**36 ± 2.4**
TEM	**>10**	**470 ± 5.6**
Gefitinib	**0.033**[c]	**47 ± 1.2**

[a] Banerjee et al., *J Med Chem* 2003; 46:5546-51.

[b] *J Pharmacol Exp Ther* 2002;303:238-46.

[c]Moulder *et al.*, *Cancer Res* 2001;61: 8887–95

[d]Banerjee *et al.*, 2006 (Chapter 5)

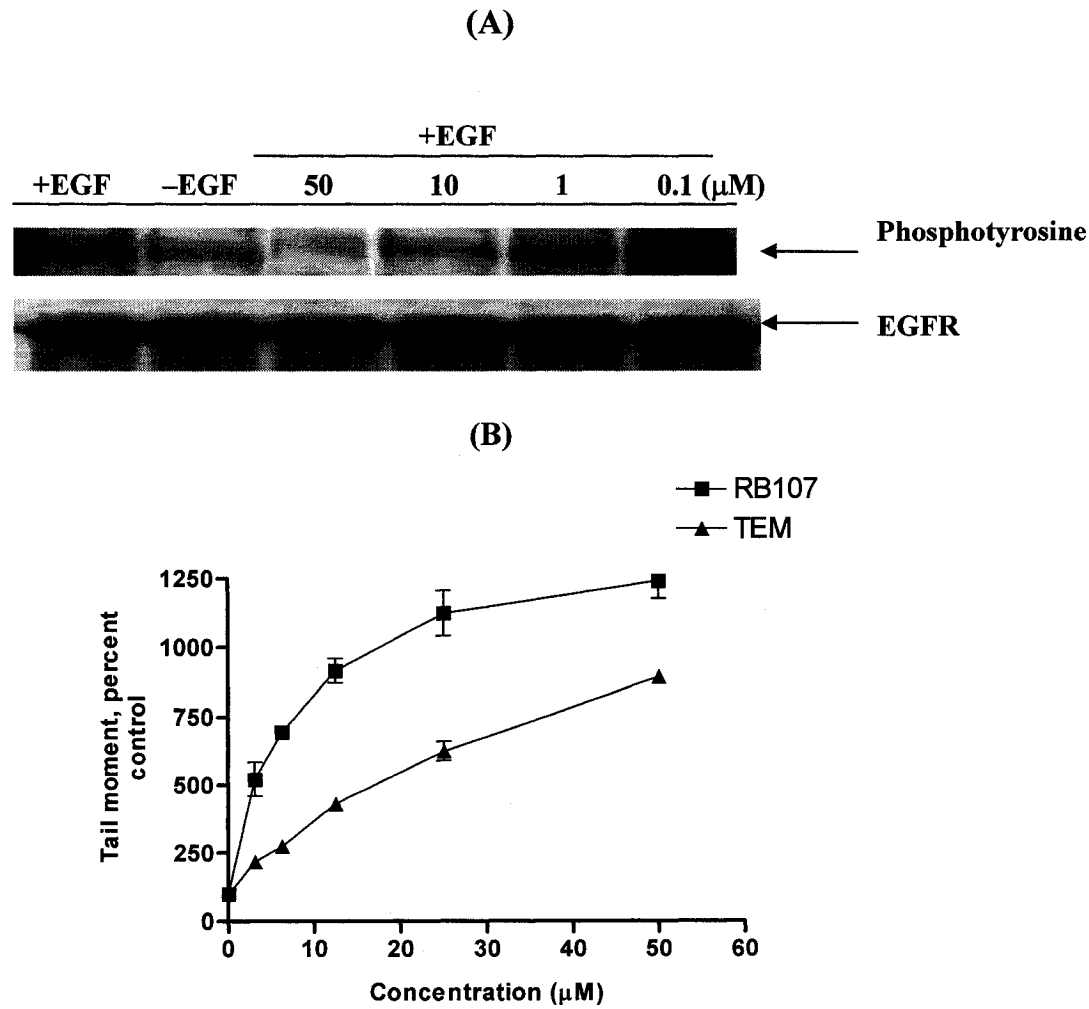


Figure 6.1. Binary targeting properties of the combi-molecule, RB107. A, inhibition of EGFR TK autophosphorylation by RB107 in DU145 cells. Serum-starved cells were pre-incubated for 2 h with the indicated concentrations of RB107 prior to stimulation with EGF (50 ng/mL) for 15 min. Equal amounts of cell lysates were analyzed by western blotting using anti-phosphotyrosine antibodies. Membranes were stripped of anti-phosphotyrosine and re-probed with anti-EGFR antibodies as a loading control. B, *In vitro* DNA damage analysis was carried out after a 2 h exposure to RB107 and TEM. Tail moment parameters were measured with ALKOMET image analysis software for at least 50 cells/dose. Each point represents at least two independent experiments.

class as RB107 (e.g. TEM) (Pegg *et al.*, 1995a; Pegg *et al.*, 1995b). The results showed that the combi-molecule, RB107, was greater than 33-fold more potent than TEM and approximately 4-fold more potent than gefitinib, indicating that the binary targeting strategy leads to more potent molecules (Table 6.1). These results suggest that the dual targeting combi-molecules are more potent than their single targeted counterparts.

6.4.1.3. Cell cycle effects

DNA alkylators are known to block the cell cycle in S and G2/M phases in sensitive tumour cells (D'Atri *et al.*, 1998; Hirose *et al.*, 2001; Hirose *et al.*, 2003). On the other hand, inhibitors of EGFR TK block the cell in the G1 phase (Lebeau *et al.*, 2003; Chang *et al.*, 2004; Gonzales and Fry, 2005). RB107 and RB24, being a combination of both classes of drugs, it was important to determine the effect of their combined properties on the cell cycle in DU145 cells. Interestingly, the results showed that both RB107 and RB24 predominantly blocked the cells in G1 and this cell cycle profile parallels that induced by their reversible inhibitory counterparts, FD105 and RB10, respectively (Fig 6.2). In contrast, the DNA damaging agent, TEM, did not induce any significant cell cycle perturbation (Fig 6.2).

6.4.2. Pharmacoanalysis

Following direct i.v. administration (100mg/kg), LC-MS characterization of compound released, at either 0 min or 5min post-injection, from RB24 confirmed the presence of RB10 (m/z 315), ZR08 (m/z 357), and RB14 (m/z 387) in plasma (Fig. 6.3a,b). Weight loss monitoring analysis showed that RB107 was well tolerated with no noticeable

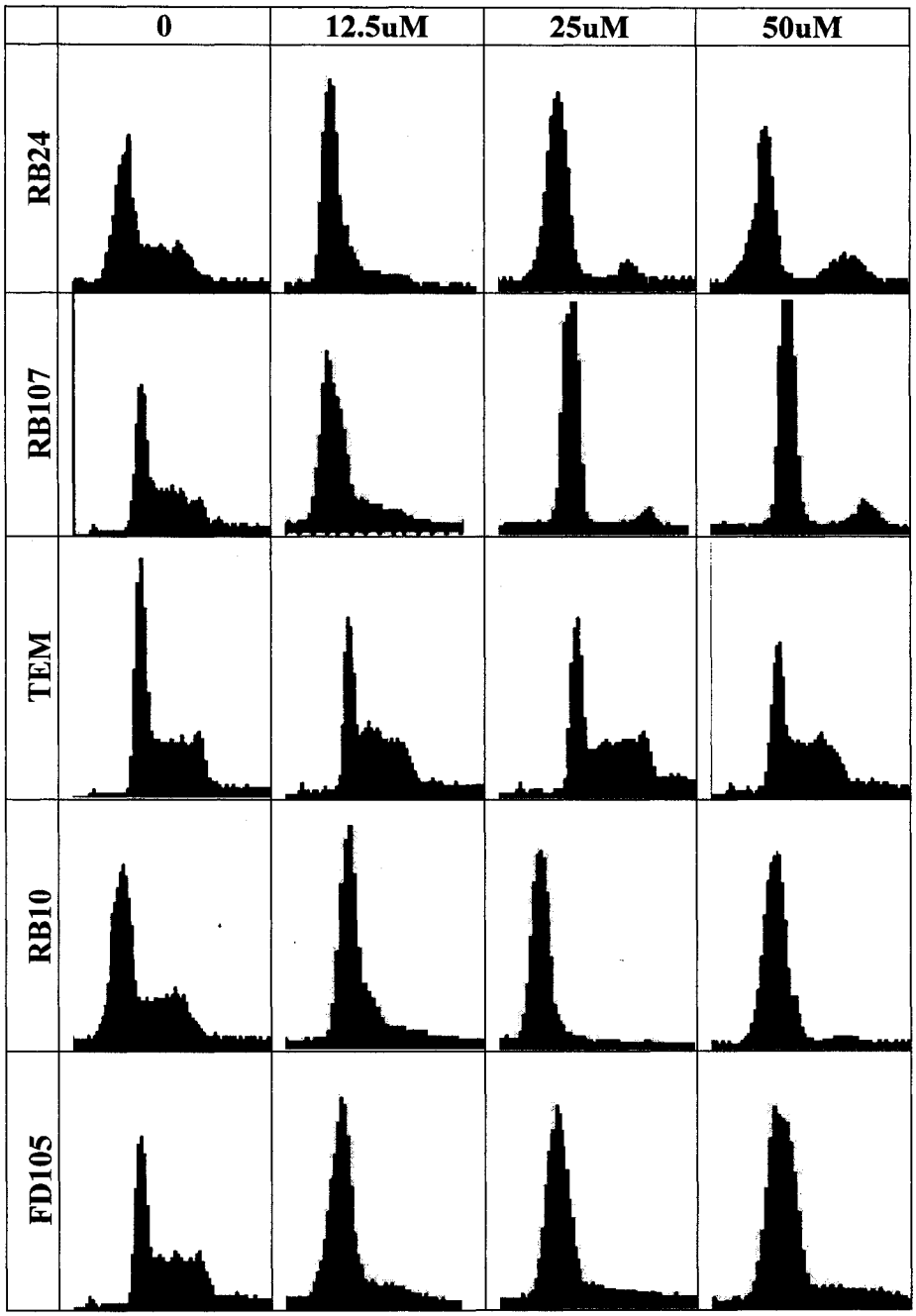


Fig. 6.2. Cell cycle effects on DU145 cells. Cell cycle profile following exposure to a dose-range of RB107, FD105 and TEM for 24 h in the DU145 cell line.

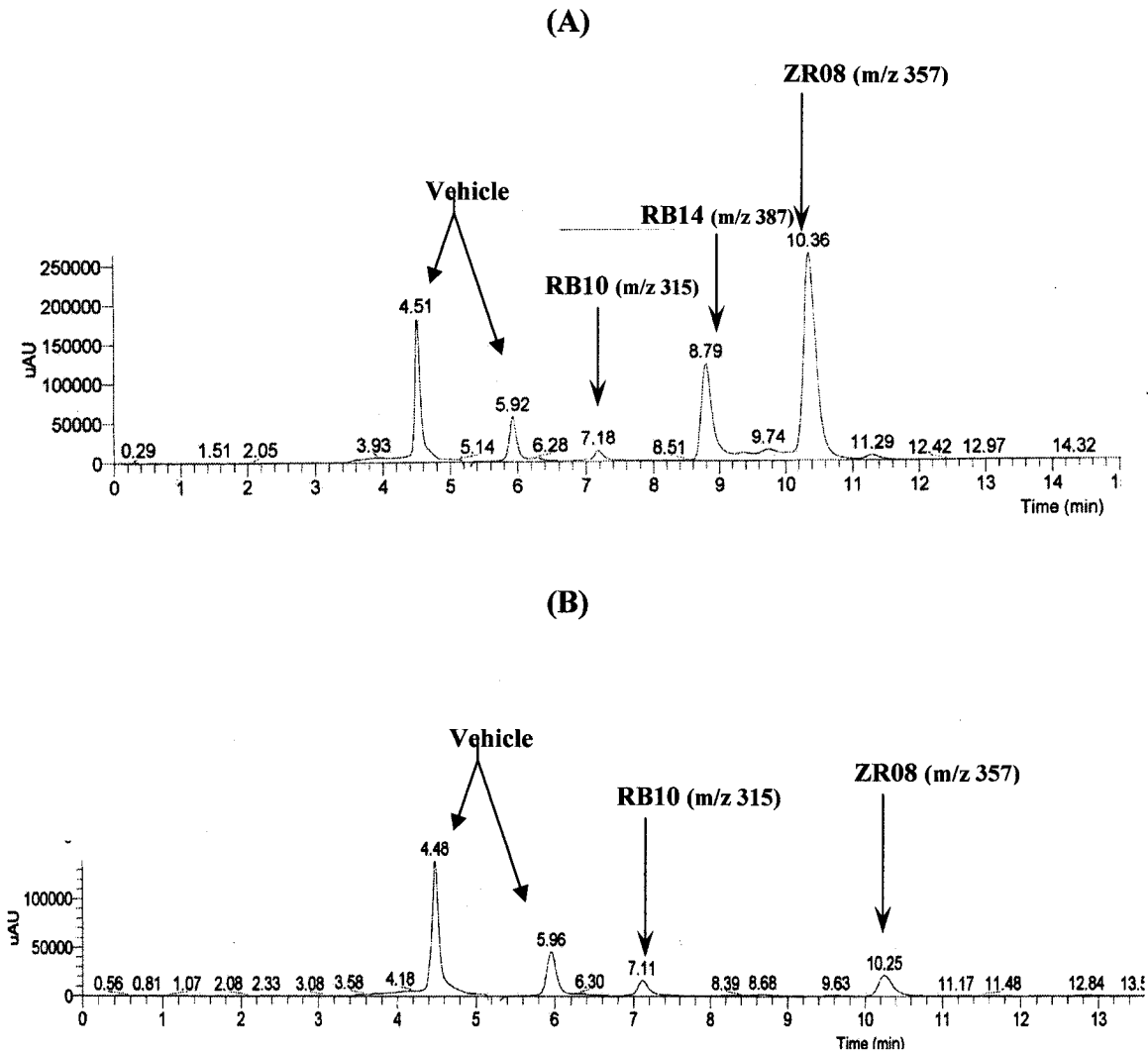


Figure 6.3. Pharmacokinetic analysis of the complex combi-molecule, RB24 in CD-1 nude mice. LC-MS analysis of the metabolites present at either A) 0 min or B) 5 min following i.v. administration of RB24 (100mg/kg).

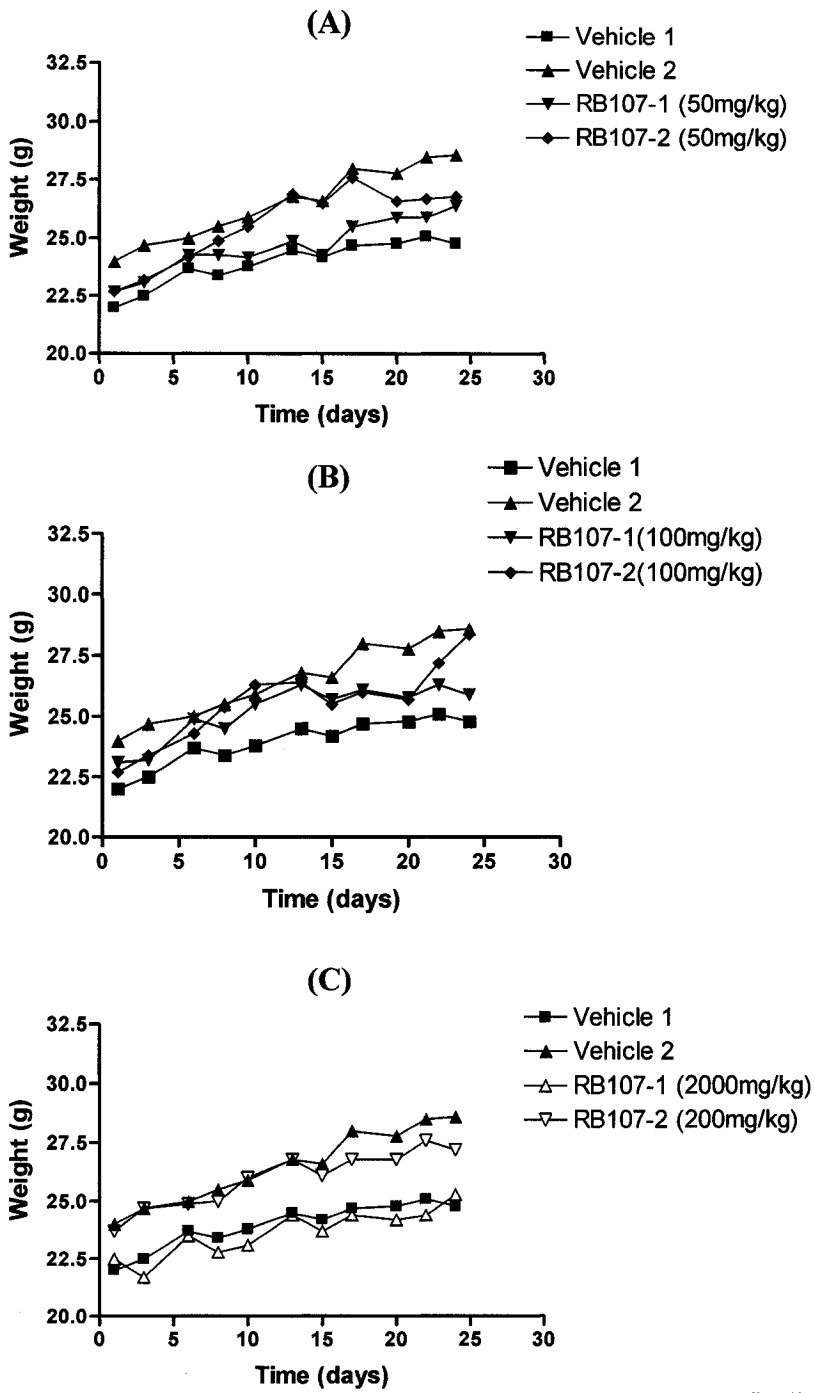


Figure 6.4.Weight loss monitoring study in CD-1 nude mice. Dose finding was performed with 2 CD-1 nude mice/group administered RB107 at A, 50 mg/kg, B, 100 mg/kg or C, 200 mg/kg. RB107 was administered i.p. every other day, for a total of 9 injections over a two week period.

weight loss 21 days after an every other day treatment schedule at doses as high as 200 mg/kg (Fig. 6.4).

6.4.3. *In vivo* efficacy

To determine whether the binary targeting properties and the *in vitro* antiproliferative activity of the compound would translate into *in vivo* activity, we analyzed the effect of RB107 on DU145 prostate tumours grafted on immunocompromised mice. The results showed significant antitumour activity 15 days post-treatment ($p<0.05$) (Fig. 6.5a). In contrast, at the same dose TEM demonstrated no significant activity (Fig. 6.5b). Similarly, under the aforementioned schedule, both gefitinib and an equi-dose combination of TEM (50 mg/kg) + gefitinib (50 mg/kg) showed no significant antitumour activity at any time point ($p>0.05$) (Fig. 6.5c,d).

6.5. Discussion

Previous studies demonstrated that RB24 could induce sustained antiproliferative activity and this was imputed to its ability to generate multiple species with EGFR TK inhibitory activity + a methyldiazonium species. Although the fluorescence property of the ultimately released metabolite RB10 permitted its identification, the short life of the other metabolites of the cascade precluded their characterization. Thus, we designed an experiment wherein RB24 was injected i.v. to CD-1 mice and the plasma collected either immediately or 5 min after injection. The rapid process permitted the detection of all predicted species, the predominant one being the monoalkyltriazene, ZR08. It should be remembered that the small difference between the half-life of RB24 ($t_{1/2}$=42 min) and RB14 ($t_{1/2}$=40 min), as well as between RB14 and ZR08 ($t_{1/2}$=38 min), indicated a rapid

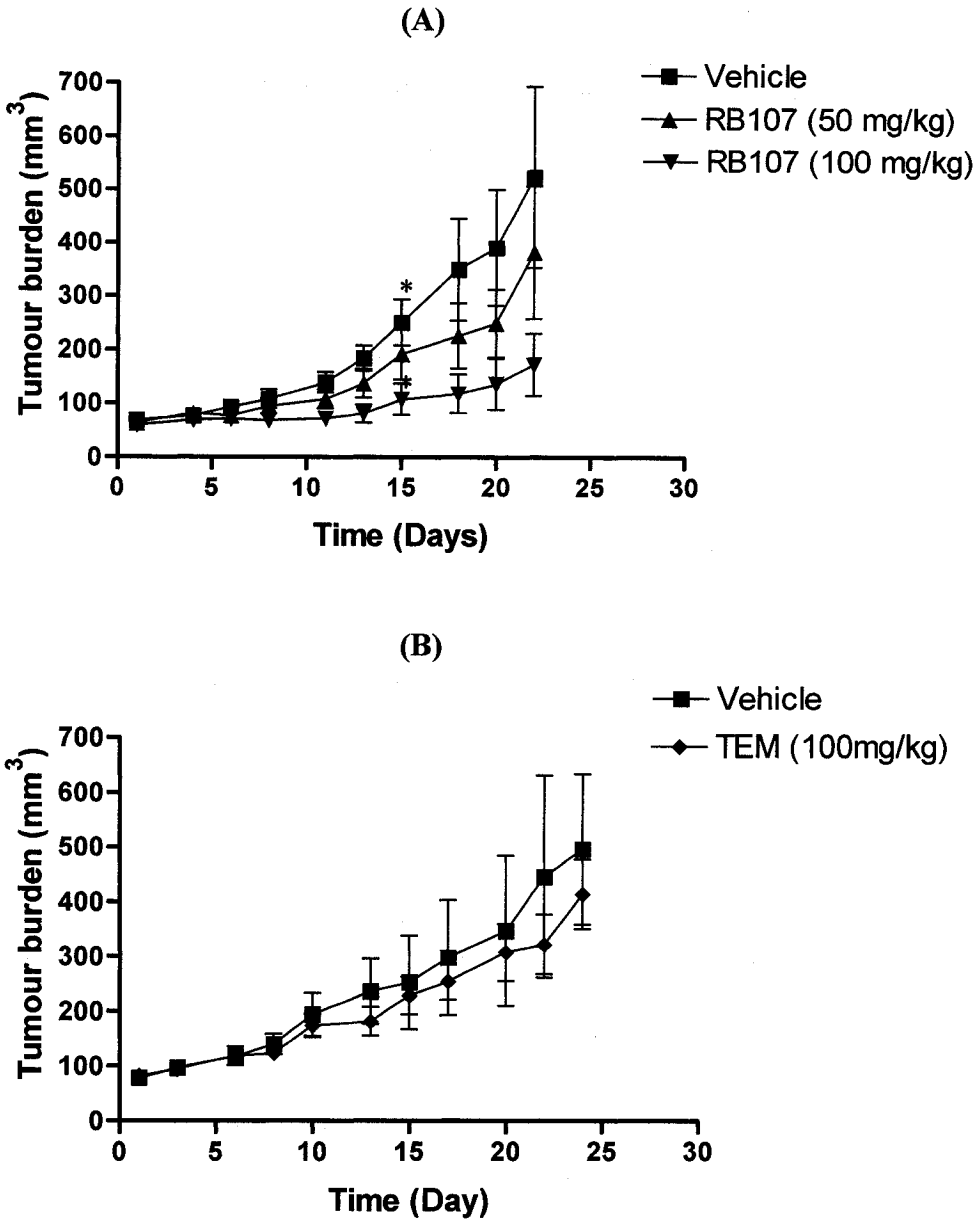
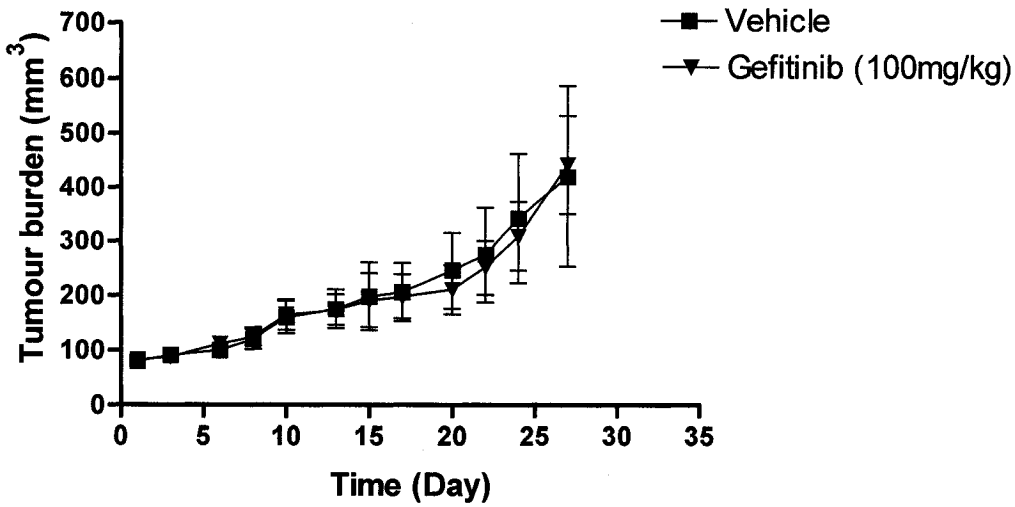


Figure 6.5. Antitumour activity of RB107 and TEM in a DU145 xenograft model. Comparison of tumour growth inhibition two weeks following i.p. administration between vehicle and A, RB107 (100 mg/kg) or B, TEM (100mg/kg) every other day in CD-1 nude male mice. Statistical analysis was performed with a two-tailed t-test. Star indicates statistical significance $p < 0.05$ (n=6); *bars*, SD.

(C)



(D)

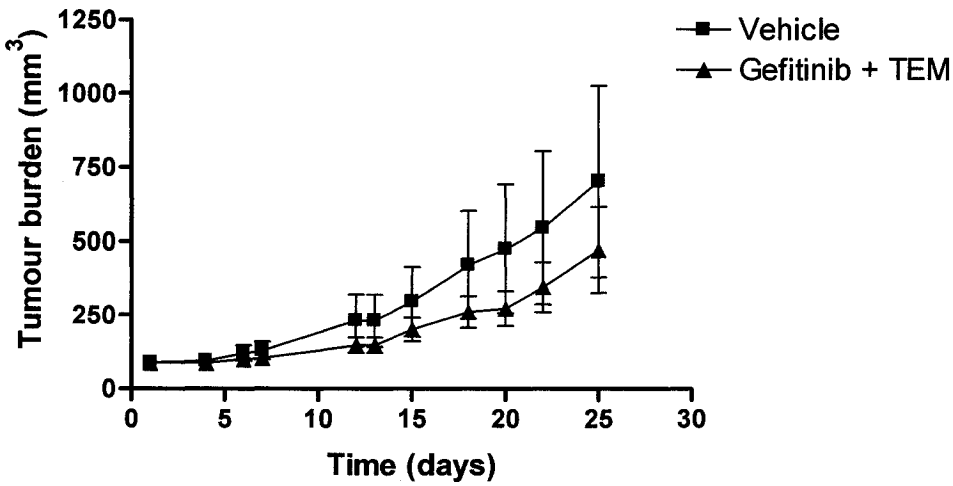


Figure 6.5. Antitumour activity of gefitinib and gefitinib + TEM in a DU145 xenograft model. Comparison of tumour growth inhibition two weeks following i.p. administration between vehicle and C, gefitinib (100 mg/kg) or D, gefitinib + TEM (50mg/kg) every other day in CD-1 nude male mice. Statistical analysis was performed with a two-tailed t-test. Star indicates statistical significance $p < 0.05$; *bars*, SD.

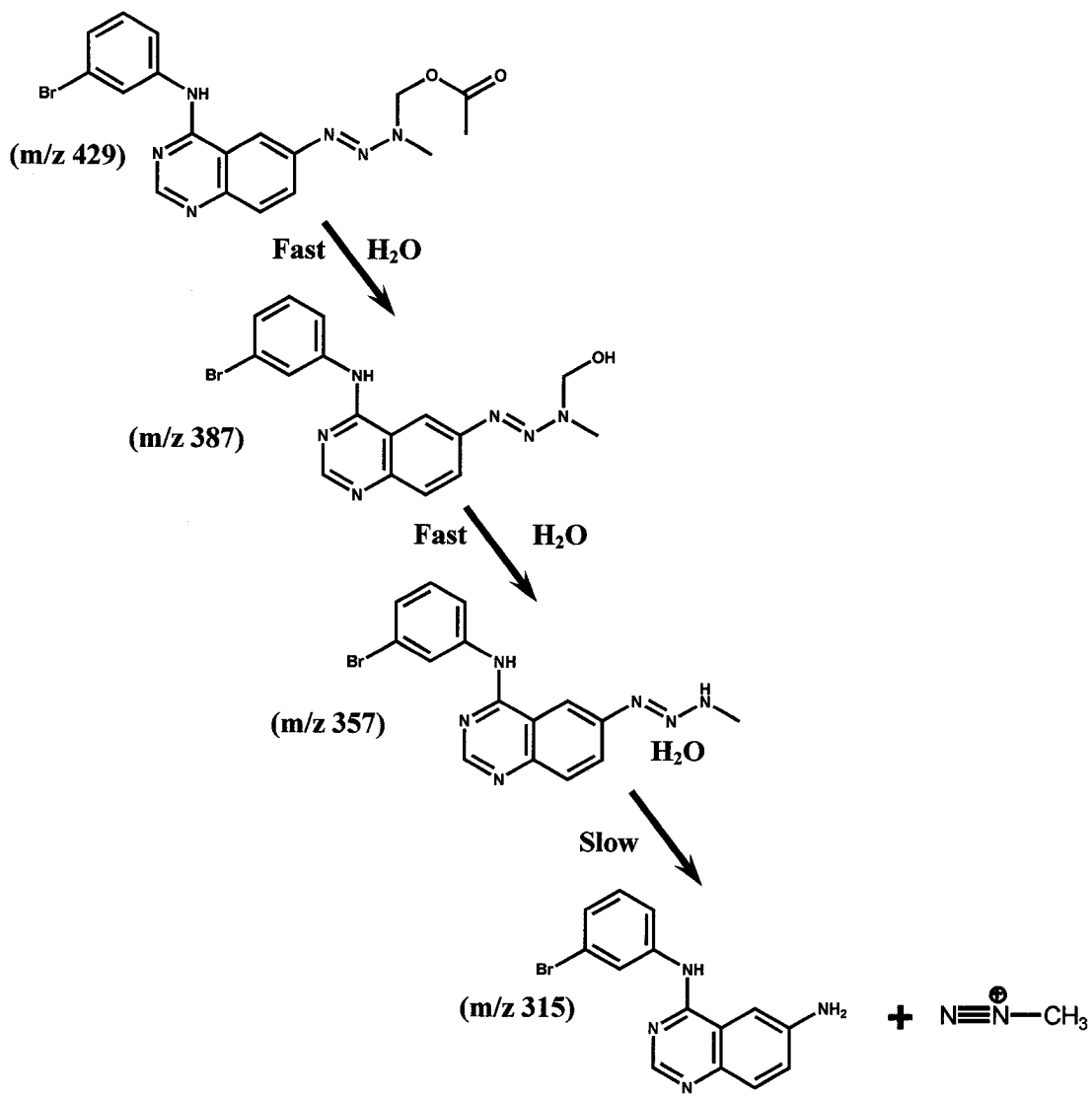


Figure 6.6. Cascade release model

transition between these elements of the cascade (Banerjee *et al.*, 2003). The stability of ZR08 is in the same range as analogous naked triazenes (e.g. SMA41, $t_{1/2}$=30 min; BJ2000, $t_{1/2}$=34 min) (Fig. 6.6). Therefore, conversion of ZR08 into RB10 at the last step of the cascade should be considered rate-limiting and RB24 or RB14, "prodrugs" of ZR08. This is further corroborated by both the appearance of a higher titer of ZR08 in the plasma immediately after RB24 injection and 5 min after administration. This indicates that indeed, ZR08 is the most stable of the transient metabolites. It should be noted that the most stable metabolite of the entire cascade is RB10, Thus, following the disappearance of all metastable metabolites, EGFR inhibition is sustained by RB10, which is a strong inhibitor of EGFR (IC_{50}=0.04μM).

Interestingly, despite the generation of these multiple species the cascade release molecule analogue, RB107, used in the *in vivo* studies, was well tolerated (MTD=200mg/kg). Their tolerance paralleled that of the cyclic methylating triazene, TEM (Brada *et al.*, 1999; Rudek *et al.*, 2004; Jones *et al.*, 2005), that is known to have an MTD > 200 mg/kg. This good tolerance also indicates that dual targeting of EGFR and DNA methylation by an alkylating agent is a non-toxic event.

Methylating agents like TEM are, in general, inactive against AGT-expressing cells *in vitro* and *in vivo* (Pegg *et al.*, 1995a; Pegg *et al.*, 1995b). In this study the combi-molecule (a methylating agent) induced superior antiproliferative potency when compared with TEM and its equi-dose combination of TEM + gefitinib. The significant potency observed with RB107, in a once every other day schedule in an AGT+ DU145

model is rather unique and may be imputed to its ability to simultaneously damage DNA and block EGFR. Indeed, previous studies with its analogue RB24, demonstrated that inhibition of EGFR activation in the DU145 cells is concomitant with down-regulation of phosphorylation of the anti-apoptotic protein Bad (Banerjee *et al.*, 2006b). In contrast to TEM, the latter molecule was capable of inducing high levels of apoptosis in the DU145 cell line. These results suggest that the single combi-molecule is a superior mimic of a two-drug combination when compared at equi-doses.

Flow cytometric analysis and fluorescence microscopy in previous studies have shown that DNA damage induced by the RB107 analogue, RB24, is consistently higher in isogenic cells that express high levels of EGFR or Her2 and that the released inhibitor RB10 (the bromo-analogue of FD105) was preferentially distributed in the perinuclear region (Banerjee *et al.*, 2006a). Further, co-administration of RB24 with its metabolite RB10 depleted levels of DNA damage and abolished EGFR targeting, indicating that the combi-molecule primarily anchors into perinuclear binding sites prior to releasing the methyldiazonium that intensively damages the DNA (Banerjee *et al.*, 2006a). Therefore, the enhanced potency of the combi-molecule when compared with combination of individual drugs, may not only be due to their binary EGFR/DNA targeting functions, but also to their ability to induce discrete distribution of the bioactive species within the various subcellular compartments (e.g. cytoplasm, perinuclear region). Qiu *et al.* (2006) have recently shown that JDA35, a fluorescent inhibitor released by the combi-molecule, JDA58, is distributed in the perinuclear region in DU145 cells analysed *in vitro* and *in vivo*, indicating that the perinuclear distribution of the cascade release combi-molecule is common to other combi-molecules.

It is important to note herein that in contrast to TEM, the cascade release molecules, RB107 and RB24, being designed to block EGFR and damage DNA, preferentially blocks the cells in G1, indicating that blockade of EGFR is playing a significant role in their antiproliferative activity. Indeed, it has already been shown that inhibitors of EGFR block the cell cycle in G1 (Lebeau *et al.*, 2003; Chang *et al.*, 2004; Gonzales and Fry, 2005). However, the combined effect of the combi-molecule on the cell cycle is seen at the highest dose (e.g. 50μM) where in addition to a strong arrest in G1 (84 %) a significant block in G2/M was also observed (i.e. 43% increase in cell number). This indicates that cells that escape the G1/S arrest may be arrested later in G2/M due to the DNA lesions inflicted by the combi-molecule.

This study conclusively demonstrated that the binary targeting property of combi-molecules capable of releasing multiple EGFR inhibitory species and DNA damaging methylating agent, translate into significant antitumour activity *in vivo*.

6.6. Acknowledgements

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CHAPTER 7

DISCUSSION

AND

CONTRIBUTION TO KNOWLEDGE

7.1. Discussion and contribution to knowledge

Depending on the progression or stage, current cancer therapy involves the combination of multiple modalities including surgery, radiation and chemotherapy. Although conventional chemotherapy with cytotoxic agents have proven to be effective in many solid tumours, their toxicity still remain a major deterrent for their clinical use. Current advances in cancer chemotherapy involve the development of therapeutics that are capable of targeting overexpressed or dysfunctional proteins implicated in the malignant phenotype (e.g. EGFR, Her2, bcr-abl).

Recent studies have shown that inhibition of EGFR or its closest homologue, Her2 (ErbB2), receptor tyrosine kinases involved in aggressive tumour progression and chemoresistance, resulted in significant anitproliferative activity in tumour cells (Lebeau *et al.*, 2003; Shelton *et al.*, 2005). Her2 is overexpressed in 20–25% of invasive breast cancers and occurs primarily through gene amplification (Slamon *et al.*, 1987; Lebeau *et al.*, 2003). Overexpression of Her2 is associated with poor disease-free survival and chemoresistance (Press *et al.*, 1997; Paik *et al.*, 1998). Therefore, Her2 has become an important therapeutic target in breast cancer (Luftner *et al.*,, 2005). Herceptin™ (trastuzumab), a recombinant humanized monoclonal antibody directed towards the extracellular domain of the Her2 receptor, has proven highly effective against breast cancer. Currently, Herceptin™ is the only Her2-targeted therapy approved by the US Food and Drug Administration for the treatment of metastatic breast cancer and is administered in combination with chemotherapies such as paclitaxel and docetaxel (Luftner *et al.*, 2005; Robert *et al.*, 2006).

Numerous small molecule inhibitors targeting the EGFR TK have been developed and have shown promising anti-tumour activity in the clinic (Baselga and Averbuch, 2000; Moulder *et al.*, 2001; Sgambato *et al.*, 2004; Martin *et al.*, 2006). However, the clinical response to this drug remains weak and in many cases, they only induce disease stabilization (Baselga and Averbuch, 2000; Moulder *et al.*, 2001; Marshall, 2006). Although these compounds have been found to be well-tolerated, they are unable to induce significant cell-killing, a characteristic that is usually associated with cytotoxic chemotherapeutic agents such as DNA alkylators and antitumour antibiotics (Ciardello *et al.*, 2000). The primary contribution of this thesis is to demonstrate that cytotoxicity can be conferred to these agents by designing compounds termed cascade release combi-molecules, "programmed" to not only release an alkylator, but also multiple inhibitors of EGFR TK. More specifically, we demonstrated that these molecules could be masked with latent functions that do not hamper EGFR inhibitory effects and rather increase the water-solubility of the designed agents, leading to a more suitable formulation. Thus, the development of this thesis can be summarized into three major discoveries: 1) design and development of cascade release molecule, 2) discovery of a novel model of EGFR and Her2 targeting by small molecules and 3) discovery of a synergistic cross-talk between MAPK, anti-apoptotic signaling and DNA repair pathways that contribute to enhanced potency.

Discovery 1: Cascade release molecules

The observation that the putative degradation products from the parent cascade combi-molecule have different biological response profiles (e.g. RB24 inducing more protracted inhibition of EGFR autophosphorylation than RB14 and ZR08) indicate that

each member of the cascade may contribute to EGFR inhibition in cells, thereby hammering the receptor to prevent its reactivation (Banerjee *et al.*, 2003, Banerjee *et al.*, 2004). More importantly, the potent and stable EGFR inhibitor released at the last step of the cascade further prolongs the EGFR inhibition and sustains antiproliferative activity. Further, the masking acetoxymethyl group of RB24, being able to promote water solubility, give the molecule the property of a water soluble envelope containing multiple species. Indeed, ZR08 that is released by the parent combi-molecule, is so insoluble in water that it is not suitable for *in vivo* studies. The fact that none of these molecules, when tested individually, showed any significant loss of EGFR TK inhibitory activity indicates that the signaling inhibitory properties of these molecules are well preserved during and after decomposition (Banerjee *et al.*, 2003). This finding can inspire the design of molecules to target other types of disease-related kinases by programming them to maintain their activity while being hydrolytically activated to generate another target-directed species. This principle is summarized by the model derived from this study and is depicted in Figure 7.1. The complex combi-molecule exemplified as I-TZ, can be designed to bind to the target 1 (e.g. EGFR, Her2), to decompose to I-TZ' directed at target 1, while being allowed to be converted to I-TZ" pointed at target 1. The latter combi-molecule can release I directed at target 1 + a species aimed at target 2. If the rate of conversion of each of the elements in the cascade can be optimized, or if conversion can lead to molecules directed at different targets (e.g. target 2, target 3, target 4), this model will provide a means to significantly ameliorate multi-targeting and sustained inhibition of kinases. Cancer being a disease arising from a complex network of interrelated biological processes within a single cell, it is critical that cancer drug development progress towards the development of

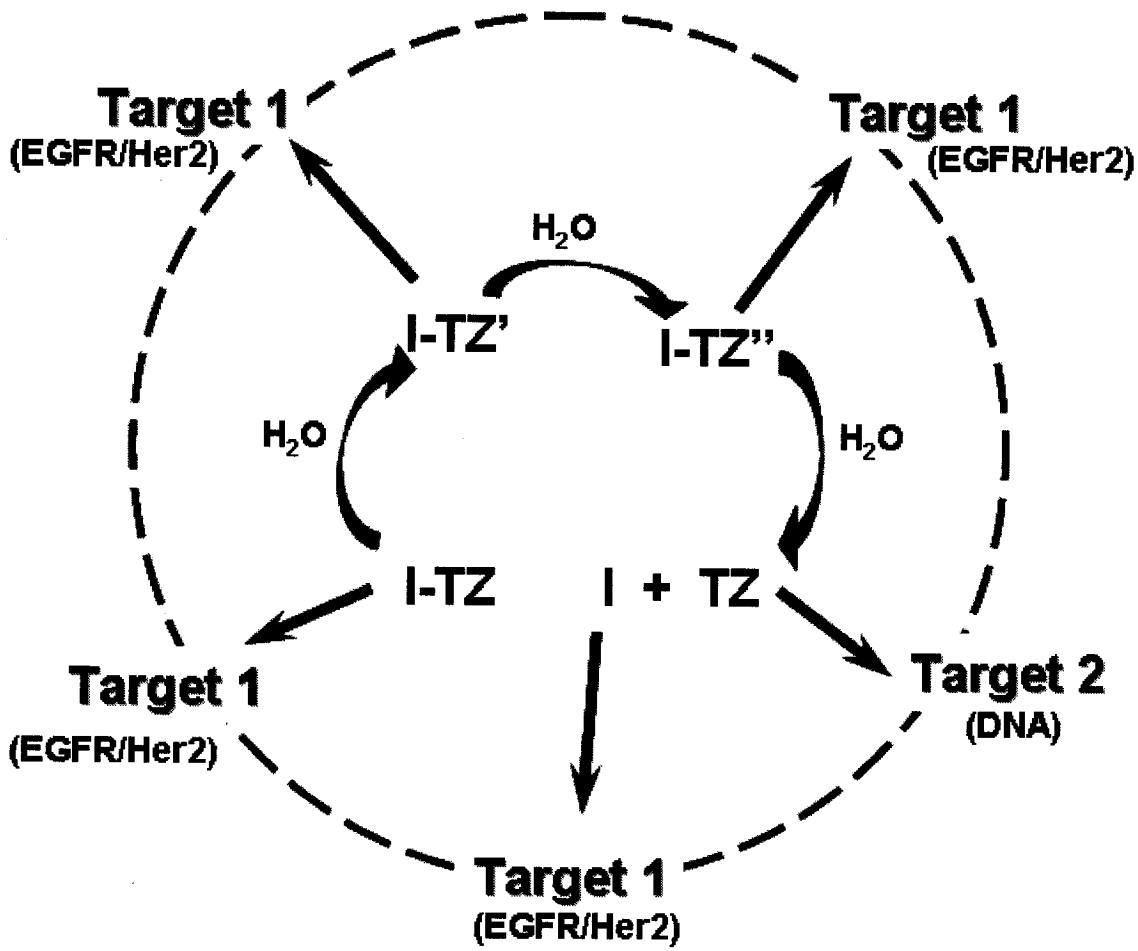

Figure7.1. The cascade release model.

single agents capable of interfering with multiple pathways. In this context, the novel model pioneered by this thesis is a demonstration of the feasibility of such agents.

Discovery 2: Discovery of a novel model of EGFR and Her2 targeting by small molecules

The literature is punctuated with molecules designed with a recognition head and DNA damaging function. As an example, estramustine was designed to contain an estradiol warhead linked to a nitrogen mustard moiety (Petrow and Padilla, 1986). Although this molecule could bind to the estrogen receptor, the compound did not damage DNA directly (von Schoultz *et al.*, 1991). Small molecule-based targeting has long been based on creating covalent linkage between the recognition head and the cytotoxic tail. Another example is the peripheral benzodiazepine receptor (PBR)-targeting agent containing a benzodiazepine moiety and a melphalan tail that did not show selectivity for tumours overexpressing PBR (Kupczyk-Subotkowska *et al.*, 1997). Here we discovered that molecules termed combi-molecules that contain a hydrolysable linkage between the recognition head and the DNA damaging tail could indeed target the DNA damage to cells expressing a specific receptor (Banerjee *et al.*, 2006a). More importantly, this is the first time that intracellular localization of the target receptor has been shown to direct the delivery of the DNA damaging agent. We believe that the combi-molecule owes this advantage to its hydrolyzable linkage. Indeed, the molecule can rapidly bind to the receptor and slowly hydrolyze to the alkylating species, allowing a bystander effect that leads to abundant release of alkylators towards the nucleus. It has now been demonstrated that EGFR is internalized following activation and is distributed in the perinuclear region (Murthy *et al.*, 1986; Khan *et al.*, 2006). Thus, this model (see

figure 4.4, p. 167) suggests that if many receptors are proven to be relocalized in the same manner, we will have discovered a mean to selectively damage DNA in cells with various receptors involved in aberrant signaling in refractory tumours.

Discovery 3: Discovery of a synergistic cross-talk between MAPK and anti-apoptotic signaling or DNA repair pathways

In principle, when growth signaling is activated in a cell, all survival pathways are alerted. This is mediated through activation of anti-apoptotic pathways, DNA repair pathways and cell cycle factors (Rowinsky et al., 1999; de Thonel and Eriksson, 2005). On the other hand, when the cells are exposed to various stressors, including radiation induced DNA damage, reactive oxygen species and DNA alkylation, many pathways leading to their rescue and those that induce their death by apoptosis are activated. The combi-molecules, RB24, described in this thesis, are capable of both blocking growth factor-mediated signaling and inducing stress through DNA damage. We have presented herein the first characterization of the multiple signaling pathways activated in response to a single drug that behaves as a binary stressor. As depicted in Figure 7.2, where the combi-molecule is exemplified by I-TZ, it appears that both the I-TZ itself, and the inhibitors that it releases, can exert blockade of two major signaling pathways: 1) the Ras/Raf/MAPK cascade and 2) the PI3K/Akt pathway. Through this mechanism, the molecule blocks growth factor-mediated signaling while down-regulating anti-apoptotic pathways (Banerjee *et al.*, 2006b). The DNA damaging component, TZ, triggers stress response mediated by the MAPK family, an event that leads to expression of the DNA repair protein, XRCC1 (Banerjee *et al.*, 2006b). The latter BER protein is also induced by receptor activation. We have discovered that concomitant

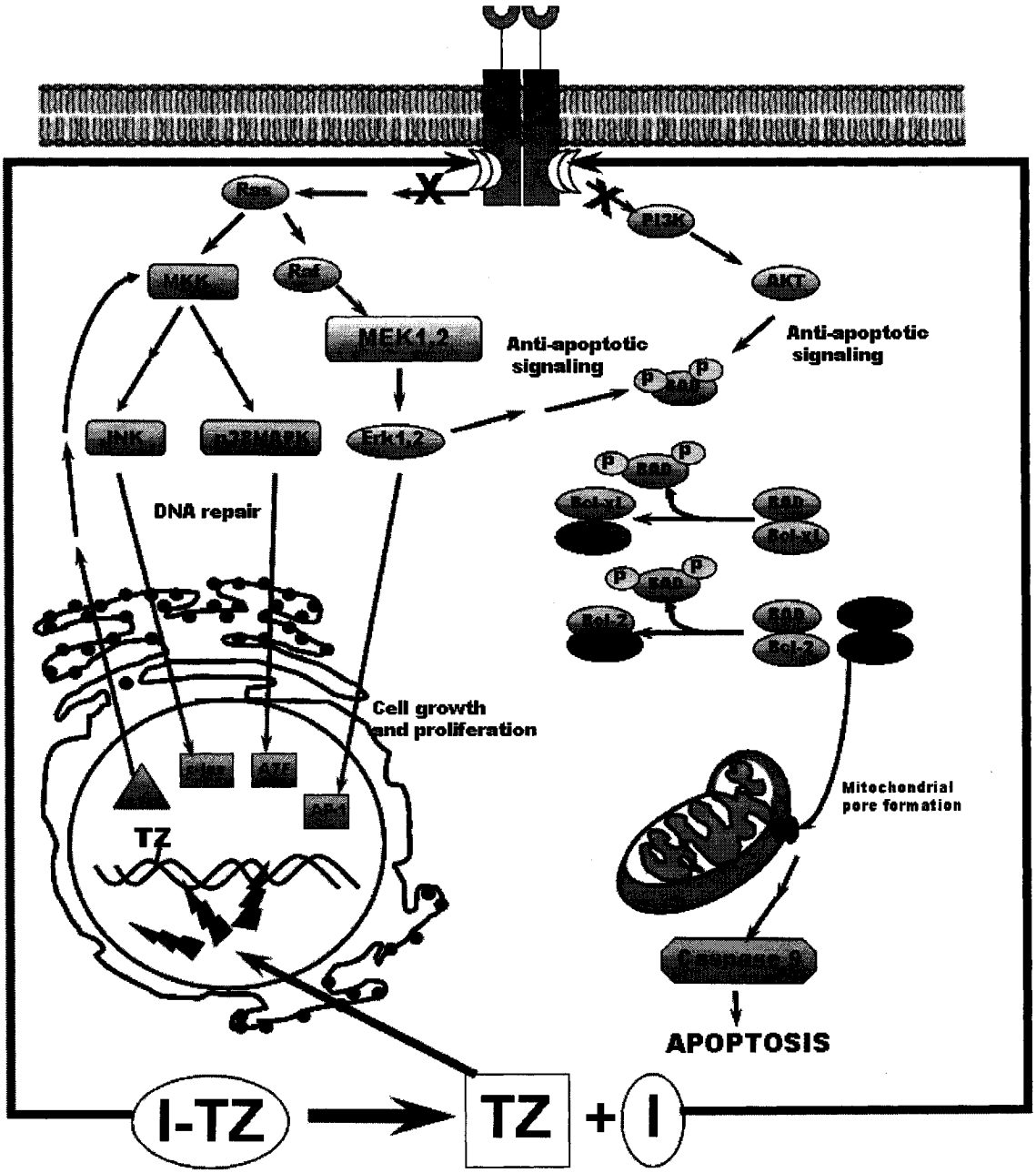

Figure 7.2. Effects of the combi-molecule on intracelullar signaling cascades.

induction of XRCC1 through DNA damage response and EGFR activation causes an elevation of the latter protein in the presence of the alkylator, TEM. This elevation of XRCC1 can only augment resistance to the latter alkylator. Conversely, the combi-molecule is capable of down-regulating the latter DNA repair protein through EGFR activation, but not through stress response. *In toto*, in contrast to TEM, by shutting down all growth factor-related signaling, the combi-molecule was capable of partially depleting XRCC1 and committing the cells to apoptosis.

On the other hand, MEK1 being at the interface of various signaling pathways, including the one that leads to induction of XRCC1, we found that its blockade could not only deplete the levels of the latter protein, but also significantly enhance apoptosis in these cells. This has led to a new formula for synergistic combination in cancer therapy. The following combinations were found markedly synergistic: 1) combi-molecule+MEK1 inhibitor and 2) clinical TKI + TEM + MEK1 (Banerjee *et al.*, 2006b). It should be noted herein that combination formulas discovered within this context of this thesis have been submitted for patent protection by McGill University Office of Technology Transfer (OTT) (Jean-Claude and Banerjee, 2005). Thus, by exploring multiple signaling pathways in response to the mixed targeted combi-molecule, we discovered a novel combination model that may not only ameliorate the potency of the combi-molecule itself, but also that of the currently used clinical drugs from both the triazene and clinical TKI classes.

In summary, we have developed novel complex combi-molecules capable of not only

interfering with multiple signaling pathways in the cell, but also selectively targeting cells with aberrant expression of the ErbB1 and ErbB2 gene products. The work performed for this thesis was deployed from the design and synthesis of the probe drugs, to the demonstration of *in vivo* potency of a novel tumour-targeting approach.

7.2 References

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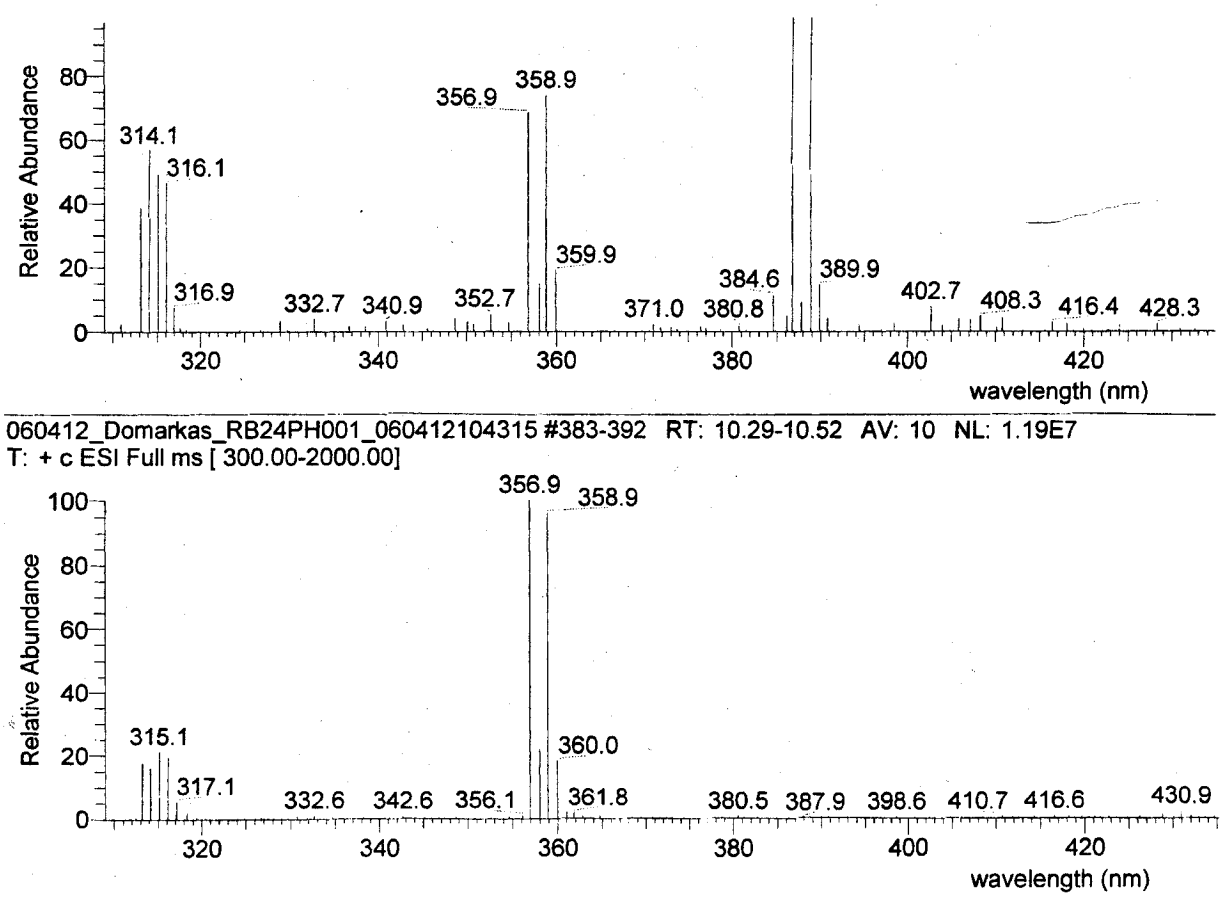
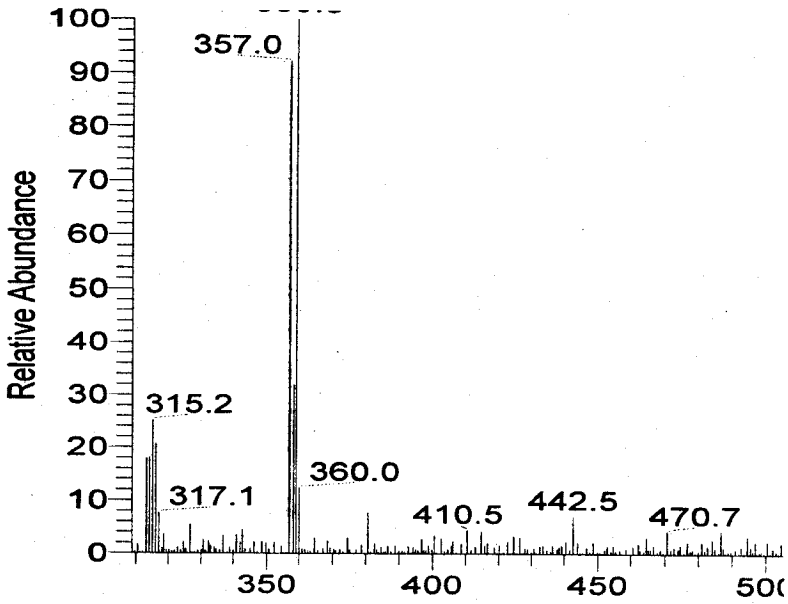
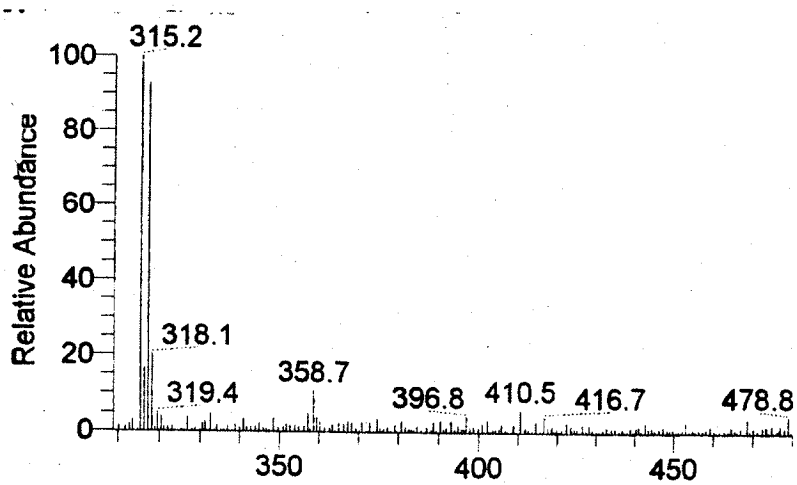
APPENDIX



Mass spectroscopy to identify the metabolites found in mouse plasma immediately following RB24 injection (Chapter 6).





Mass spectroscopy to identify the metabolites found in mouse plasma 5 min following RB24 injection (Chapter 6).

Synthesis of a Prodrug Designed To Release Multiple Inhibitors of the Epidermal Growth Factor Receptor Tyrosine Kinase and an Alkylating Agent: A Novel Tumor Targeting Concept

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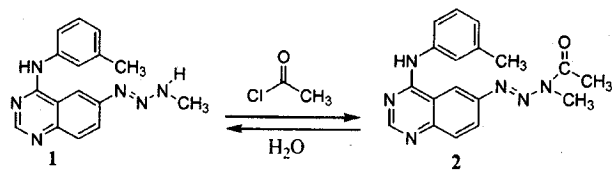
The synthesis of a novel acetoxymethyltriazene designed to be a prodrug of multiple inhibitors of the epidermal growth factor receptor (EGFR) and a methyldiazonium species is described. Studies with each of the expected metabolites demonstrated significant EGFR tyrosine kinase inhibitory activities and the released methyldiazonium was trapped with *p*-nitrobenzylpyridine. Their ability to damage genomic DNA in whole cells was demonstrated by using the single cell microelectrophoresis (comet) assay. The results suggest that this approach may well represent a novel drug combination strategy involving single molecules masking multiple bioactive agents.

Introduction

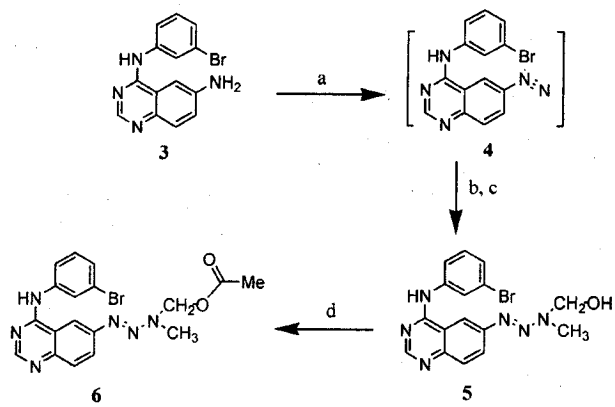
The altered protein expression and activity of receptor tyrosine kinases (TK) are implicated in the progression of various types of cancers. One such dysfunction is the overexpression of the epidermal growth factor receptor (EGFR) and its closest homologue HER2 (erbB2) that correlates with aggressive tumor progression and poor prognosis.[1] Recently, we developed a novel strategy that seeks to combine DNA-damaging properties and EGFR TK inhibitory activities into single molecules termed "combi-molecules" designed to kill EGFR-expressing tumor cells.[2−5] To enhance the EGFR inhibitory potency and stability of these compounds, we designed a novel strategy termed "cascade release" (CR) that seeks to mask the combi-molecule into a prodrug "programmed" to release the antitumor species by hydrolytic activation. Since these molecules, henceforth referred to as "cascade release molecules" (CRM), are also designed to retain significant EGFR affinity on their own, this principle leads to conditions under which three generations of inhibitors can be derived from the parent CRM.

To test the CRM principle, we initially designed structure **2** (Scheme 1) with the hope that it would not only retain affinity for EGFR but also hydrolyze to generate monoalkyltriazene **1** which, as previously demonstrated, heterolyzes into the corresponding 6-amino-4-anilinoquinazoline (an inhibitor of EGFR) + a methyldiazonium species.[2,3] Thus, CRM **2** was designed to be the prodrug of two known inhibitors of EGFR. Its synthesis proceeded according to Scheme 1. Structure **1**, obtained as described earlier,[6] was treated with acetyl chloride in methylene chloride at 0 °C to give **2** in an 18% yield. Studies of the hydrolysis of **2** under physiological conditions (i.e. incubation in serum containing media at 37 °C) demonstrated little degradation after 24 h. Further exposure for an additional 24 h led to minor degradation. Therefore, it was concluded that this molecule was not a suitable probe for the demonstration of the CRM principle.

Scheme 1



Scheme 2[a]



[a] (a) $NOBF_4$; (b) $MeNH_2/H_2CO/H^+$; (c) K_2CO_3; (d) $(CH_3CO)_2O$ in pyridine.

We subsequently designed structure **6** (Scheme 2), which would not only offer the opportunity to further mask the CRM but also to degrade according to known kinetics and mechanisms.[7,8] It has already been demonstrated that acetoxymethyltriazenes rapidly degrade to the corresponding hydroxymethyl triazene, which in turn is converted to a monoalkyltriazene of type **1**.[6] Further, heterolysis of triazene is known to generate an aromatic amine and an alkylating agent.[8] Thus, we surmised that these mechanisms of degradation might lend themselves to the demonstration of the CRM principle.

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Scheme 3

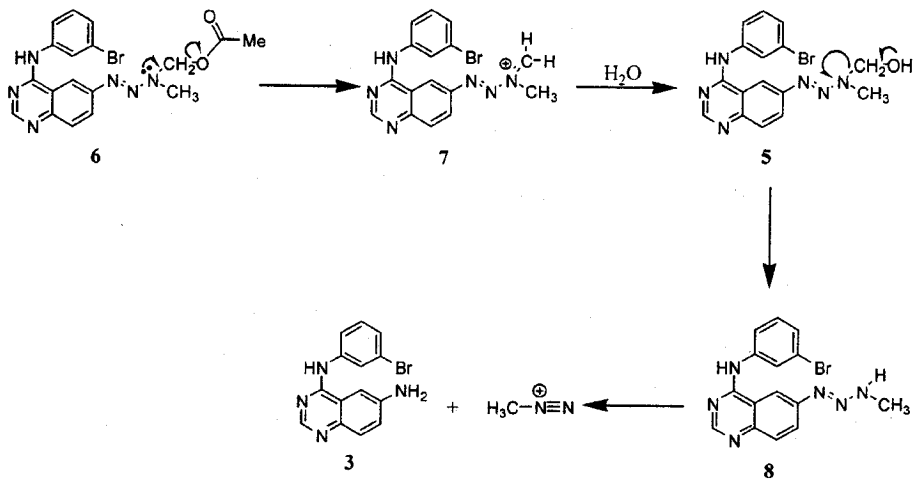


Table 1. EGFR TK Inhibition, Alkylating Activity, and Half-Lives of Compounds **1–3**, **5**, **6**, and **8**

compds	inhibn of EGFR IC_{50}, μM[a]	alkylating activity (10^{-3} min^{-1})[b]	half-life ($t_{1/2}$),[c] min RPMI	half-life ($t_{1/2}$),[c] min PBS
1	0.2	9.1	35	69
2	0.578	1.5	>200	>200
3	0.04	0.05	N/A	N/A
5	0.11	6.6	40	70
6	0.13	5.8	42	84
8	0.07	8.9	38	68
TEM	N/A	11.3	N/A	N/A

[a] EGFR TK values are means of two experiments. Alkylating activity and half-lives values were obtained from single experiments. [b] Measured as a rate of alkylation of NBP at pH 7.5. [c] Measured by UV absorbance at 290 nm.

Results and Discussion

Chemistry. The synthesis of **6** proceeded according to Scheme 2. Briefly, compound **3** was treated in acetonitrile with $NOBF_4$ to generate intermediate **4**, which was treated in situ with a 10:1 mixture of formaldehyde/methylamine. Alkalinization with aqueous K_2CO_3 led to precipitation of compound **5**, in high yield. However, this compound coprecipitated with solid K_2CO_3, which was removed by resuspension in THF followed by collection and evaporation of the filtrate. Hydroxymethyltriazene **5** was obtained in a pure state (70% yield) without requirement for further purification. Treatment of **5** with acetic anhydride in pyridine gave **6** as a pure solid. We have already reported the synthesis of **8**.[9]

The stability of **6** and that of each of its putative metabolites were studied by allowing them to degrade in both phosphate-buffered saline (PBS) and serum-containing media using UV spectrophotometric analysis. Compound **6** was much less stable than **2**, with a half-life of 42 min in serum-containing media (Table 1). Interestingly, its putative metabolite **5** (Scheme 3) showed a half-life of 40 min, suggesting that conversion of **6** to **5** may be a rapid event. Indeed, it has already been reported that acetoxymethyltriazenes are rapidly converted to the corresponding hydroxymethyltriazene in PBS.[10–12]

Attempts to monitor the formation of **5** by HPLC failed due to its instability under the separation conditions. However, when **6** was allowed to degrade overnight under physiological conditions, we could successfully detect amine **3** (Schemes 2 and 3) as the sole product, suggesting that the putative intermediary metabolites may have been formed according to the mechanism outlined in Scheme 3. This mechanism has already been proposed for similar triazenes.[11,12] Addition of water to iminium ion **7** may generate the methylol **5**, which may self-deformylate to give the monoalkyltriazene **8**. To circumvent problems associated with on-column degradation of **6** and that of its metabolite **5**, we designed a fluorescence detection assay in order to monitor the formation of **3**, the ultimately generated stable metabolite. Absorption–emission spectra of the latter showed absorption at 290 nm and emission at 450 nm. Thus, we could unambiguously analyze the kinetics of formation of **3** by spectrofluorimetry. Interestingly, the rate of conversion of **5** to **3** was slower than that of **8** to **3**. These data support a stepwise degradation of the CRM in the sequence proposed in Scheme 3. More importantly, the symmetrically inversed relationship between the degradation of the diverse metabolites and the formation of **3** suggest a near stoichiometric conversion of **6** to **3** (Figure 1). A similar result was reported for triazenoquinazolines of types **1** and **8**.[3]

Biology. Our objective being to generate multiple inhibitors from a parent one, we tested the EGFR TK inhibitory activity of each of the putative metabolites alone in a short 8-min exposure ELISA. The assay was based on the inhibition of phosphorylation of poly(L-glutamic acid-L-tyrosine, 4:1) (PGT) by EGFR tyrosine kinase. Structure–activity relationships (SAR) in the quinazoline series have already demonstrated tolerance of bulky substituents at the 6- and 7-positions.[13–15] Indeed, the triazene appendage to the 6-position of this series did not significantly alter the EGFR binding affinities when compared with **3**. As an example, the IC_{50} values for **3** and **8** were in the same range [e.g., **8** (IC_{50} = 0.07 μM), **3** (IC_{50} = 0.04 μM)] (Table 1). More importantly, previous studies have demonstrated the strong dependence of the SAR of quinazolines on the electronic character of the substituents at the 6-position.[14,15] Electron-donating groups at the 6-position enhance activity, whereas electron-withdrawing ones are deleterious to affinity. In this study, it was found that acylation of **1** decreased the affinity of the resulting

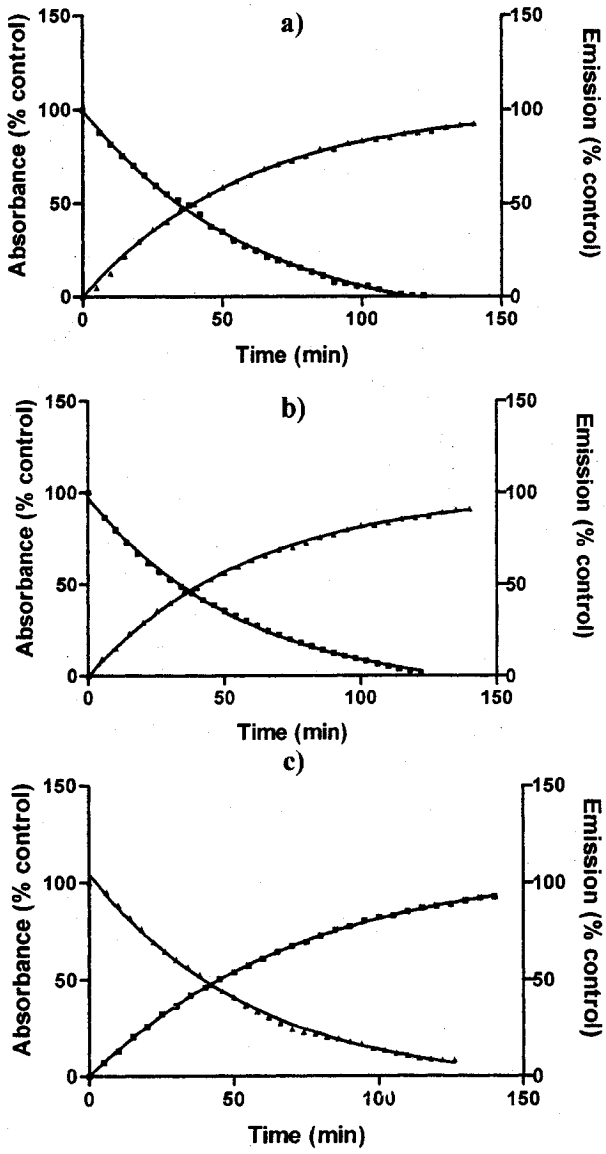


Figure 1. Formation (■) of **3** during the degradation (▲) of (a) **6**, (b) **5**, and (c) **8** in RPMI supplemented with FBS 10% at 37 °C. Degradation experiments were carried out by UV spectrophotometric analysis. The analyses were performed at 25 μM. Drugs were added to RPMI-1640 with 10% FBS (2 mL) and incubated at 37 °C.

compound **2** by 3-fold (Table 1). This may be due to the decrease in the electron-donating character of the triazene, which may deplete the partial charges on the nitrogens in the quinazoline ring. Indeed, when acylation was performed in a manner that did not affect the p-extended system of 1,2,3-triazene (as in **6**), there was little change in binding affinity when compared with the parental monomethyl triazene **8**. A significant decrease in the electron density at N1 was already demonstrated by ^{15}N spectroscopy when N3 in cyclic and acyclic triazenes are acylated.[16,17]

Previous studies demonstrated that serum stimulation of isogenic cells transfected with EGFR or the HER2 gene product represent good models for the determination of tumor selectivity of combi-molecules,[4] since they offer the advantage of growth-stimulating the two cell lines with a common growth factor. When this

Table 2. Inhibition of Serum-stimulated Growth in NIH3T3, NIH3T3/HER14, and NIH3T3/neu Cell Lines by Compounds **1–3**, **5**, **6**, **8**, **TEM**, **Ag1478** and Combination of **TEM** + **AG1478**

	IC_{50}, μM[a]		
compds	NIH3T3	NIH3T3/HER14	NIH3T3/neu
1	125 ± 1.7	28.9 ± 5.5	16.5 ± 2.3
2	200 ± 6.3	149 ± 14.2	60.6 ± 6.9
3	14.5 ± 1.9	10.4 ± 0.5	0.42 ± 0.03
5	12.8 ± 0.45	2.1 ± 0.3	0.09 ± 0.03
6	15.4 ± 0.55	3.4 ± 1.2	0.21 ± 0.05
8	15 0.5 ± 1.9	8.8 ± 0.6	0.09 ± 0.03
TEM	214.0 ± 11.1	207.2 ± 12.7	210.0 ± 16
AG1478	28.6 ± 3.6	9.8 ± 0.3	4.0 ± 0.2
TEM + AG1478	39.4 ± 5.4	6.2 ± 0.5	1.1 ± 0.06

[a] Values are means of IC_{50} values and SEMs from three experiments.

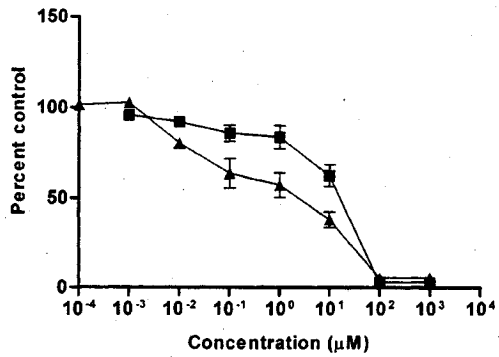
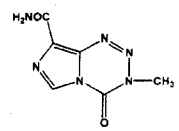


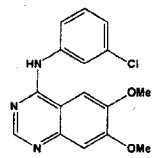
Figure 2. Effect of **6** on serum stimulated-proliferation in NIH3T3 (■) and NIH3T3/neu (▲) cells. Cells were starved for 24 h and exposed to each drug + serum for 72 h and growth inhibition was measured using SRB assay. Each point represents at least two independent experiments run in triplicate.

model was used, all compounds possessing EGFR TK inhibitory activities selectively blocked serum-stimulated growth of NIH 3T3 cells transfected with EGFR (NIH3T3/HER14) or HER2 (NIH3T3/neu), indicating translation of the EGFR TK inhibition into blockade of growth-factor-mediated proliferation (Table 2). A typical NIH3T3/NIH3T3neu differential growth response profile is shown in Figure 2. Moreover, it is noteworthy that the cascade release molecule **6** is significantly more potent than its metabolite **8** ($p < 0.001$) in the EGFR transfected cell line NIH3T3/Her14. However, the order of potency of these two molecules was inverted in the NIH3T3/neu cell line, indicating that perhaps **6** is less selective for HER2/neu than **8** and therefore may not serve as an efficiently targeted prodrug of **8** in these cells.

To further demonstrate the efficacy of the cascade combi-molecular approach, the effects of **6** were compared with those of classical combination of temozolomide (TEM) and AG1478, a known in vivo active EGFR TK inhibitor.[18] The combinations were performed at IC_{50}(TEM)/IC_{50}(AG1478) molar ratios. The results showed that **6** was 2.5- and 5-fold more potent than equieffective doses of TEM + AG1478 in NIH3T3/Her14 and NIH3T3/neu cells, respectively. Superior activity (e.g. 10-fold) when compared with the TEM + AG478 combinations was observed for **8** only in the NIH3T3/neu cells, which is in agreement with its strong activity in the latter cell line. These results support the significant antiprolif-



TEM



AG1478

erative potency of our combi-targeting and combi-molecular CRM approaches.

The hydrolysis of our CRM is expected to generate multiple inhibitors of EGFR and, at the final stage of degradation, the methyldiazonium species. Since the latter is known to be the cytotoxic DNA-alkylating metabolite of triazenes,[3-5,19] we studied the alkylating potency of the different agents using the 4-(*p*-nitrobenzyl)pyridine (NBP) test.[20] In this colorimetric assay, the alkylating species is trapped by NBP and a purple color is developed following addition of base. As a control, we compared the alkylating capacity of our compounds with that of a clinical DNA-methylating cyclic triazene, temozolomide (TEM). The NBP assay indicated that the parent compound **6** had a slower rate of alkylation when compared with the putative metabolite **5**, **8**, and TEM (Table 1). As expected, the naked inhibitor **3** demonstrated no alkylating activity, and the stable acylated compound **2** was found to have the slowest rate of alkylation in the entire triazene series.

The translation of the alkylating activity of **5** and **6** into DNA damage in human tumor cells was tested using the single cell microelectrophoresis (comet) assay in mouse fibroblast isogenic cell lines NIH 3T3 and NIH3T3/neu. In contrast to the naked inhibitor **3**, the acetoxymethyltriazene **6** was found to induce a dose-dependent increase in DNA strand breaks in both NIH 3T3 and its transfected counterpart, NIH 3T3/neu (Figure 3) confirming the binary targeting properties of the CRM.

This study conclusively demonstrates the feasibility of a molecule that may degrade in a stepwise mechanism into intermediary structures that when tested independently showed EGFR TK inhibitory activities. Although it was not possible to monitor the kinetics of formation of all the degradation products (e.g. the methylol intermediate **5** or the monoalkyltriazene **8**), their ultimate conversion to the stable metabolite **3** suggests that they may have been formed according to the mechanism outlined in Scheme 3. Further, in addition to the ability of the CRMs to release inhibitors of EGFR, their alkylating activity and their DNA-damaging potential suggest the formation of the putative methyldiazonium species. Thus, the combi-targeting and CRM principles mimic the effects of complex multidrug combinations involving modern inhibitors of EGFR TK and a classical DNA-damaging agent. More importantly, the results presented herein suggest that these effects may be selectively targeted to cells expressing the HER2 or EGFR oncogenes.

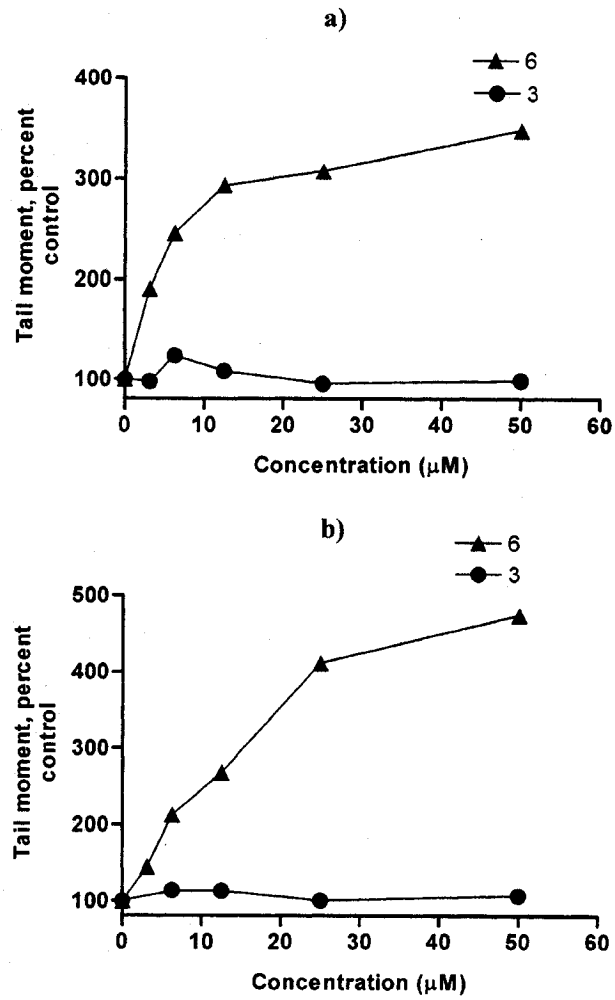


Figure 3. Quantitation of DNA damage using the alkaline comet assay. DNA damage induced by RB24 and RB10 in (a) NIH 3T3 and (b) NIH 3T3/neu cell lines. Tail moment was used as a parameter for the detection of DNA damage in NIH 3T3 and NIH 3T3/neu cells exposed to RB24 or RB10 for 30 min.

Experimental Section

Chemistry. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Varian 400 or 300 spectrometer. Mass spectroscopy was performed by the McGill University Mass Spectroscopy Center. ESI and APCI spectra were performed on a Finnigan LC QDUO spectrometer. Data are reported as *m/z* (intensity relative to base peak = 100). Melting points were determined in open capillary tubes on a Meltemp melting point apparatus and were uncorrected.

6-(3-Acetyl-3-methyltriazenyl)-4-(*m*-toluidyl)quinazoline (2). To a solution of 6-(3-methyltriazenyl)-4 anilinoquinazoline **1** (100 mg, 0.400 mmol) in methylene chloride (5 mL) was added triethylamine (0.1 mL). The mixture was stirred for 15 min at 0 °C and acetyl chloride (0.05 mL, 0.920 mmol) was added dropwise. Thereafter, the solution was evaporated under vacuum and the resulting brown residue purified on a silica gel column (40% ethyl acetate in hexane) to give **2** as a brown powder (18 mg, 16%): mp 188 °C; FABS *m/z* 335.2 (MH^+), 235 (M − 3-acetyl-3-methyltriazene) *m/z* 335.162 034 (MH^+), $C_{18}H_{18}N_6O_1$ requires 335.161 90; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.84 (s, 1H, NH), 8.58 (s, 1H), 8.14 (d, 1H, J = 2.1 Hz), 8.12 (d, 1H, J = 2.1 Hz), 7.83 (d, 1H, J = 8.7 Hz), 7.68 (s, 1H), 7.66 (s, 1H), 7.26 (t, 1H, J = 7.8 Hz), 6.95 (d, 1H, J = 8.1 Hz), 3.39 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 2.34 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.3, 158.6, 155.7,

150.7, 146.6, 139.5, 138.2, 130.0, 128.9, 125.3, 124.5, 123.6, 120.6, 120.4, 116.0, 28.4, 22.8, 22.0.

6-(3-Hydroxymethyl-3-methyltriazenyl)-4-(3′-bromophenylamino)quinazoline (5). To a solution of 4-(3′-bromophenylamino)-6-aminoquinazoline **3** (100 mg, 0.318 mmol) in acetonitrile (20 mL) kept at 0 °C on an ice bath was added nitrosonium tetrafluoroborate (74.3 mg, 0.637 mmol) dropwise. The solution was stirred for 20 min and 0.9 mL of a mixture of 40% methylamine (0.075 mL, 0.954 mmol), 37% formaldehyde (0.75 mL, 9.54 mmol), concentrated HCl (0.1 mL) was added all at once. The diazonium solution was subsequently alkalinized with potassium carbonate (400 mg, 2.86 mmol) and the precipitate was filtered to give a white solid contaminated with excess potassium carbonate. The precipitate was therefore resuspended in THF and the resulting solution filtered. The organic solvent was further evaporated to give **4** as a pure brown solid (90 mg, 73%): mp 150 °C; FABS *m/z* 387 (MH^+ with ^{79}Br), 389 (MH^+ with ^{81}Br), 359 ($M - N_2$) *m/z* 387.056 895 (MH^+), $C_{16}H_{15}N_6O_1Br$ requires 387.056 70; ^1H NMR (400 MHz, DMSO-d_6) δ 9.91 (s, 1H, NH), 8.57 (s, 1H), 8.50 (s, 1H), 8.25 (s, 1H), 7.99 (d, 1H, $J = 8.8$ Hz), 7.92 (d, 1H, $J = 7.6$ Hz), 7.76 (d, 1H, $J = 8.8$ Hz), 7.34−7.25 (m, 2H), 6.37 (s, 1H, OH), 5.17 (s, 2H, CH_2), 3.19 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.3, 158.0, 154.0, 148.9, 149.8, 130.9, 129.5, 126.3, 125.2, 124.7, 121.8, 121.2, 116.4, 116.1, 78.8, 34.0.

6-(3-Acetoxymethyl-3-methyltriazenyl)-4-(3′-bromophenylamino)quinazoline (6). 6-(3-Hydroxymethyl-3-methyltriazenyl)-4-(3′-bromophenylamino)quinazoline **5** (100 mg, 0.258 mmol) was dissolved in pyridine (2 mL), after which acetic anhydride (0.055 mL, 0.516 mmol) was slowly added. The solution was further kept on ice for 1.5 h and the pyridine evaporated as an azeotrope with toluene to give **6** as a pure brown residue (105 mg, 95%): mp 140 °C; FABS *m/z* 429 (MH^+ with ^{79}Br), 431 (MH^+ with ^{81}Br), 298 [M − (3-acetoxymethyl-3-methyltriazene)], 401 ($M - N_2$) *m/z* 429.067 460 (MH^+), $C_{18}H_{17}N_6O_2Br$ requires 429.067 28; ^1H NMR (400 MHz, DMSO-d_6) δ 10.02 (s, 1H, NH), 8.61 (s, 2H), 8.25 (s, 1H), 7.99 (d, 1H, $J = 8.4$ Hz), 7.93 (d, 1H, $J = 7.6$ Hz), 7.79 (d, 1H, $J = 9.2$ Hz), 7.35−7.26 (m, 2H), 5.86 (s, 2H, CH_2), 3.26 (s, 3H, CH_3), 2.07 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.8, 158.1, 154.4 149.5, 147.7, 141.8, 130.9, 129.7, 126.5, 125.1, 124.8, 121.8, 121.3, 117.3, 116.2, 79.4, 35.5, 21.6.

Drug Treatment. Compounds **1**, **2**, **3**, **5**, **6**, and **8** were synthesized in our laboratories according to known procedures. Temozolomide (TEM) was provided by Shering-Plough Inc. (Kenilworth, NJ). In all assays, drugs were dissolved in DMSO and subsequently diluted in RPMI-1640 containing 10% fetal bovine serum (FBS) (Wisent Inc., St-Bruno, Canada) or in DMEM containing 10% bovine calf serum (GIBCO BRL, Burlington, Canada) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

Purity of Compounds. Purity analyses were performed by high performance liquid chromatography (HPLC) using a Spectrasystem (Thermoquest) and a Waters C4 15-μm 300 × 3.9-mm column (reverse phase). The operating mode was isocratic and two different systems of solvents were used: the first one was 100% acetonitrile with a 0.35 mL/min flow rate at 254 nm and the second system was 80% 2-propanol and 20% dioxane with a 0.5 mL/min flow rate at 254 nm.

Cell Culture. The cell lines used in this study, the mouse fibroblasts NIH3T3, NIH3T3/HER14 (NIH3T3 cells stably transfected with the EGFR gene), and NIH3T3/neu (NIH3T3 cells stably transfected with HER2) were generous gifts from Dr. Moulay Aloui-Jamali of the Montreal Jewish General Hospital. NIH3T3, NIH3T3/HER14, and NIH3T3/neu cells were maintained in DMEM supplemented with 10% FBS and antibiotics. All cells were maintained in an atmosphere of 5% CO_2.

Degradation. The half-lives of **6**, **5**, and **8** under physiological conditions were studied by a UV spectrophotometer. The compounds were dissolved in a minimum volume of DMSO and diluted with RPMI-1640 supplemented with 10% FBS, and absorbances were read at 240 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).

The study of the conversion of the compounds (**6**, **5**, and **8**) to their corresponding free inhibitor (**3**) was performed by spectrofluorometry, as the latter amine is fluorescent (absorption 290 nm, emission 450 nm). Briefly, 125 μM of the drug conjugates was added to RPMI-1640 with 10% FBS and incubated for 4 h at 37 °C in a microplate spectrofluorometer (SpectraMax Gemini flourescence reader, Molecular Device, CA). The data were acquired and analyzed by SoftMaxPro (Molecular Device, CA).

HPLC analysis was performed to study the degradation of compounds **2**, **5**, and **6**. The compounds were dissolved in a minimum volume of DMSO, diluted with RPMI-1640 supplemented with 10% FBS, using a Spectrasystem (Thermoquest) and a Waters C4 15-μm 300 × 3.9-mm column (reverse phase). The operating mode was isocratic using a 60% acetonitrile and 40% water solvent system with a 0.5 mL/min flow rate at 254 nm.

EGFR Kinase Assay. Nunc Maxisorp 96-well plates 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 (Tween 20 (0.1%) in PBS). The kinase reaction was performed by using 4.5 ng/well EGFR affinity-purified from A431 cells. The compounds were added and phosphorylation was 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 following a 25 min incubation with 50 μL/well of HRP-conjugated PY20 anti-phosphotyrosine antibody (Santa Cruz Biotechnology, CA) diluted to 0.2 mg/mL in blocking buffer (3% bovine serum albumin, 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, 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).

In Vitro Growth Inhibition Assay. To study the effect of our compounds on serum-stimulated proliferation, cells were grown to 70% of confluence in 96-well plates and washed twice with PBS, after which they were exposed to serum-free media for 24 h. Cells were exposed to each drug + serum for 72 h, and cell growth was measured using the sulforhodamine B (SRB) assay. 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 the optical density was read for each well at 492 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

Alkylating Activity Assay. For each time point, 10 μL of compound 50 mM was added to 2% *p*-nitrobenzylpyridine (NBP)−ethylene glycol (100 μL) mixed with Tris pH 7.5 (27% ethanol) (70 μL) and incubated at 37 °C. The color was developed with a solution of acetone−triethylamine (50/50, v/v). Optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550).

Comet Assay for Quantitative Analysis of DNA Damage. A modified alkaline comet assay technique was used to quantitate DNA damage induced by **6** and **5** in NIH3T3 and NIH3T3/neu cells. The cells were exposed to drugs for 24 h and harvested with trypsin-EDTA. They were subsequently collected by centrifugation and resuspended in PBS. The resulting cell suspensions were diluted to 3 × 10^5 cell/ mL, and mixed with 0.75% agarose in PBS at 37 °C in a 1:9

dilution. The gels were cast on Gelband strips (Mandel Scientific, Guelph, Canada) using gel casting chambers, as previously described, and then immediately placed into a lysis buffer (2.5 M NaCl, 0.1 M tetrasodium 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 immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetrasodium 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 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 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.

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Sustained antiproliferative mechanisms by RB24, a targeted precursor of multiple inhibitors of epidermal growth factor receptor and a DNA alkylating agent in the A431 epidermal carcinoma of the vulva cell line

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Recently, with the purpose of enhancing the potency of epidermal growth factor receptor (EGFR)-based therapies, we designed a novel strategy termed 'Cascade-release targeting' that seeks to develop molecules capable of degrading to multiple tyrosine kinase (TK) inhibitors and highly reactive electrophiles, in a stepwise fashion. Here we report on the first prototype of this model, RB24, a masked methyltriazene, that in addition to being an inhibitor on its own was designed to degrade to RB14, ZR08, RB10+a DNA alkylating methyldiazonium species. The cascade degradation of RB24 requires the generation of two reactive electrophiles: (a) an iminium ion and (b) a methyldiazonium ion. Thus, we surmise that these species could alkylate the active site of EGFR, thereby irreversibly blocking its action and that DNA damage could be induced by the methyldiazonium. Using the EGFR-overexpressing human epidermoid carcinoma of the vulva cell line, A431, we demonstrate herein that (a) RB24 and its derived species (e.g. RB14, ZR08) irreversibly inhibit EGFR autophosphorylation, (b) RB24 induced significant levels of DNA strand breaks, (c) sustained inhibition of EGFR by RB24 was associated with blockade of MAPK activation and c-fos gene expression, (d) RB24 induced irreversible cell growth inhibition with a 100-fold greater potency than Temodal™, a clinical methyltriazene. The pronounced growth inhibitory potency of RB24 was attributed to its ability to simultaneously damage DNA and irreversibly block EGFR TK activity.
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Overexpression of the epidermal growth factor receptor (EGFR) family and its cognate ligand has been correlated with aggressiveness and poor prognosis in various tumours such as breast, ovarian and prostate cancer (Modjtahedi and Dean, 1994; Xie *et al*, 1995; Turner *et al*, 1996; Meden and Kuhn, 1997; Chen *et al*, 2000). The implication of these receptors in cancer progression has garnered significant attention and agents capable of blocking disordered growth signalling mediated by these proteins are now in a significant number of clinical trials against many cancers. One such agent, Iressa, a trademark of the AstraZeneca group of companies, exhibits a broad spectrum of antitumour activity against many human solid tumour xenografts of various origins including breast, lung, colorectal and head and neck (Ciardiello *et al*, 2001a; Moulder *et al*, 2001; Heimberger *et al*, 2002; Magné *et al*, 2002; Ranson *et al*, 2002). However, despite the significant activity of this compound in preclinical models, in Phase II clinical trial, it only induced a response rate of approximately 10% in a cohort of patients with non-small-cell lung cancer (NSCLC) (Magné *et al*, 2002; Dancey and Freidlin, 2003). Several possible explanations have been put forth to explain the failure to demonstrate a benefit: inadequate dosing, reduced drug delivery to tumour, lack of sustained potency and failure to select patients on the basis of having tumours in which EGFR presents a growth advantage (Dancey and Freidlin, 2003).

The lack of sustainability of the antitumour action of reversible inhibitors has stimulated the design of new irreversible inhibitors of EGFR tyrosine kinase (TK). One such compound, PD183805, bearing a 6-acrylamido group designed to alkylate cysteine 773 in the active site of the ATP-binding pocket irreversibly blocks EGFR TK and is now in Phase I development in patients with head and neck, breast and non-small cell lung carcinoma (Ciardiello and Tortora, 2001b). Other approaches to enhance the potency of EGFR inhibitor-based therapy include combinations of inhibitors with several cytotoxic agents including taxol, cytoxan and adriamycin (Ciardiello *et al*, 1999, 2001a; Magné *et al*, 2002; Dancey and Freidlin, 2003). Within the same line of idea, with the purpose of developing more potent and targeted therapies, we

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developed a novel strategy termed 'Combi-targeting' that seeks to synthesise single molecules that are capable of both blocking EGFR TK and inducing cytotoxicity by damaging DNA (Brahimi *et al*, 2002; Matheson *et al*, 2001, 2003a, 2004a; Qiu *et al*, 2003, 2004; Rachid *et al*, 2003). Two such compounds, SMA41 (Matheson *et al*, 2003a, 2004b) and BJ2000 (Brahimi *et al*, 2002), the first models designed to demonstrate the feasibility of this principle, showed significant DNA-damaging ability and irreversible block of EGFR autophosphorylation in A431 cells. More importantly, these agents induced a more sustained antiproliferative activity when compared with a reversible inhibitor of EGFR (Matheson *et al*, 2001, 2004b; Brahimi *et al*, 2002). In addition, these molecules, also termed 'Combi-molecules,' selectively induced antiproliferative activity against EGFR transfectants in isogenic models (Brahimi *et al*, 2002; Matheson *et al*, 2003a).

In order to further augment the potency of the Combi-targeting approach, we recently designed RB24 (an acetoxymethyltriazene) to generate three inhibitors of EGFR and at the final stage of degradation a methyldiazonium species capable of damaging DNA (Banerjee *et al*, 2003). This masked cluster of molecules was expected to produce more sustained antitumour effects with the prospect of inducing activities similar or superior to that of classical combinations involving a reversible EGFR inhibitor + a cytotoxic drug. Here we study the mechanism of action of RB24, the first prototype of this strategy termed 'cascade release', and demonstrate the sustainability of its antitumour activity in A431 cells, which overexpress EGFR and its cognate ligand, TGF-α (Lanzi *et al*, 1997). The ability of this cell line to aggressively proliferate by a TGF-α-mediated autocrine induction has made it an ideal model for studying the mechanism of action of EGFR TK inhibitors. In addition, these cells express *O*6-alkylguanine transferase (*O*6-AGT), a DNA repair enzyme that by repairing the *O*6-alkylguanine DNA adduct confers significant resistance to AGT + cells (Lee *et al*, 1991; Mitchel and Dolan, 1993; Pegg *et al*, 1995; Cai *et al*, 2000; Yingna *et al*, 2000; Matheson *et al*, 2003b).

MATERIALS AND METHODS

Drug treatment

RB24, RB14, ZR08 and RB10 were reported elsewhere (Banerjee *et al*, 2003). Temodal™ was provided by Shering-Plough Inc. (Kenilworth, NJ, USA). In all assays, the drug was dissolved in DMSO and subsequently diluted in RPMI-1640 containing 10% fetal bovine serum (FBS) (Wisent Inc., St-Bruno, 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, USA). The A431 cell line was maintained in RPMI-1640 supplemented with 10% FBS and antibiotics as described previously (Matheson *et al*, 2001). All cells were maintained in an atmosphere of 5% CO_2.

In vitro cytokinetic growth inhibition assay

To study the irreversible effects of our compounds on cell proliferation, 200 cells well^{-1} were plated in 96-well plates with serum-containing media. Cells were exposed to each drug + serum for either 2, 8, 12, 24 or 48 h. After each time point, the drug was removed and cells were washed 2 × with PBS and allowed to recover with fresh serum-containing media for a total of 96 h. Cell growth was measured using the sulphorhodamine B (SRB) assay. Briefly, following drug treatment and recovery, 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 coloured residue was dissolved in 200 μl of Tris base (10 mM), and optical density was read for each well at 492 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

Autophosphorylation assay

A431 cells (1×10^6) were preincubated in a six-well plate with 10% serum at 37°C for 48 h and starved 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^{-1} EGF for 15 min at 37°C. Cells 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 concentrations of protein were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were added to a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Nonspecific binding on the membrane was minimised with a blocking buffer containing nonfat dry milk (5%) in PBST. Thereafter, the membranes were incubated with primary antibodies (either antiphosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA) for the detection of phosphotyrosine, or anti-EGFR (Neomarkers, Fremont, CA, USA)) for determination of corresponding receptor levels. Blots were incubated with HRP-goat anti-mouse antibody (Bio-Rad Laboratories) and the bands visualised with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Band intensities were measured using the SynGene GeneTools software package.

To study the effects of RB24 on the activation of extraceullular signal-regulated kinases 1,2 (Erk1,2), protein lysates were obtained as described above and Western blot was performed as previously reported (Tari and Lopez-Berestein, 2000). The membrane was incubated with antiphosphorylated Erk1,2 antibodies or antibodies specific for Erk1,2 (Cell Signaling, Beverly, MA, USA).

Reverse EGFR autophosphorylation

This assay was performed as previously described (Fry *et al*, 1998). A431 cells were grown to confluence in six-well plates and then incubated in serum-free medium for 24 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 (50 ng ml^{-1}) for 15 min and extracts were made as described under the Western blotting procedure above. The other set of cells was washed free of the compound with warm 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 24 h. Cells were exposed to the indicated concentrations of the drug prior to stimulation with EGF (50 ng ml^{-1}) for 30 min. Total RNA was isolated using the High Pure RNA Isolation Kit of Roche Molecular Bochemicals (Germany), following the manufacturer's instructions. Quantitative analysis of c-fos mRNA and G3PDH mRNA (2 μg of RNA for each sample) was preformed by Titan One Tube RT-PCR Kit

(Roche Molecular Bochemicals), following the manufacturer's instructions and using the following primers: 5′ATGATGTTCTC GGGCTTC3′ (sense), 5′CTCCTGCCAATGCT CTGC3′ (antisense) for c-fos and 5′CCATGGAGAAGGCTGGGG3′ (sense), 5′CAAA GTTGTCATGGATGACC3′ (antisense) for G3PDH.

Alkaline comet assay for quantitation of DNA damage

The alkaline comet assay was performed as previously described (Matheson *et al*, 2001). The cells were exposed to drugs (RB24, RB14 or RB10) 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, 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 tetrasodium 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, 10 mM Tris-base) containing 1 mg ml^{-1} 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 20 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, USA) for 20 min. Comets were visualised at × 330 magnification and DNA damage was quantitated using the Tail Moment parameter (i.e. the distance between the barycentre 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 were analysed for each sample, using ALKOMET version 3.1 image analysis software.

RESULTS

Inhibition of EGFR autophosphorylation

Western blot analysis demonstrated that RB24 blocked EGF-induced EGFR autophosphorylation in A431 cells in a dose-dependent manner with an $IC_{50} \approx 2\ \mu M$ without affecting the levels of EGFR (Figure 1). RB24 is a degradable molecule capable of generating an extremely reactive iminium ion intermediate that may alkylate the receptor and irreversibly inhibit EGFR activity. To test this hypothesis, we used the reversibility assay previously described (Fry *et al*, 1998; Smaill *et al*, 2000) according to which the cells were treated with the drug for 90 min and the culture medium repeatedly removed and replaced three times after treatment, after which EGFR autophosphorylation was measured. As expected, RB24 at 30 μM completely suppressed EGF-dependent EGFR autophosphorylation in A431 cells immediately after drug exposure (Figure 2A). However, at 8 h post-treatment following repeated washouts in drug-free medium, EGFR autophosphorylation activity was completely inhibited in cells treated with RB24, indicating that the latter is capable of inducing irreversible inhibition of EGFR autophosphorylation. Similarly, irreversible inhibition was observed in cells exposed to the daughter molecules, RB14 and ZR08, however with a 20% recovery of the total activity (Figure 2B). In contrast, the naked inhibitor, RB10, was shown to have completely reversible inhibition of EGFR autophosphorylation (Figure 2A).

Inhibition of EGFR-mediated signalling

Antiproliferative activity induced by RB24 requires the translation of inhibition of EGFR autophosphorylation into inhibition of downstream signalling. To determine whether blockade of EGFR autophosphorylation translates into inhibition of downstream signalling, we analysed the effect of the parent compound, RB24, on EGF-induced phosphorylation of Erk1,2 and c-fos expression in A431 cells. The results showed that RB24 induced complete inhibition of Erk1,2 phosphorylation at concentrations as low as 5 μM without affecting the levels of Erk1,2 (Figure 3). Similarly, RT–PCR analysis showed that RB24 induced nearly 100% inhibition of EGF-mediated c-fos gene expression at low concentrations (1 μM) (Figure 4), indicating that inhibition of EGFR phosphorylation by RB24 is accompanied by a significant blockade of EGFR-dependent downstream signalling.

Quantitation of DNA damage

Using the alkaline comet assay, we demonstrated that like TEM (Matheson *et al*, 2001), RB24 and RB14 induced a dose-dependent DNA damage in A431 cells after a 30 min drug exposure (Figure 5). Interestingly, RB24 and RB14 induced identical levels of DNA damage, which is in agreement with the fact that the former is a prodrug of the latter. As expected, the reversible inhibitor, RB10, did not demonstrate any DNA-damaging activity.

Irreversible growth inhibitory activity

SRB assays demonstrated that RB24 and RB14 retained significant antiproliferative activity after a short 2 h exposure and a 4-day recovery (Figure 6A and B). In contrast, the free inhibitor, RB10, lost at least 95% of its activity under the same conditions (Figure 6D). The monoalkyltriazene, ZR08, showed partially reversible activity with an 85% retention of activity following 2 h drug exposure (Figure 6C). In contrast to the antiproliferative effects of RB24, increasing exposure time was associated with a significant increase in potency for all other molecules of the degradation cascade (RB14, ZR08, RB10). Despite being a potent

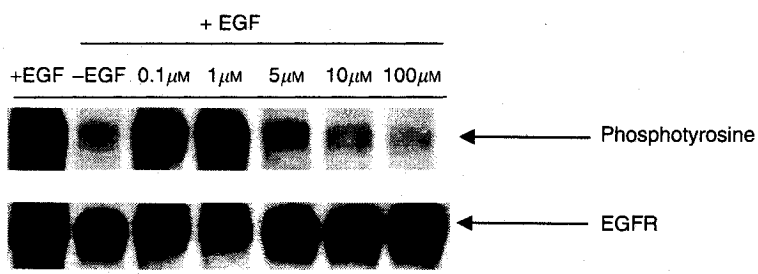


Figure 1 Selective inhibition of EGFR autophosphorylation in intact cells by RB24. Serum-starved A431 cells were preincubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF for 15 min. An equal amount of cell lysates was analysed by Western blotting using antiphosphotyrosine antibodies. Membranes were stripped of antiphosphotyrosine and reprobed with anti-EGFR antibodies as a loading control. Band intensities were measured using the SynGene GeneTools software package.

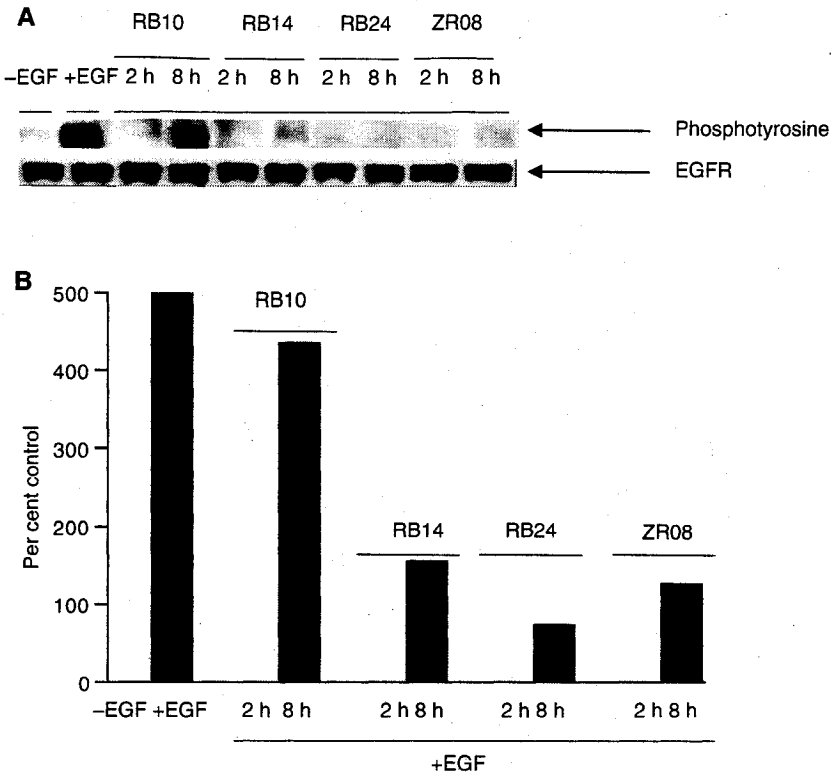


Figure 2 Reverse EGFR autophosphorylation in the presence of RB24, RB14, ZR08 and RB10 in A431 cells. (**A**) Duplicate sets of cells were treated with 30 μM of designated compound to be tested as a reversible EGFR inhibitor for 90 min. One set of cells was then stimulated with EGF for 15 min, and extracts were made as described under the Western blotting procedure. The other set of cells was washed free of the compound with serum-free media, incubated for 2 h, and further washed twice and incubated for 4 h. This set of cells was then stimulated with EGF, and extracts were made similar to the first set. (**B**) Comparison between the inhibition of autophosphorylation activity induced by RB24, RB14, ZR08 and RB10. The film was scanned and band intensities were quantified using Syngene GeneTools software. Values are percentage of control of phosphotyrosine/EGFR.

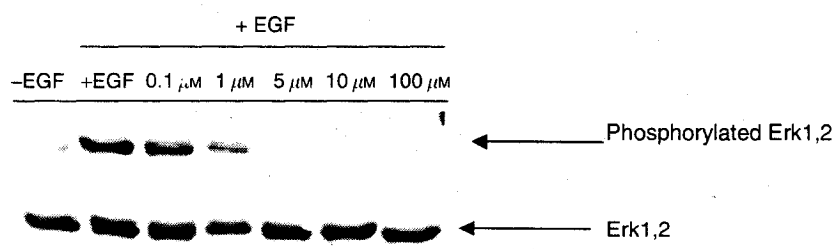


Figure 3 Effect of RB24 on Erk1,2 activation in A431 cells. Serum-starved cells were preincubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF for 15 min. Protein lysates were obtained and Western blot was performed as described by Tari and Lopez-Berestein (2000).

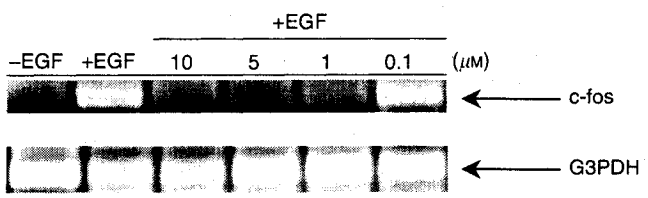


Figure 4 Effect of RB24 on c-fos gene expression in A431 cells. Serum-starved cells were preincubated for 2 h with the indicated concentrations of RB24 prior to stimulation with EGF for 30 min. Quantitative analysis of c-fos and G3PDH was preformed by RT–PCR as described in Materials and methods.

alkylating agent, the clinical triazene, Temodal™, demonstrated no significant activity at any of the exposure times in the A431 cells (Figure 6E). In summary, the strength of the retention of potency was in the following order: RB24 > RB14 > ZR08 ≫ RB10.

DISCUSSION

Mechanisms of EGFR TK inhibition

The design of our combi-molecule, RB24, was based on the premise that acetoxymethyltriazenes are known to be hydrolysed to a hydroxymethyltriazene intermediate that rapidly degrades

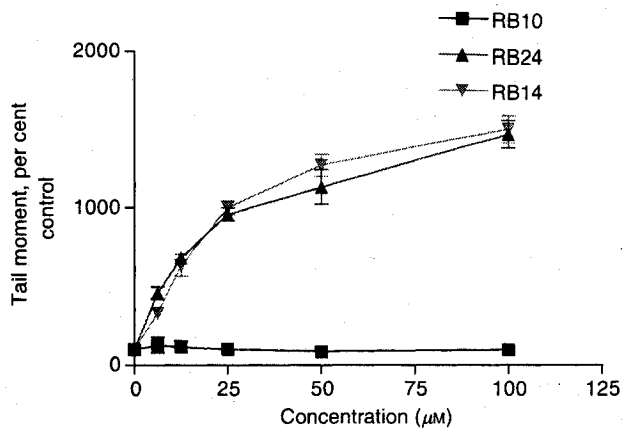


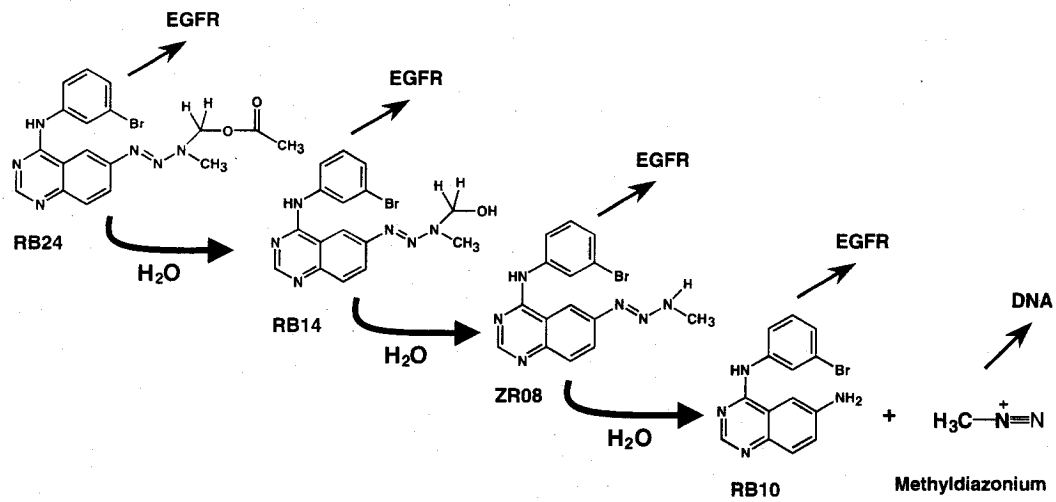
Figure 5 Quantitation of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in A431 cells exposed to RB24, RB14 and RB10 for 30 min. Each point represents at least two independent experiments.

into the corresponding monoalkyltriazene (Hemens *et al*, 1984; Cameron *et al*, 1985; Hemens and Vaughan, 1986). The latter further heterolyses to an aromatic amine + a DNA-damaging species. In a recent publication (Banerjee *et al*, 2003), we demonstrated that indeed RB24 was capable of generating the ultimate amino compound RB10, in a symmetrically inversed relationship. As depicted in Scheme 1, decomposition through the intermediates, RB14 and ZR08, is the sole mechanism by which RB24 can be converted to RB10. Thus, the significant EGFR inhibitory potency of the putative intermediates RB14, ZR08 and RB10 indicates that RB24 would maintain its TK inhibitory potency throughout the multistep degradation cascade. More importantly, RB24 and its two derivatives (RB14, ZR08) induced irreversible inhibition of EGFR TK when tested alone, a protracted inhibition that may be rationalised in the light of recent data on the mechanism of irreversible acrylamide-based inhibitors. It is now known that 4-anilinoquinazolines bearing a 6-acrylamido group react with the cysteine 773 of EGFR, thereby irreversibly blocking the receptor (Fry *et al*, 1998; Smaill *et al*, 1999, 2000). Based on the mechanism of decomposition of RB24 (Banerjee *et al*, 2003), we did not expect a direct reaction between the cysteine thiol group and the acetoxy moiety. Perhaps, if the iminium ion is formed in the active site of EGFR, it may react with the cysteine as depicted in Scheme 2. Addition of thiol group to the iminium ions of acetoxymethyltriazene has already been reported by Iley *et al* (1991) who developed this type of triazenes as lyase inhibitors. Moreover, the ultimate triazene metabolite of RB24, ZR08, may also directly alkylate the cysteine residue. Quinazolinotriazenes of the same class, BJ2000 and SMA41, have already been demonstrated to induce irreversible inhibition (Brahimi *et al*, 2002; Matheson *et al*, 2003b, 2004b).

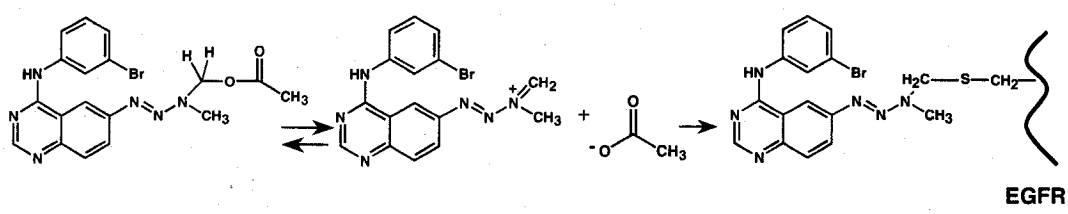
Although it was not the focus of this study to identify the site of alkylation of the receptor, the irreversible nature of TK inhibition by RB24 indicates that it may have inflicted covalent damage at a site of EGFR that is critical for its TK activity. In corroboration, RB10 that does not possess a reactive triazene tail induced reversible EGFR TK inhibitory activity.

DNA damage

The iminium ion is a stable cation that may not be reactive enough to account for the strong DNA-damaging potential of RB24. Despite the significant body of results that confirm its formation



Scheme 1



Scheme 2

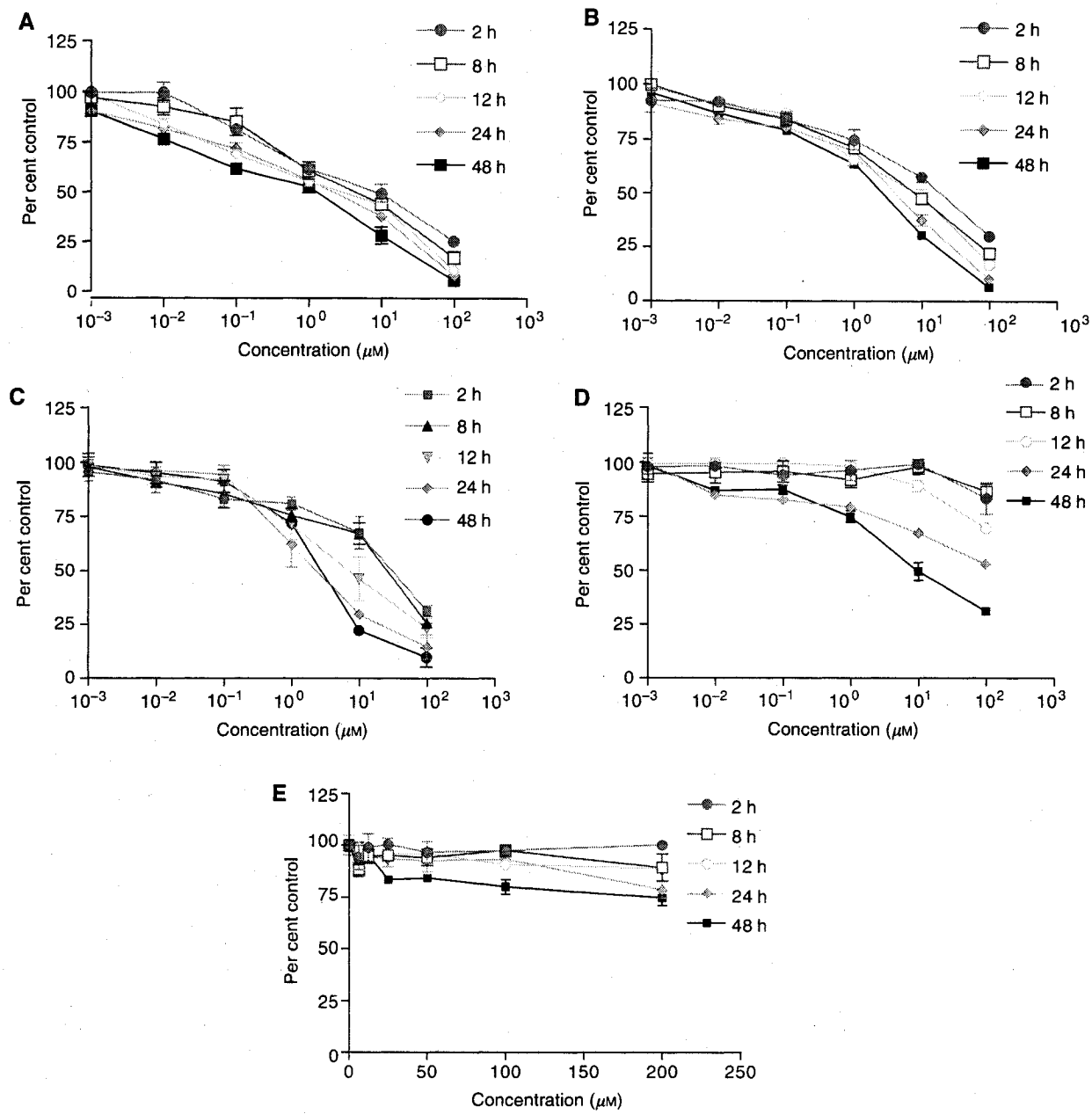


Figure 6 Irreversible growth inhibition for RB24, RB14, ZR08, RB10 and TEM in A431 cells. Cells were exposed to (**A**) RB24, (**B**) RB14, (**C**) ZR08, (**D**) RB10 or (**E**) TEM for 2, 8, 12, 24 or 48 h following recovery for a total of 96 h. Cell growth was measured using SRB assay. Each point represents at least two independent experiments run in triplicate.

during the process of hydrolysis of acetoxymethyltriazenes (Hemens *et al*, 1984; Hemens and Vaughan, 1986; Iley, 1987; Vaughan and Manning, 1988; Merrin and Hooper, 1992), its implication in the DNA-damaging properties of the latter class of compounds is yet to be demonstrated. In contrast, it is now common knowledge that the methyldiazonium is capable of inducing significantly high levels of DNA alkylation particularly at N7 and O6 positions of guanine, thereby inducing DNA damage and lethal mutations in tumour cells (Bodell *et al*, 1985; Tisdale, 1987; Baer *et al*, 1993). The promutagenic *O*6-alkylguanine adduct is considered to be the primary cytotoxic lesion induced by triazenes. Cells, like A431, that express high levels of AGT, a DNA repair enzyme that repairs the *O*6-alkylguanine adduct, are known to be resistant to alkyltriazenes. In corroboration, the A431 cells used in the study were markedly insensitive to Temodal™, a cyclic triazene known to release the methyldiazonium species upon hydrolysis (Cameron *et al*, 1985; Gibson *et al*, 1986; Baig and Stevens, 1987).

Irreversible growth inhibition

The ability of RB24 to degrade into several species with significant EGFR inhibitory activities and to damage DNA may perhaps be responsible for its sustained potency in A431 cells. More importantly, while its antiproliferative effect was maintained even 4 days after a 2 h drug exposure, under the same conditions, ZR08

induced a partially reversible growth inhibitory activity. We have already reported similar results in A431 cells for analogous quinazolinotriazenes (e.g. SMA41 and BJ2000). Thus, the marked irreversibility of the action of RB24 may be partially imputed to additive antiproliferative contribution of species generated during the degradation steps that precede the formation of ZR08. This antiproliferative contribution may result from their ability to block EGFR-mediated growth signalling, since as previously mentioned the transient iminium ion species generated during these degradation steps may not damage DNA. The partially reversible growth inhibitory potency of ZR08 (third step of the cascade) may perhaps be due to the dependence of its antiproliferative activity on the ultimately released reversible inhibitor RB10, the concentrations of which might have been drastically depleted by multiple washouts. This indicates that the ultimate antiproliferative effect of ZR08 is mediated by a combination of DNA damage inflicted by the methyldiazonium species and EGFR TK inhibitory activity induced by RB10.

A synergistic interaction between the strong EGFR TK inhibitory potency and the marked DNA-damaging potential of RB24 may also partly account for its remarkable potency against the A431 cells. Indeed, blockade of EGFR in these cells was accompanied by potent inactivation of Erk1,2 kinase and inhibition of c-fos gene expression. We now know, based on previous experiments, that blockade of EGFR-mediated signalling may not affect the levels or function of the AGT enzymes. However, Yacoub *et al* (2003) recently demonstrated that EGF upregulates the DNA repair genes XRCC1 and ERCC1 in prostate cell lines through Erk1,2 signalling. Thus, blockade of EGFR-mediated signalling may downregulate the DNA repair enzymes that are involved in the repair of DNA strand breaks, thereby exacerbating the cytotoxic effect of the latter lesions.

In conclusion, we have demonstrated that a molecule engineered to possess complex EGFR inhibitory properties and capable of further releasing DNA-damaging species could induce significantly more sustained antiproliferative activity than a reversible inhibitor of the same class (e.g. RB10). Moreover, its multiple properties have conferred antiproliferative activity in a cell line in which a classical methylating agent of the same class does not show any detectable activity. Thus this model may well represent a new strategy to ameliorate the chemotherapy of EGF-dependent refractory tumours. Further studies are ongoing to demonstrate the potency of this novel drug *in vivo*.

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

Name	Department	Job Title/Classification	Trained in the *safe Use of Biological Safety Cabinets* within the last 3 years? If yes, indicate date of attendance
Dr. Bertrand J. Jean-Claude	Medicine	Investigator	
Dr. Fouad Brahimi	Medicine	Fellow	
Dr. Qiyu Qiu	Medicine	Fellow	
Dr. Zakaria Rachid	Medicine	Fellow	
Dr. Juozas Domarkas	Medicine	Fellow	
Dr. Nuria Merayo	Medicine	Fellow	

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

The biohazardous material involved are human tissues (human tumour cell lines) and murine designated at 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
-Hand washing will be reinforced

All work with cell lines is performed in a certified containment hood, gloves and sterile procedures are followed throughout

iii) the protocol for decontaminating spills

-Spills are decontaminated using HG-7 hospital antiseptic agent
-Procedures for spills and contact persons' names and phone numbers are posted
-Autoclavable materials will be sterilized

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

NO

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

YES

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9. What precautions will be taken to reduce production of infectious droplets and aerosols?

The use of laminar flow hood for tissue culture and sterilization of work surfaces with 70% ethanol. All procedures are performed in containment hoods of a Certified Biosafety Cabinet

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10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

NO

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11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

Yes. Radioactive waste will be brought down to the Service de radioprotection du CUSM (Royal Victoria Hospital, Rm. H3.51, Responable: Christian Janicki) for proper disposal.

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12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Royal Victoria Hospital	M7.15	Microzone	Microzone	8017156	July 2, 2004