INTERACTION OF COMBINED ADMINISTRATION OF MULTI-TARGETED KINASE INHIBITORS WITH IONIZING RADIATION

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DEDICATION

This thesis is dedicated to my brother who supported me unfailingly every step of the way and to my parents who taught me to be patient, accountable and always to aim high. I am here today because of you!

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ABSTRACT

Along with surgery and chemotherapy, radiation therapy is one of the three cancer treatment modalities to treat patients. Over the recent decades, thanks to technological progress, radiation techniques have been improved drastically. However, the same effort has not been put into the biology of the tumours and how different tumours interact with radiation beams. Moreover, it has been only recently that attention has been drawn towards the chemo-radiation or combination of radiation with molecular targeting agents. In this thesis we have studied the combination of ionizing radiation with a class of small molecule inhibitors that target defects in the MAPK/PI3K pathway in breast cancer cells. These pathways are often over-activated in human malignancies including breast cancer. Two of the multiple receptors involved in these pathways are EGFR and VEGFR, which have been shown to be over-expressed in cancerous tumours and have been associated with poor prognosis as well as drug and radiation resistance.

The two inhibitors we have used in this study are ZRBA1, a combi-molecule that targets EGFR and also induces DNA lesions, and Sorafenib (Nexavar), which is an inhibitor of several RTKs including VEGFR and also Raf kinase. Using breast cancer cells, we have shown that these multi-functional inhibitors increased the sensitivity of cancer cells towards radiation as they induced a strong G2/M cell cycle arrest and apoptosis. Our *in vivo* results show that ZRBA1 and Sorafenib, if combined with radiation, can significantly increase tumour growth delay. When radiation is administered concurrently with ZRBA1, a significant tumour growth delay of 47 days is observed. Moreover, ZRBA1 in combination with radiation not only induced the DNA single and double strand breaks, but also delayed

DNA repair process contributing to its higher potency against breast cancer cells. Interestingly, Sorafenib when it is combined with radiation has more persistent anti-tumour effect in our *in vivo* model.

Overall, our results suggest that the combined administration of multi-targeting molecular inhibitors, a systemic targeted therapy, with radiation, a local and regional therapy, could be beneficial for patients as they potentiate the radiation response while not increasing adverse side effects.

The results of these preclinical studies contribute to a better understanding of how radiation interacts with small molecule inhibitors such as ZRBA1 and Sorafenib and provide the rational basis for further preclinical and clinical studies.

RÉSUMÉ

En plus de la chimiothérapie et de la chirurgie, la radiothérapie est l'une des trois modalités thérapeutiques pour les patients atteints de cancer. Au cours des dernières décennies, grâce aux progrès technologiques et techniques, la radiothérapie s'est considérablement améliorée. Cependant le même effort n'a pas été dirigé à la biologie tumourale et les modes d'interactions associées à la radiation ionisante. Récemment, l'intérêt et les recherches se sont portés sur le traitement combiné de chimio-radiothérapie et molécules ciblées. L'objectif de cette thèse est d'étudier la combinaison de la radiation ionisante associée à une classe de petites molécules inhibitrices qui ciblent de multiples défauts dans les mécanismes cellulaires de transduction du signal MAPK/PI3K des cellules cancéreuses du sein. Ces voies sont souvent surexprimées dans les cancers humains, y compris le cancer du sein. Deux des multiples récepteurs impliqués dans ces voies sont EGFR et VEGFR dont la surexpression dans les tumeurs cancéreuses a été démontrée. De plus, ces voies sont associées à un mauvais pronostic ainsi qu'à une résistance importante aux médicaments et la radiothérapie. Les deux inhibiteurs que nous avons utilisés dans cette étude sont ZRBA1, molécule combinée qui cible EGFR et induit également des lésions de l'ADN et le Sorafénib (Nexavar), inhibiteur de plusieurs RTK dont VEGFR et kinase Raf. En utilisant des cellules de cancer du sein, nous avons démontré que ces inhibiteurs multiples ont augmenté la sensibilité des cellules cancéreuses à l'irradiation car ils induisent un arrêt remarquable du cycle cellulaire en phase G2/M et en apoptose. Nos résultats *in vivo* montrent également que ZRBA1 et Sorafenib lorsque combiné à la radiothérapie peuvent augmenter l'effet cytotoxique de façon significative. Lorsque la radiation est administrée en même temps que ZRBA1, on observe un retard important de la croissance tumourale de 47 jours. Outre,

ZRBA1 en combinaison avec la radiation non seulement induit des fractures de simple brin et double brins d'ADN, mais retarde également le processus de réparation de l'ADN contribuant ainsi à son puissant effet anti-tumoural. Quand Sorafenib est combiné à la radiothérapie, on observe de manière très intéressante, une persistance de l'effet anti-tumoural dans notre modèle in vivo.

Dans l'ensemble, nos résultats suggèrent que l'administration de molécules inhibitrices multikinases en thérapie ciblée systémique combinée à la radiothérapie comme traitement locorégional pourrait être bénéfique pour les patients, car cette approche favorise une meilleure réponse à l'irradiation et est potentiellement moins toxique.

Les résultats de cette étude préclinique contribuent à mieux comprendre l'interaction de la radiation et des petites molécules inhibitrices telles que ZRBA1 et Sorafenib. Ils fournissent également une base pour de futures études précliniques et cliniques.

CONTRIBUTION OF AUTHORS

This manuscript-based thesis is composed of three manuscripts. The contributions of each author are described below.

Chapter 2: The manuscript is published in Anti-Cancer Drugs 2012, 23:525–533. Sorafenib was provided by Bayer Pharmaceuticals as part of company contribution to an investigator grant support provided to Dr. Muanza. Nada Tomic, LiHeng Liang, Dr. Slobodan Device, Dr. Francois Deblois and Joseph Holmes designed the setting and performed radiation for the *in vitro* and *in vivo* studies. I designed, performed, analyzed all the *in vitro* and *in vivo* experiments and wrote the paper. Dr. Danuta Radzioch reviewed and revised the manuscript as needed. Dr. Thierry Muanza designed the overall research plan, over saw the project and reviewed the manuscript.

Chapter 3: This paper was published in Anti-Cancer Drugs 2009, 20:659–667. The chemical synthesis and purification of ZRBA1 were performed by Dr. Zakaria Rachid research associate at Cancer Drug Research Laboratory. Atta Goudarzi and Ava Schlisser helped with Flow cytometry and comet assay experiments respectively. I designed and performed the rest of the experiments and wrote the first draft of the manuscript. Dr. Bertrand Jean-Claude, Dr. Danuta Radzioch and Dr. Thierry Muanza revised the manuscript. Dr. Thierry Muanza over saw the project and designed the overall research plan.

Chapter 4: The paper is submitted to International Journal of radiation Oncology* Biology* Physics. ZRBA1 was synthesized and purified by Dr. Zakaria Rachid. Dr. Slawomir Kumala

helped with the design and performance of Comet and γH2AX assays as well as useful discussions. Dr. Bertrand Jean-Claude and Dr. Gerald Batist reviewed the project and had constructive suggestions. I designed and performed all the *in vitro* and *in vivo* experiments and wrote the manuscript. Dr. Danuta Radzioch and Dr. Thierry Muanza reviewed the manuscript and made the necessary corrections. Dr. Thierry Muanza over saw the project and designed the overall research plan.

CONTRIBUTIONS NOT INCLUDED IN THIS THESIS

In addition to the manuscripts included in this thesis, I contributed to the following studies, which have been published:

MAPKAPK-2 signalling is critical for cutaneous wound healing. Thuraisingam T, Xu YZ, Eadie K, Heravi M, Guiot MC, Greemberg R, Gaestel M, Radzioch D. J Invest Dermatol. 2010 Jan; 130(1):278-86.

Brg-1 mediates the constitutive and fenretinide-induced expression of SPARC in mammary carcinoma cells via its interaction with transcription factor Sp1. Xu YZ, Heravi M, Thuraisingam T, Di Marco S, Muanza T, Radzioch D, Mol Cancer. 2010 Aug 5; 9:210.

Effects of DNA-dependent protein kinase inhibition by NU7026 on DNA repair and cell survival in irradiated gastric cancer cell line N87. M.T. Niazi, G.Mok, M.Heravi, T.Vuong, L. Lee, R.Aloyz, L.Panasci, T. Muanza**.** Curr Oncol, April 2014, Accepted.

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LIST OF ABBREVIATIONS

Aag	Alkyladenine DNA-glycosylase
ADCC	Antibody- dependent cell-mediated cytotoxicity
AGAT	O6-alkylguanine-DNA alkyltransferase
AGT	O6-alkylguanine-DNA alkyltransferase
AICD	Activation induced cell death
AIF	Apoptosis inducing factor
AP-1	Activator protein-1
APAF-1	Apoptotic protease-activity factor-1
APE1	Apurinic/apyrimidinic endonucleases
ATase	O6-alkylguanine-DNA alkyltransferase
ATF-2	Activating transcription factor
ATG	Autophagy-related proteins
ATR	Ataxia telangiectasia and rad3 related
ATRIP	ATR-interacting protein
BAK	Bcl2-2 killer
BAX	Bcl2-assciated X protein
Bcl2	B cell lymphoma 2
BER	Base excision repair
BID	BH3-intercating domain bromide
CI	Combination index
CML	Chronic myeloid leukaemia

CPD	Cyclobutane pyrimidine dimmers
DAMP	Danger/damage-associated molecular patterns
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DDR	DNA damage response
DEF	Dose enhancement factor
DER	Dose enhancement ratio
DFS	Disease Free Survival
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNA-PKcs	DNA dependent Protein Kinase-catalytic subunit
DSBs	Double strand breaks
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERCC1	Excision repair cross-complementation group 1
ERK	Extra cellular signal-regulated kinase1/2
FA	Fanconi anemia
FADD	Fas- associated death domain protein
FBS	Fetal bovine serum
FDA	US Food and drug administration
GADD153	Growth arrest and DNA damage inducible gene 153 (CHOP)
GBM	Glioblastoma multiforme
GGR	Genome NER Repair
GIST	Gastro-intestinal stromal tumours

Gy	Gray
HCC	Hepatocellular Carcinoma
HR	Homologous repair
HSP90	Heats hock protein 90
ICLs	Interstrand Crosslinks
JNK	C-Jun NH_2 Terminal Kinase
LET	Linear energy transfer
LOH	Loss of heterozygosity
mAbs	Monoclonal antibodies EGFR
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MDM2/HDM4	Murine/human double minute 2 and 4
MGMT	Methylguanin DNA methyltranferase
MOMP	Mitochondrial outer membrane permeablization
mRCC	Metastatic renal cell carcinoma
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium
NBS1	Nijmegen breakage syndrome 1 protein
NCCD	Nomenclature Committee on the Cell Death
NER	Nucleotide excision repair
NHEJ	Nonhemologouse end-joining
O^6 Me Gua	O^6-methylguanine

OS	Overall survival
PARP1	Poly (ADP-ribose) polymerase-1
PBS	Phosphate buffered saline
PCNA	Proliferation cell nuclear antigene
PD	Pharmacodynamics
PDGFR	Platelet-derived growth factor receptor
PE	Plating efficiency
PI	Propidium iodide
PI3K	Phosphotidylinositol 3-Kinase
PIC I	Preincision complex 1
Rb	Retinoblastoma
RBE	Relative biological effectiveness
RFC	Replication factor
RIP-1 kinase	Receptor interacting protein-1
RTKS	Receptor tyrosine kinases
Sap-1	SRF accessory protein-1
SER	Sensitivity enhancement ratio
SF	Survival fraction
SMAC	Second mitochondria-derived activator of caspases
SMKI	Small molecule kinase inhibitor
SRC	Schmidt-Ruppin-A2 viral oncogene homology
SSBs	Single strand breaks
ssDNA	Single stranded DNA

TCR	Transcription coupled NER
TKD	Tyrosine kinase domain
TN	Triple negative
TRLS	Toll-Like receptors
UV	Ultra violet
VAMP	Vincristine, Adriamycin, methotrexate, prednisone
XIAP	X-linked inhibitor of apoptosis protein
XPA	Xeroderma pigmentosum complementation group A protein
XPC-TFIIH	XPC transcription factor
XRCC1	X-ray repair cross-complementing group

CHAPTER 1

INTRODUCTION

1.1. PREFACE

Better understanding of the molecular events involved in cancer development has led to the identification of a large number of novel molecular targets and to the development of multiple approaches to anti-cancer therapy. Targeted therapy focuses on specific genetics and molecular defects associated with malignancies. This includes crucial pathways and molecules involved in cell invasion, metastasis, apoptosis, cell-cycle control, and tumour-related angiogenesis.

This chapter briefly reviews some important concepts in molecular targets discovery, different approaches taken to design molecular targeting agents as well as the basic science and the rationale behind these approaches. The importance of radiation therapy in cancer treatment and biological effect of radiation on tumour progression is also discussed.

1.2. TARGETED THERAPIES IN CANCER

In the late 1800's Paul Ehrlich introduced a new concept in the drug discovery field called "magic bullet". He described it as a chemical that specifically targeted microorganisms [1-2]. Since then, his concept has been expanded to include cancer treatment as well. During the last decades the same concept has been developed into novel cancer-treatment strategies [3]. These new strategies include monoclonal antibodies [4], small molecules, peptide mimetics and antisense oligonucleotides [5]. Monoclonal antibodies (mAbs) and small molecules have attracted the most attention in drug development programs. This review of the literature, however, focuses on the application of small molecules in cancer treatment.

1.2.1. Monoclonal antibodies

Over the past decades, the effectiveness of antibodies in treating patients with cancer has been increasingly recognized [6]. In the late 90's, first chimeric and humanized antibodies were developed and recently, with the help of advanced antibody technology, two fully human mAbs have been introduced (Panitumumab, Ofatumumab) [7]. Importantly, with the advanced technology and development of chimeric and fully humanized antibodies the limitation associated with traditional mAbs is no longer relevant [8].

During the recent years, several mAbs have been approved as treatment in cancer patients [2, 7]. These antibodies are designed to target the ligand binding site and disrupt the signalling pathways, target the tumour microenvironment by binding to the tumour secreted growth factors, or to target the immune system itself [7].

Cetuximab or Erbitux (ImClone Systems/Bristol-Myers Squibb) is a classic example of an approved chimeric IgG1 mAb which targets epithelial growth factor receptor [9] by preventing the ligand from binding to epidermal growth factor receptor (EGFR) [10] and inhibiting the receptor dimerization [11]. Similar to Cetuximab, Panitumumab (Vectibix; Amgen), a fully humanized IgG2 antibody against EGFR, also functions by a similar mechanism; however, it does not promote Antibody-dependent cell-mediated cytotoxicity (ADCC). While Panitumumab is used as the second or third line treatments for patients with metastatic colon cancer, Cetuximab is often used in combination with chemotherapies [12]. Bevacizumab [4, 13] is a humanized mAb that blocks binding of vascular endothelial growth factor (VEGF) to its receptor. It is approved for the treatment of breast, non-small-cell lung cancer and colorectal cancer in combination with cytotoxic chemotherapy [14].

Rituximab (Rituxan/Mabthera; Genentech/Roche/Biogen Idec) is a chimeric IgG against CD20 approved for treatment of non-Hodgkin lymphomas. It has been suggested that this mAb induces programmed cell death (apoptosis) in tumour cells expressing CD20 [15].

1.2.2. Small molecules

Small molecules are a fast growing class of targeted therapies and have been the focus of many drug discovery research programs during the last decades.

With the increased understanding of the key-signalling pathways in normal and cancer cells, more specific targets have been identified that may increase the therapeutic ratio of cancer therapy regiments. One of the major approaches in molecular-targeting strategies has been to target the protein kinases, an important component of major signalling pathways. Moreover, receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (non-RTKs) are crucial mediators in signalling pathways such as proliferation, differentiation, migration, angiogenesis and cell cycle regulation and often are deregulated in tumour cells. Unlike the mAbs that function through the immune system, small molecule inhibitors target the kinases by direct effects on tumour cells.

The majority of small molecule kinase inhibitors (SMKIs) are ATP mimetics. Imatinib mesylate (Glivec) [16] is a classic example and the first successful SMKI. It inhibits the kinase activity of BCR-ABL fusion protein [2, 17-19]. Glivec has been approved for chronic myeloid leukaemia (CML) and gastro-intestinal stromal tumours (GIST) [19].

Due to their small sizes, SMKIs are able to translocate through plasma membrane and interact with cytoplasmic components and intracellular signalling molecules. Using this characteristic, several SMKIs have been developed to directly target and inhibit molecules

involved in signalling pathways. Inhibitors of Ras prenylation [20], Raf-Mek kinase [21], phosphotidylinositol 3-Kinase (PI3K), the mammalian target of rapamycin (mTOR) [22] as well as heat shock protein 90 (HSP90) [23] have also been developed to target the survival and proliferation pathways. Other SMKIs are designed to inhibit cancer-cell adhesion and invasion by inhibiting SRC kinase [24], or neovascularization by inhibiting the VEGFR [25].

Several molecular targets and their recently developed inhibitors will be discussed in the next section.

1.2.2.1. Small molecule multiple kinase inhibitors

Imatinib, which selectively binds KIT, platelet-derived growth factor receptor (PDGFR), and BCR-ABL, is the first example of multikinase inhibitor. Imatinib was approved by the FDA for first line treatment of CML and GIST. Other examples are Sorafenib and Sunitinib which have been approved for metastatic renal cell carcinoma (mRCC) or hepatocellular carcinoma (HCC) and for Imatinib-resistant GIST [26] . More than a decade ago Hanahan and Weinberg [5] explained that malignant tumours are complex tissues that involve both cancer cells and their microenvironment, including: supportive cells, vascular endothelial cells, fibroblasts, immune cells and vascular pericytes [27]. Based on these ideas, Hanahan and Weinberg suggested a multi-targeted anti-cancer therapy which involves cancer cells as well as supportive tissues. Given their central role in cancer, growth receptors and their signal transduction processes represent key targets.

The rationale behind the development of multi tyrosine kinase inhibitor as opposed to nonselective cytotoxic therapies are: (I) cancer development is characterized by multiple

abnormalities rather than a single defect, and it is unlikely that a single target agent will have dramatic effects on both cancer cells and cells supporting tumour development such as endothelial cells and pericytes. Therefore, an optimal therapy may need to involve a multi-targeted TKI or combination of drugs targeting signalling pathways in both tumour cells and surrounding cells. Moreover, targeting two or more defects in a given cancer- related pathway will have greater effects than targeting a single component, e.g. inhibiting both an RTK and a downstream effector may be more effective than blocking one.

(II) It is now well-established that when a tumour has grown, it is very likely that it already contains at least a small number of clones (resistant phenotypes or cancer stem cells) that are resistant to any type of therapy. This could explain why more advanced solid tumours frequently become refractory to treatment with single-target agents or cytotoxic agents using a single mechanism of action. Additionally, new resistance mutations may arise over time [28]. In either case, primary or acquired resistance or resistance due to the mechanisms such as overexpression of key cancer pathways or drug efflux systems[26] is less likely to be an issue when agents or therapeutic regimens target multiple molecules involved in cancer development or utilize multiple mechanisms of action [28-29].

(III) The effectiveness of conventional cytotoxic therapies (or radiotherapy) may increase when combined with targeted anti-cancer agents. In the case of agents targeting angiogenesis, for example, the improvement may be attributed, at least in part, to an initial normalization of the tumour vasculature that results in improved delivery of traditional cytotoxic agents to the tumour, tumour reoxygenation and improve sensitivity to ionizing radiation [30-31].

1.2.3. Monoclonal antibodies vs. Small molecules

MAb and small molecule inhibitors have been developed in a different time line. First mAbs were developed in the early 1980's with the advanced hybrydoma technology, while small molecule inhibitors had already been introduced a decade ago [2]. Development of mAb is more time consuming and has a higher cost; however, more pharmaceutical companies are willing to invest in this sector, especially after the humanization technique was introduced. Also, humanized and chimeric Abs have a higher approval rate with almost 24% and 18% respectively [32], while small molecules have an approval rate of 5% in the field of oncology [33].

MAbs generally have a larger size which is the cause of inefficient delivery through blood-brain barrier for treatment of brain tumours. The large size of the mAbs also causes decreased tissue penetration and tumour retention as well as slower blood clearance. That may explain why most approved mAbs are used to treat the haematological malignancies.

Pharmacokinetic studies have shown that the half lives of mAbs are relatively long (3-7days in case of Cetuximab) vs. Gefitinib which has a half life of 28 hrs. This would allow for daily dosing of small molecules. Importantly, mAbs are administrated intravenously while small molecule inhibitors have the advantage of being taken orally. Small molecules and mAb have distinct mode of action biologically. MAbs target the cellular surface and secreted molecules in contrast to small molecules which can penetrate into the cellular membrane and target the intercellular molecules.

Commonly, small molecule inhibitors are considered to be less specific than mAbs. Importantly, this can be an advantage for this class of drugs as they can inhibit not only one but multiple signalling pathways at clinically relevant concentrations [34].

1.3. MOLECULAR TARGETS IN CANCER

In this section several target pathways in cancer and a number of developed targeting strategies in clinical trials/clinics will be briefly discussed.

1.3.1. MAPK Pathway

Mitogen-activated protein kinase (MAPK) pathway is one of the prominent pathways involved in the development and progression of cancer. MAPK links extra-cellular signals to the internal signal transduction pathways that control fundamental cellular processes such as growth, proliferation, apoptosis and migration [35].

In mammalian cells, three MAPK families have been clearly characterized: MAPK (also known as extra cellular signal-regulated kinase1/2 (ERK1/2)), C-Jun N terminal kinase and p38 kinase. Each MAPK family consists of three enzymes that are activated in series: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAP kinase (MAPK) (shown in figure 1-1).

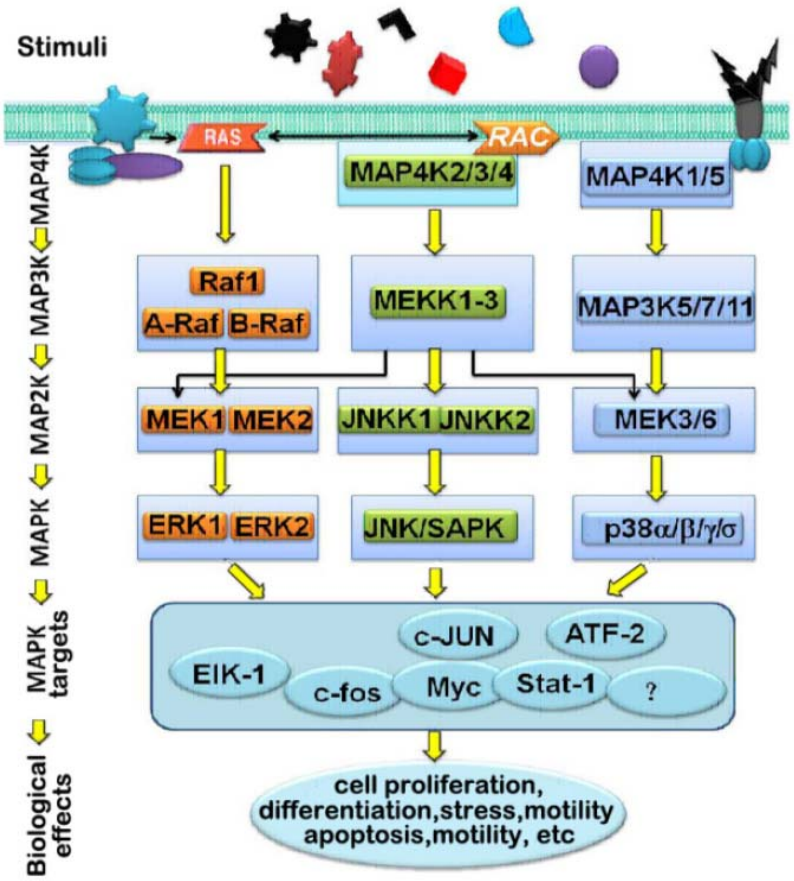

Figure 1-1. MAPK signalling pathway [36].

1.3.1.1. Receptor Tyrosine kinases (RTKs)

Among the most mature of molecular targets in the MAPK signalling pathway are the EGFR family members, VEGFR as well as downstream Raf kinases. There is a clear rationale to target these receptors and kinases as they are usually over-expressed in tumour cells. EGFR is over-expressed in a diverse array of epithelial tumours such as breast carcinomas,

prostate, lung, gliomas [37] and head and neck cancers [38] and has also been associated with activation of the AKT pathway leading to anti-apoptotic effects and reduced sensitivity to chemotherapy, resistance to ionizing radiation and poor prognosis. VEGFR over-expression has also been detected in non- small cell lung, breast, prostate, renal cell and colorectal cancers [39].

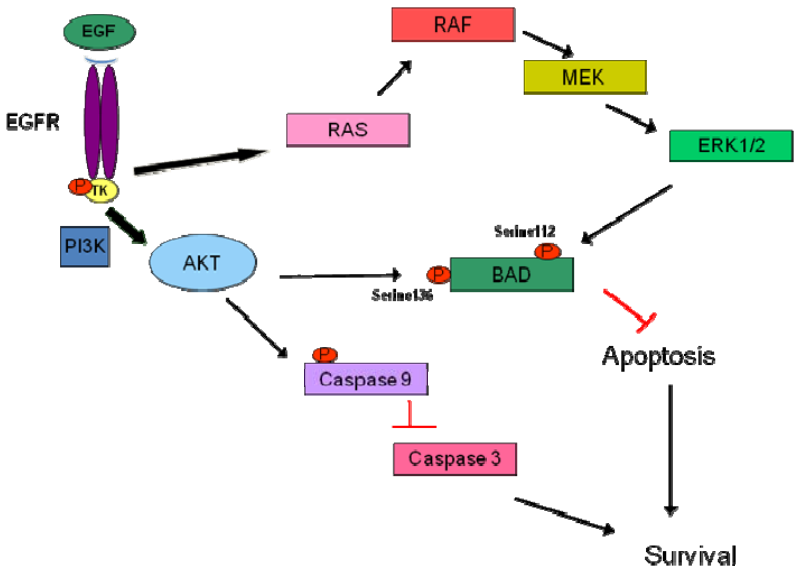

Figure 1-2. Downstream signalling by Receptor Tyrosine Kinases.

Dysregulated signalling through Raf kinase isoforms is detected in 30% of human cancers [40]. Constitutive B-Raf activity due to oncogenic mutations is prevalent in more than half of melanomas and papillary thyroid carcinomas. Importantly, wild-type Raf1 is hyperactivated in a wide range of human solid tumours as a result of hyper-activation of upstream oncogenic Ras mutants, or the over-expression of upstream growth factors and/or

their RTKs (EGFR, VEGFR). Furthermore, constitutively active Ras oncogenes (particularly K-Ras) are common in human solid tumours [40].

Blocking EGFR-mediated signalling has already been shown to induce anti-tumour activity *in-vivo* [41]; however; the observed activity is transient and reversible [42]. Recent clinical trials with anti-EGFR and angiogenic inhibitors have shown increase in progression--free survival with only minimal increase in patients' overall survival [43].

In general, RTKs inhibitors can only lead to disease stabilization and longer periods of progression-free survival as they are mainly cytostatic agents. Therefore, it may be of interest to combine them with a cytotoxic modality such as ionizing radiation or chemotherapies to achieve more sustained anti-tumour activity.

Several MAPK-targeted therapies have been approved for cancer treatment. Gefitinib for lung cancer, lapatinib for breast cancer, Sorafenib and Suntinib for renal cancer and Imatinib and Dasatinib for CML are just a few examples. Also, more clinical trials are ongoing to evaluate different schedules and combinations of existing molecular targeting agents.

The stimulation of RTKs activates MAPKs in a multistep process. A bivalent ligand interacts with two receptor molecules and effectively cross-links them into a dimeric complex. This leads to phosphorylation of the receptors themselves at the intracellular tyrosine kinase domain (TKD) resulting in activation of multiple signalling pathways.

1.3.1.2. ERK pathway

Receptor-linked tyrosine kinases are activated by extracellular ligands. Binding the receptors to the corresponding ligand activates the tyrosine kinase cavity of the cytoplasmic domain of the receptor. Docking proteins such as GRB2 contain an SH2 domain that binds to the phosphotyrosine residues of the activated receptor. GRB2 also binds to guanine nucleotides exchanged factor (SOS) through its two SH3 domains. When GRB-SOS complex docks to phosphorylated receptor, SOS becomes activated and promotes the removal of GDP from a member of Ras family. Ras (K-Ras) can bind to GTP and becomes active. Activated K-Ras stimulates protein kinase activity of Raf kinase. This leads to MEK and then ERK activation. The activated ERK translocates to the nucleus and activates transcription factors, changing gene expression to promote growth, differentiation and other fundamental cellular process [44]. ERK also phosphorylates Bad at Ser 112 which leads to the apoptosis inhibition.

1.3.1.3. JNK pathway

The C-Jun NH_2 Terminal Kinase (JNK) pathway is activated in cells by extracellular stimuli including stress, UV irradiation and cytokines [45]. A variety of receptor-associated signaling mechanisms lead to the activation of mitogen-activated protein kinase kinase kinase (MAPKKK) that are capable of activation of either MAPKK4 or 7 (MAP2K4 or MAP2K7). MAP2K4 can activate either the JNK or the p38-MAP Kinase. MAP2K7 selectively activates the JNK [46-47]. JNK activation requires dual phosphorylation of Thr183 and 185, triggering the interaction of JNKs with C-Jun component of the activator

protein-1 (AP-1) transcription factor [47]. C-Jun is phosphorylated at Ser63 and 73 and this leads to enhanced transcription activity of AP-1[48-49].

Activation of JNKs can also induce cellular apoptosis by activating an intrinsic pathway whereby Bcl-2 family of proteins promote release of pro-apoptotic molecules such as cytochrom-c from mitochondria [45].

1.3.1.4. P38 pathway

The mammalian p38 MAPK families are activated by cellular stress including UV irradiation, heat shock, high osmotic stress, LPS, protein synthesis inhibitors and proinflammatory cytokines such as IL-1 and TNF-α. Stress signals are delivered to this cascade by member of small GTPase of the Rho family including Rac, Rho and Cdc42. The signals are then transferred through MAP3K9 and MAP3K1 as well as MAP2K4 which activate/phosphorylate p38MAPK. Activated p38 phosphorylates the transcription factors such as ATF-2, Sap-1 and GADD153 [50]. P38 also regulates NF-κB dependent transcription after its translocation into the nucleus [51].

1.3.1.5. PI3K/AKT Pathway

The Phsophatidylinositol-3-kinase (PI3K) signalling pathway is critical for cell growth and survival. It has been targeted by several genomics aberrations more frequently than other pathways in human cancer, possibly after p53 and retinoblastoma (Rb) pathway [52]. PI3K pathway is also the subject of stimulation by many growth factors and regulators. Activation of PI3K through RTKs or through genetic defects, results in aberrant cell survival and

growth which can cause aggressive cell growth, metastatic ability and resistance to treatment in human malignancies.

A major PI3K associating protein is the serine/threonine kinase Akt (also called protein kinase B). Akt activates several proteins also critical to cell growth and survival. Propapototic proteins such as BAD, caspase 9 and forkhead (FOX) family of transcription factors are inhibited by Akt activation [53].

The involvement of the PI3K pathway in cancer begins with the activation of RTKs which results in recruitment of PI3K to plasma membrane anchored receptors and becomes activated. Activated PI3K phosphorylates PIP_2 to generate PIP_3 which then binds to Akt. Activated Akt phosphorylates Bad at Ser 136. Upon phosphorylation Bad disassociates from Bcl-2 and Bcl-X_L and sequesters with 14-3-3 in the cystosol leading to apoptosis inhibition [54].

The major elements of PI3K pathway have been found mutated or amplified in a broad range of cancers indicating the essential roles of PI3K in human cancer. This has made PI3K a promising target in anti-cancer drug development. There are several inhibitors that have been developed to target PI3K but only few of them have been approved. This can be due to limitation towards the identification of targets and drugs with a sufficient therapeutic index to allow clinical implementation [52]. Wortmanin and LY294002 were the first PI3K inhibitors; however, they did not go through the clinical trials due to their toxicity in preclinical models [52, 55]. Several newly developed PI3Ks have made their way to clinical trials. BEZ235 is a dual inhibitor of PI3K and mTOR in phase I and II clinical trial. The most recent PI3K inhibitor, XL147, targets class I of PI3K isoforms. It is an orally available agent and has been well-tolerated in phase I clinical trials [56-57]. Combination

studies of XL 147 are also ongoing with Gefitinib or Lapatinib and Mek inhibitor, AZD 6244 [58]. MK2206, an allosteric inhibitor of AKT1 and 2, is being clinically tested (phase I) in NSCLC and locally-advanced or metastatic tumours [55, 59].

1.3.1.6. DNA repair

One common approach in anti-cancer drug development is to inhibit the cell cycle progression and cell division and therefore to inhibit cell growth. Cell division can be targeted directly by inhibitors of the mitotic spindle. The other alternative is to inhibit the growth signals that results in entry into the cell cycle.

Targeting the cell cycle, in order to exploit the effect of DNA-damaging drugs, has been one of the frequent subjects of anti-cancer drug strategies [60]. DNA damage causes cell-cycle arrest and cell death either directly or following DNA replication during the S phase of the cell cycle. Cellular attempts to replicate damaged DNA can cause increased cell killing, therefore making DNA-damaging treatments more toxic to replicating cells than to non- replicating cells. Importantly, the toxicity of DNA-damaging drugs can be minimized by the activities of several DNA repair pathways that remove lesions before they become lethal. The efficacy of DNA damage-based cancer therapy can thus be affected by DNA repair pathways. Interestingly, some of these pathways are deactivated in some cancer types which have made the DNA repair mechanism a potential target for cancer treatments. The best example of this scenario would be the mutation or loss of heterozygosity (LOH) of BRCA1 and 2 in some breast and ovarian tumours. Targeting the DNA repair proteins such as PARP in these tumours has shown promising results in phase I and II clinical trials [(AZD2281 (AstraZeneca) and BSI-201 (BI Par)] [52, 61].

Using an alternative approach to interfere with cell cycle checkpoint signalling by modulating DNA repair activity has resulted in development of XL-844 (EXEL -9844). XL-844 is a small-molecule inhibitor of Chk1 and Chk2. This drug causes inhibition of cell-cycle arrest, progressive DNA damage, inhibition of DNA repair and, tumour cell apoptosis *in vitro* [62]. XL-844 is currently being evaluated in a phase I clinical trial in combination with gemcitabine [52].

1.3.1.7. p53

p53 is a well-known transcription factor and also a tumour suppressor. Under normal, unstressed conditions, p53 protein remains undetectable due to its short half life [63]. The protein level of p53 is controlled by its negative regulator murine/human double minute 2 and 4 (MDM2/HDM4) via ubiquitination (ubiquitin liagse, E3). p53 is responsive to a wide variety of cellular stresses including genotoxic damages, oncogene activation, and hypoxia [64-65]. It is activated through posttranslational modifications by phosphorylation, acetylation, ubiquitination, and methylation [66-67]. Activated p53 then can be functional in several processes: apoptosis, growth arrest [65, 68], angiogenesis [69-70], and autophagy regulation [71]. The major role of p53 in several important pathways makes it a good candidate to target in malignancies. In fact, p53 is inactivated in about 50% of human cancers [63].

Although p53 has good potential as a target, it may not be druggable protein according to the conventional drug discovery and development process. It is neither a receptor nor an enzyme. P53 is a homo tetrameric transcription factor and has an essential role in maintaining genomic instability and key processes in normal cells [72-73] . In

addition, p53 is inactivated through different mechanisms: inhibition of p53 by MDM2 or viral proteins, complete deletion of or/and inactivating mutations either in coding regions or regulatory regions which counts for more than 2000 different p53 mutations [74]. Therefore, due to technical challenges and also due to possible high adverse effects, targeting p53 may have not been the subject of many clinical trials. However, in recent years, some strategies have tried to restore the lost activity of p53 in some tumours.

Viral based gene therapy such as Advexin and Gendicine have been tested in phase I, II and III clinical trials in patients with head and neck and hepatocellular carcinomas. The overall results suggested that the adverse effect was not significant and the anti-tumour effect was associated with expression level of functional (wt) p53 [75-77]. ONYX-015 is an example of oncolytic adenovirus therapy and in a phase I clinical trial has shown to have expected efficacy and low adverse effects in patients with solid tumours [78].

The lost function of p53 can also be reversed by targeting the post-translational modification of p53. HLI98 is an inhibitor of HDM2 ubiquitin ligase (E3) which is responsible for the degradation of p53. Targeting HDM2 E3 activity seems to be an efficient strategy as this approach by-passes the genotoxic effect of the current cancer drugs. Recently there have been more studies on this class of drugs [79].

Nutlins belong to the class of drugs called small molecule inhibitors of protein–protein interactions (PPI). Nutlins inhibit the interaction between MDM2 and p53 and have shown a desirable safety profile with the advantage of selectively inducing cancer cell death while exerting cell cycle arrest in normal cells without initiating DNA damage [80]. R7112 is an example of this class of drugs and have reached to the phase I clinical trial for patient with haematological neoplasms [74].

1.4. RADIATION THERAPY

Radiation therapy has been in use for a century in the treatment of cancer and other diseases. Similarly to surgery and chemotherapy, radiation therapy represents one of the three major cancer treatment modalities [81]. During recent years, radiation therapy has been used in combination with other modalities in an effort to downstage locally advanced cancers, increase organ preservation and consequently improve patients' overall survival.

Radiation therapy is used to treat over 60% of all cancer patients at some point during the course of their disease. It has become a significant therapy due to the progress made in the knowledge of radiation physics, radiobiology, treatment planning, refinement of treatment machines (i.e., linear accelerator), and the use of three- and four-dimensional computer assisted treatment planning [81].

Since nearly two thirds of all cancer patients receive radiation at some point during the course of their diseases [82], it is important to constantly improve radiation therapy protocols through the introduction of technological advancements and application of knowledge gained from preclinical experimentations and clinical trials.

Although the primary goal in the administration of therapeutic radiation is to destroy malignant cells while minimizing damage to normal tissues, radiotherapy is also used to treat a fair percentage of benign diseases or non-malignant conditions: arteriovenous malformations, Graves' ophthalmopathy, orbital pseudotumour, macular degeneration, pterygium, keloids, gynecomastia, histiocytosis, heterotopic ossification and peyronie's disease [83].

Radiotherapy can be used as definitive treatment, neoadjuvant or adjuvant therapy, prophylaxis treatment and palliation [82]. Radiation protocols are designed and delivered

with different purposes depending on the type of tumour and the stage of the disease. For example, in diseases where the emphasis is on effective systemic treatment, such as small-cell lung cancer [84-85] or lymphoma [84, 86], radiotherapy has an important consolidation role.

1.5. TYPES OF RADIATION

The absorption of energy from radiation in biology material can lead to excitation or to ionization. The raising of an electron in an atom or molecule to a higher energy level without electron ejection is called excitation. However, if the radiation had enough energy to eject to one or more orbital electrons, the process is called ionizing and that radiation is called ionizing radiation [87].

There are mainly two types of ionizing radiation: 1) electromagnetic radiation that either produces x-rays (photons) from a linear accelerator or gamma emitted from a radiation source such as Co 60 or Cs137, and, 2) particulate radiation which includes particles such as alpha, beta, electrons, protons and heavy ions. Charged particles are directly ionizing while electromagnetic radiations are indirectly ionizing radiations. [88].

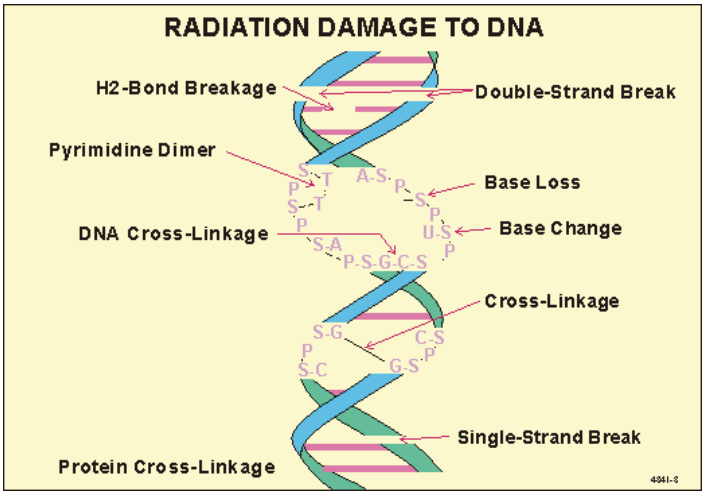

Figure 1-3. Types of radiation damage induced by ionizing radiation [89].

1.6. MECHANISM OF ACTION OF RADIATION

There is much established experimental evidence that DNA is the main target of radiation and that DNA damage is mainly responsible for radiation-induced cell death [90-91].

The types of DNA damage induced by radiation are: alteration or loss of one or more bases, destruction of hydrogen bond between base pairs, breaks in one or both strands of DNA molecule and cross-linking the strands (figure1-3). The hardest to repair and therefore most lethal type of DNA breaks are double strand breaks (DSBs). These breaks, even if they are repaired, are prone to inaccurate repair and this will lead to chromosomal abnormalities and even second malignancy in some cases [81].

Cellular effects from radiation appear to be maximum during the Mitosis and the late stage of G1 phase of the cell cycle while cells in S phase are known to be radio-resistant

[92]. This would suggest that the maximum effect from radiation should occur just before and during cell division. In addition, research by Bergonie and Tribondeau [93] has supported that the sensitivity of cells to irradiation is directly proportional to their reproductive activity and inversely proportional to their degree of differentiation.

There are other biological factors that influence cellular response to radiation such as: linear energy transfer (LET), relative biological effectiveness (RBE), oxygen effect and fractionation. In addition, Garcia-Barrons *et al.* have shown that tumour response to radiation therapy might be regulated by endothelial cell apoptosis in fibro-sarcoma and melanoma models [94].

1.6.1. Linear energy transfer

Linear energy transfer is the energy transferred per unit length of the track of a charged particle. LET, however, is only a simple way to indicate the quality of different types of radiation. It should be noted that for every charged particle, the higher the energy, the lower the LET and the biological effectiveness (in mammalian cells) [87].

1.6.2. Relative biological effectiveness

Radiation is measured and expressed in terms of the absorbed dose, a physical quantity with unit of gray or rad. In radiobiology, "dose" is a measure of energy absorbed per unit mass of a tissue. However, equal doses of different types of radiation do not have the same biological effects. It is common to use X-rays as the standard to compare different types of radiation. Therefore, RBE of some test radiation (r) is defined by the ratio D_{250}/D_r, where

D_{250} and D_r are, respectively, the doses of X-ray and the test radiation required for equal biological effect [87]. In general RBE is dependent on the following:

- Radiation Quality (LET)
- Radiation dose
- Number of dose fractions
- Dose rate
- Biological endpoints

1.6.3. Oxygen effect

The response of cells to ionizing radiation is strongly dependent upon oxygen. It has been demonstrated from rapid-mix studies that the oxygen effect occurs only if oxygen is present either during irradiation or within a few milliseconds thereafter [95].

The mechanism responsible for the enhancement of radiation damage by oxygen is generally known as the oxygen-fixation hypothesis illustrated in figure 1-4. When radiation is absorbed, free radicals are produced. These radicals are highly reactive molecules capable of breaking chemical bonds, producing chemical changes and initiating the chain of events that result in biological damage. They can be produced either directly in the target molecule (such as DNA) or indirectly in other cellular molecules and diffuse far enough to reach and damage critical targets. Most of the indirect effects occur by free radicals produced in water, as water makes up 70–80 per cent of mammalian cells. The fate of the free radicals (R•) that have been produced in the critical target is important. These R• molecules are unstable and will react rapidly with oxygen, if present, to produce RO2•, which then undergoes further reaction ultimately to yield ROOH in the target molecule. Thus we have a stable change in

the chemical composition of the target and the damage is said to be chemically "fixed." Consequently, this damage is recognized by biological pathways that participate in the DNA damage response (DDR) to invoke enzymatic processing of the lesions and perhaps their successful repair. In the absence of oxygen, or in the presence of reducing species, the unstable R• molecules have a longer half-life and can react with H^+, thus chemically restoring its original form without the need for biological and enzymatic intervention [87].

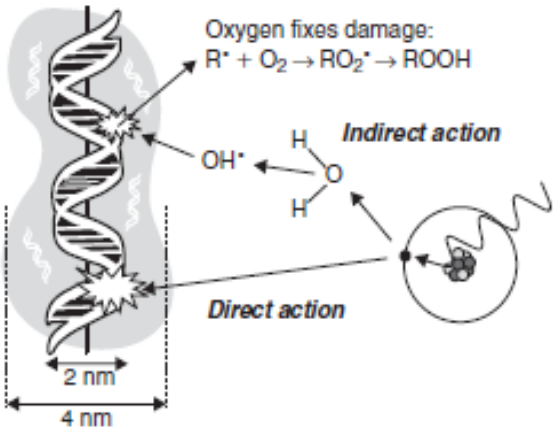

Figure 1-4. The oxygen fixation hypothesis.
Free radicals produced in DNA by either a direct or indirect action of radiation can be repaired under hypoxia but fixed in the presence of oxygen [87].

1.6.4. Fractionation

Dose fractionation in radiotherapy had begun in the first decade of the 20th century. One of the rationales for changing single-dose radiation treatments into fractionated doses was based on the correlation observed between the proliferative activity of cells and susceptibility to radiation-induced injury. Robert Kienböck first reported the higher radiosensitivity of cells with high mitotic activity in 1901 [96]. Fractionation in radiotherapy was initiated in order to spare normal tissue (by repair of sublethal damage

and repopulation from surviving cells) and also to increase the damage to the tumour (by reoxygenation of hypoxic cells and redistribution of cells along the cell cycle). The above-mentioned biological process, along with radiosensitivity**,** represent the foundation of fractionation in radiotherapy under the 5 R's of radiobiology [97]. Repair and repopulation lead to development of resistance of the irradiated tissue between two radiation doses, while redistribution and reoxygenation are expected to sensitize the tissue to a subsequent radiation dose. These four factors modify the response of a tissue to repeated doses of radiation. Different tissues (both normal and cancerous) behave differently during radiotherapy. The main factor responsible for the difference is considered to be the fifth R, radiosensitivity, as the response to radiotherapy is influenced by the sensitivity of the individual [98].

1.7. DNA DAMAGE RESPONSE PATHWAYS

DNA is the repository of genetic information of each living organism. Its integrity and stability, therefore, is of great importance to life. However, DNA is prone to lesions induced by exposure to environmental toxins and mutagens as well as events occurring during replication processes through DNA replication and cell division. Beyond the direct environment assaults, DNA can also be affected by the by-products of metabolism such as reactive oxygen produced by oxidative respiration. Macrophages and neutrophils present at the site of inflammation also produce notable amounts of reactive oxygen and nitrogen compounds [99]. Damage to DNA can disturb the cells' steady-state equilibrium and activate certain biochemical pathways that regulate cell growth and division and also

pathways that help to coordinate DNA replication with damage removal. The three types of pathways that are affected by DNA damage are DNA repair, DNA damage checkpoints, and cell death [100].

1.7.1. DNA damage mechanisms

Interestingly, it is estimated that an individual cell can face up to one million lesions per day [101]. Active compounds/agents can affect DNA by forming adducts that impair base pairing or inhibiting DNA replication and transcription, base loss or DNA single strand breaks (SSBs). In addition, when multiple SSBs are located in a close proximity, or when the DNA replication fork encounters the SSBs, double strand breaks (DSBs) are formed. DSBs are highly toxic to cells and their formation does not occur in cells as frequently as other damages [99, 102]. It has been suggested that the rate between formations of DSBs to SSBs is 1:6-8 [103-104]. One of the main agents known to induce DNA DSBs is ionizing radiation which is discussed in the previous section.

1.7.1.1. DNA base damage

Reactive oxygen species and ionizing radiation can produce DNA base damages which include O6-methylguanine, thymine glycols, and other reduced, oxidized, or fragmented bases in DNA. Ultraviolet (UV) radiation also gives rise to these species indirectly by generating reactive oxygen species, as well as producing specific products such as cyclobutane pyrimidine dimmers (CPD) and photoproducts [105-106]. Various adducts are produced by chemicals: bulky adducts formed by large polycyclic hydrocarbons or simple alkyl adducts formed by alkylating agents. The vast majority of chemotherapy drugs, including cisplatin, mitomycin C, psoralen, nitrogen mustard, and adriamycin, make base

adducts [107]. However, the major challenge still exists: how to find agents that damage DNA without invoking DNA repair or checkpoint responses in cancer cells? Combination therapy could be one possible answer. This idea has been further discussed earlier, in the section on molecular targets in cancer.

1.7.1.2. DNA backbone damage

Backbone damages include abasic sites and single- and double-strand DNA breaks. Abasic sites, also known as AP sites (apurinic/apyrimidinic site) are formed spontaneously, by the process of depurination or during the base excision repair process. Single-strand breaks are produced directly by damaging agents such as ionizing radiation, or by the byproducts of radiation such as reactive oxygen species [108]. Double-strand breaks are formed by ionizing radiation or other DNA-damaging agents such as topoisomerase I / II or bleomycin (BLM).

1.7.1.3. Cross links

Bifunctional alkylating agents such as cisplatin, nitrogen mustard and mitomycin D, form interstrand cross-links and DNA-protein cross-links. DNA-protein cross-links may also be produced by reaction of the aldehyde form of abasic sites with proteins [109].

1.7.2. DNA repair mechanism

The wide diversity of DNA-lesion types necessitates multiple, largely distinct DNA-repair mechanisms. Some lesions are subject to direct protein-mediated reversal; however, most are repaired by a sequence of catalytic events mediated by multiple proteins such as base excision repair, nucleotide excision repair, double strand breaks repair and the repair process of inter-strand cross-links.

1.7.2.1. Direct repair

There are two direct DNA repair mechanisms: photoreversal of UV-induced pyrimidine dimers by DNA photolyase and the removal of the O6-methyl group from O^6-methylguanine (O^6MeGua) in DNA by methylguanine DNA methyltransferase. The photoreversal mechanisms do not exist in humans while DNA methyltranferase is abundant in nature.

Methylguanine DNA methyltranferase (MGMT, also referred to as ATase, AGT, AGAT) is a small protein of 20 kDa that is ubiquitous in nature. MGMT is mostly located in the cytoplasm and upon alkylation it translocates into the nucleus [110]. MGMT is thought to recognize sites of damage by three-dimensional diffusion and it transfers the alkyl group from the oxygen in the DNA to a cystein residue in the catalytic pocket of MGMT, thereby restoring DNA and inactivating MGMT [107]. Interestingly, one MGMT molecule is capable of correcting one alkyl adduct and the inactivated alkylated MGMT appears to be ubiquitinated [111] and degraded by the proteasome [112]. Therefore, it is referred to as a suicide protein [107].

The removal of larger adducts such as ethyl or isopropyl groups are slower than methyl adducts. It has also been shown that MGMT is phosphorylated and the phosphorylated form of MGMT is less efficient in repairing O^6MeG [113]. Therefore, the

capacity of cells to remove DNA O^6-alkylguanine adducts is dependent on multiple factors: (1) number of existing MGMT molecules, (2) rate of MGMT synthesis (3) types of DNA adducts formed and (4) phosphorylation state of MGMT.

1.7.2.2. Base excision repair pathway (BER)

BER is the main DNA repair system in mammalian cells to eliminate small DNA base lesions. The alkylating adducts N^7MeG and N^3MeA and N^3MeG are repaired by BER [114]. Damaged bases are removed by DNA-glycosylase such as alkyladenine DNA-glycosylase (Aag), resulting in abasic sites which are recognized by apurinic/apyrimidinic endonucleases, APE1. APE1 forms cuts on the damaged strand and leaves 3-OH and 5-deoxyribose phosphate (5dRP) groups at the margins. DNA polymerase β completes DNA synthesis and fills the gap induced by APE1 by a single nucleotide and removes 5dRP groups [115-116]. The last step includes the presence of the DNA ligase I or a complex of DNA ligase III and XRCC1 which seals the nicks [117]. Poly (ADP-ribose) polymerase-1 (PARP-1), which is usually activated by the strand breaks, is participating in the last step of nick-sealing [118].

1.7.2.3. Nucleotide excision repair pathway (NER)

Nucleotide excision repair is the major repair process for eliminating the bulky DNA lesions formed by exposure to radiation and chemicals. NER pathway operates via two distinct sub-pathways which are different in recognizing the damaged sites: 1) Transcription Coupled NER (TCR) which specifically recognizes the lesions that block transcription and repairs the transcribed strands in active genes and 2) Global-Genome NER Repair (GGR) which

targets and repairs the whole genome as well as the non-transcribed strands of active genes [119]. The main feature of NER is that the damaged bases are removed by the "excision nuclease" enzyme which consists of multi sub-units. The enzyme excises the damage bases as a 22–30-base oligonucleotide, producing single stranded DNA (ssDNA) that is acted upon by DNA polymerases and associated factors before ligation ensues [120]. Excision nuclease is also capable of removing the simple single damaged bases. Excision nuclease recognizes the backbone conformational changes that have formed due to the damage rather than the specific chemical group added to DNA at the site of damage itself [107]. This non-specific recognition is referred as an indirect readout in contrast to the direct readout which recognizes the specific error in DNA sequence [121]. The basic steps in NER process are: 1) ATP independent, low specified recognition by RPA, XPA and XPC-TFIIH, 2) ATP dependent, DNA unwinding and formation of a long lasting DNA protein complex (pre-incision complex I-III), 3) dual incision by two nucleases, XPG and XPF-ERCC1 and finally 4) filling the gap by polδ/ε with the help of replication accessory proteins PCNA and RFC followed by ligation by DNA ligase III [122-124]. Since the above-mentioned three recognition proteins have moderate specificity, it is likely that they form the PIC1 at the binding site. If the binding site is not damaged, XPB and XPD hydrolyse ATP and PIC I is dissociated from the binding site. However, if the site contains damage, ATP hydrolysis by XPD and XPB unwinds the duplex by about 25 bp around the lesion, stabling preincision complex 1 (PIC1) at the damage site. Then, PICI will be further changed to PICII by XPG replacing XPC in the complex [125].

1.7.2.4. Double-strand break repair pathway

DNA double strand breaks can be produced by reactive oxygen species, ionizing radiation and chemicals that generate reactive oxygen species. There are some intrinsic factors that can cause double strand breaks as well: such as V(D)J recombination and the process of immunoglobulin class switch which usually takes place during the replication due to replication fork arrest and collapse [126-128]. The two mechanisms that correct the double strand breaks are homologous recombination (HR) or nonhomologous end-joining (NHEJ). It is still not clear how cells determine which mechanism of repair should be used to repair DSB. During the late S and G2 phases of cell cycle HR would be the main mechanism to repair the induced damage as the sister chromatids are very close. However, if there is no homolog sequence is present in the close proximity of a DSB, then NHEJ pathway seems to be the options for the cell to repair the damage. It has been shown that NHEJ can operate irrespective of the cell cycle phases [129]. Genetic data suggest that HR is crucial for the recovery of collapsed replication forks while NHEJ is essential for V(D)J recombination and is thought to be the major pathway for repair of double-strand breaks induced by ionizing radiation and radiomimetic agents [107, 130].

1.7.2.5. Non-homologous end joining repair pathway

The first step in this form of the repair starts with Ku70 and Ku 80 proteins forming a ring shape heterodimer that has a high affinity for DNA ends. Ku70/80 then recruits DNA dependent Protein Kinase-catalytic subunit (DNA-PKcs) [131]. DAN-PKcs is one of the first molecules to phosphorylate (most importantly at S2609) in response to DNA damage [132]. After loading on the damage site *p*-DNA-PKcs pushes the Ku70/80 dimer about one

helical turn inward from the end to facilitate other proteins' access. *p*-DNA-PKcs also recruits phosphorylated Artemis to the site of damage/repair [130]. The presence of *p*-Artemis in complex with DNA-PKcs generates DNA ends that can be ligated with the minimal nucleotides loss [130]. The last step in rejoining is mediated by DNA ligase IV, which is in contact with XRCC4 dimers. XRCC4 dimers stabilize DNA ligase IV and stimulate its adenylation and ligase activity [131].

The nuclease MRN complex may also have a role in NHEJ, particularly when this pathway is utilized for V(D)J recombination. In addition, the MRN complex also participates in protection of the ends from degradation. MRN complex consists of double-strand break repair protein Mre11, Rad50 homolog and Nijmegen breakage syndrome 1 protein (Nibrin or NBS1) and may be activated via Brca1/ Rad50 pathway [133].

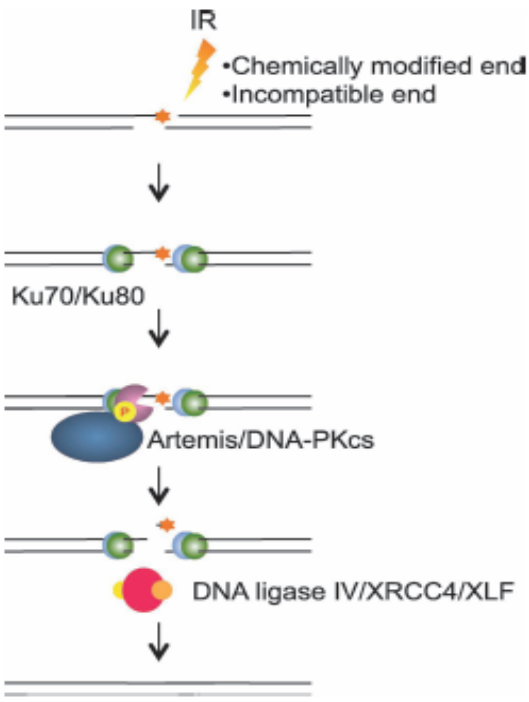

Figure 1-5. Non-homologous End Joining pathway [130].

1.7.2.6. Homologous recombination repair pathway

One of the first factors detected at DSB sites is the RAD50/MRe11 complex [134]. This complex keeps DNA molecules in close proximity before the start of the repair. Rad50/MRe11 endonuclease activity contributes to processing the several different structures induced by ionizing radiation or chemicals creating single strand ends. Rad52 is also recruited to the site of damage/repair [135-136]. In the next step of HR, the processed DNA ends are covered with a recombinase Rad51 along with BRCA 1/ 2 [137-138]. In order to complete the repair process after recombination, the crossed DNA strands have to

cut by structure specific nucleases such as XRCC3 and Rad51C that have resolvase activity [139]. Otherwise, the DNA strands can be un-crossed by co-operation of helicase and topoisoomerase [131].

1.7.2.7. Crosslink repair

The exact mechanism of crosslink repair is still not clear and there are some speculations about the molecules involved. However, there is a consensus in the published data that NER and HR have important roles in the repair of Interstrand Crosslinks (ICLs). NER is especially important for the repair of ICLs of non replicating cells (G0/1) through incisions made by XPF-ERCC1. The excision is followed by translesion synthesis and excision of the flipped out ICLs [140-141]. HR has been implicated in the repair of ICLs specifically in the replicating cells. A moving replication fork collapses at the ICL site leading to the activity of the FA (Fanconi Anemia) pathway via a group of proteins (FANCP, FANCD2, RAD51, RAD51C, BRCA2, FANCN, and BRCA1) [140]. The stalled fork can reverse to form a "chicken foot" structure, which enables NER to unhook the crosslink [140]. The endonucleases cleave the reverse fork and this generates a single-ended DSB. These breaks are substrates for RAD51-dependent HR. BRCA2 and FANCN promote RAD51 nucleoprotein filament formation and stimulate strand invasion. HR is then completed and replication can be re-established in the replication fork [142].

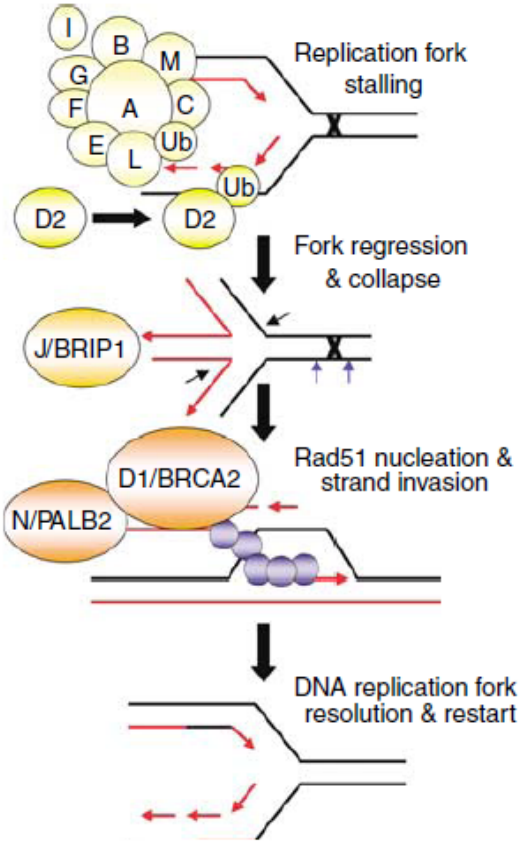

Figure 1-6. The repair mechanism of the interstrand crosslinks lesions in replicating cells [142].

1.7.3. DNA damage checkpoints

All eukaryotic cells have four cell cycle phases: G0/G1, S and G2/M which are well-defined in mammalian cells and transition from one phase to the other is closely regulated by cell cycle checkpoint proteins in both normal condition and in case of induced DNA damage. Cell cycle checkpoints are biochemical pathways operating in a normal condition during and in between the phases of the cells cycle which are up-regulated when DNA damage occurs.

However, it is still not clear if there is a required threshold for the level of DNA damage in order to activate/ up-regulate the checkpoints [107].

1.7.4. Molecular components of DNA damage checkpoints

Although many of the proteins involved in the check point regulation have roles in multiple checkpoints and also in various pathways, they may have a more prominent role in a specific checkpoint.

In general, the components of DNA damage checkpoints have been categorized as: sensors, mediators, transducers and effectors as shown in figure 7. However, there is no absolute distinction between the components of checkpoints as they may have more than one function or they may be involved in all phases of cell cycle.

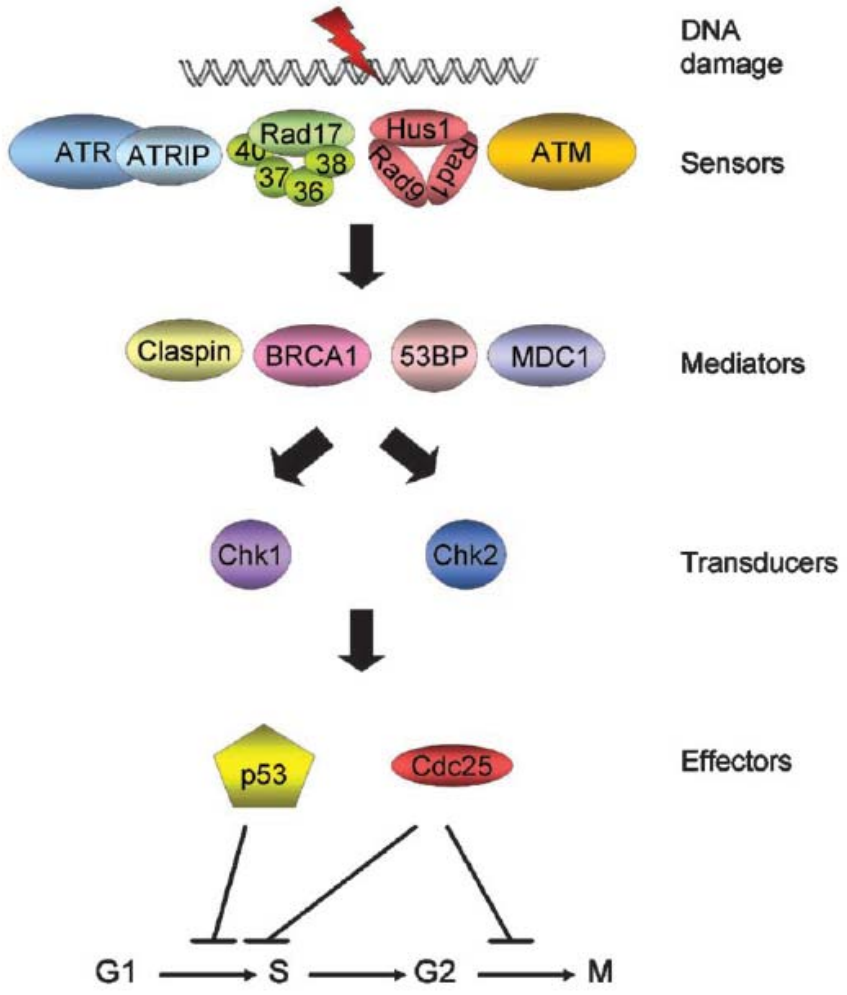

Figure 1-7. DNA-damage-induced checkpoint response pathway [107].

1.7.4.1. The G1 checkpoint

The G1/S checkpoint prevents cells from entering the S phase if DNA damage has occurred. ATM or ATR (depending on the type of the insult) is activated and phosphorylates the main targets: p53 and Chk1 and Chk2. Phosphorylation of Chk2 initiates the G1 arrest by phosphorylation, inactivation and degradation of Cdc25A. Lack of active Cdc25A results in phosphorylation of Cdk2. P-Cdk2 is the inactive form of Cdk2 and is incapable of phosphorylation of Cdc45 to initiate replication. Similarly, if the DNA damage is induced by UV light, ATR would sense the damage and through the formation of a complex with RAD17-RFC and RAD9-RAD1-HUS1 (9-1-1) resulting in phosphorylation of Chk1 by ATR. Once Chk1 is activated it phosphorylates Cdc25A leading to G1 arrest.

While Chk1 and 2 are involved in the initial arrest in G1, p53 is responsible for maintaining the G1/S arrest [143]. P53 is phosphorylated by ATM/ATR at Ser15 and by Chk1/2 at Ser 20. Phosphorylated p53 induces the transcription of its target gene p21, which binds and inactivates the S-phase-promoting Cdk2-CyclinE complex, thereby maintaining the G1/S arrest [144].

1.7.4.2. The S check point

S phase check point is up-regulated in response to DSBs induced during the S phase or by existing damages that were not repaired and escaped the G1 arrest and caused a block in the replication process [145]. The main mechanism of arrest in S phase is the inhibition of firing of late origins of replication [146-148].

When the damage is a direct DSB or a DSB formed due to a nicked or gapped DNA, ATM starts two parallel cascades to inhibit replication. Through MDC1, HAX and 53BP1, ATM

phosphorylates Chk2 on Thr68 to induce degradation of Cdc25A [149-150]. Cdc25A degradation keeps CyclinE/Cdk2 in the non-active stat (phosphorylated form) and inhibits the Cdc45 from loading on the replication origin. ATM also phosphorylates NBS1, SMC1 and BRCA1 and FANCD2 leading to the inhibition of replicative DNA synthesis [151-152].

In case of DNA damage induced by UV or the chemicals that make bulky base lesions, the main sensor is ATR-ATRIP heterodimer [153-154]. Following binding to the DNA lesions, ATR becomes activated and phosphorylates Chk1 and as a consequence down-regulates/phosphorylatesCdc25A and therefore, inhibits firing of replication origins [146].

1.7.4.3. The G2 check point

In the presence of DNA damage in G2/M phase of the cell cycle, G2/M check point prevents cells from entering mitosis. Similar to the other check points, ATM and ATR are activated by DSBs and bulky lesions/SSBs respectively. In both cases mitosis is inhibited by down-regulation of Cdc25A and up-regulation of Wee1, which controls cdc2/CyclinB activity [155]. The maintenance of G2/M arrest may have been p53 independent unlike G1/S check point. Several studies have suggested that tumour cells lacking functional p53 still accumulate in G2 in response to induction of DNA damage [156]. This could be due to the fact that p21, a target gene for p53, can also be activated by p73 [157].

1.8. CELL DEATH

As we discussed before, Eukaryotes have a well-developed and extensive mechanisms to detect different types of DNA damage and also DDR pathways. Depending on the cell type and the extent of the DNA damage, DDR can cause different responses. Mild DNA damage can be repaired through the up-regulation of Cyclin-dependent kinase inhibitors and eventually cell cycle arrest and also several repair mechanisms. More severe types of DNA damage lead the affected cells towards senescence or cell death. In addition to DNA damage, other signals/stimuli can also lead the cells towards cell death process. While chemotherapies, growth factor withdrawals and ER stress can induce cell death through mitochondria-dependent signaling, death receptor ligands such as Fas, TNF or TRAIL can trigger the death receptor signalling pathway through recruitment of Fas- associated death domain protein (FADD) [158-159].

1.9. MODES OF CELL DEATH

The Nomenclature Committee on Cell Death (NCCD) proposes that " a cell should be considered dead when any one of the following molecular or morphological criteria is observed: (1) the cell has lost the integrity of its plasma membrane, as defined by the incorporation of vital dyes (e.g., PI) *in vitro*; (2) the cell, including its nucleus, has undergone complete fragmentation into discrete bodies (which are frequently referred to as 'apoptotic bodies'); and/or (3) its corpse (or its fragments) has been engulfed by an adjacent cell *in vivo*" [160].

According to NCCD, there are two main modalities of cell death: typical or distinctive modes of cell death vs. Non-typical modes of cell death. The typical mode of cell death includes the four types of cell death that are commonly discussed in the literature: apoptosis, autophagy, cornification and necrosis. Cornification or keratinisation is a type of cell death that is specific to the skin cells; therefore it will not be discussed here. Instead, the focus will be on other common types of cell death. The atypical modes of cell death include: mitotic catastrophe, anoikis, excitotoxicity, wallerian degeneration, paraptosis, pyroptosis, pyronecrosis, and entosis. Similarly, in this section, mitotic catastrophe will be briefly discussed as it is specifically relevant to radiation and radiation biology.

1.9.1. Apoptosis

Apoptosis was first used by Kerr, Wyllie and Currie in 1972 to distinguish a distinct form of cell death [160-161]. Apoptosis occurs during several processes including development, and aging, as a homeostasis mechanism to maintain the cell population, as a defense mechanism in immune reactions or in damaged cells due to a disease or toxic agents [161]. It is a co-ordinated and energy-dependent process that might or might not involve the activation of caspases. Morphological characteristics of apoptosis are: rounding-up of the cell, reduction of cellular and nuclear volume (pyknosis), nuclear fragmentation, plasma membrane blebbing and minor modification of cytoplasmic organelles [160].

1.9.1.1. Mechanisms of apoptosis: intrinsic vs. extrinsic pathways

The intrinsic apoptotic (mitochondria pathway) stimuli such as DNA damage and ER stress or growth factor removal activate B cell lynphoma2 (Bcl-2) homology 3(BH3) only proteins

resulting in Bcl-2- associated X (BAX) protein and Bcl-2 killer (BAK) activation, and mitochondrial outer membrane permeablization (MOMP) [162-163]. This will release various proteins including cytochrom-c from mitochondria and promote caspases activation and apoptosis. After release, cytochrome c binds to apoptotic protease-activity factor-1 (APAF-1) and forms an apoptosome structure. Apoptosome then recruits and activates initiator caspases, caspase 9. Caspase 9 activates caspase 3 and 7 leading to completion of apoptosis [159]. In addition to cytochrom c, other proteins such as second mitochondria-derived activator of caspases (SMAC, aka DIABLO) neutralize X-linked inhibitor of apoptosis protein (XIAP). Interestingly, XIAP is involved in both intrinsic and extrinsic apoptotic pathways [164].

Extrinsic pathway (death cell receptor pathway) begins with the attachment of death ligands such as Fas, TRAIL or TNF to their receptors on the cell membrane and follows by the recruitment of FADD and caspase 8 to the membrane. Upon dimerization and activation of caspase 8, caspase 3 and 7 are cleaved and this will lead the cell to apoptosis [159].

Extrinsinc and intrinsic pathways can also crosstalk through activation/cleavage of BH3-only protein BH3-intercating domain death agonist (BID) into truncated form tBID by cleaved caspase 8. tBID activates BAK and BAX and leads the cell to apoptotic state [165].

Another protein being released from mitochondria upon apoptotic stimuli is apoptosis inducing factor (AIF) which leads the cell to caspase-independent apoptosis. Following the release of AIF from mitochondria, it translocates to the nucleus where it binds DNA randomly. DNA binding of AIF results in chromatin condensation and DNA fragmentation by recruitment of nucleases [166].

1.9.2. Autophagy

Macrophagy (commonly referred to as autophagy) is characterized by lack of chromatin condensation, massive vacuolization of plasma membrane, accumulation of double membrane autophagic vacuoles, little or no uptake by phagocytic cells *in vivo* [160]. In general autophagy is considered as a pro-survival mechanism through which cells adapt to stress condition by using metabolic precursors for cellular renewal and maintenance through the recycling of cellular components [167].

Autophagy is regulated by a group of proteins called autophagy-related proteins (ATG proteins). In response to a lack of amino acids or other stress, ATG1 and a member of PI3K family, VPS34, interact with beclin-1 and lead to the activation of downstream ATG factors that are involved in the initiation, elongation, and maturation of autophagy. The elongation step of autophagy is controlled by ATG 12 and LC3 and these proteins are degraded during the last step of maturation into the autolysosome [168].

There is some contradictory evidence indicating that autophagy has both cytoprotective and cytotoxic properties. It is yet to be well-defined under which conditions autophagy acts as a cytoprotective mechanism and the types of stress under which autophagy acts as a mode of cell death [169-170].

1.9.3. Necrosis

Necrotic cell death is characterized by cytoplasmic swelling (oncosis), rupture of plasma membrane, swelling of cytoplasmic organelles and moderate chromatin condensation [160]. Previously, it was thought that necrosis was an accidental uncontrolled mode of cell death but recent studies have revealed that it is tightly regulated by a set of signal transduction

pathways [171-172]. It has been shown that death receptors and Toll-Like Receptors (TLRs) are involved in necrosis especially when caspases are inhibited [173-174] and RIP-1 kinase has a crucial role as an initiator of death receptor-mediated necrosis [160, 175].

Importantly, extensive DNA damage causes hyper-activation of poly-(ADP)-ribose polymerase (PARP-1) and leads to necrotic cell death. PARP-1 contributes to the DNA damage process when DNA damage is moderate; however excessive PARP-1 activation causes depletion of NAD^+ by catalyzing hydrolysis of NAD^+ into nicotinamide and PAR, leading to ATP depletion, cellular failure and necrosis [176].

Necrosis, has a role in several signaling processes such as ovulation, the death of chondrocytes associated with the longitudinal growth of bones as well as in cellular turnover in the small and large intestine [171] and in activation-induced cell death (AICD) of T lymphocytes which is an important step for reducing T cell numbers after immune response [177]. The role of necrosis in the mentioned process is more significant when apoptosis is inhibited.

In contrast to apoptosis, the recognition and uptake of necrotic cells by macrophages is slower and less efficient and usually happens after the loss of plasma membrane integrity [175]. Therefore, a pro-inflammatory response is initiated by the release of DAMPs (danger/damage-associated molecular patterns) in response to necrosis and necrotic cells actively secret pro-inflammatory cytokines such as IL-6 due to activation of NF-κB and p38-MAPK [178].

1.9.4. Mitotic catastrophe

Mitotic catastrophe is a type of cell death occurring during or shortly after a disregulated/failed mitosis. It is characterized by micro-nucleation and multi-nucleation [160]. However, features of apoptosis and necrosis have been observed in cells undergoing mitotic failure, indicating the possibility that mitotic catastrophe might be prelude to apoptosis or necrosis rather than a cell death mechanism itself [179]. Additionally, depletion of Bcl-2 family of pro-apoptotic proteins such as Bax reduces the rate of the cell death upon mitotic catastrophe, therefore, leading to oncogenic polypoidization. [180]. It has also been shown that morphological characteristics of mitotic catastrophe can be observed in parallel with cells undergoing senescence [179].

In general it should be noted that the term ”dead cells” would be different from “dying cells” that have not yet concluded their cell death pathway. In particular, cells that are arrested in the cell cycle, such as cells undergoing senescence, should be referred to as live cells even though they might have lost their clonogenic potentials.

1.10. COMBINATION THERAPY

The origin of combination therapy goes back to 1958 when Emille Frie III published the first randomized controlled trial of combination therapy and its efficacy in cancer [181]. His trial was based on the preliminary studies by Skipper *et al.* and Law, as well as the success of combination therapies for tuberculosis. In 1952 Law had found that genetic variation is responsible for anti-folate resistance in the mouse model of cancer. Low and later Skipper *et*

al. in 1954 proposed that there might be a benefit to giving drugs in combination. The success of Emil Frie in his 1958 trial and also experimental mouse model studies by Skipper in 1964, and progress in supportive care led to the highly acceptable but not experimentally controlled adoption of the 4-drug VAMP regimen. The VAMP regimen was the first big step towards the large and potentially curative regimens that we have today [182].

Combination regimens in oncology, including drug combinations or radiation and drug combinations, may be categorized in various ways and several parameters are considered in combined strategies: for example, if the agents to be combined are already established or they are in experimental steps, and if they are known to have single-target or multi-targeting activity. Such parameters influence the ethical and regulatory requirements for development of combined regimens [183].

1.10.1. Chemotherapy combinations

The goal of cytotoxic therapy is to maximize tumour cell kill. The limited selectivity of these conventional therapies is based on their interferences with frequent cell division and DNA replication of cancer cells relative to most normal cells. Most of these cytotoxic agents deliver their effect by inhibiting synthesis of DNA precursors and also damaging the DNA or disturbing chromosomal segregation. Unfortunately, rapidly dividing normal tissues such as bone marrow, gastrointestinal tract and hair follicles are also affected by the treatment, limiting the optimum dosage required for the complete treatment. This results in reduced efficacy, drug resistance and decreased quality of life for patients [184].

For decades, oncologists have applied the strategy of combining chemotherapeutic agents in hope of curing their patients. Examples of such treatment protocols include the

combination of bleomycin, etoposide and cisplatin in germ cell tumours [185], or doxorubicin, bleomycin, vinblastine and dacarbazine in Hodgkin's lymphoma [186]. These cytotoxic combinations were usually developed simply based on single-agent activity, non-overlapping toxicities and on some preclinical evidence of synergy.

The development of cytotoxic combination for cancer is based on the following principles: 1) the drug in combination should be individually active; 2) should have different mechanism of action; 3) should have non-overlapping mechanism of resistance; 4) should have non-overlapping toxicities; and 5) should be administered at minimum tolerated doses and schedules [187].

1.10.2. Targeted therapy combinations

During the last decade of the 20th century, drug development in oncology had a paradigm shift from focusing on traditional cytotoxic to targeted therapies. Thus far, the most striking results of targeted monotherapies have been observed in cancers that are addicted to a single kinase. However, the efficacy of these targeted agents is more likely to be mitigated as most solid tumours contain multiple gene abnormalities and are not homogeneous. Therefore, targeted agents are increasingly being investigated in combination, either with cytotoxic agents including radiation or other targeted drugs [183]. In contrast to cytotoxic therapy, the goals of rational combinations of molecular-targeted agents are to achieve strong tumour control, which may lead to better therapeutic outcome through simultaneous blockade of cancer-relevant targets in properly selected patients [184]. To date, numerous molecular-targeted therapies are approved or developed. The above-mentioned principles also apply to molecular targeting drugs however, there are a few considerations to take:

1) If a molecular-targeting drug is not active as a single agent in preclinical studies, it should not be discarded but, could be rationally combined with other agents and combinations tested in preclinical models.

2) Achieving non-overlapped toxicity profile might be difficult in molecular targeting agents as combined agents targeting a specific pathway may produce greater mechanism-based or non-target specific toxicities [188].

3) Molecular targeting agents may induce limited target-specific toxicity and should thereby be dosed to maximum-biological effect on the target as opposed to maximum tolerance [187].

1.10.3. Goals of combination therapy

Treatment modalities are combined to enhance the rate or duration of clinical benefit over the existing standard of treatment or to keep the same level of efficiency with lower toxicity by using the lower doses of agents of non-overlapping toxicity. Therefore, the goals of combination therapy can be summarized below:

1) To enhance sensitivity of cancer cells to an agent through the use of another agent.
2) To reduce development of resistance.
3) To target the tumour micro-environment.

1.10.3.1. Synergy/additivity and antagonism

In oncology, the combination of drugs is usually done with the hope to achieve a beneficial pharmacodynamics interaction, which enhances cytotoxicity. The most desirable and informative effect that one can get from PD interaction is synergy. While additive effect by definition refers to the equality effect of the sum of two or more agents to each of them

separately, synergy is a general term referring to the outcome that exceeded the effect expected in the purely additive interaction. On the other hand, antagonism refers to an outcome less than expected from an additive interaction [183, 189-190].

1.10.3.2. Evaluation of combination therapy

The above terminology seems to be very simplistic and there has been some controversy in terms of definition and also whether these terminologies are transferable to the clinic.

Determination of cytotoxic synergy is based on short-term *in vitro* colorimetric assays of cell viability with the multiple data points required for mathematical analysis such MTT or SRB assays [183, 191].

There has never been a consensus on the standard definition for synergism or additivism; therefore, in 1984 Chou and Talalay introduced the scientific term "combination Index" (CI) to quantity synergism (CI<1), additive (CI=1) and antagonism (CI>1) [190, 192-193]. The following formula for calculation of the combination index was proposed by Chou and Talalay [193]:

$$CI = D_1/(Dx)_1 + D_2/(Dx)_2$$

Dx_1 and Dx_2 are concentrations for two drugs alone that will give x % inhibition.

D_1 and D_2 are the concentration of two drugs in combination that inhibit also x % (i.e., isoeffect).

In the clinical setting, however, it is not required to demonstrate synergy for a given combination. Instead, clinical investigations aim to identify drug interactions that achieve better clinical results than either the respective single agents used in the combination or other standard therapies. Importantly, synergy against cancer cells, as determined by *in vitro*

assays, is only one of the components taken into consideration when assessing clinical benefit. The key component is selective toxicity to increase the therapeutic ratio [183].

1.10.3.3. Multi-targeting agents vs. combination of multi agents

The majority of diseases including cancer are multi-factorial in nature. However, the traditional drug discovery is based on identifying a drug to act on a single target involved in the disease development or progression. So, because of the multi-factorial nature of diseases like cancer, a selective compound with a single target is less likely to achieve the desired outcome. Therefore, these single targeting compounds are combined with standard treatments or other targeted agents to improve the outcome.

As discussed before, most curative cancer treatment is based on using an effective combination of surgery with chemotherapy, radiation or targeted therapy. The successful results of combination therapies are most likely due to the fact that cancer is a heterogeneous disease among patients and even within the same patient or tumour itself. To add to the complexity of cancer biology, cancer cells adapt relatively well to new conditions due to their genotypical and phenotypical complexities, *de novo* protective mechanisms or acquired resistance to almost all agents over time (except when the disease is cured).

Multi-targeting agents may be active across a number of different cancer types. An example of this type of agents is Sorafenib or Sunitinib which targets VEGFR/PDGFR and C-Kit as well as other kinases. The spectrum of kinase inhibition within a tumour may result in greater therapeutic effect. Sunitinib with its broad targeting ability has a greater activity in renal cell carcinoma than monoclonal antibody Bevacizumab which specifically inhibits the VEGFR pathway. Using a multi-targeting agent reduces the number of drugs a patient has to

take and therefore decreases the drug-drug interaction. In addition, the multi-targeting agent may have a broader activity against the same type of tumour in different patients due to the molecular heterogeneity amongst the same type of cancer. Moreover, there are specific scientific, clinical and regulatory considerations for multi-targeted vs. relatively selective targeted combinations. For example, a single agent with multiple actions might be easier and faster to pass all the regulatory requirements for demonstrating activity and safety.

The disadvantage of a multi-targeting agent is thought to be potentially increased toxicity due to cumulative target and off-target inhibitions that are broad and less predictable [187]. In addition, in case of multikinase inhibitors, it is not feasible to develop inhibitors with a specific kinase-inhibitory profile with optimal potency and therapeutic index for each of the multiple cancer relevant targets. Therefore, even multi-targeting kinase inhibitors might need to be combined with other targeted agents for maximal therapeutic benefit [194].

1.10.3.4. Sorafenib: a dual targeting multi tyrosine kinase inhibitor

Sorafenib or (Nexavar, Bayer-Onyx) is an oral, small molecule kinase inhibitor that was initially developed as a Raf inhibitor. Further characterization of sorafenib demonstrated that it is a multikinase inhibitor of several other targets including VEGFR-2 and 3, PDGFR-b [195]. Sorafenib has been classified as an anti-angiogeneic drug due to its inhibition of VEGFR. Following by a successful phase III clinical trial, Sorafenib was approved by the Food and Drug Administration (FDA) for the treatment of mRRC in 2003. In 2008, the FDA approved sorafenib for unresectable HCC based on 2 randomized phase III studies.

It has been shown that MDA-MB-231 breast cancer models are sensitive to sorafenib treatment, with a 30 mg/kg dose producing a 42% reduction in the mean size of these

tumours after only 9 days of treatment [195]. The results in the MDA-MB-231 model indicate that sorafenib may act in breast cancer through inhibition of the MAPK pathway and inhibition of angiogenesis. More recently, Li *et al.* have shown that combination of sorafenib and radiation inhibits breast cancer stem cells by suppressing HIF-1α expression [196]. Their findings as well as others suggest the combination of Sorafenib and radiation has synergistic effects and could be a new therapeutic approach to prevent breast cancer progression by eliminating breast cancer stem cells [196-198].

1.10.3.5. Combi-molecule concept

Within the concept of developing single agents with multi-targeting properties, a series of molecules termed "Combi-molecule" have been developed by Merayo *et al*, Rachid *et al.* and Laroque *et al.* since 2003.

ZRBA1 is one of these molecules which targets EGFR and HER2 and induces DNA strand breaks [199]. ZRBA1 is designed to target the ATP binding pocket of TK domain of EGFR and HER2 and also behave as an alkylating agent to induce N, N dimethylaminoethylguanine adducts [42]. These adducts cannot be repaired/removed efficiently by MGMT in cancer cells; therefore, ZRBA1 is superior to other alkylating agents such as Temozolomide that induce mostly O^6-methylguanine adducts. In addition, targeting ATP binding site of the TK domain of EGFR provides an advantage for ZRBA1 over monoclonal Abs against EGFR such as (Cetuximab) as the mutation occurring at the extracellular ligand binding domain (EGFR vIII) will not affect the EGFR targeting moiety of ZRBA1. ZRBA1 has been designed to degrade into multiple inhibitors of EGFR, generating highly reactive intermediates (electrophiles) in a stepwise fashion a model termed

"cascade release" [200]. The formation of multiple intermediates with high affinity for EGFR results in stronger receptor inhibition and anti-proliferation activity of ZRBA1 [201]. Moreover, ZRBA1 also has a quinazoline fluorescence property which makes it possible to determine combi-molecule degradation steps in the cells and also to follow the intracellular distribution of the EGFR targeting moiety.

Although ZRBA1 has shown anti-tumour activity [42], it could be even more potent when it is combined with a cytotoxic agent such as ionizing radiation through the induction of different types of DNA lesions.

1.10.3.6. Combination with radiation

In 1979, Steel and Peckham proposed for the first time a framework to combine drugs and radiation. In their proposed framework they identified four mechanisms by which combination therapy with radiation could improve the therapeutic outcome: spatial co-operation; toxicity independence; protection of normal cells; and enhancement of tumour response.

In 2007, Bentzen *et al.* proposed the new frameworks for combining radiation and drugs which include the following mechanisms: spatial co-operation; cytotoxic enhancement; biological co-operation; temporal modulation; and normal tissue protection [202].

1.10.3.6.1. Spatial co-operation

This refers to the concept that different therapeutic modalities affect different anatomic sites of disease (i.e. chemotherapy affects the disease systemically vs., radiation affects the

disease locally) [203]. Even in cases where an effective systemic therapy exists, radiation can be used against bulky disease. Spatial cooperation may also apply to combining radiation with some non-cytotoxic agents that are effective against minimal disease. As spatial cooperation does not require an interaction between the two modalities, radiation and drug administration can be delivered at the full dose. To avoid or reduce the early cytotoxicity due to the treatment these modalities are usually administrated sequentially. The main clinical endpoint however, should include disease control both local and distance sites [202].

1.10.3.6.2. Cytotoxic enhancement

Cytotoxic enhancement refers to enhanced cell killing by inhibiting the DNA repair mechanisms. Therefore cytotoxic enhancers are able to change the *in vitro* cell survival curves [204-205]. In order to take advantage of cytotoxic enhancement, the drug should be present at the time of radiation as drugs exploiting this mechanism are directly modifying the initial stage of radiation-induced DNA damage. The main clinical endpoint using this mechanism is loco-regional control of the tumour as the drug used is an enhancer of radiation [202].

1.10.3.6.3. Biological co-operation

Biological co-operation defines the strategies that target a distinct population of cells and use different cell-killing mechanisms or delayed tumour growth. The combination could be sequential or concurrent and the main clinical end point would be loco-regional control of the disease as this mechanism is used to enhance the effect of radiation locally.

1.10.3.6.4. Temporal modulation

Temporal modulation refers to approaches that enhance the tumour response to fractionated radiation therapy. The previously discussed four R's in radiobiology are not active to the same extent on tumours and normal cells. Therefore, the total dose, the number of fraction and dose per fractions and the intervals between fractions all should be decided base on the different biological properties of tumour/normal cells. The two modalities in this approach should be delivered concomitantly or in rapid alterations. The main clinical end point is loco-regional control [202]. The rationale for combination of molecular targeting therapies with radiation is well-described using this mechanism [206].

1.10.3.6.5. Normal tissue protection

Normal tissue protection refers to the mechanisms by which several drugs protect the normal cells or modulate the cytotoxic response of normal tissue, especially radiation-induced late normal tissue toxicity [207]. Using a keratinocyte growth factor to reduce mucositis is an example of this category [208].

The clinical objective of drug-radiation combinations is to improve the rate of survival without compromising the quality of life [202]. In order to reach this objective, the risk factors of local and distant relapse as well as the early and late side effects should be carefully considered in parallel. It seems that combination therapy might be of a great help to reach the ultimate objective. Future therapeutic strategies are more likely to be rationally designed, multi-targeted and adjusted to each patient's disease. These strategies include developing future regimens exploiting spatial co-operation and temporal modulation to balance the risks and benefits of treatments [202].

1.10.3.7. Measuring radiation sensitivity

Tao *et al.* [209] used the term "Dose Enhancement Ratio" to measure radio sensitivity using clonogenic assay. DER is defined as the ratio of surviving cells with radiation alone compared to a combination of radiation and inhibitor exposures. DER=1 suggests an additive radiation effect and DER>1, a supra-additive effect as against a sub-additive effect in the case of DER<1. Similarly, Kil *et al*. [210] used the term "Dose Enhancement Factor" (DEF) to describe the radio-sensitization of temozolomide in GBM cells. They have defined DEF or SER (Sensitivity Enhancement Ratio) as the ratio of radiation dose of control cells and the radiation dose of treated cells at the survival fraction of 0.1.

1.11. HYPOTHESIS AND THE RATIONALE FOR THIS PROJECT

As discussed previously, genetic defects in cancer are interesting targets for anti-cancer treatment as the normal tissue ideally would be spared. EGFR and VEGFR or, broadly speaking, RTKs are among the most targeted receptors in anti-cancer treatment partially because they are overexpressed in several types of cancers and they have a central role in MAPK/AKT pathway which control proliferation, angiogenesis and survival and invasion processes. All of these are crucial aspects in cancer management. Importantly, 50% of breast tumours have been found to overexpress EGFR and 30% overexpressed Her-2 [211]. EGFR expression has been associated with higher tumour grade, increasing size, higher progression index, the development of distant metastasis and the incidence of death. Also, overexpression of HER-2 was significantly associated with poorer grade, higher prognostic index and local and regional recurrence [212-213]. In a multi variant study, Rimawi *et al.* have found that EGFR expressing tumours were more common in younger adults and they have a worse Disease Free Survival (DFS) and Overall Survival (OS) [213]. VEGFR also has been associated with worse outcome in breast cancer patient and overexpression of VEGFR2/ 1 has been shown in early stage breast [214], prostate , renal and colorectal cancers [39].

Our main hypothesis, for this project was that the radiation effect may be potentiated while the MAPK/ PI3K pathway is down regulated. The use of EGFR and VEGFR inhibitors, ZRBA1 and Sorafenib, in combination with radiation in this study is a fine example of exploitation of biological co-operation and temporal modulation as well as

cytotoxic enhancement, three mechanisms by which combination therapy can produce promising results.

The aim of this thesis was to evaluate and analyze the efficiency of multi-targeting inhibitors with radiation in a Triple Negative (TN) breast cancer model. In opposition to other breast cancer subtypes, patients with TN types do not have the option of targeted therapy such as anti-estrogen regimen or Herceptin due to their lack of receptor expression. Clinically, TN breast cancer is found in young patients and it is associated with early relapse and poorer long-term outcome. In consequence, there is a pressing need to develop treatment strategies to better control this type of breast cancer.

CONNECTING TEXT 1

The study presented in the next chapter focuses on the interaction of radiation with a multi-targeting small molecule inhibitor, Sorafenib. This study was completed using a TN breast cancer model. In this chapter, we have investigated the effects of radiation combined with Sorafenib treatment on cancer cell's colony survival, apoptosis, cell cycle arrest as well as *in vivo* tumour growth delay. Additionally, we aimed to study the combination of radiation and Sorafenib in multiple sequences *in vitro* and *in vivo*.

CHAPTER 2

Sorafenib in combination with ionizing radiation has a greater anti-tumour activity in a breast cancer model

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2.1. ABSTRACT

Background: High expression levels of VEGF in breast cancer patients have been associated with poor prognosis, indicating that VEGF could be linked to the efficacy of chemotherapy and radiotherapy. It has also been suggested that radiation resistance is partly due to tumour cell production of angiogenic cytokines, particularly vascular endothelial growth factor receptor (VEGF). These evidences indicate that inhibition of VEGFR might enhance the radiation response.

Sorafenib tosylate (Bay 54-9085) is an oral, small molecule multikinase inhibitor of several targets including RAF/MEK/ERK MAP kinase signaling, vascular endothelial growth factor receptor-2 (VEGFR-2), VEGFR-3, and platelet derived growth factor receptor-beta (PDGFR-b). Sorafenib has shown clinical efficacy in treating solid tumours such as renal cell and hepatocellular carcinomas. However, strategies are yet to be identified to prolong and maximize the anti- cancer effect of this multikinase inhibitor.

Objective: To determine if combination of Sorafenib and radiation will enhance the treatment response *in vitro* and *in vivo*.

Methods: The mouse mammary cancer cell line 4T1 was used in this study. Clonogenic assay was performed to assess the radio-modulating effect of Sorafenib. In addition, cell cycle analysis as well as Annexin-V apoptosis assay was performed 24 and 48 h post treatment respectively. To confirm our finding *in vitro* tumour growth delay assay was also performed.

Results: Our results showed a strong and supra-additive anti-tumour effect of radiation combined with Sorafenib *in vitro* (dose enhancement factor of 1.76). The combined therapy demonstrated a strong and significant G2/M cell cycle arrest (combined treatment vs.

irradiated alone: $P < 0.0008$). Moreover, Annexin-V staining showed a significant increase in the level of apoptosis (combined treatment vs. irradiated alone: $p < 0.0004$). Studying 4T1 syngeneic model demonstrated the superior potency of the Sorafenib combined with radiotherapy.

Conclusions: Our results demonstrate that if radiation and Sorafenib are combined, higher anti-tumour activity can be achieved.

2.2. INTRODUCTION

Radiation is a mainstay of non-surgical cancer treatment. Approximately 2/3 of cancer patients receive radiation therapy. During the last decades, radiation therapy has been advanced mainly due to technological improvements in radiotherapy planning and delivery methods; however, the efforts in understanding the biological parameters that affect the overall therapeutic outcome have not achieved the same success. Thus, radiotherapy is delivered without considering the potential differences within and between the tumours. While an understanding of the biological basis could have a significant impact in clinical radiation oncology, this knowledge could also be exploited to develop new treatment protocols and perhaps novel combined therapies.

Radiotherapy is relatively well tolerated by patients and has been successful in local tumour control [215-216]. However, the overall rate of survival of patients improves when radiation therapy is combined with chemotherapy [217]. Notably, secondary cancers, skeletal complications, radiation-induced heart disease, and lung disease are common side effects of radiation therapy [218-220]. Therefore, due to the toxicity of radiation, much focus has been placed on improving its cancer cell specificity. This includes the effort to

develop agents that sensitize cancer cells to radiation or protect normal cells from damage induced by radiation [216, 220-221].

Over the last decade, the combination of ionizing radiation with chemotherapy has led to marked improvement in local control, organ preservation and survival for locally advanced solid tumours. However, this strategy is limited by the toxicity resulting from each respective treatment and their combination. Therefore, targeting tumour specific defects should provide an advantage over conventional therapy in which the major drawback is normal tissue toxicity [222].

Sorafenib tosylate (Nexavar, Bay 54-9085) is an oral, small molecule multikinase inhibitor of several targets including vascular endothelial growth factor receptor-2 (VEGFR-2), VEGFR-3, and platelet-derived growth factor receptor-beta (PDGFR-b), RAF-1 and BRAF [223]. Sorafenib has shown clinical activity against metastatic renal cell carcinoma (mRCC) and is considered to be a standard second line therapy for mRCC patients [224-225]. Moreover, combination therapy with Sorafenib in phase I and II clinical trials has shown some promising results in melanoma patients [226]. Importantly, Sorafenib has been shown to significantly increase the overall survival rate of patients (nearly 3 months) with advanced hepatocellular carcinoma (HCC) [227]. HCC patients in hepatitis B-endemic area may also benefit from single-agent Sorafenib treatment as a phase II clinical trial has shown fairly good efficacy and acceptable tolerability in these patients [228]. However, the effect of Sorafenib is temporary and the continuous dose for Sorafenib is needed to longer inhibit the tumour growth [229].

The retrospective analysis of breast cancer patients has shown an unfavourable prognosis in patients with high expression levels of VEGF [230-231]. This indicates that

VEGF could be associated with the efficacy of chemotherapy and radiotherapy. It has also been shown that radiation resistance is partly due to tumour cell production of angiogenic cytokines, particularly VEGF, which protects endothelial cells through survival pathways [232-233]. Moreover, it has been shown that the VEGF inhibition combined with radiation enhances radiation control of bone destruction and pain associated with cancer progression in bone metastases [234].

Here we studied the efficacy of Sorafenib combined with radiation and determined whether this treatment modality could enhance the tumour growth inhibition.

2.3. MATERIALS AND METHODS

2.3.1. Reagents

The cell culture reagents were obtained from Gibco®, Invitrogen, Canada. Fetal bovine serum (FBS) was purchased from Wisent Inc., Canada. Propidium iodide (PI) was from Sigma-Aldrich, Canada. Sorafenib tosylate (Bay 54-9085) was provided by Bayer Pharmaceutical Corp. and was reconstituted in Dimethyl Sulfoxide (DMSO) for *in vitro* use and in ethanol / Cremephore L (Sigma Aldrich, Canada) (50:50) for *in vivo* use at 4x concentration. The 4x solution of Sorafenib was prepared freshly every day. The final dosing solution was prepared by diluting the 4x solution to 1x in sterile water (Gibco®, Invitrogen, Canada) every day prior to its administration to the animals. The concentrations of DMSO were kept lower than 0.2 % in all *in vitro* experiments.

2.3.2. Cell Culture

The highly metastatic mouse mammary cancer cell line, 4T1, was a generous gift from Dr Fred Miller, Karmanos Cancer Institute, Wayne State University, MI, USA. Cells were cultured in

DMEM supplemented with 10 % FBS, Penicillin-Streptomycin 1% and kept at 37°C in 95 % air/5 % CO_2.

2.3.3. Irradiation

Irradiation for *in vitro* and *in vivo* experiments was carried out at room temperature using Theratron T-780 ^{60}Co irradiator (MDS Nordion, Kanata, ON, Canada). Dose delivered to each experimental setup used in this work was verified by radiochromic film dosimetry protocol developed by Tomic *et al.* [235].

2.3.4. Colony-Forming Assay

Cells were plated at specific cell numbers in 6-well plates. At 0 Gy radiation dose, 100 cells per well were used and for each subsequent radiation dose (2, 4, 6 and 8 Gy) 200, 400, 800 and 1600 cells were seeded, respectively. They were treated with Sorafenib alone (5 and 7.5 μM for 2 h) and in combination with radiation (2, 4, 6 and 8 Gy) [236]. After 6-8 days of incubation the colonies were fixed and stained with methylene blue. Only colonies containing more than 50 cells were counted. The Plating efficiency (PE) was calculated by dividing the number of colonies formed in the untreated control plates by the number of cells plated. Survival fractions (SF) were calculated by counting the number of colonies for

each specific radiation dose and dividing by the number of cells seeded at the same dose multiplied by PE. In order to plot the survival curve, the survival fractions were normalized according to the controls (non-irradiated). Radiosensitivity was measured by determining the dose enhancement factor (DEF), which is the ratio of the radiation doses at survival fraction of 0.1 or 0.01 of non-drug treated cells to drug treated cells [210, 237].

2.3.5. Flow Cytometry Analysis

2.3.5.1. Cell cycle analysis

Cells were treated with Sorafenib (5 and 7.5 μM) and were irradiated as described. The cells were harvested and washed 24 h post-treatment after which they were fixed with ethanol, labelled with PI and analyzed by flow cytometry (BD Biosciences). Cell cycle distribution was analyzed using the Mod-Fit LT software package.

2.3.6. Analysis of apoptosis by annexin-V binding

Cells were treated with Sorafenib (5 and 7.5 μM) and were irradiated to a dose of 4 Gy. They were harvested and washed with PBS 1X (Gibco, Invitrogen) at 48 h post-treatment. They were labelled with Annexin V-FITC and PI according to the manufacturer's protocol (TACS apoptosis kit, R&D Systems). Cells were analyzed by flow cytometry (BD Bioscience) and characterized as follows: Cells appearing at the lower left quadrant of the dot plot were considered viable. Those observed at the lower right quadrant were identified as early apoptotic. The late apoptotic and necrotic cells appeared at the upper right and upper left quadrants respectively.

2.3.7. Western Blot analysis

The 4T1 cells were incubated in two sets of 6-well plates with serum-free media for 18 h and were subsequently exposed to the 5 and 20 μM of Sorafenib for 2 h and 4Gy of radiation. To test if Sorafenib is still effective in case of over-activation of RTKs such as VEGFR, one set of the plates were subsequently treated with 25 ng/ml of VEGF for 20 min and cells were harvested within 1h after which the whole cell lysates were prepared. Fifty μg of protein was loaded onto Bis-Tris gradient gels (Invitrogen, Carlsbad, CA). Western blot analysis was performed using antibodies for p-ERK 1,2 and ERK1,2 as well as tubulin (Cell signaling Technology Inc., Beverly, MA).

2.3.8. *In vivo* Tumour Model

Six to eight-week-old female BALB/c mice (Charles River Laboratories, Montréal, Canada) were used in this study. Mice were caged in groups of five or less. 4T1 tumour cells (2×10^6 cells) were injected subcutaneously (SC) into the right hind leg. All protocols were approved by the McGill University Animal Care Committee following the guidelines of the Canadian Council on Animal Care.

2.3.9. Tumour Growth Delay Assay

When tumours reached a mean volume of 144 mm^3, mice were randomized into four groups: vehicle, Sorafenib alone, irradiation (15 Gy) alone and Sorafenib plus irradiation [88]. A single dose of Sorafenib (60 mg/kg) was administered by gavage daily for 7 days [223, 238]. The drug was administered 6 h prior to local tumour irradiation (15 Gy) on day 3 (schedule

A). In case of schedule B, radiation was delivered 24 h before the start of drug treatment. To obtain tumour growth curves, perpendicular diameter measurements of each tumour were made every 2-3 days with digital callipers, and volumes were calculated using the formula $(L \times W^2)/2$. Tumours were followed until the mean tumour volume reached ~ 2,000 mm^3 after which animals were sacrificed. Relative tumour volume was calculated by dividing each individual animal's tumour volume by the mean tumour volume of the same group. Each experimental group contained 6 to 8 mice.

2.3.10. Statistical Analysis

The effects of various treatments in all experiments were compared using two-tailed *t* test (GraphPad prism 5, GraphPad software, Inc, Ca, USA). Differences with a p value < 0.05 were considered statistically significant. The data presented represent means and SEM from multiple independent experiments.

2.4. RESULTS

2.4.1. Sorafenib increases the sensitivity of 4T1 cells to radiation *in vitro*

To evaluate whether Sorafenib has an effect on the ability of cancer cells to form colonies *in vitro*, clonogenic assays were performed. As it is shown in figure 2-1, the dose enhancement factor (DEF) was as high as 1.39 and 1.76 when Sorafenib was combined with radiation at 5 and 7.5 μM concentrations, respectively.

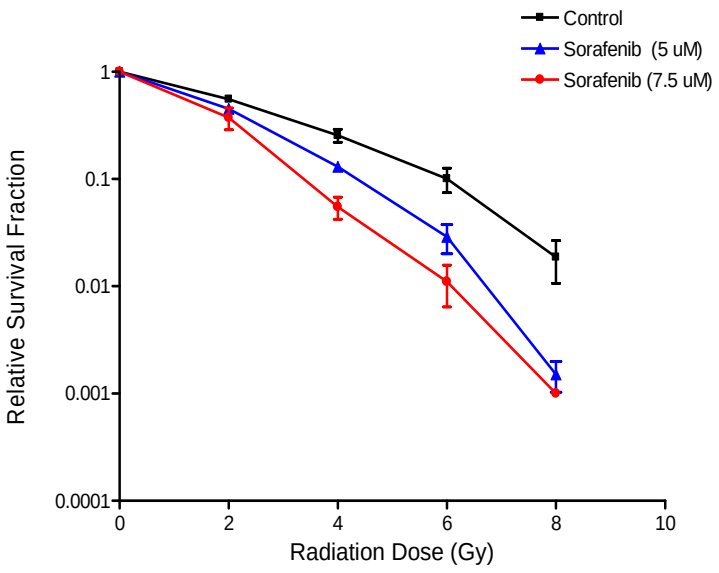


Figure 2-1 Clonogenic analysis of cell response to the combination of Sorafenib and radiation.
Cells were treated with Sorafenib (5, and 7.5 μM) with or without radiation (4 Gy). Data represent means and SEM from three independent experiments.

To test whether this effect is schedule-dependent, three schedules were employed: 1) Sorafenib 24 h before radiation, 2) Sorafenib concurrent with radiation and 3) radiation 24 h before Sorafenib administration. Interestingly, pre- treatment with Sorafenib (schedule 1) and the concurrent schedule (schedule 2) seemed to be more effective *in vitro* (figure 2-2a and 2-2b).

(a)

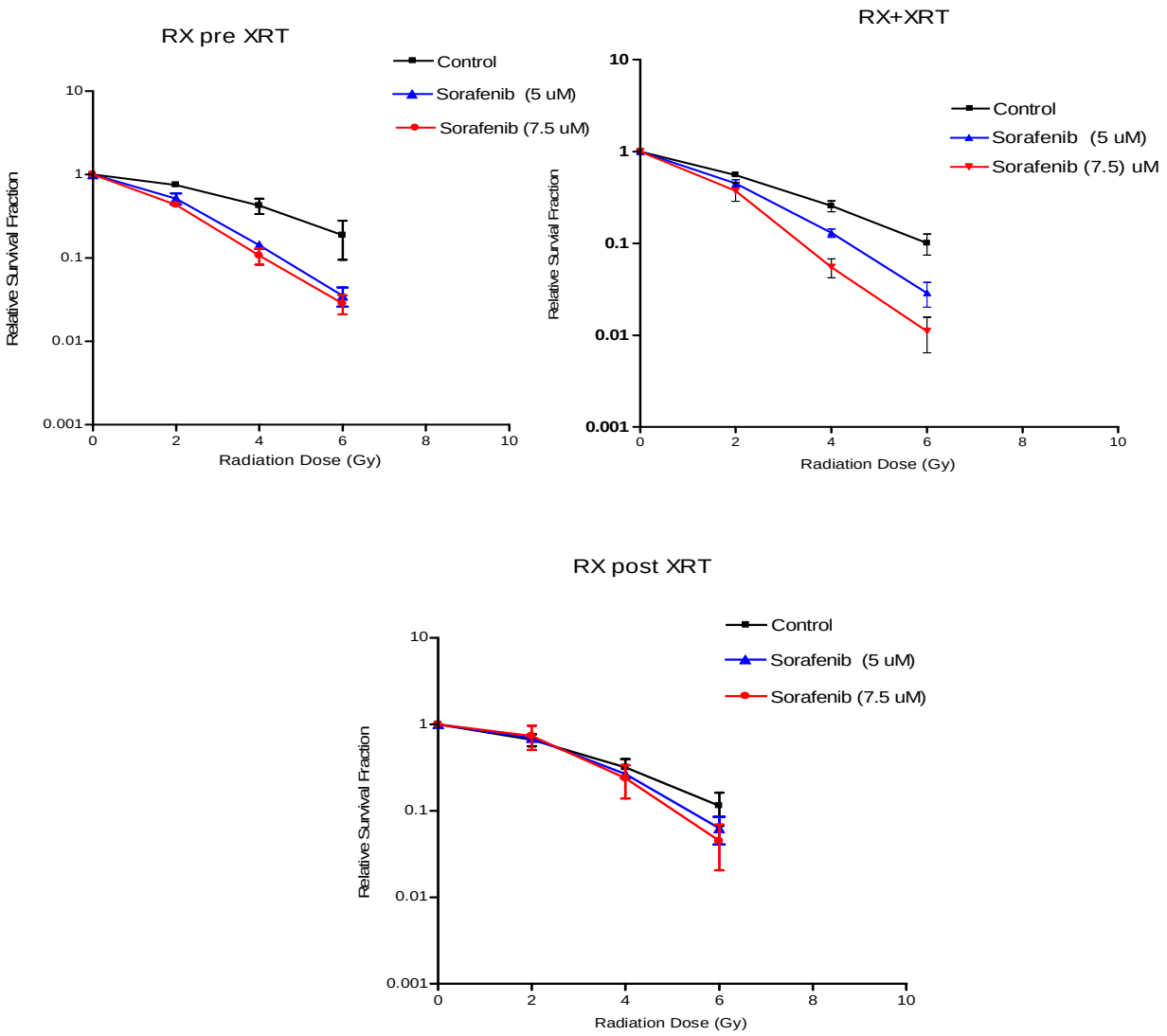
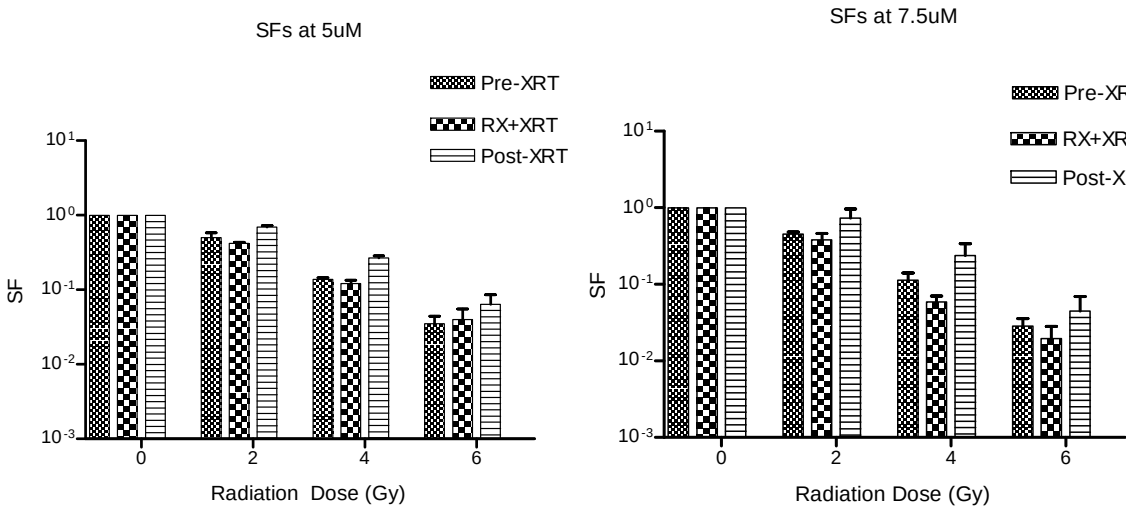


Figure 2-2. Clonogenic analysis of multiple combination schedules.

(a) Different schedules of combination (Sorafenib before radiation, Sorafenib concurrent with Sorafenib and Sorafenib 24 h after radiation) were tested on 4T1 cells.

(b)



(b) Comparison of survival fractions at the different combination schedule at specific Sorafenib doses.

2.4.2. Sorafenib combined with radiation induces G2/M arrest

To assess the effect of Sorafenib in combination with radiation on cell cycle progression, cell cycle analysis was performed. As it is shown in figure 2- 3, Sorafenib in combination with radiation had a significant and strong effect on cell cycle arrest at G2/M. Consequently, the G1 and S population was significantly decreased.

(a)

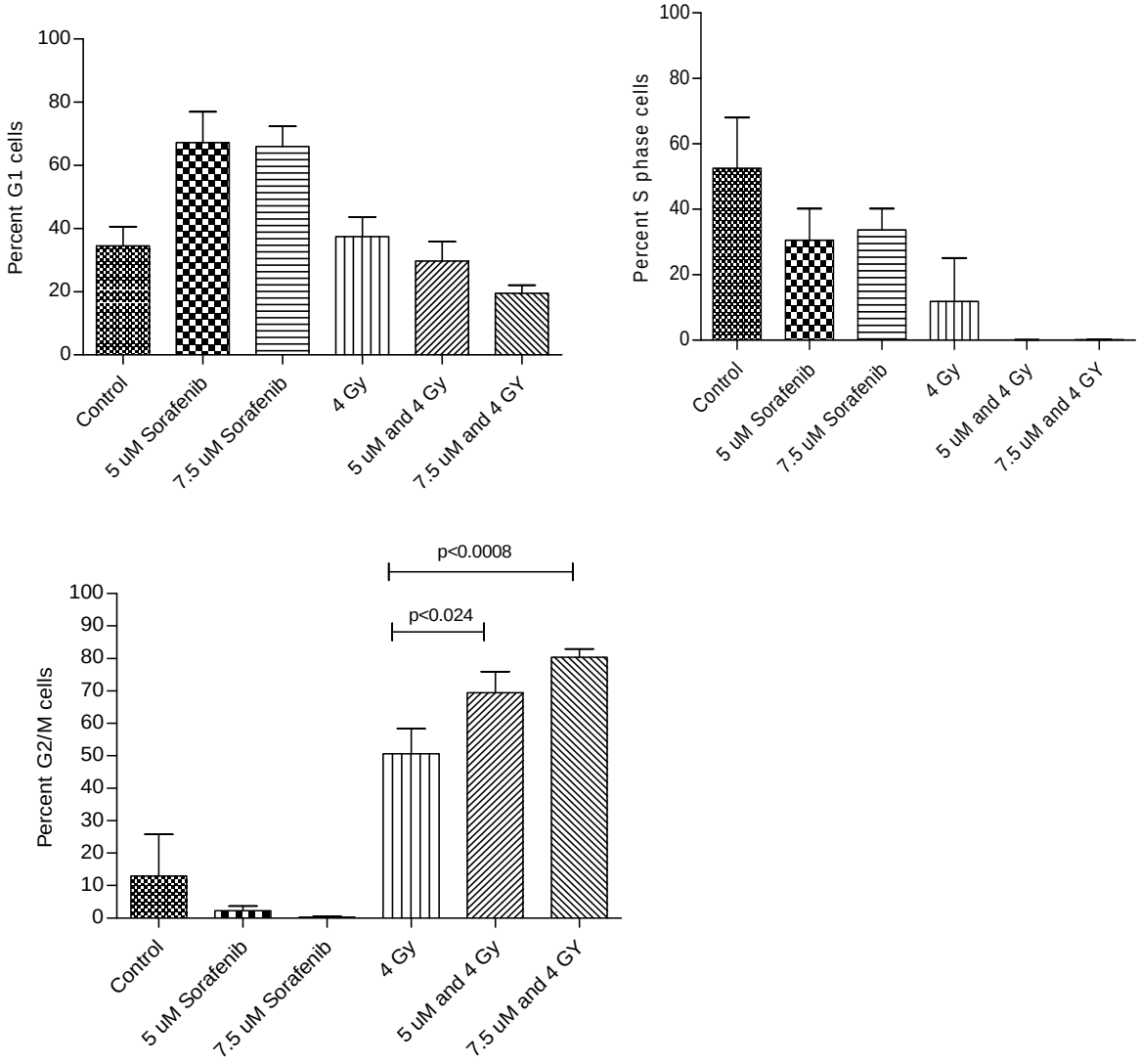
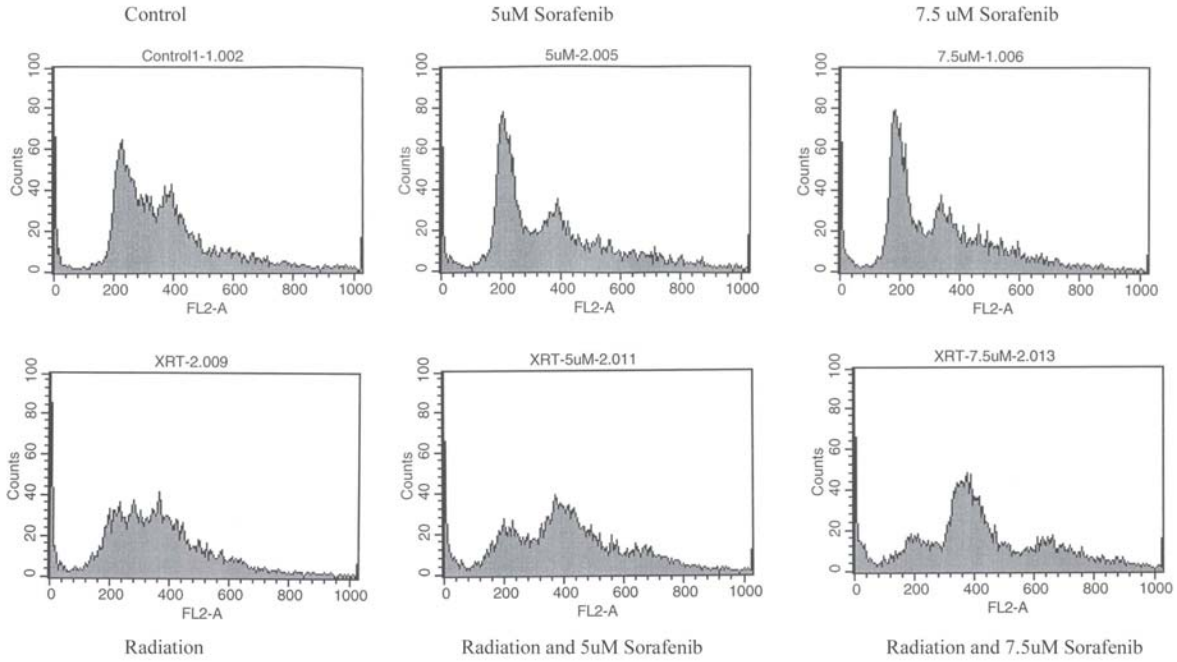


Figure 2-3. Cell cycle analysis of 4T1 cells following exposure to Sorafenib or radiation and corresponding combination.

(a) Cell distribution in G1, S and G2/M. Cells were treated with Sorafenib (5 and 7.5 μM) alone and in combination with radiation (4 Gy).

(b)



(b) A representative histogram showing the G2/M arrest in combined treatment.

2.4.3. Combination of Sorafenib and radiation enhances the level of apoptosis

In order to determine whether the multi-inhibitory activity of Sorafenib would induce high levels of apoptosis when combined with radiation, an annexin V binding assay was performed with cells exposed to radiation and Sorafenib alone or in combination. As shown in figure 2-4, Sorafenib alone induced apoptosis at levels of up to 15% and 33% at 5 and 7.5 μM, respectively, while apoptosis induced by radiation alone was approximately 10% of total analyzed cells. When Sorafenib was combined with 4 Gy of radiation the level of apoptosis reached 30% and 40% at 5 and 7.5 μM concentrations.

(a)

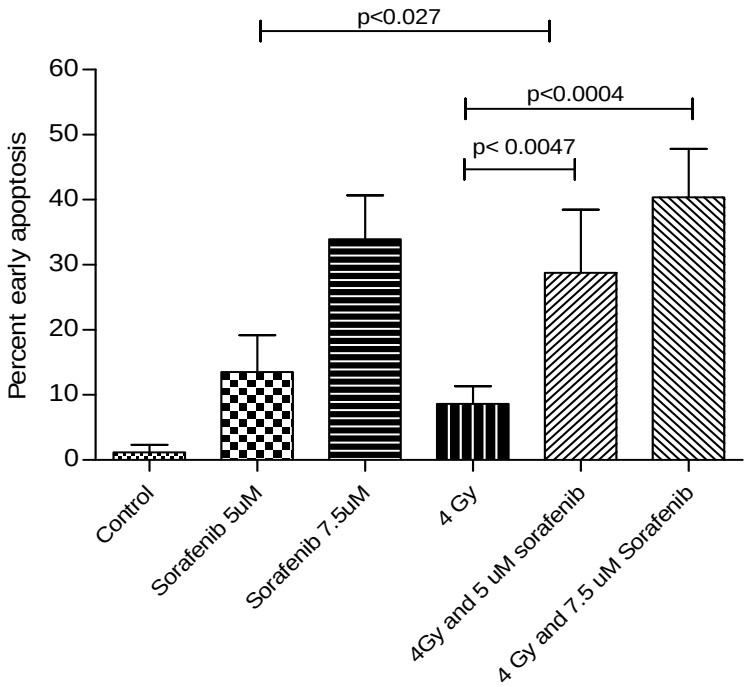
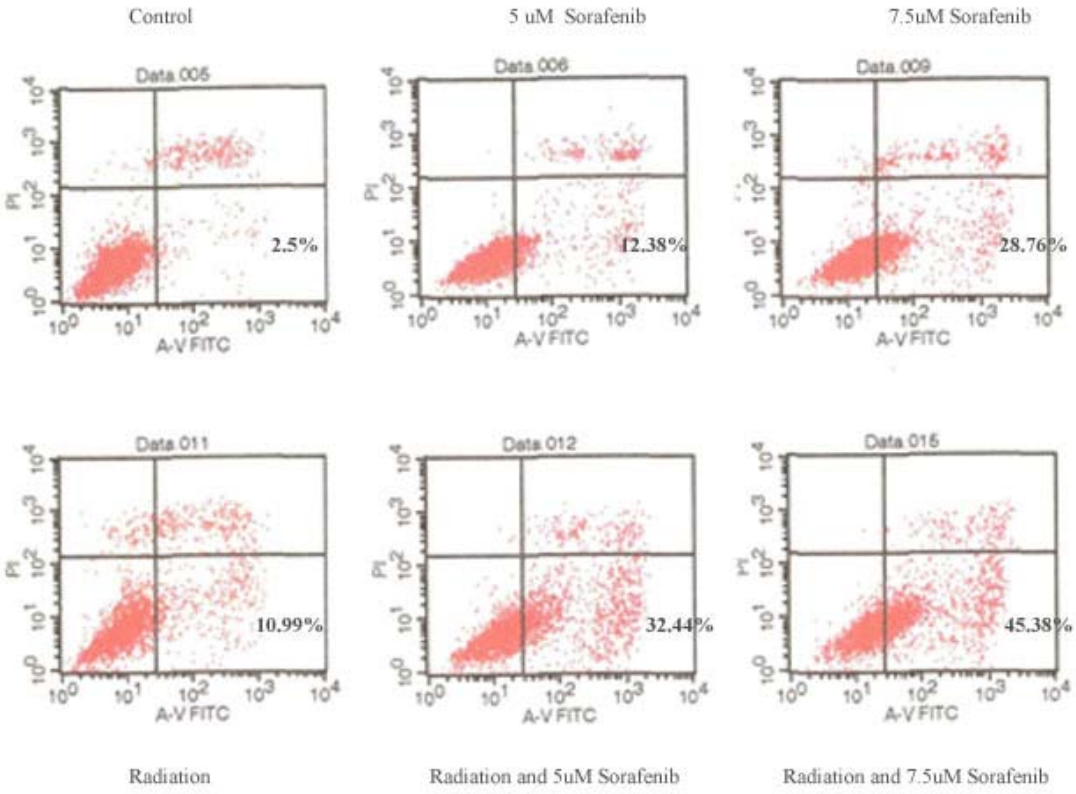


Figure 2-4. Analysis of apoptosis induced by Sorafenib or radiation and corresponding combination in 4T1.

(a) Cells were treated with Sorafenib (5 and 7.5 μM) alone and in combination with radiation (4 Gy) and were harvested at 48 h post treatment. Presented data are means and SEM of multiple independent experiments.

(b)



(b) Representatives dot plot data showing the effect of Sorafenib or /and radiation on the level of apoptosis.

2.4.4. Sorafenib with/out radiation inhibits phosphorylation of ERK1/2 downstream of RTKs

To confirm the inhibitory activity of Sorafenib in our breast cancer model, western blot analysis was performed. Since Sorafenib is a multikinase inhibitor of several RTKs, we evaluated the phosphorylation of ERK 1/2 as an indicator of activation of downstream pathway. As in figure 2- 5, Sorafenib at 5 μM completely inhibits the activation of ERK1/2 downstream of RTKs irrespective of radiation treatment.

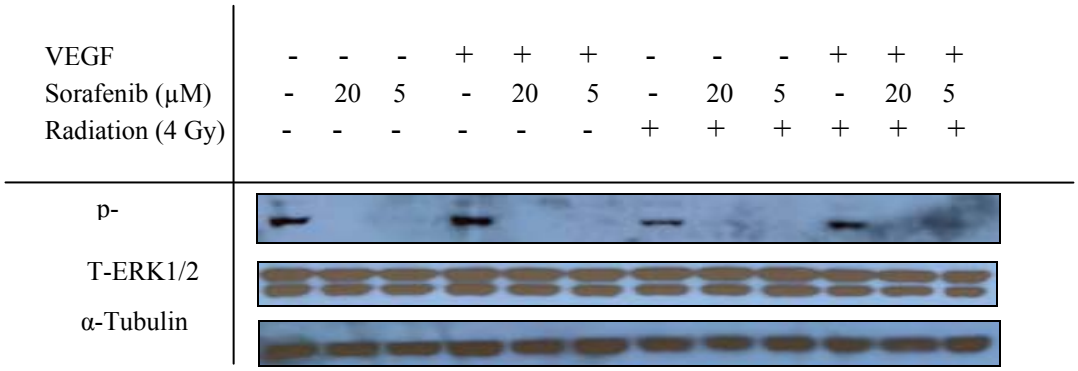


Figure 2-5. Inhibition of ERK1/2 activation.
4T1 cells were grown to 80% confluency in 6 well-plates. They were serum starved for 18 h and were treated with Sorafenib or/and radiation as it is indicated. Half of the plates were stimulated with VEGF and whole cell lysate were prepared within one hour.

2.4.5. Sorafenib increases the tumour growth delay caused by radiation as an early response

In order to evaluate and validate our *in vitro* results, we performed *in vivo* experiment with 4T1 mouse mammary cancer cells. As shown in figure 2- 6a and b, the *in vivo* results suggest that Sorafenib increases the radiation caused tumour growth delay in tumours that were treated with the combination therapy by almost 7 days. Moreover, Sorafenib combined with ionizing radiation has significantly more anti-tumour effect against 4T1 tumours than Sorafenib alone in BALB/c mice. Sorafenib was as potent as the combined treatment only until the end of the drug treatment (day 6). Soon after the end of drug treatment (day 10), tumours started to grow (Sorafenib treated group vs. combination group: $p= 0.0406$). On day 17 (figure 2-6a) the tumour volume of Sorafenib treated group was significantly larger compared to irradiated or combined treated tumours (Sorafenib vs. combination: $p= 0.0002$). The same pattern was observed when radiation was delivered 24 h before the start of Sorafenib treatment.

In schedule A (when radiation was delivered concurrently with Sorafenib treatment), the tumour growth delay was increased from 4.2 days in control group to 11 and 10.5 days in Sorafenib alone or radiation alone respectively. The growth delay, in case of combination of Sorafenib and radiation, was increased to 18 days. Similarly, in schedule B, when radiation was delivered 24h before Sorafenib treatment, the growth delay was increased from 6.5 days in control group to 13 and 14 days in Sorafenib alone and radiation alone and 20.5 days in mice treated with both modalities.

There were no significant body weight loss resulted from any of the treatments and treatments were well tolerated by the end of experimental end point (figure 2- 6.c and d).

(a) (b)

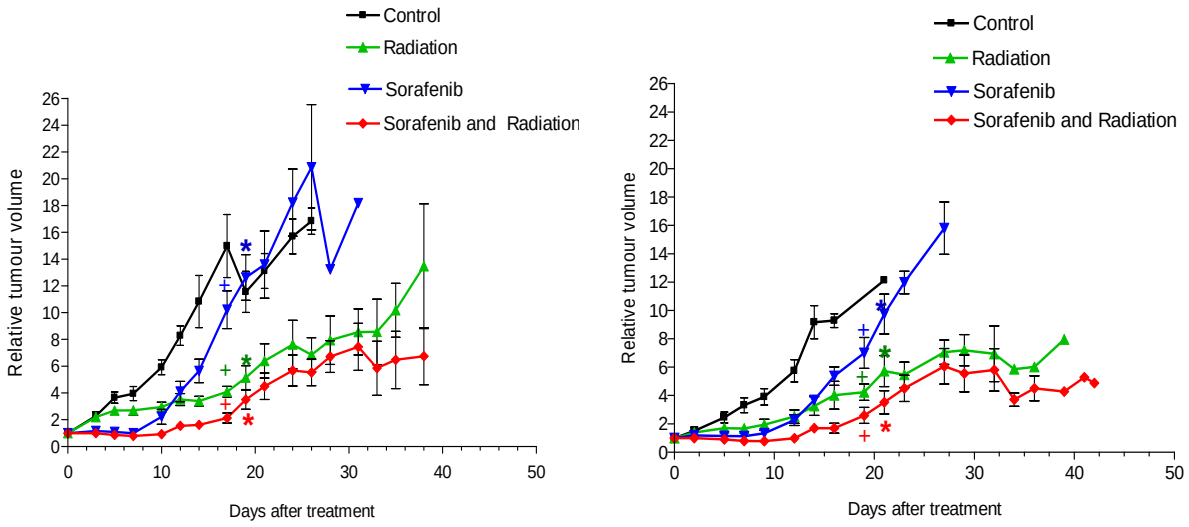
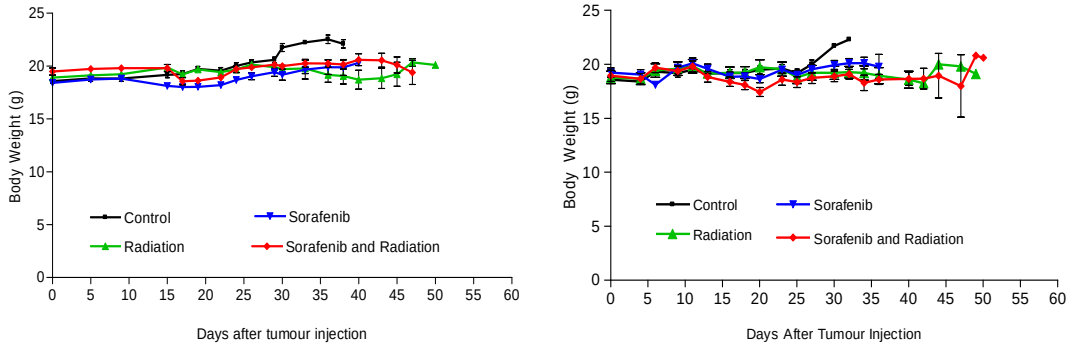


Figure 2-6. Tumour growth delay assay.

4T1 cells were injected into the right hind limb of BALB/c mice. When the mean volume of tumours reached to 144 mm^3, the animals were randomly divided into 4 groups. There were 6-8 animals per each group. Schedule A (figure 6a): Sorafenib started to be given to the animals 3 days before radiation, on the same day of irradiation and was continued for 3 days post irradiation (+: day 17, irradiated group vs. combined treatment: p= 0.0309; Sorafenib treated group vs. combination: p=0.0002; *: day 19, irradiated group vs. Combined treatment: p=0.1710; Sorafenib treated group vs. combination: p= 0.0255). Schedule B (figure 6b): 15 Gy of radiation was delivered 2h before the start of Sorafenib treatment (+: day 19, irradiated group vs. Combined treatment: p= 0.0316; Sorafenib treated group vs. combination: p=0.0255; *: day 21, irradiated group vs. Combined treatment: p=0.0815; Sorafenib treated group vs. combination: p= 0.0180). When the tumour size reached to maximum of ~2400 mm^3 the mice were euthanized. Tumour volume was calculated using: $(L \times W^2)/2$ and were normalized by dividing the tumour volume of each animal in treatment groups by the mean tumour volume of the same group. Error bars, SEM.

(b) (d)



(c and d) Variations of body weight of mice treated with Sorafenib (60mg/kg) and radiation alone and the combined treatment. Error bars, SEM.

2.5. DISCUSSION

In this study we demonstrated that Sorafenib induces a greater anti-tumour activity when it is combined with radiation in 4T1 cells, both *in vitro* and *in vivo*. 4T1 cells are highly metastatic cancer cells and they are considered to be a suitable model to study the effect of anti-angiogenesis agents *in vitro* and *in vivo* [239-242].

The increased anti-tumour activity of Sorafenib combined with radiation in 4T1 cells can be partially explained by the significant cell cycle arrest we observed at G2/M. Cancer cells show more sensitivity to ionizing radiation at the G2/M and G1 while cells residing in S stage of the cell cycle are less radiosensitive [92]. As it was shown by our result, there was a significant decrease in S phase population, which could explain the higher potency of the combined treatment.

The increased efficacy of Sorafenib in combination with radiation could also be due to the augmented apoptosis level in 4T1 cancer cells treated with both Sorafenib and radiation.

In this study, we have also shown that radiation prolongs the anti-tumour activity of Sorafenib *in vivo*. Tumours implanted in mice treated with Sorafenib alone were started to grow rapidly immediately after the stop of drug treatment (day 6-8) while the inhibitory effect of Sorafenib was longer when radiation was added (tumours started to grow gradually starting day 14). This could be of relevance as cytostatic agents as Sorafenib usually show temporary and reversible anti-tumour activity [229].

Moreover, in our *in vivo* model, Sorafenib increased the radiation response significantly as an early response (figure 2- 6 a and b). It will be interesting to evaluate different schedules and sequences of this combination to see if longer response could be achieved.

Our *in vitro* data demonstrated higher radiation response when Sorafenib was added prior or concurrent with radiation vs. post radiation. This was in disagreement with our *in vivo* results that showed no significant difference between the Sorafenib treatment post and concurrent with radiation. The difference between our *in vitro* and *in vivo* outcome could be due to the interaction of tumour cells with each other and with their microenvironment. *In vitro* assays are performed in much shorter time period than *in vivo* experiments. The difference could also be related to hypoxia and hypoxia induced radiation resistance. Although anti-angiogenic agents have been shown to stabilized neo-vasculature and improve blood perfusion, Sorafenib might not have done so resulting in forming of hypoxic regions inside the tumours and therefore reduced radiation response.

Recently, Suen *et al*. [198] and Plastaras *et al*.[238] have shown that the combination of Sorafenib and radiation enhance the radiation response in colorectal cancer cells *in vivo* and this response is schedule dependent. In their studies irradiation prior to Sorafenib treatment appears to be the most efficient schedule [198, 238]. The different outcome between their study and ours is perhaps due to the use of different tumour models and also different radiation schedules. The mice in our study were irradiated with a single radiation dose either pre or concurrent with Sorafenib administration (schedules a and b) while in the two mentioned studies fractionated radiation was used over a longer period of time.

Sorafenib being a cytostatic agent [243], it has been shown that it can induce the radiation response especially in fractionated schedules as it blocks re-growth (through its anti-angiogenic properties) between fractions [217]. Presently, in our laboratory, there are more *in vitro/in vivo* studies ongoing to test Sorafenib with fractionated radiotherapy in metastatic breast cancer models while more microenvironment studies will guide us through the complex mechanism of this combination.

There are several trials combining Sorafenib with radiation and other cytotoxic modalities [244] that are ongoing or completed in the clinical setting. Some results showed that the combination did not improve the efficacy of treatment as 40% of patients did not receive Sorafenib at all due to early disease progression. Perhaps better results can be achieved with a better design or modified combinations. Other trials including a phase I/II study of cisplatin and radiation in combination of Sorafenib in cervical cancer, a phase I/II trial of radiation therapy and Sorafenib for unrespectable liver metastases and Sorafenib combined with radiation in hepatocellular carcinoma are presently ongoing. Depending on the outcome of these clinical trials the protocol for respective patient might change

eventually and cancer patients might benefit from the new combination therapies. Nevertheless, a better understanding of mechanism of action of anti-angiogenic agents and more specifically multi-targeting agents is crucial to better design a clinical trial and to rationally choose the target patients population.

2.6. ACKNOWLEDGMENTS

We would like to thank Dr. Bassam Abdolkarim and Dr. Bertrand Jean-Claude for critical reviewing of this manuscript. This study was supported by Bayer Pharmaceuticals. TM and MH thank Foundation de la recherché du la santé Quebec (FRSQ) and McGill-CIHR Drug development program for their financial support.

CONNECTING TEXT 2

In the previous chapter we have shown that Sorafenib potentiates radiation response in our TN breast cancer model. In the third chapter we will continue investigating the combination of radiation with another type of multi-targeting inhibitor called "combi-molecule" which has been discussed in the first chapter. The combi-molecule we studied in this chapter has a cytostatic moiety (EGFR inhibitor) and a cytotoxic moiety (DNA alkylation agent) in contrast to Sorafenib which is only a cytostatic molecule (RTKI).

The combination of cytotoxic and cytostatic agents has been shown to increase the treatment response in patients as the effects of cytostatic agents can be reversible. Although the combi-molecule, ZRBA1, has two moieties, the presence of the DNA repair enzyme and other resistance mechanisms in cancer cells could still affect the treatment response. However, we hypothesized that when ZRBA1 is combined with ionizing radiation, it can induce more effective anti-tumour activity.

CHAPTER 3

Interaction of ionizing radiation and ZRBA1, a mixed EGFR/DNA targeting molecule

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3.1. ABSTRACT

ZRBA1 is a molecule termed “combi-molecule” designed to induce DNA alkylating lesions and to block EGFR TK. Because of its ability to downregulate the EGFR TK-mediated anti-apoptotic signaling and DNA repair proteins, we surmised that it could significantly sensitize cells to ionizing radiation. Using the MDA-MB-468 human breast cancer cell line in which ZRBA1 has already been reported to induce significant EGFR/DNA targeting potency, the results showed that: (a) concurrent administration of ZRBA1 and 4 Gy led to a significant decrease in cell viability, (b) the greater efficacy of the combination was sequential, being limited to conditions wherein the drug was administered concurrently with radiation or before radiation, (c) the efficacy enhancement of the combination was further confirmed by clonogenic assays from which a dose enhancement factor of 1.34 could be observed at survival fraction of 0.01. Flow cytometric analysis showed significant enhancement of cell cycle arrest in G2/M ($p<0.046$, irradiated cells vs. cells treated with ZRBA1 and radiation) and increased apoptosis when ZRBA1 was combined with radiation. Likewise, significant levels of double strand breaks were observed for the combination, as determined by neutral comet assay ($p<0.045$, irradiated cells vs. cells treated with ZRBA1 and radiation). These results *in toto* suggest that the superior efficacy of the ZRBA1+radiation combination may be secondary to the ability of ZRBA1 to arrest the cells in G2/M, a cell cycle phase in which tumour cells are sensitive to radiation. Furthermore, the increased levels of DNA damage, combined with the concomitant downregulation of EGFR-mediated signaling by ZRBA1, may account for the significant levels of cell-killing induced by the combination.

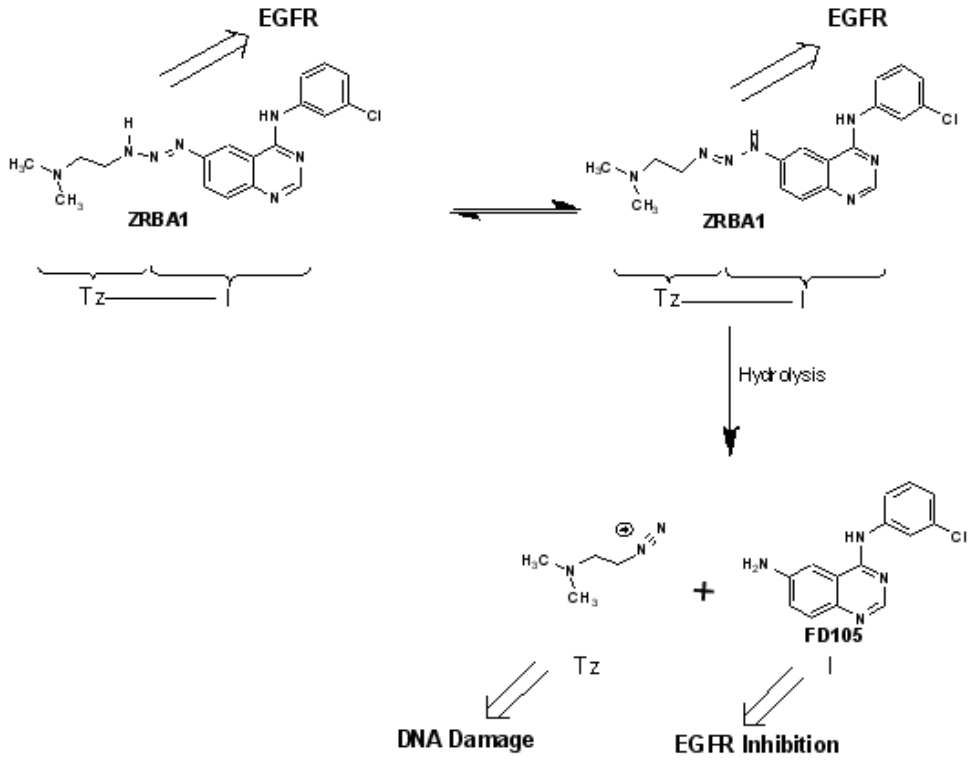
3.2. INTRODUCTION

Ionizing radiation is a mainstay of non-surgical cancer treatment. Approximately 75 % of non skin cancer patients receive radiation therapy at some time during the course of their disease. Radiotherapy has been successful in local tumour control [215]and when it is combined with chemotherapy, radiotherapy also improves overall survival of cancer patients [217]. Over the last decade, combination of ionizing radiation with chemotherapy has led to marked improvement in local control, organ preservation and survival for locally advanced solid tumours. However, this strategy is limited by the morbidity resulting from each respective treatment and their combinations. Targeting tumour specific defects should provide an advantage over conventional chemotherapy in which the major drawback is normal tissue toxicity [222].

On the other hand, acquired resistance to DNA damaging agents represents a major obstacle in the therapy of many tumours including lung, breast, ovarian and brain carcinomas. Over the past three decades, several strategies have been developed to enhance the potency of DNA damaging agents, the most common one being the use of inhibitors of DNA repair enzymes [245-247]. With the advent of molecular biology, novel markers associated with reduced sensitivity to DNA damaging agents have been identified. This includes signalling proteins such as AKT, the activation of which is related to anti-apoptotic signalling. More importantly, several receptor tyrosine kinases (RTKs) have now been identified that activate AKT-mediated anti-apoptotic signalling [245, 248-250]. One such receptor is the epidermal growth factor receptor (EGFR), which is activated by chemical and radiation-induced DNA damage. Importantly, overexpression of EGFR is associated with

aggressive tumour progression, invasion and reduced sensitivity to chemotherapy [251-253]. Recently, Yacoub *et al.* [254] showed that the activation of EGFR leads to expression of DNA repair proteins such as XRCC1 and ERCC1.

Recently, we developed a novel type of molecules termed "combi-molecules" designed to block the tyrosine kinase (TK) activity of EGFR and its subsequent adverse effect on apoptotic signalling or its ability to upregulate DNA repair proteins, while concomitantly delivering significant DNA lesions to the cells (Scheme 1) [255-256]. The combi-molecules have now been shown to indeed inflict strong DNA damage to tumour cells and to block EGFR [245, 257-259]. One such molecule, ZRBA1, induced significantly higher levels of apoptosis than the single targeted EGFR inhibitor FD105 [42]. Furthermore, its anti-proliferative activity against MDA-MB-468 breast cancer cells was more sustained than that of FD105. However, despite its significant potency, its activity was partially mitigated in cells expressing the DNA repair enzyme O6 alkylguanine transferase (AGT) e.g. SF188 AGT+ glioma cells [42]. Thus, despite the strong binary EGFR/DNA targeting potency of this agent, its activity remained to be improved in DNA repair proficient cells. However, due to its mixed EGFR/DNA targeting mechanism, it could potentially be developed as a radiopotentiator.



Scheme 3-1. Hydrolysis and binary properties of ZRBA1 under physiological condition

ZRBA1 is designed to induce N7- and O6-alkylated lesions in a manner similar to the clinical drug temozolomide, which is effective in tumours that do not express AGT. It has been shown that radiation-induced lesions potentiated the action of temozolomide in the latter type of tumours [260]. Temozolomide enhancement of radiation response was imputed to its ability to increase the degree of radiation-induced DNA double strand breaks in the cells [261]. ZRBA1, being able to induce DNA alkylating lesions of the same type as temozolomide, if combined with radiation might not only increase the levels of DNA strand breaks but also inflict different types of DNA damage, thereby delaying or complicating the

DNA repair process. Added to its ability to downregulate EGFR TK activity and its subsequent downstream effect on apoptosis and DNA repair, we proposed that the combination of ZRBA1 with radiation may translate into significant cell-killing. To verify this hypothesis, we chose to analyse the effect of ZRBA1 plus radiation in the human MDA-MB-468 breast cancer cell line that overexpresses EGFR and in which ZRBA1 has been proven to exert its binary targeting potency [42].

3.3. MATERIALS AND METHODS

3.3.1. Reagents

The cell culture reagents were from Gibco®, Invitrogen, Canada. Fetal bovine serum (FBS) was purchased from Wisent Inc., Canada. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide (PI) were from Sigma-Aldrich, Canada. The combi-molecule was synthesized according to the previously published methods [199]. The drug was reconstituted in DMSO, the concentrations of which were kept lower than 0.2 % in all experiments.

3.3.2. Cell Culture

Human MDA-MB-468 breast carcinoma cells were obtained from the National Cancer Institute, Bethesda, MD. Cells were cultured in RPMI 1640 supplemented with 10 % FBS, Penicillin-Streptomycin 1% and kept in 37°C in 95 % air/5 % CO_2.

3.3.3. Irradiation

Irradiation was carried out in our research facilities at room temperature using a 160 Kvp X-ray irradiator Faxitron FC-160 (Wheeling, Il, USA) at a dose rate of 1.5 Gy/ min.

3.3.4. Cell proliferation assay

Growth inhibition was measured using the MTT assay [262]. Cells were plated at the density of 8,000 cells/well in 96 well plates and subsequently treated with ZRBA1 or Iressa (0-100 uM) alone (for 2 or 24 h) and in combination with radiation (4 Gy). Cells were washed with drug-free media and then the fresh media was added before irradiation. Cells were incubated for 72 to 96 h depending on their schedule, after which the MTT solution was added for 3-5 h. The assay was stopped and the OD measured using a 96-well plate reader at 750nm.

3.3.5. Colony forming assay

Cells were plated at specific cell numbers in 6-well plates. They were treated with ZRBA1 or Iressa, alone for 2 h (36μM) and in combination with radiation (2, 4, 6 and 8 Gy). Cells were washed with drug-free media and then fresh media was added before irradiation. After 12 to 14 days, the colonies were fixed and stained with methylene blue. Only colonies containing more than 50 cells were counted. The Plating Efficiency (PE) was calculated by dividing the number of colonies formed in the untreated control plates by the number of cells plated. Survival Fractions (SF) were determined as colonies counted at the specific radiation dose divided by the cells seeded at the same dose multiplied by PE. In order to plot the survival curve, the survival fractions were normalized according to the controls (non-irradiated). Radiosensitivity was measured by Dose Enhancement Factor (DEF), which is the ratio between the radiation doses at survival fraction of 0.1 or 0.01 of non-drug treated cells over drug treated cells [210, 237].

3.3.6. Flow Cytometry analysis

3.3.6.1. Analysis of apoptosis by annexin-V binding

Cells were treated with ZRBA1 (50 μM) for 2 h and were irradiated for 4 Gy. They were harvested and washed with PBS 1X (Gibco, Invitrogen) at 3, 6, 12 and 24 and 48 h after treatment. They were labeled with Annexin V-FITC and PI according to the manufacturer's protocol (TACS apoptosis kit, R&D Systems). Cells were analyzed by flow cytometry (BD Bioscience) and characterized as followed. Cells appearing at the lower left quadrant of the dot plot were considered viable. Those observed at the lower right quadrant were identified as early apoptotic. The late apoptotic and necrotic cells appeared at the upper right and upper left quadrants respectively.

3.3.6.2. Cell cycle analysis

Cells were treated with ZRBA1 (25 μM) for 2 h and were irradiated as described before. They were harvested and washed 24 h post-treatment after which they were fixed with ethanol, labeled with PI and analyzed with a flow cytometer (BD Biosciences). Cell cycle distribution was analyzed using the Mod-Fit LT software package.

3.3.7. Comet assay

The modified neutral comet assay was performed as described previously [42, 263]. The cells were exposed to a dose (36 μM) of ZRBA1, Iressa or FD105 for 2 h, irradiated at 4Gy, harvested and re-suspended in PBS. Cell suspensions were diluted to approximately 10^6 cells and mixed with agarose (1%) in PBS at 37 ºC in a 1:10 dilution. The gels were casted on Gelbond strips (Mandel Scientific, Canada) using gel-casting chambers and then immediately placed into a lysis buffer (2% sarkosyl, 0.5M Na_2EDTA, 0.5 mg/ml proteinase

K (pH 8.0)) [263]. After being kept at 37°C overnight, the gels were gently rinsed with a neutral rinse buffer (90 mM Tris buffer, 90 mM boric acid, 2 mM Na_2EDTA (pH 8.5) for 30 min at 37 °C. Thereafter, the gels were submerged in fresh neutral rinse in an electrophoresis chamber and ran with 20V for 20 min. They were subsequently rinsed with distilled water, dried with 100% ethanol overnight, and stained with SYBR Gold (1/10,000 dilution in distilled H_2O, supplied from Molecular Probes, Eugene, OR) for 1 hour. 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 the comet assay IV imaging software package (Perceptive Instrument, UK).

3.3.8. Sub-cellular distribution study

MDA-MB-468 cells were plated at 70% confluency in six-well plates, allowed to adhere overnight and treated with ZRBA1 for 1 h. Cells were subsequently washed with PBS twice, and analyzed using a DAPI filter in a Leica fluorescent microscope (Leica DFC300FX camera). Pictures were obtained at a 400X magnification.

3.3.9. Statistical analysis

The effects of various treatments in all experiments were compared using two-tailed *t* test. Differences with a p value < 0.05 were considered statistically significant. The data represent means and SEM from multiple independent experiments ($\pm$ SEM).

3.4. RESULTS

3.4.1. Growth inhibitory effect

In order to determine the doses required for different combination with radiation, a dose response curve was established with ZRBA1 and radiation alone in the MDA-MB-468 cells using the MTT assay.

The IC_{50} for cell survival for ZRBA1 was 36μM and 30μM following 2 h and 24 h exposure, respectively. The dose of radiation required for killing 50% of cells was approximately 4 Gy. Thus, combinations were performed with ZRBA1 at 36 μM when doses of radiation were varied and 4 Gy when doses of ZRBA1 were altered. The results showed that concomitant exposure of a dose range of ZRBA1 to 4 Gy leads to an additive effect at the lower doses (when the two curves overlapped) while this effect is not visible at higher doses. This can be due to the limitation of the MTT assay (figure.3-1).

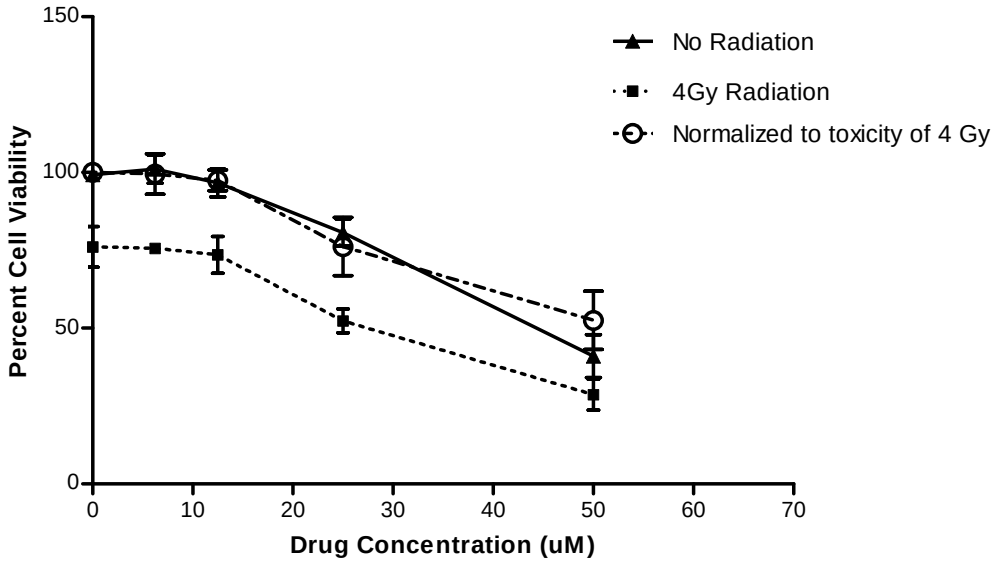


Figure 3-1. Effects of radiation and combination of radiation (4 Gy) + ZRBA1 on the viability of MDA-MB-468 cells.

The cells were treated with variable concentration of ZRBA1 and irradiated with a dose of 4 Gy. Values of treated cells with the combination of ZRBA1 and radiation were normalized to account for the toxicity induced by 4 Gy of radiation. (Data represents means and SEM from three independent experiments).

ZRBA1 being an alkylator, generated the same alkylating lesions as temozolomide, which in previous studies have been shown to enhance radiation response in human tumours in a sequence dependent manner [24]. Thus, we determined whether sequential administration of ZRBA1 and radiation would lead to different results when compared with concurrent administration. As depicted in figure 3-2, in one sequence (see A) ZRBA1 was administered for 2 h and radiation given 24 h later, followed by 72 h recovery prior to

analysis of cell viability by MTT. In another sequence (B), radiation was given first followed by a 2 hr drug exposure 24 h later and cell viability analyzed 72 h post-treatment. In the third sequence (C), cells were exposed to the drug for 2 h, after which radiation was administered and cell viability analyzed 96 hr post-treatment.

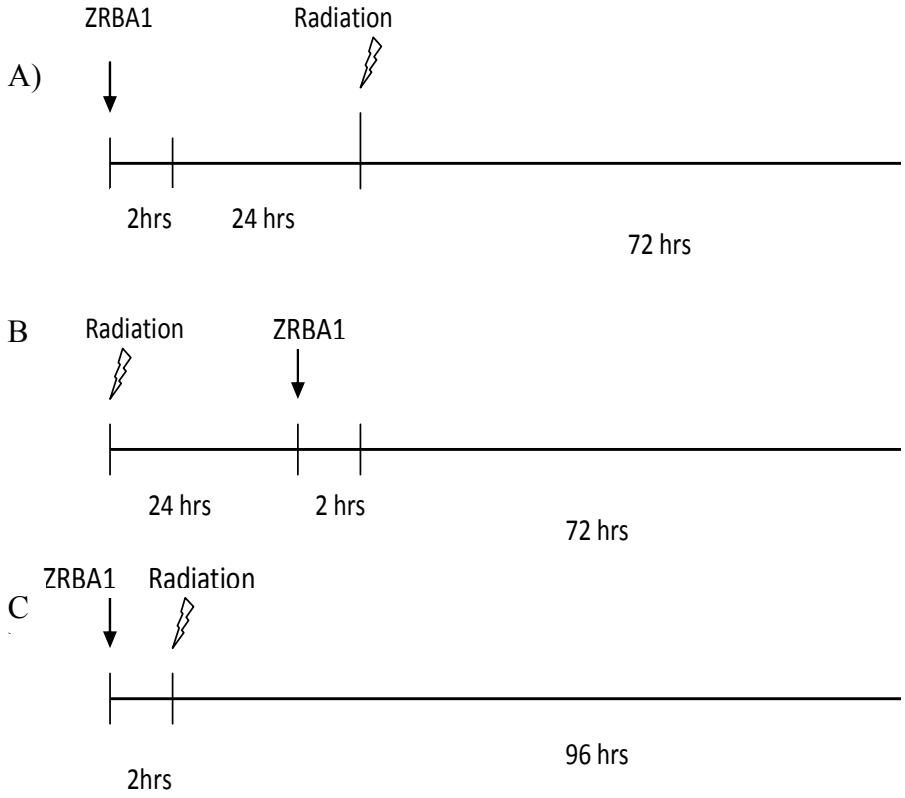


Figure 3-2. Sequences of administration of ZRBA1 and radiation in the various combinations**.**

A) MDA-MB-468 cells were treated with 36μM of ZRBA1 for 2 h and were irradiated 24 h later. They were further incubated for 72 h; B) Cells were irradiated with 4 Gy of radiation, treated with 36μM of ZRBA1 24 h and further incubated for 72 h. C) Cells were treated with 36μM of ZRBA1 for 2 h and were irradiated immediately after the drug was washed out.

The results showed that the greatest efficacy of the combination was observed when the drug was administered as depicted by sequence C (figure 3-2 and figure 3-3) according to which the drug and radiation were given concurrently. While drug administration sequence A also showed significant enhancement, sequence C showed the most effective response among the three tested protocols ($p<0.001$, B vs. C). Therefore, it appears that for effective combination, drug administration must precede or be concurrent with radiation.

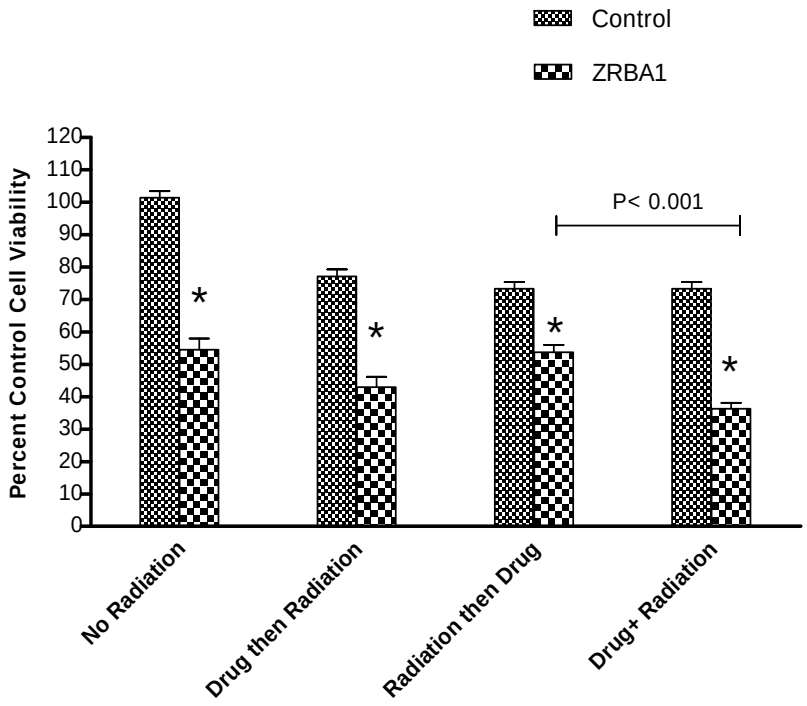


Figure 3-3. Cell Survival comparison following exposure to ZRBA1 and Radiation according to sequences A-C as determined by the MTT assay.
MDA-MB-468 cells were treated with ZRBA1 (36 μM) for 2 h with or without radiation (4 Gy) (columns: mean cell viability from three independent experiments, bars: SEM; *: $P<0.0001$).

3.4.2. Clonogenic assay

To confirm the significant results obtained from the MTT assay, a clonogenic study was performed. ZRBA1 was added (36 μM) for 2 h and the dose range of radiation applied (0 to 8 Gy). After 14 days, plating efficiency of control cells was approximately 20 %. Survival fractions at multiple radiation doses were calculated and were normalized to the controls which were the cells treated with drugs only (SF of 0.14 for ZRBA1). Normalized SF values without and with ZRBA1 at the dose of 2 Gy were 0.477, 0.351 and at the dose of 4 Gy were 0.117 and 0.062 respectively. The survival curves were plotted based on the normalized survival fractions. Radiosensitivity was measured by DEF (discussed at Materials and methods) and the values were 1.23 $\pm$ 0.073 at SF of 0.1 and 1.34 $\pm$ 0.052 at SF of 0.01 which are indication of increased sensitivity of cells treated with ZRBA1 at these survival fractions (figure 3-4). In addition, ZRBA1 was able to enhance the radiation effect more than Iressa at radiation dose of 4 Gy and higher (figure.4)

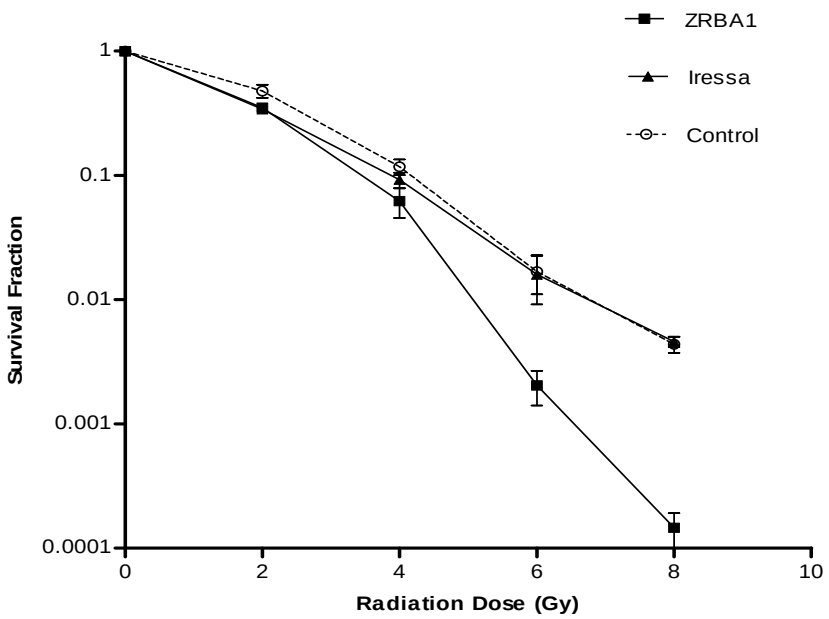


Figure 3-4. Clonogenic analysis of cell response to the combination of ZRBA1 or Iressa and radiation.

MDA-MB-468 cells were treated with ZRBA1 and Iressa (36 μM) for 2 h with or without radiation. Data represent means and SEM from three independent experiments.

3.4.3. Cell cycle effect

Considering that ZRBA1-induced DNA damage is known to be associated with cell cycle arrest in G2/M [264], it was determined whether a cell cycle rationale could be used to account for the greater efficacy of the ZRBA1+radiation combination. Indeed, 24 h after ZRBA1 administration, significant cell arrest in G2/M was observed. When radiation was given at this time point and analyzed 24 h later, an even more significant increase in cell

accumulation in G2/M phase of the cell cycle was observed ($p<0.046$, radiation vs. radiation and ZRBA1) (figure 3-5a and b). This suggests that a strong cell cycle arrest in G2/M precedes cell death following exposure to ZRBA1 plus radiation.

(a)

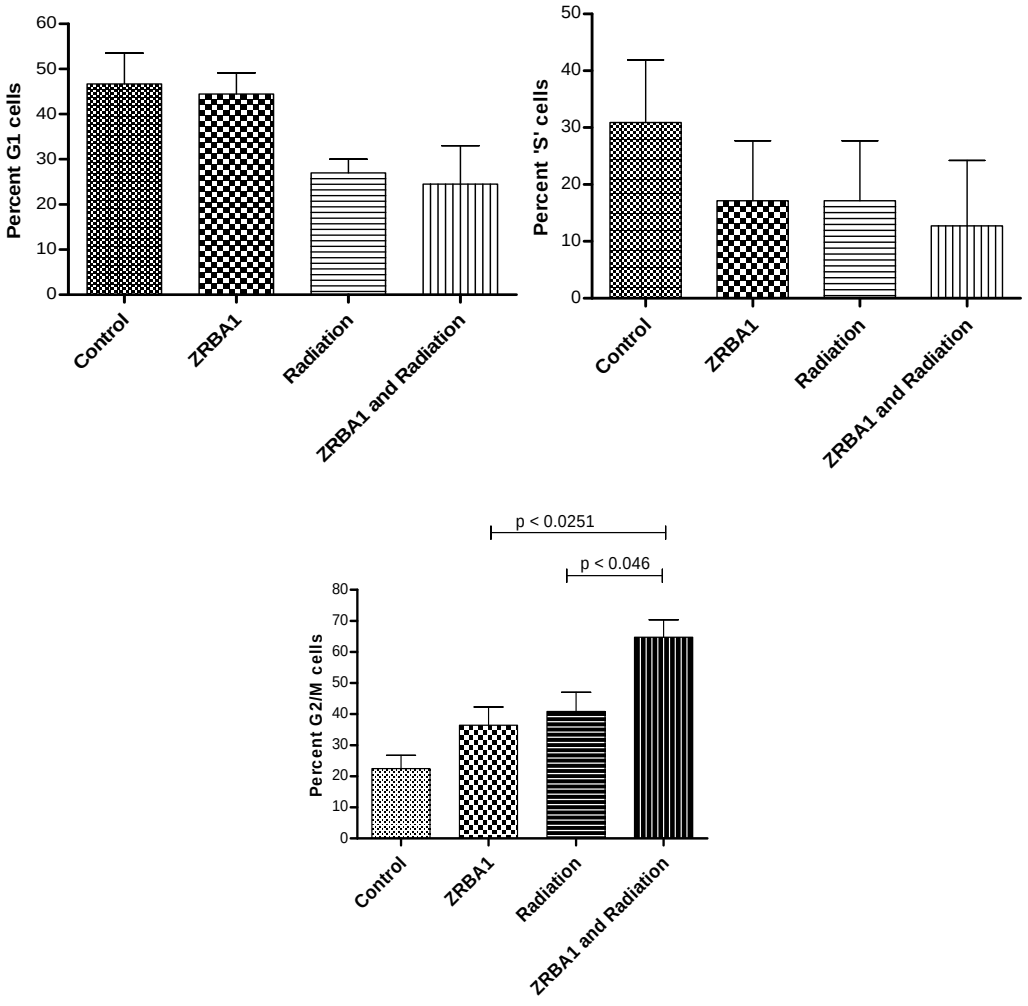
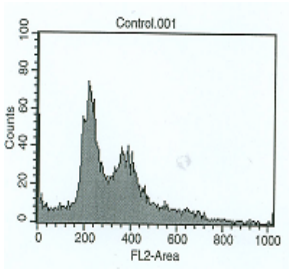


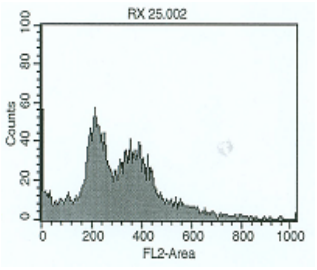
Figure 3-5. Cell cycle analysis of MDA-MB-468 cells following exposure to ZRBA1 or radiation and corresponding combination.

(a) Cell distribution in G1, S and G2/M. Cells were treated with ZRBA1 (25 μM) alone and in combination with radiation (4 Gy) and cell cycle analyzed by flow cytometry 24 h later. Data represents means and SEM from three independent experiments.

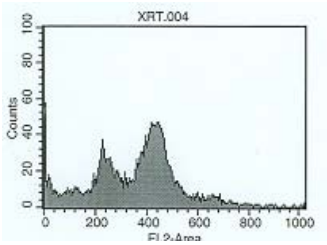
(b)



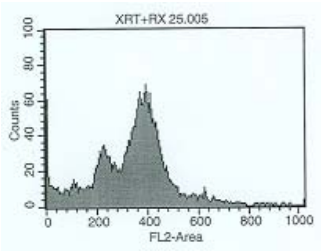
Control



ZRBA1



Radiation



ZRBA1and Radiation

(B) A representative histogram showing the G2/M arrest in combined treatment.

3.4.4. DNA damage

In order to determine whether the enhancement of G2/M arrest was associated with elevated DNA damage, a neutral comet assay was performed with cells treated with FD105, Iressa, ZRBA1 or radiation and the corresponding combination. A significant increase in levels of DNA damage was observed for the radiation plus ZRBA1 combination when compared with radiation alone ($p<0.045$) or treatment with ZRBA1 alone ($p<0.029$) (figure 3-6).

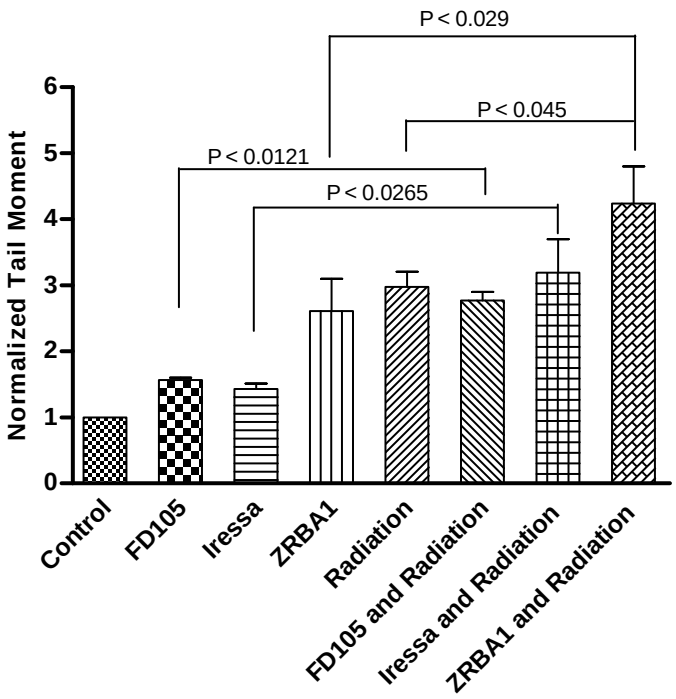


Figure 3-6. Double strand breaks induced by FD 105, Iressa, ZRBA1 or radiation and corresponding combinations as determined by a neutral comet assay.

Cells were treated with drugs (36 μM) for 2 h, irradiated (4 Gy) and analyzed by microelectrophoresis as described in the Materials and methods section. Data are means and SEM of three independent experiments.

3.4.5. Apoptosis

In order to determine whether the EGFR inhibitory activity of ZRBA1 combined with radiation-induced DNA damage, translates into high levels of apoptosis, an annexin V

binding assay was performed with cells exposed to radiation and ZRBA1 alone or in combination. As it is shown in figure 3-7, each treatment (radiation or drug alone) increased the levels of apoptosis particularly at 6 and 48 h post treatment. Although the combination of drug and radiation seemed to have higher apoptosis level compared to each treatment alone, the p-value did not reach the statistical significance.

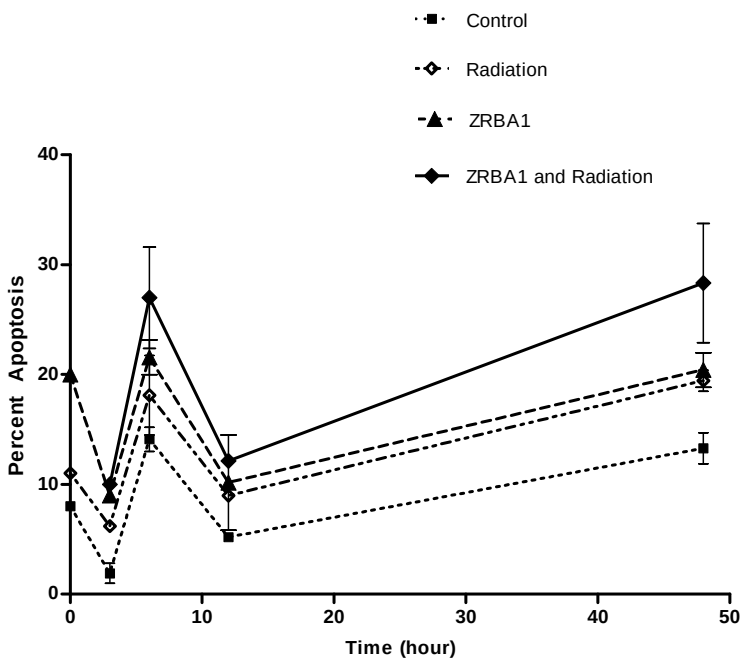
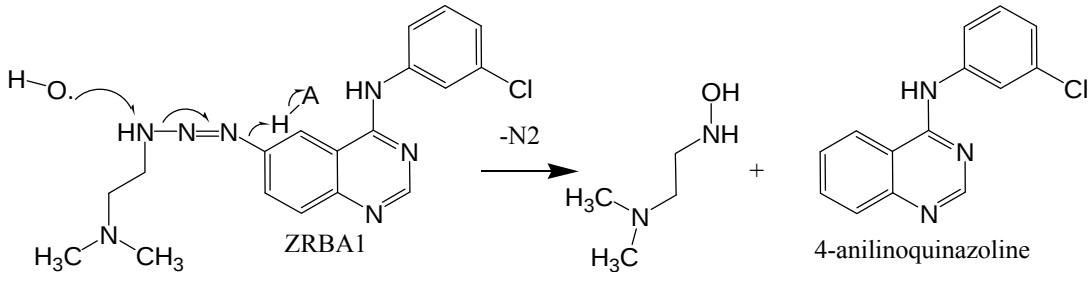


Figure 3-7. Time course analysis of apoptosis induced by ZRBA1 or radiation and corresponding combination in MDA-MB-468.

Cells were treated with ZRBA1 (50 μM) alone and in combination with radiation (4 Gy) and were harvested at the indicated time points. There are two peaks of apoptosis at 6 and 48 h post treatment. Presented data are means and SEM of three independent experiments. (At 6 h post treatment: control vs. ZRBA1, $p<0.017$; control vs. radiation, $p>0.05$; control vs. radiation and ZRBA1, $p\leq0.05$; ZRBA1 vs. radiation and ZRBA1, $p>0.05$. AT 48 h post treatment: control vs. ZRBA1, $p<0.028$; control vs. radiation, $p<0.025$; control vs. radiation and ZRBA1, $p<0.046$; ZRBA1 vs. radiation and ZRBA1, $p>0.05$).

3.4.6. ZRBA1 Biodistribution

The combi-molecule ZRBA1 is known to decompose into methyldiazonium that damages DNA, FD105 that blocks EGFR TK. Since FD105 fluoresces blue, its subcellular distribution could be characterized by fluorescence microscopy. Ionizing radiation being a radical generator, this experiment was designed to verify whether it affected the chemical decomposition of ZRBA1, thereby altering its cellular distribution. As outlined in Scheme 2, it was assumed that the hydroxyl radical generated by radiation could damage the triazene chain, thereby leading to a non-DNA alkylating moiety and the barely fluorescent unsubstituted 4-anilinoquinazoline. The results showed that the levels and localization of fluorescence intensity generated by the combi-molecule in the absence or presence of radiation were identical, suggesting that ionizing radiation does not affect the chemistry nor the localization of the drug in the cells (figure 3-8).



Scheme 3-2. A potential degradation pathway of ZRBA1 by radiation generated hydroxyl radical.

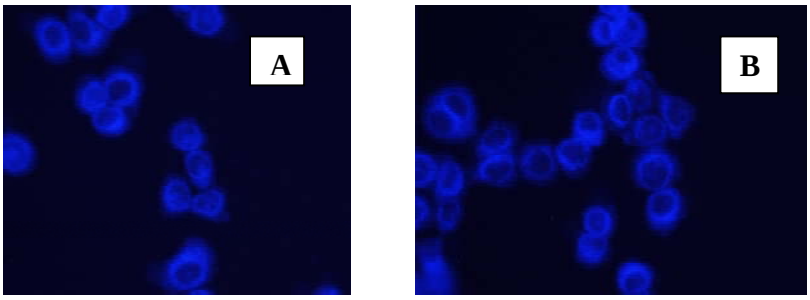


Figure 3-8. Analysis of blue fluorescence generated by ZRBA1 in the intracellular compartment by fluorescence microscopy.
A) Cells treated with ZRBA1 alone. B) Cells treated with ZRBA1and radiation (4 Gy).

3.5. DISCUSSION

The overexpression of EGFR is associated with induction of DNA repair enzymes that reverse lesions induced by cytotoxic DNA damaging agents. We have previously shown that ZRBA1 was capable of downregulating EGFR-mediated signalling, damaging DNA and inducing significant levels of apoptosis in MDA-MB-468 cells [42]. On the other hand, radiation has been shown to potentiate the action of temozolomide, an alkylating drug of the triazene class capable of inducing DNA alkylation in a manner similar to ZRBA1. Thus, it would be of interest to investigate the efficacy of the combination of ZRBA1 that can block EGFR, damage DNA by alkylation, with ionizing radiation, a DNA double strand break inducer. It is thus surmised that due to the mixed EGFR/DNA targeting potency of ZRBA1, it could behave as an efficacious radiopotentiator. Here it was shown that the combination of

ZRBA1 with radiation has a greater efficacy against MDA-MB-468 cells in comparison to single modality treatment. More importantly, the effect was sequence-dependent. ZRBA1 must be administered prior to or concurrent with radiation for greater efficacy to be observed. While the molecular mechanisms underlying the significance of these sequences remained to be elucidated, the results can be analyzed on the basis of a cell cycle rationale. We have shown herein that, ZRBA1 is capable of inducing significant cell cycle arrest in G2/M 24 h post-treatment with 40% of cells being blocked in G2/M and it is common knowledge that G2/M cells are exquisitely sensitive to radiation [265]. Therefore when radiation was added to the G2/M population 24 h post-treatment with ZRBA1, enhanced cell killing was observed. G2 being a phase of the cell cycle wherein the final DNA repair processes are triggered, inflicting double DNA strand breaks with ionizing radiation may further delay the repair, thereby committing the cells to apoptosis and death. It was also shown here that ZRBA1 induces double strand breaks approximately two times more than Iressa or FD105 and when it is combined with radiation the double strand break induction seems also to be higher than the combination of radiation with Iressa or FD105; however, the p value was not statistically significant ($p>0.05$). Furthermore, the ability of ZRBA1 to downregulate EGFR mediated signalling, as previously reported, may contribute to the enhancement of cell death observed at cytotoxic concentration (although here the observed apoptosis was not statically different when comparing single modality treatment vs. combined treatment) The results showed that the level of apoptosis induced by ZRBA1 appears to be higher than apoptosis induced by radiation alone. It is speculated that this finding is due to the significant potency of ZRBA1. This will be further clarified by studying the proportion of induced apoptosis while altering the sequence of drug administration when

combined with ionizing radiation. Downregulation of EGFR is associated with that of the AKT pathway that is known to activate the anti-apoptotic signaling. Another possibility is that the observed cell killing or the decreased colony survival in the data could be due to other modes of cell death such as mitotic catastrophe (MC).

Triazene molecules are extremely sensitive to radicals that can cleave the triazene linkage. Therefore, ZRBA1 being a triazene, was tested whether its combination with radiation known to induce the formation of hydroxyl radical would affect intracellular decomposition. Combi-molecules of the same class as ZRBA1 are known to decompose in the cells into a fluorescent aminoquinazoline (e.g. FD105). Thus, it was sought to verify whether the ZRBA1 decomposition would be affected by radiation. It appeared that the distribution of ZRBA1 in the presence or absence of radiation was identical, indicating that ionizing radiation had no effect on the chemical decomposition nor the biodistribution of ZRBA1, a debility that could affect its development as a radiomodulator. This is further corroborated by the fact that, as shown in this study, the combination increases the levels of DNA lesions incurred by the cells in the presence of radiation.

In summary, this study conclusively demonstrated that combi-molecule of the type of ZRBA1 can be used to enhance radiation-induced cytotoxicity in a sequence dependent fashion by increasing the levels of DNA damage and cell cycle arrest in G2/M. These results set premise for further investigation on the molecular mechanism underlying the observed effect and the demonstration of the efficacy of this novel type of combination in *vivo*.

ACKNOWLEDGMENT

We thank Canadian Association for Radiation Oncology (CARO) for their financial Support (RAZCER 2007 Grant). BJC thanks CIHR FR49440 for supporting the work on the chemistry of ZRBA1. We also thank Margarita Todorova for her technical assistance.

CONNECTING TEXT 3

In the previous chapter we have shown that ZRBA1 potentiates radiation response in the breast cancer cell MDA-MB-468. We have concluded that the observed anti-tumour effect was the result of the arrest in cell cycle progression and due to the increased DNA damage.

In chapter 4, we are investigating the *in vivo* effect of ZRBA1 in the TN breast cancer model. Furthermore, we are exploring the mechanism of action of combined treatment of ZRBA1 and radiation by measuring the levels of SSBs and DSBs at multiple time points, measuring the level of γH2AX formation and the rate of its disappearance and finally protein analysis on MAPK proteins as well as PARP cleavage.

CHAPTER 4

ZRBA1 a mixed EGFR/ DNA targeting molecule potentiates radiation response through delayed DNA damage repair process in a triple negative breast cancer model

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4.1. ABSTRACT

ZRBA1 is a combi-molecule designed to induce DNA alkylating lesions and to block EGFR TK domain. Since ZRBA1 downregulates the EGFR TK-mediated anti-survival signaling and induces DNA damage, it has a potential to be a radio sensitizer. The aim of this study was to further investigate the potentiating effect of ZRBA1 in combination with radiation (both *in vitro* and *in vivo*) and to elucidate the possible mechanisms of interaction of these two treatment modalities. The triple negative human breast MDA-MB-468 cancer cell line and mouse mammary cancer 4T1 cell line were used in this study. Clonogenic assay, Western blot analysis as well as DNA damage analysis were performed at multiple time points post treatment. To confirm our *in vitro* findings, *in vivo* tumour growth delay assay was also performed.

Our results show that combination of ZRBA1 and radiation: 1) increases the radiation sensitivity of both cell lines significantly with a Dose enhancement factor (DEF) of 1.56. 2) induces significant amount of DNA single and double strand breaks . 3) prolongs duration of DNA damage up to 24 h post treatment and 4) significantly, increases tumour growth delay in a syngeneic mouse model by 21 days. These data suggest that the higher efficacy of this combination could be partially due to increased DNA damage and delayed repair process as well as the inhibition of EGFR. The encouraging results of this proposed combination demonstrated potential applications of this promising class of agents in a pre-clinical as well as in clinical setting.

4.2. INTRODUCTION

Postoperative radiotherapy is a key local treatment in breast cancer. It has been proved with high level of evidence that it decreases local relapse and improves survival of patients [215-216]. Moreover, in different solid tumours, the overall survival rate of patients improves when radiation therapy is combined with chemotherapy [216-217]. However, secondary cancers, skeletal complications, radiation-induced heart and lung disease are potential side effects of radiation therapy [218-220]. Due to the toxicity of radiation, recent efforts have been placed on improving its cancer cell specificity through developing agents that sensitizes cancer cells to radiation or protect normal cells from damage induced by radiation [216, 220-221]. Even though the combination of ionizing radiation with chemotherapy has shown significant improvement in local control, organ preservation and survival for locally advanced solid tumours, this strategy is limited by the toxicity resulting from both single therapies and their combination. Therefore, targeting specific features of tumour cells should provide an advantage over conventional therapy in which the major drawback is normal tissue toxicity [222].

The mechanism by which radiation eliminates the cells involves induction of multiple types of DNA damage in cells. Thus, adding such agents that form additional DNA damage or inhibit or delay the DNA repair process in tumour cells would improve the efficiency of radiation as well as the therapeutic ratio. Moreover, the acquired resistance to DNA damaging agents represents a major obstacle in the therapy of many tumours including lung, breast, ovarian and brain carcinomas [245-246, 266].

With the advancement of molecular biology, various novel markers associated with reduced sensitivity to DNA damaging agents have been identified including signalling

proteins such as AKT and several receptor tyrosine kinases (RTKs) which activate anti-apoptotic signalling [245, 248, 266]. One of these receptors is the epidermal growth factor receptor (EGFR), which could be activated by chemical and radiation-induced DNA damage [267-268]. Importantly, overexpression of EGFR is associated with aggressive tumour progression, invasion and reduced sensitivity to chemotherapy [251-252, 269-271].

It has been shown that targeting more than one characteristics/defects in a tumour cell could result in a better tumour control and treatment response. A better treatment response can also be achieved by combining different drugs although the main drawbacks would be increased toxicity. Other major obstacles of combining different compounds are the difficulty of conducting clinical trials combining two investigational drugs due to the necessity of sharing the intellectual property between the companies, more complicated design since the dosage of two modality need to be determined and also an unforeseen side effects from the combination therapy [272].

We have developed a binary targeting molecule "ZRBA1" to block TK domain of EGFR that also induces DNA breaks. The superior activity of ZRBA1 has already been shown in comparison to similar investigational or clinical EGFR inhibitors *in vitro* and *in vivo* [273]. However, the activity of such molecule can be limited by DNA repair mechanisms. Our previous publication has shown that ZRBA1 combined with ionizing radiation has higher potency *in vitro* compared to radiation or ZRBA1 alone. ZRBA1 is designed to induce N7- and O6-alkylated lesions in a manner similar to the clinical drug temozolomide. In addition, it has already been shown in multiple models that Temozolomide is able to potentiate the radiation response [210]. Temozolomide enhancement of radiation response is thought to be due to its ability to increase the degree of

radiation-induced DNA double strand breaks in the cells [261]. Since ZRBA1, induces DNA alkylating lesions of the similar type as temozolomide, we hypothesized that its combination with radiation will increase the levels of DNA strand breaks and delay or complicate the DNA repair process.

Here we aimed to further elucidate the mechanism of combination of ionizing radiation and ZRBA1 in our breast cancer model *in vitro* and *in vivo*.

4.3. MATERIALS AND METHODS

4.3.1. Reagents

The cell culture reagents were obtained from Gibco®, Invitrogen, Canada. Fetal bovine serum (FBS) was purchased from Wisent Inc., Canada. ZRBA1 was synthesized according to the previously described methods [274] and was reconstituted in DMSO for *in vitro* use and in ethanol / Cremephore L (Sigma Aldrich, Canada) (50:50) for *in vivo* use at 4x concentration. The 4x solution of ZRBA1 was prepared freshly every time. The final dosing solution was prepared by diluting the 4x solution to 1x in sterile water (Gibco®, Invitrogen, Canada) prior to its administration to the animals. The concentrations of DMSO were kept lower than 0.2 % in all *in vitro* experiments.

4.3.2. Cell Culture

Human MDA-MB-468 breast carcinoma cells were obtained from the National Cancer Institute, Bethesda, MD. Highly metastatic mouse mammary cancer cell line, 4T1, was a

generous gift from Dr Fred Miller, Karmanos Cancer Institute, Wayne State University, MI, USA. Cells were cultured in DMEM supplemented with 10 % FBS, Penicillin-Streptomycin 1% and kept at 37°C in 95 % air/5 % CO_2.

4.3.3. Irradiation

Irradiation for *in vitro* and *in vivo* experiments was carried out at room temperature using Theratron T-780 ^{60}Co irradiator (MDS Nordion, Kanata, ON, Canada). Dose delivered to each experimental setup used in this work was verified by radiochromic film dosimetry protocol developed by Tomic et al. [235].

4.3.4. Colony-Forming Assay

Cells were plated at specific cell numbers in 6-well plates. They were treated with ZRBA1 alone (18 and 22 μM in MDA-MB-468 cells and 60 and 75 μM in 4T1) for 2 h and in combination with radiation (2, 4 and 6 or 8 Gy) [273]. After 6-10 days of incubation the colonies were fixed and stained with methylene blue. Only colonies containing more than 50 cells were counted. Plating efficiency (PE) was calculated by dividing the number of colonies formed in the untreated control plates by the number of cells plated. Survival fractions (SF) were calculated by counting the number of colonies for each specific radiation dose and dividing by the number of cells seeded at the same dose multiplied by PE. In order to plot the survival curve, the survival fractions were normalized according to the controls (non-irradiated). Radiosensitivity was measured by determining the dose enhancement

factor (DEF), which is the ratio of the radiation doses at survival fraction of 0.1 or 0.01 of non-drug treated cells to drug treated cells [210, 237].

4.3.5. Western Blot analysis

The cells were incubated in two sets of 6-well plates with serum-free media for 18 h and were subsequently exposed to the 5, 10 μM (MDA-MB-468 cells) and 70 μM (4T1 cells) of ZRBA1 and 4 Gy of radiation respectively for 2 h. To test if ZRBA1 is still effective in case of over-activation of RTKs such as EGFR, one set of the plates (MDA-MB-468 cells) were subsequently treated with 25 ng/ml of EGF for 20 min and cells were harvested within 30 min after which the whole cell lysates were prepared. Twenty μg of protein was loaded onto Bis-Tris gradient gels (Invitrogen, Carlsbad, CA). Western blot analysis was performed using antibodies against p-Tyr, EGFR, p-ERK1,2, ERK1,2, p-Akt, Akt and p-Bad, Bad as well as cleaved PARP and tubulin (Cell signaling Technology Inc., Beverly, MA).

4.3.6. DNA damage analysis (Comet assay)

The alkaline and modified neutral comet assays were performed as described previously [263] . The MDA-MB-468 and 4T1 cells were exposed to a dose of (22 μM and 70 μM respectively) of ZRBA1 for 2 h, irradiated at 4 Gy, harvested and re-suspended in PBS. Cell suspensions were diluted to approximately 10^6 cells and mixed with agarose (1%) in PBS at 37 ºC in a 1:10 dilution. The gels were casted on Gelbond strips (Mandel Scientific, Canada) using gel-casting chambers and then immediately placed into an alkaline or neutral buffer lysis buffer according to the previous published protocols [263]. Thereafter, the gels were submerged in fresh running buffer in an electrophoresis chamber and ran with 20V for 20

min. They were subsequently rinsed with distilled water, dried with 100% ethanol overnight, and stained with SYBR Gold (1/10,000 dilution in distilled H_2O, supplied from Molecular Probes, Eugene, OR) for 1 hour. Comets were visualized at 330X magnification and DNA damage was quantified 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 or comets were analyzed for each sample, using the comet assay IV imaging software package (Perceptive Instrument, UK).

4.3.7. DNA Damage analysis (Flow cytometry)

MDA-MB-468 cells were treated with ZRBA1 and /or radiation as before and trypsinized at 1 and 24 h post-treatment. They were washed with PBS, fixed with 1% formaldehyde and permeabilized with 0.2 % Triton-X. After washing, cells were incubated with anti-γH2AX (1:100) for 1 h at room temperature followed by addition of the FITC conjugated secondary antibody. After incubation with the secondary antibody cells were washed and re-suspended in PBS and were analyzed by BD FACS Caliber (BD Biosciences).

4.3.8. DNA Damage analysis (Immunofluorescent staining)

MDA-MB-468 cells were grown on chamber slides and were treated as mentioned before. They were fixed with 2% formaldehyde and permeablized by 2% triton-X for 10 min at room temperature. After washing steps, anti-γH2AX was added to the chambers (1:200) for 1 h and subsequently FITC conjugated secondary antibody was added (1:500). The cells

were also counter stained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride), cover slips were placed and the slides were analyzed by Fluorescent microscope.

4.3.9. *In vivo* Tumour Model

Six to eight-week-old female BALB/c mice (Charles River Laboratories, Montréal, Canada) were used in this study. Mice were caged in groups of five or less. 4T1 tumour cells (1×10^6 cells) were injected subcutaneously (SC) into the right hind leg. All protocols were approved by the McGill University Animal Care Committee following the guidelines of the Canadian Council on Animal Care.

4.3.10. Tumour Growth Delay Assay

When tumours reached a mean volume of 100 mm^3, mice were randomized into four groups: vehicle, ZRBA1 alone, irradiation (3 x 5 Gy) alone and ZRBA1 plus irradiation. A single dose of ZRBA1 (75 mg/kg) was administered by gavage for 9 days every other day. The mice were irradiated locally with the total dose of 15 Gy in 3 fractions (3x 5 Gy) on day 6, 7 and 8 of treatment. To obtain tumour growth curves, perpendicular diameter measurements of each tumour were made every 2-3 days with digital callipers, and volumes were calculated using the formula $(L \times W^2)/2$. Tumours were followed until the mean tumour volume reached ~ 1,500 mm^3 after which animals were sacrificed. Relative tumour volume was calculated by dividing each individual animal's tumour volume by the mean tumour volume of the same group. Each experimental group contained 5 mice.

4.3.11. Statistical Analysis

The effects of various treatments in all experiments were compared using two-tailed *t* test (GraphPad prism 5, GraphPad software, Inc, Ca, USA). Differences with a p value < 0.05 were considered statistically significant. The data presented represent means and SEM from multiple independent experiments except otherwise is stated.

4.4. RESULTS

4.4.1. ZRBA1 increases the sensitivity of MDA-MB-468 and 4T1 cells to radiation *in vitro*

To evaluate whether ZRBA1 can decrease the ability of cancer cells to form colonies *in vitro*, clonogenic assays were performed. As it is shown in figure 1a and 1b, the dose enhancement factor (DEF) was as high as 1.56 and 1.50 when ZRBA1 was combined with radiation in MDA-MB-468 (18 μM) and 4T1 (60 μM) respectively. As demonstrated in figure 4-1, MDA-MB-468 cells generally show more sensitivity towards radiation and ZRBA1 compared to 4T1 murine cell line *in vitro*.

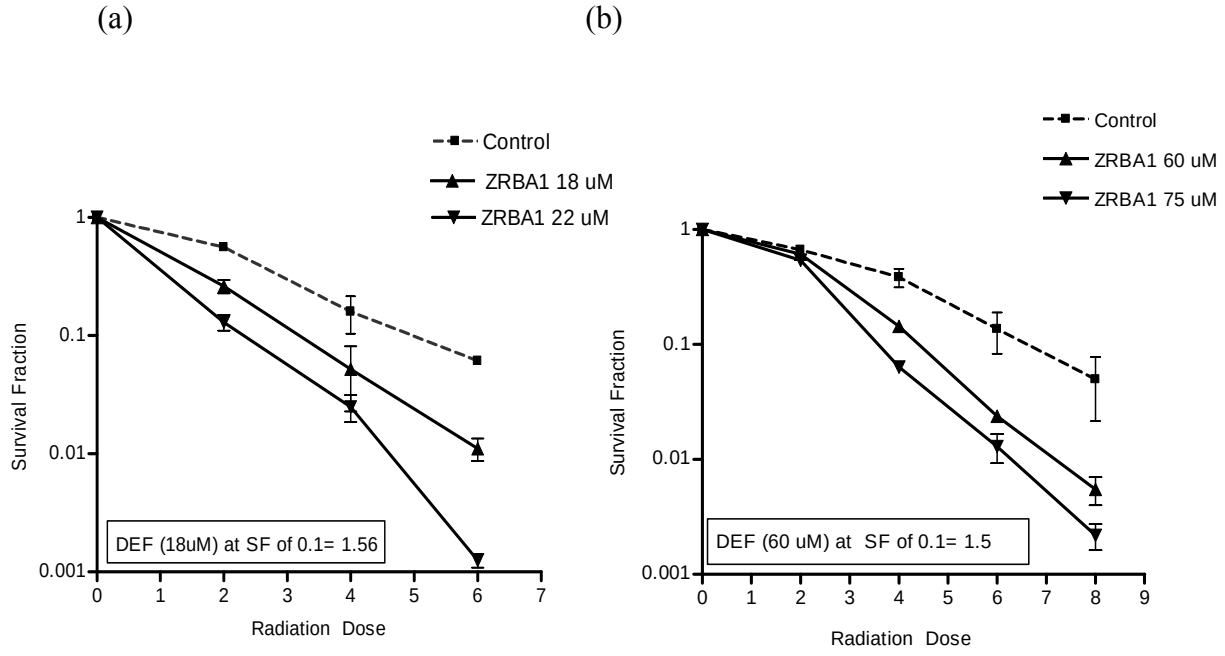


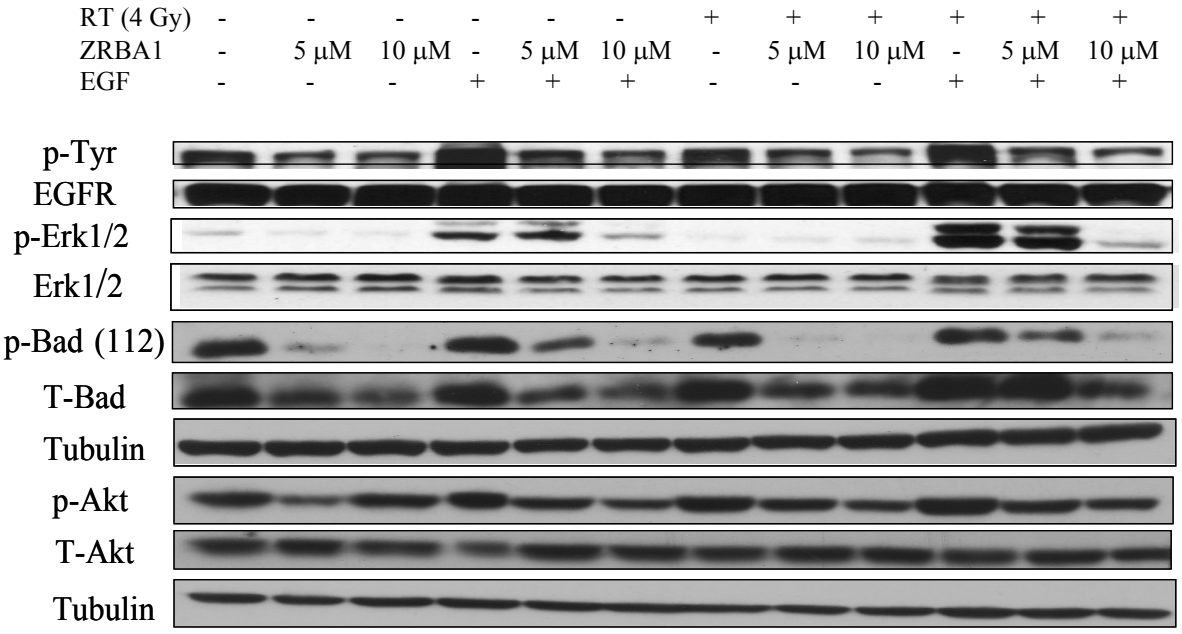
Figure 4-1. Radio modulating effect of ZRBA1 in MDA-MB-468 and 4T1 cells.

(a) Analysis of MDA-MB-468 cells response to the combination of ZRBA1 and radiation using a clonogenic assay. Cells were treated with ZRBA1 (18, and 22 μM) with or without radiation (4 Gy). (b) Clonogenic assay using4T1 cells with ZRBA1 (60, and 75 μM) with or without radiation. Data represent means and SD from three independent experiments.

4.4.2. ZRBA1 with/out radiation inhibits MAPK pathway and induces apoptosis

To evaluate the inhibitory activity of ZRBA1with radiation in our breast cancer model, western blot analysis was performed. As shown in figure 4-2a, ZRBA1 inhibited the phosphorylation of EGFR, ERK1, 2 and also Bad and AKT in a dose dependent manner downstream of EGFR even in the case of radiation induced activity of EGFR. Furthermore, as shown in figure 4-2b and c, ZRBA1 enhanced apoptosis as a relatively late respond after 48 h in MDA-MB-48 cells while 4T1 cells already gone under apoptosis after 24 h.

(a)



(b)

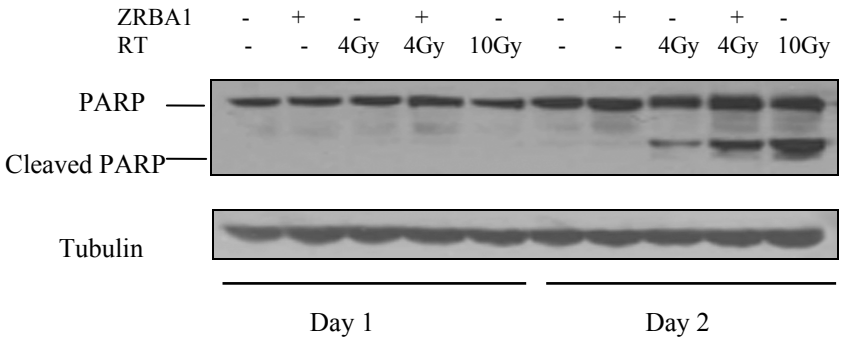
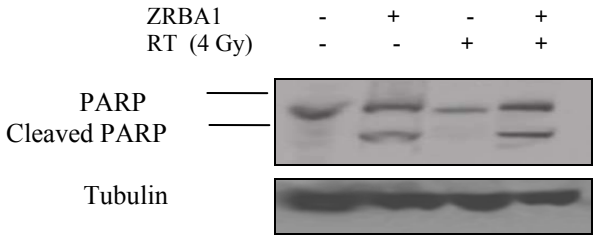


Figure 4-2. Western blot analysis.

(a and b) MDA-MB-468 cells were grown to 80% confluency in 6 well-plates. They were serum starved for 18 h and were treated with ZRBA1 or/and radiation as it is indicated. Half of the plates were stimulated with EGF and whole cell lysate were prepared within one hour and at different time point (day 1 and day 2).

(c)



(c) 4T1 cells were grown to 70% confluency and were treated with ZRBA1 (60 μM) or/and radiation. Whole cell lysate were prepared 24 hr after the treatment completion.

4.4.3. ZRBA1 combined with radiation induces DNA double and single strand breaks

In order to examine the effect of ZRBA1 and/or radiation on the induction of DNA damage, Neutral and Alkaline comet assay were performed to measure single strand and double strand DNA breaks respectively. The cells were lysed 1 h after the completion of treatment. As it is shown in figure 4-3 (a and b), cells treated with ZRBA1 or radiation alone have 2 folds more DNA double strand breaks compared to the non-treated cells both in MDA-MB-

468 and 4T1 cells. Interestingly, the combined treatment with ZRBA1 and radiation induced almost double number of breaks up to 2 to 4 folds higher breaks as compared to the non-treated cells in both cell lines.

The alkaline comet assay showed the similar results as neutral assay. MDA-MB-468 cells treated with both ZRBA1 and radiation had significant higher single strand breaks compared to ZRBA1 or radiation alone treated cells up to six times higher than non-treated cells.

The effect of two treatment modalities on the MDA-MB-468 cells were also demonstrated by Immunofluorescent staining for γH2AX as a marker for induction and repair of double strand breaks. As it has been shown in figure 3, cells exposed to ZRBA1 and radiation both show more γH2AX foci. When cells were exposed to the combined treatment the number of the foci and also the numbers of cells that express the foci were increased.

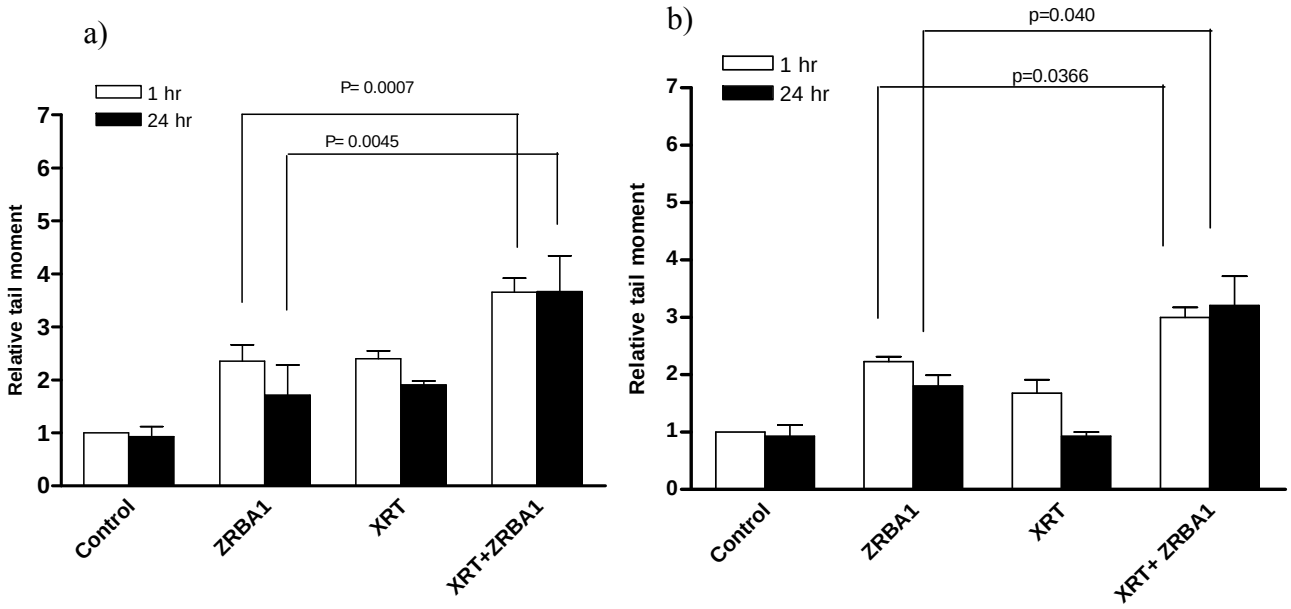
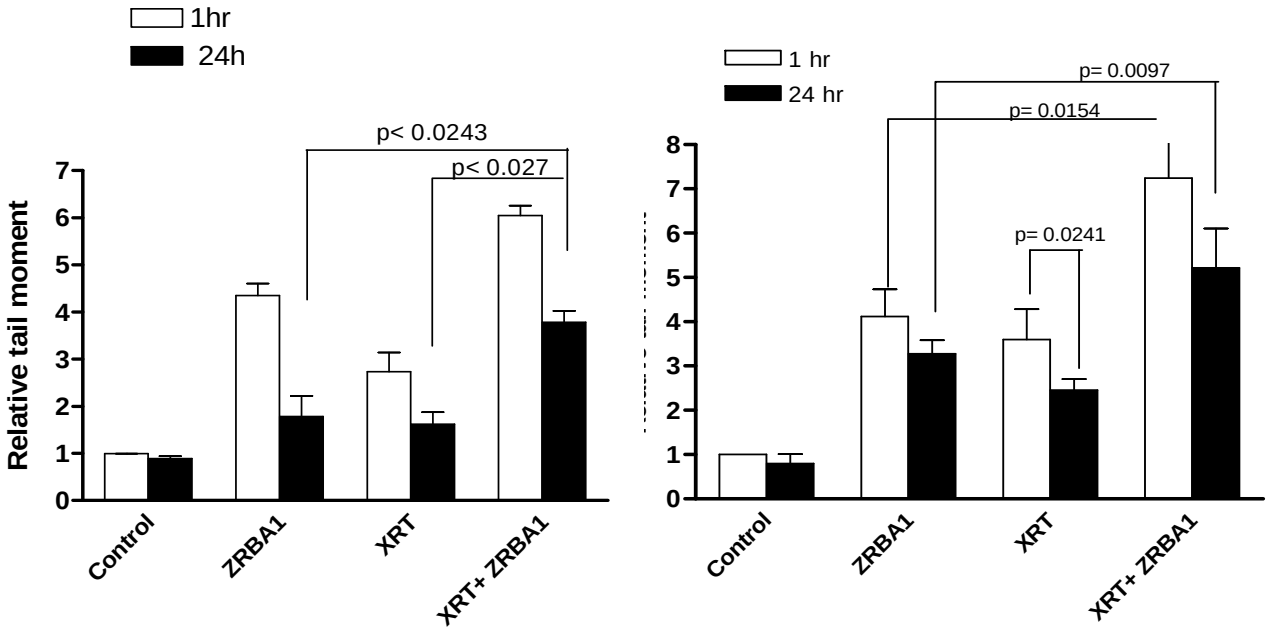


Figure 4-3. DNA damage analysis.

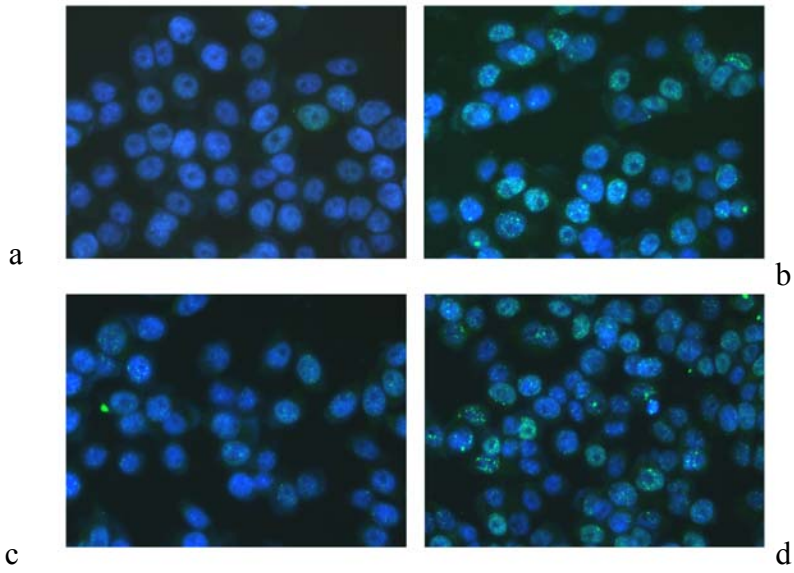
a and b) Double strand breaks induced by ZRBA1 or radiation and combination of both as determined by neutral comet assays. a) MDA-MB-468 and b) 4T1 Cells were treated with ZRBA1 for 2 h, irradiated (4 Gy) and analyzed by microelectrophoresis as described in the Materials and methods section. Data are means and SD of three independent experiments.

(d)

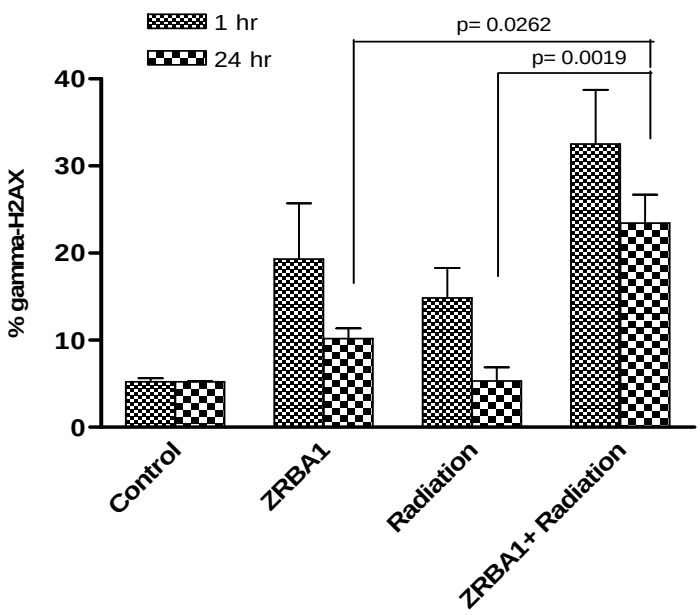


c and d) Single and double strand breaks induced by ZRBA1 or radiation and combination of both as determined by alkaline comet assays in c) MDA-MB-468 and d) 4T1 Cells.

(e)



(f)



e) Immunofluorescence staining of MDA-MB-468 cells treated with ZRBA1 and/ or radiation showing the γH2AX foci. f) Flow cytometry analysis showing the induced γH2AX percentage

in MDA-MB-468 cells being treated with ZRBA1or/and radiation at 1 and 24 h post-treatment.

4.4.4. Combination of ZRBA1 and radiation also delays the DNA repair process

In order to study the DNA repair process alkaline and neutral assays were also performed at 24 h post-treatment. Interestingly, we were able to show that the higher levels of DNA double strands were remained at high even after 24 h when cells were treated with both ZRBA1 and radiation.

This effect was more prominent in the neural comet assay showing double strand breaks. However, there was a significant difference between the DNA breaks of combined treated cells at 24 h post-treatment vs. cells treated with either ZRBA1 or radiation in both neutral and alkaline conditions.

To confirm our comet assay data, flow cytometry analysis was also performed to quantitatively measure the level of γH2AX induction at 1 vs. 24 h post-treatment. As it is demonstrated in figure 4-3b, the intensity of γH2AX formation is not only higher in the combines treated group compared to the ZRBA1 or radiation alone but also remains almost at the same level after 24 hr.

4.4.5. ZRBA1 increases the tumour growth delay caused by radiation

In order to evaluate and validate our *in vitro* results, we performed *in vivo* experiment with 4T1 mouse mammary cancer cells. As shown in figure 4-4a, the *in vivo* results suggest that ZRBA1 increases the radiation caused tumour growth delay in tumours that were treated with the combination therapy by 21 days. Moreover, ZRBA1 combined with ionizing

radiation has significantly more anti-tumour effect against 4T1 tumours than ZRBA1or radiation alone in BALB/c mice as the tumour free rate was 20% for each single modality treatments and was 60% for the combined treatment (figure 4-4b). Tumour-free rate is expressed as the fraction of the number of the mice without the tumour at 48 days after the treatment out of the total number of mice with the tumour before the treatment in each group.

ZRBA1 had slightly better anti-tumour activity than Iressa (Gefitinib) when it was used alone however, in combination with radiation, ZRBA1 showed a significant efficiency against the 4T1 tumours compared with Iressa (radiation+ ZRBA1 vs. radiation+ Iressa: $p= 0.0461$) (figure. 4c).

(a)

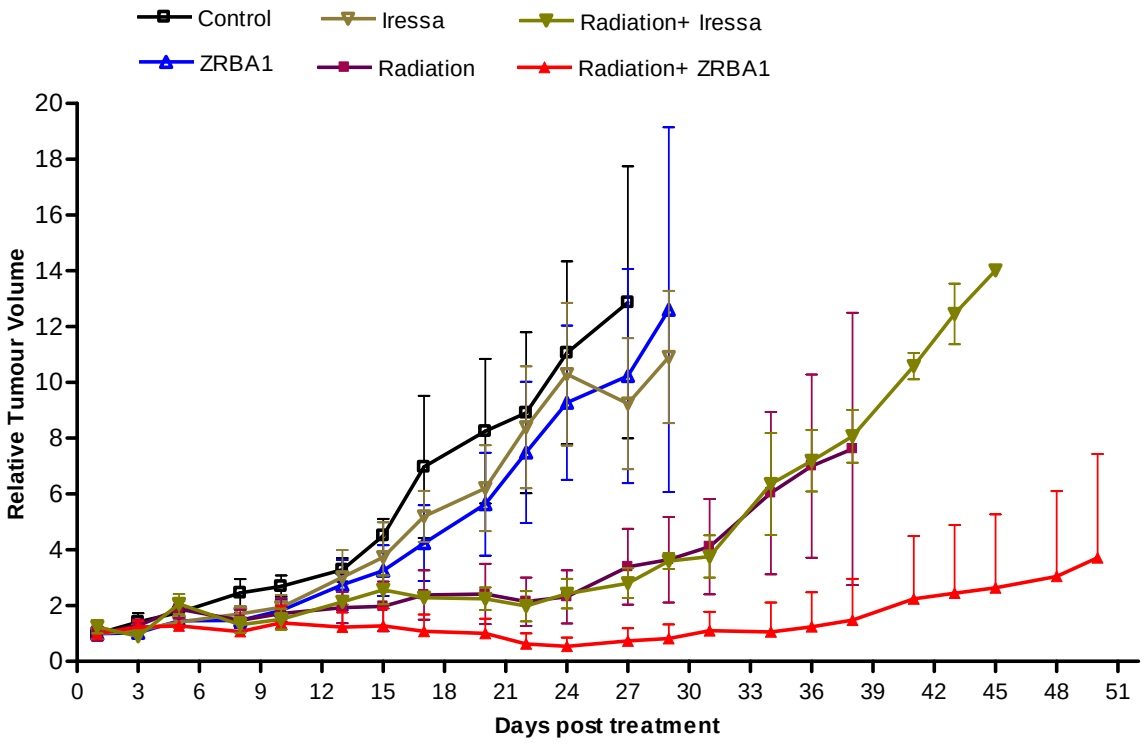
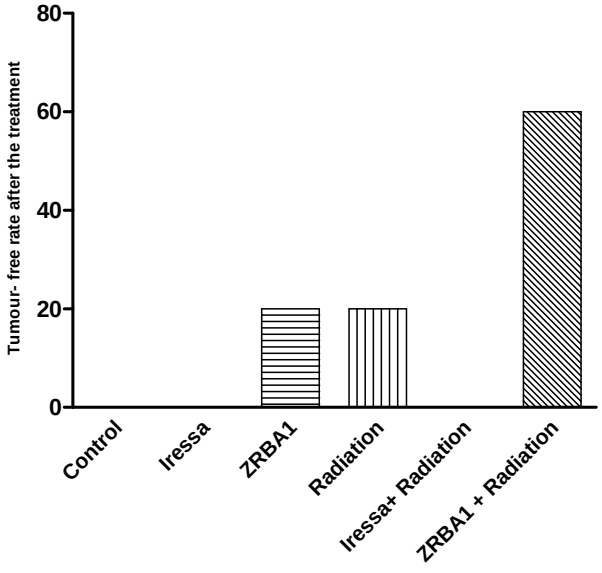


Figure 4-4. Tumour growth delay assay.

a) 4T1 cells were injected into the right hind limb of BALB/c mice. When the mean volume of tumours reached to 100 mm^3, the animals were randomly divided into 6 groups. There were 5 animals per each group. ZRBA1 started to be given to the animals 3 days before radiation, on the same days of irradiation and was continued for 1 day post irradiation When the tumour size reached to maximum of ~1500 mm^3 the mice were euthanized. Tumour volume was calculated using: $(L \times W^2)/2$ and were normalized by dividing the tumour volume of each animal in treatment groups by the mean tumour volume of the same group. Error bars, SEM.

(b)



(b) Tumour free rate after the treatment.

Moreover, the tumour growth delay was increased from 11.5 days in control group to 14, 13 days in ZRBA1, Iressa alone and 26 days in radiation alone groups respectively. The growth delay, in case of combination of ZRBA1 and Iressa, was increased to 47 and 27.5 days. There were no significant body weight loss resulted from any of the treatments and treatments were well tolerated by the end of experimental end point (figure 4-4 d).

Table 4-1. Tumour growth delay of each treatment group. Mice which received the combined treatment had a growth delay almost twice and three times more than the irradiated only and ZRBA1 only treated groups respectively (47 vs. 26 days and 14 days).

Treatment group	Day after treatment
Control	11.5
Iressa	13
ZRBA1	14
Radiation	26
Radiation + Iressa	27.5
Radiation + ZRBA1	47

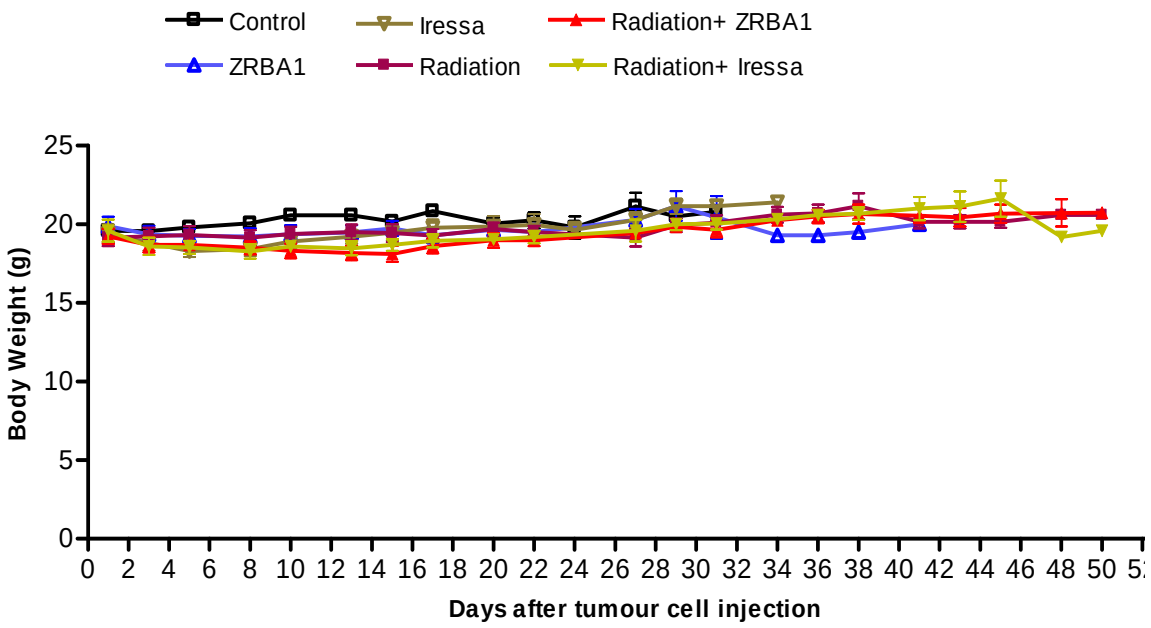
Table 4-2. p-values of multiple experimental groups at day 27, 29 and 31st after the completion of treatment. Combination of ZRBA1 and radiation in all of the cases is significantly more effective compared to the radiation or ZRBA1 or Iressa treated groups.

Day 27	
Treatment group	P- value
Radiation+ ZRBA1 vs. radiation + Iressa	0.0033
Radiation + ZRBA1 vs. radiation	0.0492
Radiation + Iressa vs. radiation	0.3165
Radiation + ZRBA1 vs. ZRBA1	0.0375
Radiation + ZRBA1 vs. control	0.0153
Radiation + Iressa vs. control	0.0537
Radiation + Iressa vs. Iressa	0.0299
Radiation + Iressa vs. radiation	0.6987

Day 29	
Treatment group	P- value
Radiation+ ZRBA1 vs. radiation + Iressa	0.0075
Radiation + ZRBA1 vs. radiation	0.0486
Radiation + Iressa vs. radiation	0.9823

Day 31	
Treatment group	P- value
Radiation+ ZRBA1 vs. radiation + Iressa	0.0461
Radiation + ZRBA1 vs. radiation	0.0456
Radiation + Iressa vs. radiation	0.4888

(c)



(c) Variations of body weight of mice treated with ZRBA1 or Iressa (75mg/kg) and radiation alone and the combined treatment. Error bars, SEM.

4.5. DISCUSSION

ZRBA1 is designed to induce N7- and O6-alkylated lesions in a manner similar to temozolomide, which is effective in tumours that do not express AGT. It has been shown that radiation-induced lesions potentiated the action of temozolomide in the latter type of tumours [210, 273]. Temozolomide enhancement of radiation response is thought to be due to its ability to increase the degree of radiation-induced DNA double strand breaks in the cells [210, 261] . As ZRBA1, is designed to induces DNA alkylating lesions of the same type as temozolomide, if combined with radiation, we surmised that not only the level of DNA strand breaks will increases but also induces different types of DNA damage. In addition, ZRBA1 has an advantage over temozolomide as it contains a polar N, Ndimethylaminoethyl group on the alkylating moiety. Therefore ZRBA1 forms N,N-dimethylaminoethylguanine adducts which are poor substrates for the AGT compared with temozolomide induced O6-methylguanine. This would result in delayed or complicated DNA repair process even in AGT positive cells and therefore makes ZRBA1 an interesting compound to study further. Furthermore, based on ZRBA1 ability to downregulate EGFR TK activity and its subsequent downstream effect on apoptosis and DNA repair, we proposed that the combination of ZRBA1 with radiation may translate into significant improvement in cancer cell-killing.

In this study we have shown that ZRBA1 potentiates the radiation response in breast cancer model *in vitro* and *in vivo*. The mechanism involved in this optimized combination could be partially explained by the induction of DNA damage (both single and double strands) and also inhibition of MAPK pathway through the inhibition of EGFR and the downstream pathway that both lead to the higher cell death mainly apoptosis in this case.

As it is demonstrated in current study and also in our previous publication [273], we have confirmed that apoptosis is the main mode of cell death when cells were treated with both ZRBA1 and radiation. Notably, apoptosis was delayed for 2 days in MDA-MB-468 cells. In contrast, in 4T1 cells apoptosis is visible just after 24 hr after the treatment. This could be due to the shorter cell cycle in 4T1 cells.

We had previously shown that the combination of ZRBA1 and radiation arrests the cell cycle at the G2/M phase 24 h after the treatment [273]. Here we are demonstrating that cells undergoing the combined treatment have significantly higher DNA breaks (single and double strands). Importantly, these DNA breaks cannot be repaired by 24 h and there is a significant delay in the repair process. This could partially explain the cell cycle arrest at 24 h and delayed apoptosis detected starting day 2 post-treatment in MDA-MB-468 cells.

Similarly, γH2AX analysis (figure 4-3a and b) showed the highest level of γH2AX induction at 1 hr and also at 24h after the treatment. The IF study (figure 4-3a), interestingly, demonstrated that the combination therapy not only increases the number of the foci but also the majority of cells express these foci at high level in case of combined treatment.

As it is shown in figure 4-3c, while the single strand breaks are reduced/repaired after 24 hr post-treatment (although still higher than any single therapy), the level of double strand breaks stays at the same level at 1 and 24 h. One explanation for this could be that the extensive single strand breaks induced by ZRBA1 were not repaired and as cells progressed through the cell cycle from G1 to S and G2 phase majority of these single strand breaks have transformed into double strand breaks through the replication process. Hence, the high level of double strand breaks is still visible at 24 hr compared to radiated only group which the

breaks tent to be repaired as there is a significant difference at 1 h vs. 24 h (MDA-MB-468 cells, p= 0.0132; 4T1 cells, p= 0.216).

Next, we investigated if the combined treatment would effectively inhibit tumour growth *in vivo*. 4T1 cells were used as a mouse syngeneic cancer model. 4T1 mouse mammary cell line has been in use in various studies and is one of the appropriate models of human breast cancer. Moreover, its molecular characteristics are comparable to the triple negative (TN) breast tumour cells such as MDA-MB-468 [275-276]. 4T1 cells do not express HER2, ER or PR and are highly aggressive mouse mammary cancer cells. As it is presented in the figures, 4T1 cells also show some level of sensitivity towards higher doses of ZRBA1. 4T1 cells do not over-express EGFR and this could be the reason for the lower sensitivity towards ZRBA1. However, as it is shown in figures 3c and d ZRBA1 induces high levels of DNA single and double strand breaks in 4T1 cells similar to MDA-MB 468.

In this study, we applied a fractionated schedule for radiation delivery *in vivo* as it would be more clinically relevant. The *in vivo* data (figure 4-4) showed a significant increase in the tumour growth delay in the mice treated with radiation and ZRBA1 and importantly we observed 60% complete response. As it is shown in figure 4-4a, ZRBA1 or Iressa only treated group did not show any significant sensitivity towards the treatments. The group receiving radiation and combination of radiation and Iressa also did not show any significant difference in terms of tumour growth, suggesting that addition of Iressa did not sensitize the 4T1 cell towards the radiation. As presented in our *in vitro* data, there is a higher level of response towards this novel combination therapy in MDA-MB-468 cells. Therefore, it would be interesting to see the tumour response in MDA-MB-468 human cancer model using nude mice. Also, based on our DNA damage data one could suspect that

ZRBA1 might be able to induce more damage than only strand breaks and this need to be investigated further. The future experiments with this molecule will open the doors to better understand the mechanism of action in details and also to be able to optimize this promising combi-molecule.

With the advancement of cellular and molecular biology, targeting molecules for cancer therapy has moved rapidly in the broad context of oncology therapeutics. Notably, EGFR has been the most studied molecular targeting in oncology drug development over the past decade. Despite all the efforts and rapid progress in developing EGFR therapeutics there are still many patients that do not respond to EGFR inhibitors or eventually will have a disease progression due to intrinsic resistance or acquired to therapy. More recently, new promising drugs have been introduced as multi-targeting agents [277]. Theoretically, these multi-targeting inhibitors would have the advantage over the classical inhibitors as they can also be effective in subtypes of cancer patients with different mutations in the pathway, patient with non homogenous tumours and patients with acquired resistance towards EGFR inhibition therapy.

Although it is crucial to study the tumour specific defects in order to develop new therapeutic approaches but it is even more needed at this time to understand the mechanism by which tumour cells develop resistance towards the existing therapeutics and also towards radiation. This will illuminate new strategies to improve the therapeutic ratio of this promising class of agents and to help the cancer patients more efficiently.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1. GENERAL DISCUSSION AND CONTRIBUTION TO KNOWLEDGE

With advances in the molecular biology of cancer, emerging molecular targeting therapeutics are being developed with the hope of treating a certain population of patients. MAPK cascade represents one of the most frequently-targeted molecular pathways in cancer therapeutics. The MAPK signaling pathway is highly conserved between different eukaryotic cell types [278]. It includes multiple levels of kinase families such as MAP4K, MAP3 K, MAP2K and MAPK.

Upon activation these kinases are phosphorylated, triggering activation of components at the next level (discussed in the first chapter of this thesis). Considerable evidence has been reported in the literature documenting the cross-talk between the components of this cascade, by which certain signals can be transmitted through cooperation of different components of this cascade [278-280].

Given the importance of this cascade in cancer biology and numerous deregulated signals which seem to be associated both with cancer development and its progression, therapeutically targeting this cascade has been at the centre of attention and the subject of intense research.

Targeted treatment modalities have the advantage of inducing less normal tissue toxicity and therefore improving the therapeutic ratio; however, adding an additional type of treatment would increase the efficiency of anti-cancer treatment particularly if the combined modalities have different modes of action [281].

Radiation being one of the main therapeutic options for cancer patients would be a clinically relevant additional component in the combination treatment protocol. However,

there are only a few studies focusing on evaluating the efficiency of the combination of radiation and multi-targeting molecules, probably due to the uncertainties regarding the possible toxicities and concerns that the combination with radiation may negatively affect the therapeutic ratio.

In this thesis, it is demonstrated that combined treatments with radiation and multi-targeting agents can safely be delivered and evaluated. Two multiple-targeting SMTKIs, Sorafenib and ZRBA1 were investigated separately in combination with single and multiple fractions of radiation.

In general, the present thesis has made the following contributions to knowledge advancement:

5.1.1. CONTRIBUTION 1

It has been shown that radiation resistance is partially due to the tumour cell production of angiogenic cytokines, particularly VEGF that protects endothelial cells through survival pathways [281-282]. The aim of this chapter was to investigate whether radiation response in breast cancer cells is enhanced through inhibition of p-VEGFR2/PDGFR-b by Sorafenib and whether the response is time and sequence-dependent.

Sorafenib, being an inhibitor of VEGFR, PDGFR, and Raf kinase, is considered a cytostatic agent, meaning that it has a transient effect and upon removal the remaining viable tumour cells could repopulate and cause recurrence and/or metastasis. According to our results, adding radiation therapy to the treatment protocol against triple negative breast

cancer tumours significantly prolonged the effect of Sorafenib against tumours. In addition, Sorafenib increases the radiation response due to prolonged inhibition of the RTKs and downstream of MAPK pathway.

Initially, in our *in vivo* experiment with Sorafenib, we had hypothesized that irradiation after treatment with Sorafenib would increase the tumour response to the combined treatment. However, our data did not show a significant difference when Sorafenib was given before or concurrently with radiation. Since Sorafenib is also a VEGFR inhibitor, it is thought to normalize the irregular vasculature existing in the surroundings and within the tumours and therefore it provides better tumour oxygenation. It is known that the well-oxygenated tumours respond better not only to chemotherapy but also to radiation therapy. Therefore, we expected to see significant tumour response when they were treated with Sorafenib before the addition of radiation. One explanation for not seeing a significant difference in anti-tumour effect at different schedules could be that Sorafenib needed to be administered for a longer period and so tumour cells would have enough time to normalize the abnormal vasculature.

In summary, in this chapter we have shown that multikinase inhibitors such as Sorafenib could be exploited as radiation enhancers without increasing toxicity. This chapter emphasizes on the fact that timing and treatment scheduling in combination treatment should be considered when preclinical and clinical trials are being designed.

5.1.2. CONTRIBUTION 2

EGFR is often overexpressed in human malignancies and it is associated with the activation of the AKT pathway, leading to anti-apoptotic effects. This also includes the lack of

sensitivity of some tumours to several existing cytotoxic therapies such as radio- and chemotherapies. Therefore, it is of great importance to develop novel anti-cancer drugs effective against EGFR-overexpressing cancer cells.

ZRBA1 is a binary-targeting molecule, which not only blocks EGFR at the TK domain but also induces DNA breaks. However, the binary property of this molecule can be limited by DNA repair mechanisms active in cancer cells. Therefore to further increase the efficacy of treatment, we combined ZRBA1 with ionizing radiation.

Previously it had been shown that ZRBA1 is capable of EGFR inhibition, inducing single strand breaks and also apoptosis in breast cancer cells [42, 199]. In this chapter we have investigated the interaction of ZRBA1 with ionizing radiation against triple negative breast cancer cells *in vitro*. We have demonstrated that ZRBA1 potentiates radiation response *in vitro*. ZRBA1, being a dual targeting molecule, has been designed to inhibit EGFR's TK domain and also to induce DNA lesions by adding alkyl adducts to position O6 and N7 similar to Temozolamide. Temozolamide is an alkylating drug that is the standard of care for gliomas in combination with radiation. Here we have also shown that ZRBA1 is able to increase the level of DNA double strand breaks formed by ionizing radiation. Induced DNA breaks as well as cell cycle arrest at G2/M, which represents the radiosensitive phase of the cell cycle, could explain the superior potency of the combined treatment in MDA-MB-468 cells. Moreover, ZRBA1, being a triazene is extremely sensitive to radicals that can cleave the triazene linkage. Therefore we also tested if its intracellular decomposition might be affected due to hydroxyl radical formed by radiation. As presented

in Chapter 2, ZRBA1 decomposition and bio distribution was not affected by ionizing radiation.

In summary, in this chapter we have demonstrated, *in vitro,* that combi-molecules such as ZRBA1 can be used as radiation enhancers.

5.1.3. CONTRIBUTION 3

ZRBA1 is a molecule with two distinct functions: EGFR inhibitor (cytostatic) and also DNA alkylator (cytotoxic). While the effect of ZRBA1 is not transient due to cytotoxic moiety of ZRBA1, adding radiation therapy increases both the number of DNA breaks and also distinct types of DNA damage leading to significant tumour cell killing and better treatment response. In this chapter, we have further studied the effect of ZRBA1 combined with radiation in triple negative breast cancer models; MDA-MB-468 and 4T1 cells *in vitro* as well as *in vivo*. It is suggested in the literature that one possible reason for resistance towards radiotherapy could be the activation and increase in EGFR phosphorylation in response to radiation through the stress response pathway. However, ZRBA1 is capable of inhibiting EGFR activity as well as ERK1/2, BAD and to some extent AKT activation is also down regulated in a dose-dependent manner even when cells have been irradiated.

In chapter 3 we have demonstrated that the radiation potentiation effect of ZRBA1 is not limited to MDA-MB-468 cells which over-express EGFR. 4T1 mouse mammary cancer cells, also triple negative and highly metastatic, showed sensitivity towards ZRBA1 and radiation combination with a DEF of 1.7. Furthermore, ZRBA1 and radiation-induced single and double strand breaks in both cell lines and, interestingly, the repair process is delayed

even after 24 hr post treatment. This could explain the high level of G2/M arrest and apoptosis seen in MDA-MB-468 breast cancer cells in the previous chapter.

An important part of this chapter focuses on the *in vivo* effect of this combination therapy. Mice treated with combined modalities showed significant tumour growth delay up to 47 days while ZRBA1 mono therapy did not show significant delay in growth rate. Intriguingly, the tumour-free rate in the combined treated group was as high as 60% when mice were followed up to 84 days post treatment. This high response rate could be due to the induced DNA damage of different and more complex types compared to single modality treatment with radiation, ZRBA1 or other alkylating agents such as Temozolomide.

In addition, ZRBA1's activity in MDA-MB-468 cells, which express MGMT [283-284], demonstrates that the effect of this combi-molecule is independent from the MGMT status of the cancer cells. Therefore, it would be of great interest to investigate the effect of this dual targeting agent using glioma models.

In summary, in this chapter we have demonstrated a possible mechanism of interaction of ZRBA1 and radiation. The radiation potentiation effect is due to increased single and double strand breaks, delayed DNA repair process and accumulation of cells at the G2/M phase of cell cycle, which leads to further increase in cell death and ultimately significant tumour growth inhibition. Importantly, we have also shown that ZRBA1 not only exerts radio-potentiating effect *in vitro* but also it is a strong radio-enhancer *in vivo*.

5.1.4. CLAIMS TO ORIGINALITY

To date, there have been only a limited number of studies focusing on the combination of multi-targeting agents with radiation therapy. In this thesis, we have shown that combining this class of agents with radiation therapy in TN breast cancer treatment is a feasible strategy and should be studied further. Ultimately, clinical trials could be designed to evaluate the efficacy of these combinations in the clinic.

We have shown, in this thesis, for the first time that:

1- Multi-targeting agents such as ZRBA1 or Sorafenib can improve radiation response in triple negative breast cancer models *in vitro* and *in vivo.*

2- The data generated during this research demonstrates that the anti-tumour effects of Sorafenib and ZRBA1 are prolonged when they are combined with radiation therapy.

3- This work contributes to the elucidation of the mechanism of interaction of ionizing radiation with multi-targeting agents such as ZRBA1.

4- This thesis provides fundamental evidence to further study combination of multi-targeting molecules with ionizing radiation in preclinical and clinical settings and directly contributes to the demonstration of the potential applications of combination therapy in treating cancer patients especially triple negative breast cancer tumours.

We are at a turning point in radiation oncology with the techniques having been optimized for more precise delivery. Now we need the insight of molecular biology and genetics to further refine radiation targeting and its effectiveness. With the help of molecular biology, anti-tumour therapy can be delivered more specifically to each group of patients by

indentifying the most important targets that can increase the therapeutic ratio when combined with radiation therapy. This could ultimately translate into a specific treatment protocol for individuals based on their sub-type of disease. The development of new approaches and their implementation in clinical practice will again require an integrated effort between clinicians, physicists and biologists.

Radiation oncology will continue to be one of the key modalities in the treatment and management of cancer, as it is a non-invasive killing force that can be focused and enhanced with pharmacological agents. The ultimate goal of radiation oncology for the future is to direct cancer from an acute disease to a chronic disease that can be treated or controlled for a prolonged period of time.

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APPENDIX

Appendix 1. Paper published in Anti-Cancer Drugs: 2012, 23:525–533.

Appendix 2. Paper published in Anti-Cancer Drugs: 2009, 20:659–667.

Sorafenib in combination with ionizing radiation has a greater anti-tumour activity in a breast cancer model

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High expression of vascular endothelial growth factor (VEGF) in patients with breast cancer has been associated with a poor prognosis, indicating that VEGF could be linked to the efficacy of chemotherapy and radiotherapy. It has also been suggested that radiation resistance is partly due to tumour cell production of angiogenic cytokines, particularly VEGF receptor (VEGFR). This evidence indicates that inhibition of VEGFR might enhance the radiation response. Sorafenib tosylate (Bay 54-9085) is an oral, small-molecule multikinase inhibitor of several targets including RAF/MEK/ERK MAP kinase signalling, VEGFR-2, VEGFR-3 and platelet-derived growth factor receptor-beta. Sorafenib has shown clinical efficacy in treating solid tumours such as renal cell and hepatocellular carcinomas. However, strategies are yet to be identified to prolong and maximize the anticancer effect of this multikinase inhibitor. The objective of this study was to determine whether a combination of Sorafenib and radiation will enhance the treatment response *in vitro* and *in vivo*. Radio-modulating effect of Sorafenib was assessed by performing clonogenic assays. In addition, cell cycle analyses as well as annexin-V apoptosis assays were performed 24 and 48 h after treatment, respectively. To confirm our in-vitro results, tumour growth delay assays were performed. Our results showed a strong and supra-additive antitumour effect of radiation combined with Sorafenib *in vitro* (dose enhancement factor of 1.76). The combined therapy demonstrated a strong and significant G2/M cell cycle arrest (combined treatment vs. irradiated alone: $P<0.0008$). Moreover, annexin-V staining showed a significant increase in the level of apoptosis (combined treatment vs. irradiated alone: $P<0.0004$). Study of the syngeneic model demonstrated the superior potency of the Sorafenib combined with radiotherapy. Our results demonstrate that higher antitumour activity can be achieved when radiation and Sorafenib are combined. *Anti-Cancer Drugs* 23:525–533 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Radiation is a mainstay of nonsurgical cancer treatment. Approximately two-thirds of cancer patients receive radiation therapy. During the last decades, radiation therapy has advanced mainly due to technological improvements in radiotherapy planning and delivery methods; however, efforts made towards understanding the biological parameters that affect the overall therapeutic outcome have not achieved the same success. Thus, radiotherapy is delivered without considering the potential differences within and between the tumours. Although an understanding of the biological basis could have a significant impact on clinical radiation oncology, this knowledge could also be exploited to develop new treatment protocols and perhaps novel combined therapies.

Radiotherapy is relatively well tolerated by patients and has been successful in local tumour control [1,2]. However, the overall rate of patient survival improves when radiation therapy is combined with chemotherapy [3]. Notably, secondary cancers, skeletal complications, radiation-induced heart disease and lung disease are the common side effects of radiation therapy [4–6]. Therefore, due to the toxicity of radiation, considerable focus has been placed on improving its cancer cell specificity. This includes the effort to develop agents that sensitize cancer cells to radiation or protect normal cells from damage induced by radiation [2,6,7].

Over the last decade, the combination of ionizing radiation with chemotherapy has led to marked improvement in local control, organ preservation and survival for locally advanced solid tumours. However, this strategy is limited by the toxicity resulting from each respective treatment and their combination. Therefore, targeting tumour-specific defects should provide an advantage over conventional therapy in which the major drawback is normal tissue toxicity [8].

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Sorafenib tosylate (Nexavar, Bay 54-9085) is an oral, small-molecule multikinase inhibitor of several targets including vascular endothelial growth factor receptor-2 (VEGFR-2), VEGFR-3 and platelet-derived growth factor receptor-beta, RAF-1 and BRAF [9]. Sorafenib has shown clinical activity against metastatic renal cell carcinoma and is considered to be a standard second-line therapy for patients with metastatic renal cell carcinoma [10,11]. Moreover, combination therapy with Sorafenib in phase I and II clinical trials has shown some promising results in melanoma patients [12]. Importantly, Sorafenib has been shown to significantly increase the overall survival rate of patients (nearly 3 months) with advanced hepatocellular carcinoma (HCC) [13]. Patients with HCC in hepatitis B-endemic areas may also benefit from single-agent Sorafenib treatment as a phase II clinical trial has shown fairly good efficacy and acceptable tolerability in these patients [14]. However, the effect of Sorafenib is temporary and the continuous dose for Sorafenib is needed to inhibit tumour growth for longer [15].

Retrospective analysis of patients with breast cancer has shown an unfavourable prognosis in patients with high expression levels of VEGF [16,17]. This indicates that VEGF could be associated with the efficacy of chemotherapy and radiotherapy. It has also been shown that radiation resistance is partly due to tumour cell production of angiogenic cytokines, particularly VEGF, which protects endothelial cells through survival pathways [18,19]. Moreover, it has been shown that VEGF inhibition combined with radiation enhances radiation control of bone destruction and the pain associated with cancer progression in bone metastases [20].

Here, we studied the efficacy of Sorafenib combined with radiation and determined whether this treatment modality could enhance tumour growth inhibition.

Materials and methods

Reagents

The cell culture reagents were obtained from Gibco, Invitrogen (Burlington, Ontario, Canada). Foetal bovine serum was purchased from Wisent Inc., (St Bruno, Quebec, Canada). Propidium iodide (PI) was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Sorafenib tosylate (Bay 54-9085) was provided by Bayer Pharmaceutical Corp. (West Haven, Connecticut, USA) and was reconstituted in dimethyl sulfoxide for in-vitro use and in ethanol/Cremephore L (Sigma-Aldrich) (50 : 50) for in-vivo use at 4 × concentration. The 4 × solution of Sorafenib was freshly prepared every day. The final dosing solution was prepared by diluting the 4 × solution to 1 × in sterile water (Gibco, Invitrogen) every day before its administration to the animals. The concentrations of dimethyl sulfoxide were maintained lower than 0.2% in all in-vitro experiments.

Cell culture

The highly metastatic mouse mammary cancer cell line, 4T1, was a generous gift from Dr Fred Miller, Karmanos Cancer Institute, Wayne State University, Michigan, USA. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, penicillin–streptomycin 1% and kept at 37°C in 95% air/5% CO_2.

Irradiation

Irradiation for in-vitro and in-vivo experiments was carried out at room temperature using a Theratron T-780 ^{60}Co irradiator (MDS Nordion, Kanata, Ontario, Canada). The dose delivered in each experimental set-up used in this work was verified by the radiochromic film dosimetry protocol developed by Tomic *et al.* [21].

Colony-forming assay

Cells were plated at specific cell numbers in six well plates. At a 0 Gy radiation dose, 100 cells per well were used and for each subsequent radiation dose (2, 4, 6 and 8 Gy), 200, 400, 800 and 1600 cells were seeded, respectively. They were treated with Sorafenib alone (5 and 7.5 μmol/l for 2 h) and in combination with radiation (2, 4, 6 and 8 Gy) [22]. After 6–8 days of incubation, the colonies were fixed and stained with methylene blue. Only colonies containing more than 50 cells were counted. The plating efficiency was calculated by dividing the number of colonies formed in the untreated control plates by the number of cells plated. Survival fractions were calculated by counting the number of colonies for each specific radiation dose and dividing by the number of cells seeded at the same dose multiplied by plating efficiency. In order to plot the survival curve, the survival fractions were normalized according to the controls (nonirradiated). Radiosensitivity was measured by determining the dose enhancement factor, which is the ratio of the radiation doses at a survival fraction of 0.1 or 0.01 of non-drug-treated cells to drug-treated cells [23,24].

Flow cytometry analysis

Cell cycle analysis

Cells were treated with Sorafenib (5 and 7.5 μmol/l) and were irradiated as described. The cells were harvested and washed 24 h after treatment, after which they were fixed with ethanol, labelled with PI and analysed by flow cytometry (BD Biosciences, San Jose, California, USA). Cell cycle distribution was analysed using the Mod-Fit LT software package (Verity Software House, Topsham, Maine, USA).

Analysis of apoptosis by annexin-V binding

Cells were treated with Sorafenib (5 and 7.5 μmol/l) and were irradiated to a dose of 4 Gy. They were harvested and washed with PBS 1 × (Gibco, Invitrogen) at 48 h after treatment. They were labelled with annexin V–FITC and PI according to the manufacturer's protocol (TACS apoptosis kit; R&D Systems, Minneapolis, Minnesota,

USA). Cells were analysed by flow cytometry (BD Bioscience) and characterized as follows: cells appearing at the lower left quadrant of the dot plot were considered viable. Those observed at the lower right quadrant were identified as early apoptotic. The late apoptotic and necrotic cells appeared at the upper right and the upper left quadrants, respectively.

Western blot analysis

The 4T1 cells were incubated in two sets of six well plates with serum-free media for 18 h and were subsequently exposed to the 5 and 20 μmol/l of Sorafenib for 2 h and 4 Gy of radiation. To test whether Sorafenib is still effective in the case of overactivation of receptor tyrosine kinases (RTKs) such as VEGFR, one set of the plates was subsequently treated with 25 ng/ml of VEGF for 20 min and cells were harvested within 1 h, after which the whole cell lysates were prepared. Fifty micrograms of protein was loaded onto Bis-Tris gradient gels (Invitrogen, Carlsbad, California, USA). Western blot analysis was performed using antibodies for p-Erk 1,2 and Erk 1,2 as well as tubulin (Cell signaling Technology Inc., Beverly, Massachusetts, USA).

In-vivo tumour model

Six to eight-week-old female BALB/c mice (Charles River Laboratories, Montreal, Canada) were used in this study. Mice were caged in groups of five or less. 4T1 tumour cells (2×10^6 cells) were injected subcutaneously into the right hind leg. All protocols were approved by the McGill University Animal Care Committee following the guidelines of the Canadian Council on Animal Care.

Tumour growth delay assay

When tumours reached a mean volume of 144 mm^3, mice were randomized into four groups: vehicle, Sorafenib alone, irradiation (15 Gy) alone, and Sorafenib plus irradiation [25]. A single dose of Sorafenib (60 mg/kg) was administered by gavage daily for 7 days [9,26]. The drug was administered 6 h before local tumour irradiation (15 Gy) on day 3 (schedule A). In case of schedule B, radiation was delivered 24 h before the start of drug treatment. To obtain tumour growth curves, perpendicular diameter measurements of each tumour were made every 2–3 days with digital callipers, and volumes were calculated using the formula $(L \times W^2)/2$. Tumours were followed until the mean tumour volume reached $\sim$2400 mm^3, after which the animals were sacrificed. The relative tumour volume was calculated by dividing each individual animal's tumour volume by the mean tumour volume of the same group. Each experimental group included six to eight mice.

Statistical analysis

The effects of various treatments in all experiments were compared using a two-tailed *t*-test (GraphPad prism 5; GraphPad software Inc., California, USA). Differences with a *P* value less than 0.05 were considered statistically significant. The data presented are means and SEM from multiple independent experiments.

Results

Sorafenib increases the sensitivity of 4T1 cells to radiation *in vitro*

To evaluate whether Sorafenib has an effect on the ability of cancer cells to form colonies *in vitro*, clonogenic assays were performed. As shown in Fig. 1, the dose enhancement factor was as high as 1.39 and 1.76 when Sorafenib was combined with radiation at 5 and 7.5 μmol/l concentrations, respectively. To test whether this effect is schedule-dependent, three schedules were used: (a) Sorafenib 24 h before radiation, (b) Sorafenib concurrent with radiation and (c) radiation 24 h before Sorafenib administration. Interestingly, pretreatment with Sorafenib (schedule A) and the concurrent schedule (schedule B) seemed to be more effective *in vitro* (Fig. 2a and b).

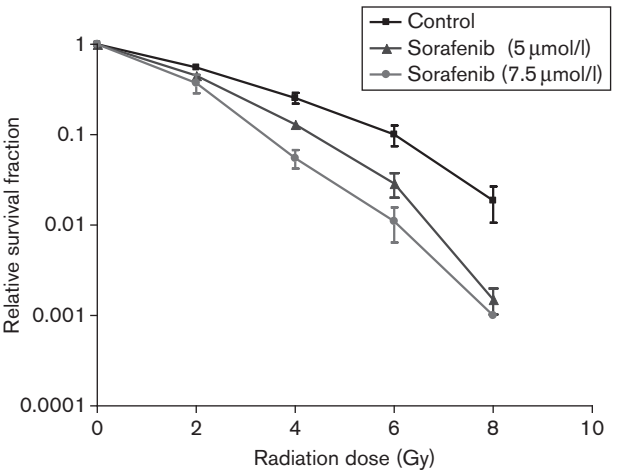
Sorafenib combined with radiation induces G2/M arrest

To assess the effect of Sorafenib in combination with radiation on cell cycle progression, cell cycle analysis was performed. As shown in Fig. 3, Sorafenib in combination with radiation had a significant and strong effect on cell cycle arrest at G2/M. Consequently, the G1 and S population was significantly decreased.

Combination of Sorafenib and radiation enhances the level of apoptosis

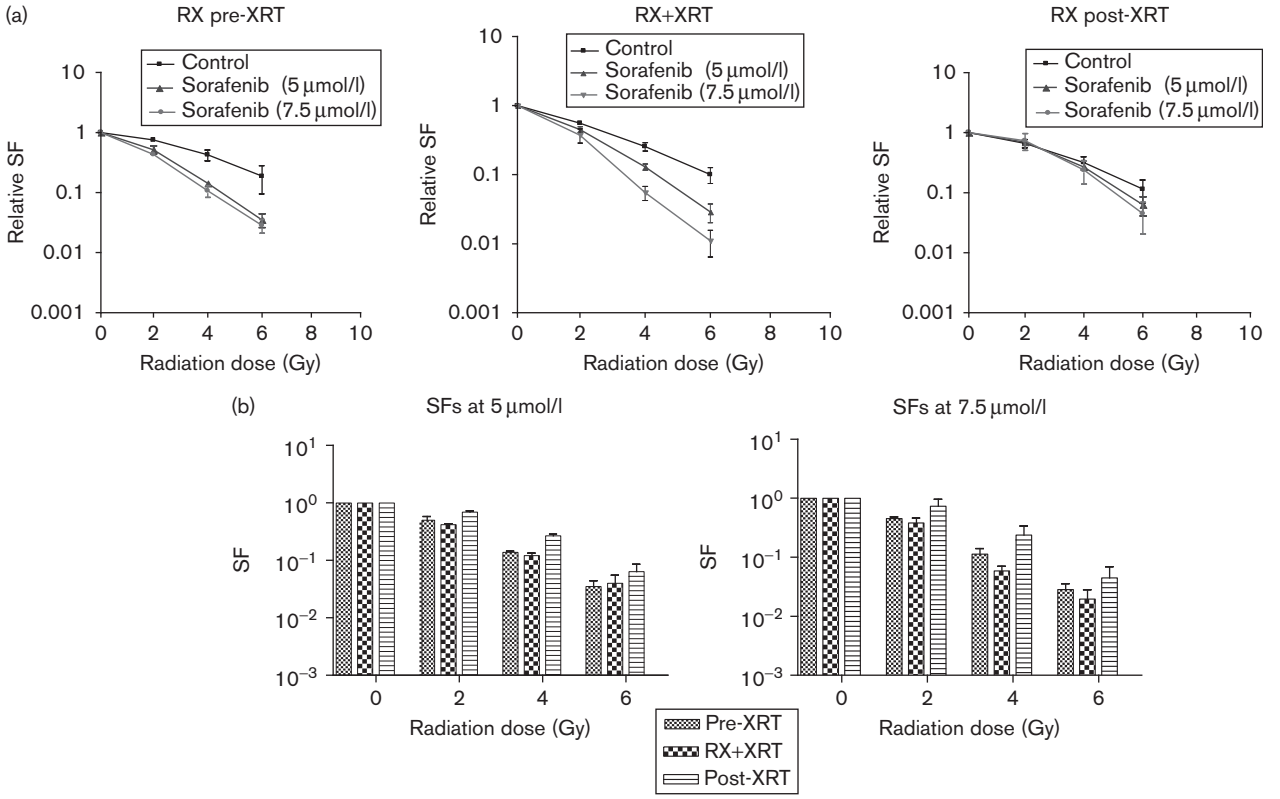
In order to determine whether the multi-inhibitory activity of Sorafenib would induce high levels of apoptosis when combined with radiation, an annexin-V binding

Fig. 1



Analysis of cell response to the combination of Sorafenib and radiation using a clonogenic assay. Cells were treated with Sorafenib (5 and 7.5 μmol/l) with or without radiation (4 Gy). Data represent means and SEM from three independent experiments.

Fig. 2



(a) Different schedules of combination (Sorafenib before radiation, Sorafenib concurrent with Sorafenib and Sorafenib 24 h after radiation) were tested on 4T1 cells. (b) Comparison of survival fractions (SFs) at the different combination schedules at specific Sorafenib doses. RX: drug (Sorafenib); XRT: radiation.

assay was performed with cells exposed to radiation and Sorafenib alone or in combination. As shown in Fig. 4, Sorafenib alone induced apoptosis at levels of up to 15 and 33% at 5 and 7.5 µmol/l, respectively, whereas apoptosis induced by radiation alone was approximately 10% of the total analysed cells. When Sorafenib was combined with 4 Gy of radiation, the level of apoptosis reached 30 and 40% at 5 and 7.5 µmol/l concentrations.

Sorafenib with/out radiation inhibits phosphorylation of Erk1/2 downstream of receptor tyrosine kinases

To confirm the inhibitory activity of Sorafenib in our breast cancer model, a western blot analysis was performed. As Sorafenib is a multikinase inhibitor of several RTKs, we evaluated the phosphorylation of Erk 1/2 as an indicator of activation of the downstream pathway. As in Fig. 5, Sorafenib at 5 µmol/l completely inhibits the activation of Erk1/2 downstream of RTKs irrespective of radiation treatment.

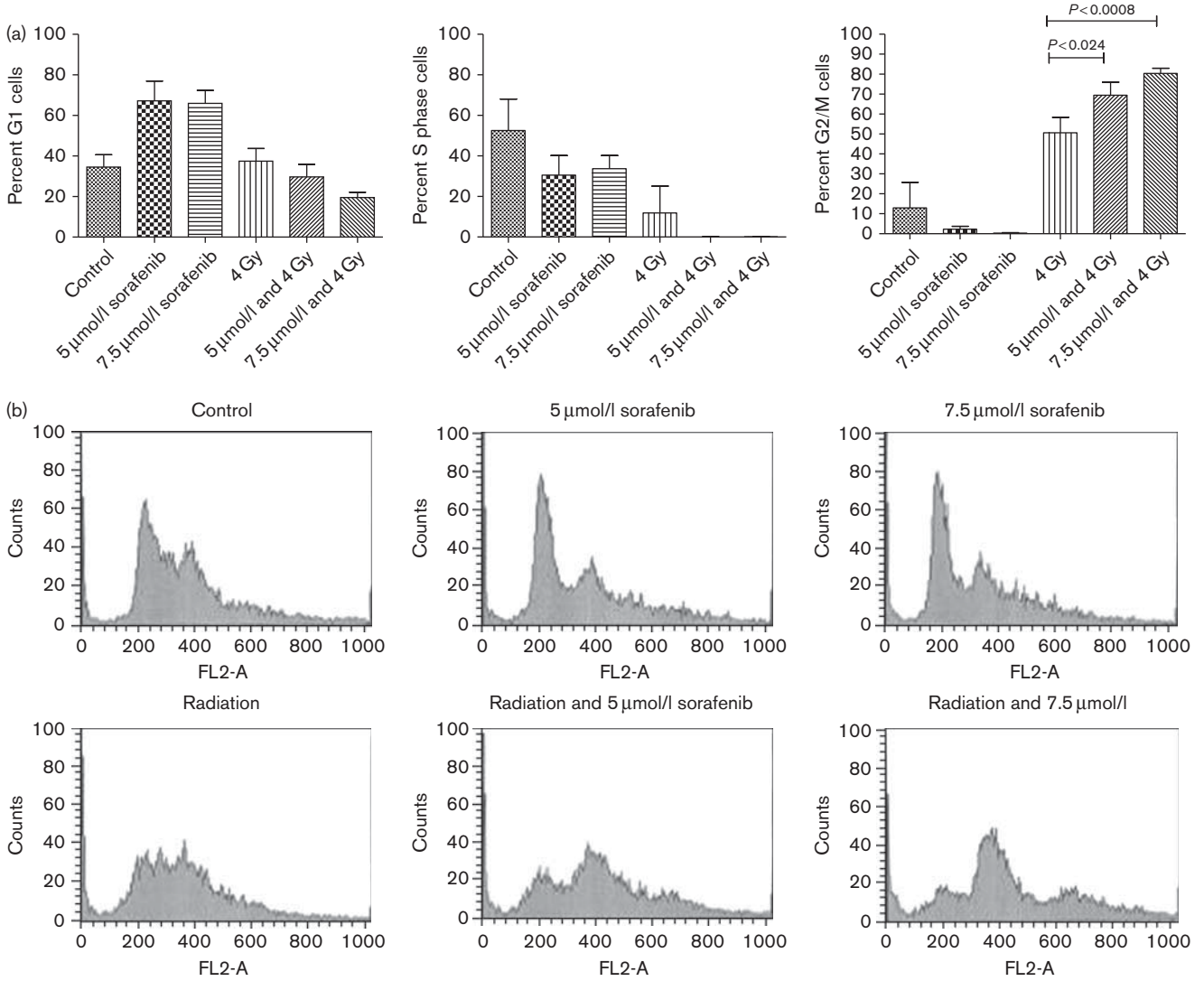
Sorafenib increases the tumour growth delay caused by radiation as an early response

In order to evaluate and validate our in-vitro results, we performed an in-vivo experiment with 4T1 mouse mammary cancer cells. As shown in Fig. 6a and b, the in-vivo results suggest that, in tumours treated with the combination therapy, Sorafenib increases the delay in tumour growth caused by radiation by almost 7 days. Moreover, Sorafenib combined with ionizing radiation has significantly more antitumour effect against 4T1 tumours than Sorafenib alone in BALB/c mice.

Sorafenib was as potent as the combined treatment only until the end of the drug treatment (day 6). Soon after the end of the drug treatment (day 10), tumours started to grow (Sorafenib-treated group vs. combination group: $P = 0.0406$). On day 17 (Fig. 6a), the tumour volume of the Sorafenib-treated group was significantly larger compared with the irradiated or the combined treated tumours (Sorafenib vs. combination: $P = 0.0002$). The same pattern was observed when radiation was delivered 24 h before the start of Sorafenib treatment.

In schedule A (when radiation was delivered concurrently with Sorafenib treatment), the tumour growth delay was increased from 4.2 days in the control group to 11 and 10.5 days in Sorafenib alone or radiation alone, respectively. The growth delay, in the case of the combination of Sorafenib and radiation, was increased to 18 days.

Fig. 3



Cell cycle analysis of 4T1 cells following exposure to Sorafenib or radiation and the corresponding combination. (a) Cell distribution in G1, S and G2M. Cells were treated with Sorafenib (5 and 7.5 µmol/l) alone and in combination with radiation (4 Gy) and cell cycle analysed by flow cytometry 24 h later. Data represent means and SEM from three independent experiments. (b) A representative histogram showing the G2M arrest in combined treatment.

Similarly, in schedule B, when radiation was delivered 24 h before Sorafenib treatment, the growth delay was increased from 6.5 days in the control group to 13 and 14 days in Sorafenib alone and radiation alone and 20.5 days in mice treated with both modalities.

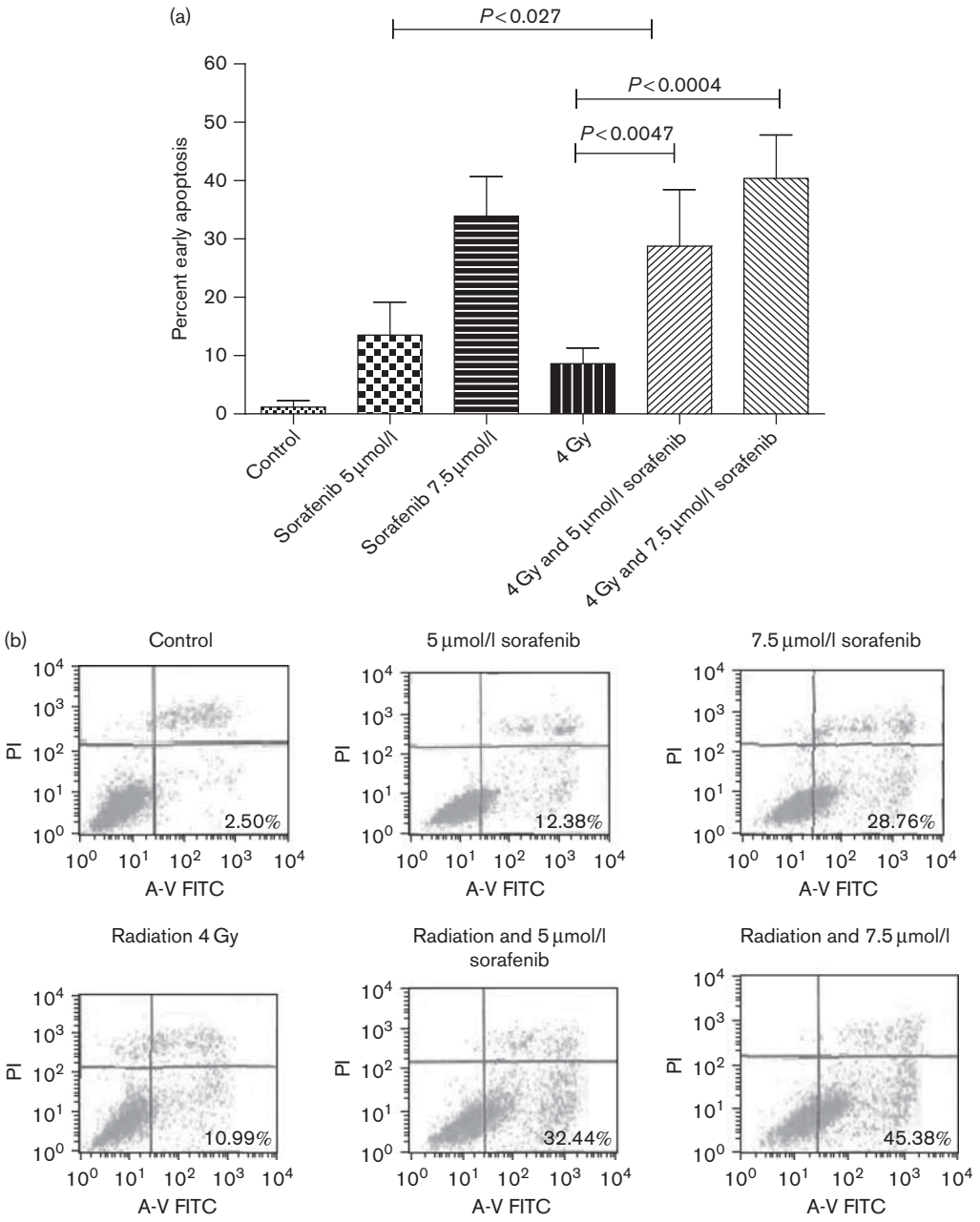
No significant loss of body weight resulted from any of the treatments and the treatments were well tolerated by the end of the experiment (Fig. 6c and d).

Discussion

In this study, we demonstrated that Sorafenib induces a greater antitumour activity when it is combined with radiation in 4T1 cells, both *in vitro* and *in vivo*. 4T1 cells are highly metastatic cancer cells and are considered to be a suitable model to study the effect of antiangiogenesis agents *in vitro* and *in vivo* [27–30].

The increased antitumour activity of Sorafenib combined with radiation in 4T1 cells can be partially explained by the significant cell cycle arrest we observed at G2/M. Cancer cells show more sensitivity to ionizing radiation at the G2/M and G1 whereas cells residing in the S stage of the cell cycle are less radiosensitive [31]. As was shown by our result, there was a significant decrease in the S-phase population, which could explain the higher potency of the combined treatment.

Fig. 4

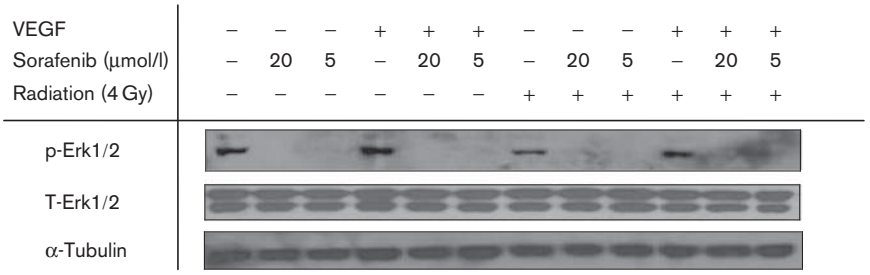


Analysis of apoptosis induced by Sorafenib or radiation and the corresponding combination in 4T1. (a) Cells were treated with Sorafenib (5 and 7.5 µmol/l) alone and in combination with radiation (4 Gy) and were harvested at 48 h after treatment. Presented data are means and SEM of multiple independent experiments. (b) Representatives dot-plot data showing the effect of Sorafenib or/and radiation on the level of apoptosis. PI, propidium iodide.

The increased efficacy of Sorafenib in combination with radiation could also be due to the augmented apoptosis level in 4T1 cancer cells treated with both Sorafenib and radiation.

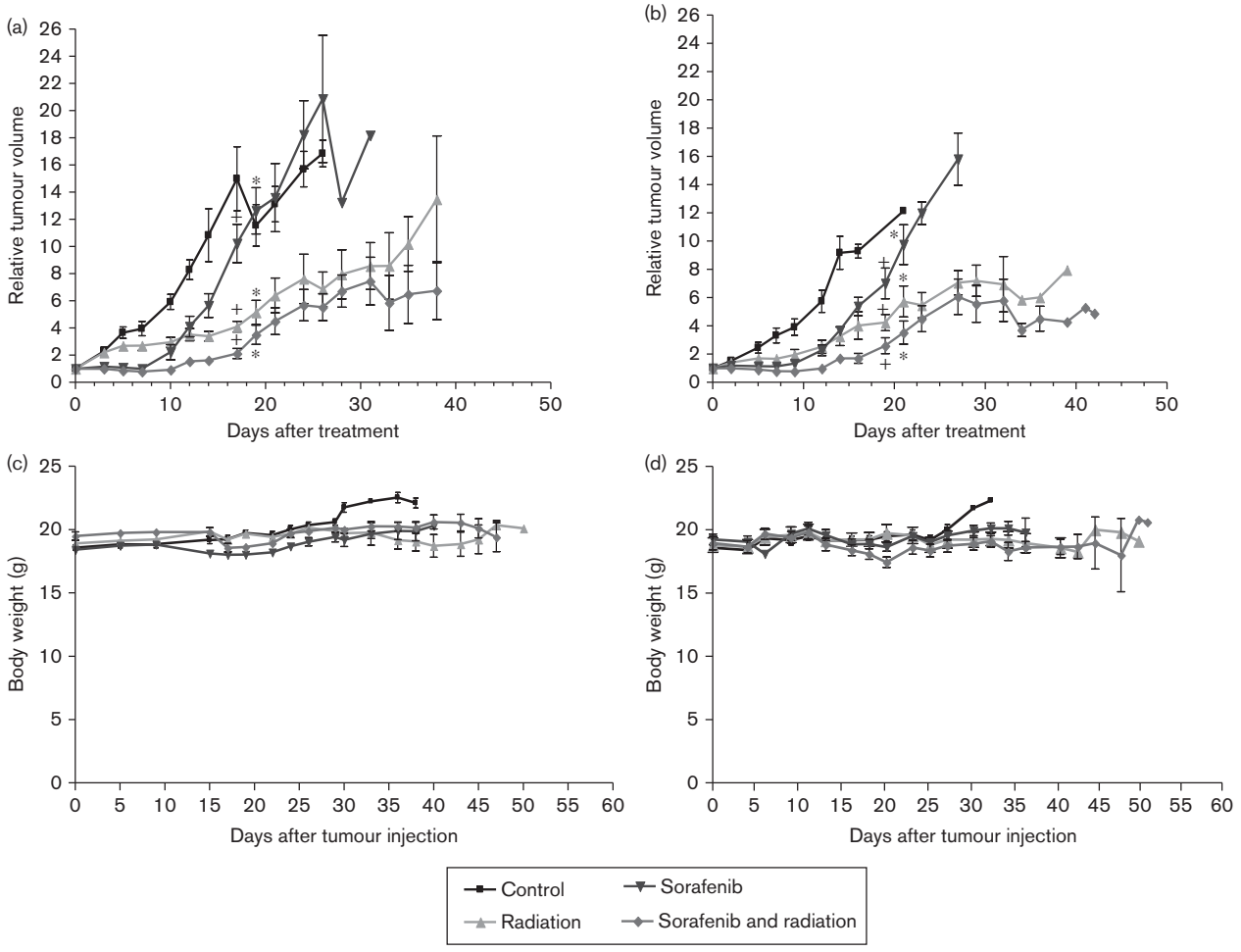
In this study, we have also shown that radiation prolongs the antitumour activity of Sorafenib *in vivo*. Tumours implanted in mice treated with Sorafenib alone started to grow rapidly immediately after the drug treatment was stopped (day 6–8) whereas the inhibitory effect of Sorafenib was longer when radiation was added (tumours started to grow gradually starting day 14). This could be of relevance as cytostatic agents such as Sorafenib usually show temporary and reversible antitumour activity [15].

Fig. 5



Western blot analysis. 4T1 cells were grown to 80% confluency in six well plates. They were serum starved for 18 h and were treated with Sorafenib or/and radiation as indicated. Half of the plates were stimulated with vascular endothelial growth factor (VEGF) and whole-cell lysates were prepared within 1 h.

Fig. 6



(a, b) Tumour growth delay assay. 4T1 cells were injected into the right hind limb of BALB/c mice. When the mean volume of tumours reached 144 mm^3, the animals were randomly divided into four groups. There were six to eight animals in each group. Schedule A (a): Sorafenib was given to the animals 3 days before radiation, on the same day as irradiation and was continued for 3 days after irradiation (+: day 17, irradiated group vs. combined treatment: $P=0.0309$; Sorafenib-treated group vs. combination: $P=0.0002$; *: day 19, irradiated group vs. combined treatment: $P=0.1710$; Sorafenib-treated group vs. combination: $P=0.0255$). Schedule B (b): 15 Gy of radiation was delivered 2 h before the start of Sorafenib treatment (+: day 19, irradiated group vs. combined treatment: $P=0.0316$; Sorafenib-treated group vs. combination: $P=0.0255$; *: day 21, irradiated group vs. combined treatment: $P=0.0815$; Sorafenib-treated group vs. combination: $P=0.0180$). When the tumour size reached a maximum of 2400 mm^3, the mice were euthanized. Tumour volume was calculated using: $(L \times W^2)/2$ and was normalized by dividing the tumour volume of each animal in the treatment groups by the mean tumour volume of the same group. Error bars, SEM. (c, d) Variations of body weight of mice treated with Sorafenib (60 mg/kg) and radiation alone and the combined treatment. Error bars, SEM.

Moreover, in our in-vivo model, Sorafenib increased the radiation response significantly as an early response (Fig. 6a and b). It will be interesting to evaluate different schedules and sequences of this combination to determine whether a longer response can be achieved.

Our in-vitro data demonstrated a higher radiation response when Sorafenib was added before or concurrent with radiation versus after radiation. This was in disagreement with our in-vivo results, which showed no significant difference between the Sorafenib treatment after and concurrent with radiation. The difference between our in-vitro and in-vivo outcome could be due to the interaction of tumour cells with each other and with their microenvironment. In-vitro assays are performed in a much shorter time period than in-vivo experiments. The difference could also be related to hypoxia and hypoxia-induced radiation resistance. Although antiangiogenic agents have been shown to stabilize neovasculature and improve blood perfusion, Sorafenib might not have done so, resulting in the formation of hypoxic regions inside the tumours and therefore reduced radiation response.

Recently, Suen *et al.* [32] and Plastaras *et al.* [26] have shown that the combination of Sorafenib and radiation enhances the radiation response in colorectal cancer cells *in vivo* and this response is schedule dependent. In their studies, irradiation before Sorafenib treatment appears to be the most efficient schedule [26,32]. The different outcome between their study and ours is perhaps due to the use of different tumour models and also the different radiation schedules. The mice in our study were irradiated with a single radiation dose either before or concurrent with Sorafenib administration (schedules A and B) whereas in the two mentioned studies, fractionated radiation was used over a longer period of time.

It has been shown that Sorafenib, being a cytostatic agent [33], can induce radiation response especially in fractionated schedules as it blocks regrowth (through its antiangiogenic properties) between fractions [3]. Presently, in our laboratory, more in-vitro/in-vivo studies are ongoing to test Sorafenib with fractionated radiotherapy in metastatic breast cancer models while more microenvironment studies will guide us through the complex mechanism of this combination.

There are several trials combining Sorafenib with radiation and other cytotoxic modalities [34] that are ongoing or have been completed in the clinical setting. Some results showed that the combination did not improve the efficacy of treatment as 40% of the patients did not receive Sorafenib at all due to early disease progression. Perhaps better results can be achieved with a better design or modified combinations. Other trials including a phase I/II study of cisplatin and radiation in combination with Sorafenib in cervical cancer, a phase I/II trial of radiation therapy and Sorafenib for unrespectable liver metastases and Sorafenib combined with radiation in HCC are ongoing. Depending on the outcome of these clinical trials, the protocol for patients might change and patients with cancer might benefit from the new combination therapies. Nevertheless, a better understanding of the mechanism of action of antiangiogenic agents and, more specifically, multitargeting agents is crucial to better design a clinical trial and to rationally choose the target patient population.

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Conflicts of interest

This study was supported by an investigator grant from Bayer Pharmaceuticals corp.

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Interaction of ionizing radiation and ZRBA1, a mixed EGFR/DNA-targeting molecule

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ZRBA1 is a molecule termed 'combi-molecule' designed to induce DNA-alkylating lesions and to block epidermal growth factor receptor (EGFR) tyrosine kinase. Owing to its ability to downregulate the EGFR tyrosine kinase-mediated antiapoptotic signaling and DNA repair proteins, we inferred that it could significantly sensitize cells to ionizing radiation. Using the MDA-MB-468 human breast cancer cell line in which ZRBA1 has already been reported to induce significant EGFR/DNA-targeting potency, the results showed that: (i) concurrent administration of ZRBA1 and 4 Gy radiation led to a significant decrease in cell viability, (ii) the greater efficacy of the combination was sequential, being limited to conditions wherein the drug was administered concurrently with radiation or before radiation, and (iii) the efficacy enhancement of the combination was further confirmed by clonogenic assays from which a dose enhancement factor of 1.34 could be observed at survival fraction of 0.01. Flow cytometric analysis showed significant enhancement of cell cycle arrest in G_2/M ($P<0.046$, irradiated cells vs. cells treated with ZRBA1 and radiation) and increased apoptosis when ZRBA1 was combined with radiation. Likewise, significant levels of double-strand breaks were observed for the combination, as determined by neutral comet assay ($P<0.045$, irradiated cells vs. cells treated with ZRBA1 and radiation). These results *in toto* suggest that the superior efficacy of the ZRBA1 plus radiation combination may be secondary to the ability of ZRBA1 to arrest the cells in G_2/M, a cell cycle phase in which tumor cells are sensitive to radiation. Furthermore, the increased levels of DNA damage, combined with the concomitant downregulation of EGFR-mediated signaling by ZRBA1, may account for the significant levels of cell killing induced by the combination. *Anti-Cancer Drugs* 20:659–667 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: apoptosis, cell cycle, clonogenic survival, combi-molecule, comet assay, epidermal growth factor receptor tyrosine kinase inhibitor, ionizing radiation

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Introduction

Ionizing radiation is a mainstay of nonsurgical cancer treatment. Approximately, 75% of nonskin cancer patients receive radiation therapy at some time during the course of their disease. Radiotherapy has been successful in local tumor control [1], and when it is combined with chemotherapy, radiotherapy also improves overall survival of cancer patients [2]. Over the last decade, the combination of ionizing radiation with chemotherapy has led to marked improvement in local control, organ preservation, and survival for locally advanced solid tumors. However, this strategy is limited by the morbidity resulting from each respective treatment and their combinations. Targeting tumor-specific defects should provide an advantage over conventional chemotherapy in which the major drawback is normal tissue toxicity [3].

In contrast, acquired resistance to DNA-damaging agents represents a major obstacle in the therapy of many tumors, including lung, breast, ovarian, and brain carcinomas. Over the past three decades, several strategies have been developed to enhance the potency of DNA-damaging agents, the most common one being the use of inhibitors of DNA-repair enzymes [4–6]. With the advent of molecular biology, novel markers associated with reduced sensitivity to DNA-damaging agents have been identified. This includes signaling proteins, such as AKT, the activation of which is related to antiapoptotic signaling. More importantly, several receptor tyrosine kinases (TKs), which activate AKT-mediated antiapoptotic signaling, have now been identified [4,7–9]. One such receptor is the epidermal growth factor receptor (EGFR), which is activated by chemical and radiation-induced DNA damage. Importantly, overexpression of EGFR is associated with aggressive tumor progression, invasion, and reduced sensitivity to chemotherapy [10–12]. In addition Yacoub *et al.* [13] have shown that the activation of EGFR leads to expression of DNA-repair proteins, such as XRCC1 and ERCC1.

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Recently, we developed a novel type of molecule termed 'combi-molecule' designed to block the TK activity of EGFR and its subsequent adverse effect on apoptotic signaling or its ability to upregulate DNA-repair proteins, while concomitantly delivering significant DNA lesions to the cells (Fig. 1) [14,15]. The combi-molecules have now been shown to indeed inflict strong DNA damage to tumor cells and to block EGFR [4,16–18]. One such molecule, ZRBA1, induced significantly higher levels of apoptosis than the single-targeted EGFR inhibitor, FD105 [19]. Furthermore, its antiproliferative activity against MDA-MB-468 breast cancer cells was more sustained than that of FD105. However, despite its significant potency, its activity was partially mitigated in cells expressing the DNA-repair enzyme O6-alkylguanine transferase (AGT), for example, SF188 AGT + glioma cells [19]. Thus, despite the strong binary EGFR/DNA-targeting potency of this agent, its activity remained to be improved in DNA-repair proficient cells. However, owing to its mixed EGFR/DNA-targeting mechanism, it could potentially be developed as a radiopotentiator.

ZRBA1 is designed to induce N7-alkylated and O6-alkylated lesions in a manner similar to the clinical drug temozolomide, which is effective in tumors that do not express AGT. It has been shown that radiation-induced lesions potentiated the action of temozolomide in the latter type of tumors [20]. Temozolomide enhancement of radiation response was imputed to its ability to increase the degree of radiation-induced DNA double-strand breaks in the cells [21]. ZRBA1, being able to induce DNA-alkylating lesions of the same type as temozolomide, if combined with radiation might not only increase the levels of DNA strand breaks but also inflict different types of DNA damage, thereby delaying or complicating the DNA-repair process. In addition to its ability to downregulate EGFR TK activity and its subsequent downstream effect on apoptosis and DNA repair, we proposed that the combination of ZRBA1 with radiation might translate into significant cell killing. To verify this hypothesis, we chose to analyze the effect of ZRBA1 plus radiation on the human MDA-MB-468 breast cancer cell line that overexpresses EGFR, and in which ZRBA1 has been proven to exert its binary targeting potency [19].

Materials and methods

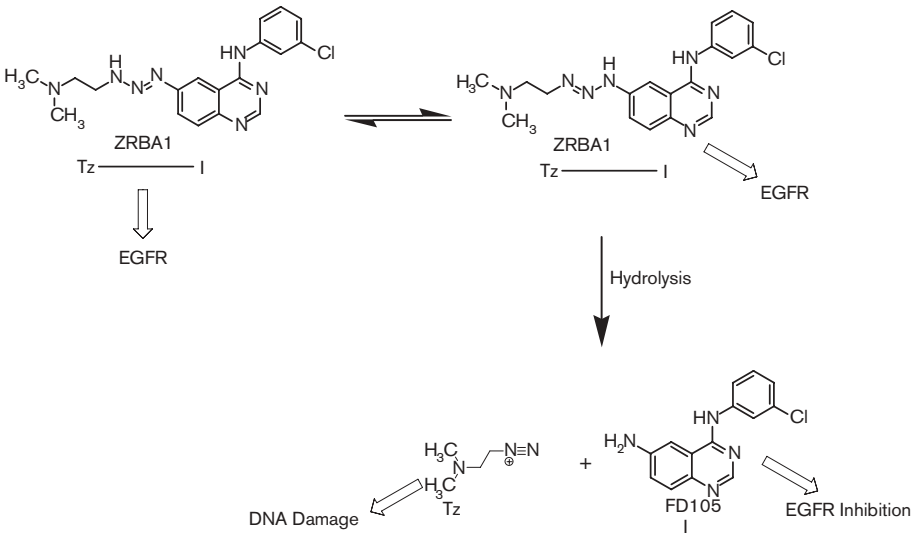
Reagents

The cell culture reagents were from Gibco, Invitrogen, Burlington, Ontario, Canada. Fetal bovine serum was purchased from Wisent Inc., St-Bruno, Quebec, Canada. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide (PI) were from Sigma-Aldrich, Oakville, Ontario, Canada. The combi-molecule was synthesized according to the previously published methods [22]. Iressa (AstraZeneca, Mississauga, Ontario, Canada) was purchased from the Royal Victoria Hospital pharmacy in Montreal and was extracted in B.J.C.s laboratory. The drugs were reconstituted in dimethyl sulfoxide, the concentrations of which were kept lower than 0.2% in all experiments.

Cell culture

Human MDA-MB-468 breast carcinoma cells were obtained from the National Cancer Institute (Bethesda, Maryland, USA). Cells were cultured in RPMI 1640 (Gibco, Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum, penicillin–streptomycin 1% and kept at 37°C in 95% air/5% CO_2.

Fig. 1



Hydrolysis and binary properties of ZRBA1 under physiological conditions.

Irradiation

Irradiation was carried out in our research facilities at room temperature using a 160 kVp X-ray irradiator Faxitron FC-160 (Wheeling, Illinois, USA) at a dose rate of 1.5 Gy/min.

Cell proliferation assay

Growth inhibition was measured using the MTT assay [23]. Cells were plated at the density of 8000 cells/well in 96-well plates and subsequently treated with ZRBA1 or Iressa (0–100 μmol/l) alone (for 2 or 24 h) and in combination with radiation (4 Gy). Cells were washed with drug-free media and then the fresh media was added before irradiation. Cells were incubated for 72–96 h depending on their schedule, after which the MTT solution was added for 3–5 h. The assay was stopped and the optical density was measured using a 96-well plate reader at 750 nm.

Colony-forming assay

Cells were plated at specific cell numbers in six-well plates. They were treated with ZRBA1 or Iressa, alone for 2 h (36 μmol/l) and in combination with radiation (2, 4, 6, and 8 Gy). Cells were washed with drug-free media and then fresh media was added before irradiation. After 12–14 days, the colonies were fixed and stained with methylene blue. Only colonies containing more than 50 cells were counted. The plating efficiency (PE) was calculated by dividing the number of colonies formed in the untreated control plates by the number of cells plated. Survival fractions (SFs) were determined as colonies counted at the specific radiation dose divided by the cells seeded at the same dose multiplied by PE. To plot the survival curve, the SFs were normalized according to the controls (nonirradiated). Radiosensitivity was measured by dose enhancement factor, which is the ratio of the radiation doses at SF of 0.1 or 0.01 of nondrug-treated cells to drug-treated cells [24,25].

Flow cytometry analysis

Analysis of apoptosis by annexin-V binding

Cells were treated with ZRBA1 (50 μmol/l) for 2 h and were irradiated at 4 Gy. They were harvested and washed with PBS 1X (Gibco, Invitrogen, Burlington, Ontario, Canada) at 3, 6, 12, and 24 and 48 h after treatment. They were labeled with Annexin-V–fluorescein isothiocyanate and PI according to the manufacturer's protocol (TACS apoptosis kit; R&D Systems, Minneapolis, Minnesota, US). Cells were analyzed by flow cytometry (BD Bioscience, Mississauga, Ontario, Canada). Data was collected using logarithmic amplification of both FL1 (FITC) and FL2 (PI) channels. Cells were characterized as apoptotic when they were positive for Annexin-V or Annexin-V and PI. Collected data was then analyzed by CellQuest software (BD Bioscience, Mississauga, Ontario, Canada).

Cell cycle analysis

Cells were treated with ZRBA1 (25 μmol/l) for 2 h and were irradiated as described before. They were harvested and washed 24-h post-treatment after which they were fixed with ethanol, labeled with PI, and analyzed with a flow cytometer (BD Biosciences, Mississauga, Ontario, Canada). Cell cycle distribution was analyzed using the Mod-Fit LT software package (Verity software house, Topsham, Maine, US).

Comet assay

The modified neutral comet assay was performed as described earlier [19,26]. The cells were exposed to a dose (36 μmol/l) of ZRBA1, Iressa, or FD105 for 2 h, irradiated at 4 Gy, harvested 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, Ontario, Canada) using gel-casting chambers and then immediately placed into lysis buffer [2% sarkosyl, 0.5 mol/l Na_2EDTA, 0.5 mg/ml proteinase K (pH 8.0)] [26]. After being kept at 37°C overnight, the gels were gently rinsed with a neutral rinse buffer [90 mmol/l Tris buffer, 90 mmol/l boric acid, 2 mmol/l Na_2EDTA (pH 8.5)] for 30 min at 37°C. Thereafter, the gels were submerged in fresh neutral rinse in an electrophoresis chamber and ran at 20 V for 20 min. They were subsequently rinsed with distilled water, dried with 100% ethanol overnight, and stained with SYBR Gold (1/10 000 dilution in distilled H_2O, supplied from Molecular Probes, Eugene, Oregon, USA) for 1 h. Comets were visualized at × 330 magnification and DNA damage was quantitated using the 'tail moment' parameter (i.e. the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet). A minimum of 50 cells/comet were analyzed for each sample, using the comet assay IV imaging software package (Perceptive Instrument, Haverhill, Suffolk, UK).

Subcellular distribution study

MDA-MB-468 cells were plated at 70% confluency in six-well plates, allowed to adhere overnight and treated with ZRBA1 for 1 h. Cells were subsequently washed with PBS twice, and analyzed using a DAPI filter in a Leica fluorescent microscope (Leica DFC300FX camera; Leica, Richmond Hill, Ontario, Canada). Pictures were obtained at a × 400 magnification.

Statistical analysis

The effects of various treatments in all experiments were compared using two-tailed *t*-test. Differences with a *P* value of less than 0.05 were considered statistically significant. The data are represented as means and SEM from multiple independent experiments (± SEM).

Results

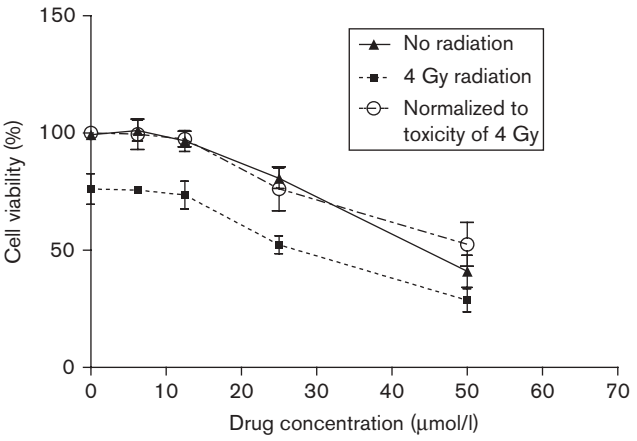
Growth inhibitory effect

To determine the doses required for different combination with radiation, a dose–response curve was established with ZRBA1 and radiation alone in the MDA-MB-468 cells using the MTT assay.

The half maximal inhibitory concentration for cell survival for ZRBA1 was 36 and 30 μmol/l after 2 and 24 h exposure, respectively. The dose of radiation required for killing 50% of the cells was approximately 4 Gy. Thus, combinations were performed with ZRBA1 at 36 μmol/l when doses of radiation were varied, and 4 Gy when doses of ZRBA1 were altered. The results showed that concomitant exposure of a dose range of ZRBA1 to 4 Gy leads to an additive effect at the lower doses (when the two curves overlapped), whereas this effect is not visible at higher doses. This can be because of the limitation of the MTT assay (Fig. 2).

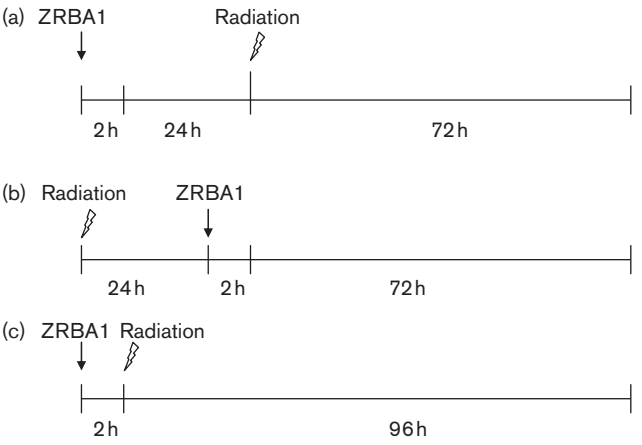
ZRBA1, being an alkylator, generated the same alkylating lesions as temozolomide, which in earlier studies have been shown to enhance radiation response in human tumors in a sequence-dependent manner [24]. Thus, we determined whether sequential administration of ZRBA1 and radiation would lead to different results when compared with concurrent administration. As depicted in Fig. 3, in one sequence (Fig. 3a) ZRBA1 was administered for 2 h and irradiated 24 h later, followed by 72 h recovery before analysis of cell viability by MTT. In the second sequence (Fig. 3b), first the cells were irradiated and then 24 h later ZRBA1 was administered for 2 h. Cell viability was measured 72 h after drug treatment. In the third sequence (Fig. 3c), cells were exposed to the drug for 2 h, and then they were irradiated. Cell viability was analyzed 96-h post-treatment.

Fig. 2



Effects of radiation and combination of radiation (4 Gy) and ZRBA1 on the viability of MDA-MB-468 cells. The cells were treated with variable concentrations of ZRBA1 and irradiated with a dose of 4 Gy. Values of treated cells with the combination of ZRBA1 and radiation were normalized to account for the toxicity induced by 4 Gy of radiation (data are represented as means and SEM of three independent experiments).

Fig. 3



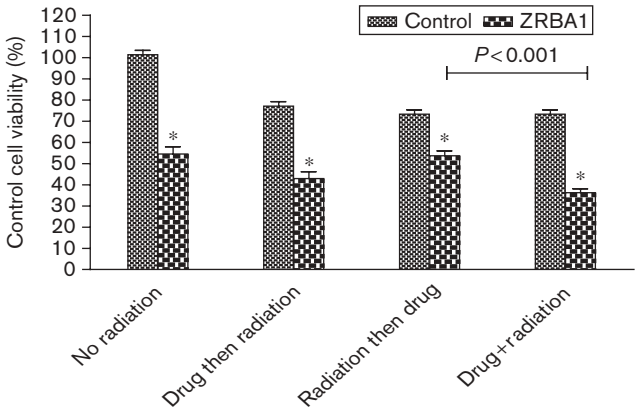
Sequences of administration of ZRBA1 and radiation in the various combinations. (a) MDA-MB-468 cells were treated with 36 μmol/l of ZRBA1 for 2 h and were irradiated 24 h later. They were further incubated for 72 h. (b) Cells were irradiated at 4 Gy of radiation. Twenty-four hours later they were treated with 36 μmol/l of ZRBA1 for 2 h, and further incubated for 72 h. (c) Cells were treated with 36 μmol/l of ZRBA1 for 2 h and were irradiated immediately after the drug had been washed out.

The results showed that the greatest efficacy of the combination was observed when the drug was administered as depicted in the third sequence (Figs 3c and Fig. 4) according to which the drug and radiation were administered concurrently. Although drug administration sequence one (Fig. 3a) also showed significant enhancement, the third sequence (Fig. 3c) showed the most effective response among the three tested protocols ($P < 0.001$, first vs. third sequence; Fig. 3b and c). Therefore, it seems that for effective combination, drug administration must precede or be concurrent with radiation.

Clonogenic assay

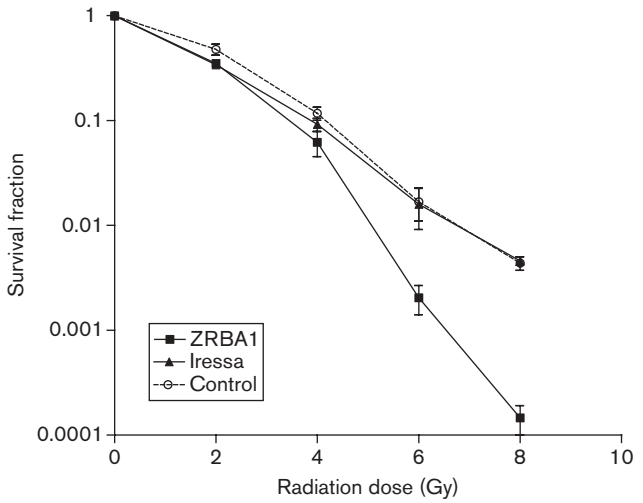
To confirm the significant results obtained from the MTT assay, a clonogenic study was conducted. ZRBA1 was administered (36 μmol/l) for 2 h and the dose range of radiation was applied (0–8 Gy). After 14 days, PE of control cells was approximately 20%. SFs at multiple radiation doses were calculated and were normalized to the controls, which were the cells treated with drugs only (SF of 0.14 for ZRBA1). Normalized SF values without and with ZRBA1 at the dose of 2 Gy were 0.477 and 0.351, respectively, and at the dose of 4 Gy were 0.117 and 0.062, respectively. The survival curves were plotted based on the normalized SFs. Radiosensitivity was measured by dose enhancement factor (discussed in the Materials and methods) and the values were 1.23 ± 0.073 at SF of 0.1 and 1.34 ± 0.052 at SF of 0.01, which are

Fig. 4



Comparison of cell survivals after exposure to ZRBA1 and radiation according to the sequences as determined by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Fig. 3a–c). MDA-MB-468 cells were treated with ZRBA1 (36 μmol/l) for 2 h with or without radiation (4 Gy). (Data are represented as mean and SEM of three independent experiments, $*P<0.001$).

Fig. 5



Analysis of cell response to the combination of ZRBA1 or Iressa and radiation using a clonogenic assay. MDA-MB-468 cells were treated with ZRBA1 and Iressa (36 μmol/l) for 2 h with or without radiation. Data are represented as means and SEM of three independent experiments.

indication of increased sensitivity of cells treated with ZRBA1 at these SFs (Fig. 5). In addition, ZRBA1 was able to enhance the radiation effect more than Iressa at radiation dose of 4 Gy and higher (Fig. 5).

Cell cycle effect

Considering that ZRBA1-induced DNA damage is known to be associated with cell cycle arrest in G_2/M [27], it was determined whether a cell cycle rationale could be used to account for the greater efficacy of the ZRBA1 plus radiation combination. Indeed, 24 h after ZRBA1 administration, significant cell arrest in G_2/M was observed. When irradiation was performed at this time point and analyzed 24 h later, an even more significant increase in cell accumulation in G_2/M phase of the cell cycle was observed ($P < 0.046$, radiation vs. radiation and ZRBA1) (Fig. 6a and b). This suggests that a strong cell cycle arrest in G_2/M precedes cell death after exposure to ZRBA1 plus radiation.

DNA damage

To determine whether the enhancement of G_2/M arrest was associated with elevated DNA damage, a neutral comet assay was performed with cells treated with FD105, Iressa, ZRBA1 or radiation, and the corresponding combination. A significant increase in levels of DNA damage was observed for the radiation plus ZRBA1 combination when compared with radiation alone ($P < 0.045$) or treatment with ZRBA1 alone ($P < 0.029$) (Fig. 7).

Apoptosis

To determine whether the EGFR inhibitory activity of ZRBA1 combined with radiation-induced DNA damage translates into high levels of apoptosis, an annexin-V-binding assay was performed with cells exposed to radiation and ZRBA1 alone or in combination. As it is shown in Fig. 8, each treatment (radiation or drug alone) increased the levels of apoptosis particularly at 6 and 48 h post-treatment. Although the combination of drug and radiation seemed to have higher apoptosis level compared with each treatment alone, the P value did not reach the statistical significance.

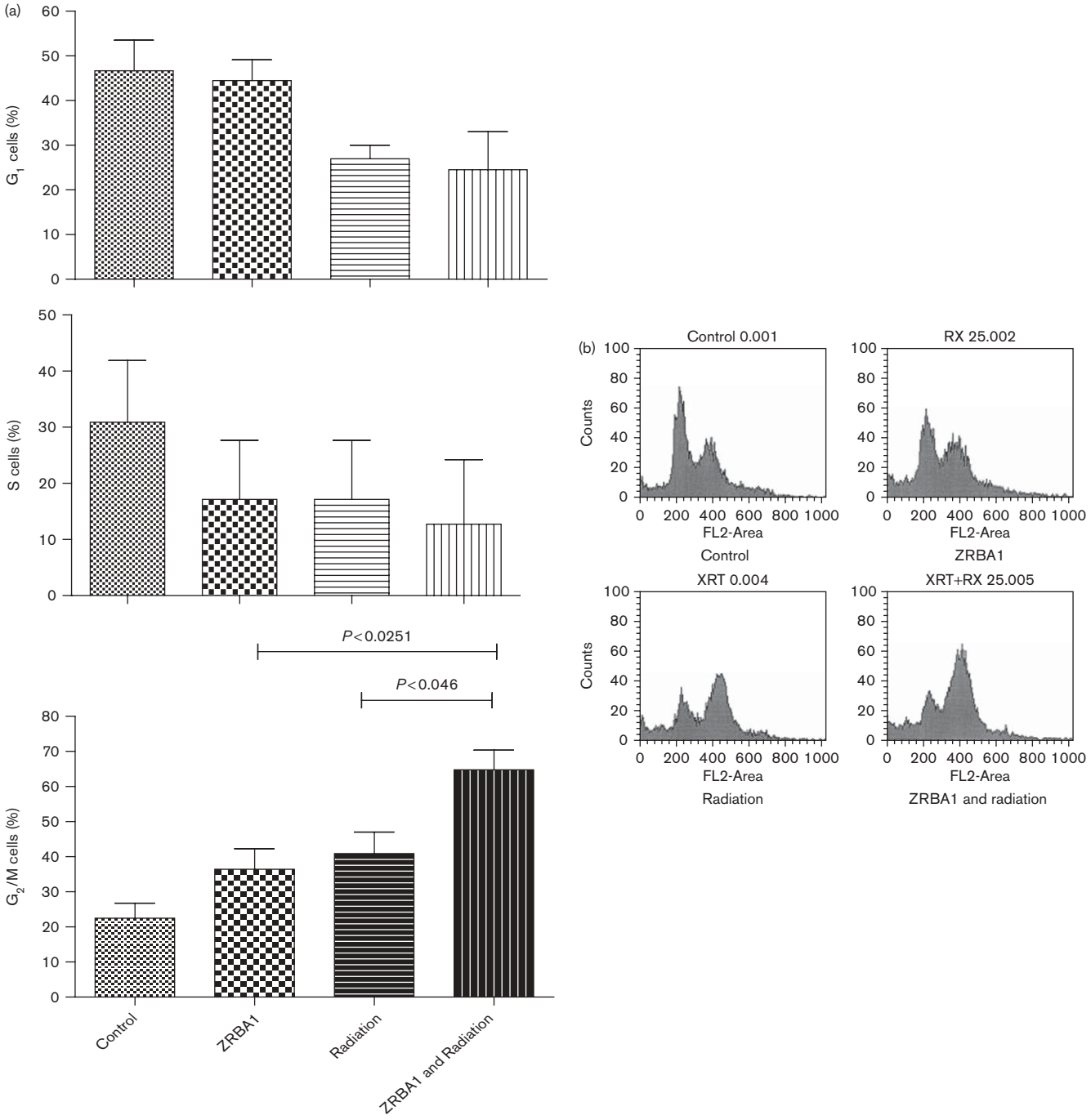
ZRBA1 biodistribution

The combi-molecule ZRBA1 is known to decompose into methyldiazonium, which damages DNA, and FD105, which inhibits EGFR TK. As FD105 fluoresces blue, its subcellular distribution could be characterized by fluorescence microscopy. Ionizing radiation being a radical generator, this experiment was designed to verify whether it affected the chemical decomposition of ZRBA1, thereby altering its cellular distribution. As outlined in Fig. 9, it was assumed that the hydroxyl radical generated by radiation could damage the triazene chain, thereby leading to a non-DNA-alkylating moiety and the barely fluorescent unsubstituted 4-anilinoquinazoline. The results showed that the levels and localization of fluorescence intensity generated by the combi-molecule in the absence or presence of radiation were identical, suggesting that ionizing radiation neither affects the chemistry nor the localization of the drug in the cells (Fig. 10).

Discussion

The overexpression of EGFR is associated with induction of DNA repair enzymes that reverse lesions induced by

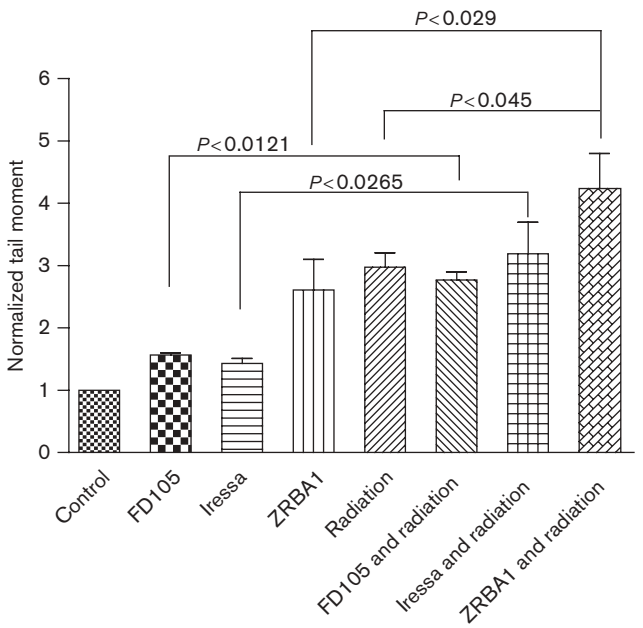
Fig. 6



Cell cycle analysis of MDA-MB-468 cells after exposure to ZRBA1 or radiation and corresponding combination. (a) Cell distribution in G_1, S, and G_2/M. Cells were treated with ZRBA1 (25 μmol/l) alone and in combination with radiation (4 Gy), and cell cycle was analyzed by flow cytometry 24 h later. Data are represented as means and SEM of three independent experiments. (b) A representative histogram showing the G_2/M arrest in combined treatment.

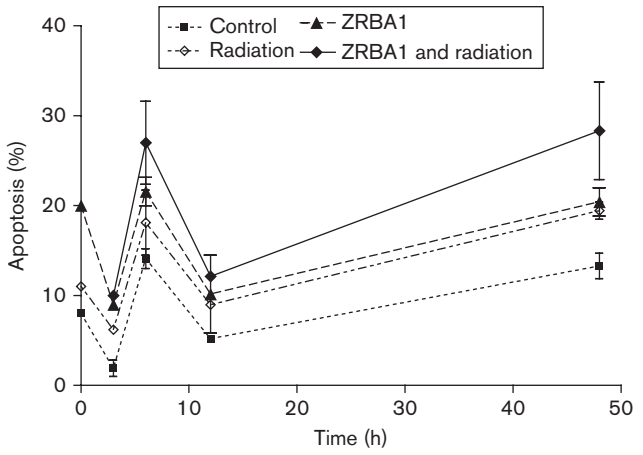
cytotoxic DNA-damaging agents. We have shown earlier that ZRBA1 was capable of downregulating EGFR-mediated signaling, damaging DNA and inducing significant levels of apoptosis in MDA-MB-468 cells [19]. In contrast, radiation has been shown to potentiate the action of temozolomide, an alkylating drug of the triazene class capable of inducing DNA alkylation in a manner similar to ZRBA1. Thus, it would be of interest to investigate the efficacy of the combination of ZRBA1 that can block EGFR, damage DNA by alkylation, with

Fig. 7



Double-strand breaks induced by FD105, Iressa, ZRBA1 or radiation, and corresponding combinations as determined by a neutral comet assay. Cells were treated with drugs (36 μmol/l) for 2 h, irradiated (4 Gy), and analyzed by microelectrophoresis as described in the Materials and methods. Data are represented as means and SEM of three independent experiments.

Fig. 8

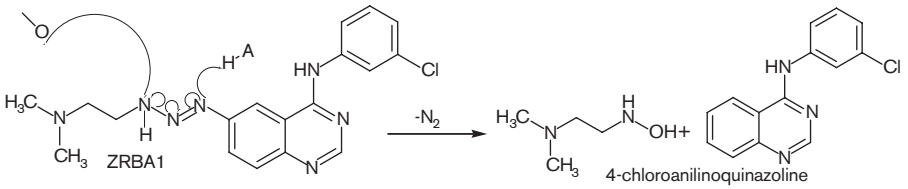


Time course analysis of apoptosis induced by ZRBA1 or radiation and corresponding combination in MDA-MB-468. Cells were treated with ZRBA1 (50 μmol/l) alone and in combination with radiation (4 Gy) and were harvested at the indicated time points. There are two peaks of apoptosis at 6 and 48 h post-treatment. Presented data are means and SEM of three independent experiments (at 6 h post-treatment: control vs. ZRBA1, $P<0.017$; control vs. radiation, $P>0.05$; control vs. radiation and ZRBA1, $P \leq 0.05$; ZRBA1 vs. radiation and ZRBA1, $P>0.05$. AT 48 h post-treatment: control vs. ZRBA1, $P<0.028$; control vs. radiation, $P<0.025$; control vs. radiation and ZRBA1, $P<0.046$; ZRBA1 vs. radiation and ZRBA1, $P>0.05$).

ionizing radiation, a DNA double-strand break inducer. It is thus inferred that owing to the mixed EGFR/DNA-targeting potency of ZRBA1, it could behave as an efficacious radiopotentiator. Here, it was shown that the combination of ZRBA1 with radiation has a greater efficacy against MDA-MB-468 cells in comparison with single-modality treatment. More importantly, the effect was sequence-dependent. ZRBA1 must be administered before or concurrent with radiation for greater efficacy to be observed. Although the molecular mechanisms underlying the significance of these sequences remained to be elucidated, the results can be analyzed on the basis of a cell cycle rationale. We have shown herein that, ZRBA1 is capable of inducing significant cell cycle arrest in G_2/M 24-h post-treatment with 40% of cells being blocked in G_2/M and it is common knowledge that G_2/M cells are exquisitely sensitive to radiation [28]. Therefore, enhanced cell killing was observed when the G_2/M population of cells was irradiated 24 h post ZRBA1 treatment. G_2 being a phase of the cell cycle wherein the final DNA-repair processes are triggered, inflicting double DNA strand breaks with ionizing radiation may further delay the repair, thereby committing the cells to apoptosis and death. It was also shown here that ZRBA1 induces double-strand breaks approximately two times more than Iressa or FD105, and when it is combined with radiation the double-strand break induction also seems to be higher than the combination of radiation with Iressa or FD105; however, the P value was not statistically significant ($P > 0.05$). Furthermore, the ability of ZRBA1 to downregulate EGFR-mediated signaling, as reported earlier, may contribute to the enhancement of cell death observed at cytotoxic concentration (although here the observed apoptosis was not statically different when comparing single-modality treatment vs. combined treatment). The results showed that the level of apoptosis induced by ZRBA1 seems to be higher than apoptosis induced by radiation alone. It is speculated that this finding is because of the significant potency of ZRBA1. This will be further clarified by studying the proportion of induced apoptosis while altering the sequence of drug administration when combined with ionizing radiation. Downregulation of EGFR is associated with that of the AKT pathway that is known to activate the antiapoptotic signaling. Another possibility is that the observed cell killing or the decreased colony survival in the data could be because of other modes of cell death, such as mitotic catastrophe.

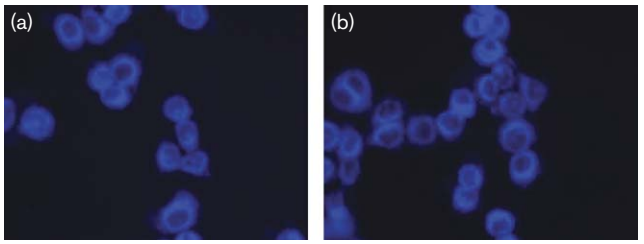
Triazene molecules are extremely sensitive to radicals that can cleave the triazene linkage. Therefore, ZRBA1 being a triazene was tested whether its combination with radiation known to induce the formation of hydroxyl radical would affect intracellular decomposition. Combimolecules of the same class as ZRBA1 are known to decompose in the cells into a fluorescent aminoquinazoline (e.g. FD105). Thus, we sought to verify whether the

Fig. 9



A potential degradation pathway of ZRBA1 by radiation-generated hydroxyl radical.

Fig. 10



Analysis of blue fluorescence generated by ZRBA1 in the intracellular compartment by fluorescence microscopy. (a) Cells treated with ZRBA1 alone. (b) Cells treated with ZRBA1 and radiation (4 Gy).

ZRBA1 decomposition would be affected by radiation. It seemed that the distribution of ZRBA1 in the presence or absence of radiation was identical, indicating that ionizing radiation had no effect on the chemical decomposition or the biodistribution of ZRBA1, a debility that could affect its development as a radiomodulator. This is further corroborated by the fact that, as shown in this study, the combination increases the levels of DNA lesions incurred by the cells in the presence of radiation.

In summary, this study conclusively showed that a combi-molecule of the type of ZRBA1 can be used to enhance radiation-induced cytotoxicity in a sequence-dependent manner by increasing the levels of DNA damage and cell cycle arrest in G_2/M. These results set premise for further investigation on the molecular mechanism underlying the observed effect and the demonstration of the efficacy of this novel type of combination *in vivo*.

Acknowledgements

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