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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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

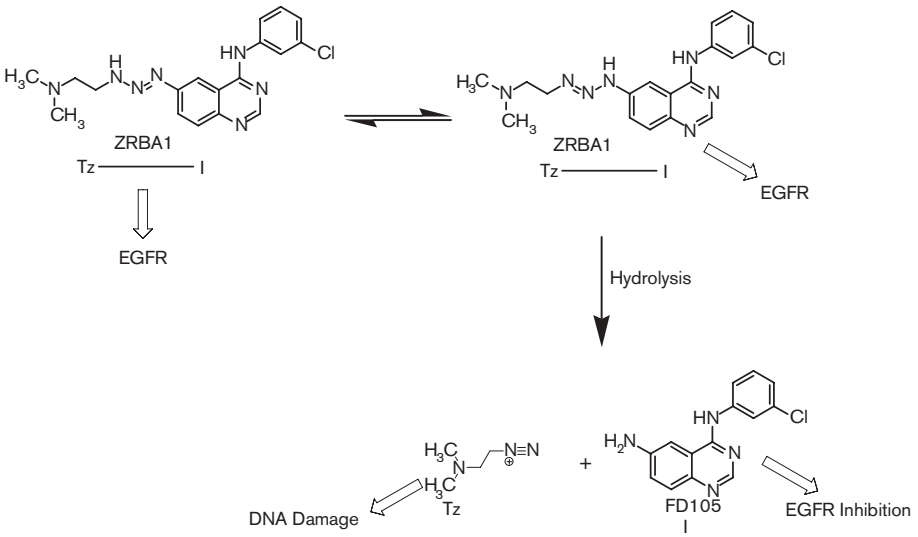
Reagents

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

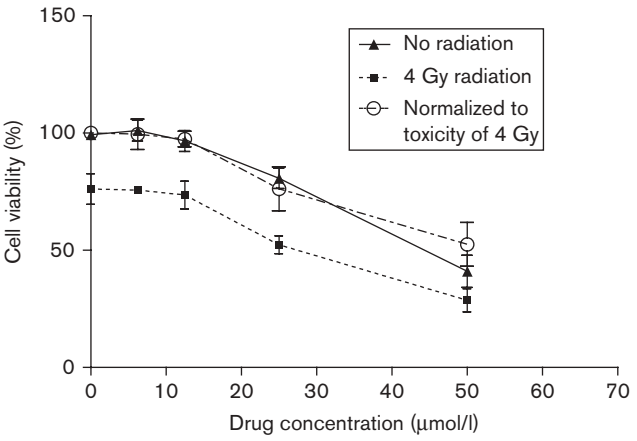
Growth inhibitory effect

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).

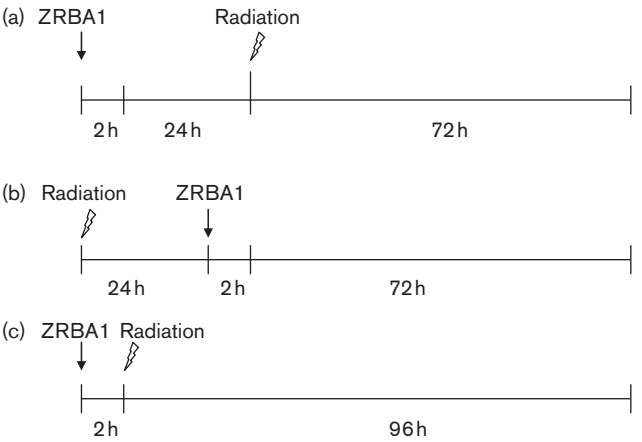
ZRBA1, being an alkylator, generated the same alkylating lesions as temozolomide, which in earlier studies have been shown to enhance radiation response in human tumors in a sequence-dependent manner [24]. Thus, we determined whether sequential administration of ZRBA1 and radiation would lead to different 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



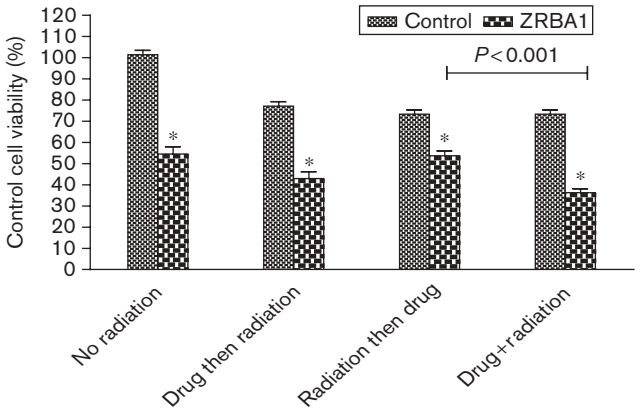
Sequences of administration of ZRBA1 and radiation in the various combinations. (a) MDA-MB-468 cells were treated with 36 μmol/l of ZRBA1 for 2 h and were irradiated 24 h later. They were further incubated for 72 h. (b) Cells were irradiated at 4 Gy of radiation. Twenty-four hours later they were treated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

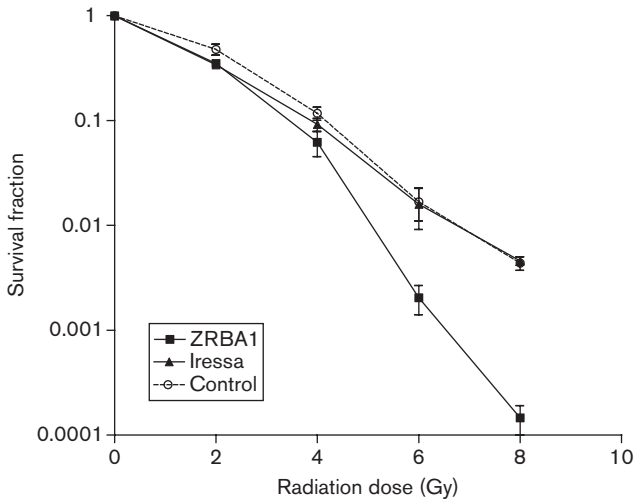
To confirm the significant results obtained from the MTT assay, a clonogenic study was conducted. ZRBA1 was administered (36 μmol/l) for 2 h and the dose range of radiation was applied (0–8 Gy). After 14 days, PE of control cells was approximately 20%. SFs at multiple radiation doses were calculated 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.

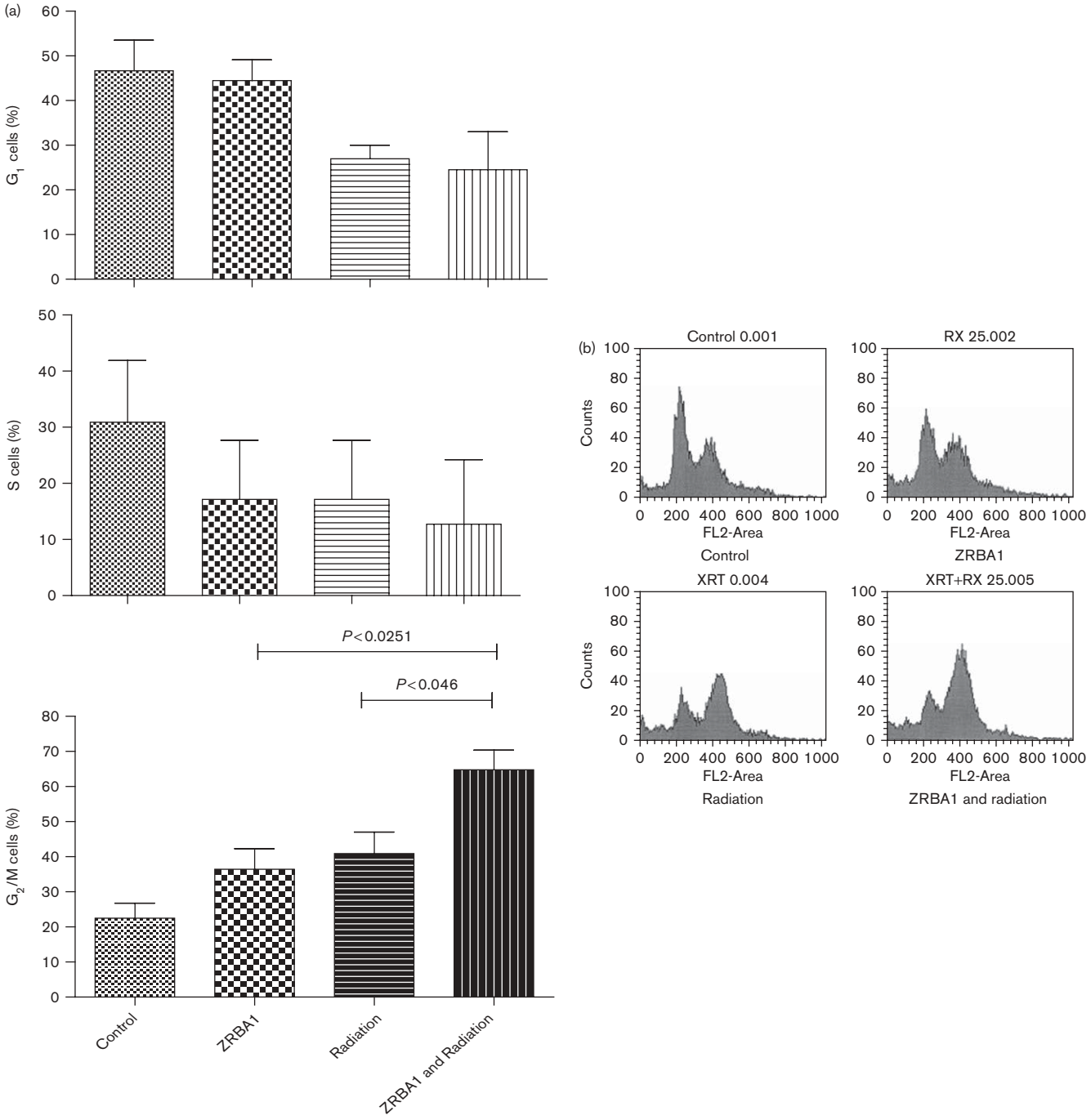
ZRBA1 biodistribution

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

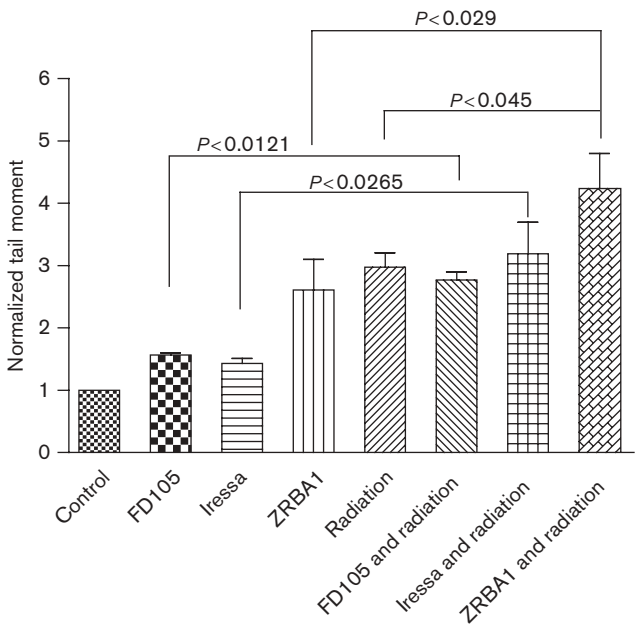
Fig. 6



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.

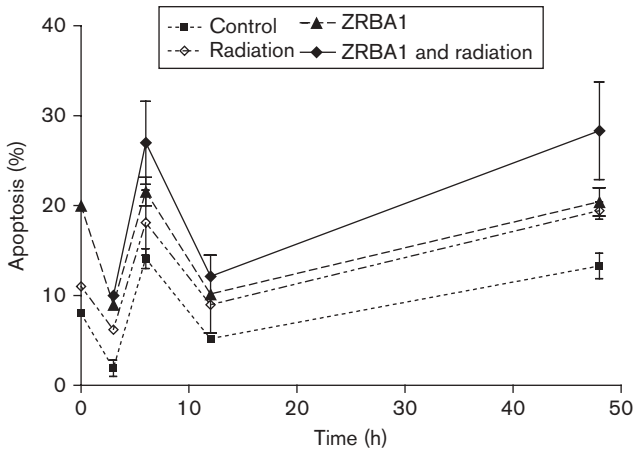
cytotoxic DNA-damaging agents. We have shown earlier that ZRBA1 was capable of downregulating EGFR-mediated signaling, damaging DNA and inducing significant levels of apoptosis in MDA-MB-468 cells [19]. In contrast, radiation has been shown to potentiate the action of temozolomide, an alkylating drug 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

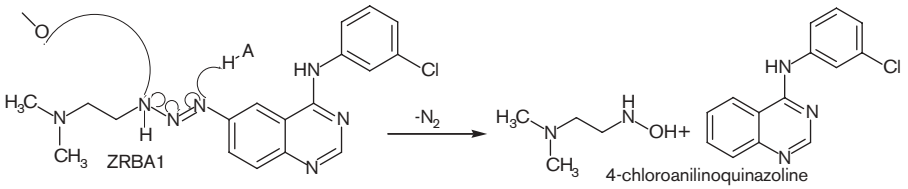


Time course analysis of apoptosis induced by ZRBA1 or radiation and corresponding combination in MDA-MB-468. Cells were treated with ZRBA1 (50 μmol/l) alone and in combination with radiation (4 Gy) and were harvested at the indicated time points. There are two peaks of apoptosis at 6 and 48 h post-treatment. 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.

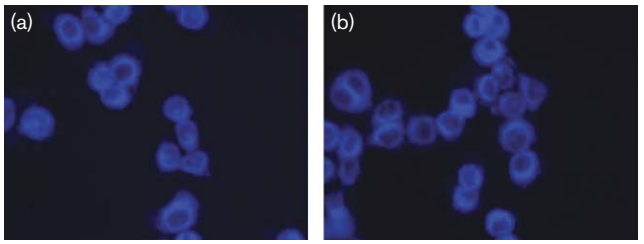
Triazene molecules are extremely sensitive to radicals that can cleave the triazene linkage. Therefore, ZRBA1 being a triazene was tested whether its combination with radiation known to induce the formation of hydroxyl radical would affect intracellular decomposition. Combimolecules of the same class 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*.

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