**Summary**

In this report, using a triple negative breast cancer model, we show that ZRBA1 could be used as a radiosensitizer. We demonstrate that the radiopotentiation effect of ZRBA1 is due to the increased level of DNA damage, delayed DNA damage repair process, and enhancement in apoptosis.

**Purpose:** ZRBA1 is a combi-molecule designed to induce DNA alkylating lesions and to block epidermal growth factor receptor (EGFR) TK domain. Inasmuch as ZRBA1 down-regulates the EGFR TK-mediated antisurvival signaling and induces DNA damage, we postulated that it might be a radiosensitizer. The aim of this study was to further investigate the potentiating effect of ZRBA1 in combination with radiation and to elucidate the possible mechanisms of interaction between these 2 treatment modalities.

**Methods and Materials:** 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, and DNA damage analysis were performed at multiple time points after treatment. To confirm our in vitro findings, in vivo tumor growth delay assay was performed.

**Results:** Our results show that a combination of ZRBA1 and radiation increases the radiation sensitivity of both cell lines significantly with a dose enhancement factor of 1.56, induces significant numbers of DNA strand breaks, prolongs higher DNA damage up to 24 hours after treatment, and significantly increases tumor growth delay in a syngeneic mouse model.

**Conclusions:** Our data suggest that the higher efficacy of this combination could be partially due to increased DNA damage and delayed DNA repair process and to the...
Introduction

The mechanism by which radiation eliminates the cells involves induction of multiple types of DNA damage. Thus, adding agents that form additional DNA damage or inhibit or delay the DNA repair process in tumors would improve the efficiency of radiation and the therapeutic ratio. Moreover, the acquired resistance to DNA damaging agents represents a major obstacle in the therapy of many tumors, including lung, breast, ovarian, and brain carcinomas (1-3).

With the advancement of molecular biology, various novel markers associated with reduced sensitivity to DNA damaging agents have been identified, including several receptor tyrosine kinases (RTKs) (1, 2, 4). One of these receptors is the epidermal growth factor receptor (EGFR), which could be activated by chemical and radiation-induced DNA damage (5, 6). Additionally, its overexpression has been associated with aggressive tumor progression, invasion, and reduced sensitivity to chemotherapy (7-11).

In locally advanced solid tumors, the combination of cytotoxic treatments such as chemotherapy and radiation has been shown to improve local control, organ preservation, and long-term survival. Furthermore, it has been shown that targeting more than 1 defect in a tumor cell could result in better tumor control and treatment response. Therefore, we have developed a binary targeting molecule, ZRBA1, designed to block EGFR TK activity and to induce DNA alkylating lesions (12). We have already demonstrated that the combination of ZRBA1 and radiation has higher potency in vitro compared with either treatment alone or with clinical EGFR inhibitors (12). It has already been shown that DNA alkylating agents are able to potentiate the radiation response because of their ability to increase the degree of radiation-induced DNA double strand breaks (DSBs) in the cells (13, 14). Inasmuch as ZRBA1 was designed to induce alkylated DNA lesions of N,N-dimethylaminoethylguanine, we hypothesized that its combination with radiation will increase the levels of DNA strand breaks and influence the DNA repair process.

Here we aimed to elucidate the mechanism of action of the combined treatment of radiation with ZRBA1 in triple negative breast cancer models in vitro and in vivo.

Methods and Materials

Reagents

The cell culture reagents were obtained from Gibco, Invitrogen. Fetal bovine serum (FBS) was purchased from Wisent Inc, ZRBA1 was synthesized according to the previously described methods (15), and Iressa (Gefitinib, ZD 1839) was purchased from Selleck Chemicals LLC. ZRBA1 was reconstituted in dimethylsulfoxide for in vitro use.

Cell culture

Human MDA-MB-468 breast carcinoma cells were obtained from the American Type Culture Collection, #HTB-132, Manassas, VA. The highly metastatic mouse mammary cancer cell line, 4T1, was a generous gift from Dr Fred Miller (Karmanos Cancer Institute, Wayne State University, MI) (16). The cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin 1% and kept at 37°C in 95% air/5% CO2.

Irradiation

Irradiation for in vitro and in vivo experiments was carried out at room temperature using Theratron T-78060Co irradiator (MDS Nordion, Kanata, ON, Canada). The dose delivered (0.65 Gy/min) to each experimental setup used in this work was verified by radiochromic film dosimetry (17).

Colony-forming assay

Cells were plated at specific cell numbers treated with ZRBA1 (18 μM) and 2 hours later irradiated and analyzed as described previously (12). Radiosensitivity was measured by determining the dose enhancement factor (DEF), which is the ratio of the radiation doses at survival fraction of 0.1 of non–drug-treated cells to drug treated cells (Supplementary Methods, available online at www.redjournal.org) (12, 13, 18).

Western blot analysis

The MDA-MB-468 and 4T1 cells were treated with 18 and 60 μM of ZRBA1, respectively, for 2 hours, or irradiated (4 Gy) or received both treatments. The cells were harvested, and the whole cell lysates were separated on acrylamide gels, transferred to PVDF membranes, and incubated with primary antibodies as described (Supplementary Methods, available online at www.redjournal.org).
DNA damage analysis

Comet assay
The MDA-MB-468 and 4T1 cells were treated as above and harvested, and the alkaline (pH $\geq 13$) and modified neutral (pH = 8) comet assays were performed as described (19).

Flow cytometry
MDA-MB-468 cells were treated as before and washed with PBS, and the flow cytometry protocol was followed (Supplementary Methods, available online at www.redjournal.org). Flow cytometry was performed using BD FACSCaliber (BD Biosciences) and analyzed by FlowJo software (FlowJo).

In vivo tumor growth delay assay
Female BALB/c mice (Charles River Laboratories, Montréal, Canada), 6 to 8 weeks old, were caged in groups of 5 or fewer. The 4T1 tumor cells (1 x $10^6$ cells) were injected subcutaneously into the upper right hind leg. When tumors reached a mean volume of 100 mm$^3$, mice were randomized into 6 groups according to the treatment they were scheduled to receive (Supplementary Methods, available online at www.redjournal.org). Tumors were followed up until the mean tumor volume reached $\sim$1500 mm$^3$, after which the animals were killed. The relative tumor volume was calculated by dividing each individual animal’s tumor volume by the mean tumor volume of the same group.

Results

ZRBA1 increases the sensitivity of MDA-MB-468 and 4T1 cells to radiation in vitro
To determine the effects of ZRBA1 on breast tumor cell radiosensitivity, clonogenic survival analysis was performed on the MDA-MB-468 and 4T1 cells. Pretreatment of cells with ZRBA1 (Fig. 1) increased the radiosensitivity of both types of cells in a dose-response manner. As demonstrated, the DEF was as high as 1.56 and 2.08 in MDA-MB-468 cells (at 18 and 22 $\mu$M) and 1.50 and 1.80 in 4T1 cells (60 and 75 $\mu$M), respectively. The MDA-MB-468 cells generally showed more sensitivity towards ZRBA1 compared with the 4T1 murine cell line, which ZRBA1 radiosensitizes the cells and induces DNA breaks (21).

ZRBA1 combined with radiation induces DNA double and single strand breaks
To examine the effect of ZRBA1, radiation, or both on the induction of DNA damage, alkaline and neutral comet assays were performed to measure single (SSBs) and double strand breaks (DSBs), respectively. As shown in Figure 3a and b, cells treated with ZRBA1 or radiation alone had twofold more DNA double strand breaks at 1 hour compared with the nontreated cells, in both MDA-MB-468 and 4T1 cells. Interestingly, the combined treatment with ZRBA1 and radiation induced almost fourfold more breaks compared with the nontreated cells in both cell lines.

Combination of ZRBA1 and radiation delays the DNA repair process
To study the DNA repair process, alkaline and neutral assays were also performed 24 hours after treatment. Interestingly, we were able to show that the levels of DNA breaks remained at a high level even after 24 hours when cells were treated with both ZRBA1 and radiation.

Although there was a significant difference between the DNA breaks of combined treated cells 24 hours after treatment versus cells treated with either ZRBA1 or radiation alone in both neutral and alkaline conditions, this effect was more prominent in the neural comet assay showing DSBs (Fig. 3a, b).

Furthermore, to better understand the mechanism by which ZRBA1 radiosensitizes the cells and induces DNA DSBs, we measured the level of phosphorylation of ATM (Ser1981) and histone H2AX (Ser193-γH2AX), which are known surrogate markers of DNA damage response and DSBs formation, respectively (21). The combination of ZRBA1 and radiation increases the phosphorylation of both ATM and H2AX (Fig. 4a) when compared with 2 agents alone and remains almost stable after 24 hours (ZRBA1 + IR, 1 hour vs 24 hours, $P$ = .062 and $P$ = .158 for ATM and H2AX, respectively). Moreover, immunofluorescent staining for γH2AX revealed that cells exposed to combination treatment contained more γH2AX foci per cell, and the number of cells that retained the foci increased compared with both treatments alone (Fig. E1, available online at www.redjournal.org).

These results all indicate that ZRBA1 radiosensitizes cells by induction of additional DNA damage, possible
delay of DNA DSBs repair, or both. Therefore, we examined the activity of DNA-PKcs, a key component of the NHEJ pathway. In response to DNA damage, DNA-PK is activated or phosphorylated at multiple sites, including autophosphorylation at Ser2056 (22). ZRBA1 and radiation treatment resulted in a significant increase in DNA-PKpS2056 (Fig. 4a), especially in the combined treatment group, where it remained unchanged up to 24 hours after treatment ($P= .91$ for 1 hour vs 24 hours). Interestingly, after 24 hours of incubation, the majority of the DNA-PKcs activity was localized in the S and G2/M phases of the cell cycle and correlated with the levels of ATM-pSer1981 and γH2AX (Fig. 4b). Additionally, the elevated activity of DNA-PKcs in S and G2/M phases of the cell cycle suggests that the NHEJ pathway is highly activated in response to ZRBA1/radiation treatment. To examine homologous recombination (HR) activity, Western blot analysis was performed to observe the effect of ZRBA1 treatment on the level of RAD51, BRCA1, and BRCA2 proteins (23). Our results showed increased expression of BRCA2 protein 6 hours and 24 hours after the ZRBA1/radiation treatment, but no changes in levels of Rad51 or BRCA1 were detected (Fig. 4c).

**ZRBA1 increases the tumor growth delay caused by radiation**

To evaluate and validate our in vitro results, we performed an in vivo experiment with 4T1 mouse mammary cancer cells. The combination of ZRBA1 and radiation was the most effective treatment in controlling tumor growth, not only in comparison with either treatment alone but also in comparison with the combination of radiation and Iressa (Fig. 5a) (Table E1, available online at www.redjournal.org). The in vivo results suggest that ZRBA1 enhances the tumor growth delay caused by radiation in mice treated with the combination therapy by 21 days (Table 1). Furthermore, ZRBA1 combined with ionizing radiation had significantly more antitumor effect against 4T1 tumors than ZRBA1 or

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**Fig. 1.** Analysis of response of (a) MDA-MB-468 cells and (b) 4T1 cells to the combination of ZRBA1 and radiation using clonogenic assays. Before irradiation, cells were exposed for 2 hours to 18 μM ZRBA1, and colony-forming efficiency was determined as described in Methods and Materials. Data represent means and standard deviation from 3 independent experiments. DEF = dose enhancement factor.

**Fig. 2.** ZRBA1 inhibits EGFR phosphorylation and downstream MAPK pathway signaling. MDA-MB-468 cells were serum starved for 18 hours and treated with ZRBA1, radiation (RT), or both, or stimulated with epidermal growth factor (EGF) as indicated. Cell lysates were prepared within 1 hour and analyzed by Western blot.
radiation alone, inasmuch as the tumor-free rate (fraction of the number of the mice without the tumor at 48 days after the treatment out of the total number of mice with the tumor before the treatment) was 20% for groups that received single-modality treatments and 60% for the combined treatment group. Interestingly, in the group that received Iressa alone or in combination with radiation, we did not observe any tumor elimination.

As summarized in Table 1, tumor growth delay was increased from 11.5 days in the control group to 14 days in the ZRBA1-alone group, 26 days in the radiation-alone group, and 47 days in the group with combined ZRBA1/radiation. No significant body weight loss resulted from any of the treatments, and treatments were well tolerated by the end of the experiment (Fig. 5b).

**Discussion**

ZRBA1 is designed to induce N7- and O6-alkylated lesions in a manner similar to temozolomide, which has been shown to radiosensitize cells mainly because of its ability to increase the degree of radiation-induced DNA DSBs (13, 14). Therefore, we hypothesized that the combination of ZRBA1 with radiation might not only increase the level of DNA damage but also, because of its EGFR TKI activity, significantly improve cancer cell killing.

In this study we have shown that ZRBA1 potentiates the radiation response in breast cancer model both in vitro and in vivo. The mechanism involved in this optimized combination could be partially explained by the induction of DNA damage (SSBs and DSBs) and inhibition of MAPK pathway through the inhibition of EGFR and its downstream signaling cascade (Figs. 2 and 3).

We previously reported that the combination of ZRBA1 and radiation arrests the cell cycle at the G2/M phase and increases the level of apoptosis (12). Notably, apoptosis was delayed for 2 days in MDA-MB-468 cells, whereas in 4T1 cells it was visible already 24 hours after the treatment (Fig. E2, available online at www.redjournal.org). Here, we show that cells that underwent the combined treatment after 24 hours had a significantly higher level of DNA breaks, especially DSBs as measured by comet assay and the
induction of γH2AX. Additionally, FACS and microscopic analysis show that 1 hour after treatment, the level of γH2AX was highest in the group receiving combined treatment and remained almost unchanged up to 24 hours after treatment. This could explain the observed G2/M cell cycle arrest and delayed apoptosis detected starting on day 2 after treatment in MDA-MB-468 cells.

As mentioned above, although the SSBs were partially reduced or repaired 24 hours after treatment (although still higher than any single therapy), the level of DSBs stayed the same for the entire time of the assay. The most probable explanation could be that although ZRBA1 as an alkylating agent does not directly induce DSBs, these lesions can be formed during processing of DNA alkylating lesions,

**Fig. 4.** DNA double-strand breaks repair analysis. (a) Flow cytometric analysis of level of phosphorylated ATM (Ser1981), H2AX (Ser193), and DNA-PKcs (ser2056) in MDA-MB-468 cells. Fluorescence intensity indicates the relative amount of phosphorylation of proteins 1 hour and 24 hours after treatment. (b) Distribution of ATM (Ser1981), H2AX (Ser193), and DNA-PKcs (ser2056) throughout the cell cycle 24 hours after treatment. (c) Analysis of the same cells by Western blot to determine levels of BRCA1, BRCA2, and Rad51 proteins.

**Fig. 5.** (a) Tumor growth delay assay. ZRBA1 started to be given to the animals 3 days before radiation, on the same days of irradiation, and continued for 1 day after irradiation. Radiation was delivered in 3 fractions of 5 Gy. Each experimental group contained 5 mice. Tumor volume was calculated by \((L \times W^2)/2\) and normalized by dividing the tumor volume of each animal in treatment groups by the mean tumor volume of the same group. (b) Variations of body weight of mice treated with ZRBA1 or Iressa and radiation alone and the combined treatments. Error bars = standard equivalent of the mean.
leading to the formation of SSBs, which subsequently, during cell division, are transformed into DSBs (24). Hence, the high level of DSBs is still visible at 24 hours in the groups receiving combined and ZRBA1 treatment compared with the group receiving radiation only, in which the breaks tend to be repaired (Figs. 3a, 4). Additionally, as a confirmation in both S and G2/M cell cycle stages, we observed an increased activation of DNA damage response and DNA DSB repair as measured by ATMSer1981 and DNA-PKcsSer2056 phosphorylation, respectively. Moreover, the increased activity of DNA-PK in the S phase suggests that the majority of the damage induced by the combined treatment is repaired by the NHEJ pathway. Our findings are consistent with previously published results and the presence of fast and slow repair components and their contribution in the repair of DNA DSBs (25).

Using Western blot analysis, we did not observe any changes in RAD51 and BRCA1 levels. However, this does not rule out involvement of the HR pathway in the repair of DSBs caused by ZRBA1 and radiation, as we have shown increased levels of BRCA2, especially after 6 hours and 24 hours (Fig. 4c). Although BRCA2 has shown to be the specific protein involved in the HR pathway, BRCA1 is involved in several pathways, and thereby its expression can be affected by several factors. Moreover, as demonstrated, deficiency in BRCA1 does not change the radiosensitivity of cells, whereas Brca2-deficient cells are sensitive to radiation (26, 27).

Furthermore, we cannot exclude the possibility that the observed delay in DNA repair process could be related to EGFR inhibition. As reported previously, EGFR translocates to the nucleus in response to radiation and interacts with and augments the activity of DNA-PKcs (28). Additionally, Kriegs et al (29) demonstrated that EGFR modulates DSB repair by regulating NHEJ via the MAPK signaling pathway. Importantly, this regulation was affected only when ERK1/2 was inhibited but not when AKT was knocked down. In fact, as we have demonstrated, ZRBA1 efficiently inhibits phosphorylation of EGFR and its downstream signaling, including ERK1/2 and BAD, and only to some extent AKT (Fig. 2).

Next, we investigated whether the combined treatment would effectively inhibit tumor growth in vivo. 4T1 cells, a syngeneic mouse cancer model, have been used in various studies and are considered to be among the appropriate models of human breast cancer. Moreover, their molecular characteristics are comparable to those of the triple negative breast tumor cells, such as MDA-MB-468 cells with normal levels of expression of EGFR (30, 31). Unlike other breast cancer subtypes, triple negative types do not have the option of targeted therapy such as an antiestrogen regimen or Herceptin because of their lack of receptor expression. Clinically, triple negative breast cancer is associated with early relapse and poorer long-term outcome. Therefore, there is a pressing need to develop treatment strategies to better control this type of breast cancer.

In this study, we applied a fractionated schedule for radiation delivery in vivo because it would be more clinically relevant. The in vivo data (Fig. 5a) showed a significant increase in tumor growth delay in the mice treated with radiation and ZRBA1; importantly, we observed 60% complete response. As shown in Figure 5a, the groups treated with ZRBA1 or Iressa only did not show any significant sensitivity to the treatments. The group receiving radiation and a combination of radiation and Iressa also did not show any significant difference in terms of tumor growth, suggesting that the addition of Iressa did not sensitize the 4T1 cell to the radiation.

With the advancement of cellular and molecular biology, targeting molecules for cancer therapy has moved rapidly in the broad context of oncology therapeutics. Despite all the efforts and rapid progress in the development of novel therapeutics, many patients still do not respond to molecular inhibitors or eventually will experience disease progression because of their intrinsic or acquired resistance to therapy. However, the use of multitargeting inhibitors would have the advantage over the classic inhibitors because they can also be effective in patients having subtypes of cancer with different mutations, patients with nonhomogenous tumors, and patients with acquired resistance to monотargeting therapy (32). In conclusion, as we have shown in this study, the combined therapy of multitargeting agents similar to ZRBA1 with radiation can enhance cytotoxic therapy and tumor response, and the treatment is well tolerated. Our encouraging preclinical results demonstrate a significant response in the combined modality arm and a strong indication for confirmatory xenograft studies and in phase I clinical trials in patients with triple negative breast cancer.

References


Table 1 Tumor growth delay of each treatment group

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Days after treatment</th>
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<tbody>
<tr>
<td>Control</td>
<td>11.5</td>
</tr>
<tr>
<td>Iressa</td>
<td>13</td>
</tr>
<tr>
<td>ZRBA1</td>
<td>14</td>
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<tr>
<td>Radiation</td>
<td>26</td>
</tr>
<tr>
<td>Radiation + Iressa</td>
<td>27.5</td>
</tr>
<tr>
<td>Radiation + ZRBA1</td>
<td>47</td>
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Mice that received the combined treatments had a growth delay almost 2 times and 3 times more than the irradiated-only and ZRBA1-only treated groups, respectively (47 vs 26 days and 14 days).


