The Combi-Targeting Concept: Selective Targeting of the Epidermal Growth Factor Receptor- and Her2-Expressing Cancer Cells by the Complex Combi-Molecule RB24

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ABSTRACT

Within the context of a new tumor-targeting strategy termed “combi-targeting,” we designed RB24 to inhibit epidermal growth factor receptor (EGFR) or Her2 phosphorylation and to further degrade upon hydrolysis to 4-(3’-bromophenylamino)-6-aminoquinazoline (RB10; another EGFR/Her2 inhibitor) plus a strong DNA-alkylating species. 6-(3-Acetoxyethyl-3-methyltriazenyl)-4-(3’-bromophenylamino)quinazoline (RB24) showed significant antiproliferative activity against human breast cancer cells, and transfection of one such cell line, MDA-MB-435, with ErbB1 or ErbB2 (Her2) dramatically enhanced cell death by apoptosis. RB24 was capable of releasing 2- to 3-fold higher levels of RB10 in the transfectants than in their wild-type counterparts. More importantly, RB10 was abundantly distributed in the perinuclear region of the cells, and its elevated levels in the ErbB transfectants were concomitant with increased levels of DNA lesions in the latter cells. This selectivity could be abolished by coincubation of the cells with exogenous RB10, suggesting that the entire combi-molecule may bind primarily to its cognate perinuclear sites before degradation. This localization may exert a bystander effect, allowing the alkylating species to be abundantly propagated into the nucleus. Cell response to this novel targeting mechanism was mediated by 1) activation of c-Jun NH2-terminal kinase in response to DNA damage and 2) down-regulation of Bad through blockade of EGFR tyrosine kinase activity; two events that cooperatively converged into enhancement of apoptosis in the oncogene-transfected cells.

Members of the epidermal growth factor (EGF) family of receptors, including EGFR, Her2 (ErbB2), and EGFRvIII (truncated EGFR), are overexpressed in 20 to 40% of breast cancers (Tang et al., 2000). The Her2 receptor, the closest EGFR homolog, is overexpressed in 20 to 25% of overall breast cancers, 60 to 70% of ductal carcinoma in situ, and is often associated with highly aggressive tumor phenotypes and poor disease-free survival (Slamon et al., 1989; Arteaga, 2001). For example, the average survival of breast cancer patients with Her2-positive tumors is 2 to 3 years compared with 6 to 7 years of Her2-negative tumors (Slamon et al., 1987; Borg et al., 1990). More importantly, this dysfunction is also associated with resistance to endocrine therapy and chemotherapy (Slamon et al., 1987). The significant implication of the ErbB family members in tumor progression has made them important targets for breast cancer therapy. Indeed, blockade of EGFR or Her2 has proven highly effective in breast xenograft models and in the clinic (Ciardiello et al., 1999; Molder et al., 2001). Although gefitinib (Iressa, ZD1839) that targets the EGFR tyrosine kinase (TK) has shown moderate activity in breast cancer models, trastuzumab (Herceptin), a monoclonal antibody directed toward Her2, has demonstrated significant activity in advanced
breast carcinomas (von Minckwitz et al., 2005; Tokunaga et al., 2006). Its effectiveness is such that it is now being evaluated for adjuvant breast cancer therapy. However, despite its significant potency against Her2-positive breast tumors, its efficacy in patients who express EGFRvIII with loss of PTEN function remains to be demonstrated. As a corollary to the great potency of trastuzumab, strategies are now being developed to enhance its potency in breast tumors using combinations with cytotoxic drugs (Ciardiello et al., 2006).

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

Activation of EGFR and Her2 leads to the recruitment of multiple adaptor proteins such as Grb2, followed by activation of the Ras-Raf mitogen-activated protein kinase pathway and subsequent expression of genes that regulate cellular growth and proliferation (McCubrey et al., 2006). More importantly, activation of the latter receptors prevents apoptosis through induction of the phosphatidylinositol 3-kinase (PI3K) and its downstream target Akt, a sequence of events that leads to inactivation of the proapoptotic protein Bad, a member of the Bcl-2 family of proteins (Stauffer et al., 2005; McCubrey et al., 2006).

In contrast, cell exposure to cytotoxic agents is often associated with induction of apoptotic signaling through stress-activated protein kinases such as c-Jun NH2-terminal kinase (JNK) that is known to interact with members of the Bcl-2 family (Huang et al., 1999; Ohtsuka et al., 2003). Based upon these mechanisms, we surmised that molecules capable of inducing DNA damage while blocking receptor-mediated signaling should induce significant levels of apoptosis and cell killing in cancer cells expressing these receptors. It is more important to note that the molecules being designed to penetrate the cell intact can bind to the ATP site of the TK domain before releasing their DNA-damaging fragments, thereby targeting them to cells that overexpress these receptors. Here, we study the mechanism of action of one such molecule, RB24, which possesses the following properties: 1) it has a half-life comparable with that of the clinical methylating agent temozolomide [Temodal; (TEM) (t1/2 = 45 min in serum)]; 2) it blocks EGFR TK activity both in enzyme and whole-cell phosphorylation assays; and 3) it has been shown to induce significant levels of DNA damage in tumor cells. This study sought to demonstrate its ability to selectively induce antiproliferative activity in cancer cells that express or are transfected to express the target receptors and to analyze the implication of cell signaling in its targeting mechanism. RB24 is the 3′-bromo analog of SMA41 (Scheme 1) that, like TEM, has been shown to alkylate DNA at the O6- and N7-positions of guanine (Matheson et al., 2003, 2004). As outlined in Scheme 1, RB24 is hydrolyzed to ZR08 (Scheme 1) that further degrades into RB10 (an inhibitor of EGFR) and a methyl diazonium species that alkylates DNA (Banerjee et al., 2003). Although TEM can generate the methyl diazonium species, it cannot inhibit EGFR.

It is to be noted that a controversy is still ongoing about the origin of one of the cell line MDA-MB-435 used in this study. It is suggested that the latter cell type is not a breast cancer cell line but a melanoma cell type based on expression array studies (Rae et al., 2007). However, a recent analysis by Chambers (2009) suggests that the MDA-MB-435 cell line

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<td>Antiproliferative effects of RB24, RB10, gefitinib, and TEM in ErbB-negative cell line MDA-MB-435 and the ErbB-expressing cell lines MDA-MB-468 and MDA-MB-453</td>
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<td>Compound</td>
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N.D., not determined.
Fig. 1. Selective delivery of the combi-molecule RB24 in ErbB-transfected MDA-MB-435 breast cancer cell lines. A, MDA-MB-435, MDA-MB-435/EGFR, and MDA-MB-435/ErbB2 cells were preincubated for 2 h with either RB24 or TEM, and levels of DNA damage were quantitated using the alkaline comet assay. Tail moment (i.e., the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet) was used as a parameter for the detection of DNA damage. \( P < 0.05 \) (Student’s t test). Data represent means of three independent experiments; bars are S.D. B, MDA-MB-435 and MDA-MB-435/ErbB2 cells were exposed to serum alone (a and e) or serum + RB24 (25 \( \mu M \)) (c and g) for 2 h before observation of levels of RB10 by fluorescence microscopy (400× magnification). C, UV flow cytometric analysis of intracellular levels of RB10 released in MDA-MB-435, MDA-MB-435/EGFR, and MDA-MB-435/ErbB2 cells after 2-h exposure to RB24. Points are mean percentage of untreated cells (\( n = 2 \)); bars are S.D.

TABLE 2


For MDA-MB-435, MDA-MB-435/EGFR, and MDA-MB-435/ErbB2, values are means of IC\(_{50}\) values and S.E.M.s from three experiments.

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<td>RB24</td>
<td>7 ± 2.4</td>
<td>1 ± 0.4</td>
<td>7</td>
<td>0.8 ± 0.2</td>
<td>8.8</td>
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<tr>
<td>RB10</td>
<td>41 ± 2.1</td>
<td>9 ± 1.4</td>
<td>4.5</td>
<td>14 ± 1.3</td>
<td>3</td>
</tr>
<tr>
<td>TEM</td>
<td>18 ± 1.3</td>
<td>&gt;50</td>
<td>4.4</td>
<td>&gt;100</td>
<td>3</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>48 ± 1.9</td>
<td>11 ± 2.1</td>
<td>4.4</td>
<td>21 ± 1.8</td>
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Mechanism of Selective Targeting by Combi-Molecules
is an aggressive, poorly differentiated breast cancer cell line expressing epithelial and melanocytic markers. Nevertheless, in this study, the MDA-MB-435 cell line is used for not expressing a DNA enzyme that repairs $O^6$-methylating lesion in DNA, the $O^6$-methylguanine DNA methyl transferase and for being PTEN-proficient: two properties that characterize sensitivity to methylating agents and EGFR inhibitors, respectively.

Materials and Methods

Drug Treatment

RB24, RB10, and JDA41 were synthesized in our laboratory according to known procedures (Banerjee et al., 2003; Domarkas et al., 2006). Temozolomide was provided by Schering Plough (Kenilworth, NJ). Gefitinib was provided by AstraZeneca Pharmaceuticals LP (Wilmington, DE). In all assays, drug was dissolved in DMSO and subsequently diluted in DMEM containing 10% fetal bovine serum (FBS) (Wisent Inc., St-Bruno, QC, Canada) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

Cell Culture

The cell lines used in this study, the human carcinoma cells MDA-MB-435, MDA-MB-435/EGFR (MDA-MB-435 cells stably transfected with the EGFR gene), and MDA435/HER2 (MDA-MB-435 cells stably transfected with the HER2 gene) were generous gifts from Dr. Moulay Aloui-Jamali (Montreal Jewish General Hospital, Montreal, QC, Canada). The human breast cancer cell lines MDA-MB-468 and MDA-MB-453 were obtained from the American Type Culture Collection (Manassas, VA). All MDA-MB-435 cells (transfected and nontransfected) were maintained in DMEM supplemented with 10% fetal bovine serum. MDA-MB-468 and MDA-MB-453 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics as described previously (Matheson et al., 2001). All cells were maintained in an atmosphere of 5% CO$_2$.

Growth Inhibition Assay

For nonstimulated cell growth inhibition, approximately $10^4$ cells/well were plated in 96-well plates. After a 24-h incubation at 37°C in a humidified environment of 5% CO$_2$, cell monolayers were exposed to different concentrations of each drug continuously for 6 days, and all growth inhibitory activities were evaluated using the sulforhodamine B assay (Skehan et al., 1990). In brief, after drug treatment, cells were fixed using 50 μl of ice-cold trichloroacetic acid (50%) for 60 min at 4°C, washed four times with tap water, and stained for 30 min at room temperature with sulforhodamine B (0.4%) dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid and allowed to air dry. The resulting colored residue was dissolved in 200 μl of Tris base (10 mM), and optical density was read for each well at 540 nm using a microplate reader (model 2550; Bio-Rad Laboratories, Hercules, CA). Each point represents the average of at least two independent experiments run in triplicate.

Autophosphorylation Assay

EGFR or Her2 Activation. MDA-MB-435/EGFR or MDA-MB-435/ErbB2 cells ($1 \times 10^6$) were plated in six-well plates with 10% serum at 37°C for 24 h and starved overnight for 24 h, after which they were exposed to a range of drug for 2 h and subsequently treated for 15 min with 50 ng/ml EGF or 50 ng/ml HGF, respectively. Cells were washed with PBS and resuspended in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 mM NaF, 1 mM Na$_3$VO$_4$, and protease inhibitor tablet (Roche Biochemicals, Laval, QC, Canada)]. The lysates were kept on ice for 30 min and collected by centrifugation at 10,000 rpm for 20 min at 4°C. The concentrations of protein were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories). Equal amounts of protein were added to a 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Nonspecific binding on the membranes was minimized with a blocking buffer containing nonfat dry milk (5%) in PBS-Tween 20. Thereafter, the membranes were incubated with primary anti-phosphotyrosine antibody (Millipore) for the detection of phosphotyrosine. Anti-phospho-EGFR (Tyr1068) was used to detect EGFR autophosphorylation and anti-phospho-ErbB2 (Tyr1221/1222) for analyzing direct phosphorylation of ErbB2. Membranes were stripped and reprobed with anti-EGFR (Neomarkers, Fremont, CA) or anti-ErbB2 (Millipore) for determination of corresponding receptor levels. Phosphospecific Blots were incubated with horseradish peroxidase goat anti-mouse antibody (1:1000 dilution; Cell Signaling Technology Inc., Danvers, MA), and the bands were visualized.
with an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) (Matheson et al., 2001).

**AKT and Bad Activation.** Inhibition of Bad activation was determined by exposing serum-starved MDA-MB-435/EGFR or MDA-MB-435/ErbB2 cells for 2 h with the indicated concentrations of RB24 or TEM before stimulation with EGF (50 ng/ml) or HRGα (50 ng/ml), respectively. Equal amounts of cell lysates were analyzed by Western blot using anti-phospho-Bad (Ser136) or anti-phospho-Akt (Ser473), antibodies (Cell Signaling Technology Inc.). Membranes were stripped of antibody and reprobed with anti-Bad antibodies (Cell Signaling Technology Inc.).

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

**Flow Cytometric Analysis of Intracellular Fluorescence and the Cell Cycle**

MDA-MB-435, MDA-MB-435/EGFR, and MDA-MB-435/ErbB2 cells were seeded in DMEM with 10% FBS for 24 h and grown in six-well plates (1 × 10⁶/well). Cells were then treated with varying concentrations of RB24 and 10% FBS at 37°C for 2 h. Thereafter, cells were harvested with trypsin-EDTA, subsequently collected by centrifugation, and then resuspended in PBS. Fluorescence levels were measured using the BD LSR flow cytometer (BD Biosciences, Oakville, ON, Canada).

For cell cycle analysis, the cells were exposed to either RB24 (25 μM) or TEM (25 μM) for 24 h and stained with propidium iodide (PI) after fixation with 70% ethanol. Fluorescence levels were detected by flow cytometry and cell cycle phase distribution analyzed using the ModFit software (Verity Software House, Topsham, ME).

**Fluorescence Microscopy Imaging for Intracellular Release of the Amine**

MDA-MB-435, MDA-MB-435/EGFR, and MDA-MB-435/ErbB2 cells were seeded in DMEM with 10% FBS and grown in six-well plates for 24 h (1 × 10⁶/well), and drug-containing media were replaced for fresh media in each well. Thereafter, plates were analyzed using an LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY), and cells were excited at 405 nm and emission was at 505 nm.

Colocalization experiments were performed using fluorescent EGFR and ErB2 antibodies in the MDA-MB-435/EGFR and MDA-MB-435/ErbB2 (Fig. 4, right) cells. In brief, the cells were exposed to 25 μM RB24 for 2 h and fixed with 3.7% paraformaldehyde, blocked with 5% normal goat serum, and stained with anti-EGFR and anti-ErbB2 primary antibody, followed by incubation with

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**Fig. 3.** Effects of competitive binding between RB24 and a non-DNA-damaging EGR TK inhibitor in the MDA-MB-435 isogenic cell line panel. A, MDA-MB-435, MDA-MB-435/EGFR, and MDA-MB-435/ErbB2 cells were preincubated for 2 h with a dose range of RB24 in combination with RB10 (40 μM), and the levels of DNA damage were quantitated using the alkaline comet assay. Tail moment (i.e., the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet) was used as a parameter for the detection of DNA damage. Points are mean percentage of untreated cells (n = 3); bars are S.D.
anti-rabbit-cy3 antibody for detection. A direct PE-labeled antibody was used to detect EGFR. The images were observed by fluorescence microscopy and merged with Photoshop (Adobe Systems, Mountain View, CA).

Alkaline Comet Assay for Quantitation of DNA Damage

The alkaline comet assay was performed as described previously (Matheson et al., 2001). The cells were exposed to drugs (RB24, RB10, TEM, or RB24 + RB10) for 2 h, harvested with trypsin-EDTA, subsequently collected by centrifugation, and then resuspended in PBS. Cell suspensions were diluted to approximately $10^6$ cells and mixed with agarose (1%) in PBS at 37°C in a 1:10 dilution. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, ON, Canada) using gel casting chambers, as described previously (McNamee et al., 2000), and then immediately placed into a lysis buffer (2.5 M NaCl, 0.1 M tetra-sodium EDTA, 10 mM Tris-base, 1% (w/v) N-lauryl sarcosine, 10% (v/v) DMSO, and 1% (v/v) Triton X-100, pH 10.0). After being kept on ice for 30 min, the gels were gently rinsed with distilled water and immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetrasodium EDTA, and 10 mM Tris-base) containing 1 mg/ml proteinase K for 60 min at 37°C. Thereafter, the gels were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and then electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed in 1 M ammonium acetate for 30 min. Thereafter, they were soaked in 100% ethanol for 2 h, dried overnight, and stained with SYBR Gold (1/10,000 dilution of stock supplied from Invitrogen, Carlsbad, CA) for 20 min. Comets were visualized at 330× magnification, and DNA damage was quantitated using the Tail Moment parameter (i.e., the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet). A minimum of 50 cell comets were analyzed for each sample, using ALKOMET version 3.1 image analysis software (Richard Brancker Research Ltd, Ottawa, ON, Canada).

Apoptosis and Cell Cycle Analysis

Cells were grown in six-well plates until confluence and then incubated with the compounds for 24 h. The cells were then harvested, washed twice with PBS, and then centrifuged. For apoptosis assay, cells ($10^6$) were treated with annexin V-FITC and PI using the apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA) and the supplier’s protocol. Annexin V-FITC and PI binding were analyzed by flow cytometry. Data were collected using logarithmic amplification of both the FL1 (FITC) and FL2 (PI) channels. Quadrant analysis of coordinate dot plots was performed with CellQuest software (BD Biosciences, San Jose, CA). Unstained cells were used to adjust the photomultiplier voltage and for compensation setting adjustment to eliminate spectral overlap between the FL1 and FL2 signals. For cell cycle analysis, cells were fixed with 70% ethanol after drug incubation and

Fig. 4. A, intracellular localization of RB10 released after 2 h exposure to RB24 alone (25 μM) or in combination with the nonfluorescent EGFR inhibitor JDA41 (40 μM) in MDA-MB-435/ErbB2 cells (400× magnification). B, MDA-MB-435/EGFR (left) and MDA-MB-435/ErbB2 (right) cells exposed to 25 μM RB24 for 2 h were fixed with 3.7% paraformaldehyde, blocked with 5% normal goat serum, and then stained with direct PE-labeled anti-EGFR and anti-ErbB2 primary antibody, followed by incubation with anti-rabbit-Cy3 antibody for detection. The images were observed by fluorescence microscopy, and overlay was analyzed by Photoshop.
stained with PI. Samples were run by flow cytometer and cell cycle profiles were analyzed by ModFit software.

**Results**

**Antiproliferative Activity of RB24, RB10, Gefinitib, and TEM.** The combi-molecule RB24 is designed to hydrolyze into various derivatives that inhibit EGFR TK activation, the most stable metabolite being RB10. As demonstrated previously, at the last stage of the decomposition pathway, concomitantly with the release of RB10, a methylidyldiazonium is formed that damages DNA (Banerjee et al., 2003). Likewise, TEM is also hydrolyzed in a stepwise manner to release the latter species that methylates DNA. However, its transient metabolites, 5-(3-methyltriaz-1-yl)imidazole-4-carboxamide (Scheme 1, MTIC) and 5-aminomidazole-6-carboxamide (Scheme 1, AIC), do not have any biological target, and we have already demonstrated that TEM is deprived of EGFR inhibitory activity (Mattheson et al., 2001). Therefore, TEM represents an appropriate comparative tool for our combi-molecule because it can only damage DNA but not block EGFR activation. Alternatively, gefitinib, a clinical EGFR TK inhibitor that does...

![Fig. 5](image-url)
not have any DNA-damaging potential (Giocanti, 2004), was used as an appropriate mechanistic control for this study. Thus, we first examined the antiproliferative activities of all these compounds, including the expected degradation product for RB24, RB10, in a panel of established breast cancer cell lines and directed the mechanistic studies at a subset of isogenic cancer cells, MDA-MB-435 and its ErbB transfectants MDA-MB-435/EGFR and MDA-MB-435/ErbB2, that do not express the DNA enzyme 6-methylguanine DNA methyl transferase and that are PTEN-proficient: two properties that characterize sensitivity to methylating agents and EGFR inhibitors, respectively. The results showed that RB24 is selectively more potent in cells overexpressing EGFR or Her2, with IC_{50} values ranging from 0.8 to 2.5 μM (Table 1). Evidence of this selectivity was strengthened by the response of the

Fig. 6. Effects of EGFR/Her2 inhibition by RB24 on anti-apoptotic signaling in ErbB-transfected MDA-MB-435 cell lines. a, serum-starved MDA-MB-435/EGFR or MDA-MB-435/ErbB2 cells were exposed to the indicated concentrations of RB24 for 2 h and subsequently stimulated with EGF (25 ng/ml) or HRGs (50 ng/ml), respectively. Equal amounts of lysate (100 μg) were analyzed by Western blotting using anti-phosphotyrosine, anti-EGFR, and anti-ErbB2 antibodies. b, effects of RB24 on Bad activation after a 2-h exposure in serum-starved MDA-MB-435/ErbB2 or MDA-MB-435/EGFR cells before stimulation with EGF (25 ng/ml) or HRGα (50 ng/ml), respectively. c, serum-starved MDA-MB-435/ErbB2 or MDA-MB-435/EGFR cells were exposed to a dose range of TEM for 2 h before EGF (25 ng/ml) or HRGα (50 ng/ml) stimulation. Equal amounts of cell lysates were analyzed by Western blot using anti-phospho-Bad (Ser136) or anti-Bad antibodies.
isogenic cell line panel to the various drugs. Indeed, RB10 and gefitinib selectively targeted the EGFR and Her2 transfectants, with IC_{50} values ranging from 9 to 21 μM (Table 2). It is interesting to note that the multitargeted RB24 was 8- to 13-fold more potent against the transfectants than its monotargeted EGFR inhibitory counterparts RB10 or gefitinib and more than 20-fold more potent than TEM (Table 2). In contrast, TEM selectivity targeted the nontransfected wild-type cancer cells, MDA-MB-435 (IC_{50} = 18 μM) and was found to be inactive in the ErbB transfectants (IC_{50} > 50 μM) (Table 2).

**Mechanism of Target Selectivity of the Combi-Molecule.** For the combi-molecule being designed to target both EGFR and DNA, various factors such as DNA repair enzymes and nonspecific binding may affect their potency in established cell lines. Therefore, we chose to pursue our studies on the mechanism of target selectivity of the combi-molecule in the transfectants (e.g., MDA-MB-435, MDA-MB-435/EGFR, and MDA-MB-435/ErbB2) that do not express O^6^-alkylguanine DNA alkyltransferase, a DNA repair protein known to mitigate cellular response to the DNA lesions induced by TEM or the combi-molecules (Pegg et al., 1995; Matheson et al., 2003). First, comet assay analysis was performed to determine the extent of DNA damage in the isogenic cell panel. The results showed that RB24 induced significantly higher levels of DNA damage in the ErbB transfectants (EGFR and ErbB2) than in the wild type (p < 0.05) (Fig. 1A). DNA damage induced in the parental cell line may be receptor-independent because TEM did not exhibit any selectivity for the transfectants, with equal levels of DNA damage in all three cell lines (Fig. 1A). It is more important that RB24 was a 3.1- and 1.9-fold more potent DNA-damaging agent than TEM in MDA-MB-435/EGFR and MDA-MB-435/ErbB2, respectively. Likewise, RB24 induced a 25% higher number of MDA-MB-435/EGFR cells than MDA-MB-435 wild type to arrest in the S phase. In the MDA-MB-435/ErbB2 cells, the strong S-phase arrest was accompanied by more cells in G1 than for its wild-type counterpart, indicating a mixture of G1 and S arrest (Fig. 2, A–C). In contrast, cell cycle perturbation by TEM was moderate in these cells, suggesting that despite generating the same type of lesions, the two agents have a distinct mechanism of action.

The ability of our compound to induce superior levels of DNA damage than TEM in these cells and furthermore, to selectively damage DNA in cell expressing EGFR and ErbB2, stimulated our interest in determining the mechanism underlying this selectivity. Having found that the released inhibitor RB10 was fluorescent, we undertook a fluorescence microscopy analysis of its subcellular distribution after cell exposure to RB24. The results showed that intracellular levels of RB10 released by RB24 degradation were concentrated in the perinuclear region, and, interestingly, the fluorescence intensity was higher in the ErbB transfectants than in the wild type (Fig. 1B). This result was further confirmed by UV flow cytometric quantitation that showed significant difference (2–3-fold) between the wild type and the transfectants (Fig. 1C). Thus, these results lead to the hypothesis that the enhanced DNA damage inflicted by RB24 may be due to a bystander effect, whereby RB24 anchors in its cognate sites in the perinuclear region from which the methyldiazonium is released. To test this hypothesis, we designed a competitive experiment whereby RB10 was given in combination with RB24 to prevent the interactions of the latter with its binding sites.

In theory, if binding of RB24 to its cognate sites around the nucleus played a bystander effect, we would expect competitive binding of RB10 to deplete the levels of DNA damage induced by RB24. Indeed, as depicted in Fig. 2A, combined treatment with RB10 + RB24 significantly depleted the levels of DNA damage induced by RB24 in all MDA-MB-435 cells. It is more important to note that the difference was significantly enhanced in the cells expressing Her2 (ErbB2) or EGFR, indicating that the latter receptors may significantly contribute to the enhanced DNA damage observed with RB24. It also should be noted here that the levels of DNA damage induced by the combination of RB10 + RB24 were similar to that of TEM and more interestingly that selectivity for the ErbB transfectants was abolished (Fig. 3). This is further corroborated by the depletion and delocalization of the fluorescence associated with RB24 upon coincubation with JDA41 (a nonfluorescent inhibitor of EGFR with IC_{50} competitive binding within the same range as RB24) (Fig. 4Ae). Further analysis using EGFR and ErbB2 antibodies showed that the fluorescence generated through the decomposition of RB24 was colocalized with the PE- and Cy3-labeled antibodies (see magenta, Fig. 4B).

**Mechanism of Selective Cell Killing.** Although it was obvious that RB24 could induce substantially higher levels of DNA damage than TEM in these cells, the translation of the latter effect into cell killing remained to be elucidated. Thus, the levels of JNK phosphorylation, a signaling protein known to be activated by DNA damage and to be involved in apoptosis, were analyzed (Huang et al., 1999; Ohtsuka et al., 2003). The results showed that JNK was activated at concentrations of RB24 that induced significant DNA damage (25–100 μM) (Fig. 5A). In contrast, at the same concentrations (e.g., 50 and 100 μM) TEM did not induce significant DNA damage, and no JNK activation was apparent. To further ascertain the correlation between DNA damage and JNK activation, the analysis of TEM was carried out at a higher dose at which it induces similar levels of DNA damage as RB24 (e.g., 200 μM) (Fig. 7).
It is interesting to note that, as in RB24-treated cells, JNK activation was detected for TEM at its DNA-damaging dose. Furthermore, the proapoptotic role of JNK in these cells was corroborated by analysis of levels of apoptosis induced in the presence of its potent inhibitor SP600125. Indeed, annexin V flow cytometric analysis showed that SP600125 reduced the levels of apoptosis induced in cells treated with RB24 and TEM by approximately 33 to 35% (Fig. 5B).

It is now known that inactivation of apoptosis is well regulated by EGFR- or Her2-mediated signaling and that JNK activation promotes apoptosis in response to DNA damage. Therefore, we surmise that the lack of sensitivity of the ErbB transfectant cells to TEM may be mediated by EGFR or Her2. Thus, we analyzed the activation of related signaling pathways in response to RB24 and TEM. Activation of EGFR and Her2 correlated with Bad phosphorylation in the transfectants (Fig. 6, A–C). However, treatment with RB24 led to a dose-dependent inhibition of both EGFR and Her2 phosphorylation, and this was accompanied by down-regulation of Bad phosphorylation in these cells. We also showed that RB24 was capable of inhibiting autophosphorylation of EGFR by using an anti-phospho-Tyr1068 antibody. Strong inhibition of phosphorylation of Her2 at its Tyr1221/1222 was observed when cells were exposed to RB24. These effects were accompanied by down-regulation of AKT, indicating an alleviation of the anti-apoptotic effects of ErbB receptors (Fig. 7, A and B). In contrast, TEM was not capable of depleting levels of Bad phosphorylation at a concentration as high as 50 μM, which is in agreement with its inability to induce neither JNK nor to block EGFR TK-mediated signaling (Fig. 6C).

We further determined whether the ability of the combi-molecule to selectively block signaling and damage DNA would translate into ErbB-selective apoptosis. It is interesting to note that RB24 induced approximately 2-fold higher level of apoptosis in the ErbB transfectants than in the wild type (58–73% 24 h after treatment) (Fig. 8). Inversely, TEM was only capable of inducing significant levels of apoptosis in the wild-type cells (Fig. 8). RB10, an inhibitor of EGFR, induced moderate levels of apoptosis in the ErbB-expressing cells (25–30%) (Fig. 8).

**Discussion**

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

The first significant observation of this study was the differential cytotoxicity and DNA damage induction produced by the combi-molecule in the isogenic panel. In contrast to
TEM, levels of DNA damage induced by RB24 appeared to correlate with receptor levels. The correlation of DNA damage with the levels of receptors in these cells suggests that their mechanism of intracellular distribution may differ from that of TEM. Based upon our results, it appears that the combi-molecule may enter the cells by passive diffusion as previously described for other combi-molecules (Qiu et al., 2004; Matheson et al., 2004a) and bind to its cognate sites (e.g., nascent or internalized EGFR proteins or related proteins) located in the endoplasmic reticulum or the perinuclear region (Offterdinger et al., 2004; Khan et al., 2006) (Fig. 5). We recently made the same observation with AL237, a fluorescence-labeled probe that allowed us to localize the DNA-alkylating moiety by fluorescence microscopy. It is more important that by using red-labeled EGFR antibody, we demonstrated that the released FD105, the chloro analog of RB10, was colocalized with EGFR (Todorova et al., 2010). It also should be noted that, in addition to our recent imaging work, there is now ample evidence of the perinuclear and even nuclear translocation of EGFR (Lo et al., 2006; Dittmann et al., 2009).

The loss of selective delivery of DNA alkylation upon competitive binding with an EGFR inhibitor indicates that the entire combi-molecule may bind to the perinuclear region before degradation and release of the methyl diazonium ion. Our model is further supported by 1) the lower levels of DNA damage induced by RB24 in the wild-type cells line compared with the transfected counterparts; and 2) the lower levels of DNA damage induced by TEM, a non-EGFR targeting molecule (Matheson et al., 2001), in all the cell lines. It is also important to mention here that RB24 induced slightly higher levels of DNA damage than TEM in the wild-type cell line. This may be due to unspecific binding of RB24.

To elucidate the contribution of the high levels of DNA damage to cell killing, we examined various pathways related to the receptor and in response to DNA alkylation. On one hand, it is known that EGFR and Her2 exert antiapoptotic effects through the phosphatidylinositol-3-kinase/Akt signaling cascade that ultimately leads to the inhibition of mitochondrial pore formation via Bax oligomerization (McCubrey et al., 2006). On the other hand, proapoptotic effects in response to DNA damage is mediated by the stress-activated protein kinase JNK (Huang et al., 1999; Ohtsuka et al., 2003). Thus, we analyzed key elements of these pathways (e.g., Bad and JNK activation)
In the transfectants. Bad being downstream of EGFR or Her2 activation, its observed inactivation by the combi-molecule is evidence that inhibiting the receptors alleviates their antiapoptotic effects. Activation of JNK in response to DNA damage is proapoptotic; therefore, receptor inactivation and induction of DNA lesions are two cooperative events that can contribute to cell cycle arrest and enhanced apoptosis. It is more important to note that activation of JNK was only seen at doses that correspond to significant levels of DNA damage by both RB24 and that TEM provides an indirect link between levels of DNA damage and apoptosis in these cells. Indeed, substantially higher levels of apoptosis were observed in the transfectants compared with their wild-type counterpart.

In summary, the results presented here can allow us to propose a model for selective targeting and cell killing of cancer cells expressing ErbB receptors by the combi-molecule concept. As depicted in Fig. 9, binding of a fraction of the combi-molecule (see I-TZ) to the TK domain of the receptors at the membrane induces blockade of downstream signaling, as proven by our analysis of the downstream proteins Akt, Bad, and JNK. After internalization, binding of a large fraction of the combi-molecules I-TZ to EGFR/Her2 or related protein in the perinuclear or perhaps a small fraction in the nucleus may play a bystander effect that directs the alkylating species methyl diazonium released form the combi-molecules I-TZ toward genomic DNA. The elevated levels of DNA damage induced by the combi-molecule enhace apoptosis through activation of JNK and down-regulation of alternative pathways associated with the antiapoptotic effect of EGFR/Her2 receptor-mediated signaling.

References

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