Type II combi-molecules: design and binary targeting properties of the novel triazolinium-containing molecules JDD36 and JDE05

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We recently designed molecules termed 'type II combi-molecules' to block the epidermal growth factor receptor and to damage DNA without the requirement for hydrolytic cleavage. Here, we studied two such combi-molecules (JDD36 and JDE05), containing a novel quinazoline-linked chloroethyltriazolinium system. The epidermal growth factor receptor-targeting potential of these novel structures was studied by ELISA for isolated epidermal growth factor receptor and by Western blotting for whole-cell assays. DNA damage was analyzed using the single-cell microelectrophoresis comet assay. Antiproliferative effects were determined by the sulforhodamine B assay. JDD36 showed an IC_{50} of 0.6 μmol/l in the ELISA for epidermal growth factor receptor tyrosine kinase, a dose-dependent inhibition of epidermal growth factor receptor phosphorylation and significant levels of DNA damage in the human DU145 prostate cancer cell line. JDD36 was an overall 2- to 15-fold stronger antiproliferative agent than JDE05 that showed potent epidermal growth factor receptor inhibitory activity (IC_{50} epidermal growth factor receptor, 0.035 μmol/l) but weak DNA-damaging potential. In a panel of LNCaP erbB transfectants, in contrast to JDE05, JDD36 showed remarkable and selective potency against the LNCaPerbB2 transfectant. The results *in toto* suggest that the overall superior potency of JDD36 when compared with JDE05 may be imputed to its balanced binary epidermal growth factor receptor–DNA-targeting properties that may induce a tandem blockade of epidermal growth factor receptor-mediated mitogenic signaling while depleting alternative survival mechanism by damaging DNA. *Anti-Cancer Drugs* 18:171–177 © 2007 Lippincott Williams & Wilkins.

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Introduction

In the advanced stage of progression, many solid tumors overexpress a variety of growth factor receptors and in many cases they coexpress the ligands that activate these receptors, leading to autocrine induction. Prostate carcinomas often harbor one such growth factor receptor, epidermal growth factor receptor (EGFR), and its closest homolog, erbB2, the overexpression of which is associated with aggressive proliferation, reduced drug sensitivity and poor prognosis [1–3]. The reduced chemosensitivity of these tumors is often imputed to resistance to apoptosis and expression of DNA repair enzymes. Recently, activation of DNA repair genes such as XRCC1 has directly been linked to EGFR-mediated signaling. Yacoub *et al.* [4] demonstrated that blockade of EGFR tyrosine kinase (TK) with the quinazoline inhibitor Iressa (AstraZeneca, Toronto, Ontario, Canada), led to down-regulation of XRCC1 and ERCC1, and impairment of DNA repair [5].

Recently, for the purpose of enhancing the potency of DNA alkylators in cells overexpressing the erbB oncogenes, we developed a novel strategy termed 'combi-targeting' that seeks to design a novel type of molecule capable of blocking TK-mediated signaling while inducing significant DNA damage [6–10]. The principle is based on the equilibria outlined in Scheme 1. A molecule termed TZ-I, in which TZ represents the DNA-damaging tail and I the EGFR TK recognition moiety, is designed to bind to the ATP site of EGFR on its own (TZ-I-EGFR) or to be hydrolyzed under physiological conditions to give TZ, a DNA-damaging species + I, another inhibitor of EGFR leading to adducted DNA (TZ-DNA) and further blockade of EGFR (see I-EGFR). These molecules, also

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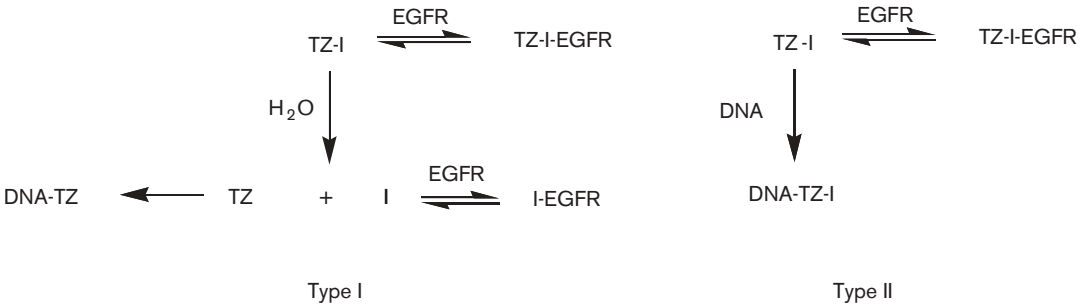
Scheme 1



designated as 'combi-molecules', designed to degrade to generate the DNA-damaging species, are termed 'type I' and those capable of blocking EGFR TK and damaging DNA without the requirement for hydrolysis are termed 'type II'. On the basis of their ability to downregulate genes required to rescue the cells through blockade of EGFR, while damaging DNA, they are expected to be significantly more potent than a TK inhibitor or a classical DNA-damaging compound used alone. More importantly, we recently demonstrated that combi-molecules of the triazene class showed 3- to 5-fold greater antiproliferative activity than the classical two-drug combinations of a TK inhibitor with a DNA-damaging agent [6–10].

In the current study, we examine the binary targeting potency of the first example of cyclic triazenes, classified as 'type II combi-molecules', that do not require hydrolysis to exert their binary targeting potency. The development of the novel compounds JDD36 and JDE05 (Scheme 2) was inspired by the previously described in-vivo activity of 5-[3,3-bis(2-chloroethyl)-1-triazene] imidazole-carboxamide (NSC-82196, BCTIC) [11], which also exists as a cyclic triazolinium form (cycl-BCTIC) (Scheme 2). The latter drug contains a triazene chain capped with a nitrogen mustard moiety that is believed to be oxidized *in vivo* into a mono-chloroethyl-triazene moiety [12], a species known to be prone to hydrolyze to a DNA alkylating chloroethylating species. The cyclic chlorotriazolinium form cycl-BCTIC (see Scheme 2), however, is inactive. The combi-molecules JDD36 and JDE05 present a 2-choroethyltriazolinium moiety, but they also contain a quinazoline ring to bind to the ATP site of EGFR. Another noticeable advantage of these novel structures is the positive charge on the triazolinium cycle that may enhance their hydrophilicity. As electron-withdrawing groups (e.g. the positively charged triazolinium group) are known to decrease the EGFR inhibitory activity of the quinazoline ring [13], JDE05 was designed to keep the charge away from the latter ring through an aminobenzoic acid spacer. In the current study, we analyzed the binary targeting potency of the two combi-molecules in isogenic NIH3T3 mouse fibroblasts and human prostate cancer cells transfected with erbB1 (EGFR) or with its closest homolog erbB2 (HER2).

Materials and methods

Cell culture

The cell lines used in this study, the human prostate cancer DU145, LNCaPW.T and NIH3T3 cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). The androgen-sensitive prostate cancer cell lines LNCaPEGFR, LNCaPerbB2 (LNCaPerbB2 cells stably transfected with the erbB2 gene), mouse fibroblast cell line NIH3T3her14 (transfected with erbB1 or EGFR) and NIH3T3neu (transfected with erbB2) were provided by Dr Moulay Aloui-Jamali (Montreal Jewish General Hospital, Montreal, Canada). All the cell lines were maintained in RPMI 1640 (Wisent, St Bruno, Canada) or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics as previously described [6]. Cells were maintained in a monolayer at 37°C in a humidified environment of 5% CO_2. The cultures were maintained in logarithmic growth by harvesting with a trypsin-ethylenediaminetetraacetic acid (EDTA) solution containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA and replating before confluence. In all assays, the cells were plated for 24–48 h before drug administration.

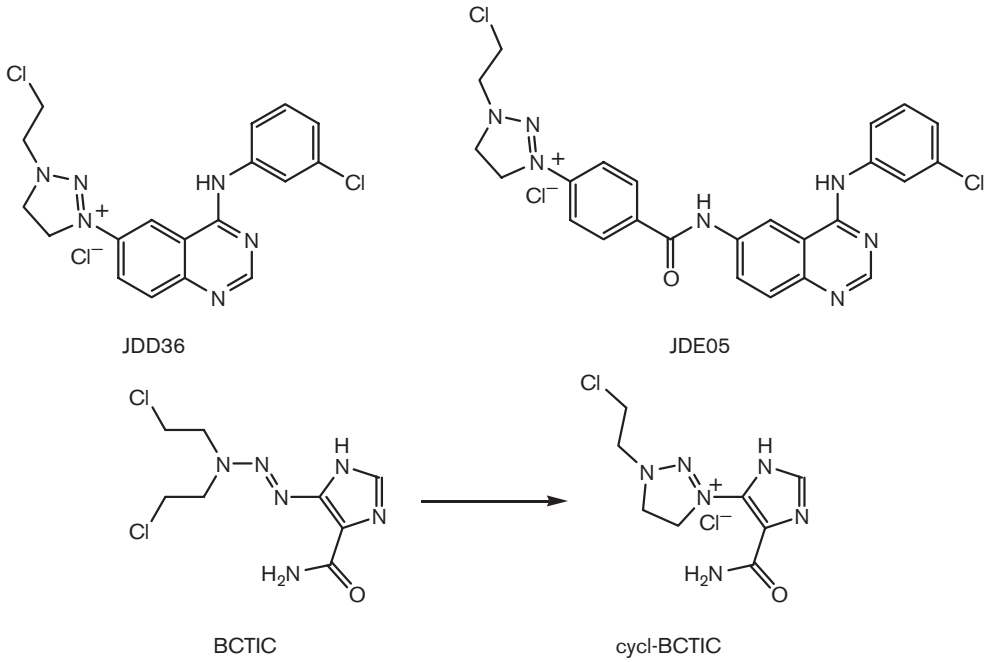
Drug treatment

JDE05, JDD36 and BCTIC were synthesized in our laboratory. In all assays, the drug was dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in sterile RPMI-1640 media containing 10% fetal bovine serum immediately before the treatment of cell cultures (concentration of DMSO never exceeded 0.2% (v/v)).

Kinase enzyme assay

The EGFR kinase assay is similar to the one described previously [10,14–16]. MaxiSorp 96-well plates (Nalge Nunc International, Naperville, Illinois, USA) were

Scheme 2



Scheme 2

incubated overnight at 37°C with 100 µl/well of 0.25 ng/ml poly[L-glutamic acid–L-tyrosine, 4:1] (PGT) in phosphate-buffered saline (PBS). Excess PGT was removed and the plates were washed three times with wash buffer (0.1% Tween 20 in PBS). The kinase reaction was performed by using 4.5 ng/well EGFR affinity-purified from A431 cells [17]. The compound was added and phosphorylation initiated by the addition of ATP (20 µmol/l). After 8 min at room temperature with constant shaking, the reaction was terminated by aspiration of the reaction mixture and rinsing the plate four times with wash buffer. Phosphorylated PGT was detected after a 25-min incubation with 50 µl/well of horseradish peroxidase-conjugated PY20 antiphosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted to 0.2 mg/ml in blocking buffer (3% bovine serum albumin and 0.05% Tween 20 in PBS). Antibody was removed by aspiration and the plate was washed four times with wash buffer. The signals were developed by the addition of 50 µl/well 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) and after blue color development, 50 µl of H_2SO_4 (0.09 mol/l) was added per well to stop the reaction. The plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550; Bio-Rad, Hercules, California, USA).

Growth inhibition assay

For nonstimulated cell growth inhibition, approximately 10×10^3 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. All growth inhibitory activities were evaluated using the sulforhodamine B (SRB) assay [18–20]. Briefly, following drug treatment, cells were fixed using 50 µl of cold trichloroacetic acid (50%) for 60 min at 4°C, washed four times with tap water and stained for 30 min at room temperature with SRB (0.4%) dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid and allowed to air dry. The resulting colored residue was dissolved in 200 µl of Tris base (10 mmol/l) and optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

Autophosphorylation assay

DU145 cells (1×10^6) were preincubated in a six-well plate with 10% serum at 37°C for 24 h and starved overnight for 18 h, after which they were exposed to a dose range of each drug for 2 h and subsequently treated with 50 ng/ml EGF for 15 min at 37°C. Cells were washed

with PBS and resuspended in cold lysis buffer [50 mmol/l Tris–HCl pH 7.5; 150 mmol/l NaCl; 1% Nonidet P-40, 1 mmol/l EDTA; 5 mmol/l NaF; 1 mmol/l Na_3VO_4; protease inhibitor tablet (Roche Biochemicals, Laval, Canada)]. The lysates were kept on ice for 30 min and collected by centrifugation at 10 000 r.p.m. for 20 min at 4°C. The concentrations of protein were determined using the Bio-Rad protein assay kit (Bio-Rad). Equal amounts of protein were added to a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene diflouride membrane (Millipore, Bedford, Massachusetts, USA). Nonspecific binding on the membranes was minimized with a blocking buffer containing nonfat dry milk (5%) in 0.1% Tween 20 in PBS. Thereafter, the membranes were incubated with primary anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, New York, USA) for the detection of phosphotyrosine. Membranes were stripped and reprobed with anti-EGFR (Neomarkers, Fremont, California, USA) for the determination of corresponding receptor levels. Blots were incubated with horseradish peroxidase–goat antimouse antibody (1 : 1000 dilution; Cell Signaling Research, Beverly, Massachusetts, USA) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK) [21,22].

Alkaline comet assay for quantitation of DNA damage

The modified alkaline comet assay was performed as described previously. Briefly, cells were treated with drugs for 2 h, collected by centrifugation and resuspended in PBS. Cell suspensions were diluted to approximated 1×10^6 cells, and mixed with agarose (1%) at 37°C in a 1 : 10 dilution. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel casting chambers, as described previously [23] and then immediately placed into a lysis buffer [2.5 mol/l NaCl, 0.1 mol/l tetra-sodium EDTA, 10 mmol/l 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 overnight at 4°C, the gels were gently rinsed with distilled water and immersed in a second lysis buffer (2.5 mol/l NaCl, 0.1 mol/l tetra-sodium EDTA, 10 mmol/l Tris-base containing 1 mg/ml proteinase K) for 60 min at 37°C. Thereafter, they were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C and electrophoresed at 300 mA for 20 min. The gels were subsequently rinsed with distilled water and placed in 1 mol/l ammonium acetate for 30 min. Thereafter, they were soaked in 100% ethanol for 2 h, dried overnight and stained with SYRB Gold (1/10 000 dilution of stock supplied from Molecular Probes, Eugene, Oregon, USA) for 20 min. Comets were visualized at × 330 magnification and the DNA damage was quantitated using the tail moment parameter (i.e. the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet). A minimum of 50 cell comets was analyzed for each sample, using ALKOMET 3.1 version image analysis software, (Richard Branker Research Ltd, Ottawa, Ontario, Canada) and values represent calculated means of tail moments for the entire cell population.

Results

Inhibition of epidermal growth factor receptor phosphorylation

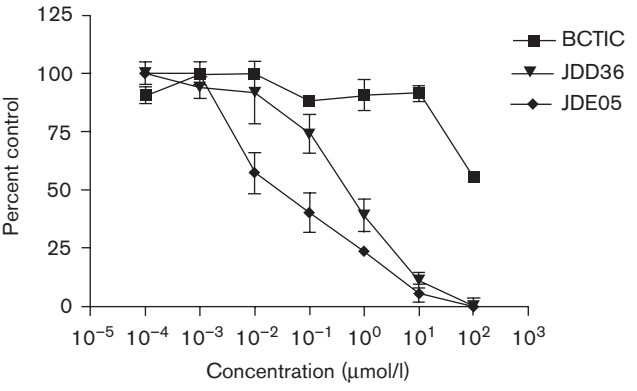
Enzyme assay

The ability of the combi-molecules to block EGFR TK activity was tested in an ELISA using isolated EGFR. JDD36 ($IC_{50} = 0.6$ μmol/l) was 17-fold less potent than JDE05, in which the cyclized mustard function is separated from the quinazoline ring by a benzoic acid species ($IC_{50} = 0.034$ μmol/l) (Fig. 1). As expected, BCTIC was inactive ($IC_{50} > 100$ μmol/l).

Whole-cell assay

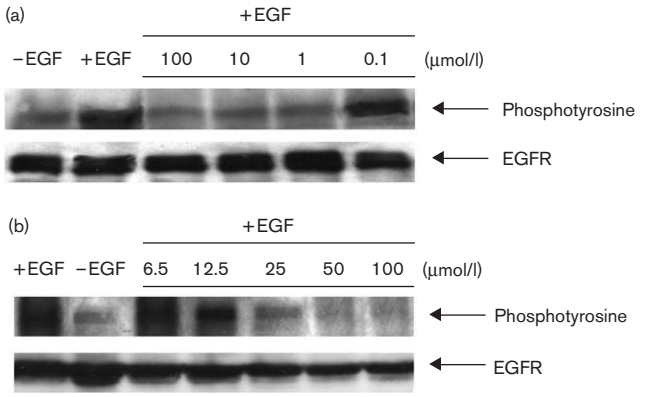
JDE05 was designed to shift the positive charge of the triazolinium away from the quinazoline ring to attenuate its electron-withdrawing effect on the pyrimidine moiety of the bicyclic system. Although this translated into a greater potency as measured in the EGFR TK isolated enzyme assay, it was important to determine whether this difference was maintained in whole cells, as the chemical stability of the triazolinium ring might be different in the intracellular milieu. Analysis of EGF-induced EGFR autophosphorylation showed that the superior potency of JDE05 was maintained in the whole-cell assay. Fifty percent inhibition of EGFR phosphorylation was achieved in the range of 0.5 μmol/l in cells treated with JDE05 (Fig. 2a). In contrast, to achieve this level of

Fig. 1



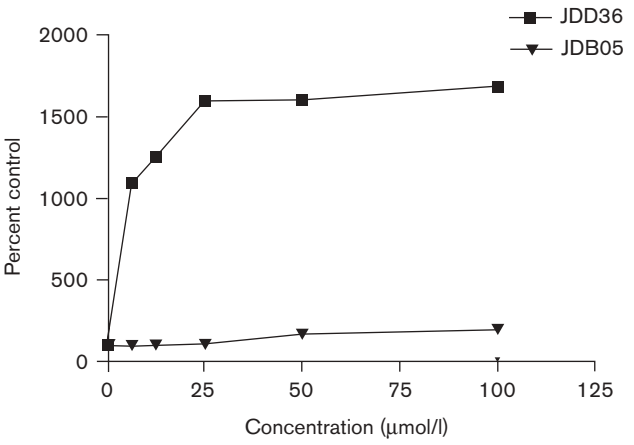
Competitive binding to epidermal growth factor receptor by JDE05, JDD36 and 5-[3,3-bis(2-chloroethyl)-1-triazene]imidazole-carboxamide (BCTIC). Poly[L-glutamic acid–L-tyrosine, 4 : 1] substrate phosphorylation was detected using an antiphosphotyrosine antibodies. each point represents at least two experiments run in triplicate.

Fig. 2



Inhibition of epidermal growth factor (EGF)-stimulated EGF receptor (EGFR) autophosporylation by JDD36 (a) and JDE05 (b) in the DU145 cell line. Cells were starved for 24 h and 50 ng/ml EGF was added for 15 min with different concentrations for 2 h. Western blotting was performed with an antiphosphotyrosine antibody (1 : 1000 diluted). The same polyvinylidene difluoride membrane was stripped and EGFR detected with anti-EGFR antibody.

Fig. 3



Quantification of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in DU145 cells exposed to JDE05 and JDD36 for 2 h. Each point represents at least two independent experiments.

inhibition with JDD36, a concentration of around 11 μmol/l was required (Fig. 2b).

DNA damage

The ability of JDD36 and JDE05 to induce DNA damage was studied by single-cell microelectrophoresis comet assay. Interestingly, despite the marked similarity between these two structures, their DNA-damaging potential significantly differed. JDD36 was able to induce maximum levels of DNA strand breaks at 25 μmol/l. In contrast, even at 100 μmol/l, JDE05 induced barely detectable levels of DNA damage (Fig. 3).

Antiproliferative activity

The translation of the binary EGFR/DNA-targeting property of these two agents into antiproliferative activity was studied in a panel of isogenic NIH3T3 transfectants and human prostate cancer cells. We have already demonstrated that NIH3T3 transfectants represent a good model for testing the selectivity of combi-molecules [8,10]. Their ability to block the growth of NIH3T3 erbB transfectants has already been shown to correlate directly with their IC_{50} for EGFR TK inhibition [10].

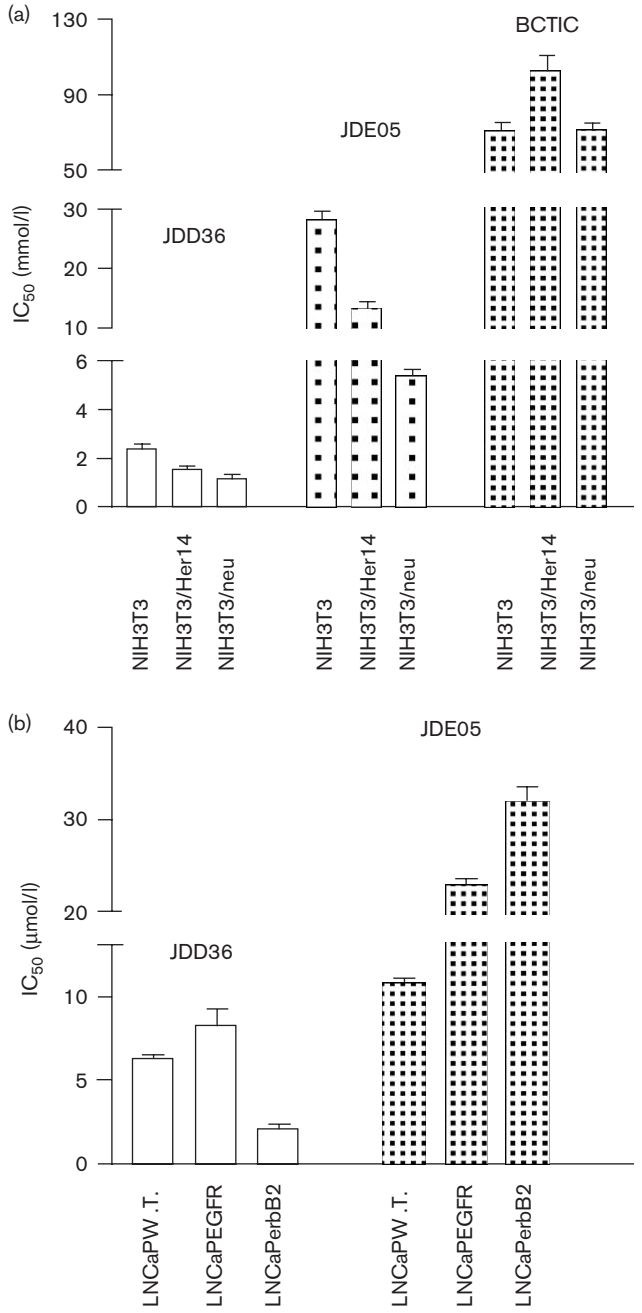
In this model, JDE05 showed 2-fold selectivity for the EGFR-transfected NIH3T3/HER14 and 5-fold for the HER2-transfected cells (Fig. 4a). The selective growth inhibitory potency of JDD36 was less than that of JDE05 with only 1.5-fold selectivity for NIH3T3/HER14 and 2-fold for NIH3T3/neu (Fig. 4a). As expected, BCTIC did not show any selectivity for NIH3T3/neu or for NIH3T3/HER14.

The potency of JDE05 and JDD36 was further compared in a panel of prostate cancer cells that includes the androgen-sensitive LNCaP, its erb1 or erb2 transfectants and the androgen-independent DU-145 cells. The LNCaP cells express EGFR, but were transfected with erbB1 to elevate the levels of the receptor (LNCaPEGFR). LNCaP (wild-type) cells were also transfected to co-express erbB2 (LNCaPerbB2). The DU-145 cells express EGFR and its ligand transforming growth factor-α, thereby growing by autocrine induction. The results showed that JDD36 was 1.7- to 14-fold more potent than JDE05 in all the cell lines. Among the LNCaP transfectants, the erb2-transfected cells were the most sensitive to JDD36 ($IC_{50} = 2.2$ μmol/l) (Fig. 4b). It is noteworthy that JDD36 was 5-fold more potent than JDE05 against the DU145 cell line, indicating that its bifunctional EGFR/DNA-directed mechanism of action may confer enhanced potency (Fig. 5).

Discussion

Classical alkylators are mostly single-targeted agents, the potency of which is often mitigated by DNA repair enzymes [14–17]. To circumvent problems associated with the lack of potency of these agents, a number of drug combination regimens have been developed that included various agents of different action mechanisms. Owing to the toxicity and sometimes failure of these combinations, the search for new targets for antitumor therapy has intensified over the past two decades and TK-mediated signaling has become the most investigated target for antitumor therapy. The majority of agents (e.g. Iressa) resulting from these drug discovery programs,

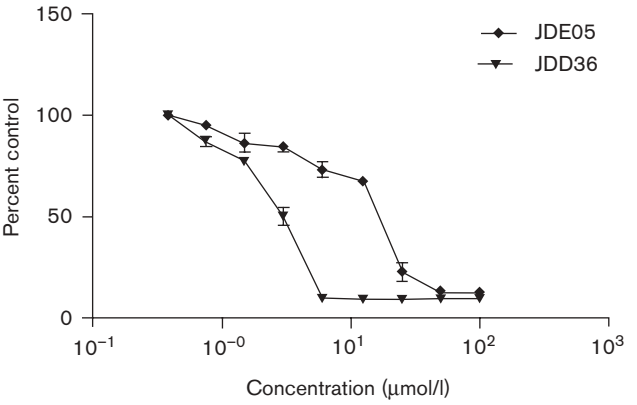
Fig. 4



Antiproliferative effects of JDD36, JDE05 and 5-[3,3-bis(2-chloroethyl)-1-triazene]imidazole-carboxamide (BCTIC) on NIH3T3, NIH3T3Her14, NIH3T3neu cells (a) and prostate cancer cell lines LNCaPW.T, LNCaPEGFR, LNCaPerbB2 (b). Cells were treated with drugs for 5 days of continuous exposure and growth inhibition was measured using sulforhodamine B assay. Each point represents at least two independent experiments run in triplicate.

however, remained single-targeted and show rather weak response in the clinic as single agents. To ameliorate their potency, combinations with classical antitumor drugs have become an actively explored strategy [21,24–26]. Within this context, the combi-targeting principle is a novel

Fig. 5



Inhibition of cell growth by JDD36 and JDE05 in DU145 cell line. After 6 days of continuous exposure, cell growth inhibition was measured using sulforhodamine B assay. Each point represents at least two independent experiments run in triplicate.

approach that seeks to develop single agents with multiple targeting properties. In this study, we designed molecules in which a 2-chloroethyltriazolinium ring was fused with a quinazoline inhibitor of EGFR. This led to the synthesis of two single molecules with different levels of binary targeting potency. Here, one agent exhibited EGFR TK inhibitory activity in the micromolar range and strong DNA-damaging properties and the other with strong EGFR TK inhibitory (submicromolar range) with weak DNA-damaging potential. This represents the first case in which two molecules carrying the same pharmacophores, but with almost inverse binary EGFR/DNA targeting potential, could be analyzed.

Interestingly, in the isogenic NIH3T3 cells, the results demonstrate that JDE05, the drug with the stronger EGFR TK inhibitory potency, was more selective. This result is in line with previous studies demonstrating that the antiproliferative potency of combi-molecules against NIH3T3 erb-transfectant linearly increased with EGFR inhibitory potency [10]. In the isogenic human prostate cancer cell line LNCaP, the selective potency of these agents did not, however, follow the same trend. LNCaP cells transfected with erbB2 were exquisitely sensitive to JDD36 and the strongest antiproliferative effect observed for JDE05 was against the wild-type LNCaP. These interesting results can be analyzed in light of the fact that, the erbB gene transfection being the sole abnormality of the NIH3T3 cells, their sensitivity is directly correlated with the EGFR or HER2 inhibitory potency of the molecules. Hence, JDE05 being a strong inhibitor, induces selective potency against the transfectant. In contrast, in the human prostate cancer cells wherein EGFR inhibition does not suffice to achieve significant

growth inhibition, its activity was mitigated. A cooperative interaction between the EGFR or HER2 inhibitory potency of JDD36 and its DNA-damaging potential may account for its ability to induce significant cell killing in the LNCaPerbB2 transfectant and the DU145 cells. Downregulation of the DNA repair gene through inhibition of EGFR or HER2 activation and induction of high levels of DNA strand breaks may explain its selectively high antiproliferative potency against LNCaP-erbB2 cells and the DU145 cells. Indeed, Yacoub *et al.* [4] have already shown that EGFR activation in these cells (LNCaP and DU145) is accompanied by elevation of the DNA repair proteins XRCC1 and ERCC1. Further, we have recently demonstrated that downregulation of DNA repair protein XRCC1 and inactivation of EGFR-mediated antiapoptotic pathways could be the possible mechanism of enhanced potency of a combi-molecule against the DU145 cells (R. Banerjee, Q. Qiu, J. McNamee, B.J. Jean-Claude, in preparation). Thus, in the human cancer cells, selective targeting by combi-molecules may perhaps be based on a synergistic or cooperative effect between the two mechanistic components of the combi-molecule.

The chemical basis of the reduced DNA-damaging potential of JDE05 when compared with JDD36, despite possessing an identical DNA-damaging pharmacophore, is not known. On the basis of nuclear magnetic resonance data, it appears that the positive charge in JDE05 may, however, be predominantly located on the *N*-alkyl nitrogen, whereas in JDD36 it may be on the *N*-aryl nitrogen, a charge distribution that may markedly affect the reactivity of these species towards electrophiles.

The results presented herein demonstrate that binary EGFR/DNA targeting can be achieved with a chloroethyltriazolinium moiety directly appended to the quinazoline ring. Addition of a benzoic acid spacer between the two moieties ameliorates EGFR inhibitory potency, but depletes DNA-damaging potential. Given the strong potency of JDD36 against the human prostate carcinoma cell lines, further chemical and mechanistic studies are ongoing to ameliorate the potency of this novel type of anticancer drug candidates.

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