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ORIGINAL ARTICLE

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The combi-targeting concept: a novel 3,3-disubstituted nitrosourea with EGFR tyrosine kinase inhibitory properties

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Abstract *Purpose*: To study the dual mechanism of action of FD137, a 3,3-disubstituted nitrosourea designed to block signaling mediated by the epidermal growth factor receptor (EGFR) on its own and to be hydrolyzed to an inhibitor of EGFR plus a DNA-damaging species. *Materials and methods*: HPLC was used to determine the half-life ($t_{1/2}$) of FD137 and to characterize its derived metabolite FD110. The dual mechanisms of DNA damaging and EGFR tyrosine kinase (TK) targeting were ascertained by the comet assay for DNA damage and by inmunodetection of phosphotyrosine in an ELISA and a whole-cell assay for EGFR-mediated signaling. The antiproliferative effects of the different drugs and their combinations were determined by the sulforhodamine B (SRB) assay. *Results*: In contrast to BCNU, FD137 significantly blocked EGF-induced EGFR autophosphorylation (IC_{50} 4 μM) in the human solid tumor cell line A431. DNA damage induced by FD137 could only be observed after 24 h exposure, but the level of DNA damage remained 3.6-fold lower than that induced by BCNU. This difference was rationalized by the 160-fold greater stability of FD137 when compared with BCNU in serum-containing medium. Further, degradation of FD137 was accompanied by the slow release of FD110, an extremely potent inhibitor of EGFR TK [IC_{50} (EGFR autophosphorylation) < 0.3 μM]. The complex properties of FD137 translated into a 55-fold greater antiproliferative activity than BCNU against the EGFR-overexpressing A431 cells that coexpresses the O^6-alkylguanine transferase (AGT). Depletion of AGT in these cells by the use of O^6-benzylguanine (O^6-BG) enhanced their sensitivity to BCNU by 8-fold, but only by 3-fold to FD137. *Conclusions*: The results overall suggest that the superior antiproliferative activity of FD137 when compared with BCNU may be associated with its ability to behave as a combination of many species with different mechanisms of action. However, the enhancement of its potency by O^6-BG suggests that its antiproliferative effect was at least partially mitigated by AGT and perhaps it may be largely dominated by its signal transduction inhibitory component.

Keywords Nitrosourea · Signal transduction · Tyrosine kinase · EGFR · BCNU

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Introduction

The nitrosoureas are among the most potent alkylating agents used in the clinic in the therapy of lymphoproliferative diseases, glioma, small-cell lung carcinoma, melanoma and gastrointestinal cancer. However, despite their broad spectrum of activity, nitrosoureas are inactive against tumors expressing the O^6-alkylguanine transferase (AGT) enzyme [1, 2], a DNA-repair protein capable of repairing the O^6-alkylguanine DNA lesion. This limitation stems from the very mechanism of action of these drugs that are believed to kill tumor cells by generating, upon hydrolysis, a chloroethyldiazonium species responsible for the alkylation of guanine at the O^6-position [3]. The resulting O^6-(2-chloroethyl)guanine adduct alkylates a base (e.g. the 7-position of guanine or the cytosine amino group) in the complementary DNA strand, thereby inducing a DNA crosslink that is believed to be the lethal lesion induced by bifunctional nitrosoureas [1, 2, 4]. Tumor cell resistance to chloroethylnitrosoureas is imputed to the AGT-mediated repair of the primary O^6-alkylguanine lesion, which

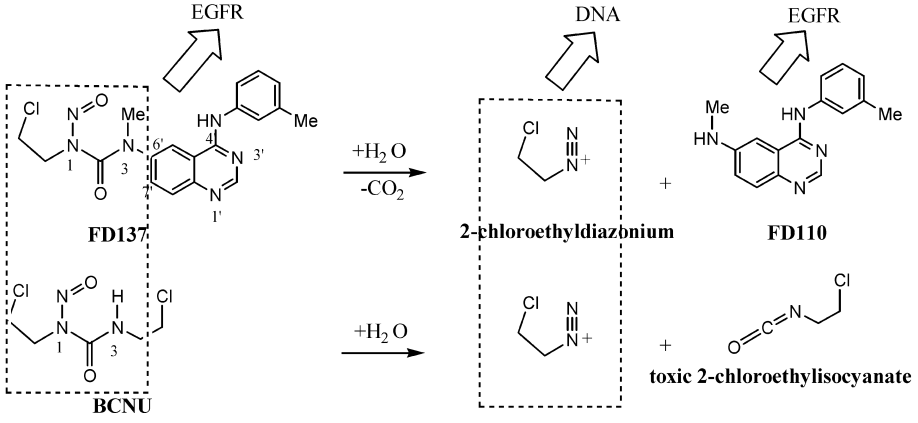
prevents the formation of the lethal DNA crosslink. This mechanism is corroborated by the use of O^6-benzylguanine (O^6-BG), an irreversible inhibitor of AGT that significantly sensitizes tumor cells to the action of 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (BCNU) (Scheme 1) in a number of animal models [5, 6]. However, recent observations of acquired resistance to repeated doses of BCNU plus O^6-BG in tumor cells [7, 8, 9] and the significant sensitivity of bone marrow cells to the latter combination [10] cast doubt on its ultimate clinical efficacy. Chemoprotection of the bone marrow using stem cells transfected with an O^6-BG-insensitive mutant form of AGT is an active field of research [8, 11]. Thus, the development of strategies designed to enhance the clinical efficacy of bifunctional nitrosoureas remains a burgeoning field.

Our laboratory is currently studying a novel approach termed "combi-targeting" that consists of imprinting into the structure of cytotoxic drugs, new molecular features allowing them to interfere with mitogenic signal transduction, while retaining their ability to damage DNA [12, 13, 14]. It is expected that the hydrolytic cleavage of these molecules termed "combi-molecules" (C-molecules) will generate other bioactive derivatives, the antiproliferative effects of which may add to or synergize with each other. We designed FD137 (Scheme 1) that contains a 1-chloroethyl-1-nitrosourea function and a 3-methyl-3-(4′-anilinoquinazolin-6′-yl) moiety. Each of the substituents around the ureido function is designed to play a specific role following the degradation of the C-molecule: (a) the 1-chloroethyl-1-nitrosourea moiety is responsible for the postdegradative generation of the DNA-damaging 2-chloroethyldiazonium species, (b) the methyl group is designed to prevent the formation of a toxic isocyanate following cleavage of the ureido function, and (c) the 4′-anilinoquinazoline moiety contains all the structural requirements for competitively blocking of ATP binding to the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR).

EGFR is a transmembrane glycoprotein with an external domain that binds activating ligands such as EGF and tumor growth factor α (TGF-α). It contains an intracellular TK domain, that upon activation (by binding of EGF or TGF-α) phosphorylates both the receptor itself and a variety of effector proteins that ultimately triggers DNA synthesis and proliferation of EGFR-proficient cells [15, 16]. Overexpression of EGFR and often coexpression of cognate ligands (e.g. TGF-α, amphiregulin) that leads to autocrine growth, are associated with aggressive tumor proliferation and invasiveness. Agents like the anilinoquinazolines that are capable of blocking EGFR-mediated signaling have already been shown to induce significant antitumor activity in vivo [17, 18]. In this study, we chose to combine our nitrosourea moiety with the pharmacophores of anilinoquinazolines because of their known tolerance of substituents at the 6′- and 7′-positions (see Scheme 1) which in their binding mode to EGFR are located at the entrance of the binding cleft. Indeed, a variety of compounds with bulky side chains on the 6′- and 7′-positions have been synthesized and found to retain significant affinity for the EGFR ATP binding site [17, 19, 20, 21].

Most of the known quinazolines are reversible inhibitors of EGFR that require repeated dosing schedules for sustained inhibition in vivo and the irreversible ones are specifically directed at EGFR or its closest homologue $p185^{neu}$ [17, 18]. Thus, these agents target a single mechanism of cell growth, leaving all alternative growth pathways unaffected. Recently, synergistic combinations of EGFR TK inhibitors with other cytotoxic drugs have been evoked as an alternative to the lack of sustained growth inhibition induced by EGFR TK inhibitors when used as single agents [22]. This is consistent with our strategy that not only seeks to combine these two mechanisms in a single molecule (C-molecule), but also to target the latter to EGFR.

As shown in Scheme 1, the C-molecule (FD137) is designed to target EGFR on its own and to further decompose into the cytotoxic 2-chloroethyldiazonium species directed at DNA plus another less bulky inhibitor (e.g. FD110) capable of inducing stronger EGFR TK inhibitory activity than its parent C-molecule. Conversely, its classical counterpart BCNU does not possess an intracellular target on its own and is merely designed to generate the DNA-damaging chloroethyldiazonium plus a toxic isocyanate upon hydrolysis (see Scheme 1). In the study reported here, we



Scheme 1 C-molecule FD137 degradation to DNA-damaging 2-chloroethyldiazonium, the less bulky inhibitor FD110 and the toxic 2-chloroethylisocyanate

demonstrated that the C-molecule FD137, a nitrosourea tethered to a 4-anilinoquinazoline was capable of: (a) generating a less bulky, more potent EGFR inhibitor FD110 upon hydrolysis, (b) blocking EGFR TK activity and EGF-induced EGFR autophosphorylation in whole cells, and (c) inducing DNA damage perhaps through the generation of a chloroethyldiazonium species. The levels of DNA damage induced by FD137 were about 3-fold less than those induced by BCNU. However, FD137 was 55-fold more potent than BCNU against the A431 cell line that coexpresses EGFR and AGT.

Materials and methods

Drug treatment

FD137 and FD110 were synthesized in our laboratory according to known procedures [19, 20]. O^6-BG was a generous gift from Dr. Robert Moschel (National Cancer Institute, USA). BCNU was purchased from Sigma Chemical Company (Mississauga, Canada). In all experiments, drugs were dissolved in DMSO and subsequently diluted in sterile RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Life Technologies, Burlington, Canada) prior to addition to the cell culture medium. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

Cell culture

A431 cells (American Type Culture Collection, Manassas, Va.) and SF126 cells (generous gift from Mrs. Dolores Dougherty, Brain Tumor Research Center, University of California, Calif.) were maintained in RPMI-1640 supplemented with FBS (10%) and ciprofloxacin 10 μg/ml (Mediatech, Herndon, Va.). The cultures were kept in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA and replating before confluence. All cell lines used repeatedly tested negative for mycoplasma contamination.

HPLC determinations of FD137 degradation to FD110

A volume of 25 μl of FD137 (24 m*M*) was added to RPMI-1640 supplemented with 10% FBS (1 ml) and incubated for various periods (6–168 h) at 37°C. Proteins were precipitated by addition of acetonitrile (1.75 ml) and the supernatant collected by centrifugation. HPLC analyses were performed with a Hewlett Packard 1090 liquid chromatograph, using a Waters C4 15-μm 300×3.9-mm column (reverse phase) to characterize and quantify the products resulting from the degradation of FD137. The concentration of FD110 deriving from the degradation of FD137 was calculated using a standard curve obtained from the serial dilution of independently synthesized FD110. The operating mode was isocratic and two solutions, A (53% acetonitrile) and B (47% water) were used with a flow rate of 0.5 ml/min and an injection volume of 10 μl. Under these conditions, the retention times of independently synthesized FD110 and FD137 were 7.6 and 11.9 min, respectively.

Combination studies

For the combination of FD110 and BCNU, the drugs were mixed at their IC_{50} ratio (FD110/BCNU, 1:125), serially diluted and added to the monolayers for 96 h. IC_{50} values were determined using the median effect equation as described by Perez et al. [23]. The results of drug interactions were determined using Eq. 1 where CI_{50} values >1, $=1$, and <1 indicate antagonism, additivity and synergism, respectively. Cell growth-inhibitory activities were evaluated using the sulforhodamine B (SRB) assay [24].

$$CI_{50} = \frac{IC_{50}(\text{FD110 in combination})}{IC_{50}(\text{FD110 alone})} + \frac{IC_{50}(\text{BCNU in combination})}{IC_{50}(\text{BCNU alone})}$$

Enzyme assay

The EGFR kinase assay was similar to the one described previously [13]. Nunc Maxisorp 96-well plates were incubated overnight at 37°C with poly(L-glutamic acid/L-tyrosine, 4:1; PGT) in phosphate-buffered saline (PBS). The kinase reaction was performed using 4.5 ng/well EGFR affinity-purified from A431 cells. Following drug addition, phosphorylation was initiated by the addition of ATP. A typical assay was performed at 0.001–100 μ*M*. The reaction was terminated by aspiration of the reaction mixture and phosphorylated substrate was detected with anti-phosphotyrosine antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, Calif.). The signals were developed by the addition of 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersburg, Md.) and H_2SO_4 (0.09 *M*) was added to stop the reaction. The plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550).

Autophosphorylation assay

A431 cells (1×10^6) were preincubated in a six-well plate with 10% serum at 37°C overnight for 48 h and starved for 24 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 20 min at 37°C. Cells were washed with PBS and resuspended in cold lysis buffer. 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, Hercules, Calif.). Equal amounts of protein (80 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). Nonspecific binding on the membrane was minimized with a blocking buffer containing nonfat dried milk (3%) in PBS Tween. Thereafter, the primary antibodies were incubated with membranes, either anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, N.Y.) for the detection of phosphotyrosine, or anti-EGFR (Neomarkers, Fremont, Calif.) for the determination of corresponding receptor levels. Blots were incubated with HRP-goat anti-mouse antibody (BioRad Laboratories) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK). Band intensities were measured using the SynGene GeneTools software package.

Alkaline comet assay for quantification of DNA damage

The alkaline comet assay technique was used to quantify DNA damage induced by FD137, FD110 and BCNU as previously described [12, 25]. Briefly, A431 cells were exposed to drugs for 2 or 24 h, harvested with trypsin-EDTA, subsequently collected by centrifugation and resuspended in PBS. Cell suspensions were diluted to approximately 10^6 cells, and mixed with agarose (1%) in PBS at 37°C at a dilution of 1:10. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel-casting chambers, as previously described [25], and then immediately placed into a lysis buffer (2.5 *M* NaCl, 0.1 *M* tetrasodium EDTA, 10 m*M* 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,

10 m*M* Tris-base) containing 1 mg/ml proteinase K for 60 min at 37°C. The gels were then rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37°C, and 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. They were then soaked in 100% ethanol for 2 h, dried overnight, and stained with SYBR Gold (1:10,000 dilution of stock supplied by Molecular Probes, Eugene, Ore.) 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 cells/comet were analyzed for each sample, using ALKOMET version 3.1 image analysis software. For the determination of interstrand crosslink levels, the tail moments of irradiated control cells (4000 rad) were compared with those of irradiated drug-treated cells and the percentage decrease in tail moment was calculated as described by Hartley et al. [26] according to the formula:

$$\%\ \text{decrease in tail moment} = \left(1 - \left\{\frac{\text{Tm(treat} - \text{irr)} - \text{Tm(unirr)}}{\text{Tm(irr)} - \text{Tm(unirr)}}\right\}\right) \times 100$$

where Tm(treat-irr) is the tail moment of the drug-treated irradiated sample, Tm(unirr) is the tail moment of the untreated unirradiated control, and Tm(irr) is the tail moment of the untreated irradiated control.

Results

Degradation of FD137

The decomposition of FD137 was analyzed by monitoring the variations of the area under the HPLC chromatogram peaks corresponding to FD137 and FD110, as a function of time. As expected, an inverse relationship was observed between the disappearance of FD137 and the appearance of FD110. From these results, a $t_{1/2}$ of 41 h was calculated using a one-phase exponential decay curve fit (Fig. 1). This $t_{1/2}$ was 160-fold slower than that reported for BCNU, a nitrosourea known to be extremely unstable in serum-containing medium ($t_{1/2}$ 15 min) [3, 27, 28]. The retention time of FD110 was ascertained by HPLC analysis of an independently synthesized standard. After complete degradation (72 h), the overall yield of FD110 was approximately 80%.

Binary targeting properties of FD137

Inhibition of substrate phosphorylation. The EGFR TK-inhibitory activity of FD137 was demonstrated through an ELISA assay whereby the drug was incubated with the isolated full-length EGFR for a short 8-min period and through an EGF-induced autophosphorylation assay in A431 cells involving a 2-h exposure. As shown in Fig. 1, the kinetics of degradation ($t_{1/2}$ 41 h) indicated that within these short exposure periods (8 and 120 min), the entire structure remained intact in the medium. Thus, in this time-frame the analysis of EGFR TK-inhibitory activity of FD137 was not complicated by that of the concomittant generation of FD110. In the ELISA assay, FD137 blocked PGT substrate phosphorylation in a dose-dependent manner (IC_{50} 1 μ*M*) but its activity was 20-fold lower than that of FD110 (IC_{50} 0.05 μ*M*; Fig. 2), indicating that it is a prodrug of a more potent inhibitor of EGFR TK. As expected, the EGFR TK-inhibitory activity of BCNU was negligible.

Inhibition of EGF-induced autophosphorylation. In further analysis, the cells were starved for 24 h and stimulated with EGF in the presence of the different drugs for 2 h. Inhibition of autophosphorylation of EGFR was analyzed by Western blotting. The observed order of potency from this whole cell assay paralleled that of the ELISA. In contrast to BCNU, FD137 blocked EGFR autophosphorylation in a dose-dependent manner (IC_{50} 4 μ*M*; Fig. 3a, b), but its potency was more than 14-fold less than that of FD110 which induced an almost 100% inhibition of EGFR TK at concentrations as low as 0.3 μ*M* (Fig. 3b). This further confirms that FD137 is a stable carrier of a more potent inhibitor.

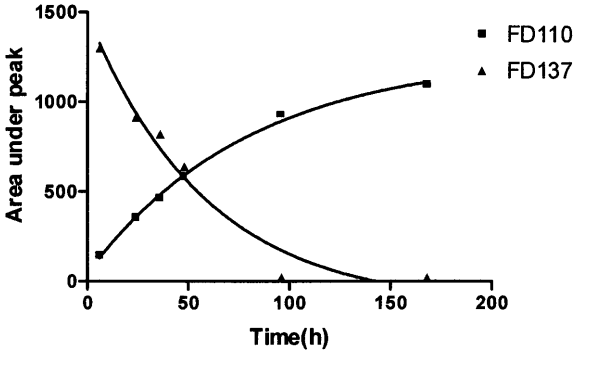


Fig. 1 Conversion of FD137 (*triangles*) to FD110 (*squares*) in RPMI supplemented with 10% FBS at 37ºC. Protein precipitation and HPLC determination were performed as described in Materials and methods

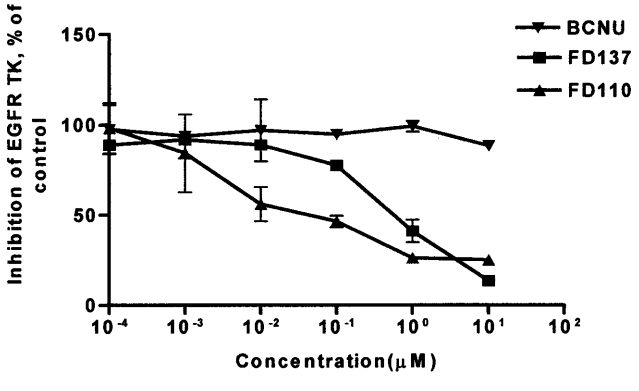


Fig. 2 Competitive binding to EGFR by BCNU (*down triangles*), FD137 (*squares*) and FD110 (*up triangle*). PGT substrate phosphorylation was detected using an anti-phosphotyrosine antibody. Each point represents at least two experiments run in triplicate

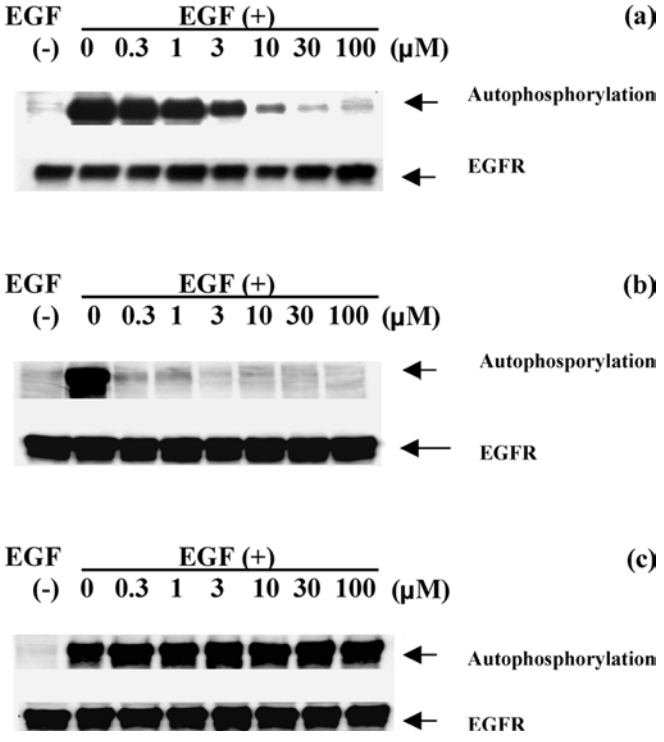


Fig. 3a–c Inhibition of EGF-stimulated EGFR autophosphorylation by FD137 (**a**), FD110 (**b**) and BCNU (**c**) in the A431 cell line. Cells were starved for 24 h and 50 ng/ml EGF (20 min) was added with different concentrations of compounds for 2 h. Western blotting was performed with an anti-phosphotyrosine antibody (diluted 1:1000). The same PVDF membrane was stripped and EGFR detected with anti-EGFR antibody

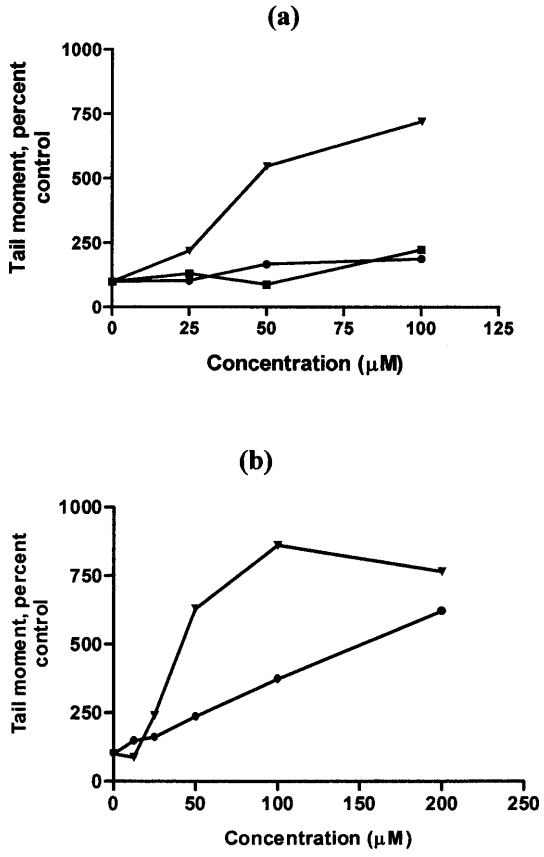


Fig. 4a, b Alkaline comet assay for quantitation of DNA damage. The levels of DNA damage were measured as tail moments in A431 cells exposed (**a**) to FD137 (*circles*), FD110 (*squares*), BCNU (*triangles*) for 2 h and (**b**) to FD137 (*circles*) or BCNU (*triangles*) for 24 h. Tail moment parameters (formula shown in Materials and methods) were measured with ALKOMET image analysis software for at least 50 cells per dose

DNA-damaging property of FD137

While the released FD110 was directed at EGFR, the concomitantly generated chloroethyldiazonium (see Scheme 1) was designed to damage DNA. It is noteworthy that the latter species, due to its short life, was not detectable by our available methods. Thus, we surmised that induction of DNA damage by FD137 would be an indirect evidence of the formation of the latter. We used the single-cell microelectrophoresis (comet assay) to characterize DNA strand breaks from A431 cells following exposure to all three drugs. This assay permits the microscopic observation and quantitation of DNA damage from whole cell nuclei [29]. When we used the 2-h drug exposure time normally allowed for analysis of DNA damage by nitrosoureas [27, 30], BCNU induced a dose-dependent increase in strand breaks (Fig. 4a). In contrast, levels of DNA damage inflicted by FD137 were similar to those of FD110 which, as expected, induced negligible levels of DNA lesions. However, when the cells were exposed to drug for 24 h (Fig. 4b), in contrast to BCNU which exhibited a saturation curve, FD137 induced a dose-dependent linear increase in DNA strand breaks with no appearance of a plateau at concentrations as high as 100 μ*M*.

Calculations using the slope of the linear part of these curves showed that BCNU was a 3.6-fold stronger DNA-damaging agent than FD137, a result that is in line with the order of stability of these two nitrosoureas. Likewise, a crosslink assay involving comparison between tail moments of γ-irradiated drug-treated and control samples (4000 rad) showed that at 25–50 μ*M*, BCNU induced 6–20% decrease in tail moment, indicating detectable levels of crosslinks at these doses. In contrast, in cells treated with FD137, the same levels of crosslinks could only be observed at twice higher concentrations (50–100 μ*M*; Fig. 5). This further confirms that BCNU is a much stronger DNA-damaging agent than FD137.

Cell growth inhibition of FD137

Comparative effects of the three agents. To demonstrate whether the binary properties of FD137 translate into enhanced antiproliferative activities when compared with its nitrosourea counterpart BCNU, we evaluated the effects of each drug on cell proliferation using a

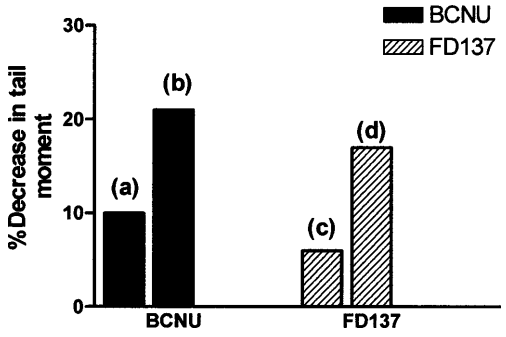


Fig. 5 Comparison between levels of DNA interstrand crosslinks induced by BCNU (*a* 25 μ*M*, *b* 50 μ*M*) and FD137 (*c* 50 μ*M*, *d* 100 μ*M*) as measured in terms of the percentage decrease in tail moments of γ-irradiated control and treated cells

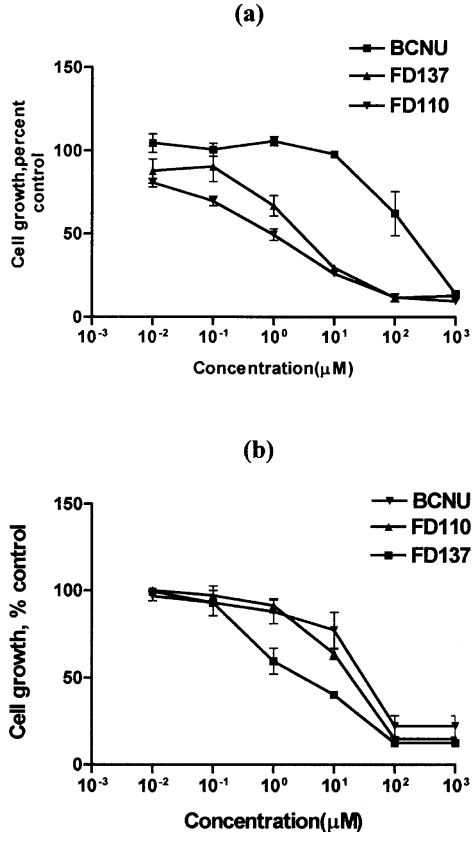


Fig. 6a, b Comparison between the antiproliferative activity of BCNU, FD137 and FD110 (**a**) in the AGT/EGFR-proficient A431 cell line and (**b**) in the AGT/EGFR-deficient SF126 glioma cell line, as determined by the SRB assay. Each point represents at least three independent experiments run in triplicate

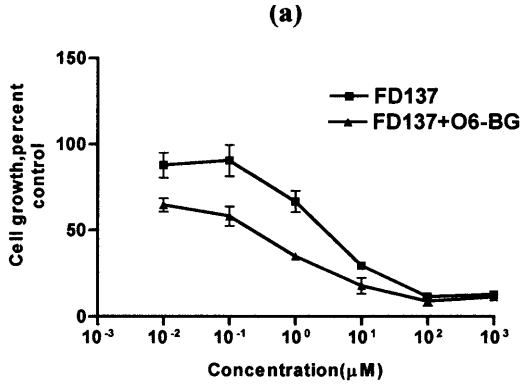
4-day SRB assay in the A431 cells that overexpress EGFR and coexpress AGT. More importantly, it has already been demonstrated that antiproliferative activities of quinazolines strongly correlate with their ability to block EGF autophosphorylation in this cell line [31, 32]. Our results showed a 2.3-fold superior potency of FD110 (IC_{50} 1.11 μ*M*) when compared with FD137 (IC_{50} 2.57 μ*M*; Fig. 6a). However, the latter was more than 50-fold more potent than its nitrosourea counterpart BCNU. Interestingly when these drugs were tested in the glioma cell line SF126 that is deprived of both AGT and EGFR, a different order of potency was observed. FD137 (IC_{50} 1.7 μ*M*) was approximately 8-.fold more potent than FD110 (IC_{50} 13 μ*M*; Fig. 6b). Interestingly, the latter was almost equipotent with BCNU (IC_{50} 17 μ*M*) in this AGT/EGFR-deficient glioma cell line.

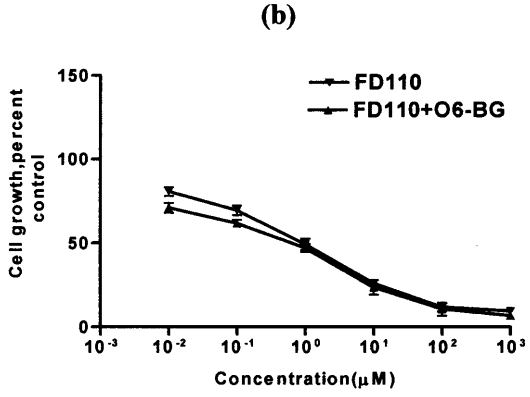
Effects of AGT depletion in A431 cells. From the DNA damage studies, it appeared that in contrast to BCNU, FD137 could induce detectable DNA damage at concentrations 20–50 times higher than its IC_{50} (25–100 μ*M*). Under the conditions of the comet assay, the type of lesions observed may probably be the alkali labile *N*7-alkylpurines. Despite their abundance in cells treated with *N*-alkyl-*N*-nitrosoureas, these lesions are far less cytotoxic than the O^6-alkylguanine adducts, as proven by the significant sensitization of AGT-proficient cells to BCNU when the activity of AGT is depleted [33]. Thus, we surmised that if the chloroethyldiazonium ion was indeed released by FD137 or that DNA alkylation at O^6-guanine was induced, its activity should be enhanced by coadministration of O^6-BG. Indeed depletion of AGT activity by its inhibitor O^6-BG sensitized the cells to BCNU by 8-fold (Fig. 7c) and by 3-fold to FD137 (Fig. 7a). As expected, O^6-BG had no effect on cell sensitivity to FD110 that does not possess DNA-damaging properties (Fig. 7b). This indicates that the cytotoxic effects of the O^6-alkylguanine DNA adducts were indeed induced by FD137, but may have been at least partly mitigated by AGT.

Comparison with classical two-drug combinations. FD137 being a unimolecular combination of two antiproliferative properties combining inhibition of the EGFR pathway with DNA damage, we thought it of interest to compare the potency of this form of combination with that of two different drugs representing the two antiproliferative mechanisms under study. We surmised that the closest mimic was a combination of FD110 (the metabolite resulting from FD137) with BCNU (a prodrug of the 2-chloroethyldiazonium species; Fig. 8a). From IC_{30} to IC_{80}, we only observed near-additive to subadditive interactions (Fig. 8b). As an example, the CI_{50} at the 50% effect for FD110 plus BCNU was 0.85, indicating a subadditive interaction. More importantly, FD137 was 23-fold more potent than the latter combination against the A431 cells (Fig. 8a).

Discussion

The combi-targeting concept postulates that a molecule (C-molecule) possessing receptor affinity on its own and that is able to further degrade into another inhibitor of the same target and DNA-damaging species should induce significantly sustained antiproliferative activity in cells expressing these receptors. The results presented here demonstrated that FD137 was indeed capable of





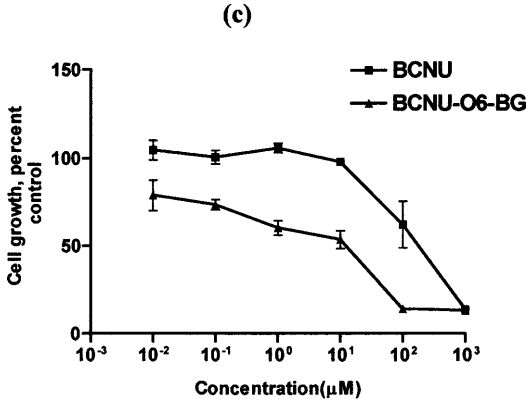
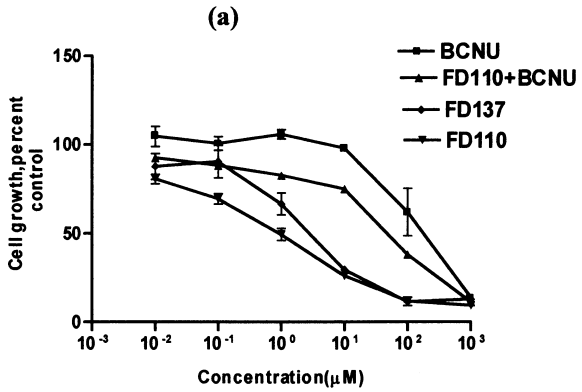


Fig. 7a–c Effect of O^6-BG (AGT inhibitor) on (**a**) FD137-, (**b**) FD110- and (**c**) BCNU-induced cell growth inhibition in A431 cells treated with the different drugs and their corresponding combinations with O^6-BG for 96 h. Cell growth control percentages were determined by the SRB assay. Each data point represents an average of triplicate samples from two separate experiments



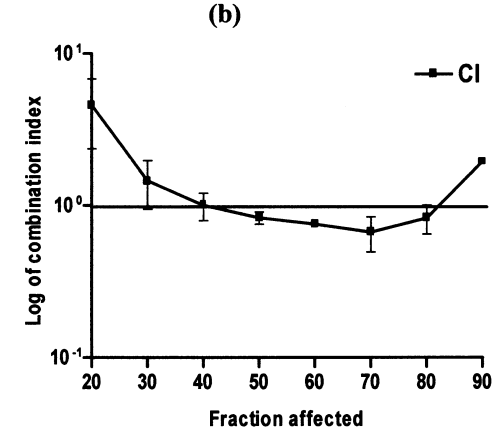


Fig. 8 **a** Comparison of the antiproliferative effects of FD110 plus BCNU (*up triangles*) with those of FD110 (*down triangles*) and BCNU (*squares*) and FD137 (*diamonds*) alone. FD110 and BCNU were mixed at their IC_{50} ratio (1:125) and each point represents a combination of two independent experiments. **b** Log of combination index calculated from the dose-response curves of **a** as a function of the effects expressed as a percentage. Combination index values <1, $=1$, and >1 indicate synergism, additivity and antagonism, respectively

slowly degrading to FD110 with a $t_{1/2}$ of nearly 41 h in serum-containing medium at 37°C, a rate of degradation markedly slower than that of BCNU ($t_{1/2}$ 15 min in serum). The superior stability of FD137 is primarily due to the bulkiness of the 3,3-disubstituted nitrosourea function wherein both the 3-methyl and the 4′-anilino-quinazolin-6′-yl groups significantly hinder the carbonyl of the ureido moiety, thereby decreasing the rate of hydrolysis of the latter. More importantly, the stability of this molecule is superior to that of SarCNU, an experimental 3,3-disubstituted nitrosourea that is known to exhibit a $t_{1/2}$ of 2.69 h in serum-containing medium [34]. The 3-methyl group hindering the urea moiety was included in the structure design to prevent the formation of a toxic isocyanate function following hydrolysis of the urea moiety. It is now known that the chloroethylisocyanate species derived from the hydrolysis of BCNU is responsible for its carbamoylating power and perhaps may increase its in vivo toxicity [35].

The stability of FD137 was in agreement with the barely detectable levels of DNA damage observed following a 2-h drug exposure. In contrast, BCNU ($t_{1/2}$ about 15 min in serum) [3, 28, 29, 36] induced significant DNA damage within the same exposure period. Perhaps, during this short period, the concentration of the generated chloroethyldiazonium species was insufficient

to induce detectable levels of DNA damage. Likewise, the slow release of the chloroethyldiazonium species accounts for the 3-fold lower levels of DNA damage induced by FD137 when compared with BCNU, even after a 24-h continuous exposure. Indeed, our kinetic analysis suggests that only 29% of the C-molecule was converted into FD110 by 24 h. Complete degradation of FD137 was observed only after 72 h in the culture medium, an exposure time considered too long for quantitation of DNA damage due to complications introduced by DNA repair and cell loss.

It is noteworthy that the slow rate of degradation of the C-molecule created conditions under which the cells were exposed to high but decreasing concentrations of intact FD137 during the first 48 h and increasingly high doses of FD110 during the subsequent 48 h. With the assumption that the release of each mole of FD110 is associated with one equivalent of the metastable chloroethyldiazonium, the DNA lesions may have been slowly but continuously inflicted in the presence of FD110 and FD137 gradients, forming then a three-drug-like combination over a long period. We clearly characterized the target of each of the constitutive elements of FD137 in the human carcinoma of the vulva cell line A431 that overexpresses EGFR and proliferates by a TGF-α-mediated autocrine induction [31]. More importantly, the observation that FD110 was a more than 20-fold stronger inhibitor of EGFR TK than its parent FD137, confirms the postulate according to which the C-molecule is designed to block EGF-induced EGFR autophosphorylation on its own and to further degrade to a smaller molecule with significantly higher affinity for the ATP binding site of EGFR. Thus, FD137 represents a complex model of a three-drug combination wherein decreasing and increasing concentrations of two agents directed at the same target but possessing different levels of affinity are generated over a long period. In addition, the decreasing concentration of the parent agent (the C-molecule) may be accompanied by the slow release of a DNA-alkylating species.

Having demonstrated the events proposed in Scheme 1, we characterized their effects on antiproliferative activities in the A431 cells using the SRB assay. The advantages of the unimolecular combinations were first determined by comparison with BCNU and FD110 alone. FD137 was clearly more potent than BCNU but less potent than FD110. It is interesting to note that despite being a precursor of FD110, FD137 was 2.3-fold less potent than the latter. This can be rationalized in light of its kinetics of degradation that showed a plateau concentration for FD110 only 72 h after treatment. In contrast, when administered alone, the cells were exposed for 96 h to a steady concentration of FD110, a 20-fold more potent EGFR inhibitor than FD137. More importantly, the order of potency of these drugs in the SRB assay paralleled that of their EGFR TK-inhibitory IC_{50} values (BCNU > FD137 > FD110). Since it is well established that the potency of compounds of the anilinoquinazoline class against A431 cells increases with increasing EGFR TK-inhibitory activity [31, 32], these results may also indicate that the antiproliferative activity of FD137 may be largely dominated by its effect on EGFR-mediated signaling. Thus, what is the contribution of the chloroethyldiazonium-induced DNA lesions to the overall antiproliferative activity?

Our comet analysis demonstrated that, although at supratoxic concentrations, FD137 was capable of inducing DNA crosslinks. Assuming that the FD137-derived chloroethyldiazonium species induced the primary cytotoxic O^6-alkylguanine DNA adduct, the use of an inhibitor to deplete the activity of the DNA repair enzyme AGT should potentiate its action. It is now well established that inhibition of AGT significantly sensitizes cells to BCNU and other related bifunctional nitrosoureas [33, 36, 37]. We found that exposure of the cells to nontoxic 25 μM O^6-BG dramatically sensitized them to the effect of the C-molecule. Indeed coadministration of O^6-BG decreased FD137 IC_{50} to a slightly lower value than that of FD110 which is a 20-fold stronger EGFR TK inhibitor. This is an indirect evidence of the generation of the chloroethyldiazonium and that its effect is somewhat mitigated by AGT. More importantly, these results suggest that blockade of EGFR-mediated signaling may not downregulate or affect the activity of AGT in the cells.

Further evidence of absence of crosstalk between AGT levels and EGFR-mediated signaling was provided by the absence of a synergistic interaction between an isoeffective combination of BCNU and FD110. The two effects were found to be mostly additive. Interestingly, the C-molecule, a single agent encompassing the two antiproliferative mechanisms of these two drugs was 23-fold more effective than their individual combinations. Perhaps the ability of the stable and lipophilic C-molecule to penetrate the cells prior to releasing the DNA damaging species plus the EGFR TK inhibitors may account for its superior antiproliferative activity when compared with the classical two-drug combination of the two agents BCNU (unstable) plus FD110 (a polar free amine).

The results overall suggest that the growth-inhibitory activity of the C-molecule in the A431 cells was chiefly achieved through inhibition of EGFR and any contribution from the DNA-damaging component should originate from cytotoxic lesions other than O6-alkylguanine (e.g. N7-alkylguanine, N3-alkyladenine). Thus, the AGT-dependence of FD137 antiproliferative activity suggests that its mechanism of cell killing may be similar to that of BCNU or SarCNU, two nitrosoureas bearing the *N*-(2-chloroethyl)-*N*-nitrosourea moiety.

FD137 is the first nitrosourea possessing TK-inhibitory properties ever reported. While our model clearly demonstrated its ability to damage DNA, the impact of the generated lesions appeared at least partially mitigated by the presence of AGT in these cells. However, this stable nitrosourea could induce 55-fold stronger antiproliferative activity than BCNU in AGT-expressing cells perhaps due to an alternative mechanism by which

it can block EGF- or TGFα-mediated mitogenic signaling. Hence, this is the first generation of ''smart'' nitrosoureas that may primarily behave like multidrug combinations in EGF-dependent tumor cells and like classical nitrosoureas in the absence of EGFR and AGT. In order to enhance the potency of the approach, our laboratory is currently optimizing the EGFR TK affinity of FD137 and improving its water solubility prior to the demonstration of its activity in vivo.

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