The Combi-Targeting Concept: *In vitro* and *In vivo* Fragmentation of a Stable Combi-Nitrosourea Engineered to Interact with the Epidermal Growth Factor Receptor while Remaining DNA Reactive

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Abstract

**Purpose:** JDA58 (NSC 741282), a “combi-molecule” optimized in the context of the “combi-targeting concept,” is a nitrosourea moiety tethered to an anilinoquinazoline. Here, we sought to show its binary epidermal growth factor receptor (EGFR)/DNA targeting property and to study its fragmentation *in vitro* and *in vivo*.

**Experimental Design:** The fragmentation of JDA58 was detected in cells *in vitro* and *in vivo* by fluorescence microscopy and tandem mass spectrometry. EGFR phosphorylation and DNA damage were determined by Western blotting and comet assay, respectively. Tumor data were examined for statistical significance using the Student’s *t* test.

**Results:** JDA58 inhibited EGFR tyrosine kinase (IC50, 0.2 μmol/L) and blocked EGFR phosphorylation in human DU145 prostate cancer cells. It induced significant levels of DNA damage in DU145 cells *in vitro* and *in vivo* and showed potent antiproliferative activity both *in vitro* and in a DU145 xenograft model. In cell-free medium, JDA58 was hydrolyzed to JDA35, a fluorescent amine that could be observed in tumor cells both *in vitro* and *in vivo*. In tumor cells *in vitro* or *in vivo*, or in plasma collected from mice, the denitrosated species JDA41 was the predominant metabolite. However, mass spectrometric analysis revealed detectable levels of the hydrolytic product JDA35 in tumor cells both *in vitro* and *in vivo*.

**Conclusions:** The results *in toto* suggest that growth inhibition *in vitro* and *in vivo* may be sustained by the intact combi-molecule plus JDA35 plus JDA41, three inhibitors of EGFR, and the concomitantly released DNA-damaging species. This leads to a model wherein a single molecule carries a complex multitargeted-multidrug combination.

Prostate tumors are characterized by the amplification and overexpression of the epidermal growth factor receptor (EGFR). Expression of EGFR and its cognate ligands may lead to autocrine stimulatory loops and constitutive receptor activation that translate into aggressive tumor progression and poor patient prognosis (1–3). The nitrosoureas are among the most potent alkylating agents used in the clinical therapy of gliomas, lymphoproliferative diseases, and non–small cell lung carcinoma (4, 5). However, despite their broad spectrum of activity, nitrosoureas are inactive against tumors expressing the O6-alkylguanine transferase (AGT) enzyme and are seldom used in the therapy of prostate carcinomas (6, 7).

In search for other strategies to sensitize AGT tumors to nitrosourea, we developed a novel approach, termed the “combi-targeting” concept, to target tumor cells overexpressing receptor tyrosine kinase or growing by an autocrine stimulatory loop. The basic premise of the combi-targeting concept is that, given receptor or target heterogeneity in tumor cells, molecules “programmed” to block multiple cellular targets and/or to release multiple bioactive species should induce sustained antitumor activity in refractory tumor cells. As outlined in Fig. 1A, if the combi-molecule is represented by I-TZ wherein I is the quinazoline moiety and TZ is the nitrosourea tail, it may penetrate the cells and bind to the ATP site of EGFR to form I-TZ-EGFR or degrade in the intracellular compartment to generate TZ, a DNA-reactive species plus another inhibitor I that can further block EGFR-mediated signaling (I-EGFR). In brief, this strategy seeks to synthesize chimeric molecules referred to as “combi-molecules” that possess the ability to (a) inhibit receptor tyrosine kinase on their own and (b) generate, following hydrolytic scission under physiologic conditions,

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another inhibitor of the same receptor tyrosine kinase and a cytotoxic DNA-alkylating agent. The combined effects of these multiple species (combi-molecule plus receptor tyrosine kinase inhibitor plus alkylating agent) are expected to induce significant antiproliferative activity in cells expressing receptor tyrosine kinase (Fig. 1A and B).

Previous studies designed to test the feasibility of the approach using a nitrosourea probe (FD137) showed that, in cells exposed to the drug, significant levels of DNA damage were induced whereas phosphorylation of EGFR was markedly inhibited after 2 h of exposure (8, 9). However, the poor water solubility, weak EGFR tyrosine kinase inhibitory activity (IC_{50}-
1 μmol/L, and long chemical half-life (1/2; 41 h) of this probe compromised its further development as a useful drug. To circumvent these problems, we exploited a known structure-activity relationship in the quinazoline series for enhancing the affinity of the probe for the ATP-binding site of EGFR (10). Thus, the bulky 3'-methyl group of the quinazoline moiety was replaced by a smaller 3'-Cl group and this has led to JDA58 (see structure, Fig. 1B), a 5-fold stronger inhibitor of EGFR tyrosine kinase, a more water soluble, and a more readily hydrolyzed nitrosourea than FD137. In the current study, the optimized combi-molecule JDA58 is used to verify the postulates of the combi-targeting concept in vitro and in vivo.

Materials and Methods

**Drug treatment**

JDA58, JDA35, and JDA41 were synthesized in our laboratory. BCNU was purchased from Sigma Chemical Co. (Mississauga, Ontario, Canada). In all assays, the drug was dissolved in DMSO and subsequently diluted in sterile RPMI 1640 containing 10% fetal bovine serum (Wisent, St. Bruno, Quebec, Canada) immediately before the treatment of cell cultures [concentration of DMSO never exceeded 0.2% (v/v)].

**Cell culture**

The cell lines used in this study were the human prostate cancer DU145, obtained from the American Type Culture Collection (Manassas, VA), and the androgen-sensitive prostate cancer cell lines LNCaP and LNCaP/BeB2 (stably transfected with erbB2 gene), which were generous gifts from Dr. Moulay Aloui-Jamali (Montreal Jewish General Hospital, Montreal, Quebec, Canada). All the cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics as described previously (10, 11). Cells were maintained in a monolayer at 37°C in a humidified environment of 5% CO2, 95% air. The cultures were maintained 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. In all assays, the cells were plated for 24 to 48 h before drug administration.

**Degradation**

The study of the conversion of JDA58 to JDA35 was done by spectrophotometry as the latter amine was fluorescent (absorption, 270 nm; emission, 450 nm). A 50 mmol/L stock solution of JDA58 in DMSO was added to RPMI 1640 with 10% of fetal bovine serum and incubated for 100 h at 37°C in a microplate spectrophotometer. The data were analyzed using the SoftMaxPro and GraphPad software packages.

**Kinase enzyme assay**

The EGFR kinase assay is similar to the one described previously (12–14). MaxiSorp 96-well plates (Nalge Nunc International, Naperville, IL) were incubated overnight at 37°C with 100 μL/well of 0.25 ng/mL poly(-glutamic acid-i-tyrosine) (4:1) in PBS. Excess poly(-glutamic acid-i-tyrosine) was removed and the plate was washed thrice with wash buffer (0.1% Tween 20 in PBS). The kinase reaction was done by using 4.5 mg/well EGFR affinity purified from A431 cells (15–17). The compound was added and phosphorylation was 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 poly(-glutamic acid-i-tyrosine) was detected after a 25-min incubation with 50 μL/well of horseradish peroxidase–conjugated PY20 anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 0.2 μg/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, MD). After blue color development, 50 μL per well of H2SO4 (0.09 mol/L) were added to stop the reaction. The plates were read at 490 nm using a Bio-Rad ELISA reader (model 2550; Bio-Rad Laboratories, Hercules, CA).

**Growth inhibition assay**

For nonstimulated cell growth inhibition, approximately 10 × 104 cells per well were plated in 96-well plates. After 24 h of incubation at 37°C, cell monolayers were exposed to different concentrations of each drug continuously for 6 days. All growth-inhibitory activities were evaluated using the sulforhodamine B assay (18–20). Briefly, following drug treatment, cells were fixed using 50% 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 mmol/L), and absorbance 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 × 104) were preincubated in a six-well plate with 10% serum at 37°C for 24 h and starved overnight 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 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% NP-40, 1 mmol/L EDTA, 5 mmol/L NaF, 1 mmol/L NaVO4, protease inhibitor tablet (Roche Biochemicals, Laval, Quebec, 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-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, 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 (Upstate Biotechnology, Lake Placid, NY) for the detection of phosphotyrosine. Membranes were stripped and reprobed with anti-EGFR (NeoMarkers, Fremont, CA) for determination of corresponding receptor levels. Blots were incubated with horseradish peroxidase-goat anti-mouse antibody (1:1,000 dilution; Cell Signaling Research, Beverly, MA), and the bands were visualized with an enhanced chemiluminescence system (Amersham Pharmaica Biotech, Buckinghamshire, United Kingdom; refs. 21, 22).

**Alkaline comet assay for quantitation of DNA damage**

The modified alkaline comet assay was done as described previously (23, 24). For in vitro analysis, the cells were exposed to a dose range of drug (JDA58, JDA41, JDA35, or BCNU) for 24 h, harvested with trypsin-EDTA, washed with PBS, and collected by centrifugation. For in vivo analysis, CD-1 nude male mice (Charles River Laboratories, Senneville, Quebec, Canada) bearing DU145 xenografts were injected i.p. with vehicle or JDA58 (100 mg/kg) and tumors were excised 3 h following treatment and kept on ice. They were further homogenized with a mortar, and the resulting mixture was centrifuged to remove tissue fragments. The supernatant was collected and the cells were washed thrice with cold PBS.
For comet analysis, cell suspensions were diluted to approximately 1 x 10⁶ cells and mixed with agarose (1%) at 37°C in a 1:10 dilution. The gels were cast on GelBond strips (Mandel Scientific, Guelph, Ontario, Canada) using gel casting chambers as described previously (25) and then immediately placed into a lysis buffer [2.5 mol/L NaCl, 0.1 mol/L tetrasodium EDTA, 10 mmol/L Tris-base, 1% (w/v) N-lauryl sarcosine, 10% (v/v) DMSO, 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 tetrasodium 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 SYBR Gold [1/10,000 dilution of stock solution supplied by Molecular Probes (Eugene, OR)] 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 was analyzed for each sample using ALKOMET version 3.1 image analysis software, and values represent calculated means of tail moments for the entire cell population.

**UV fluorescence microscopy analysis**

**In vitro analysis.** Cells were plated on two-well chamber slides (Nalge Nunc International) in complete medium and allowed to grow until confluency. Following a 2- or 24-h treatment with JDA58 or JDA35 at 37°C, the medium was removed and cells were washed with PBS. Subsequently, cells were fixed with formaldehyde (3.7% formaldehyde in PBS; 1 mmol/L MgCl₂) for 30 min at room temperature. Thereafter, the slides were washed thrice with PBS containing 1 mmol/L MgCl₂, and coverslips were added using the SlowFade Light Antifade kit (Molecular Probes). The slides were examined with fluorescence microscope at an excitation wavelength of 250 nm and magnification of ×400 (23).

**In vivo analysis.** The animals (male CD-1 nude mice) bearing DU145 xenografts were given 100 mg/kg doses i.p. Following 3 or 24 h, the tumors were excised and homogenized and the cells were isolated as described previously. Thereafter, they were resuspended in serum and observed directly without fixation using an inverted fluorescence microscope.

**Pharmacological analysis**

The drug was given i.p. to CD-1 male mice (three per group per time point) at a 100 mg/kg dose in 0.4 mL of a Tween 80 (6%)/ethanol (6%) saline solution. At each time point, blood samples were collected in heparinized tubes and centrifuged for 8 min at 3,000 rpm and plasma was separated with a micropipette and stored at −80°C. Plasma aliquots (50 μL) were mixed with acetonitrile (100 μL) to precipitate the proteins, and the mixture was centrifuged for 5 min at 6,000 rpm. Standard curves were done using JDA05 [6-nitro-4-(m-toluidyl)quinazoline] as an internal standard. All solutions were filtered before injection into the high-performance liquid chromatography. Analyses were done with a Discovery C18 Supelco column (3 x 150 mm, 5-μm particle size). Elutions were achieved with a 40% acetonitrile in water solution at a 0.45 mL/min flow rate. The injection volumes were kept at 20 μL throughout the study.

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**Fig. 2.** A, internalization of JDA58 and JDA35 in DU145 cells as revealed by fluorescence microscopy. Drugs (50 μmol/L) were given for either 2 or 24 h before observation. Magnification, ×400. B, fluorescence microscopy images for the in vivo intracellular localization of JDA35. Cells were collected from DU145 xenografts 3 or 24 h following i.p. injection of JDA58 (100 mg/kg).
Mouse xenograft studies (DU145)

Severe combined immunodeficient male mice (Charles River) were maintained as per McGill animal safety protocols. Dose finding was done with two severe combined immunodeficient mice per group, and the maximum tolerated dose was defined as the dose that does not induce >15% weight loss over a period of at least 14 days. For xenograft studies, mice were treated with $1 \times 10^6$ cells suspended in 0.2 mL PBS injected s.c. into the flank of each mouse. Treatments began when tumors became palpable. The animals were placed into two treatment groups of six mice each and one control group that received the vehicle [Tween 80 (6%)/ethanol (6%) saline solution]. The treatment groups were given JDA58 i.p. (50 mg/kg) in 0.4 mL of the vehicle every other day. Tumor burdens were measured before each injection, and tumor volume was calculated using the formula $TV = \frac{(tumor\ width + tumor\ length)}{4}^3$. Biodistribution

Analysis of cell extracts in vitro. Cells were treated with various concentrations of drugs at 37°C for 2 h and then immediately washed with ice-cold PBS to remove extracellular drug. Cells were scraped from the bottoms of the six-well plates and transferred to microcentrifuge tubes. The samples were then resuspended in 100 µL of acetonitrile and centrifuged at 10,000 rpm for 6 min. Supernatant (50 µL) was collected for in vitro liquid chromatography-mass spectrometry analysis. The pellets in the microcentrifuge tube were digested with lysis buffer for 30 min on ice and centrifuged at 10,000 rpm for 20 min, and the concentrations of protein were determined using the Bio-Rad protein assay kit. All analyses were done in duplicate.

Analysis of tumor content. The animals (male CD-1 nude mice, three per group) bearing DU145 xenografts were given 100 mg/kg doses by i.p. injection. Three hours after injection, the tumors were excised, weighed, and homogenized. The homogenates were resuspended in acetonitrile and centrifuged at 10,000 rpm for 8 min at 4°C, and the supernatants were collected. Following an evaporation to dryness, the samples were reconstituted in acetonitrile and analyzed by liquid chromatography-mass spectrometry. Purified standards [analytes, including metabolites and internal standard (JDA05)] were fragmented, and the appropriate product ions were selected for multiple reaction monitoring. Linear standard curves ($R > 0.99$) were obtained between

<table>
<thead>
<tr>
<th>Drug</th>
<th>EGFR, $IC_{50}$ ($\mu$mol/L)</th>
<th>Continuous exposure* (6 d), $IC_{50}$ ($\mu$mol/L)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LNCAp (wild-type)</td>
</tr>
<tr>
<td>JDA58</td>
<td>0.204</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>JDA35</td>
<td>0.004</td>
<td>11.7 ± 0.3</td>
</tr>
<tr>
<td>JDA41</td>
<td>0.081</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>BCNU</td>
<td>167.9</td>
<td>90 ± 2</td>
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*Each point represents at least three independent experiments run in triplicate.

Table 1. EGFR tyrosine kinase inhibition and growth-inhibitory activity of JDA58, JDA35, JDA41, and BCNU on DU145, LNCAp (wild-type), and LNCApPerB2 cell lines

Fig. 3. Inhibition of EGFR phosphorylation in DU145 cells. Serum-starved cells were preincubated for 2 h with the indicated concentrations of JDA58 (A), JDA35 (B), or JDA41 (C) before stimulation with EGF (50 ng/mL) for 15 min. Equal amounts of cell lysates were analyzed by Western blotting using anti-phosphotyrosine antibodies. Membranes were stripped of anti-phosphotyrosine and reprobed with anti-EGFR antibodies as a loading control.
Samples were injected on a Zorbax C18 (150 x 4.6 mm, 5 μm) column, and the effluent was introduced into a Sciex API III mass spectrometer. All samples were quantitated relative to standard curves.

**Results**

**In vitro analyses**

**Intracellular and extracellular hydrolysis of JDA58.** The hydrolytic cleavage of JDA58 was investigated by spectrofluorometry because JDA35, the expected degradation product, fluoresces at 450 nm on excitation at 270 nm, a wavelength at which JDA58 cannot be excited. After monitoring degradation over a long period (100 h; Fig. 1C), calculations gave a $t_{1/2}$ of 21 h, showing that JDA58 was 2-fold less stable than its predecessor FD137 for which the reported $t_{1/2}$ was 41 h (9). It is important to note that the nearly complete conversion of JDA58 to JDA35 occurred after 2 days, indicating that maximum levels of the released inhibitor and alkylating species are achieved within this period in cell culture medium.

We further exploited the fluorescence properties of JDA35 to study its intracellular release and subcellular localization by fluorescence microscopy. Fluorescence was observed as early as 2 h after exposure, reaching high detectable intensity levels at 24 h after treatment with clear distribution in the perinuclear region (Fig. 2A). The detection of fluorescence after a 2-h exposure seems rapid given the long extracellular $t_{1/2}$ of JDA58.

**Binary EGFR/DNA targeting.** If the conversion of JDA58 occurred as confirmed by intracellular release of JDA35, one should expect significant inhibition of EGFR phosphorylation and considerable levels of DNA damage induced by concomitantly released alkylating species. Thus, we determined the EGFR phosphorylation status 2 h after drug exposure to largely reflect the EGFR inhibitory potency of intact JDA58. DNA damage was analyzed 24 h after treatment to allow maximal intracellular decomposition.

A preliminary EGFR tyrosine kinase inhibition assay was done with isolated EGFR, and the results showed IC$_{50}$ values of 0.2 μmol/L for JDA58 and 0.004 μmol/L for JDA35 (Table 1), suggesting that JDA58 is the prodrug of a 50-fold more potent inhibitor of EGFR. Thus, we subsequently analyzed the ability of these two drugs to block EGFR tyrosine kinase in DU145 cells. JDA58 blocked EGFR phosphorylation in a dose-dependent fashion (IC$_{50}$, 4 μmol/L; Fig. 3A) and induced 1.00 and 100 ng for each standard. Samples were injected on a Zorbax C18 (150 x 4.6 mm, 5 μm) column, and the effluent was introduced into a Sciex API III mass spectrometer. All samples were quantitated relative to standard curves.

Nevertheless, significantly higher fluorescence intensity was observed in the cells after a 24-h exposure, indicating that, at 2 h after treatment, JDA58 hydrolysis was not at its maximum level. As expected, JDA35 given directly to the cells showed observable fluorescence as early as 2 h after treatment. In all cases, the fluorescence seemed mostly localized in the perinuclear region, an area that contains the endoplasmic reticulum and the Golgi apparatus.

**Fig. 4.** A, quantitation of DNA damage (alkaline comet assay) in vitro after 24-h exposure to JDA58, BCNU, JDA35, and JDA41. B, quantitation of DNA damage (alkaline comet assay) in vivo 3 h after treatment. Tumor-bearing mice were injected i.p. with vehicle or JDA58 (100 mg/kg). Tail moments were measured with ALKOMET image analysis software for at least 50 cells per dose. Each point represents at least two independent experiments run in three mice per group. Statistical analysis was done with a two-tailed $t$ test. Asterisk, statistical significance $P < 0.01$ ($P = 0.0009$).

**Fig. 5.** Analysis of combi-molecule and its metabolites in tumor cells in vitro and in tumor-bearing mice. A, drugs detected in the cells following in vitro administration of JDA58, JDA41, and JDA35 at 100 μmol/L. B, intratumoral content of different metabolites following in vivo administration of JDA58 at 100 mg/kg. For in vitro study, data are expressed as ng of drug per ng of protein in cell extracts. For tumor distribution analysis, data are expressed as ng of drug per g of tumor.

In all cases, the fluorescence seemed mostly localized in the perinuclear region, an area that contains the endoplasmic reticulum and the Golgi apparatus.
levels of DNA damage similar to that of the classic alkylating agent BCNU (Fig. 4A). Similarly, direct administration of JDA35 induced a dose-dependent blockade of EGF-induced EGFR phosphorylation but with a greater potency than JDA58 (~100% inhibition was achieved at a concentration as low as 1.6 μmol/L; Fig. 3B). At this dose, JDA41 induced <100% inhibition (Fig. 3C). As expected, JDA35 and JDA41 did not induce any DNA damage (Fig. 4A). We have already shown that the classic alkylating agent BCNU does not inhibit EGFR phosphorylation (9). Based on enzyme and whole-cell assay results, the order of EGFR inhibitory potency of all the agents tested was the following: JDA35 > JDA41 > JDA58 > BCNU.

Antiproliferative activities in human tumor cells in vitro. Given the kinetics and a binary mechanism of action of JDA58, antiproliferative studies were done at a long exposure period (6 days) to allow the maximum conversion to JDA35. We have chosen to test the potency of different molecules against human prostate cancer cell lines LNCaP (wild-type), LNCaPerbB2, and DU145 that have already been shown to be sensitive to antiproliferative agents targeting EGFR in vitro and in vivo (8, 27, 28). The DU145 cells express elevated levels of EGFR and high levels of the DNA repair enzyme AGT (11, 29) that confers a resistance to chloroethynitrosoureas. The results showed that the combi-molecule JDA58 was 2- to 6-fold more potent than JDA35 given alone, despite the superior EGFR tyrosine kinase inhibitory potency of the latter, and was more than 20- to 40-fold more potent than BCNU (IC50 >90 μmol/L) in all cell lines (Table 1), indicating that, perhaps, the mixed EGFR/DNA targeting activity of the combi-molecule may contribute to an enhancement of its activity.

To determine whether the superior potency of the combi-molecule was due to its enhanced cellular uptake, we analyzed the levels of each drug in the cells following incubation at various doses. Because the combi-molecule degrades inside the cells, for simplicity only the results obtained from cell exposure at 100 μmol/L are shown (Fig. 5A). The trend was that JDA41 possessed a superior penetration with a detected amount of 67.5 ng/ng of protein when compared with JDA35 (17.5 ng/ng of protein). Interestingly, the combi-molecule was fragmented in the cells to two metabolites JDA41 (10.4 ng/ng of protein) and JDA35 (0.32 ng/ng of protein). We also observed that, at the high concentrations (e.g., 100 μmol/L), a small amount of JDA35 (0.3 ng/ng of protein) could be detected from the intracellular hydrolysis of JDA41 (Fig. 5A). Based on the overall levels of intact JDA58 in the cells and those of its two major metabolites, it can be estimated that its cellular penetration is in the same range as that of JDA35. Overall, the metabolite JDA41, which seemed to have increased cellular penetration, and JDA35, which showed approximately the same level of uptake as JDA58, were 1.8- to 6-fold less potent than the latter in the cell panel. Therefore, the potency of the three compounds does not seem to correlate with their levels of cellular uptake.

In vivo translation

Pharmacokinetics. As in vitro analysis showed that JDA58 was converted to JDA35 under physiologic conditions, we investigated whether this reaction was achievable in vivo and would translate into tumor growth-inhibitory activity. The drug was given i.p. to CD-1 male mice in its more soluble hydrochloride salt form and analyzed in the plasma at various time points. Following i.p. administration of JDA58 (Fig. 6A), an unknown metabolite was detected as early as 5 min after injection, reaching its maximum in the range of 25 μmol/L at 30 to 45 min (Fig. 6B). Liquid chromatography-mass spectrometry analysis showed that this was JDA41, the denitrosated product of JDA58 and not JDA35. Lack of formation of JDA35 may be due to the slow rate of chemical decomposition of JDA58 (t1/2 = 21 h) in plasma, allowing the latter to be rapidly denitrosated by metabolic enzymes [e.g., glutathione transferases (30)] before hydrolytic cleavage.

Intratumoral analysis: subcellular localization and DNA damage. The failure to detect JDA35 in plasma prompted us to investigate the status of this critical metabolite within tumor cells in vivo. Despite the absence of detectable amounts of
JDA35 from plasma, a significant level of fluorescence was observed in cells taken from tumors in vivo 3 h after injection, a time point chosen to allow a sufficient absorption of JDA58. More importantly, fluorescence was observed in the cells even 24 h after injection, indicating a significant tumor retention of the released fluorescent amine (Fig. 2B). Further liquid chromatography-mass spectrometry analysis confirmed the presence of JDA35 in the cells. In a manner similar to in vitro analysis, when JDA58 was given at a 100 mg/kg dose, the intact combi-molecule, its denitrosated metabolite JDA41, and the hydrolytic product JDA35 were observed with the following distribution: JDA58, 14 ng/g of tumor; JDA41, 1,952 ng/g of tumor; and JDA35, 150 ng/g of tumor (Fig. 5B).

Because fluorescence associated with JDA35 was observed in vivo, we surmised that the DNA-alkylating fragment might have also been concomitantly released in tumor cells as predicted by the chemical mechanism of degradation. Thus, we analyzed the levels of DNA damage induced by the combi-molecule in vivo using the comet assay 3 h after injection at a 100 mg/kg dose. Interestingly, significant levels of DNA damage (P < 0.05) were observed in treated animals (Fig. 4B).

In vivo efficacy. First, we undertook a dose finding study comparing JDA58 with BCNU by monitoring weight loss over a period of 18 days in groups of two animals. From this study, a 50 mg/kg dose was defined as the maximum tolerated dose for JDA58, BCNU being toxic at the latter dose. JDA58 induced significant antitumor activity (P < 0.05) 15 days after treatment (Fig. 6C). The in vivo potency of JDA58 is a significant result because, in our study and that of others (7), BCNU was inactive in vivo against DU145 cells (Table 1).

Discussion

The combi-targeting concept postulates that a molecule possessing EGFR inhibitory activity on its own and capable of being further hydrolyzed into a DNA-damaging agent and another inhibitor of EGFR should induce sustained antiproliferative activity in EGFR-dependent refractory tumors. Thus, the potency of the approach chiefly depends on the rate of hydrolytic scission of the combi-molecule under physiologic conditions. The stability of the combi-molecule will enable it to cross the cell membrane and interact with the ATP site of EGFR. Here, we showed that JDA58 is a relatively stable nitrosourea that decomposes at a slow rate in serum-containing medium, a property that may allow it to penetrate the cells intact and block EGFR phosphorylation before degradation into JDA35 plus the chloroethylating species. Indeed, our results show that it can induce a tandem blockade of EGFR phosphorylation and significant levels of DNA damage in a dose-dependent manner in DU145 cells. More importantly, despite its slow rate of decomposition in serum-containing medium (t<sub>1/2</sub> = 21 h), detectable levels of fluorescence associated with JDA35 could be observed in the cells as early as 2 h after drug exposure, indicating that the combi-molecule decomposes in the intracellular milieu at a faster rate than predicted by its t<sub>1/2</sub>. This agrees with previous studies on trisubstituted nitrosourea 1,3-dimethyl-3-phenyl-1-nitrosourea that showed a t<sub>1/2</sub> of 220 days in phosphate buffer (31, 32). However, the latter molecule was capable of producing significant genotoxicity in the Chinese hamster V79-E cells after a 1-h drug exposure (33). It was shown in the latter study that the hydrolysis of the nitrosourea could be accelerated by intracellular serine hydrolases, which were shown to catalyze the hydrolysis of the nitrosourea function in a manner similar to the mechanism proposed for JDA58 (Fig. 1B).

We have already reported on the accelerated intracellular decomposition of another combi-molecule SMA41 (a triazene) in tumor cells. This drug decomposed four times faster intracellularly than in cell culture medium ex vivo (34).

Given the short life of the chloroethylating species, the remarkable stability of JDA58 in extracellular serum-containing medium is a major advantage. Rapid exogenous decomposition would perhaps abolish its DNA-damaging property. It has already been shown that addition of exogenous porcine liver carboxylesterase that is unable to cross cell membrane in cell culture medium completely depleted the genotoxicity of trisubstituted nitrosoureas (33), indicating that the released alkylating species may be too unstable to permeate the cells and further interact with DNA.

The degradation of JDA58 in tumor cells in vivo presented characteristics that markedly distinguished it from decomposition in cell-free medium. Interestingly, the exogenous decomposition of JDA58 in cells and in the mice is not primarily mediated by hydrolytic cleavage but by denitrosation, a process that leads to JDA41, the major metabolite. It should be noted herein that the in vivo denitrosation of nitrosoureas is a known process already reported for BCNU [t<sub>1/2</sub>, 17 min in plasma (35)] and other analogues (36). For JDA58, because of the stability of the nitrosourea moiety, denitrosation is the predominant metabolic pathway. However, it is noteworthy that detectable levels of intact JDA58 were observed in plasma 30 to 45 min after treatment, indicating that the bioavailability of the latter molecule was sufficiently high to permit its tumor uptake. This is further corroborated by the substantial amount of JDA58 (14 ng/g) observed in the tumors even 3 h after injection. Furthermore, the quantitation and characterization of JDA35 can be considered a direct evidence of the intracellular hydrolytic cleavage of JDA58. Despite the abundant formation of the denitrosated metabolite both intracellularly and metabolically, the results suggest that the level of endogenous hydrolysis of the nitrosourea moiety is sufficient for the generation of DNA damage. This agrees with the significant levels of DNA damage observed both in vitro and in vivo.

It was clearly shown that JDA58 and its degradation products or metabolites JDA35 and JDA41 are strong inhibitors of EGFR. Hence, in accordance with the combi-targeting postulates, JDA58, whether it was given in vitro or in vivo, degraded into two types of agents: EGFR inhibitors and DNA-damaging species. Their combined effects translated into significant antiproliferative activity as evidenced by its 1.8- to 47-fold superior potency when compared with single inhibitors and the classic nitrosourea BCNU. We have already shown that FD137, a 5-fold less potent analogue of JDA58, was more potent than a two-drug combination involving an EGFR inhibitor plus BCNU (9). It is to be noted that the latter drug was too toxic to be compared at equidose in vivo with JDA58, which showed significant antitumor activity in the DU1145 xenograft. It should also be remembered that DU1145 cells (a) overexpress EGFR (37) and (b) express the DNA repair enzyme AGT (7) and (c) EGFR activation in these cells is associated with elevated levels of the base excision repair protein, XRCC1, and nucleotide excision repair protein, ERCC1, which confer significant resistance to alkylating agents (38, 39). The significant in vivo
activity of JDA58 as well as its 47-fold superior antiproliferative activity when compared with BCU1 against this cell line set premise for further investigation on this novel type of combin-nitrosoureas engineered to damage DNA and block EGFR tyrosine kinase.

In summary, the results in toto have allowed not only to verify the basic postulates of the combi-targeting concept but also to propose a model that reconciles the premise for further investigation on this novel type of combi-targeting concept. JDA58 plus JDA41 plus JDA35 can contribute to sustained inhibition of EGF tyrosine kinase and, based on microscopic observation, may be competing for localization in the perinuclear region, perhaps interacting with nascent or internalized EGF. Indeed, it is now known that EGF is relocated to the perinuclear region following activation (40, 41). Thus, JDA58 represents a single molecule capable of emulating the effect of a complex multidrug combination in the cells. The significant in vivo and in vitro potency observed for JDA58 in the highly resistant prostate tumor cells suggests that the combi-targeting model may offer a new alternative to the lack of sensitivity of EGF and AGT-overexpressing tumors to classic nitrosoureas.

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The Combi-Targeting Concept: In vitro and In vivo 
Fragmentation of a Stable Combi-Nitrosourea Engineered to 
Interact with the Epidermal Growth Factor Receptor while 
Remaining DNA Reactive 

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