Subcellular Distribution of a Fluorescence-Labeled Combi-Molecule Designed to Block Epidermal Growth Factor Receptor Tyrosine Kinase and Damage DNA with a Green Fluorescent Species

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Abstract

To monitor the subcellular distribution of mixed epidermal growth factor (EGF) receptor (EGFR)–DNA targeting drugs termed combi-molecules, we designed AL237, a fluorescent prototype, to degrade into a green fluorescent DNA damaging species and FD105, a blue fluorescent EGFR inhibitor. Here we showed that AL237 damaged DNA in the 12.5 to 50 μmol/L range. Despite its size, it blocked EGFR phosphorylation in an enzyme assay (IC50 = 0.27 μmol/L) and in MDA-MB468 breast cancer cells in the same concentration range as for DNA damage. This translated into inhibition of extracellular signal-regulated kinase 1/2 or BAD phosphorylation and downregulation of DNA repair proteins (XRCC1, ERCC1). Having shown that AL237 was a balanced EGFR-DNA targeting molecule, it was used as an imaging probe to show that (a) green and blue colors were primarily colocalized in the perinuclear and partially in the nucleus in EGFR- or ErbB2-expressing cells, (b) the blue fluorescence associated with FD105, but not the green, was colocalized with anti-EGFR red-labeled antibody, (c) the green fluorescence of nuclei was significantly more intense in NIH 3T3 cells expressing EGFR or ErbB2 than in their wild-type counterparts (P < 0.05). Similarly, the growth inhibitory potency of AL237 was selectively stronger in the transfectants. In summary, the results suggest that AL237 diffuses into the cells and localizes abundantly in the perinuclear region and partially in the nucleus where it degrades into EGFR and DNA targeting species. This bystander-like effect translates into high levels of DNA damage in the nucleus. Sufficient quinazoline levels are released in the cells to block EGF-induced activation of downstream signaling.

Introduction

The epidermal growth factor (EGF) receptor (EGFR) and its closest family member p185Neu, the product of the HER2 gene, are transmembrane receptor tyrosine kinases (TK), which transduce signals associated with tumor cell proliferation (1–4). The variety of approaches currently used to target EGFR includes small monoclonal antibody strategy to block ligand binding and TK inhibitors (5–7). Over the past 10 years, molecules that inhibit receptor autophosphorylation and downstream intracellular signaling have been developed and have shown significant antitumor activity in vitro (8–12). Several of them including gefitinib (Iressa), erlotinib (Tarceva), and lapatinib (Tykerb/Tyverb) have been approved for clinical use (13, 14). However, none of these drugs are used in the clinic as single agents in the therapy of advanced cancers (15, 16). Despite their ability to block growth signaling associated with EGFR or p185Neu, cancer cells have the ability to evade the growth inhibitory effect of these drugs by activating alternative signaling pathways. Moreover, these drugs are reversible inhibitors, indicating that the tumor cells may resume proliferation following drug clearance. Therefore, for an effective therapy, combination with a drug capable of killing the cell by a different mechanism (e.g., DNA damage or inhibition of DNA synthesis) is required. However, despite this overwhelming reality of cancer therapy, the development of mono-targeted drugs through high throughput screening or rational drug design remains the most generally adopted strategy. Over the past several years, we have developed a paradigm shifting strategy that seeks to design a single molecule with multiple functions...
termed combi-molecule (17–22). These molecules, despite their combination-based design, were not developed with the purpose of eventually replacing the traditional chemotherapy but rather complementing it. Albeit, we showed that many prototypes (e.g., SMA41, FD137) showed stronger antiproliferative activity than classic combinations of drugs with the same mechanism of action (20, 23–26).

As outlined in Fig. 1A, the combi-molecules (see I-Alk) were designed to bind to EGFR on their own and to decompose into another EGFR inhibitor (I) plus a DNA alkylating species (Alk). Previous studies from our laboratory have shown that indeed the combi-molecules (e.g., SMA41; Fig. 1A) could directly block EGFR in short exposure assay in vitro at room temperature in serum-containing media (18). Additionally, we showed that they are capable of blocking EGFR phosphorylation and significantly damaging DNA in human tumor cells in vitro and in vivo, indicating that our combi-molecules induce a bifunctional activity in whole cells (22, 27). Using the fluorescence of the generated inhibitor I (excitation, 294 nm; emission, 451 nm) and 14C-radiolabeled alkyl moiety of SMA41, we previously confirmed that the combi-molecule could indeed decompose in the intracellular environment.

Figure 1. A, schematic representation of the combi-targeting concept. B, stepwise decomposition of AL237. I-Alk-Dansyl, to generate EGFR inhibitor (I, FD105) and dansylated alkylating DNA species (Alk-Dansyl). The entire AL237 molecule and the dansylated DNA damaging species both emit at 525 nm (green).
compartment into an EGFR inhibitor (I) and a methyl diazonium species (Alk) that damages DNA (27, 28). Whereas the fluorescence property of the aminoquinazoline (I) permitted the observation of its subcellular distribution or localization, that of the short-lived [14C]methyl diazonium could not be imaged (28). Here we designed a novel probe, AL237, in which the fluorescent dansyl tag is attached to a 3-alkyl triazene moiety (Fig. 1B; I-Alk-Dansyl), which when hydrolysed will release a fluorescent alkylating agent (see Alk-Dansyl). Alkylation of DNA by this fluorescent alkylating molecule (see Fig. 1B) would lead to green nuclei (excitation, 340 nm; emission, ~525 nm), and the release of the aminoquinazoline (I, FD105; excitation, 294 nm; emission, 451 nm) would generate blue areas in the cells. Thus, fluorescence microscopy would allow us to image the complete fragmentation of the combi-molecule and its colocalization with one of its targets, EGFR, using immunofluorescence. Here, we test these hypotheses with AL237 and correlate its biodistribution profile with its dual mechanism of action. For purpose of comparison, a nonconjugated dansylated alkyl agent, N-dansylaziridine, was used (see structure in Fig. 5C). The latter, as previously reported, can only alkylate nucleic acids (29). It is to be noted that this study does not seek to establish the growth inhibitory potency of AL237 but rather to show its binary EGFR-DNA targeting property and to be used as a probe to image the subcellular localization of the two bioactive degradation products responsible for its EGFR-DNA binary targeting mechanism.

Materials and Methods

Cell culture

MDA-MB-468 human breast carcinomas were obtained from American Type Culture Collection. Mouse fibroblast cells NIH 3T3 used as control or NIH 3T3her14 (transfected with erbB1/EGFR gene) and NIH 3T3neu (transfected with erbB2 gene) were provided by Dr. Moulay Aloui-Jamali (Montreal Jewish General Hospital). All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 10 mmol/L HEPES, 2 mmol/L L-glutamine, and antibiotics (all reagents were purchased from Wisent, Inc.) as previously described (18). Cells were maintained at exponential growth at 37°C in a humidified environment with 5% CO2. In all assays cells were plated 24 h before drug administration.

Drug treatment

AL237 and JDA41 were synthesized in our laboratory. The methods used for AL237 and JDA41 complete synthesis were described elsewhere (22, 30). N-dansylaziridine was purchased from Biomol, and Temozolomide and Iressa were purchased from the hospital pharmacy and extracted from pills in our laboratory. EGF was obtained from Roche Molecular Diagnostics. In all assays, drugs were dissolved in DMSO and subsequently diluted in phenol red/fetal bovine serum–free DMEM before added to cells. The concentration of DMSO never exceeded 0.2% (v/v) during treatment.

Growth inhibition assay

Cells were plated in 96-well flat-bottomed microtiter plates at 5,000 cells per well (NIH 3T3her14, NIH 3T3neu, MDA-MB-468) or 10,000 cells per well (NIH 3T3). After 24 h cells were exposed to different drug concentrations for 4 d. Briefly, following drug treatment, cells were fixed with 10% ice-cold trichloroacetic acid for 60 min at 4°C, stained with sulforhodamine B (0.4%) for 4 h at room temperature, rinsed with 1% acetic acid, and allowed to dry overnight (31). The sulforhodamine B absorbance was recorded at 492 nm using a Bio-Rad microplate reader. The results were analyzed by GraphPad Prism (GraphPad Software, Inc.), and the sigmoidal dose response curve was used to determine IC50. Each point represents the average of at least three independent experiments run in triplicate.

In vitro enzyme assay

The EGFR and src kinase assays are similar to one described previously (27). Briefly, the kinase reaction was done in 96-well plates using 4.5 ng/well EGFR or src (Biomol). Following drug addition (range, 0.0001–10 μmol/L), phosphorylation of the EGFR was initiated by supplementing the reaction with ATP. The phosphorylated substrate was detected using a horseradish peroxidase–conjugated anti-phospho-tyrosine antibody (Santa Cruz Biotechnology), and the colorimetric reaction was monitored at 450 nm using a Bio-Rad reader. The results were analyzed by GraphPad Prism, and IC50 was calculated.

Western blot analysis

Cells were grown to 80% confluence in six-well plates and serum starved for 24 h (serum-free DMEM), followed by a 2-h incubation with AL237 at the indicated concentrations. Cells were washed from the drug with PBS, and then cells were stimulated with EGF (50 ng/mL) for 15 min. Cells were collected and lysed in ice-cold protein extraction buffer for 30 min [20 mmol/L Tris-HCl (pH 7.5), 1% NP40, 10 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L NaF, 1 mmol/L Na vanadate, complete protease inhibitor cocktail (Roche Molecular Diagnostics)]. Equal amounts of proteins were separated on 10% SDS-polyacrylamide gels and then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Membranes were blocked with 5% milk in TBST (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween 20) for 3 h. Primary antibodies used for immunodetection were dissolved in antibody buffer [5 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% (v/v) Tween 20, 0.05% (w/v) Na azide, 0.25% (w/v) gelatin] or TBST buffer as follows: anti-phospho-tyrosine (clone 4G10, Upstate; 1:1,000), anti-EGFR (sc-03, Santa Cruz; 1:1,000), anti-phospho-EGFR (Tyr1068, 1:1,000), anti-XRCC1 (33-2-5, Millipore)] or TBST.
ThermoFisher Scientific; 1:1,000), anti-ERCC1 (clone 3H11, ThermoFisher Scientific; 1:1,000), and anti-phospho-γH2AX (1:1,000, Abcam). Anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2; Thr202/Tyr204; 1:4,000), anti-ERK1/2 (p44/p42 mitogen-activated protein kinase; 1:2,500), anti-phospho-BAD (Ser112; 1:1,000), anti-phospho-γH2AX (1:1,000), anti-BAD (1:250) were obtained from Cell Signaling Technology. Anti-tubulin-α (clone DM1A, NeoMarkers; 1:2,000) was used as a loading control. Secondary horse–radish peroxidase–conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories. The bands were visualized using enhanced chemiluminescence (Amersham Bioscience). Western blot experiments were done at least twice from two independent cell treatments.

**Alkaline comet assay**

Cells were exposed to AL237 or N-dansylaziridine (0, 6.25, 12.5, 25, 50, 100 μmol/L) for 2 h, and the alkaline comet assay was done as previously described (27, 32). During this procedure, cells were protected from direct light to minimize DNA damage. Comets were visualized at 10× magnification using Leica microscope after staining with SYBR Gold (1:10,000, Molecular Probes). DNA damage was quantified using Comet Assay IV software (Perceptive Instruments), and the degree of DNA damage was expressed as tail moments. A minimum of 50 comets were analyzed for each cell treatment and the mean tail moments were calculated from three independent experiments.

**Neutral comet assay**

Cells were treated and collected as in the alkaline comet assay, embedded in agarose at the same cell density, and lysed with a neutral buffer for 2 h at room temperature [154 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.8), 10 mmol/L EDTA, 0.5% (v/v) N-Lauroyl-sarcosine (pH 8.0)]. Gels were soaked for 30 min in neutral Tris-borate EDTA buffer [90 mmol/L Tris-HCl (pH 7.8), 90 mmol/L boric acid, 2 mmol/L EDTA] and electrophoresed for 20 min at 20 V (33, 34). Modification of the assay permitted to retain the fluorescent dansyl tag to the sites of DNA adducts and allowed to observe nuclei without using a specific DNA staining dye.

**Intracellular fluorescence by UV flow cytometry**

NIH 3T3, NIH 3T3her14, and NIH 3T3nu were plated at 0.5 × 10^6 cells per well in six-well plates, allowed to adhere overnight, and treated with AL237 (0, 6.25, 12.5, 25, 50, 100 μmol/L) for 45 min. Cells were collected, washed, and resuspended in 300 μL PBS supplemented with 1% fetal bovine serum to minimize cell clumping. Cellular fluorescence levels were measured using a Becton Dickinson LSR flow cytometer (BD Biosciences). Cells were excited with 340-nm light emitting laser, and the AL237 hydrolyzed fragments were detected as follows: the aminquinazoline emitted at 451 nm (blue) and the dansylated DNA damaging species emitted at 525 nm (green). Fluorescence levels determined by fluorescence-activated cell sorting were analyzed with GraphPad Prism software, and the accumulated fluorescence for each cell line was expressed as percentage of mean fluorescence over control. Four independent experiments were done in duplicate.

**Nuclear fluorescence UV flow cytometry**

NIH 3T3, NIH 3T3her14, and NIH 3T3nu were plated at 0.5 × 10^6 cells per well in six-well plates, incubated overnight, and exposed for 45 min to AL237 or N-dansylaziridine (0, 6.25, 12.5, 25, 50, 100 μmol/L). After washing with PBS, cells were incubated in 300 μL of Vindelov solution for 15 min at 37°C [10 mmol/L Tris-HCl (pH 7.5); 10 mmol/L NaCl, 0.1% Nonidet P40 (v/v); 100 μg/mL; 50 units/mL RNaseA; ref. 35]. Nuclei were analyzed on a BD LSR flow cytometer as described earlier. A minimum of 10,000 cell nuclei were acquired per sample, and each drug concentration was done in duplicate. The fluorescence levels were quantified with CellQuest Pro software (Becton Dickinson), and results are reported as percentage fluorescence over control from three independent experiments.

**Immunofluorescence of EGFR and phospho-tyrosine**

MDA-MB-468 cells were plated at 60% confluence on a microcover glass (VWR) placed in a 24-well plate. Cells were starved overnight, followed by treatment with 25 μmol/L AL237 for 2 h, and then stimulated with 50 ng/mL EGF for 15 min. Subsequently, cells were washed twice with PBS, fixed with 100% ice-cold methanol at −20°C for 5 min, followed by 1 h blocking with 5% normal goat serum. Double immunostaining was done using directly-coupled mouse anti-phospho-tyrosine-FITC (1:100), mouse anti-EGFR-PE (1:100), or the appropriate IgG-FITC– or IgG-PE–conjugated controls (1:100, purchased from Santa Cruz Biotechnology, Inc.). Thereafter, cells were washed twice with PBS, stained with 5 ng/mL 4′,6-diamidino-2-phenylindole solution (Sigma), and mounted with a gel mounting media (Fisher Scientific). Immunofluorescence images were captured with Leica microscope (Leica) using the appropriate filters and analyzed with Leica application suite software.

**Live cell fluorescence imaging of AL237 in NIH 3T3 cells**

NIH 3T3, NIH 3T3her14, and NIH 3T3nu were plated at 70% confluence in six-well plates, allowed to adhere overnight, and treated with 25 μmol/L AL237 for 2 h. At the indicated time points, cells were washed with PBS twice and images were saved with Leica DFC300FX camera with the appropriate filters.

**Immunofluorescence of phospho-γH2AX**

MDA-MB-468 cells were plated on slides and incubated overnight, followed by a treatment with 25 and 50 μmol/L of AL237 for 2 h. Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with...
0.1% Triton X-100 for 10 min, followed by 1 h blocking with 5% normal goat serum. Slides were incubated with mouse anti-phospho-γH2AX antibody (Abcam) for 3 h and antimouse FITC-labeled secondary antibody (Sigma) for 1 h. Typical for double-strand DNA breaks, phospho-γH2AX foci were observed on a fluorescent Leica microscope at 40×.

**Annexin V/propidium iodide binding assay**

MDA-MB-468 cells were plated in a six-well plate and treated with a dose range of each drug for 48 h. Thereafter, cells were harvested and incubated with Annexin V–FITC and propidium iodide (PI) using the apoptosis detection kit (BenderMedSystems, Inc.) following the protocol provided by the supplier. Annexin V–FITC
and PI binding were analyzed with a Becton Dickinson FACScan. Data were collected using logarithmic amplification of both the FL1 (FITC) and FL2 (PI) channels. Quadrant analysis of coordinate dot plots was done with CellQuestPro software.

Results

Analysis of binary EGFR-DNA targeting potentials

Inhibition of EGFR phosphorylation. For AL237, being a bulky molecule, we first determined whether the long spacer attached to the 6-position of the quinazoline ring affects its ability to inhibit receptor phosphorylation in purified EGFR enzyme assay. Our results showed that AL237 was capable of inhibiting EGFR phosphorylation with an IC50 of 0.27 μmol/L. Moreover, we have addressed AL237 selective EGFR binding by measuring inhibition of tyrosine phosphorylation on a src kinase. The result indicates that, despite its significant bulkiness, AL237 binds strongly to the ATP-binding site of EGFR and is a selective EGFR inhibitor (Fig. 2A). Indeed, various of similar compounds with bulky side chains have been synthesized in our laboratory and have been shown to retain strong EGFR binding affinity (23, 36, 37). It is also known that most anilinoquinazolines show unspecific binding to the ErbB2 gene product, which is the closest family member of EGFR (13). More importantly, to verify the ability of AL237 to block EGFR phosphorylation in whole cells, we analyzed phospho-EGFR levels when cells were exposed to 100 μmol/L Temozolomide (9%), 25 μmol/L N-dansylaziridine (12.5%), or combinations of 25 μmol/L Temozolomide and 25 μmol/L Iressa (35%; see Supplementary Fig. S1). Thus, the combined EGFR TK inhibition and DNA damaging properties of AL237 were sufficient to confer high levels of apoptosis in MDA-MB-468 cells. Next, we used AL237 to determine its selective growth inhibition in EGFR/ErbB2-expressing cells and to image the release of its bioactive species in the intracellular compartment.

Induction of DNA damage in MDA-MB-468 breast cancer cells. Annexin V–FITC and PI staining were used to distinguish viable (PI−/FITC−), early apoptotic (PI+/FITC−), late apoptotic (PI+/FITC+), and necrotic (PI+/FITC−) cells after 48 hours of exposure to the combi-molecule. We observed a strong and a dose-dependent increase in apoptosis by AL237 at IC50 range reaching as high as 60% apoptotic cells 48 hours posttreatment (Fig. 3C). In contrast, much lower levels were observed when cells were exposed to 100 μmol/L Temozolomide (9%), 25 μmol/L N-dansylaziridine (12.5%), or combinations of 25 μmol/L Temozolomide and 25 μmol/L Iressa (35%; see Supplementary Fig. S1). Thus, the combined EGFR TK inhibition and DNA damaging properties of AL237 were sufficient to confer high levels of apoptosis in MDA-MB-468 cells.

Imaging of AL237 in MDA-MB-468 human breast cancer cells and NIH 3T3 transfectants

Imaging of MDA-MB-468 cells treated with AL237. Fluorescence emission released by AL237 was analyzed in MDA-MB-468 cells at different time points using 25 μmol/L of the drug. The two hydrolyzed degradation products were detectable in the cells as early as 5 minutes after addition, reaching a maximum 20 minutes later (Fig. 3D, top). We observed the blue fluorescence associated with the aminoquinazoline FD105 (451 nm) and the green fluorescence with the DNA damaging dansylated moiety (525 nm) in the cytoplasm and in the vicinity of the nucleus. Whereas the entire molecule can still fluoresce in green, the detection of blue FD105 fragment is indicative of a degradation of the molecule, which as reported elsewhere has a half-life of 22 minutes (30). Whether the combi-molecule was partially or completely decomposed, the green fluorescence was consistently localized in the perinuclear area (Fig. 3D, bottom left). To challenge the EGFR-directed localization of the
Figure 3. DNA damage induced by AL237 in MDA-MB-468. A, cells were exposed to AL237 for 2 h, followed by assessment of drug-induced DNA damage using an alkaline comet assay. Comet tail moments were quantitated by comet IV software (Perceptive Instruments). Columns, average comet tail moment calculated from 50 comets based on three independent experiments for each concentration (0, 6.25, 12.5, 25, 50, 100 μmol/L). Representative images of DNA comets stained with SYBR Gold dye and visualized by fluorescence microscopy at 10× were shown for each dose. B, extracts from cells treated for 2 h with increasing concentrations of AL237 were analyzed by Western blot with anti-phospho-γH2AX antibody. Membrane was probed with anti-tubulin antibody as a loading control. MDA-MB-468 cells were preincubated on a slide, treated with 0, 25, or 50 μmol/L of AL237, and analyzed for phospho-γH2AX foci. Cells were observed at Leica fluorescent microscope (40×) by indirect immunofluorescence using primary mouse anti-phospho-γH2AX antibodies, followed by FITC-labeled antimouse antibody. C, Annexin V/PI-stained MDA-MB-468 cells were analyzed by fluorescence-activated cell sorting 48 h after treatment with AL237 and N-dansylaziridine. D, fluorescence distribution of AL237-EGFR-binding aminoquinazoline species at 25 μmol/L was imaged over time in MDA-MB-468 cells (top). Fluorescence distribution of AL237 hydrolyzed degradation products were observed either alone (bottom left) or in the presence of equimolar concentrations (25 μmol/L) with a competitive EGFR binding nonfluorescent molecule JDA41 (bottom right) at 20 min.
A

phase contrast  451 nm AL237  525 nm AL237  overlay

anti-EGFR-PE  overlay EGFR-PE/451 nm AL237  overlay EGFR-PE/525 nm AL237  enlarged

B

NIH 3T3  NIH 3T3her14

451 nm  525 nm

overlay

C

Blue fluorescence of AL237

NIH 3T3neu  NIH 3T3her14  NIH 3T3

Fluorescence, % Control

Concentration (μmol/L)

Green fluorescence of AL237

NIH 3T3neu  NIH 3T3her14  NIH 3T3

Fluorescence, % Control

Concentration (μmol/L)

D

- EGF  anti-pTyrosine-FITC  anti-EGFR-PE

+ EGF

+EGF +AL237
combi-molecule in the perinuclear region, we used JDA41 (IC$_{50}$ = 0.081 mmol/L), a nonfluorescent EGFR TK inhibitor (22). The results showed that competitive exposure of AL237 (IC$_{50}$ = 0.27 mmol/L) with JDA41 delocalized the latter into the cytoplasm, indicating that the perinuclear colocalization may be directed by EGFR binding (Fig. 3D, bottom right).

**Qualitative analyses of isogenic NIH 3T3 EGFR/ErB2 transfectants.** AL237-released green and blue fluorescence were also observed in an isogenic context using NIH 3T3, NIH 3T3her14 (transfected with EGFR), and NIH 3T3neu (transfected with erbB2). As we have shown on the merged images in MDA-MB-468 cells (Fig. 4A, top right), in NIH 3T3 cells, the blue and green fluorescence produced by AL237 or its decomposition products were similarly colocalized in the perinuclear area or in the vicinity of the nucleus (Fig. 4B). Interestingly, in the isogenic cells, AL237 or its degradation products were colocalized, with a more pronounced perinuclear distribution in EGFR- and ErbB2-expressing cells than in their wild-type counterpart. Moreover, quantitative flow cytometric analysis of NIH 3T3, NIH 3T3her14, and NIH 3T3neu cells further confirmed at a single-cell level that AL237 decomposed in the cells and released its two fluorescent degradation products: blue fluorescence corresponding to the aminoquinazoline moiety (Fig. 4C, top graph) and green fluorescence due to the entire molecule and/or the released dansylated DNA damaging species (Fig. 4C, bottom graph).

**Colocalization of EGFR and AL237 in MDA-MB-468 cells.** To verify whether AL237 binds to EGFR, we used direct immunofluorescence by staining MDA-MB-468 cells with PE-labeled EGFR and FITC-labeled phospho-tyrosine antibodies. After EGFR stimulation, we observed activated EGFR at the plasma membrane (Fig. 4D, middle), which was strongly inhibited after the cells were exposed for 2 hours to 25 μmol/L of AL237, a dose at which EGFR phosphorylation was also shown to be significantly depleted using Western blot analysis (see Fig. 2B and C). Whereas in EGF-stimulated cells, the EGFR showed a more membrane localization, in AL237-treated cells, it was redistributed in the cytoplasm in endosome-like structures primarily concentrated around the perinuclear region (Fig. 4A and D, bottom right). The localization of the fluorescence is in agreement with the ability of the released blue aminooquinazoline (see Fig. 1) to bind to EGFR TK and also with that of the green dansylated alkylating species to alkylate DNA.

**Selective nuclear localization and growth inhibition**

**Quantitative and qualitative nuclear analysis.** When total subcellular fluorescence was analyzed in the isogenic cells, although the trend was toward greater fluorescence in cells transfected with EGFR and its closest homologue ErbB2, the differences in fluorescence intensity were not statistically significant when compared with the wild type (P > 0.05; Fig. 4C). However, we believed that if the primary localization of AL237 in the perinuclear region was partially due to its binding to EGFR or related proteins, concomitantly released dansylated alkylating species that covalently bind to DNA might induce high levels of green fluorescence in the nuclei of these cells. Hence, we attempted to detect the levels of green fluorescence directly bound to DNA using a neutral comet assay and flow cytometric analysis of nuclei isolated by the Vindelov method (35). Under neutral conditions, we expected the alkylated dansyl species to remain bound to nuclei, thereby allowing direct imaging of the added DNA. For purpose of comparison, N-dansylaziridine, a dansylated alkylating agent deprived of the quinazoline moiety required for binding to EGFR, was used. As depicted in Fig. 5, strong green fluorescence intensity was observed from nuclei of cells treated with AL237 (Fig. 5A, left), with higher intensity in EGFR and ErbB2-transfected cells. Intensities were lower with N-dansylaziridine and not selectively stronger in the transfectants (Fig. 5A, right). Quantitative flow cytometric analysis confirmed that AL237 released significantly higher levels of fluorescence in the NIH

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**Figure 4.** Cellular fluorescence of AL237 and EGFR in MDA-MB-468, NIH 3T3, NIH 3T3her14, and NIH 3T3neu cells. A, AL237 was hydrolyzed to an aminooquinazoline fragment that emitted at 451 nm (blue), whereas the entire AL237 molecule and the dansylated DNA damaging species both emitted at 525 nm (green). Direct drug fluorescence was analyzed in MDA-MB-468 cells after 2 h of drug treatment with 25 μmol/L AL237 (top). Each fluorescent fragment was detected using individual filters on Leica fluorescent microscope (40× magnification), and the overlaid image was generated to observe colocalization of the two hydrolyzed AL237 fragments (top right). Following drug treatment, MDA-MB-468 cells were immuno-stained with EGFR PE-labeled antibody (red, bottom left). Merged images of AL237 (blue, EGFR inhibitor fragment) with EGFR (red) were assembled to outline the area of colocalization (magenta), and an enlarged cell is represented (bottom right). The DNA-targeting fragment of AL237 (green) was also overlaid with EGFR to observe the level of colocalization at the perinuclear and nuclear regions. B, NIH 3T3 and NIH 3T3her14 live cells were imaged to observe biodistribution of the molecule in EGFR-expressing and non-EGFR-expressing cells. This qualitative analysis was complemented by a quantitative fluorescence measurement of each AL237 species at a single-cell level. C, NIH 3T3, NIH 3T3her14, and NIH 3T3neu cells were exposed to the drug for 45 min and washed with PBS, and thereafter, each fragment fluorescence was measured by flow cytometry: The accumulated drug-associated fluorescence was measured from 10,000 cells and then normalized over the background cell fluorescence in the control. Blue fluorescence of the aminooquinazoline species (top) and green fluorescence of the dansylated alkylidiazonium fragment (bottom) were quantified for each cell type. Each point is the average value of three independent experiments done for each cell line in duplicate at each drug concentration. D, MDA-MB-468 cells were treated with 25 μmol/L AL237 for 2 h, followed by EGFR stimulation, methanol fixation, and directly stained with anti-EGFR-PE and anti-phospho-tyrosine-FITC antibodies. 4′,6-Diamidino-2-phenylindole stain was used to define nuclei (top and middle), but it was omitted from the samples that were drug treated because 4′,6-diamidino-2-phenylindole-stained nuclei also emitted strong green fluorescence, which interfered with AL237 fluorescence (bottom).
3T3her14 and NIH 3T3neu nuclei than in their NIH 3T3 wild-type counterpart (Fig. 5B). Two- to 3-fold differences in green fluorescence intensity were observed between NIH 3T3 and ErbB2 or EGFR. Statistical analysis was done with a two-tailed unpaired t test between NIH 3T3 and ErbB2 (at 50 μmol/L, $P = 0.0256$; 100 μmol/L, $P = 0.0175$) and between NIH 3T3 and EGFR (at 50 μmol/L, $P = 0.0281$; 100 μmol/L, $P = 0.0121$). Similarly, higher blue fluorescence intensity in the nuclei of the transfectants was observed and the
difference when compared with the wild-type cells was statistically significant at the highest dose (100 μmol/L, P = 0.0287 and P = 0.0234). Importantly, no selective green fluorescence distribution was observed in nuclei from isogenic cells treated with N-dansylaziridine (Fig. 5C) that does not contain an EGFR targeting moiety. This is an indirect evidence supporting the implication of EGFR in the selective nuclear accumulation of AL237.

Selective growth inhibition of EGFR-expressing MDA-MB-468 and NIH 3T3 cells. To determine whether the binary EGFR/DNA targeting property of AL237 that showed selective biodistribution in EGFR/ErbB2 transfectants would translate into increased growth inhibitory potency on EGFR-expressing cells, we tested its growth inhibitory effect on MDA-MB-468 and NIH 3T3 transfectants (Fig. 6). AL237 showed 5-fold stronger inhibition on EGFR- and ErbB2-expressing cells than in control NIH 3T3 cells (P < 0.05, unpaired t test; Fig. 6A). AL237 also induced strong growth inhibition in the MDA-MB-468 cells that overexpress EGFR (Fig. 6B).

Discussion
The combi-molecules are a novel type of structures designed to block divergent targets in tumor cells. The growth of refractory tumors is driven by multiple signaling disorders that often cannot be blocked by the use of a single drug. The combi-molecule approach is the first that seeks to create molecules capable of blocking at least two divergent targets in the cells by allowing the intact molecules to block one target on their own and to degrade into other species directed at the same or different targets (18, 20, 22, 23, 26, 40, 41). To gain insight into the subcellular distribution and degradation of combi-molecules, we designed a new prototype termed AL237 to (a) be fluorescent on its own and (b) degrade under physiologic conditions to FD105 (an EGFR inhibitor) that fluoresces in the blue and a DNA alkylating fragment that fluoresces in the green (see Fig. 1). Thus, this was designed to not only allow us to visualize the release of the EGFR inhibitor in the cells but also to image...
the concomitantly generated DNA damaging species. Because the green fluorescence released from the dansyl moiety attached to the intact combi-molecule is undistinguishable from that emitted by the alkylating dansyl species, the colocalization of blue and green fluorescence suggests that both the intact molecule and its dissociated DNA alkylating dansylated species may be released concomitantly and primarily in the same locations. The high intensity of the green fluorescence in the perinuclear area indicates that the combi-molecules might be primarily localized and decompose to release both the aminoquinazoline inhibitor of EGFR FD105 and the DNA damaging species therein. A fraction of the combi-molecule might also decompose in the nucleus, because blue fluorescence was also detected therein. The fact that good colocalization was seen between the blue fluorescence of FD105 and red fluorescence associated with anti-EGFR antibody lends support to the ability of the released blue FD105 to bind to EGFR. Correspondingly, the poor colocalization observed for the green fluorescence associated with the dansylated alkylating DNA damaging agent with EGFR was in agreement with the inability of the latter species to bind to EGFR but rather to DNA in the nuclei.

Previous work with the $^{14}$C-methyl labeled combi-molecules showed preferential perinuclear distribution of SMA42, an analogue of FD105, released from the model combi-molecule SMA41 (24). The corresponding $^{14}$C-labeled alkyl group was bound to all three major macromolecules of the cells (DNA, RNA, and protein; ref. 27). Here, the fluorescence-labeled alkyl group presents the advantage of being observable by fluorescence microscopy and being quantified by fluorescence intensity per cell or nucleus by flow cytometry. The results obtained from immunofluorescence and flow cytometric analyses indicate that the combi-molecule and its derived species, despite being abundantly distributed in the perinuclear or partially in the nuclear region, is available at high enough concentrations to block EGFR TK activity at the level of the plasma membrane, downregulate the mitogen-activated protein kinase pathway, and prevent downstream induction of DNA repair genes, such as the XRCC1 and ERCC1. Thus, it suggests that the combi-molecules simultaneously damages DNA and impairs its repair mechanism and also the phosphorylation of the proapoptotic protein BAD. Unfortunately, we were not able to image the membrane localization of AL237, perhaps due to insufficiently high population of bound combi-molecules in the latter area. However, the fact that phosphorylation of EGFR was strongly inhibited by AL237 is an indirect evidence of the presence of a fraction of the intact molecule or its derived quinazoline at the level of the membrane. It should be noted that, whereas the data suggest that EGFR overexpression is associated with elevated nucleus binding of the dansylated DNA moiety, the compound through its DNA damaging moiety is capable of damaging tumor cells that do not express EGFR although to a lesser extent as exemplified by the NIH 3T3 wild type. This suggests that subpopulation of non-EGFR-expressing cells present in heterogeneous tumors may also be killed by the combi-molecule through its DNA damaging arm.

Importantly, we showed that the dansyl moiety is strongly bound to the nucleus, which is in agreement with the high levels of DNA damage observed by the comet assay and a slight increase in γH2AX phosphorylation and foci formation. The rather moderate increase in γH2AX phosphorylation is due to the inability of alkylating lesions to induce significant levels of double-strand DNA breaks. In term of nuclear staining, the most striking observation was the significant difference in fluorescence intensity observed between the NIH 3T3 transfectants and their wild-type counterpart. This is consistent with our previous observation of selectively high levels of DNA damage induced by the combi-molecule SMA41 in NIH 3T3 cells transfected with EGFR when compared with its wild type (42). A similar observation was made in MDA-MB-435 cells transfected with EGFR or ErbB2 (43). In this study, nuclei of cells transfected with EGFR or its closest homologue ErbB2 emitted higher green and blue fluorescence intensity than those of NIH 3T3 wild type. This can be rationalized in light of the high levels of EGFR observed in the perinuclear region. Perhaps EGFR and related proteins localized in the perinuclear region serve as anchorage from which the free dansyl alkyldiazonium moiety (see Fig. 1) can diffuse toward genomic DNA. Indeed, many reports not only described perinuclear distribution of EGFR but also its nuclear translocation (44–48). Recent studies by Dittmann et al. (48) showed that EGFR translocates to the nucleus in response to radiation-induced DNA lesions, and more importantly, the nuclear EGFR is shown to be involved in DNA repair. Our observations that N-dansylaziridine, which does not contain a quinazoline EGFR targeting moiety, does not emit higher fluorescence intensity in the transfectants is an indirect evidence of the implication of EGFR and related proteins in the selectivity of nuclear staining by AL237.

This study conclusively showed that the combi-molecule AL237 is indeed an agent that (a) penetrates the cells and primarily localizes in the perinuclear region, (b) releases species that block signaling associated with EGFR activation, (c) damages DNA, and (d) significantly inhibits tumor cell growth. Thus, it behaved as a valid agent to image the distribution of not only the EGFR inhibitory but also the DNA binding species.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

Subcellular Distribution of a Fluorescence-Labeled Combi-Molecule Designed to Block Epidermal Growth Factor Receptor Tyrosine Kinase and Damage DNA with a Green Fluorescent Species


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