Cancer Chemother Pharmacol (2003) 51: 11–20
DOI 10.1007/s00280-002-0525-4

ORIGINAL ARTICLE

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Differential responses of EGFR-/AGT-expressing cells to the "combi-triazene" SMA41

Received: 19 May 2002 / Accepted: 23 August 2002 / Published online: 22 November 2002

Abstract *Purpose*: Previous studies have demonstrated enhanced potency associated with the binary [DNA/epidermal growth factor receptor (EGFR)] targeting properties of SMA41 (a chimeric 3-(alkyl)-1,2,3-triazene linked to a 4-anilinoquinazoline backbone) in the A431 (epidermal carcinoma of the vulva) cell line. We now report on the dependence of its antiproliferative effects (e.g. DNA damage, cell survival) on the EGFR and the DNA repair protein O6-alkylguanine DNA alkyltransferase (AGT) contents of 12 solid tumor cell lines, two of which, NIH3T3 and NIH3T3 HER14 (engineered to overexpress EGFR), were isogenic. *Methods*: Receptor type specificity was determined using ELISA for competitive binding, as well as growth factor-stimulation assays. DNA damage was studied using single-cell microelectrophoresis (comet) assays, and levels of EGFR were determined by Western blotting. The effects of SMA41 on the cell cycle of NIH3T3 cells were investigated using univariate flow cytometry. *Results*: Studies of receptor type specificity showed that SMA41: (a) preferentially inhibited the kinase activity of EGFR over those of Src, insulin receptor and protein kinase C (PKC, a serine/threonine kinase), (b) induced stronger inhibition of growth stimulated with EGF than of growth stimulated with platelet-derived growth factor (PDGF) or fetal bovine serum (FBS). Despite the EGFR specificity of SMA41, there was an absence of a linear correlation between the EGFR status of our solid tumor cell lines and levels of DNA damage induced by the alkylating component. Similarly, EGFR levels did not correlate with IC_{50} values. The antiproliferative activities of SMA41 correlated more with the AGT status of these cells and paralleled those of the clinical triazene temozolomide (TEM). However, throughout the panel, tumor cell sensitivity to SMA41 was consistently stronger than to its closest analogue TEM. Experiments performed with the isogenic cells showed that SMA41 was capable of inducing twofold higher levels of DNA damage in the EGFR transfectant and delayed cell entry to G_2/M in both cell types. When the cells were starved and growth-stimulated with FBS (conditions under which both cell types were growth-stimulated), in contrast to TEM, SMA41 and its hydrolytic metabolite SMA52 exhibited approximately nine- and threefold stronger inhibition of growth of the EGFR transfectant. *Conclusions*: These results suggest that, in addition to its ability to induce DNA damage and cell cycle perturbations, SMA41 is capable of selectively targeting the cells with a growth advantage conferred by EGFR transfection. When compared with the monoalkyltriazene prodrug TEM, its potency may be further enhanced by its ability to hydrolyze to another signal transduction inhibitor (SMA52) plus a DNA alkylating agent that may further contribute to chemosensitivity. Thus, our new "combi-targeting" strategy may well represent a tandem approach to selectively blocking receptor tyrosine kinase-mediated growth signaling while inducing significant levels of cytotoxic DNA lesions in refractory tumors.

Keywords Signal transduction · DNA damage · Cell cycle perturbation · Receptor-type specificity · O6-alkylguanine DNA alkyltransferase (AGT)

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Introduction

We have designed a novel strategy, termed the "combi-targeting" concept, to selectively target tumor cells

overexpressing receptor tyrosine kinases (RTK) or growing by an autocrine stimulatory loop. This strategy seeks to synthesize chimeric molecules referred to as "combi-molecules" (C-molecules) that possess the ability to: (a) inhibit RTK on their own, (b) generate, following hydrolytic scission under physiological conditions, another inhibitor of the same RTK and a cytotoxic DNA alkylating agent [1]. The combined effects of these three species (C-molecule, RTK inhibitor and alkylating agent) would be expected to induce significant antiproliferative activity in cells expressing RTK. Recently, we have demonstrated the feasibility of this approach by synthesizing our first C-molecule, SMA41 (Scheme 1), a masked combination of SMA52 (targeted to EGFR, see arrow) and a 3-methyl-1,2, 3-triazene (precursor of the DNA-damaging methyldiazonium species), that shows over tenfold greater potency than the clinical triazene temozolomide (TEM) in the carcinoma of the vulva cell line A431 [1]. More importantly, the C-molecule shows fourfold greater activity than the combination of equitoxic doses of TEM and the "naked" inhibitor SMA52.

Since a large number of human solid tumors express high levels of EGFR, and many coexpress EGFR and O6-alkylguanine DNA alkyltransferase (AGT), we thought it of interest to study the correlation between the EGFR/AGT status of established tumor cell lines and their sensitivity to the C-molecule. The latter was designed to retain significant affinity for its cognate receptor, thereby creating conditions under which cell recognition would be assisted by its affinity for RTK, and further degradation into a secondary RTK inhibitor plus a DNA-damaging fragment would take place in the cytosol. Since SMA41 showed (a) a 0.2 μ*M* affinity for EGFR, (b) the ability to degrade in serum-containing medium to generate 81% of SMA52 (IC_{50} EGFR inhibition 1 μ*M*), and (c) DNA-damaging properties, we thought it of interest to study its differential effects on a panel of cell lines with various levels of AGT and EGFR expression.

Here, we report a complex relationship between the EGFR/AGT status of established cells and selective targeting of EGFR in an isogenic pair of NIH 3T3 and NIH 3T3-HER14 cells (engineered to overexpress EGFR). All cells lacking AGT were remarkably sensitive to SMA41 and TEM regardless of their EGFR status. While all cells expressing AGT were resistant to TEM, the antiproliferative activities of SMA41 were consistently stronger (10- to 50-fold) than those of TEM whether AGT was expressed singly or coexpressed with EGFR, confirming the superior antiproliferative properties of the C-molecule principle. More importantly, using conditions under which the sole difference between two NIH 3T3 clones was the EGFR gene, we demonstrated that, in contrast to TEM, the C-molecule selectively blocked serum-induced growth of the EGFR transfectant.

Materials and methods

Drug treatment

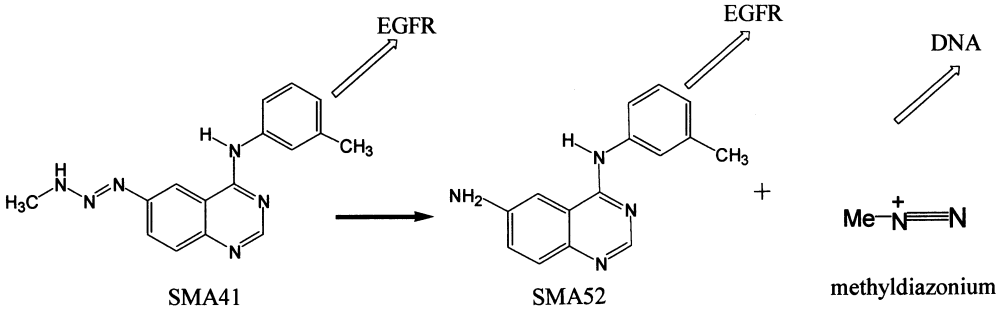
SMA41 and SMA52 were synthesized in our laboratories according to known procedures [2, 3]. TEM was provided by Schering-Plough (Kenilworth, N.J.). In all assays, the drug was dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in sterile RPMI-1640 medium containing 10% fetal bovine serum (FBS; Life Technologies, Burlington, Canada) immediately prior to the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% v/v.

Cell culture

The human tumor cell lines MDA-MB-435, MDA-MB-453, MDA-MB-468, MCF-7 (breast carcinoma), PC-3, DU-145 (prostate carcinoma), SF-126, and SF-188 (glioma) (ATCC, Manassas, Va.) were maintained in RPMI-1640 supplemented with FBS (10%), gentamicin (50 mg/ml), and HEPES (12.5 m*M*) (Wisent, St. Bruno, Canada). The mouse fibroblast cell line NIH 3T3, and its stable EGFR transfectant NIH 3T3/HER14 (generous gift from Dr. Moulay Aloui-Jamali), as well as MCF-10A (a normal breast cell line) were maintained in DMEM supplemented with donor calf serum (10%), gentamicin (50 mg/ml), and HEPES (12.5 m*M*) (Wisent). All cells were maintained in monolayer culture at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were maintained in logarithmic growth by harvesting with a trypsin-EDTA solution containing 0.5 mg/ml of trypsin and 0.2 mg/ml of EDTA and replating before confluence. In all assays, the cells were plated for 24 h before drug administration.

Growth inhibition studies

For nonstimulated cell growth, approximately 5000 cells were plated per well in 96-well plates. Following a 24-h incubation, cell monolayers were exposed to different concentrations of drug continuously for 7 days. For growth factor- and serum-stimulated growth, approximately 5000 cells were plated in each well of a 48-well dish. After 48 h, the cells were washed three times with phosphate-buffered saline (PBS), and serum-free medium without



Scheme 1 C-molecule SMA41 and its degradation into a secondary RTK inhibitor (SMA52) plus a DNA-damaging fragment (methyldiazonium)

phenol red was added. Cells were serum-deprived for 24 h, after which growth factor (12 ng/ml EGF, 25 ng/ml TGF-α, or 50 ng/ml PDGF) or FBS (10%) were added with various concentrations of each drug. Growth inhibitory activities for both stimulated and nonstimulated growth were evaluated using the sulforhodamine B (SRB) assay [4]. Briefly, following drug treatment, cells were fixed using 50 µl cold trichloroacetic acid (50%) for 60 min at 4°C, washed five times with tap water, and stained for 30 min at room temperature with SRB (0.4%) dissolved in acetic acid (0.5%). The plates were rinsed five times with 1% acetic acid, and allowed to air-dry. The resulting colored residue was dissolved in 200 µl Tris-base (10 m*M*), and the optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

EGFR, Src, and insulin receptor binding assays

Nunc MaxiSorp 96-well plates were incubated overnight at 37°C with 100 µl per well of 0.25 mg/ml poly(L-glutamic acid-L-tyrosine) (4:1, PGT) in PBS. Excess PGT was removed and the plate was washed three times with Tween 20 (0.1%) in PBS. The kinase reaction was performed as previously described using 15 ng/well of EGFR affinity-purified from A431 cells [5] (generous gift from Pfizer, N.J., and commercial supplies from Biomol, Plymouth Meeting, Pa.). The compound was added and phosphorylation initiated by the addition of ATP. 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 (0.1% Tween 20 in PBS). Phosphorylated PGT was detected following a 25-min incubation with 50 µl per well of PY54 anti-phosphotyrosine antibody conjugated with horseradish peroxidase (HRP) diluted to 0.2 µg/ml in blocking buffer comprising 3% bovine serum albumin (BSA) and 0.05% Tween 20. Antibodies were removed by aspiration, and the plate washed four times with wash buffer. The signals were developed by the addition of 50 µl per well of tetramethylbenzidine (TMB) peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersburg, Md.) and, following blue color development, 50 µl H_2SO_4 (0.09 *M*) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550). For the Src assay, 1.2 U/well of protein were used (Biomol), and 100 m*M* of EDTA was added to stop the phosphorylation reaction. For the Ins-R assay, 15 ng/well of protein was used (Biomol), and to stop the reaction, EDTA (250 m*M*) was added. In both cases, known inhibitors were used to calibrate the assay.

Protein kinase C assay

Myelin basic protein (MBP)-coated 96-well plates (UBI, Lake Placid, N.Y.) were reconstituted with PBS prior to the addition of drug diluted in assay dilution buffer II (UBI), protein kinase C (PKC) lipid activator, Mg/ATP cocktail, and purified PKC 25 ng/well (UBI). The reaction was allowed to occur at room temperature with constant shaking for 15 min. The plate was washed three times with PBS, and blocking buffer was added (1% BSA in PBS) for 45 min. Anti-phospho-MBP antibodies (1 µg/ml in blocking buffer; UBI) and goat anti-mouse HRP secondary antibodies (1:5000 in blocking buffer; UBI) were used to detect phosphorylated substrate. The plates were washed seven times with PBS and the signals developed by the addition of 75 µl per well of TMB peroxidase substrate. Following blue color development, 75 µl H_2SO_4 (0.09 *M*) was added per well, and plates were read at 450 nm using a Bio-Rad ELISA reader (model 2550).

Alkaline comet assay for quantitation of DNA damage

A modified alkaline comet assay technique [1, 6] was used to quantitate DNA damage induced by SMA41, SMA52, and TEM. Cells were exposed to drugs for 30 min and harvested with trypsin-EDTA. The cells were subsequently collected by centrifugation and resuspended in PBS. The resulting cell suspension was diluted to approximately 10^6 cells/ml, and mixed with agarose (1%) in PBS at 37ºC at a dilution of 1:10. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel-casting chambers, as previously described [1, 6], 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).

After being kept on ice for 30 min, the gels were gently rinsed with distilled water and then 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 37ºC. Thereafter, they were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37ºC, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed into 1 *M* ammonium acetate for 30 min. They were further soaked in 100% ethanol for 2 h, dried overnight and subsequently stained with SYBR Gold (1:10,000 dilution of stock supplied by Molecular Probes, Eugene, Ore.) for 20 min. For evaluation of comets, DNA damage was assessed using the tail moment parameter (i.e. the product of the distance between the barycenters of the head and the tail of the comet multiplied by the percentage DNA in the tail region). A minimum of 50 cells/comet were analyzed for each sample, using ALKOMET v3.1 software, and the values presented are average tail moments for the entire cell population.

Flow cytometry for cell cycle analysis

Cells were grown in six-well plates in a monolayer until confluence, washed three times with PBS, and grown in serum-free medium without phenol red for 24 h. Thereafter, they were harvested with trypsin-EDTA, collected by centrifugation and washed with PBS. Vindelov's solution (0.01 *M* Tris-base, 10 m*M* NaCl, 700 U RNAse, 7.5×10^{-5} *M* propidium iodide, 0.1% NP-40) was added (400 µl) for 10 min at 37°C and the fluorescence (FL2) detected using a Becton-Dickinson FACScan. When G_1 cell synchronization was satisfactory, drugs plus FBS (10%) were added for an additional 24 h, after which analyses were performed as described.

Western blotting

NIH 3T3/HER14 cells were preincubated in a six-well plate (1×10^6 cells/well) with 0.1% serum at 37°C 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 30 min at 37°C. All other cell lines were grown in six-well plates (1×10^6 cells/well) in medium containing 10% serum until confluence. Thereafter, they were washed with PBS and resuspended in cold lysis buffer (50 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 1% NP40, 1 mM EDTA, 5 m*M* NaF, 1 m*M* Na_3VO_4, protease inhibitor tablet; Roche Biochemicals, Laval, Canada). The lysates were kept on ice for 30 min and collected by centrifugation at 10,000 rpm for 20 min at 4°C. The protein concentrations were determined against a standardized control using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). Equal amounts of protein (40 µg/ml) from each lysate were added to a 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, Mass.). Nonspecific binding on the PVDF membrane was minimized with a blocking buffer containing 3% nonfat dried milk in PBS. The membrane was incubated with the following primary antibodies: anti-EGFR (Neomarkers, Fremont, Calif.) for determination of corresponding receptor levels, anti-β-tubulin (Neomarkers) for the detection of equal loading, or anti-AGT (Pharmingen, Toronto, Canada). Thereafter, blots were incubated with HRP-goat anti-mouse antibody (1:200 dilution; UBI) 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 (Cambridge, UK).

Results

Receptor type specificity

Prior to determining whether the mixed targeting properties of SMA41 translated into selective targeting of EGFR-overexpressing cells, we thought it of interest to analyze its receptor type specificity. This was determined using two lines of assays: (1) ELISA-based competitive binding and (2) growth factor- or serum-stimulated growth of solid tumor cells. As shown in Table 1, SMA41 did not inhibit insulin or Src tyrosine kinase activities ($IC_{50} > 100$ μM), nor did it inhibit PKC serine/threonine kinase activity. More importantly, as shown in Fig. 1, when the cells were starved and stimulated with various growth factors or serum, SMA41 preferentially blocked growth of NIH 3T3/HER14 cells stimulated with EGF over those stimulated with platelet-derived growth factor (PDGF) or serum, indicating some degree of selectivity for EGFR-mediated growth.

Correlation with EGFR expression

Since SMA41 showed preferential affinity for the intracellular ATP binding site of EGFR, we hypothesized that this was likely to translate into selective targeting of EGF-dependent cells. More importantly, the ATP binding site being located in the cytosol, we surmised that methyldiazonium species generated by the in situ hydrolysis of EGFR-bound SMA41 may diffuse away from the membrane towards the nucleus, thereby inducing an EGFR-assisted elevation of the levels of DNA damage in EGFR-overexpressing cells. This was also expected to confer a direct dependence of levels of DNA damage or antiproliferative activity on the EGFR content of the cells. Growth inhibitory studies using the NCI SRB assay [4] under a 7-day continuous exposure in a panel of ten established cell lines expressing various levels of EGFR (Table 2; Fig. 2), demonstrated little correlation between IC_{50} values and EGFR levels. Also, single-cell microelectrophoresis (comet) assays for DNA damage in all these cell lines revealed no linear correlation between levels of DNA lesions induced by TEM and EGFR status of the cells (data not shown). Similarly, no linear correlation was apparent between the IC_{50} values of the naked inhibitor SMA52 and EGFR levels.

Table 1 Inhibition of tyrosine and serine/threonine kinases by SMA41 and SMA52 in an ELISA-based competitive ATP binding assay. Each value was calculated by nonlinear regression analysis using GraphPad Prism software (GraphPad Software, San Diego, Calif.), and represents the results of at least two independent experiments run in duplicate

Tyrosine kinase	IC_{50}, competitive binding (μM)	
	SMA41	SMA52
PKC	>100	>100
Src	>100	>100
Insulin receptor	>100	>100
EGFR	0.1	1

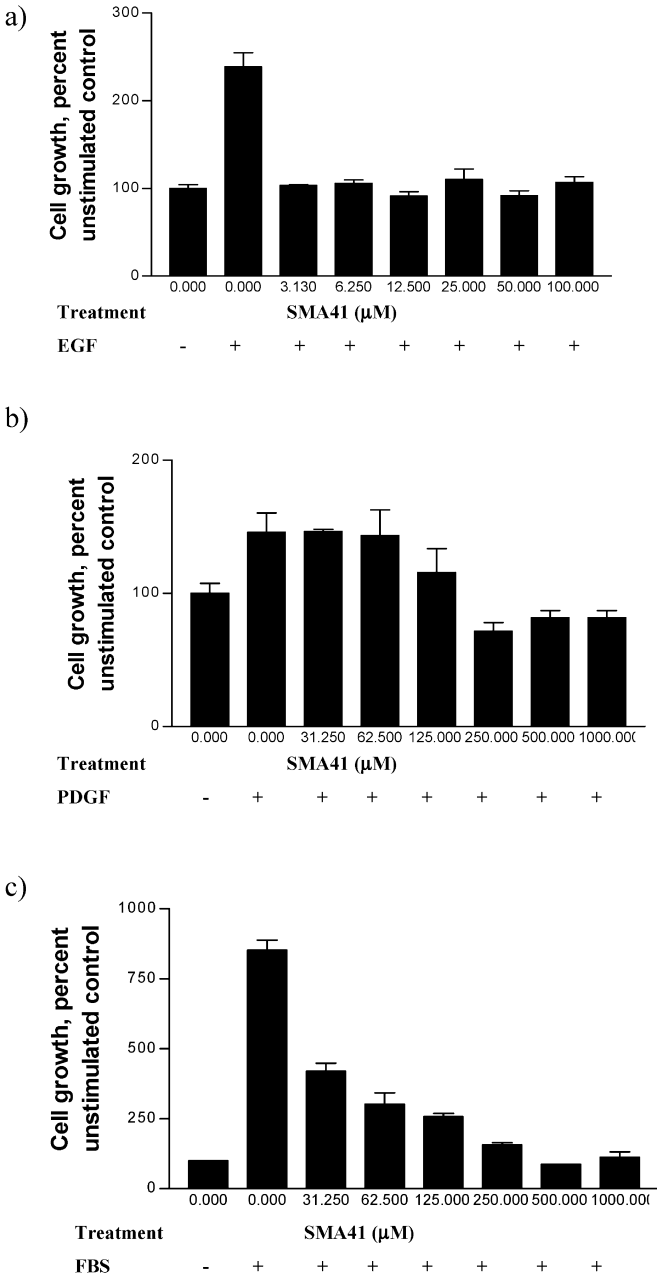


Fig. 1a–c Inhibition of growth factor-stimulated cell growth by SMA41. NIH 3T3/HER14 cells were serum-deprived for 24 h prior to the addition of (**a**) EGF (12 ng/ml), (**b**) PDGF (50 ng/ml), or (**c**) serum (10%) plus SMA41 at the indicated doses. Each point represents the average results from at least two independent experiments run in triplicate

The IC_{50} values for SMA41 varied from 1.1 to 24 μM (Table 2), with the lowest value observed for AGT-deficient cells. This differential response profile paralleled that for TEM. Linear regression analyses showed a significant correlation between the IC_{50} values of SMA41 and the AGT levels of the cells (Pearson

Table 2 AGT/EGFR levels and IC_{50} values for cell growth inhibition by SMA41, SMA52 and TEM in a panel of human tumor cell lines. Each IC_{50} value (μM) represents the results from at least two independent experiments performed in triplicate. AGT and EGFR levels were determined by Western blotting. AGT values were normalized by subtracting the band intensity ratio (AGT/tubulin) of SF-126 from those of all other cells, leaving AGT values for the latter as 0. Similarly, the SF-188 band intensity ratio was considered the background for normalizing EGFR levels

Cell line	IC_{50}			AGT	EGFR
	SMA41	SMA52	TEM	(AGT/tubulin)	(EGFR/tubulin)
MCF-10A	21.0	16.4	92.4	1.15	0.04
A431	21.6	8.6	604.9	0.20	2.46
PC-3	21.8	24.4	573.3	0.76	1.20
DU-145	24.3	26.2	197.8	0.81	1.64
SF-126	2.0	26.9	12.5	0.0	0.10
SF-188	19.8	33.8	412.6	0.36	0.0
MCF-7	24.1	39.6	699.6	1.29	0.51
MDA-MB-435	3.0	29.3	12.0	0.06	0.23
MDA-MB-453	1.1	8.1	451.0	0.56	1.54
MDA-MB-468	28.6	39.5	125.0	0.65	1.22

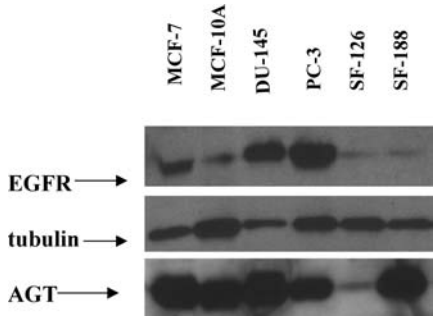


Fig. 2 Expression of EGFR and AGT in selected cell lines from the panel. Tubulin was used as a loading control for quantitation of EGFR and AGT levels

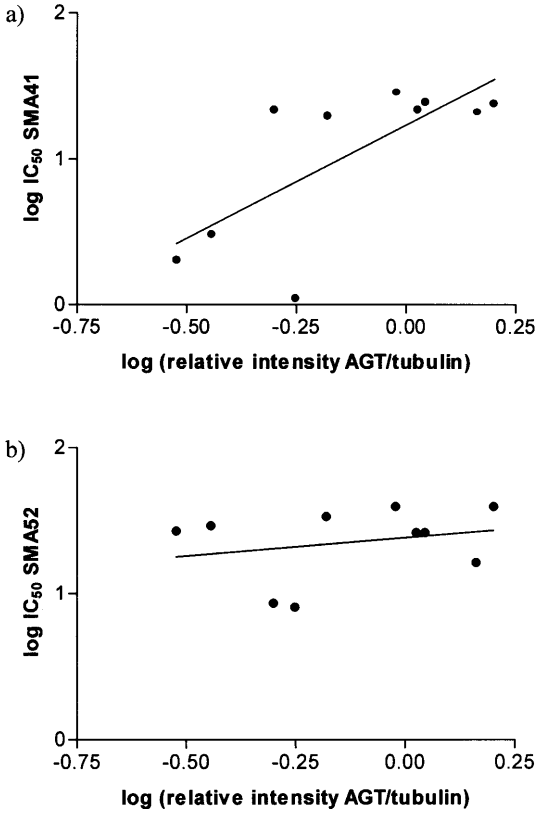


Fig. 3a, b Correlation of AGT levels with IC_{50} values for (**a**) SMA41 and (**b**) SMA52. Pearson correlation coefficients were calculated as $r^2 = 0.7241$ ($P < 0.05$) for SMA41, and $r^2 = 0.2472$ ($P > 0.05$) for SMA52

coefficient $r^2 = 0.7$, $P < 0.05$). As expected, this correlation was not significant for SMA52 ($r^2 = 0.2472$, $P > 0.05$; Fig. 3). Thus, responses to SMA41 seem to depend more on the AGT than the EGFR status of the cells. The sole SMA41-sensitive cell line with significant levels of AGT was the breast carcinoma MDA-MB-453. Since the latter line was also exquisitely sensitive to SMA52, we believe that this may have been due to its expression of high levels erb2 and erb3 [7], the two closest homologues of EGFR.

It is noteworthy that despite being a monomethylating agent, the IC_{50} values of SMA41 (average 16.7 ± 3.3 μM) were more than 19-fold lower than those of TEM (average 318 ± 82 μM) throughout the panel, indicating a consistently greater potency of SMA41 when compared with TEM.

Differential DNA damage induced by SMA41 in the NIH 3T3 isogenic pair of cell lines

Various factors, such as DNA repair status, dependence of cell growth on factors other than EGF, and nonspecific binding to unidentified receptors, may affect the correlation between EGFR and levels of DNA lesions or antiproliferative effects. Thus, we chose to pursue the study in an isogenic pair of cell lines (NIH 3T3 and NIH 3T3/HER14 cells, engineered to overexpress EGFR), the sole difference between which was the transfected EGFR gene. Using the comet assay, we demonstrated that SMA41 induced twofold higher levels of DNA damage in the EGFR transfectant when compared with its parental NIH 3T3 cell line (Fig. 4). However, this did not

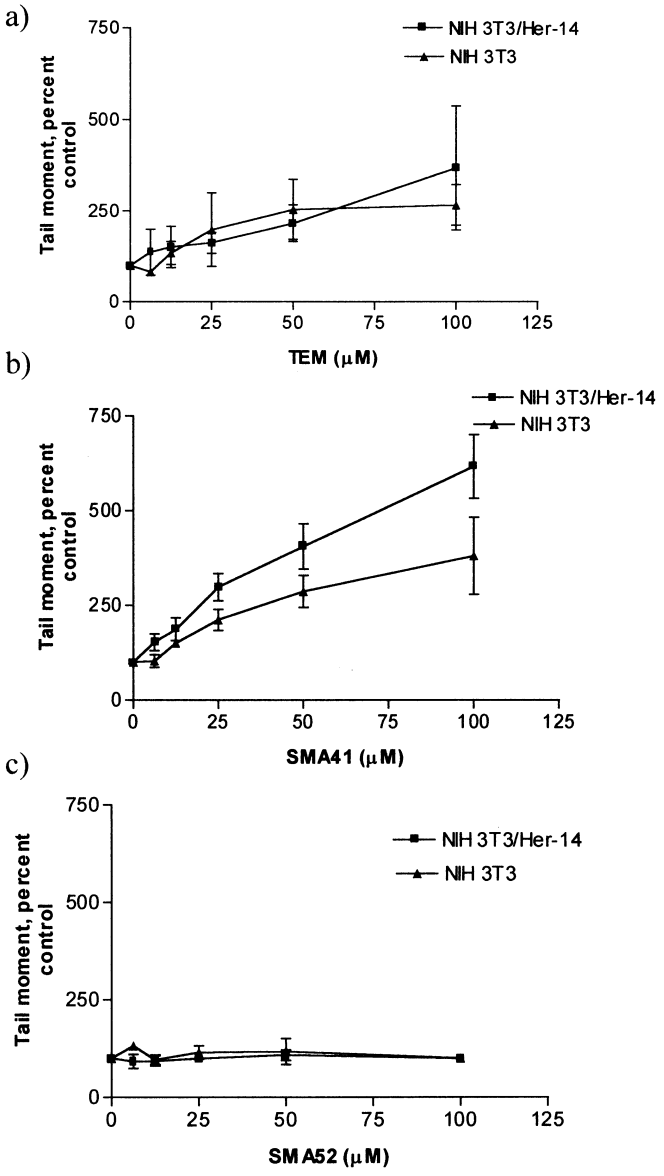


Fig. 4a–c Differential DNA damage induced by (**a**) TEM, (**b**) SMA41, and (**c**) SMA52 in NIH 3T3 and NIH 3T3/HER14 cells as measured by the comet assay following a short 30-min exposure. Each point represents the average results from at least two independent experiments

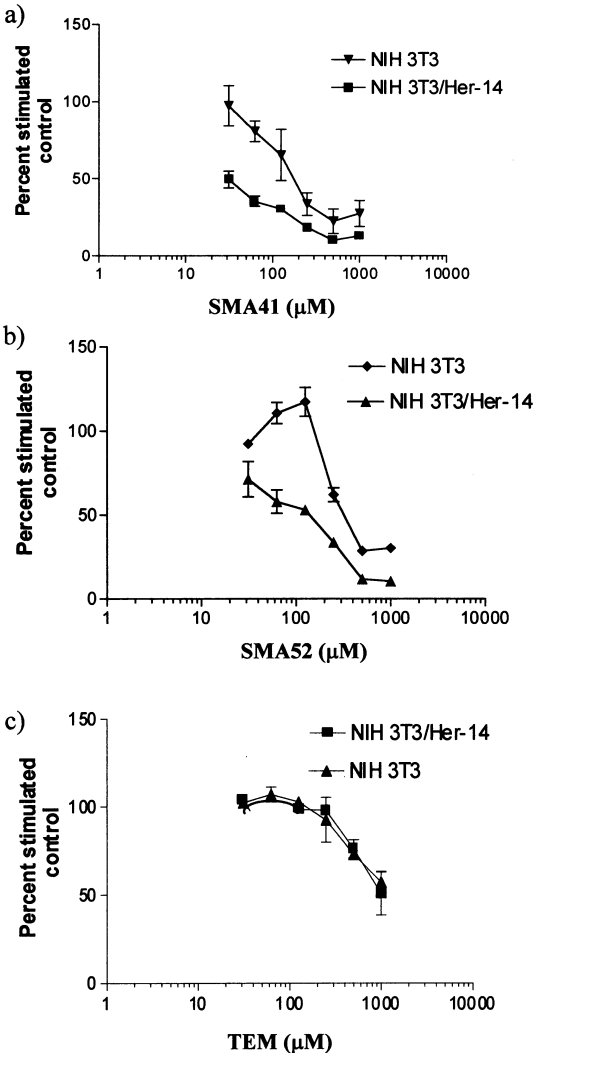


Fig. 5a–c Inhibition of serum-stimulated growth of NIH 3T3 and NIH 3T3/HER14 cells by (**a**) SMA41, (**b**) SMA52, and (**c**) TEM. Each point represents the average results from at least two independent experiments run in triplicate

translate into any differential effect in the SRB assay, which showed a barely detectable difference in the antiproliferative activities of SMA41 in the two cell types (data not shown).

Growth stimulation assays with NIH 3T3/HER14 and NIH 3T3 isogenic pair of cell lines

Having observed an absence of correlation under both poly- and isogenic conditions, we sought to determine whether the EGFR transfection had conferred a growth advantage on these cells under conditions in which their proliferation was serum-stimulated. Further, we hypothesized that if this growth advantage were associated with EGFR-mediated signaling, abrogation of EGFR tyrosine kinase activity would selectively sensitize the EGFR-transfected cells. Thus, we designed an experiment in which the cells were starved for 24 h and growth-stimulated with a dose range of serum. Interestingly, it was found that the EGFR transfectant grew approximately threefold faster than its parent non-transformed NIH 3T3 cell line, confirming that the EGFR transfection had conferred a growth advantage on these cells. With this model, we tested the effects of SMA41 and SMA52 in comparison with that of TEM using the serum concentration at which maximum growth stimulation was observed (10%). The results (Fig. 5) showed that TEM induced no differential response between the two cell types. In contrast, SMA41 selectively induced fivefold stronger serum-stimulated growth inhibitory activity in the EGFR transfectant. Similarly, the naked inhibitor SMA52 induced threefold

stronger inhibition of serum-mediated growth in NIH 3T3/HER14 cells.

EGF- and serum-induced cell cycle effects

The mechanism of this significant selectivity was further investigated by flow cytometric analyses of SMA41-, SMA52- and TEM-induced cell cycle perturbation under serum-driven growth stimulation. It should be remembered that SMA41 possesses mixed signal transduction and DNA tar geting properties, and it is now well established that inhibition of signaling and induction of DNA damage are associated with significant cell cycle perturbations. More specifically, DNA lesions induced by alkyltriazenes of the same class as SMA41 (e.g. monomethyltriazenylimidazole-4-carboxamide, MMTIC) [8, 9], and its prodrug TEM) cause significant cell cycle arrest in S or G_2/M and inhibitors of EGFR arrest the cell cycle in G_1 [5]. Since SMA41 is a combination of these two types of mechanism, a complex cell cycle distribution was expected.

Indeed, when the cells were starved by serum deprivation, a significant synchronization in G_1 was observed (Fig. 6). The addition of serum to control cells induced significant cell transition to S and G_2/M 24 h later. Coaddition of SMA41 did not significantly block cell exit from G_1, but rather induced a dose-dependent arrest in S phase with a noticeable accumulation of cells in G_1/S at the highest dose of 100 μM. At the latter concentration, a barely detectable number of cells had reached G_2/M 24 h after serum stimulation. More importantly, cell cycle perturbations were more pronounced in the NIH 3T3/HER14 than in its nontransformed counterpart NIH 3T3. This was clearly observable at the highest dose at which cell entry to G_2/M was delayed at earlier phases in NIH 3T3/HER14 cells (arrest in G_1/S, early) than in their parental NIH 3T3 cells (arrest in mid-S and late S; Fig. 6). Similarly, SMA52 delayed cell entry to G_2/M, but the effects were weaker than those of SMA41. In contrast, no significant cell cycle arrest was induced by TEM. Even at the highest dose, the G_2/M peaks were similar to those of control cells. These results provide further evidence of the markedly distinct mechanism of action of SMA41 when compared with those of SMA52 and TEM.

Discussion

Solid tumors are often characterized by the expression of DNA repair enzymes, that confer resistance to chemotherapy, and the overexpression or dysfunction of proteins directly implicated in mitogenic signaling [10, 11, 12, 13]. The expression of DNA repair enzymes, such as AGT, significantly decreases the chemosensitivity of these tumors to alkylating agents of the nitrosourea and triazene classes [14, 15, 16, 17, 18]. On the other hand, amplification and overexpression of receptors of the erb family (e.g. EGFR, $p185^{neu}$) have been detected in subpopulations of many types of human tumors and are experimentally associated with aggressive tumor progression, poor patient survival and more importantly, reduced chemosensitivity [19, 20]. The binding of our growth factor of interest, EGF, to its cognate receptor EGFR is followed by a cascade of activation events that ultimately induces the transcription of genes associated with proliferation (e.g. early response genes such as c-*fos*

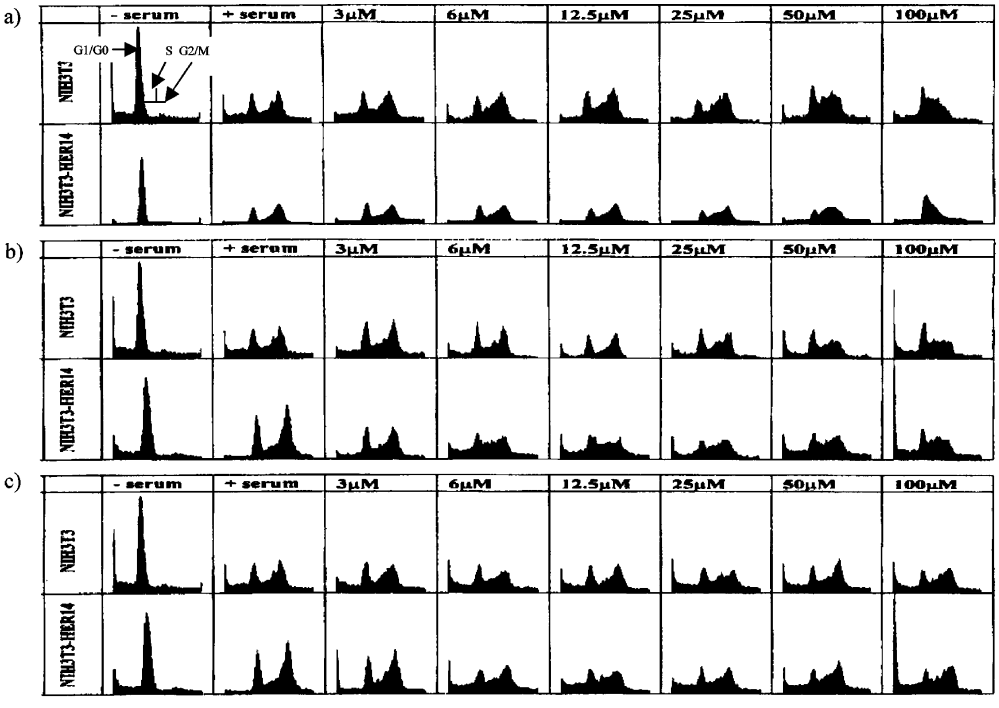


Fig. 6a–c Cell cycle effects of (**a**) SMA41, (**b**) SMA52 and (**c**) TEM on NIH 3T3 and NIH 3T3/HER14 cells. Cells were synchronized in G_1 by serum deprivation for 24 h, after which they were treated with 10% serum plus a range of drug doses. The cell cycle phases G_1/G_0, S, and G_2/M are labeled

and c-*jun*) [21, 22]. More recently, aberration of c-*fos* expression has been associated with greater cell sensitivity to *N*-methyl-*N*-nitrosoguanidine, a methylating agent generating the same type of lesions as SMA41 [22]. This observation lends support to approaches like the combi-targeting concept that seeks to combine blockade of EGF-mediated signal transduction with methylating DNA lesions in order to induce enhanced chemosensitivity and perhaps chemoselectivity in refractory tumors. The current study was designed to determine the potency of the approach in a panel of 12 cell lines with various levels of expression of EGFR and AGT, the latter being a DNA repair enzyme that confers resistance to triazenes of the same class as our C-molecule.

We have already demonstrated that SMA41 is the precursor of two major species: SMA52 that inhibits EGFR tyrosine kinase (TK) (IC_{50} 1 μM), and a metastable methyldiazonium whose presence, due to its short life, could only be indirectly confirmed by the high levels of alkali-labile lesions that it induced in A431 cells [1]. Thus, we compared the antiproliferative effects of C-molecule SMA41 with those of independently synthesized SMA52 and with the clinical methyldiazonium generator TEM. It should be remembered that SMA41 generates 81% of SMA52 following degradation in serum-containing medium [1]. In the present study, the IC_{50} values of SMA52 for cell growth inhibition were consistently in the same range in almost all the cell lines as determined by a 7-day continuous exposure SRB assay. Only values for A431 and MDA-MB-453 cells were threefold lower than the average (Table 2). Since A431 growth is driven by an aggressive autocrine growth mediated by TGF-α/EGFR [23], prolonged exposure and consequently sustained inhibition of EGFR TK may translate into significant antiproliferative activity. As for MDA-MB-453 cells which, despite their significant AGT content, were the cells most sensitive to SMA41, strong dependence of growth on erb2 or erb3 gene products [7] and nonspecific binding to these receptors (the closest homologues of EGFR) may account for the superior sensitivity of these cell lines. As expected, there was no dependence of SMA52 activity on the AGT status of the cells since, as previously reported, it does not alkylate DNA. In contrast, throughout the panel, the IC_{50} values of the methylating agent TEM were quite high, but significantly low in AGT-null cells. This is in agreement with a great number of reports of a direct correlation between the antiproliferative activity of TEM and the AGT status of human tumor cells [14, 16].

Since SMA41 is a combination of the two lines of mechanisms of action described above (i.e. inhibition of TK activity, methylation of DNA), antiproliferative activities superior to those of SMA52 and TEM alone were expected. Surprisingly, the IC_{50} values for SMA41 were in the same range as those of SMA52 in almost all cells expressing AGT except the MDA-MB-453, but were 9- to 25-fold stronger in AGT-null cells. Coexpression of EGFR and AGT did not seem to affect the levels of antiproliferative activities induced by SMA41 in the cell panel. However, despite the absence of correlation with EGFR status, the antiproliferative activities of SMA41 were superior to those of TEM in all cells tested, regardless of their AGT status. These results suggest overall that, where AGT is expressed, repair of O6-methylguanine may at least partially compromise the cytotoxic effects of the DNA-damaging component of the dual mechanism of action of SMA41, leaving an antiproliferative activity that is mostly the result of the 81% conversion of the C-molecule to SMA52. The contribution of the growth-inhibitory property of SMA52 may also explain the overall superior antiproliferative effects of the C-molecule SMA41 when compared with those of its clinical counterpart TEM.

The absence of correlation between the EGFR status of our cell panel and IC_{50} values for cell survival may be primarily explained by the DNA repair status of the cells on which depend the cytotoxic effects of all SMA41-induced DNA lesions. However, cell growth may be dependent on factors or hormones other than EGF that are available in the serum-containing medium. Therefore, we presumed that EGFR selectivity should be analyzed in isogenic clones that differ only in their EGFR content but possess identical DNA repair status. More importantly, EGFR should confer a growth advantage to its host clone. This is in line with recent study by Bishop et al. [24] who showed in the NCI panel that only EGFR-expressing cells with an intact EGF-mediated mitogenic signaling pathway were significantly sensitive to anilinoquinazolines. Thus the study was further carried out in NIH 3T3 and NIH 3T3/HER14 (engineered to overexpress EGFR).

We first demonstrated the binary targeting properties of SMA41 in these cells by analyzing levels of DNA damage and inhibition of EGF-induced growth in the EGFR-expressing transfectant (Fig. 4). Interestingly, DNA damage appeared more enhanced in the EGFR transfectant when compared with its parental line. These results lend support to the hypothesis that methyldiazonium ions generated from EGFR-bound SMA41 may diffuse towards the nucleus, thereby enhancing the levels of DNA damage. Since this did not translate into EGFR-selective antiproliferative activity in the continuous exposure SRB assay, we surmise that the EGFR-targeting component of SMA41 is more likely to have been responsible for the differential response between the two cell types. As we have demonstrated, EGF plays a role in mitogenic signaling in these cells. However, for the parental NIH 3T3 cells, being insensitive to EGF-induced growth, FBS (10%) was considered the most appropriate stimulation medium as it stimulated the growth of both cell types and in addition represented a good mimic of an in vivo medium. SMA41 showed ninefold selective inhibition of FBS-induced growth in the EGFR transfectant when compared with the parental cell line NIH 3T3. Similarly, its derived metabolite SMA52 induced threefold selectivity for NIH 3T3/HER14. The greater selectivity of SMA41 when compared

with SMA52 is in line with the order of affinity of these two drugs for EGFR, suggesting the possible dominance of the EGFR-inhibitory component of SMA41 in this selective activity.

The mechanism of selectivity of SMA41 under growth stimulation can be explained in light of recent observations by He et al. [25], who showed using NIH 3T3 cells that serum or PDGF activation of p21-activated kinase (PAK), a family of threonine kinases involved in cell motility and directed migration, requires EGFR. Specific inhibitors of both PDGFR and EGFR TK block the activation of PAK. Abolition of EGFR using fibroblasts from $EGFR^{-/-}$ mice shows complete depletion of PAK activation, suggesting a key role for EGFR in this process. Although the implication of these types of cooperative interactions between PDGF and EGFR in cell proliferation has not yet been fully elucidated, we clearly observed that EGFR transfection conferred a significant growth advantage on the NIH 3T3/HER14 transfectant over its parental line under serum stimulation. Since at concentrations of SMA41 at which significant serum-induced growth was inhibited by 50% (e.g. 32.5 μM), no inhibition of PDGF-induced proliferation was observed, its selective growth inhibition is likely to be based on blockade of EGFR TK which at this concentration was inhibited by 100%.

It could be argued that rapidly growing cells are more sensitive to cytotoxic agents than slowly growing ones. However, this can be refuted by the absence of differential cytotoxic activity produced by TEM in the two cell types. More importantly, SMA52 which showed the same levels of activity as SMA41 in basal growth assays induced only a threefold difference between the two cell types. The preferential inhibition of serum-induced growth stimulation in the EGFR transfectant is a clear demonstration of the tumor targeting role of the signal transduction inhibitory component of our C-molecule.

In order to gain an insight into the effects of the detected DNA lesions induced by SMA41 in these cells, we analyzed the effects of the latter on cell cycle progression. In contrast to TEM, SMA41 significantly delayed cell cycle progression of G_1-synchronized cells 24 h after growth stimulation by serum with a slightly more pronounced effect in the EGFR transfectant NIH 3T3/HER14. Since inhibition of growth stimulation would cause a protracted arrest in G_1/G_0, the complex cell cycle profiles induced by our C-molecule [e.g. arrests in S(early) and S(middle)] when compared with SMA52 or TEM may primarily have been due to the DNA-damaging component or the results of nonspecific inhibition of cell cycle proteins. It is not clear at this point how the induced cell cycle delays correlate with cell death or with the overall antiproliferative activity.

In previous studies we have demonstrated the feasibility of a C-molecule with mixed EGFR- and DNA-targeting properties. In the current study we conclusively demonstrated that the EGFR-targeting component plays a significant role in blocking growth stimulation not only by EGF alone, but also by serum which contains a variety of growth factors, the most abundant of which is PDGF. We have also demonstrated that the EGFR component may assist in the selective induction of DNA damage in isogenic clones. Our studies in a heterogeneous panel of cells led to the conclusion that, while combining a DNA-damaging triazene with an EGFR-inhibitory moiety confers significantly greater potency to the resulting C-molecule when compared with the classical triazenes, the DNA repair status of the cells still remains a significant barrier to optimal potency. To this end, other polyfunctional cytotoxic moieties that may enhance the potency of the DNA-damaging component of the C-molecules are now being investigated in our laboratory. Moreover, work is underway to transfect the AGT-null and AGT-proficient cells with EGFR in order to better outline the effects of coexpression of AGT and EGFR on their sensitivity to C-molecules.

Acknowledgements We thank the Cancer Research Society for financial support. S.L.M. is grateful to the McGill Faculty of Medicine for a graduate scholarship.

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