The Combi-Targeting Concept: Chemical Dissection of the Dual Targeting Properties of a Series of "Combi-Triazenes"

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The combi-targeting concept postulates that a molecule termed a "combi-molecule" designed to interact with an oncoreceptor on its own and allowed to further degrade to another more stable inhibitor of the latter receptor + a DNA-damaging species should be more potent than the individual combination of the same inhibitor with a DNA-damaging agent in cells expressing the targeted receptor. Recently, using the epidermal growth factor receptor (EGFR) as a target, we demonstrated the feasibility of combi-molecules with dual EGFR/DNA-targeting properties and with the ability to degrade to another potent inhibitor of EGFR. However, despite a clear demonstration of their superior potency when compared with classical combinations in EGFR-expressing cells, the true contribution of each fragment of the combi-molecules to their overall antiproliferative activity remained elusive. Here, we report a structure−function approach whereby a series of quinazoline-based "combi-triazenes" were altered to either abrogate the affinity of the EGFR-targeting quinazoline head or to suppress the DNA-damaging property of the triazene tail. The results showed that (a) inactivation of the quinazoline head by appending an *N*-methylaniline group to its 4-position reduced EGFR tyrosine kinase (TK) inhibitory activity by ca. 200-fold and decreased the ability of the combi-molecule to block serum-induced growth stimulation in c-erbB2 transfected NIH3T3 cells by ca. 10-fold, (b) abrogation of the alkylating activity or the DNA-damaging potential of the triazene tail by forming 3,3-dimethyltriazenes did not suppress EGFR TK inhibitory affinity but decreased the antiproliferative activity in basal growth assays, and (c) the antiproliferative activities of the monoalkyltriazenes that possessed binary EGFR TK inhibitory and alkylating activities were superior to those of their monotargeted counterparts. The results in toto suggest that each component of the dual targeting property of combi-triazenes plays a critical role in their overall antiproliferative activity.

Introduction

Overexpression of EGFR and its closest homologue $p185^{neu}$, the HER2 (erbB2) gene product, is known to induce aggressive tumor progression and reduced sensitivity to antitumor drugs.[1−4] We recently reported a novel tumor-targeting strategy termed the "combi-targeting concept" that sought to combine inhibitors of EGFR tyrosine kinase (TK) with DNA-damaging agents in order to irreversibly block EGF-dependent tumor progression. The combi-targeting approach consists of synthesizing agents termed "combi-molecules" that are not only capable of inhibiting EGFR-mediated signaling on their own but also to be hydrolyzed under physiological conditions to release another EGFR TK inhibitor + a cytotoxic DNA-alkylating species.[5−8] This multiple "knock out" of the EGFR TK activity added to the DNA damage induced by the alkylating species is expected to culminate in a sustained antiproliferative activity in EGFR-overexpressing cells. It should be remembered that while a great number of highly specific and potent inhibitors of EGFR TK have now been synthesized, the emphasis is now placed on their combination with other cytotoxic drugs in order to enhance their efficacy in vivo.[9]

The fundamental premises outlining the combi-targeting concept are extensively discussed elsewhere.[6] Briefly, the combi-targeting approach is based on equilibria outlined in Scheme 1. In EGFR-overexpressing cells, the combi-molecule (**TZ**−**I**) enters the cells by passive diffusion and in the cytosol (**TZ**−**I**) can either directly bind to the EGFR ATP-binding site (path 1) or degrade to a cytotoxic **TZ** + an EGFR TK inhibitor **I** (path 2). While the **TZ** will exert cytotoxic activity by damaging DNA, the generated inhibitor **I** will further induce EGF-dependent (autocrine or paracrine) growth inhibition via binding to EGFR. Recent studies demonstrated the irreversible EGFR TK inhibitory effects of a **TZ**−**I** (BJ2000) of the triazene class,[6] suggesting that the **TZ** may also damage the receptor as in path 3. These effects may lead to enhanced antiproliferative activity of the **TZ**−**I** conjugate in high EGFR-expressing cells. In contrast, in non-EGF-dependent cells, because of the absence of an inhibitory target for **TZ**−**I** and **I**, these cells will be less sensitive to the action of **TZ**−**I** conjugates. Thus, the combi-targeting concept is a tandem approach to chemosensitivity and chemoselectivity.

The feasibility of the combi-targeting concept has now been demonstrated with two molecular probes of the triazene class SMA41[5] (**9**) and BJ2000[6] (**10**) that showed a dual ability to block signaling mediated by the

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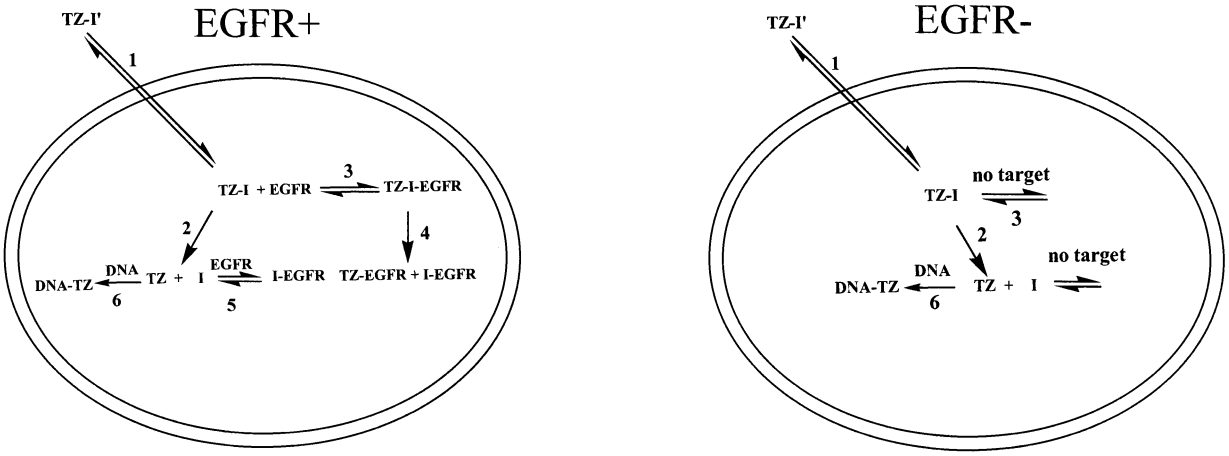
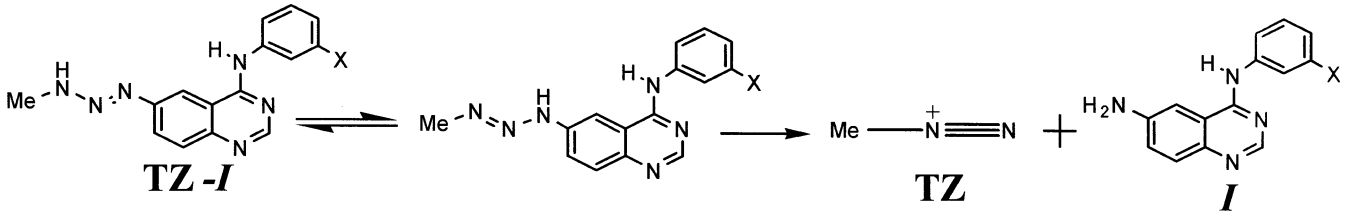
Scheme 1



Scheme 2



epidermal growth factor receptor (EGFR) and to significantly damage DNA in high EGFR-expressing human carcinoma of the vulva A431 cells. As depicted in Scheme 2, in these models the **TZ**−**I** represents the triazenoquinazoline, **TZ** the released methyldiazonium, and the **I** the released aminoquinazolines. The primary models focused on 3-monoalkyltriazenes, since 3,3-dialkyltriazenes, despite their superior stability and clinical activity, require metabolic activation to ultimately generate the cytotoxic alkyldiazonium.[10−12] This type of compound (**12**−**14**) will be used in this study as examples of non-DNA-damaging combi-molecules.

A significant body of work has accumulated to suggest that, on one hand, combi-molecules block EGFR-mediated signaling like other known EGFR inhibitors and, on the other hand, significantly damage DNA according to a mechanism similar to that of classical triazenes.[5−7] However, the precise contribution of the triazene tail and the quinazoline head to the mixed potency of the combi-molecules remained elusive. Here, we report a structure−function approach designed to define the role of each of the two major targeting components (i.e. DNA-damaging triazene and the EGFR inhibitory quinazoline) in the overall antiproliferative activity of a series of combi-triazenes. To clearly dissect the effects of their reactive alkylating DNA-damaging component from that of their EGFR inhibitory one, we synthesized three categories of agents: (a) molecules on which the DNA-damaging function is abrogated by appending a 3,3-dimethyltriazene moiety to the 6-position of the quinazolines, (b) molecules in which the quinazoline moiety is tethered to an *N*-methylaniline or benzylamine at the 4-position in order to attenuate the EGFR-inhibitory properties, and (c) dual targeting monoalkyltriazenes with various substituents on both the anilinoquinazoline backbone and the 3-position of the monoalkyltriazene side chain. The latter class of combi-molecules is designed to degrade as outlined in Scheme 2.

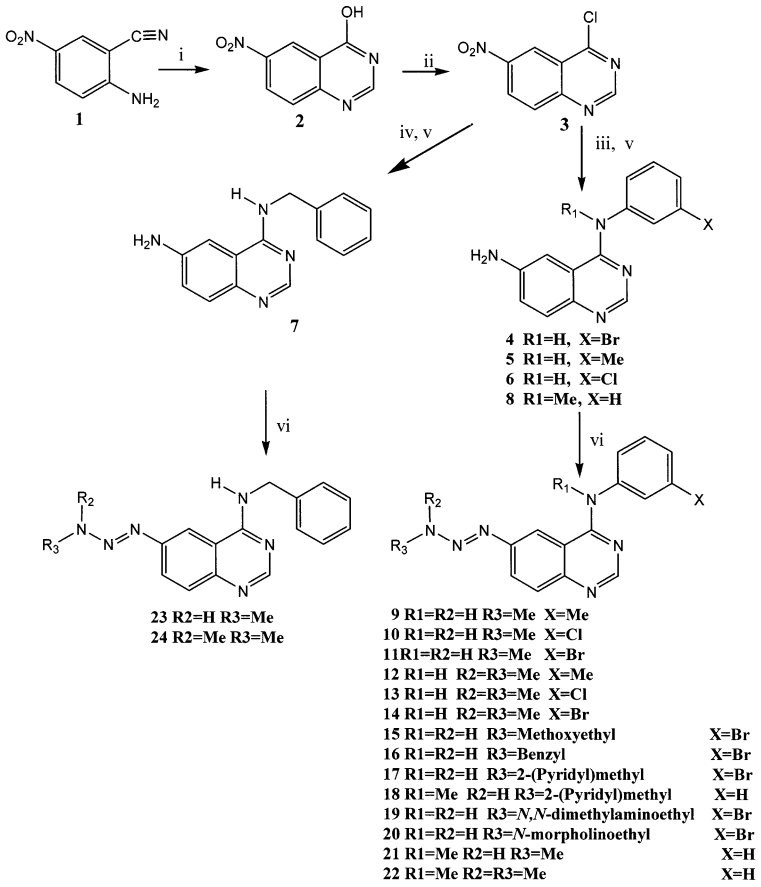
Results and Discussion

Chemistry. The synthesis of these compounds proceeded as described in Scheme 3. Briefly, compound **2** was obtained as described by Roth et al.[13] by treating the 5-nitroanthranilonitrile **1** with a sulfuric acid/formic acid mixture. The resulting quinazoline **2** was heated with phosphorus pentachloride to provide **3**, which was treated with benzylamine or substituted anilines. The nitro groups of the resulting compounds were reduced by catalytic hydrogenation to give 6-aminoquinazolines **4**−**8**. Amine **4** bearing X = Br was obtained by reduction with Fe in ethanol as previously described.[14] Diazotization of amines **4**−**8** using $NOBF_4$ in acetonitrile followed by addition of a series of primary or secondary amines and neutralization with triethylamine gave **9**−**24** as pure solids after purification by column chromatography on basic alumina. Their structures were confirmed by ^1H, ^{13}C NMR and high-resolution mass spectrometry (HRMS).

It is important to note that monoalkyltriazenes are unstable molecules that rapidly decompose under acidic conditions. The 6-alkyltriazene-substituted quinazolines reported herein degraded on silica gel and even neutralization of the eluent with triethylamine did not improve the yield of purification. It was found that only a rapid elution on basic alumina could provide pure compounds in a reasonable yield. On the other hand, diazotization of the aminoquinazoline in acetonitrile with $NOBF_4$ has proven more effective than aqueous reaction with $NaNO_2/H^+$, perhaps due to a greater stability of the diazonium tetrafluoroborate complex in an organic solvent.

Biology. Effects of the Quinazoline Head. One of the premises of the combi-targeting concept is to design the combi-molecule to be small enough to interact with the receptor on its own prior to degradation. Thus, we tested the ability of each agent of the series to block

Scheme 3[a]

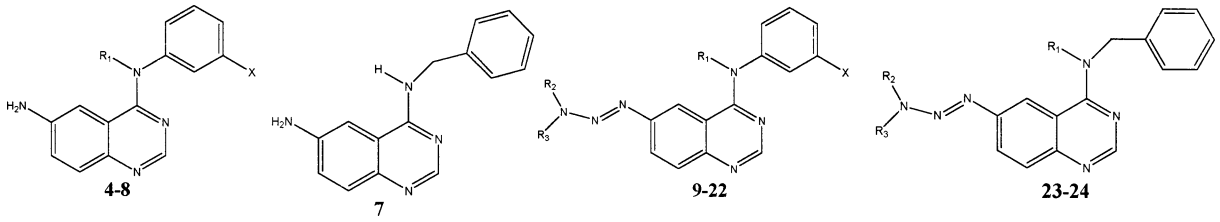


[a] Reagents: (i) H_2SO_4/formic acid/heat; (ii) PCl_5/heat; (iii) $ArNH_2$/^{i}PrOH/rt; (iv) $BnNH_2$/^{i}PrOH/rt; (v) H_2/Pd−C or Fe/ethanol/AcOH/heat; (vi) $CH_3CN/NOBF_4$/−5 °C/ether/Et_3N/alkylamine.

isolated EGFR tyrosine kinase in a short 8 min exposure assay at room temperature in order to prevent partial degradation of the combi-molecules prior to binding to the ATP site of EGFR. It is important to note that the significant number of structure−activity relationship (SAR) studies on anilinoquinazolines[14−16] and pyrido[d]pyrimidines[15,16] as EGFR TK inhibitors are consistent with their binding to the ATP site of EGFR. Molecular modeling suggests that the N-1 atom accepts an H-bond from Met-769 and N-3 from the side chain of Thr-766. The anilino moiety binds in an adjacent hydrophobic pocket and the 6- and 7-positions are positioned at the entrance of the binding cleft. The SAR in the current series of 6-triazene-substituted quinazolines paralleled those of previously reported quinazolines[14−16] (Table 1). Compounds with X = Me were less active than their bromo or chloro analogues. As an example, in the monomethyltriazene series **9**−**11**, a 0.039 μM IC_{50} was observed for the bromo analogue **11**, whereas the 3′-Me substituted combi-molecule **9** was 5 times less active (IC_{50} = 0.2 μM). Although electron-donating substituents at the 6-position are known to significantly enhance binding affinity,[14−16] the 3-mono- and 3,3-dialkyltriazenoquinazolines of these series showed surprisingly higher EGFR TK inhibitory potency than their parental 6-aminoquinazolines (**4**−**8**). The amino group being a stronger electron-donating group than the 3-alkyl-1,2,3-triazene moiety, one would expect the former to confer a greater EGFR affinity than the latter. We believe that the conformation of the triazene tail is such that it may stabilize the binding of the structure to the ATP site. While compounds substituted with a benzylamino group at the 4-position (**7**, **23**, **24**) retain EGFR TK inhibitory activity (IC_{50} = 0.3−0.5 μM), those substituted with an *N*-methylanilino group (**18**, **21**, **22**) were virtually inactive (IC_{50} = 8−13 μM). A similar effect has already been reported for 4-(*N*-methylanilino)quinazolines in earlier structure optimization studies.[14] Thus, these series of molecules have provided a sufficiently broad spectrum of EGFR TK activities to test the role of the EGFR-targeting component in the antiproliferative activity of combi-triazenes.

The modulation of the EGFR targeting component of the series was further tested in serum-stimulated growth assays using NIH3T3 and NIH3T3neu cells transfected with the HER2 (erbB2) gene, a close homologue of the EGFR gene that confers a significant growth advantage to its host transfectant. We have already demonstrated that inhibition of serum-stimulation in this pair of isogenic cell lines was a good cell-based assay to correlate EGFR TK inhibitory activities with inhibition of growth signaling.[7] Interestingly, a statistically significant correlation (Pearson r = 0.8, p < 0.001) was obtained between IC_{50} values of EGFR TK inhibition and those for inhibition of serum-stimulated growth in the NIH3T3neu (Figure 1 and Table 2). More importantly, when IC_{50} values for growth inhibition of NIH3T3 wild type were compared with those of its transfected counterpart NIH3T3neu (a test for selective targeting), the combi-molecules possessing affinity for the ATP site

Table 1. EGFR Tyrosine Kinase (TK) Inhibition and Antiproliferative Data for Combi-Molecules of the Triazene Class



					IC_{50} (μM)	
no.	R1	R2	R3	X	EGFR enzyme assay[a]	inhibition of A431 basal growth[b]
4	H			Br	0.044	45
5	H			Me	1	65
6	H			Cl	0.2	47
7					0.508	25
8	Me			H	8.4	115.9
9 (SMA41)	H	H	Me	Me	0.2	23.22
10 (BJ2000)	H	H	Me	Cl	0.1	16.67
11	H	H	Me	Br	0.039	16
12	H	Me	Me	Me	0.024	93.97
13	H	Me	Me	Cl	0.014	100
14	H	Me	Me	Br	0.011	100
15	H	H	methoxyethyl	Br	0.071	40
16	H	H	benzyl	Br	0.025	20
17	H	H	(2-pyridyl)methyl	Br	0.238	11.40
18	Me	H	(2-pyridyl)methyl	H	9.3	25.14
19	H	H	*N*,*N*-dimethylaminoethyl	Br	0.038	12.40
20	H	H	*N*-morpholinoethyl	Br	0.064	33
21	Me	H	Me	H	8	89.46
22	Me	Me	Me	H	13.35	29.76
23	H	H	Me	H	0.335	20
24	H	Me	Me	H	0.482	50.29

[a] IC_{50} (mM) to inhibit the phosphorylation of poly(L-glutamic acid-L-tyrosine) by EGFR isolated form A431 carcinoma cells. See the Experimental Section for details. [b] IC_{50} (mM) of inhibition of A431 carcinoma cells basal growth. Cell growth was measured using SRB assay. See the Experimental Section for details. Values are the average of two independent experiments run in triplicate. Variation was generally ±1%.

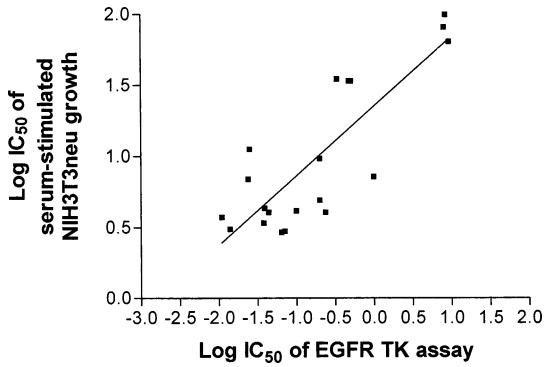


Figure 1. Correlation between the IC_{50} values for EGFR TK inhibition and blockade of serum-stimulated growth of NIH3T3neu (NIH3T3 transfected with erbB2 gene). Pearson correlation coefficient $r = 0.834$, $p < 0.001$. See the Experimental Section for details.

of EGFR were selectively more potent against the transfectant and selectivity (expressed as IC_{50} NIH3T3/IC_{50} NIH3T3neu) linearly increased with EGFR affinity of the drugs. A statistically significant correlation (Pearson $r = 0.8$, $p < 0.001$) was obtained (Figure 2 and Table 2). The significant substituent effects on EGFR TK IC_{50} values, selectivity, and subsequent inhibition of growth stimulation by the combi-molecules is strong evidence of the targeting role of the quinazoline head in their ultimate antiproliferative activities.

Effects of the Triazene Tail. As per Scheme 2, the monoalkyltriazenes (**9**−**11**, **15**−**21**, **23**) are designed to be hydrolyzed to a cytotoxic alkyldiazonium and a 6-aminoquinazoline.[5,6] In contrast, dialkyltriazenes (e.g. **12**−**14**) are stable molecules that require oxidative demethylation in order to generate the corresponding monoalkyltriazene and the ultimate DNA-damaging alkyldiazonium.[10−12] Thus, the dimethyltriazenes were tested as isosteric and perhaps isolectronic non-DNA-damaging analogues of the combi-triazenes. Their inability to damage DNA under the conditions of the study was confirmed by comparing the DNA-damaging potential of **14** with that of **11**. A comet assay demonstrated that in contrast to **11**, which showed a dose-dependent increase in DNA strand breaks following a 30 min drug exposure, the dimethyltriazene **14** did not show any DNA damage in the A431 carcinoma cells (Figure 3). We have already demonstrated in a similar fashion that 6-aminoquinazolines do not damage DNA.[5−8] Thus **4**−**8** were studied as the nonisoelectronic, non-DNA-damaging EGFR inhibitors deriving from aqueous degradation of combi-triazenes. The release of aminoquinazolines from SMA41 and BJ2000 has already been confirmed by HPLC on earlier studies.[5,6] In this study the conversion of the combi-triazenes to their corresponding aminoquinazolines was determined by spectrofluorometer as the latter amines were fluorescent (absorption 290 nm, emission 450 nm) and the rates of amine released were in the range of $0.6-2.5 \times 10^{-2}$ min^{-1} (see **11**, **16**, **19**, Figure 4). These results confirmed that regardless of the 3-alkyl substituents, the combi-triazenes are indeed prodrugs of the aminoquinazolines **4**−**8**, and even after their degradations, another stable inhibitor

Table 2. Selective Inhibition of Serum-Stimulated Growth by Combi-Molecules of the Triazene Class in NIH3T3 Isogenic Cells

no.	R1	R2	R3	X	inhibition of serum-stimula ted growth, IC_{50} (μM)[a] NIH3T3neu	NIH3T3	IC_{50} NIH3T3/ IC_{50} NIH3T3neu
4	H			Br	4.10	52.89	12.92
5	H			Me	7.20	90	12.51
6	H			Cl	4.93	39.19	7.94
7					33.79	96.74	2.86
8	Me			H	99.37	71.35	0.72
9 (SMA41)	H	H	Me	Me	9.69	68.29	7.05
10 (BJ2000)	H	H	Me	Cl	4.16	32.09	7.71
11	H	H	Me	Br	4.32	33.03	7.64
12	H	Me	Me	Me	6.94	50.75	7.31
13	H	Me	Me	Cl	3.09	60.38	19.53
14	H	Me	Me	Br	3.74	52.45	14.02
15	H	H	methoxyethyl	Br	2.98	42.94	14.41
16	H	H	benzyl	Br	11.23	60.18	5.36
17	H	H	(2-pyridyl)methyl	Br	4.06	30.55	7.53
18	Me	H	(2-pyridyl)methyl	Br	64.09	39.42	0.62
19	H	H	*N*,*N*-dimethylaminoethyl	Br	3.40	16.71	4.91
20	H	H	*N*-morpholinoethyl	Br	2.92	22.24	7.61
21	Me	H	Me	H	81.05	30.46	0.38
22	Me	Me	Me	H	31.64	47	1.49
23	H	H	Me	H	34.91	50.27	1.44
24	H	Me	Me	H	33.90	67.29	1.98

[a] Cell growth was measured using the SRB assay. See the Experimental Section for details. Values are the average of two independent experiments run in triplicate. Variation was generally ±1%. [b] IC_{50} ratio of inhibition of serum-stimulated growth in NIH3T3/NIH3T3neu.

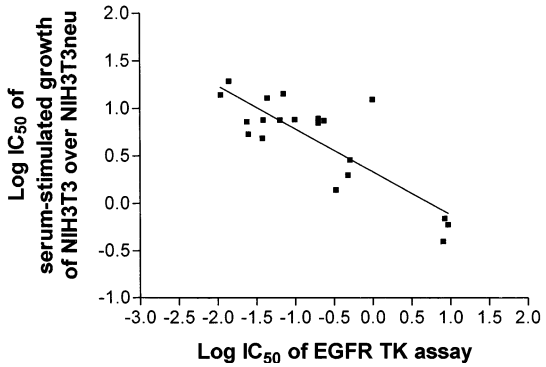


Figure 2. Correlation between the ratio IC_{50} NIH3T3 (wild type)/IC_{50} NIH3T3neu (erbB2 transfectant) for inhibition of serum-stimulated growth (selective targeting) and IC_{50} values for EGFR TK inhibition. Pearson correlation coefficient $r = 0.827$, $p < 0.001$. See the Experimental Section for details.

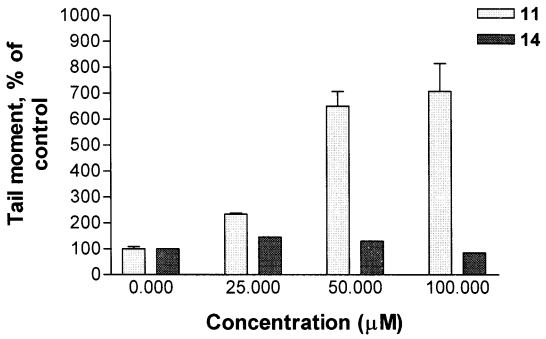


Figure 3. Quantitation of DNA damage using the alkaline comet assay. Tail moment was used as a parameter for the detection of DNA damage in A431 cells exposed to **11** and **14** for 30 min. See the Experimental Section for details.

(the released aminoquinazoline) may remain in the cell culture medium.

Using the A431 carcinoma of the vulva cell line that coexpresses EGFR and its ligand TGFα and aggressively proliferates by autocrine induction, we found that the aminoquinazolines (**4**−**8**) and dimethyltriazenes

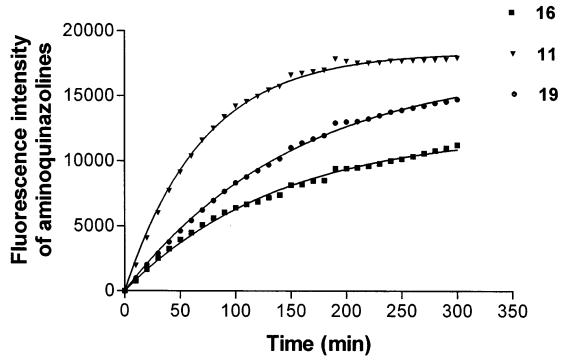


Figure 4. Formation of fluorescent aminoquinazolines from combi-triazenes (**11**, **16**, **19**). Experiments were carried out by spectrofluorometry as described in the Experimental Section. The rates of amine released from **11**, **16**, and **19** were 1.4×10^{-2}, 0.71×10^{-2}, and 0.69×10^{-2} min^{-1}, respectively.

(**12**−**14**, **22**, and **24**), whose activities were solely dependent on their ability to block EGFR-mediated cell signaling, were in the 50−100 μM range in a basal growth assay. In contrast, all monoalkyltriazenes were more potent than their blocked counterpart (Table 1). As depicted in Figure 5, where only compounds with methylating properties are compared, agents such as Temozolomide (TEM),[17−19] methylnitrosourea (MNU), or **21**, which only possess methylating properties, had little antiproliferative activity against A431 cells that express O6-methylguanine transferase, a DNA-repair enzyme capable of repairing the cytotoxic O6-methylguanine adduct. In contrast, the methylating combi-triazenes **9**−**11** and **23**, which possess mixed targeting properties, were significantly more potent in this refractory tumor cell line. Thus, combi-triazenes that combine multiple properties appear to be more potent than their monotargeted counterparts.

The Combi-Molecules. The current study demonstrated that the combination of an alkyltriazene with an aminoquinazoline in order to simultaneously target DNA and EGFR is an at least additive process in which

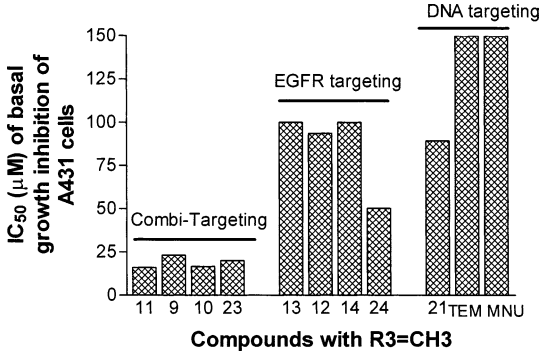


Figure 5. Comparison of the antiproliferative activities of monotargeted methylating agents (EGFR-targeting or DNA-targeting) with mixed DNA/EGFR-targeting methylating combi-molecules (combi-targeting) in the A431 human carcinoma of the vulva cell line. See the Experimental section for details.

each individual component **TZ** and **I** plays a distinct antiproliferative role. This study also demonstrated that, as postulated, molecules designed to enhance both chemoselectivity and chemosensitivity in growth factor-dependent tumors can be achieved. Although the impact of each of these components may vary with the cell phenotype, the combi-molecular approach possesses significant pharmacological advantages over mono-targeted agents, since where DNA repair enzymes deplete the cytotoxic activity associated with DNA alkylation, the signaling inhibitory component will exert significant antiproliferative effects. Where DNA repair status is deficient and EGFR/erbB2 confers a growth advantage, a tandem block can be inflicted to both growth factor-stimulated proliferation and uncontrolled DNA replication in refractory tumor cells. More importantly, this combination of two major antiproliferative properties are imprinted into single and small structures: the "combi-molecules".

Experimental Section

Chemistry. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Varian 400.14 or 300.06 spectrometer. Chemicals shifts are expressed in parts per million (ppm) relative to the internal standard tetramethylsilane. Mass spectrometry was performed by the McGill University Mass spectroscopy Center. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) spectra were performed on Finnigan LC QDUO spectrometers. High-resolution mass spectra (HMRS) were obtained from a ZAB-E4F analytical mass spectrometer. Data are reported as *m*/*z* (intensity relative to base peak = 100). The compounds were purified by flash chromatography using Caledon 50−200 μm alumina (basic). Melting points were determined in open capillary tubes on a Meltemp melting point apparatus and were uncorrected. Intermediates **2**−**8** were synthesized as described previously.[14−16]

4-Hydroxy-6-nitroquinazoline (2). 5-Nitroanthranilonitrile **1** (25 g, 0.15 mol) was added by portions over 1 h to a mildly refluxing mixture of 88% formic acid (300 mL) and sulfuric acid (18 mL). After an additional 30 min, the mixture was cooled to 0 °C and poured into ice−water. The resulting precipitate was collected by filtration, washed with water, and dried under vacuum to give **2** (34 g, 86%) as a yellow solid: mp 283−285 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.72 (br s, 1H, OH), 8.76 (d, 1H, J = 3.0 Hz, H-5), 8.50 (m, 1H, H-7), 8.28 (s, 1H, H-2), 7.83 (d, 1H, J = 8.7 Hz, H-8); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.7, 152.5, 148.6, 144.6, 128.7, 128.0, 122.4, 121.6.

6-Amino-4-[(3-bromophenyl)amino]quinazoline (4). General Example of the Coupling Procedure. To a solution of **3**[14] (2 g, 9.5 mmol) in 2-propanol was added 3-bromoaniline (1 mL, 9.5 mmol) and the resulting mixture stirred at room temperature overnight under argon. The precipitate that formed was filtered and washed with water and ether, after which it was dried under vacuum to give 6-nitro-4-(3-bromophenylamino)quinazoline (4 g, 82%). This compound was found to be sufficiently pure to be used for the next step: mp 267−270 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.95 (br s, 1H, NH), 9.87 (d, 1H, J = 2.6 Hz, H-5), 9.0 (s, 1H, H-2), 8.73 (dd, 1H, J = 12.2, 2.6 Hz, H-7), 8.15 (d, 1H, J = 12.2 Hz, H-8), 8.04 (s, 1H, H-2′), 7.79 (d, 1H, J = 10.4, H-6′), 7.16−7.14 (m, 2H, H-4′,5′); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.2, 158.0, 153.2, 145.2, 140.7, 131.1, 130.0, 127.6, 127.5, 125.5, 122.0, 121.9, 121.5, 115.0.

To a solution of the isolated 6-nitro-4-(3-bromophenylamino)quinazoline (5 g, 0.015 mol) in aqueous ethanol (1:2, 300 mL) and acetic acid (15 mL) was added Fe (10 g. 0.18 mol), and the resulting cloudy mixture was kept under reflux for 1 h. The reaction was cooled to room temperature, alkalinized with concentrated ammonia, and filtered through Celite. The filtrate was evaporated and the resulting solid washed with water and further dried to give **4** (5.7 g, 80%) as a yellow solid: mp 204−206 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.44 (s, 1H, NH), 8.37 (s, 1H, H-2), 8.20 (s, 1H, H-2′), 7.85 (d, 1H, J = 8.0 Hz, H-6′), 7.53 (d, 1H, J = 8.8 Hz, H-8), 7.32−7.20 (m, 4H, H-5,7,4′,5′), 5.66 (s, 2H, NH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.4, 156.2, 148.9, 143.7, 143.3, 131.5, 128.7, 124.9, 123.9, 121.8, 118.4, 114.9, 114.1, 99.5.

6-Amino-4-[(3-methylphenyl)amino]quinazoline (5). A suspension of 6-nitro-4-[(3-methylphenyl)amino]quinazoline obtained as previously described[14] (5.49 g, 0.02 mol) and palladium (5% charcoal, 1 g, 9.4 mmol) in MeOH (100 mL) was hydrogenated at 2 bar for 1 h at room temperature. Filtration, evaporation, and recrystallization from neat ethyl acetate gave **5** (5 g, 90%): mp 245−248 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.18 (br s, 1H, NH), 8.30 (s, 1H, H-2), 7.65−7.63 (m, 2H, H-5,7), 7.5 (d, 1H, J = 8.8 Hz, H-8), 7.37 (s, 1H, H-2′), 7.24−7.21 (m, 2H, H-4′,5′), 6.86 (d, 1H, J = 7.6 Hz, H-6′), 5.49 (br s, 2H, NH_2); 2.31 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.7, 150.5, 147.8, 143.1, 140.5, 138.1, 129.3, 128.8, 124.3, 124.2, 122.9, 119.7, 117.4, 101.9, 22.1.

6-Amino-4-[(3-chlorophenyl)amino]quinazoline (6). 6-Aminoquinazoline **6** was obtained as described for **5** (4.5 g, 80%): mp 235−237 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.5 (br s, 1H, NH), 8.56 (s, 1H, H-2), 8.00 (br s, 1H, H-2′), 7.74 (dd, 1H, J = 8, 0.8 Hz, H-4′), 7.68 (d, 1H, J = 8.8 Hz, H-8), 7.56 (d, 1H, J = 1.6 Hz, H-5), 7.42−7.35 (m, 2H, H-7,5′), 7.21 (dt, 1H, J = 8.0, 1.0 Hz, H-6′), 5.50 (br s, 2H, NH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.8, 149.5, 147.7, 140.4, 135.3, 133.3, 130.8, 125.7, 125.0, 124.3, 123.4, 122.4, 116.6, 102.3.

6-Amino-4-[(phenylmethyl)amino]quinazoline (7). 6-Aminoquinazoline **7** was obtained as described for **5** (3 g, 85%): mp 170 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.65 (br s, 1H, NH), 8.43 (s, 1H, H-2), 7.56−7.23 (m, 8H, ArH), 5.90 (br s, 2H, NH_2), 4.81 (d, 2H, J = 4.0 Hz, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.2, 149.0, 148.6, 139.3, 129.2, 129.0 (2C overlap), 128.0 (2C overlap), 127.6, 124.8, 124.2, 116.0, 102.4, 44.8.

6-Amino-4-[(*N*-methyl-N-phenyl)amino]quinazoline (8). 6-Aminoquinazoline **8** was obtained as described for **5** (3.5 g, 75%): mp 220−222 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.66 (s, 1H, H-2), 7.59 (d, 1H, J = 9.0 Hz, H-7), 7.45−7.12 (m, 7H, ArH), 5.93 (s, 2H, NH_2), 3.58 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.7, 148.3, 147.8, 147.6, 139.6, 130.4 (2C overlap), 126.8, 126.3, 125.3 (2C overlap), 124.8, 118.2, 105.1, 43.4.

1-{4-[(3-Bromophenyl)amino]-6-quinazolinyl}-3-methyltriazene (11). 6-Aminoquinazoline **4** (100 mg, 0.28 mmol) was stirred in dry acetonitrile (10 mL) under argon. Thereafter, it was cooled to −5 °C and nitrosonium tetrafluoroborate (67 mg, 0.58 mmol) in acetonitrile was added. The resulting solution was stirred for 1 h at −5 °C and added dropwise to a mixture of cold ether (10 mL), water (2 mL), triethylamine (0.5 mL), and methylamine (2.0 M solution in THF, 1.1 mL, 2.24 mmol). The mixture was kept at 0 °C for 2 h more and

further was extracted with ethyl acetate. The organic layer was dried over potassium carbonate and evaporated to give **11** as a brown residue, which was purified by flash chromatography on basic alumina (1:4 cyclohexane−AcOEt). Triazene **11** was obtained as an oil that solidified upon addition of 1:1 ether/petroleum ether (100 mg, 88%): mp 167−169 °C; FABMS *m/z* 356.9 (MH^+ with ^{79}Br), 358.9 (MH^+ with ^{81}Br), 329 (M − N_2, 10.7), 315 (M − methyldiazonium, 19.8), 299 (M − methyltriazene, 11.9) *m/z* 357.046 331 (MH^+), $C_{15}H_{13}N_6Br$ requires *m/z* 357.046 200; ^1H NMR (400 MHz, DMSO-d_6) δ 9.88 (br s, 1H, −N═N−NH), 9.28 (s 1H, NH), 8.57 (s, 1H, H-2), 8.45 (d, 1H, J = 1.6 Hz, H-2′), 8.27 (s, 1H, H-5), 8.00−7.94 (m, 2H, H-7,6′), 7.74 (d, 1H, J = 9.2 Hz, H-8), 7.32−7.26 (m, 2H, H-5′,4′), 3.08 (d, 3H, J = 3.6 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.0, 153.7, 149.6, 148.7, 141.8, 130.9, 129.5, 126.4, 125.5, 124.6, 121.8, 121.1, 116.3, 115.2, 31.5.

1-{4-[(3-Methylphenyl)amino]-6-quinazolinyl}-3,3-dimethyltriazene (12). 6-Aminoquinazoline **5** (100 mg, 0.4 mmol) was stirred in dry acetonitrile (10 mL) under argon. Thereafter, it was cooled to −5 °C, and nitrosonium tetrafluoroborate (94 mg, 0.8 mmol) in acetonitrile was added. The resulting solution was stirred for 1 h at −5 °C and added dropwise to a mixture cold of ether (10 mL), water (2 mL), triethylamine (0.5 mL), and aqueous dimethylamine (40%) (0.3 mL). The mixture was further kept at 0 °C, and 2 h later, the precipitate was filtered and washed with ether and AcOEt to give **12** as a brown powder (80 mg, 65%): mp 165−167 °C; FABMS 307 (MH^+), 235 (M − 3,3-dimethyltriazene, 36.2) *m/z* 307.167120 (MH^+), $C_{17}H_{18}N_6$ requires *m/z* 307.167 000; ^1H NMR (400 MHz, DMSO-d_6) δ 9.87 (s, 1H, NH), 8.69 (s, 1H, H-2), 8.00 (d, 1H, J = 11.8 Hz, H-7), 7.86 (d, 1H, J = 11.8 Hz, H-8), 7.80 (s, 1H, H-5), 7.56−7.53 (m, 2H, H-4′,2′), 7.29 (t, 1H, J = 9.9 Hz, H-5′), 6.98 (d, 1H, J = 9.9 Hz, H-6′), 3.56 (br, 3H, $N(Me)_2$), 3.29 (br, 3H, $N(Me)_2$), 2.41 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.2, 153.9, 149.1, 148.5, 139.9, 138.1, 129.3, 128.8, 125.1, 124.8, 123.4, 120.1, 116.4, 115.2, 43.8, 36.9, 22.1.

1-{4-[(3-Chlorophenyl)amino]-6-quinazolinyl}-3,3-dimethyltriazene (13). As described for **12,** triazene **13** was obtained as a pale brown powder (84 mg, 70%), using **6** (100 mg, 0.4 mmol) and aqueous dimethylamine (40%) (0.97 mL, 2.8 mmol) in ether: mp 192−194 °C; FABMS 327 (MH^+), 255 (M − 3,3-dimethyltriazene, 39.9) *m/z* 327.112497 (MH^+), $C_{16}H_{15}N_6Cl$ requires *m/z* 327.112 520; ^1H NMR (400 MHz, DMSO-d_6) δ 9.87(s, 1H, NH), 8.56 (s, 1H, H-2), 8.41 (d, 1H, J = 2.8 Hz, H-5), 8.14 (t, 1H, J = 1.4 Hz, H-2′), 7.96 (dd, 1H, J = 2.8, 11.9 Hz, H-7), 7.88 (dd, 1H, J = 1.4, 10.8 Hz, H-4′), 7.74 (d, 1H, J = 11.9 Hz, H-8), 7.38 (t, 1H, J = 10.9 Hz, H-5′), 7.13 (dd, 1H, J = 1.4, 10.9 Hz, H-6′), 3.56 (br, 3H, $N(Me)_2$), 3.23 (br, 3H, $N(Me)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.9, 153.6, 149.2, 148.5, 141.6, 133.3, 130.7, 129.4, 125.3, 123.5, 121.8, 120.8, 116.4, 115.0, 43.9, 37.1.

1-{4-[(3-Bromophenyl)amino]-6-quinazolinyl}-3,3-dimethyltriazene (14). As described for **12**, triazene **14** was obtained as a brown powder (85 mg, 72%), using **4** (100 mg, 0.32 mmol) and dimethylamine (40%) (0.3 mL) in ether: mp 179−180 °C; FABMS *m/z* 371.1 (MH^+ with ^{79}Br), 373.0 (MH^+ with ^{81}Br), 299 (M − dimethyltriazene, 39.6) *m/z* 371.061981 (MH^+), $C_{16}H_{15}N_6Br$ requires *m/z* 371.061 980; ^1H NMR (400 MHz, DMSO-d_6) δ 8.72 (s, 1H, NH), 8.09 (s, 1H, H-2), 8.00−7.51 (m, 5H, H-8,7,5,6′,2′), 7.26 (m, 2H, H-4′,5′), 3.58 (s, 3H, $N(Me)_2$), 3.29 (s, 3H, $N(Me)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.9, 153.6, 149.2, 148.5, 141.7, 130.9, 129.4, 126.3, 125.3, 124.6, 121.8, 121.1, 116.3, 115.0, 43.8, 37.0.

1-{4-[(3-Bromophenyl)amino]-6-quinazolinyl}-3-(2-methoxyethyl)triazene (15). As described for **11**, triazene **15** was obtained as a brown powder (100 mg, 79%), using **4** (100 mg, 0.32 mmol) and 2-methoxyethylamine (0.21 mL, 2.24 mmol) in ether: mp 141−143 °C; FABMS *m/z* 401 (MH^+ with ^{79}Br), 403 (MH^+ with ^{81}Br), 373 (M − N_2, 9.0), 315 (M − methoxyethyldiazonium, 42.1), 299 (M − methoxyethyltriazene, 38.3) *m/z* 401.072546 (MH^+), $C_{17}H_{17}N_6OBr$ requires *m/ z*, 401.072 420; ^1H NMR (400 MHz, $CDCl_3$) δ (−N═N−NH signal not observed) 8.73 (s, 1H, NH), 8.10 (s, 1H, H-2), 7.90 (s, 1H, H-2′), 7.82−7.41 (m, 4H, H-5,7,8,6′), 7.26−7.24 (m, 2H, H-4′,5′), 3.91 (m, 2H, $CH_2CH_2OCH_3$), 3.72 (t, 2H, J = 6.8 Hz, $CH_2CH_2OCH_3$), 3.43 (s, 3H, $CH_2CH_2OCH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.9, 153.7, 149.5, 148.7, 141.8, 130.9, 129.4, 126.3, 125.4, 124.6, 121.8, 121.1, 116.3, 115.2, 68.7, 58.8, 44.0.

1-{4-[(3-Bromophenyl)amino]-6-quinazolinyl}-3-benzyltriazene (16). As described for **11**, triazene **16** was obtained as a pale brown powder (90 mg, 65%), using **4** (100 mg, 0.32 mmol) and benzylamine (0.24 mL, 2.24 mmol) in ether: mp 132−133 °C; FABMS *m/z* 433 (MH^+ with ^{79}Br), 435 (MH^+ with ^{81}Br), 405 (M − N_2, 5.6), 315 (M − benzyldiazonium, 16.0), 299 (M − benzyltriazene, 13.9) *m/z* 433.077631 (MH^+), $C_{21}H_{17}N_6Br$ requires *m/z* 433.077 730; ^1H NMR (400 MHz, DMSO-d_6) δ (−N═N−NH signal not observed) 9.80 (s, 1H, NH), 8.73 (s, 1H, H-2), 8.07−7.21 (m, 13H, ArH), 4.86 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.0, 153.8, 149.3, 148.8, 141.8, 137.1, 130.9 (2C overlap), 129.5, 129.1 (2C overlap), 128.7, 127.9, 126.4, 125.6, 124.7, 121.8, 121.2, 116.3, 115.3, 48.1.

1-{4-[(3-Bromophenyl)amino]-6-quinazolinyl}-3-(2′-pyridylmethyl)triazene (17). As described for **11**, triazene **17** was obtained as a brown powder (80 mg, 58%), using **4** (100 mg, 0.32 mmol) and 2-(aminomethyl)pyridine (0.23 mL, 2.24 mmol) in ether: mp 145−147 °C; FABMS *m/z* 433.92 (MH^+ with ^{79}Br), 435.95 (MH^+ with ^{81}Br), 406 (M − N_2, 3.7), 315 (M − pyridylmethyldiazonium, 21.5), 297 (M − pyridylmethyltriazene, 3.8) *m/z* 434.072880 (MH^+), $C_{20}H_{16}N_7Br$ requires *m/z* 434.072 820; ^1H NMR (400 MHz, DMSO-d_6) δ (−N═N−NH signal not observed) 9.89 (s, 1H, NH), 8.55 (d, 1H, J = 9.2 Hz, Py-H), 8.53 (s, 1H, H-2), 8.39 (s, 1H, H-2′), 8.23 (s, 1H, H-5), 7.94−7.90 (m, 2H, H-7, 6′), 7.76−7.74 (m, 2H, H-8, Py-H), 7.36−7.24 (m, 4H, H-5′, H4′, Py-H), 4.89 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.0, 156.7, 153.8, 149.7, 149.1, 148.8, 141.7, 137.4, 130.9, 129.5, 126.4, 125.6, 124.7, 123.0, 122.7, 121.8, 121.2, 116.3, 115.5, 49.6.

1-{4-[(*N*-Methyl-N-phenyl)amino]-6-quinazolinyl}-3-(2′-pyridylmethyl)triazene (18). As described for **11**, triazene **18** was obtained as a brown powder (80 mg, 58%), using **8** (100 mg, 0.32 mmol) and 2-(aminomethyl)pyridine (0.23 mL, 2.24 mmol) in ether: mp 145−147 °C; FABMS 370 (MH^+), 342 (M − N_2, 49.4), 251 (M − pyridylmethyldiazonium, 100.0), 235 (M − pyridylmethyltriazene, 47.6) *m/z* 370.178019 (MH^+), $C_{21}H_{19}N_7$ requires *m/z* 370.178 150; ^1H NMR (400 MHz, DMSO-d_6) δ (−N═N−NH signal not observed) 8.77 (s, 1H, H-2), 8.5−7.18 (m, 12H, ArH), 4.81 (s, 2H, CH_2), 3.66 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.1, 153.8, 149.4, 149.2, 141.7, 137.1, 133.3, 130.7, 129.5, 129.2 (2C overlap), 129.1 (2C overlap), 127.9, 125.7, 123.6, 121.9, 120.9, 116.3, 115.3, 48.2.

1-{4-[(3-Bromophenyl)amino]-6-quinazolinyl}-3-(2-*N*,*N*-dimethylaminoethyl)triazene (19). As described for **11**, triazene **19** was obtained as a pale brown powder (100 mg, 76%) using **4** (100 mg, 0.32 mmol) and *N*,*N*-dimethylethylenediamine (200 mg, 2.24 mmol) in ether: mp 130−132 °C; FABMS *m/z* 414 (MH^+ with ^{79}Br), 416 (MH^+ with ^{81}Br), 386 (M − N_2, 1.5), 315 (M − *N*,*N*-dimethylaminoethyldiazonium, 16.7), 299 (M − *N*,*N*-dimethylaminoethyltriazene, 7.6) *m/z* 414.104180 (MH^+), $C_{18}H_{20}N_7Br$ requires *m/z* 414.103 990; ^1H NMR (400 MHz, DMSO-d_6) δ 9.88 (br s, 1H, N═N−NH), 8.57 (s, 1H, NH), 8.41 (s, 1H, H-2), 8.26 (br s, 1H, H-2′), 7.95−7.73 (m, 3H, H-8, 7,6′), 7.32−7.24 (m, 3H, H-5,5′,4′), 3.65 (br, 2H, $CH_2CH_2N(CH_3)_2$), 2.56 (t, 2H, J = 6.8 Hz, $CH_2CH_2N(CH_3)_2$), 2.19 (s, 6H, $N(CH_3)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.1, 152.8, 148.8, 147.8, 141.0, 130.1, 128.6, 125.5, 124.6, 123.8, 121.0, 120.3, 115.5, 114.3, 55.3, 54.2 (2C overlap), 41.5.

1-{4-[(3-Bromophenyl)amino]-6-quinazolinyl}-3-[2-(4-morpholino)ethyl]triazene (20). As described for **11**, triazene **20** was obtained as a brown powder (95 mg, 66%), using **4** (100 mg, 0.32 mmol) and 4-(2-aminoethyl)morpholine (229 mg, 2.24 mmol) in ether: mp 150−153 °C; FABMS *m/z* 456 (MH^+ with ^{79}Br), 458 (MH^+ with ^{81}Br), 429 (M − N_2, 1.5), 328 (M − morpholinoethyltriazene, 2.0), 317 (M − morpholinoethyldiazonium, 6.4) *m/z* 456.114745 (MH^+), $C_{20}H_{22}N_7OBr$ requires *m/z* 456.114 620; ^1H NMR (400 MHz, DMSO-d_6) δ

(−N═N−NH signal not observed) 8.74 (s, 1H, NH), 8.60 (s, 1H, H-2), 8.10−7.53 (m, 5H, H-8, 7, 5, 6′, 2′), 7.27−7.25 (m, 2H, H-5′, 4′), 3.77−3.74 (m, 6H, $3CH_2$), 2.74 (t, 2H, J = 6.0 Hz, CH_2), 2.53 (br, 4H, $2CH_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.0, 153.8, 149.6, 148.7, 141.8, 131.0, 129.5, 126.4, 125.5, 124.6, 121.8, 121.2, 116.3, 115.1, 67.0 (2C overlap), 55.3, 54.1 (2C overlap), 41.5.

1-{4-[(*N*-Methyl-N-phenyl)amino]-6-quinazolinyl}-3-methyltriazene (21). As described for **11**, triazene **21** was obtained as a brown powder (90 mg, 77%) using **8** (100 mg, 0.4 mmol) and methylamine (2.0 M solution in THF, 1.4 mL, 2.8 mmol) in ether: mp 152−154 °C; FABMS *m/z* 293.1 (MH^+), 250 (M − methyldiazonium, 42.3), 235 (M − methyltriazene, 26.8) *m/z* 293.151470 (MH^+), $C_{16}H_{16}N_6$ requires *m/z* 293.151 380; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (br s, 1H, N═N−NH), 8.75 (s, 1H, H-2), 7.70−6.85 (m, 8H, ArH), 3.54 (s, 3H, CH_3), 2.85 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.0, 151.2, 149.0, 147.0, 143.5, 129.3 (2C overlap), 129.1, 126.0, 116.9, 115.3, 112.3 (2C overlap), 112.0, 43.9, 32.5.

1-{4-[(*N*-Methyl-N-phenyl)amino]-6-quinazolinyl}-3,3-dimethyltriazene (22). As described for **12**, triazene **22** was obtained as a pale brown powder (55 mg, 45%) using **8** (100 mg, 0.4 mmol) and dimethylamine (40%) (0.4 mL, 2.8 mmol) in ether: mp 140−142 °C; FABMS *m/z* 307 (MH^+), 235 (M − dimethyltriazene, 92.8), *m/ z* 307.167000 (MH^+), $C_{16}H_{16}N_6$ requires *m/z* 307.167 120; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.66 (s, 1H, H-2), 7.53−7.17 (m, 8H, ArH), 3.54 (s, 3H, CH_3), 3.35 (br, 3H, $N(Me)_2$), 3.10 (br, 3H, $N(Me)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.6, 153.5, 149.6, 148.5, 147.6, 130.7 (2C overlap), 129.3, 127.0, 126.9, 126.3 (2C overlap), 117.3, 115.8, 43.2, 43.0, 37.4

1-{4-[(Phenylmethyl)amino]-6-quinazolinyl}-3-methyltriazene (23). As described for **11**, triazene **23** was obtained as a pale brown powder (97 mg, 83%), using **7** (100 mg, 0.4 mmol) and methylamine (2.0 M solution in THF, 1.4 mL, 2.8 mmol) in ether: mp 144−146 °C; FABMS *m/z* 293 (MH^+), 265 (M−N_2, 2.9), 250 (M − methyldiazonium, 30.2), 235 (M − methyltriazene, 13.2) *m/z* 293.151470 (MH^+), $C_{16}H_{16}N_6$ requires *m/z* 293.151 380; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.58 (br, s, 1H, N═N−NH), 8.86 (br, s, 1H, NH), 8.34 (s, 1H, H-2), 8.20 (s, 1H, H-5), 7.84 (d, 1H, J = 11.4 Hz, H-7), 7.62 (d, 1H, J = 11.4 Hz, H-8), 7.33−7.20 (m, 5H, ArH), 4.75 (d, 2H, J = 6.8 Hz, CH_2), 3.04 (d, 3H, J = 3.2 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.0, 154.7, 149.0, 148.0, 140.2, 129.1, 128.9 (2C overlap), 127.9 (2C overlap), 127.3, 125.1, 116.0, 114.9, 44.3, 31.4.

1-{4-[(Phenylmethyl)amino]-6-quinazolinyl}-3,3-dimethyltriazene (24). As described for **12**, triazene **24** was obtained as a brown powder (97 mg, 83%), using **7** (90 mg, 0.36 mmol) and dimethylamine (40%) (0.3 mL) in ether: mp 144 °C; FABMS *m/z* 307 (MH^+), 235 (M − dimethyltriazene, 30.9), *m/z* 307.167000 (MH^+), $C_{16}H_{16}N_6$ requires *m/z* 307.167 120; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.83 (t, 1H, J = 6.0 Hz, NH), 8.34 (s, 1H, H-2), 8.17 (d, 1H, J = 2.0 Hz, H-5), 7.84 (dd, 1H, J = 2.0, 8.9 Hz, H-7), 7.62 (d, 1H, J = 8.9 Hz, H-8), 7.35−7.20 (m, 5H, ArH), 4.75 (d, 2H, J = 6.0 Hz, CH_2), 3.52 (br, 3H, $N(Me)_2$), 3.18 (br, 3H, $N(Me)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.9, 154.6, 148.6, 147.9, 140.2, 129.0, 128.9 (2C overlap), 127.9 (2C overlap), 127.3, 125.0, 116.0, 114.6, 44.3, 43.9, 37.5.

Cell Culture. The human epidermoid carcinoma of the vulva A431 was obtained from the American Type Culture Collection (Manassas, VA). The mouse fibroblasts NIH3T3 and NIH3T3neu (NIH3T3 cells stably transfected with ErbB2 gene) were generous gifts from Dr. Moulay Aloui-Jamali of the Montreal Jewish General Hospital. The A431 cell line was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics as described previously.[6] NIH3T3 and NIH3T3neu cells were maintained in DMEM supplemented with 10% FBS and antibiotics. All cells were maintained in an atmosphere of 5% CO_2.

Purity of Compounds. Purity analyses were performed by high performance liquid chromatography (HPLC) using a Spectrasystem (Thermoquest) and a Waters C4 15-μm 300 × 3.9-mm column (reverse phase). The operating mode was isocratic and two different systems of solvents were used: the first one was 57% acetonitrile and 43% water, and the second system was 70% methanol and 30% water (pH8) with a 0.5 mL/min flow rate at 250 nm.

Degradation. The study of the conversion of the combitriazenes to their corresponding aminoquinazolines was performed by spectrofluorometer as the latter amines were fluorescent (absorption 290 nm, emission 450 nm). Briefly, 125 μM of the combi-triazenes was added to RPMI-1640 with 10% FBS and incubated for 4 h at 37 °C in a microplate spectrofluorometer (SpectraMax Gemini fluorescence reader, Molecular Device, CA). The data were analyzed by the SoftMaxPro (Molecular Device, CA) and GraphPad software packages.

Tyrosine Kinase Assays. This assay is similar to the one described previously.[6] Nunc Maxisorp 96-well plates were incubated overnight at 37 °C with 100 μL/well of 0.25 mg/mL PGT in PBS. The kinase reaction was performed by using 4.5 ng/well EGFR affinity-purified from A431 cells. The compound was added and phosphorylation initiated by the addition of ATP (50 μM). After 8 min at room temperature with constant shaking, the reaction was terminated by aspiration mixture and by rinsing the plate four times with wash buffer. Phosphorylated PGT was detected following a 25 min incubation with 50 μL/well of HRP-conjugated PY20 anti-phosphotyrosine antibody (Santa Cruz Biotechnology) diluted to 0.2 μg/mL in blocking buffer (3% bovine serum albumin, 0.05% Tween 20 in PBS). Antibody was removed by aspiration, and the plate washed four times with wash buffer. The signals were developed by the addition of 50 μL/well of 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersberg, MD), and following blue color development, 50 μL of 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).

In Vitro Growth Inhibition Assay. (A) To study the effect of our compounds on serum stimulated-proliferation, cells (NIH3T3 and NIH3T3neu) were grown to 70% of confluence in 96-well plates and washed twice with PBS after which they were exposed to serum-free medium for 18 h. Cells were exposed to each drug and serum for 72 h and cell growth measured using the sulforhodamine B (SRB) assay.[20]

(B) To study the antiproliferative effects of our compounds in basal growth condition and under continuous exposure, A431 cells were grown to 70% of confluence in 96-well plates for 24 h. Thereafter, cells were exposed to different concentrations of each drug for 96 h. Growth inhibition was evaluated using the SRB assay.

Alkaline Comet Assay for Quantitation of DNA Damage. The alkaline comet assay was performed as previously described.[6] The cells were exposed to drugs for 30 min, harvested with trypsin−EDTA, and resuspended in PBS. Cell suspensions were diluted to approximately 10^6 cells and mixed with agarose (1%) in PBS at 37 °C in a 1:10 dilution. The gels were cast on Gelbond strips (Mandel Scientific, Guelph, Canada) using gel casting chambers and then immediately placed into a lysis buffer [2.5 M NaCl, 0.1 M tetrasodium-EDTA, 10 mM Tris-base, and 1% (v/v) Triton X-100, pH 10.0]. After being kept on ice for 30 min, the gels were gently rinsed with distilled water and immersed in a second lysis buffer (2.5 M NaCl, 0.1 M tetrasodium-EDTA, 10 mM Tris-base) containing 1 mg/mL proteinase K for 60 min at 37 °C. Thereafter, the gels were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37 °C, and electrophoresed at 19 V for 20 min. The gels were subsequently rinsed with distilled water and placed in 1 M ammonium acetate for 30 min. Thereafter, they were soaked in 100% ethanol for 2 h, dried overnight, and stained with SYBR Gold (1/10000 dilution of stock supplied from 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 were

analyzed for each sample, using ALKOMET version 3.1 image analysis software.

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Supporting Information Available: NMR spectra, HRMS data, and HPLC chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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