Synthesis of a Prodrug Designed To Release Multiple Inhibitors of the Epidermal Growth Factor Receptor Tyrosine Kinase and an Alkylating Agent: A Novel Tumor Targeting Concept

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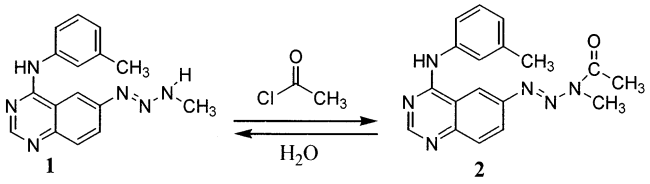
The synthesis of a novel acetoxymethyltriazene designed to be a prodrug of multiple inhibitors of the epidermal growth factor receptor (EGFR) and a methyldiazonium species is described. Studies with each of the expected metabolites demonstrated significant EGFR tyrosine kinase inhibitory activities and the released methyldiazonium was trapped with *p*-nitrobenzylpyridine. Their ability to damage genomic DNA in whole cells was demonstrated by using the single cell microelectrophoresis (comet) assay. The results suggest that this approach may well represent a novel drug combination strategy involving single molecules masking multiple bioactive agents.

Introduction

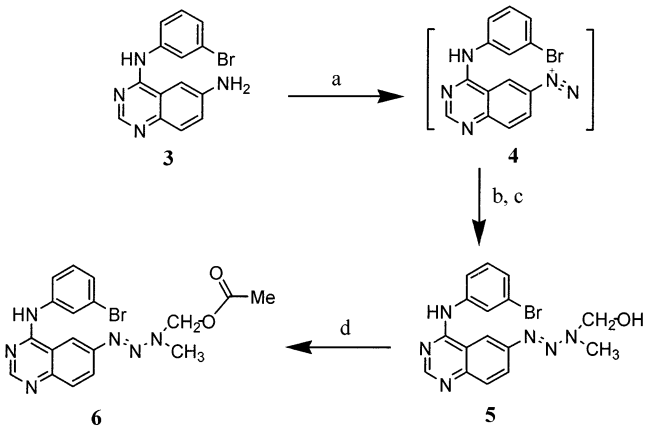
The altered protein expression and activity of receptor tyrosine kinases (TK) are implicated in the progression of various types of cancers. One such dysfunction is the overexpression of the epidermal growth factor receptor (EGFR) and its closest homologue HER2 (erbB2) that correlates with aggressive tumor progression and poor prognosis.[1] Recently, we developed a novel strategy that seeks to combine DNA-damaging properties and EGFR TK inhibitory activities into single molecules termed "combi-molecules" designed to kill EGFR-expressing tumor cells.[2−5] To enhance the EGFR inhibitory potency and stability of these compounds, we designed a novel strategy termed "cascade release" (CR) that seeks to mask the combi-molecule into a prodrug "programmed" to release the antitumor species by hydrolytic activation. Since these molecules, henceforth referred to as "cascade release molecules" (CRM), are also designed to retain significant EGFR affinity on their own, this principle leads to conditions under which three generations of inhibitors can be derived from the parent CRM.

To test the CRM principle, we initially designed structure **2** (Scheme 1) with the hope that it would not only retain affinity for EGFR but also hydrolyze to generate monoalkyltriazene **1** which, as previously demonstrated, heterolyzes into the corresponding 6-amino-4-anilinoquinazoline (an inhibitor of EGFR) + a methyldiazonium species.[2,3] Thus, CRM **2** was designed to be the prodrug of two known inhibitors of EGFR. Its synthesis proceeded according to Scheme 1. Structure **1**, obtained as described earlier,[6] was treated with acetyl chloride in methylene chloride at 0 °C to give **2** in an 18% yield. Studies of the hydrolysis of **2** under physiological conditions (i.e. incubation in serum containing media at 37 °C) demonstrated little degradation after 24 h. Further exposure for an additional 24 h led to minor degradation. Therefore, it was concluded that this molecule was not a suitable probe for the demonstration of the CRM principle.

Scheme 1



Scheme 2[a]



[a] (a) $NOBF_4$; (b) $MeNH_2/H_2CO/H^+$; (c) K_2CO_3; (d) $(CH_3CO)_2O$ in pyridine.

We subsequently designed structure **6** (Scheme 2), which would not only offer the opportunity to further mask the CRM but also to degrade according to known kinetics and mechanisms.[7,8] It has already been demonstrated that acetoxymethyltriazenes rapidly degrade to the corresponding hydroxymethyl triazene, which in turn is converted to a monoalkyltriazene of type **1**.[6] Further, heterolysis of triazene is known to generate an aromatic amine and an alkylating agent.[8] Thus, we surmised that these mechanisms of degradation might lend themselves to the demonstration of the CRM principle.

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Scheme 3

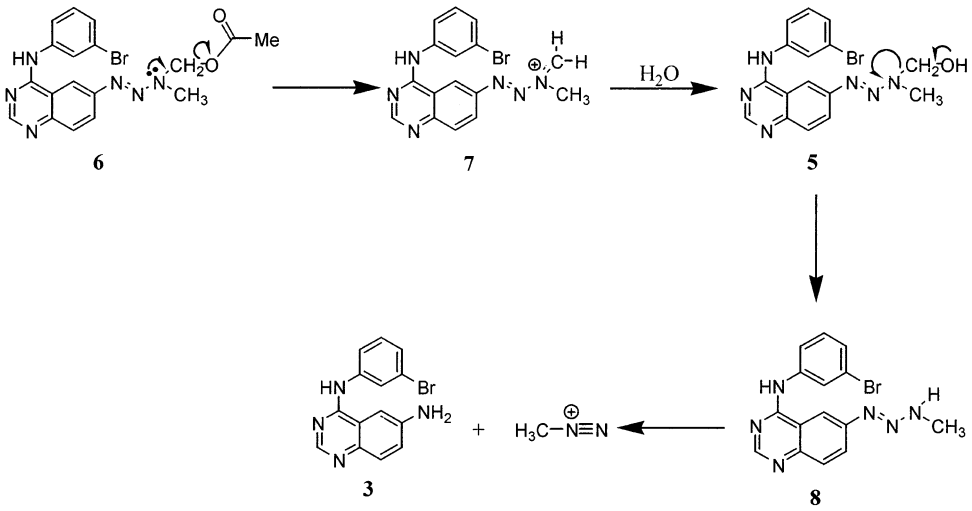


Table 1. EGFR TK Inhibition, Alkylating Activity, and Half-Lives of Compounds **1**−**3**, **5**, **6**, and **8**

			half-life ($t_{1/2}$),[c] min	
compds	inhibn of EGFR IC_{50}, μM[a]	alkylating activity (10^{-3} min^{-1})[b]	RPMI	PBS
1	0.2	9.1	35	69
2	0.578	1.5	>200	>200
3	0.04	0.05	N/A	N/A
5	0.11	6.6	40	70
6	0.13	5.8	42	84
8	0.07	8.9	38	68
TEM	N/A	11.3	N/A	N/A

[a] EGFR TK values are means of two experiments. Alkylating activity and half-lives values were obtained from single experiments. [b] Measured as a rate of alkylation of NBP at pH 7.5. [c] Measured by UV absorbance at 290 nm.

Results and Discussion

Chemistry. The synthesis of **6** proceeded according to Scheme 2. Briefly, compound **3** was treated in acetonitrile with $NOBF_4$ to generate intermediate **4**, which was treated in situ with a 10:1 mixture of formaldehyde/methylamine. Alkalinization with aqueous K_2CO_3 led to precipitation of compound **5**, in high yield. However, this compound coprecipitated with solid K_2CO_3, which was removed by resuspension in THF followed by collection and evaporation of the filtrate. Hydroxymethyltriazene **5** was obtained in a pure state (70% yield) without requirement for further purification. Treatment of **5** with acetic anhydride in pyridine gave **6** as a pure solid. We have already reported the synthesis of **8**.[9]

The stability of **6** and that of each of its putative metabolites were studied by allowing them to degrade in both phosphate-buffered saline (PBS) and serum-containing media using UV spectrophotometric analysis. Compound **6** was much less stable than **2**, with a half-life of 42 min in serum-containing media (Table 1). Interestingly, its putative metabolite **5** (Scheme 3) showed a half-life of 40 min, suggesting that conversion of **6** to **5** may be a rapid event. Indeed, it has already been reported that acetoxymethyltriazenes are rapidly converted to the corresponding hydroxymethyltriazene in PBS.[10−12]

Attempts to monitor the formation of **5** by HPLC failed due to its instability under the separation conditions. However, when **6** was allowed to degrade overnight under physiological conditions, we could successfully detect amine **3** (Schemes 2 and 3) as the sole product, suggesting that the putative intermediary metabolites may have been formed according to the mechanism outlined in Scheme 3. This mechanism has already been proposed for similar triazenes.[11,12] Addition of water to iminium ion **7** may generate the methylol **5**, which may self-deformylate to give the monoalkyltriazene **8**. To circumvent problems associated with on-column degradation of **6** and that of its metabolite **5**, we designed a fluorescence detection assay in order to monitor the formation of **3**, the ultimately generated stable metabolite. Absorption−emission spectra of the latter showed absorption at 290 nm and emission at 450 nm. Thus, we could unambiguously analyze the kinetics of formation of **3** by spectrofluorimetry. Interestingly, the rate of conversion of **5** to **3** was slower than that of **8** to **3**. These data support a stepwise degradation of the CRM in the sequence proposed in Scheme 3. More importantly, the symmetrically inversed relationship between the degradation of the diverse metabolites and the formation of **3** suggest a near stoichiometric conversion of **6** to **3** (Figure 1). A similar result was reported for triazenoquinazolines of types **1** and **8**.[3]

Biology. Our objective being to generate multiple inhibitors from a parent one, we tested the EGFR TK inhibitory activity of each of the putative metabolites alone in a short 8-min exposure ELISA. The assay was based on the inhibition of phosphorylation of poly(L-glutamic acid-L-tyrosine, 4:1) (PGT) by EGFR tyrosine kinase. Structure−activity relationships (SAR) in the quinazoline series have already demonstrated tolerance of bulky substituents at the 6- and 7-positions.[13−15] Indeed, the triazene appendage to the 6-position of this series did not significantly alter the EGFR binding affinities when compared with **3**. As an example, the IC_{50} values for **3** and **8** were in the same range [e.g., **8** (IC_{50} = 0.07 μM), **3** (IC_{50} = 0.04 μM)] (Table 1). More importantly, previous studies have demonstrated the strong dependence of the SAR of quinazolines on the electronic character of the substituents at the 6-position.[14,15] Electron-donating groups at the 6-position enhance activity, whereas electron-withdrawing ones are deleterious to affinity. In this study, it was found that acylation of **1** decreased the affinity of the resulting

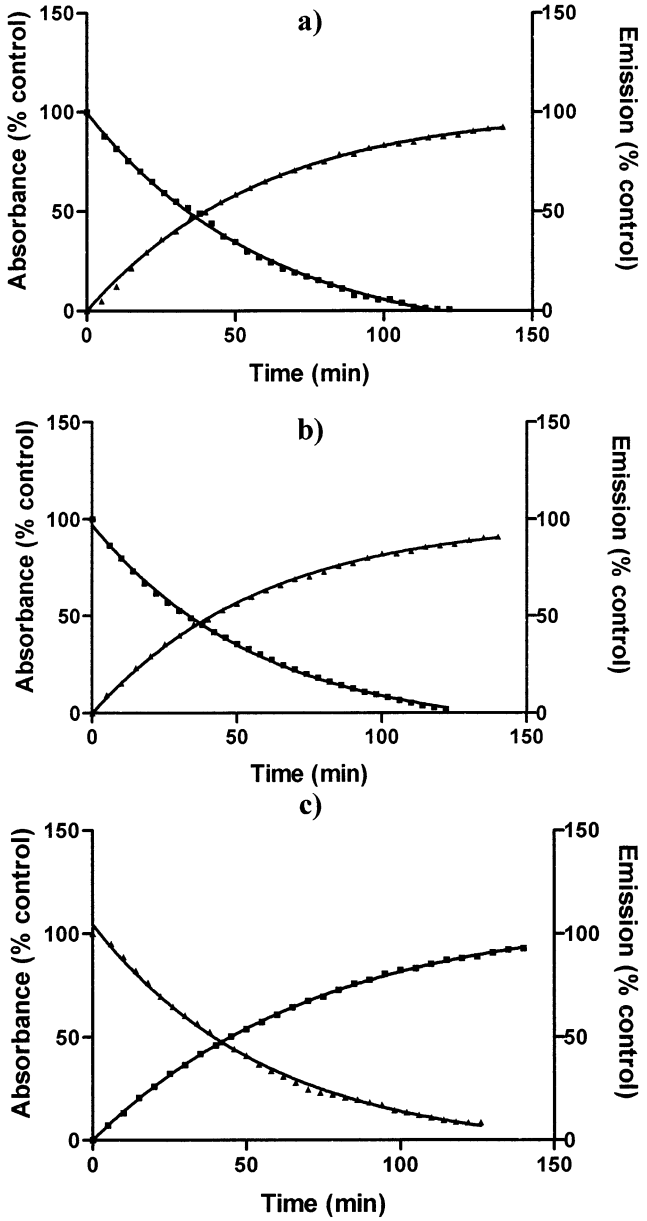


Figure 1. Formation (■) of **3** during the degradation (▲) of (a) **6**, (b) **5**, and (c) **8** in RPMI supplemented with FBS 10% at 37 °C. Degradation experiments were carried out by UV spectrophotometric analysis. The analyses were performed at 25 μM. Drugs were added to RPMI-1640 with 10% FBS (2 mL) and incubated at 37 °C.

compound **2** by 3-fold (Table 1). This may be due to the decrease in the electron-donating character of the triazene, which may deplete the partial charges on the nitrogens in the quinazoline ring. Indeed, when acylation was performed in a manner that did not affect the p-extended system of 1,2,3-triazene (as in **6**), there was little change in binding affinity when compared with the parental monomethyl triazene **8**. A significant decrease in the electron density at N1 was already demonstrated by ^{15}N spectroscopy when N3 in cyclic and acyclic triazenes are acylated.[16,17]

Previous studies demonstrated that serum stimulation of isogenic cells transfected with EGFR or the HER2 gene product represent good models for the determination of tumor selectivity of combi-molecules,[4] since they offer the advantage of growth-stimulating the two cell lines with a common growth factor. When this

Table 2. Inhibition of Serum-stimulated Growth in NIH3T3, NIH3T3/HER14, and NIH3T3/neu Cell Lines by Compounds **1**−**3**, **5**, **6**, **8**, **TEM**, **Ag1478** and Combination of **TEM** + **AG1478**

	IC_{50}, μM[a]		
compds	NIH3T3	NIH3T3/HER14	NIH3T3/neu
1	125 ± 1.7	28.9 ± 5.5	16.5 ± 2.3
2	200 ± 6.3	149 ± 14.2	60.6 ± 6.9
3	14.5 ± 1.9	10.4 ± 0.5	0.42 ± 0.03
5	12.8 ± 0.45	2.1 ± 0.3	0.09 ± 0.03
6	15.4 ± 0.55	3.4 ± 1.2	0.21 ± 0.05
8	15 0.5 ± 1.9	8.8 ± 0.6	0.09 ± 0.03
TEM	214.0 ± 11.1	207.2 ± 12.7	210.0 ± 16
AG1478	28.6 ± 3.6	9.8 ± 0.3	4.0 ± 0.2
TEM + AG1478	39.4 ± 5.4	6.2 ± 0.5	1.1 ± 0.06

[a] Values are means of IC_{50} values and SEMs from three experiments.

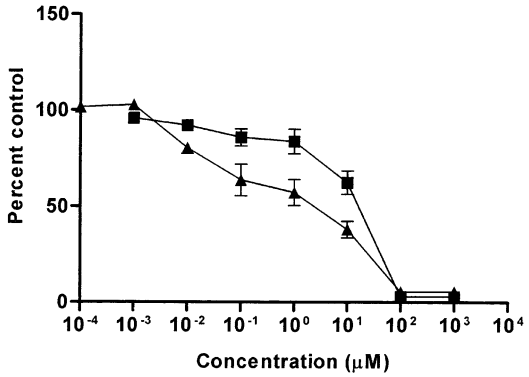
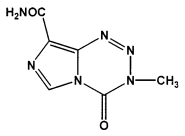


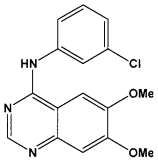
Figure 2. Effect of **6** on serum stimulated-proliferation in NIH3T3 (■) and NIH3T3/neu (▲) cells. Cells were starved for 24 h and exposed to each drug + serum for 72 h and growth inhibition was measured using SRB assay. Each point represents at least two independent experiments run in triplicate.

model was used, all compounds possessing EGFR TK inhibitory activities selectively blocked serum-stimulated growth of NIH 3T3 cells transfected with EGFR (NIH3T3/HER14) or HER2 (NIH3T3/neu), indicating translation of the EGFR TK inhibition into blockade of growth-factor-mediated proliferation (Table 2). A typical NIH3T3/NIH3T3neu differential growth response profile is shown in Figure 2. Moreover, it is noteworthy that the cascade release molecule **6** is significantly more potent than its metabolite **8** ($p < 0.001$) in the EGFR transfected cell line NIH3T3/Her14. However, the order of potency of these two molecules was inverted in the NIH3T3/neu cell line, indicating that perhaps **6** is less selective for HER2/neu than **8** and therefore may not serve as an efficiently targeted prodrug of **8** in these cells.

To further demonstrate the efficacy of the cascade combi-molecular approach, the effects of **6** were compared with those of classical combination of temozolomide (TEM) and AG1478, a known in vivo active EGFR TK inhibitor.[18] The combinations were performed at IC_{50}(TEM)/IC_{50}(AG1478) molar ratios. The results showed that **6** was 2.5- and 5-fold more potent than equieffective doses of TEM + AG1478 in NIH3T3/Her14 and NIH3T3/neu cells, respectively. Superior activity (e.g. 10-fold) when compared with the TEM + AG478 combinations was observed for **8** only in the NIH3T3/neu cells, which is in agreement with its strong activity in the latter cell line. These results support the significant antiprolif-



TEM



AG1478

erative potency of our combi-targeting and combi-molecular CRM approaches.

The hydrolysis of our CRM is expected to generate multiple inhibitors of EGFR and, at the final stage of degradation, the methyldiazonium species. Since the latter is known to be the cytotoxic DNA-alkylating metabolite of triazenes,[3−5,19] we studied the alkylating potency of the different agents using the 4-(*p*-nitrobenzyl)pyridine (NBP) test.[20] In this colorimetric assay, the alkylating species is trapped by NBP and a purple color is developed following addition of base. As a control, we compared the alkylating capacity of our compounds with that of a clinical DNA-methylating cyclic triazene, temozolomide (TEM). The NBP assay indicated that the parent compound **6** had a slower rate of alkylation when compared with the putative metabolite **5**, **8**, and TEM (Table 1). As expected, the naked inhibitor **3** demonstrated no alkylating activity, and the stable acylated compound **2** was found to have the slowest rate of alkylation in the entire triazene series.

The translation of the alkylating activity of **5** and **6** into DNA damage in human tumor cells was tested using the single cell microelectrophoresis (comet) assay in mouse fibroblast isogenic cell lines NIH 3T3 and NIH3T3/neu. In contrast to the naked inhibitor **3**, the acetoxymethyltriazene **6** was found to induce a dose-dependent increase in DNA strand breaks in both NIH 3T3 and its transfected counterpart, NIH 3T3/neu (Figure 3) confirming the binary targeting properties of the CRM.

This study conclusively demonstrates the feasibility of a molecule that may degrade in a stepwise mechanism into intermediary structures that when tested independently showed EGFR TK inhibitory activities. Although it was not possible to monitor the kinetics of formation of all the degradation products (e.g. the methylol intermediate **5** or the monoalkyltriazene **8**), their ultimate conversion to the stable metabolite **3** suggests that they may have been formed according to the mechanism outlined in Scheme 3. Further, in addition to the ability of the CRMs to release inhibitors of EGFR, their alkylating activity and their DNA-damaging potential suggest the formation of the putative methyldiazonium species. Thus, the combi-targeting and CRM principles mimic the effects of complex multidrug combinations involving modern inhibitors of EGFR TK and a classical DNA-damaging agent. More importantly, the results presented herein suggest that these effects may be selectively targeted to cells expressing the HER2 or EGFR oncogenes.

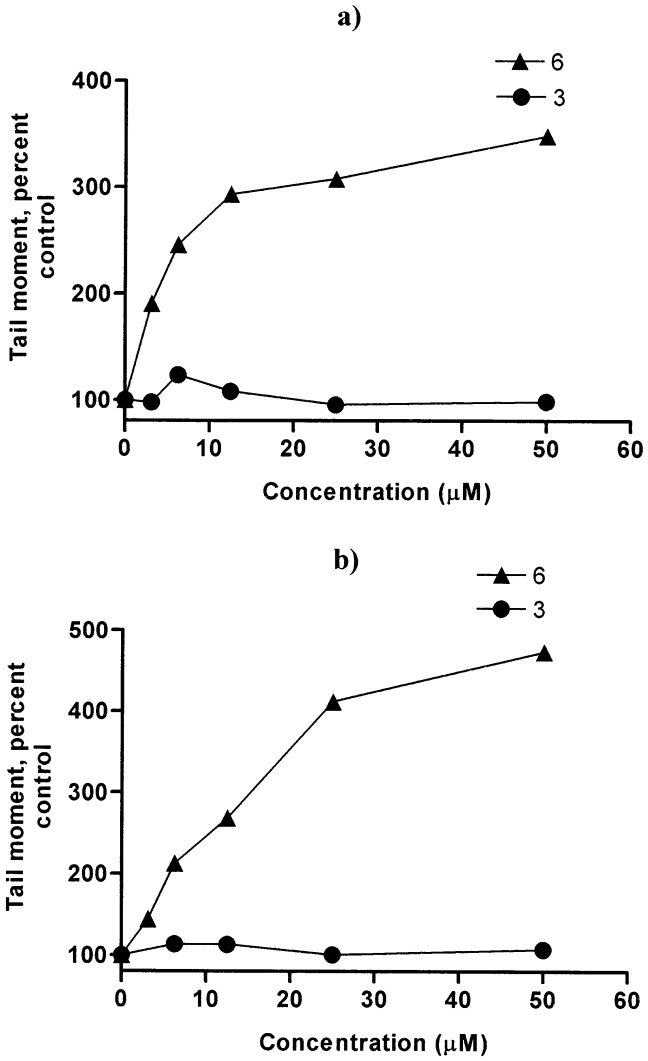


Figure 3. Quantitation of DNA damage using the alkaline comet assay. DNA damage induced by RB24 and RB10 in (a) NIH 3T3 and (b) NIH 3T3/neu cell lines. Tail moment was used as a parameter for the detection of DNA damage in NIH 3T3 and NIH 3T3/neu cells exposed to RB24 or RB10 for 30 min.

Experimental Section

Chemistry. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Varian 400 or 300 spectrometer. Mass spectroscopy was performed by the McGill University Mass Spectroscopy Center. ESI and APCI spectra were performed on a Finnigan LC QDUO spectrometer. Data are reported as *m*/*z* (intensity relative to base peak = 100). Melting points were determined in open capillary tubes on a Meltemp melting point apparatus and were uncorrected.

6-(3-Acetyl-3-methyltriazenyl)-4-(*m*-toluidyl)quinazoline (2). To a solution of 6-(3-methyltriazenyl)-4 anilinoquinazoline **1** (100 mg, 0.400 mmol) in methylene chloride (5 mL) was added triethylamine (0.1 mL). The mixture was stirred for 15 min at 0 °C and acetyl chloride (0.05 mL, 0.920 mmol) was added dropwise. Thereafter, the solution was evaporated under vacuum and the resulting brown residue purified on a silica gel column (40% ethyl acetate in hexane) to give **2** as a brown powder (18 mg, 16%): mp 188 °C; FABS *m*/*z* 335.2 (MH^+), 235 (M − 3-acetyl-3-methyltriazene) *m*/*z* 335.162 034 (MH^+), $C_{18}H_{18}N_6O_1$ requires 335.161 90; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.84 (s, 1H, NH), 8.58 (s, 1H), 8.14 (d, 1H, J = 2.1 Hz), 8.12 (d, 1H, J = 2.1 Hz), 7.83 (d, 1H, J = 8.7 Hz), 7.68 (s, 1H), 7.66 (s, 1H), 7.26 (t, 1H, J = 7.8 Hz), 6.95 (d, 1H, J = 8.1 Hz), 3.39 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 2.34 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.3, 158.6, 155.7,

150.7, 146.6, 139.5, 138.2, 130.0, 128.9, 125.3, 124.5, 123.6, 120.6, 120.4, 116.0, 28.4, 22.8, 22.0.

6-(3-Hydroxymethyl-3-methyltriazenyl)-4-(3′-bromophenylamino)quinazoline (5). To a solution of 4-(3′-bromophenylamino)-6-aminoquinazoline **3** (100 mg, 0.318 mmol) in acetonitrile (20 mL) kept at 0 °C on an ice bath was added nitrosonium tetrafluoroborate (74.3 mg, 0.637 mmol) dropwise. The solution was stirred for 20 min and 0.9 mL of a mixture of 40% methylamine (0.075 mL, 0.954 mmol), 37% formaldehyde (0.75 mL, 9.54 mmol), concentrated HCl (0.1 mL) was added all at once. The diazonium solution was subsequently alkalinized with potassium carbonate (400 mg, 2.86 mmol) and the precipitate was filtered to give a white solid contaminated with excess potassium carbonate. The precipitate was therefore resuspended in THF and the resulting solution filtered. The organic solvent was further evaporated to give **4** as a pure brown solid (90 mg, 73%): mp 150 °C; FABS *m/z* 387 (MH^+ with ^{79}Br), 389 (MH^+ with ^{81}Br), 359 ($M - N_2$) *m/z* 387.056 895 (MH^+), $C_{16}H_{15}N_6O_1Br$ requires 387.056 70; ^1H NMR (400 MHz, DMSO-d_6) δ 9.91 (s, 1H, NH), 8.57 (s, 1H), 8.50 (s, 1H), 8.25 (s, 1H), 7.99 (d, 1H, $J = 8.8$ Hz), 7.92 (d, 1H, $J = 7.6$ Hz), 7.76 (d, 1H, $J = 8.8$ Hz), 7.34−7.25 (m, 2H), 6.37 (s, 1H, OH), 5.17 (s, 2H, CH_2), 3.19 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.3, 158.0, 154.0, 148.9, 149.8, 130.9, 129.5, 126.3, 125.2, 124.7, 121.8, 121.2, 116.4, 116.1, 78.8, 34.0.

6-(3-Acetoxymethyl-3-methyltriazenyl)-4-(3′-bromophenylamino)quinazoline (6). 6-(3-Hydroxymethyl-3-methyltriazenyl)-4-(3′-bromophenylamino)quinazoline **5** (100 mg, 0.258 mmol) was dissolved in pyridine (2 mL), after which acetic anhydride (0.055 mL, 0.516 mmol) was slowly added. The solution was further kept on ice for 1.5 h and the pyridine evaporated as an azeotrope with toluene to give **6** as a pure brown residue (105 mg, 95%): mp 140 °C; FABS *m/z* 429 (MH^+ with ^{79}Br), 431 (MH^+ with ^{81}Br), 298 [M − (3-acetoxymethyl-3-methyltriazene)], 401 ($M - N_2$) *m/z* 429.067 460 (MH^+), $C_{18}H_{17}N_6O_2Br$ requires 429.067 28; ^1H NMR (400 MHz, DMSO-d_6) δ 10.02 (s, 1H, NH), 8.61 (s, 2H), 8.25 (s, 1H), 7.99 (d, 1H, $J = 8.4$ Hz), 7.93 (d, 1H, $J = 7.6$ Hz), 7.79 (d, 1H, $J = 9.2$ Hz), 7.35−7.26 (m, 2H), 5.86 (s, 2H, CH_2), 3.26 (s, 3H, CH_3), 2.07 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.8, 158.1, 154.4 149.5, 147.7, 141.8, 130.9, 129.7, 126.5, 125.1, 124.8, 121.8, 121.3, 117.3, 116.2, 79.4, 35.5, 21.6.

Drug Treatment. Compounds **1**, **2**, **3**, **5**, **6**, and **8** were synthesized in our laboratories according to known procedures. Temozolomide (TEM) was provided by Shering-Plough Inc. (Kenilworth, NJ). In all assays, drugs were dissolved in DMSO and subsequently diluted in RPMI-1640 containing 10% fetal bovine serum (FBS) (Wisent Inc., St-Bruno, Canada) or in DMEM containing 10% bovine calf serum (GIBCO BRL, Burlington, Canada) immediately before the treatment of cell cultures. In all assays, the concentration of DMSO never exceeded 0.2% (v/v).

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 100% acetonitrile with a 0.35 mL/min flow rate at 254 nm and the second system was 80% 2-propanol and 20% dioxane with a 0.5 mL/min flow rate at 254 nm.

Cell Culture. The cell lines used in this study, the mouse fibroblasts NIH3T3, NIH3T3/HER14 (NIH3T3 cells stably transfected with the EGFR gene), and NIH3T3/neu (NIH3T3 cells stably transfected with HER2) were generous gifts from Dr. Moulay Aloui-Jamali of the Montreal Jewish General Hospital. NIH3T3, NIH3T3/HER14, and NIH3T3/neu cells were maintained in DMEM supplemented with 10% FBS and antibiotics. All cells were maintained in an atmosphere of 5% CO_2.

Degradation. The half-lives of **6**, **5**, and **8** under physiological conditions were studied by a UV spectrophotometer. The compounds were dissolved in a minimum volume of DMSO and diluted with RPMI-1640 supplemented with 10% FBS, and absorbances were read at 240 nm in a UV cell maintained at 37 °C with a circulating water bath. The half-life was estimated by a one-phase exponential decay curve-fit method using the GraphPad software package (GraphPad software, Inc., San Diego, CA).

The study of the conversion of the compounds (**6**, **5**, and **8**) to their corresponding free inhibitor (**3**) was performed by spectrofluorometry, as the latter amine is fluorescent (absorption 290 nm, emission 450 nm). Briefly, 125 μM of the drug conjugates was added to RPMI-1640 with 10% FBS and incubated for 4 h at 37 °C in a microplate spectrofluorometer (SpectraMax Gemini flourescence reader, Molecular Device, CA). The data were acquired and analyzed by SoftMaxPro (Molecular Device, CA).

HPLC analysis was performed to study the degradation of compounds **2**, **5**, and **6**. The compounds were dissolved in a minimum volume of DMSO, diluted with RPMI-1640 supplemented with 10% FBS, using a Spectrasystem (Thermoquest) and a Waters C4 15-μm 300 × 3.9-mm column (reverse phase). The operating mode was isocratic using a 60% acetonitrile and 40% water solvent system with a 0.5 mL/min flow rate at 254 nm.

EGFR Kinase Assay. Nunc Maxisorp 96-well plates were incubated overnight at 37 °C with 100 μL/well of 25 ng/mL PGT in PBS. Excess PGT was removed and the plate was washed three times with wash buffer (Tween 20 (0.1%) in PBS). The kinase reaction was performed by using 4.5 ng/well EGFR affinity-purified from A431 cells. The compounds were added and phosphorylation was initiated by the addition of ATP (20 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, CA) diluted to 0.2 mg/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. To study the effect of our compounds on serum-stimulated proliferation, cells were grown to 70% of confluence in 96-well plates and washed twice with PBS, after which they were exposed to serum-free media for 24 h. Cells were exposed to each drug + serum for 72 h, and cell growth was measured using the sulforhodamine B (SRB) assay. Briefly, following drug treatment, cells were fixed using 50 μL of 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 of Tris base (10 mM), and the optical density was read for each well at 492 nm using a Bio-Rad microplate reader (model 2550). Each point represents the average of at least two independent experiments run in triplicate.

Alkylating Activity Assay. For each time point, 10 μL of compound 50 mM was added to 2% *p*-nitrobenzylpyridine (NBP)−ethylene glycol (100 μL) mixed with Tris pH 7.5 (27% ethanol) (70 μL) and incubated at 37 °C. The color was developed with a solution of acetone−triethylamine (50/50, v/v). Optical density was read for each well at 540 nm using a Bio-Rad microplate reader (model 2550).

Comet Assay for Quantitative Analysis of DNA Damage. A modified alkaline comet assay technique was used to quantitate DNA damage induced by **6** and **5** in NIH3T3 and NIH3T3/neu cells. The cells were exposed to drugs for 24 h and harvested with trypsin-EDTA. They were subsequently collected by centrifugation and resuspended in PBS. The resulting cell suspensions were diluted to 3×10^5 cell/ mL, and mixed with 0.75% agarose in PBS at 37 °C in a 1:9

dilution. The gels were cast on Gelband strips (Mandel Scientific, Guelph, Canada) using gel casting chambers, as previously described, and then immediately placed into a lysis buffer (2.5 M NaCl, 0.1 M tetrasodium EDTA, 10 mM 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 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, they were rinsed with distilled water, incubated in alkaline electrophoresis buffer for 30 min at 37 °C, and electrophoresed at 300 mA for 60 min. The gels were subsequently rinsed with distilled water and placed 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 (1:10000 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.

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