Novel Nitrogen Mustard-Armed Combi-Molecules for the Selective Targeting of Epidermal Growth Factor Receptor Overexperessing Solid Tumors: Discovery of an Unusual Structure−Activity Relationship

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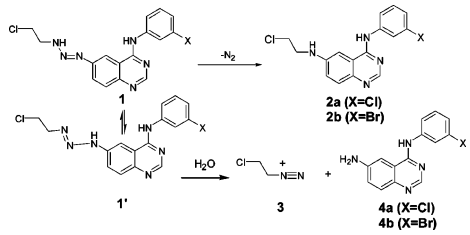
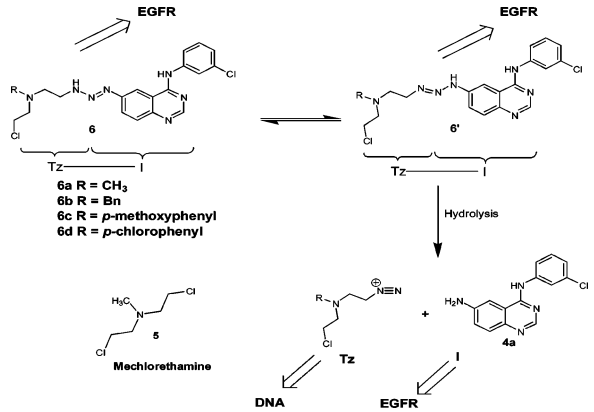
Abstract: To enhance the potency of "combi-molecules", we designed **6a**−**d** and **18** to release an inhibitor of EGFR TK and a bifunctional alkylator. The combi-molecules blocked EGFR TK with potency increasing with the basicity of the mustard moiety. They selectively killed cells transfected with EGFR and were potent against the DU145 prostate cancer cells. Combi-molecule **6a** blocked EGFR phosphorylation in an irreversible manner, induced DNA-cross-links, and arrested the cells in mid-S.

The combi-targeting concept postulates that a molecule termed "combi-molecule" designed to interact with the epidermal growth factor receptor (EGFR) on its own and allowed to further degrade into another more stable inhibitor of the latter receptor plus a DNA damaging species should be more potent than combination of individual molecules involving EGFR inhibition or DNA damage. Over the past 5 years, we demonstrated the feasibility of this principle with triazene carrying a methylating agent designed to alkylate guanine at the O-6 and N-7 positions.[1] Methylating combi-molecules showed significant EGFR−DNA binary targeting potency. However, their antiproliferative activities were shown to be mitigated by DNA repair enzymes.[4] The major advantage of these triazene-based molecules was their high affinity for the EGFR ATP binding site ($IC_{50} = 0.05-0.1\ \mu M$). Therefore, their structures remained a good platform for structural modification to enhance the potency of the combi-targeting strategy. Accordingly, we designed **1** (Scheme 1) to contain a chloroethyl group, which upon hydrolysis of the triazene chain would lead to the formation of the known cross-linking chlorethylating species **3**. It is well-known that this kind of compound can form DNA interstrand cross-links and show significantly greater cytotoxic activity than their methyltriazene counterparts.[2−4] Unfortunately, all attempts to synthesize such a molecule failed because of the instability of the designed structure **1**. It was rapidly converted to the corresponding chloroethylaminoquinazolines **2** through rapid loss of nitrogen at room temperature (Scheme 1). Therefore, it could never be isolated. Fortunately, **2a** and **2b** were found to be potent EGFR inhibitors.[5] Thus, the syntheses of combi-molecules of the triazene class capable of releasing chloroethyldiazo species remained an unresolved issue.

Scheme 1. Stability of Triazenes Designed To Generate DNA Cross-Linking Species



Scheme 2. Decomposition of **6** under Physiological Conditions



Recently, to circumvent problems associated with the direct attachment of the chloroethyl group to the N3 nitrogen of the triazene ring, we designed **6** (Scheme 2) to contain a chloroethylaminoethyl group that will confer a second alkylating function to the released alkyldiazonium species (see TZ, Scheme 2). Thus, as per the combi-targeting principles,[6] the combi-molecule (TZ-I) was designed to generate a nitrogen mustard analogue (TZ) capable of mimicking the alkylating properties of mechlorethamine (**5**) (Scheme 2) and **4a**, another inhibitor of EGFR (I). Here, we report on the first combi-molecules of the triazene class capable of releasing bifunctional alkylating species. This study led to the discovery of a novel structure−activity relationship based on the basicity of the central nitrogen of the mustard moiety, which was elucidated by molecular modeling. We also demonstrated the potent binary EGFR−DNA targeting properties of these novel mixed mustard−triazene combi-molecules.

The synthesis of **4a** proceeded as described by Roth et al.[7−10] and that of amines **11**, **13**, and **16** as in Scheme 3. Briefly, **7** obtained from the treatment of 2-bromoethylamine hydrobromide with benzyl chloroformate was mixed with 2-(methylamino)ethanol or *N*-benzylethanolamine in the presence of potassium iodide in DMF to provide **14.** The latter amino alcohol was chlorinated with $POCl_3$ to afford **15**. Hydrolysis of **15** in 6 N HCl gave the desired amines **16**. The synthesis of aniline derivatives proceeded as described in Scheme 3. Amines **8** were treated with ethylene oxide in the presence of zirconium tetrachloride to give amino alcohols **9**, which were chlorinated with $POCl_3$ to give **10**. The amino group of **10** was unveiled by hydrolysis in 6 N HCl to provide the desired amines **11**. Amino alcohol **12** was prepared in a similar fashion using propylene oxide instead of ethylene oxide to generate the amino

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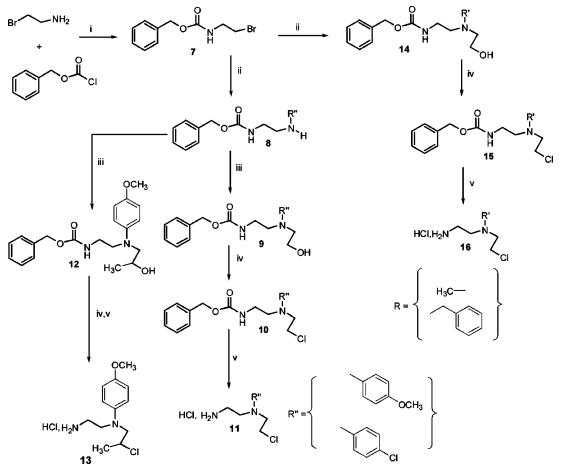
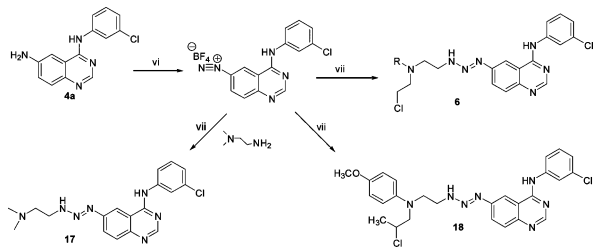
Scheme 3[a]



[a] Reagents and conditions: (i) DMF, Et_3N, 0 °C; (ii) KI, DMF, $RNHC_2H_5OH$ or $ArNH_2$, rt; (iii) ethylene oxide, $ZrCl_4/CH_2Cl_2$, rt; or propylene oxide, anhydrous EtOH; (iv) $POCl_3$ at 0 °C , then refluxing; (v) 6 N HCl, heat.

Scheme 4[a]



[a] Reagents and conditions: (vi) $NOBF_4$, 0 °C, CH_3CN; (vii) Et_3N, Et_2O, corresponding amine.

alcohol **12**, which was chlorinated and hydrolyzed to give the amine **13**. Following the synthesis of their corresponding amines, the bifunctional combi-molecules were synthesized as outlined in Scheme 4. Diazotization of the aminoquinazoline **4a** using $NOBF_4$ in dry acetonitrile followed by addition of the amine **11**, **13**, or **16** and neutralization with triethylamine gave **6a**−**d** (Scheme 2) and **17** and **18** (Scheme 4) as pure solids after purification by column chromatography on basic alumina. The structures of the combi-molecules were confirmed by ^1H and ^{13}C NMR and mass spectrometry, and their purity was confirmed by elemental analysis (see Supporting Information).

Having demonstrated the feasibility of "combi-triazenes" containing a pro-nitrogen mustard, we then determined whether this structural modification that introduced a more bulky side chain to the 6-position of the quinazoline ring negatively affected EGFR inhibitory activities. Interestingly, the IC_{50} for the different compounds varied between 0.0044 and 3.96 μM (Table 1).

The most potent agents were compounds **6a** and **6b** with the most basic monoalkyl side chain (IC_{50} = 0.0044−0.071 μM). When the nitrogen of the bifunctional moiety was directly attached to a phenyl ring, EGFR inhibitory activity was considerably depleted. Thus, to rationalize these results, we first correlated the IC_{50} values with the basicity of the corresponding amine side chains.

A linear correlation (R = 0.83) was obtained between the relative pK_a of the amines (determined as that of amines related to chloroethylamino moiety (see Supporting Information)) and log(IC_{50}) for inhibiting the EGFR TK activity by compounds **6a**−**d**, **17**, and **18** (Figure 1). This indicated that the central nitrogen of the bifunctional moiety might be involved in some

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

	IC_{50} (μM)			
compd	EGFR[a] enzyme assay	growth inhibition in DU145 cells[b]	inhibition of serum-stimulated growth in NIH3T3[b]	inhibition of serum-stimulated growth in NIH3T3 HER14[b]
4a	0.2	34.14	38.38	3.19
6a	0.0044	2.6	27.18	1.31
6b	0.071	10.55	15.19	2.93
6c	3.96	7.95	9.068	6.224
6d	2.2	4.7	27.56	11.95
17[13]	0.037	37.23	39.50	1.67
18	0.97	11.4	21.53	5.75
5		7.6		

[a] Polysubstrate (L-glutamic acid/L-tyrosine, 4:1) phosphorylation by EGFR was detected using an antiantiphosphotyrosine antibody. [b] Cell growth was measured using SRB assay.[14] Values are the average from at least two independent experiments. Variation was generally ±5%.

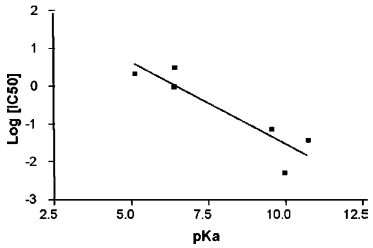


Figure 1. Linear correlation between log(IC_{50}) of **6a**−**d**, **17**, and **18** and the pK_a values.

form of interaction with their cognate ATP site. In an effort to elucidate this structure−activity relationship, we undertook a molecular modeling study whereby all the combi-molecules were modeled into the ATP site of EGFR using the MOI7[11] software package. The X-ray structure of a 4-anilinoquinazoline inhibitor bound to EGFR (PDB[a] code 1m17)[12] was used as a template to manually construct starting structures for **6a**−**d**, **17**, and **18** in the EGFR pocket. The 4-anilinoquinazoline moiety of **6a**−**d**, **17**, and **18** was assumed to bind in the same mode as the 4-anilinoquinazoline moiety in the PDB structure. The water molecule bridging N1 to the receptor was retained during the modeling process, as it was crucial for obtaining more precise refined geometries. Each starting structure was minimized to a gradient of 0.001 kcal/A in the presence of the fixed protein using the MMFF94x force field as implemented in MOE. A systematic conformational search was performed on each ligand, and the resulting conformations were reminimized in the EGFR pocket with the MMFF94x force field to a gradient of 0.001 kcal/Å. The conformation with the lowest combined strain and receptor interaction energy was chosen as the docked pose. It was found that 6-substituted structures possessed the appropriate length to permit the orientation of the central nitrogen of the bifunctional moiety toward Asp776, suggesting that, as depicted in Figure 2, upon protonation it may exert an electrostatic interaction with the carboxylate of Asp776 (Figure 3).

Thus, increased basicity of the nitrogen as in **6a** and **6b** may account for the superior potency of the latter compounds when compared with the combi-molecules carrying aniline mustards (**6c**−**d**, **18**). It is known that the basicity of nitrogen mustards correlate with their reactivity. Thus, we investigated whether the most basic **6a** could react with the receptor, thereby inducing

[a] Abbreviations: PDB, protein data bank; MOE, molecular operating environment; MMFF94X, molecular mechanics force field 94X; ERCC1, excision repair cross-complementing, group 1; rt, room temperature.

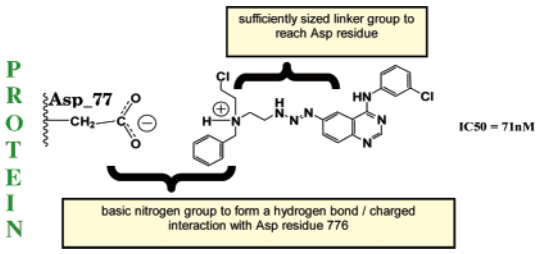


Figure 2. Requirements for a good binder.

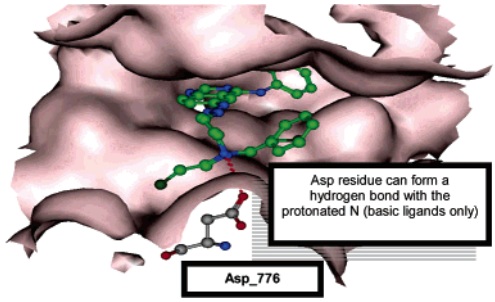


Figure 3. Molecular modeling in the EGFR pocket of the basic ligands **6a** and **6b**.

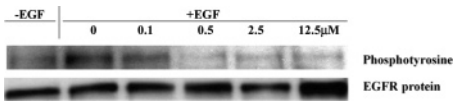


Figure 4. Inhibition of EGFR phosphorylation by **6a** in DU145 cell line. Serum-starved cells were preincubated for 2 h with the indicated concentrations of **6a** prior to stimulation with EGF for 10 min. Equal amounts of cell lysates were analyzed by Western blot using an antiphosphotyrosine antibody.

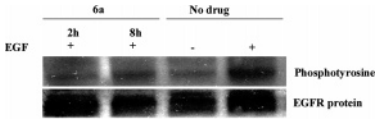


Figure 5. Reverse EGFR phosphorylation in the presence of **6a** in DU145. Duplicate sets of cells were treated with the drug (2 μM) for 2 h. One set of cells was then stimulated with EGF. The other set of cells was stimulated with EGF after two washouts at 2 h intervals and an additional 4 h recovery. Cell lysates were analyzed by Western blot using antiphosphotyrosine or anti-EGFR antibodies.

irreversible inhibition of the latter. Using the human prostate cancer cell line DU145, we showed that **6a** could block EGFR phosphorylation in a dose-dependent manner (Figure 4).

When the cells were exposed to **6a** for 2 h followed by multiple washouts, inhibition of phosphorylation was still maintained 8 h post-treatment. In contrast, in nondrug treated cells, EGFR phosphorylation was significantly recovered. Thus, **6a** blocked EGFR phosphorylation with an at least partially irreversible mechanism (Figure 5). This is in agreement with previous study that showed a partially irreversible inhibition for **17** and similar combitriazenes.[13]

To determine whether the potency of the combi-molecules translates into selective targeting of cells expressing EGFR, we examined their antiproliferative activity in an isogenic pair of NIH3T3 cells, one of which is transfected with the EGFR gene and the established human prostate cancer cell line DU145. The results showed that selective targeting of the combi-molecules was directly proportional to the strength of the EGFR inhibitory potency of the combi-molecules, with the most selective agent being **6a** (20-fold selectivity for the EGFR transfectant) (Table 1).

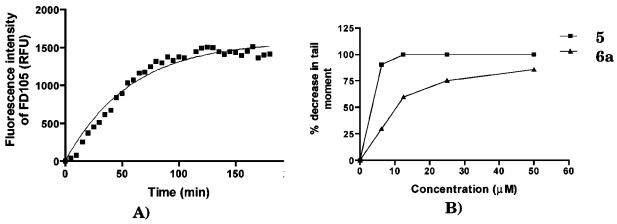


Figure 6. (A) Formation of fluorescent aminoquinazoline **4a** from **6a**. A microplate spectrofluorometer was used to detect the released **4a** from **6a** added to media with 10% of FBS and incubated for 4 h at 37 °C (excitation 270 nm, emission 451 nm). (B) DNA interstrand cross-linking expressed as % decrease in comet tail moment following irradiation of DU145 cells treated for 2 h with **6a** or **5**.

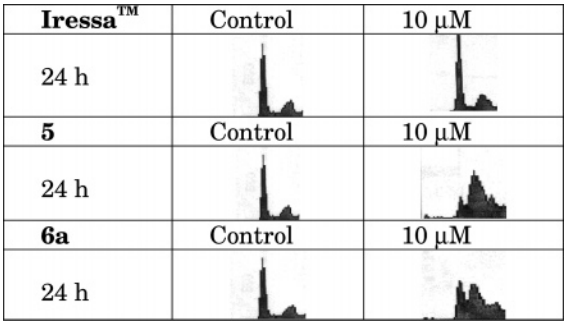


Figure 7. Cell cycle effect of **6a**, **5**, and Iressa on DU145. Cells were treated with the indicated concentrations of each drug for 24 h. Cells were stained with PI and analyzed by flow cytometry.

It is known that the basicity of nitrogen mustards correlate with their cytotoxic activity. This effect was not observed here because the control drug **5** that has similar basicity when compared with **6a** was approximately 3-fold less potent than the latter against the DU145 cells. Likewise, its potency was not markedly superior to that of the less basic aniline mustards **6c**,**d.** Interestingly, **17**, whose structure is similar to that of **6a** but deprived of the chloroethylamino moiety, was more than 10-fold less potent than the latter, indicating that the chloroethyl function confers significant antiproliferative advantage.

Having shown that the novel combi-molecules could achieve selective killing of solid tumor cells, it was critical to determine its mode action. UV fluorescence spectroscopy and HPLC analysis showed that the most potent compound **6a** decomposes with a $t_{1/2}$ of 7 min to generate **4a** (Figure 6A).

Because of their short half-lives, we were not able to detect the released nitrogen mustards. However, the single cell gel electrophoresis (comet) assay[15] showed that **6a** was capable of inducing marked dose-dependent levels of DNA cross-links, with potency almost in the same range as that of **5**, indicating that the DNA cross-linking species may have been generated by these molecules (Figure 6B).

More importantly, at 10 μM, combi-molecule **6a** induced significant cell cycle arrest in mid-S, with cell cycle phase distribution profile similar to that of **5** (IC_{50} = 7.6 μM). This indicates that in addition to its EGFR inhibitory function, its DNA damaging properties play an antiproliferative role and it also induces sufficient levels of DNA damage to block cell cycle progression. It is also noted that the EGFR inhibitor Iressa used as a control in this study only induced cell cycle arrest in G1. This suggests that the cell cycle distribution profile of **6a** may be a combination of cells arrested in G1 and in mid-S. Indeed, the proportion of cells arrested in G1 by **6a** was superior to that observed for **5** (Figure 7).

In summary, this work has allowed us to circumvent the problem associated with the instability of chloroethyltriazene

moiety when linked to the quinazoline ring. Using the 1,2,3-triazene as a carrier of the bulky mustard has allowed us to overcome steric hindrance of the chloroethyaminoethyl group. However, the most critical elements for the enhanced potency of the combi-molecules remain the mustard nitrogen that, as suggested by molecular modeling, may interact with the ATP site through a protonated nitrogen and its chloroethyl function that confers the ability to induce DNA cross-links, irreversible EGFR inhibition, and cell cycle arrest in mid-S. Thus, this study has permitted the development of a unique molecule with multiple antiproliferative mechanisms. A study describing its synergistic mechanism of action (i.e., down-regulation of the DNA repair protein ERCC1 through EGFR inhibitory function and delayed DNA repair) will be reported elsewhere.

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Supporting Information Available: Experimental section, NMR, mass, and elemental analysis data, and details of the modeling programs. This material is available free of charge via the Internet at http://pubs.acs.org.

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