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Design and synthesis of new stabilized combi-triazenes for targeting solid tumors expressing the epidermal growth factor receptor (EGFR) or its closest homologue HER2

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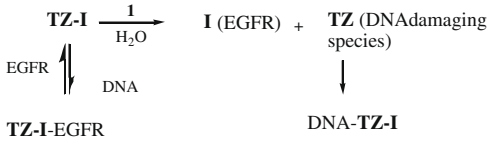
ABSTRACT

The monoalkyltriazene moiety lends itself well to the design of combi-molecules. However, due to its instability under physiological conditions, efforts were directed towards stabilizing it by grafting a hydrolysable carbamate onto the 3-position. The synthesis and biological activities of these novel *N*-carbamyl triazenes are described.

Combi-molecules are agents designed to block multiple targets in cancer cells. One such target the epidermal growth factor receptor (EGFR) also known as erbB1 or HER1, is overexpressed in many solid tumors and this is associated with aggressive tumor progression and reduced sensitivity to antitumor agents. Blockade of the tyrosine kinase (TK) activity of EGFR and the erbB2 or HER2 gene product p185neu induces significant antitumor activity in vivo and Iressa™, an aminoquinazoline that binds to the ATP site of EGFR has proven an extremely potent agent against many human tumors in nude mice. This drug is now approved for the treatment of lung cancer. A specific mutation in the EGFR site (deletion in exon 19)[1] is associated with the sensitivity of lung tumors to Iressa™.[2,3] However these tumors that initially responded to Iressa develop resistance to the drug and this is believed to be associated with the occurrence of an additional EGFR mutation in exon 20.[4] In many cases, inhibition of EGFR does not suffice to eradicate the tumor. Thus, we recently developed a novel class of molecules termed 'combi-molecules' or TZ-I (Scheme 1) capable of not only blocking EGFR-mediated signaling by binding to its ATP site (see TZ-I-EGFR), but also damaging DNA (DNA-TZ-I). The combi-molecules (TZ-I) require hydrolysis to generate the DNA damaging species TZ and an additional EGFR inhibitor (see I, Scheme 1). Thus, the degradation of this class of compounds is critical for their antitumor activity. Importantly, we have also shown that these combi-molecules induced sustained inhibition of EGFR which we believe is due to their ability to alkylate the receptor, thereby inducing a partially irreversible activity.[5,6] It has now been shown that irreversible inhibitors such as CI1033 induce sustained inhibition of the erb family and some of them are shown to be pan-erbB inhibitors.[7] Recently, in order to delay the degradation of the comi-molecules, we designed a novel type of structures termed 'cascade release' combi-molecules. Two such molecules RB24[8] and RB107[9] (Scheme 2) are designed to loose their acetoxy group upon hydrolysis to give a methylol derivative that rapidly degrades to the corresponding monomethyltriazene TZ-I″ (Scheme 2).



Scheme 1.

The latter species is subsequently hydrolyzed to an alkyl diazonium species and a free EGFR inhibitor I. When compared with the rate of degradation of monoalkyltriazenes [e.g., BJ2000[10] (Scheme 2) or ZRBA1[11] (Scheme 4)], it was found that the addition of the

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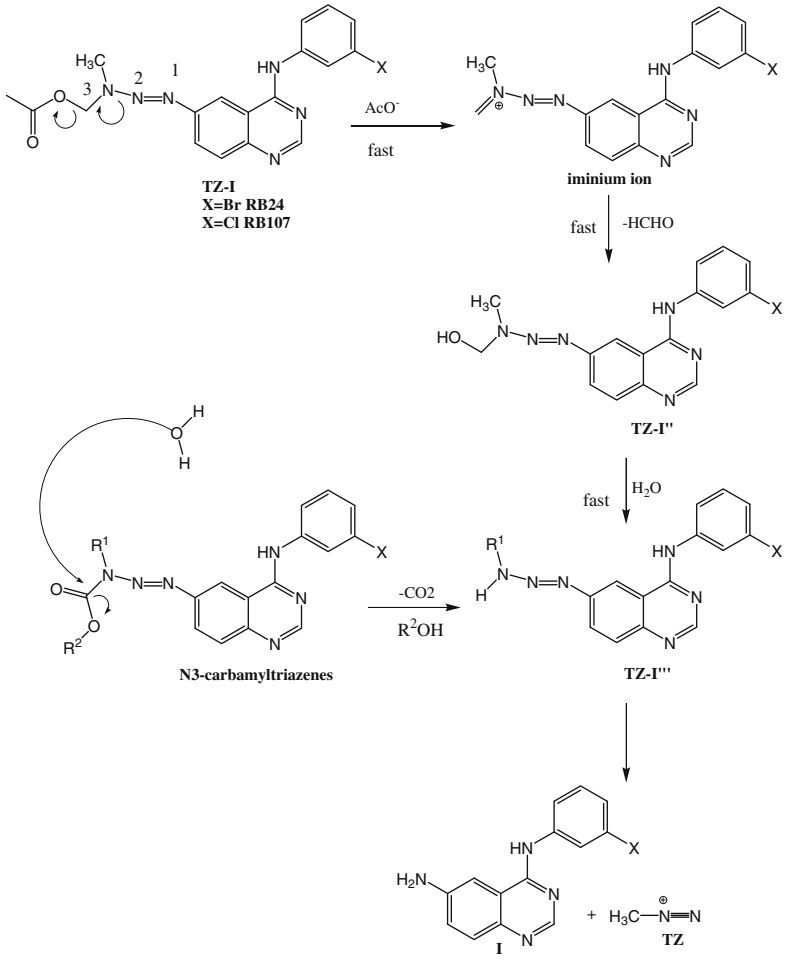


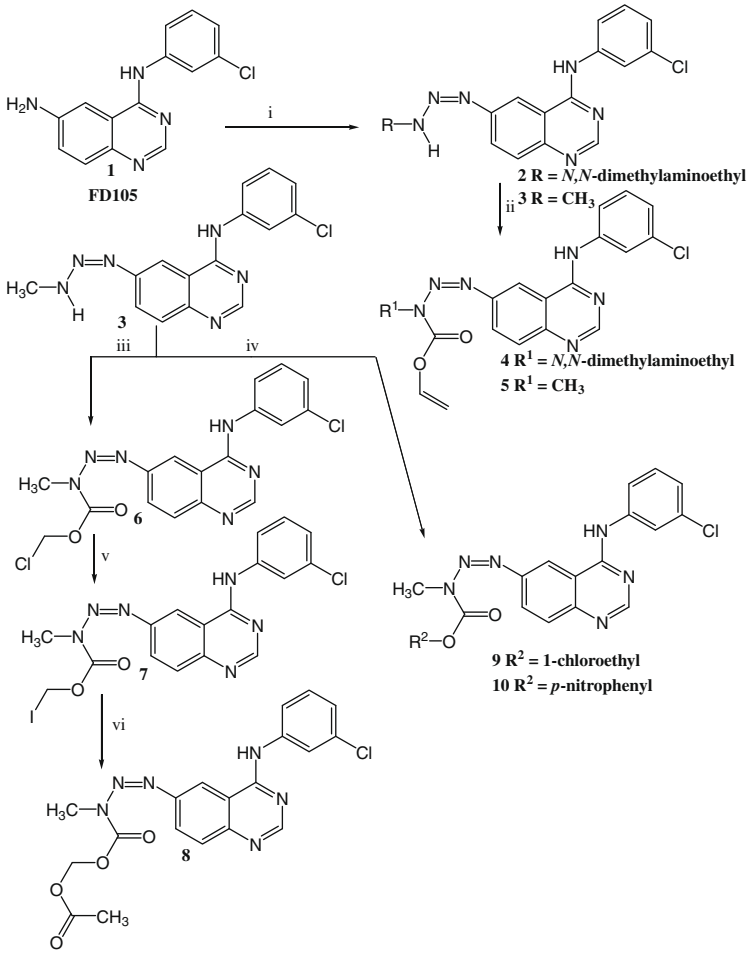
Scheme 2.

acetoxymethyl group to N3 only delayed the degradation of the monomethyltriazene by approximately 5 min. We further attempted to increase the bioavailability of combi-molecules by designing stable 3,3-dimethyltriazene prodrugs to be metabolically activated. These types of molecules failed to generate adequate levels of monoalkyltriazenes in vivo.[12]

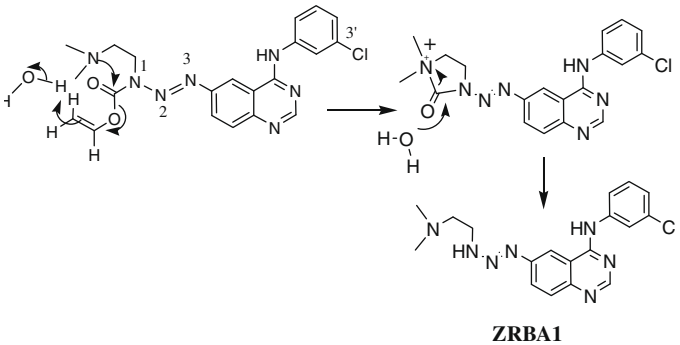
Here, we report on a novel approach that seeks to mask the monoalkyltriazene with carbamates whose hydrolysis can be rate limiting. The stability of the various carbamates (vinyl, chloroalkyl and acetoxymethyl, *p*-nitrophenyl carbamates) was modulated by altering the leaving group designed to depart following water addition on the carbonyl moiety (see N3-carbamyltriazene, Scheme 2). The synthesis of the 3-carbamyl triazenes (see general structure TZ-I′, Scheme 2) proceeded as described in Scheme 3. With the purpose of identifying a leaving group that would confer optimal stability to the combi-molecule, we designed three types of carbamates: (a) three with an electron-deficient leaving group [e.g., vinyl (**4** and **5**) or *p*-nitrophenyl (**10**), (b) two with a degradable chloroalkyl function (**6** and **9**) and (c) one with an acetoxymethyl moiety (**8**).

Compound **1** was obtained as described by Tsou et al.[13–17] Diazotization of aminoquinazolines **1** using NOBF4 in acetonitrile, followed by addition of *N,N*-dimethyl ethylenediamine or methylamine gave **2** and **3**[11,18] as pure solids after neutralization with triethylamine and purification by column chromatography on basic alumina. Compounds **4** and **5** were synthesized by treatment of **2** and **3** with the commercially available vinyl chloroformate. By the same procedure, we obtained **9** and **10** using *p*-nitrophenyl chloroformate and 1-chloroethyl chloroformate. The combi-molecule **8** was obtained by treatment of the methyltriazene **3** with chloromethyl chloroformate and pyridine in acetonitrile at cold temperature to give **6**. Exchange of the Cl atom using potassium iodide in dry acetone, gave the corresponding iodomethyloxycarbonyl-methyltriazene **7**.[19] The latter intermediate was treated with silver acetate in situ to provide **8** as a pure solid after purification by preparative TLC alumina plates. The structures of all the combi-molecules were confirmed by ^{1}H, ^{13}C NMR and mass spectrometry (ESI). The stability of the resulting structures was studied by measuring the rate of evolvement of blue fluorescence (Exct 340, Emission 451 nm) from drugs dissolved and kept in serum-containing media at 37 °C. As outlined in Scheme 2 and previously demonstrated,[20,21] the ultimate degradation product of each of the synthesized drug is an aminoquinazoline FD105 (**1**) that fluoresces in the blue. The chloromethylcarbamate ZRL1 decomposed at the same rate as RB107 and was the least stable of the series. The most stable compound was ZRL2 (**5**) containing a vinyl carbamate. ZRS1 (**8**) and ZRL5 (**10**) had approximately the same level of stability. ZRL3 was less stable than its 1-chloroethyl counterpart.

These results in toto can be rationalized in light of the chemistry of the carbamates. ZRL3 (**9**) being a 1-chloroethyl-1-substituted carbamate may perhaps form a more stable oxonium ion intermediate than its ZRL4 (**6**) counterpart, thereby leading to a faster rate of hydrolysis. However, the case of ZRL1 (**4**), a vinyl carbamate which is twofold less stable than its counterpart ZRL2 (**5**) (the most stable of the series) deserves special attention. Although being



Scheme 3. Reagents and conditions: (i) $CH_3CN/NOBF_4/-5$ °C/ether then addition of correspondent amine, 0 °C; (ii) vinyl chloroformate, ACN/Pyr/0 °C; (iv) *p*-nitrophenyl chloroformate or 1-chloroethylcholroformate, ACN/Pyr/0 °C; (iii) chloromethyl chloroformate, ACN/Pyr/0 °C; (v) acetone anhydrous/KI (1.1 equiv) at room temperature; (vi) silver acetate.



Scheme 4.

electron deficient, the vinyl group does not present the extension of conjugation offered by the *p*-nitrophenyl of **10** nor the ability to lose a chloro through formation of aldehyde as in **6** and **9**. However, as depicted in Scheme 4, in ZRL1, the hydrolysis of the carbamate may be assisted by the neighboring dimethylamino group that catalyzes the hydrolysis of the carbamate group, thereby accelerating its rate of decomposition into ZRBA1.[3]

The EGFR tyrosine kinase (TK) inhibitory potency of the series did not correlate with stability. Because of their bulkiness, none of the structures were expected to show greater EGFR TK inhibitory potency than RB107 or Iressa™. However, surprisingly, ZRL1 showed a level of potency similar to that of RB107 and was the most potent of the series. To rationalize these results, molecular modeling was used to compare ZRL1 with its closest analogue ZRL2, a vinyl carbamate containing a methyl group in lieu of the dimethylethylamino group and that is more than three times less potent than ZRL1 (Fig. 1). The experimental structure of the tyrosine-kinase domain of the EGFR complexed with a 4-anilinoquinazoline inhibitor was downloaded from the PDB (PDB code 1M17)[22] and used as the target structure in this study. The 1M17 PDB file was loaded into the MOE software package version 2008.10.[23] All crystallographic waters were deleted except for one water molecule near the N3 nitrogen in the quinazoline ring. This water is conserved in both the complex (1M17) and the apo (PDB code 1M14) PDB structures. It forms a hydrogen bond with the N3 quinazoline nitrogen in the bound complex. The protonation state of the system was set using the Protonate3D function in MOE, which resulted in the expected deprotonation of the solvent-exposed ASP 776 residue near the opening of the 1M17 binding pocket. ZRL1 was constructed in the binding pocket using the X-ray ligand as a template, followed by minimization of the new ligand in the field of the fixed protein atoms. The MMFF94s forcefield was used with the Born salvation model.

The results showed that both compounds retain the essential quinazoline interactions with the receptor—namely the H-bond between N1 and M_769 and the water-mediated interaction between N3 and T_766—but only ZRL1 can form an interaction with ASP_776. This additional interaction significantly increases its

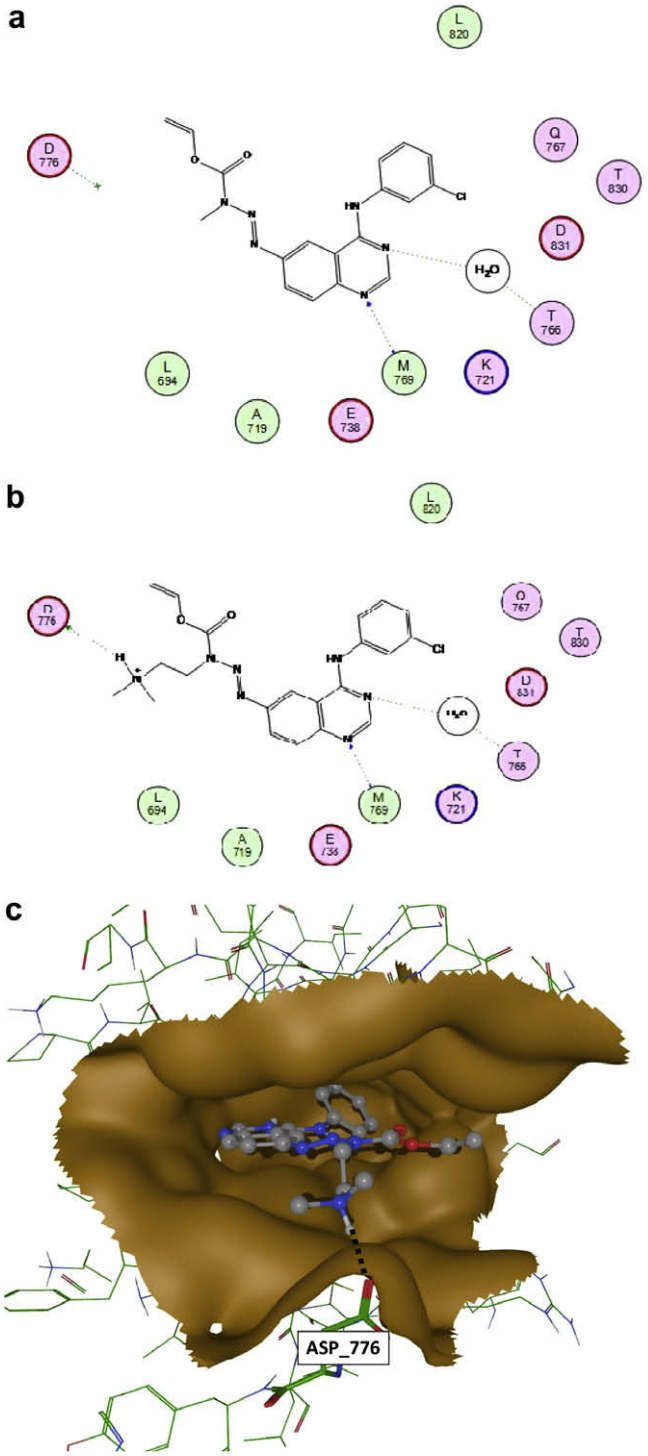


Figure 1. The 2D protein–ligand interaction diagram of ZRL2 (a) compared with ZRL1 (b); (c) A Gaussian-Connolly surface is drawn around the binding pocket, and the residue ASP_776 is labeled. The position of the 4-anilinoquinazoline core is essentially unchanged from the X-ray structure, while the *N*,*N*-dimethylaminoethyl is of sufficient length to interact with ASP_776 (indicated by the dotted line).

EGFR TK inhibitory potency when compared with ZRL2 and the other analogues (see Table 1).

A complete analysis of EGFR-DNA targeting properties of the molecules in a human cell line is reported elsewhere.[24] Nevertheless it is worth mentioning that all compounds were capable of damaging DNA, except the most stable drug ZRL2, which did not induce a dose-dependent increase in DNA damage. A comparison

Table 1
Rate of formation of FD105 from and biological properties of combi-triazenes

Compounds	EGFR TK Inhibition[a] IC_{50}, μM ± SEM	Rate of formation of FD105 $\times 10^{-3}$	Growth inhibition IC_{50}, μM[b] NIH3T3neu
Iressa	0.033		0.54 ± 0.019
RB107	0.24[b]	7.2	ND
4, ZRL1	0.18 ± 0.08	7.0	2.28 ± 0.17
5, ZRL2	0.72 ± 0.21	2.9	2.37 ± 0.18
9, ZRL3	0.50 ± 0.11	5.6	2.07 ± 026.
8, ZRS1	0.63 ± 0.23	5.1	1.50 ± 0.16
6, ZRL4	0.39 ± 0.22	4.7	4.94 ± 0.82
10, ZRL5	0.77 ± 0.23	4.6	3.79 ± 0.18

[a] IC_{50} for inhibition of the phosphorylation of poly(L-glutamic acid-L-tyrosine) and data are averages of two independent experiments run in triplicates.
[b] IC_{50} for growth inhibition of NIH3T3 cells transfected with the erbB2 gene.

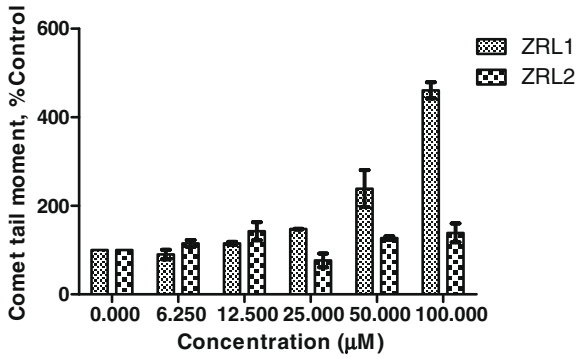


Figure 2. Comparison between tail moments induced by ZRL1 and ZRL2 in human MDA-MB468 breast cancer cells.

between the DNA damaging potency of ZRL2 and ZRL1 is showed in Figure 2.

To compare the effect of the different agents on cell growth, we chose the NIH3T3neu cell line, a HER2 transfectant that has already been shown to be extremely sensitive to EGFR inhibitors and a direct correlation was established between EGFR TK IC_{50} values for EGFR inhibitors and growth inhibitory potency against the latter cell line.[25] Our positive control Iressa being a strong inhibitor of EGFR, showed the greatest potency of all combi-triazenes tested. The drug with the closest potency level when compared with Iressa™ was ZRS1 that despite being an almost 20-fold weaker EGFR inhibitor showed an IC_{50} value only threefold lower than that of Iressa™ against NIH3T3neu. The purpose of this work being to identify a stabilizing strategy for combi-triazenes, the results suggest that based on its stability, its EGFR inhibitory property and its strongest growth inhibitory effect in the series, ZRS1 can be considered the optimized structure.

This study conclusively demonstrated that combi-molecules stabilized by grafting a carbamate to the N3 of the triazene chain can achieve greater stability when compared with our previously reported carboxymethyl-N3 strategy[8,9] exemplified by **5**. More importantly, we showed that stability could be achieved without any significant loss in the binary EGFR-DNA targeting property of the identified lead molecule ZRS1 (**8**). Further studies are ongoing to demonstrate their and potency in vivo.

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