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Synthesis of Pyrimidinopyridine–Triazene Conjugates Targeted to abl Tyrosine Kinase

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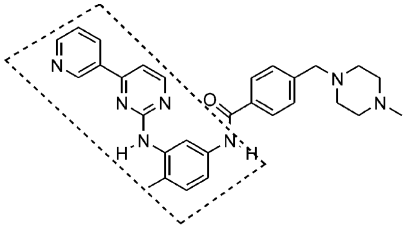
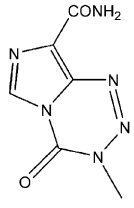
Abstract—The synthesis and abl tyrosine kinase inhibitory activities of alkyltriazenes conjugated to phenylaminopyrimidines are described. Significant abl inhibitory activities were observed only when a benzamido spacer was inserted between the 1,2,3-triazene chain and the 2-phenyaminopyridopyrimidine moiety.

The alkyltriazenes represent one of the most potent classes of cytotoxic drugs used in the clinic in the chemotherapy of many cancers including melanoma, gliomas and leukemias.[1,2] Dimethyltriazenes have superior stability when compared with monoalkyltriazenes.[3] They require metabolic activation to generate the cytotoxic monoalkyltriazene.[4,5] As outlined in Scheme 1, the clinical drug dacarbazine has been shown to be oxidized in vivo to its corresponding hydroxymethyl metabolite which following loss of formaldehyde gives the potent methyltriazenylimidazole-4-carboxamide (MTIC), a monoalkyltriazene that is further hydrolyzed to a methyldiazonium species and 5-aminoimidazole-4-carboxamide.[6,7] Recently, the cyclic triazene temozolomide (TEM), a stable prodrug of MTIC was developed that does not require metabolic oxidation to generate the cytotoxic species.[8] TEM is now used in the clinic in the therapy of many malignancies including melanoma and brain tumours.[2,9] However, the toxicity of these compounds which is believed to be associated with their lack of tumor selectivity, is a major deterrent for their use in cancer therapy. Despite the significant potency of triazenes in the clinic, little effort is directed at ameliorating their tumor selectivity.



Gleevec™



TEM

Recently, we developed a novel strategy termed 'Combi-Targeting' that seeks to synthesize chimeric molecules designed to simultaneously block tyrosine kinase-mediated growth signaling while inducing cytotoxic DNA damage.[10–13] More importantly, the resulting molecules also termed 'combi-molecules' were designed to degrade to another inhibitor of the same tyrosine kinase under physiological conditions. This was expected to culminate into selective and sustained antiproliferative activity in cells whose growth depend on the targeted tyrosine kinase. Here, we report the first attempt to apply these principles to the targeting of bcr-abl, a tyrosine kinase proven to be the cause of chronic myelogenous leukemia (CML).[14,15]

The bcr-abl oncoprotein is a 210 KDa fusion protein that is believed to play an anti-apoptotic role and blockade of its abl kinase activity has now been shown to translate into significant antitumor activity in both mouse models and in the clinic.[14–16] STI571 or Gleevec™,[16] one of the most potent in vitro and in vivo inhibitors of abl

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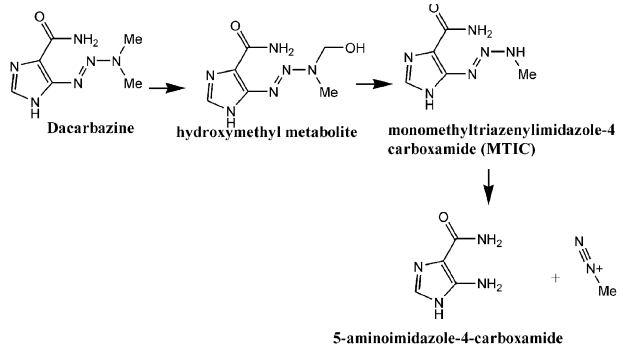
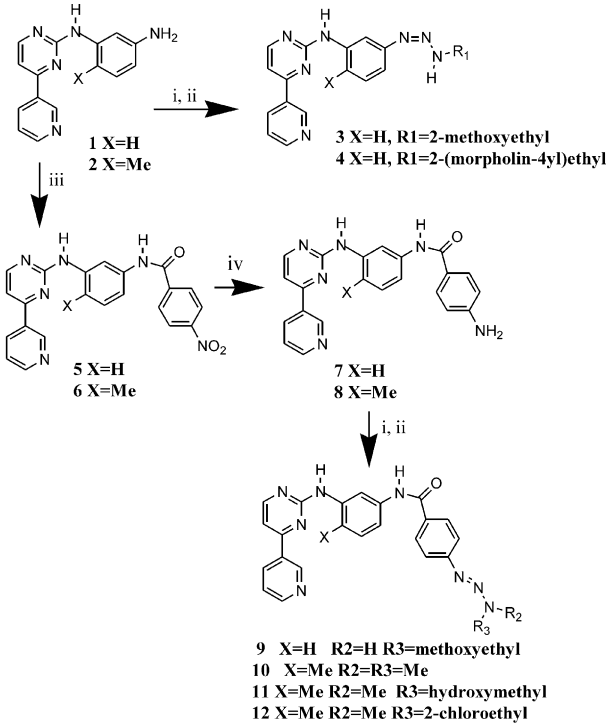


Scheme 1. Metabolic activation of dacarbazine.

kinase has now been approved by the Food and Drug Administration for the therapy of CML. Unfortunately, clinical studies with Gleevec™ showed rapid development of resistance in CML patients.[16] To circumvent this problem, the combination of this potent c-abl inhibitor with cytotoxic agents has become an actively explored strategy.[17,18] Here we report the first attempt to combine these two major mechanisms of antitumour actions into single molecules termed: 'combi-molecules'.

The design of our combi-molecules was first based on appending the triazene tail to the 2-phenylaminopyrimidinopyridine moiety (see square on the structure of STI571) since derivatives of the latter group retained significant bcr-abl kinase inhibitory activities.[19] Thus, the synthesis of the phenylaminopyrimidines **1** and **2** (Scheme 2) proceeded according to strategy described by Zimmermann et al.[20] Triazenes directly branched onto the 2-phenylaminopyrimidopyridine moiety were obtained by diazotization of **1** or **2** with nitrosonium tetrafluoborate in acetonitrile followed by addition of the desired amine and neutralization with triethylamine to give **3** and **4**. Compound **11** was synthesized by addition of a 1/10 mixture of aqueous methylamine (40%)/aqueous formaldehyde (37%).

The abl inhibitory activities of these compounds were tested in an enzyme immunosorbent assay (ELISA) using poly(L-glutamic acid-L-tyrosine, 4/1)(PGT) as a substrate for isolated v-abl kinase.[20] In this assay, STI571 showed an $IC_{50} = 0.04$ μM. This assay permits a short 10-min drug exposure, thereby avoiding substantial degradation of the triazenes prior to binding to the ATP site of abl kinase. Compounds **3** and **4** with the triazenes directly attached to the phenylaminopyrimidine ring showed IC_{50} values in range 8–10 μM. In view of the rather weak activities of these triazenes, we decided to insert the benzamide spacer to our conjugates as in **9**–**12**. This was achieved by treating amines **1** and **2** with *p*-nitrobenzoyl chloride to give **5** and **6** which were reduced with Fe in ethanol to provide **7** and **8.** Diazotization of these amines (**7**–**8**) with nitrosonium tetrafluoborate in acetonitrile followed by addition of the desired amine and neutralization with triethylamine gave triazene conjugates **9**–**12**. While **10**[27] and **12** were stable molecules, compounds **9** and **11** had half-lives of 16 and approximately 30 min, respectively, as determined by spectrophotometric monitoring of disappear-



Scheme 2. (i) CH_3CN, $NOBF_4$, −5 °C; (ii) Et_2O, Et_3N, alkylamine or 1/10 mixture of aqueous methylamine (40%)/aqueous formaldehyde (37%); (iii) *p*-nitrobenzoylchloride, pyridine, DMAP, rt overnight; (iv) Fe, EtOH, H_2O, AcOH, refluxing 4 h.

ing peaks at 315 and 365 nm. Interestingly, the bcr-abl kinase inhibitory activities of the latter compounds increased by more than 10-fold when compared with those of **1**, **3** and **4** (see Table 1).

These results can easily be rationalized in light of previous structure–activity relationships and X-ray structures of STI571 or its variants[21,22] co-crystallized with c-abl that showed a hydrogen bonding role for the benzamido moiety. Indeed, recent studies showed that

Table 1. Cytotoxicity and v-abl tyrosine kinase inhibitory activities for compounds **1**–**8** and **9**–**12**

Compd	Inhibition of abl kinase IC_{50}, μM[a]	IC_{50}, μM[b] K562 cells	Alkylating activity[c] × 10^{-4}
STI571	0.04	0.11	ND
TEM	>100	156.1	13
1	>10	21.69	
3	8.6	5.39	
4	10	10.11	
7	4.89	9.88	
8	>10	68	2
9	0.903	16.18	2
10	0.1946	2.164	2
11	1.057	3.151	15
12	0.8496	17.35	1.4

ND, not determined.
[a]IC_{50} values were determined by the v-abl kinase binding assay.[20] Results are means of two separate experiments.
[b]IC_{50} values were determined by the MTT assay[24] after 72 h of continuous drug exposure. Values are means of two separate experiments.
[c]Values are expressed by OD (540 nm)/mM of drug) measured following addition of triethylamine/acetone (v/v) to solutions of drug (0.1 mM) + NBP pre-incubated in at pH7.4 TRIS buffer solution for 2 h at 37 °C.[26]

in addition to hydrogen bonds between Met 318 amide backbone and the pyridine nitrogen, between the pyrimidine NH and the Thr 315 OH group, the amide bond of the benzamido moiety was involved in a donor–acceptor interaction wherein the oxygen of Glu 286 formed a hydrogen bond with the benzamide NH and the oxygen of the latter accepted a hydrogen bond from the amide NH of Asp 381. The monoalkyltriazenes were designed with the hope that the benzamide hydrogen could be replaced by the hydrogen of the non-conjugated tautomeric form as depicted in Figure 1. The lack of abl kinase inhibitory activity may be due to the notorious instability of this tautomer, the structure of which resembles the transition state for protolysis of monoalkyltriazenes.[23]

Furthermore, the contribution of the piperazine ring to the potency of STI571 is now known. It is located in a partially hydrophobic pocket forming van der Waals interactions with Val 289, Phe 359 and Asp 381.[22] Since these interactions would be unavailable in our conjugates, we did not expect our benzamide-containing conjugates to exhibit bcr-abl inhibitory activities superior or in the same range as STI571. However, surprisingly the dimethyltriazene showed activity in the submicromolar range (Table 1). More importantly, the hydroxymethyl derivative that differs from the dimethyltriazenes by only a hydroxy group was 5-fold less active. This may be explained by the disfavored orientation of the polar hydroxy group towards the partially hydrophobic pocket of abl.

To rationalize these results, we overlaid the MM2 optimized structures with the X-ray determined conformation[23] of c-abl-bound STI571, using the Chem3D software package. (Fig. 2) The model showed that of all the triazenes (**3–4**, **9–12**), the dimethyltriazene **10** adopted the conformation found the closest to that of STI571. The dimethyl group appears in an orientation where it may form, like the piperazinyl ring STI571, van der Waals interactions with the partially hydrophobic pocket of c-abl. The presence of the polar hydroxy group of **11** may decrease such interactions leading, as observed, to a lesser affinity for the ATP site. It is noteworthy that while the activity of **11** was 5-fold less than that of **10**, it was 5–10-fold more potent than derivatives **1–4** (devoid of the benzamido spacer) and amines **7–8**.

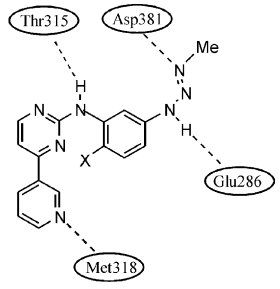


Figure 1. Predicted interactions of the non-conjugated tautomers of the monoalkyltriazenes with the active site of c-abl (dotted lines indicate possible hydrogen bonds).

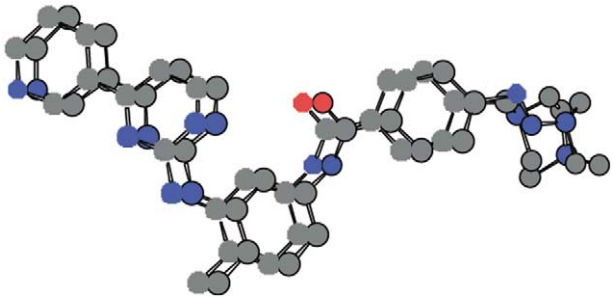


Figure 2. Overlay of STI571 with dimethyltriazene conjugate **10**.

As outlined in Scheme 1, dialkyltriazenes like dacarbazine are known to be metabolized by oxidation of their methyl group to generate a methylol intermediate that is rapidly converted to the cytotoxic monoalkyltriazene. The latter ultimately decomposes to an aromatic amine and an alkylating agent.[6,7] Similarly, our dimethyltriazene **10** will require metabolic oxidation to generate its putative metabolite **11**, the precursor of an alkylating species. Indeed, alkylating test using the *p*-nitrobenzylpyridine (NBP) assay,[25] showed that in contrast to dimethyltriazene **10**, that is devoid of alkylating activity, hydroxymethyltriazene **11** strongly alkylated NBP (Table 1). The translation of these properties into cytotoxic activity was studied in K562 promyelocytic leukemia cells. Triazenes **3** and **4** showed significant cytotoxic activities; however, this must not be rationalized on the basis of their bcr-abl inhibitory activities in these cells as their IC_{50} values for abl TK inhibition were extremely high (8.6–10 μM). In contrast, dimethyltriazene **10** that does not generate methyldiazonium owes its strong cytotoxic effect solely to its abl kinase inhibitory activity. Interestingly, the hydroxymethyltriazene analogue **11,** despite being a 5-fold weaker inhibitor of abl kinase than **10** was only 2-fold less cytotoxic than the latter. This may well be due to the combination of effects associated with the abl inhibitory activity and its ability to alkylate DNA. Indeed, it was found that compound **11** damage DNA in K562 cells (data not shown). Detailed mechanism of the cytotoxic effects of **10** and **11** are reported elsewhere.

From the current studies, we have identified a potent abl inhibitor of the dimethyltriazene class that does not generate alkylating species in vitro and synthesized its putative metabolite **11** that showed significant bcr-abl tyrosine kinase inhibitory activity and alkylating activity comparable with that of temozolomide, a prodrug of the dacarbazine-derived monoalkyltriazene.[7] Further studies are required in vivo to demonstrate the metabolic conversion of **10** to **11** and to establish the pharmacological advantages of these novel chimeric molecular approach.

Acknowledgements

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27. Experimental data for combi-molecule **10**. Amine **8** (100 mg, 0.25 mmol) was dissolved in dry acetonitrile (10 mL) and after cooling to $-5\,^{\circ}C$, a solution of nitrosonium tetrafluoroborate (60 mg, 0.5 mmol) suspended in acetonitrile was added. The clear solution was stirred for 1 h at $-5\,^{\circ}C$ and a mixture of ether (10 mL), water (2 mL), triethylamine (0.5 mL) and dimethylamine (40%) (0.3 mL) was added dropwise. The mixture was subsequently stirred at $0\,^{\circ}C$ for 2 h and the precipitate that formed filtered and washed with ether/ethyl acetate to give **10** (75 mg, 66%) as a pure solid: mp $165\,^{\circ}C$; APCI m/z 452.9 (MH^+), 380 (M-dimethyltriazene, 76.7); ^1H NMR (400 MHz, DMSO-d_6) δ 10.10 (s, 1H, CONH), 9.25 (s, 1H, NH), 8.97 (s, 1H, ArH), 8.66 (d, 1H, $J = 4.8$ Hz, ArH), 8.49–8.44 (m, 2H, ArH), 8.05 (d, 1H, $J = 2.0$ Hz, ArH), 7.93 (d, 2H, $J = 8.8$ Hz, ArH), 7.52–7.46 (m, 2H, ArH), 7.4 (d, 2H, $J = 8.8$ Hz, ArH), 7.18 (d, 2H, $J = 8.4$ Hz, ArH), 3.52 (br s, 3H, $N(Me)_2$), 3.18 (br s, 3H, $N(Me)_2$), 2.21 (s, 3H, CH3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.5, 162.2, 160.1, 153.5, 152.0, 148.8, 138.4, 137.9, 135.1, 132.9, 131.7, 130.7, 129.4 (2C), 128.2, 124.5, 120.3 (2C), 117.9, 117.4, 108.2, 43.8, 36.8, 18.5