Potential Tumor-Selective Nitroimidazolylmethyluracil Prodrug Derivatives: Inhibitors of the Angiogenic Enzyme Thymidine Phosphorylase

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Received June 14, 2002

Abstract: Thymidine phosphorylase (TP) is an angiogenic growth factor and a target for anticancer drug design. Molecular modeling suggested that 2′-aminoimidazolylmethyluracils would be potent inhibitors of TP. The novel 5-halo-2-aminoimidazolylmethyluracils (**4b**/**4c**) were very potent inhibitors of *E. coli* TP ($IC_{50} \sim 20$ nM). Contrastingly, the corresponding 2′-nitroimidazolylmethyluracil (as bioreductively activated) prodrugs (**3b**/**3c**) were 1000-fold less active (IC_{50} 22−24 μM). This approach may be used to selectively deliver TP inhibitors into hypoxic regions of solid tumors where TP is overexpressed.

Introduction. An essential stage in the growth and metastasis of solid tumors is the development of new blood vessels (angiogenesis). Platelet-derived endothelial cell growth factor (PD-ECGF) has been implicated in a variety of angiogenic effects by promoting endothelial cell proliferation in a range of tumor cell types.[1] PD-ECGF is identical to the enzyme thymidine phosphorylase (TP, dThdPase, EC 2.4.2.4).[2] TP catalyses the reversible phosphorylation of thymidine to thymine and 2-deoxyribose-1-phosphate (see Supporting Information), and it is proposed that the dephosphorylated 2-deoxyribose is responsible for the angiogenic stimulus of TP.[3]

TP/PD-ECGF has chemotactic activity in vitro, and angiogenic effects in vivo, and its expression is an adverse prognostic indicator in breast, bladder, ovarian, and colorectal tumors.[4] Griffiths and colleagues showed that TP/PD-ECGF is regulated by hypoxia and is focally expressed in the hypoxic regions of solid tumors.[5] Thus, the hypoxic up-regulation of TP in many tumors and its angiogenic activity makes TP an attractive target for cancer chemotherapy.[6] By inhibiting TP, tumor growth can be inhibited.

The development of TP inhibitors has primarily centered on uracil-type analogues with various substituents at the C-5 and/or C-6 positions.[7−10] The 6-amino-5-bromouracil (**1**, 6A5BU) is commonly used as the benchmark,[11] and the potent inhibitor 5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl]uracil hydrochloride (**2**, TPI) caused a substantial reduction in tumor growth rate when given continuously to mice carrying experimental tumors (for structures, see Figure 1).[12]

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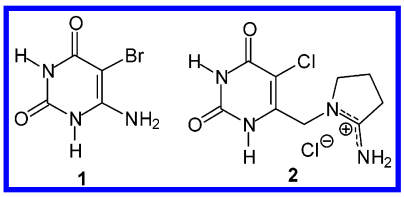


1 **2**

Figure 1. Known TP inhibitors.

As TP is expressed at high levels in platelets and other normal tissues, such as the brain, there would be substantial advantage in selectively inhibiting TP in the tumors where it is generating its angiogenic effects by promoting tumor growth.[13] Here, we report the design and synthesis of novel tumor-activated TP prodrugs that are likely to meet this criterion. From a homology model of human TP based on the *Escherichia coli* structure (in the open conformation), we identified important residues such as Ser 217, Asp 209, and Leu 148 in the cavity where thymine/thymidine is presumed to bind.[14] Nitroimidazolylmethyluracil analogues (**3**, as potentially hypoxia-mediated bioreductively activated TP inhibitors) and their corresponding amino derivatives (**4**) have been synthesized. The presence of hypoxia in tumors will cause bioreductive "activation" of the nitro prodrug to form the active amino species (Table 1) in areas of the tumor where TP is most highly expressed. The activation mechanism in hypoxia is catalyzed by reductive enzymes such as cytochrome P450 reductase. There are many examples in the literature of bioreductive activation of a nitro prodrug to the active amino derivative for selective delivery of cytotoxic agents, providing confidence in such an approach.[15−17]

The compounds were designed using molecular modeling by quantitative docking studies on the proposed compounds, and their energies were calculated in the docked states. To prove the principle that TP can discriminate between the nitro and amino analogues, the compounds were synthesized and evaluated for inhibition of recombinant purified *E. coli* TP (which shows a 69% sequence similarity to the active site of human TP) (purchased from Sigma Chemical Company, UK).[14] The experimental IC_{50} values were compared with those of the known inhibitors 6A5BU and TPI.

Molecular Modeling. As previously described, the known inhibitors 6A5BU (**1**) and TPI (**2**) showed good positioning within the active site of TP, with several H-bonds formed to active site residues. In particular, interactions with Ser 217, Leu 148, and Asp 209 appeared important for achieving low binding energy by **2**.[14]

The amino compounds **4** (X = H, Cl, Br) were designed from docking studies on the *E. coli* enzyme and a human similarity model.[14] The Autodock energies for the bound amino derivatives revealed that they were more highly stabilized than their corresponding nitro prodrug derivatives **3** (X = H, Cl, Br) within the active site of TP. The most highly populated binding conformation of **4c** generated by Autodock (see Figure 2) had several H-bonds with active-site residues including Ser 217 and Asp 209, which was similar to **TPI**. The docking

10.1021/jm020964w CCC: $25.00 © 2003 American Chemical Society
Published on Web 12/12/2002

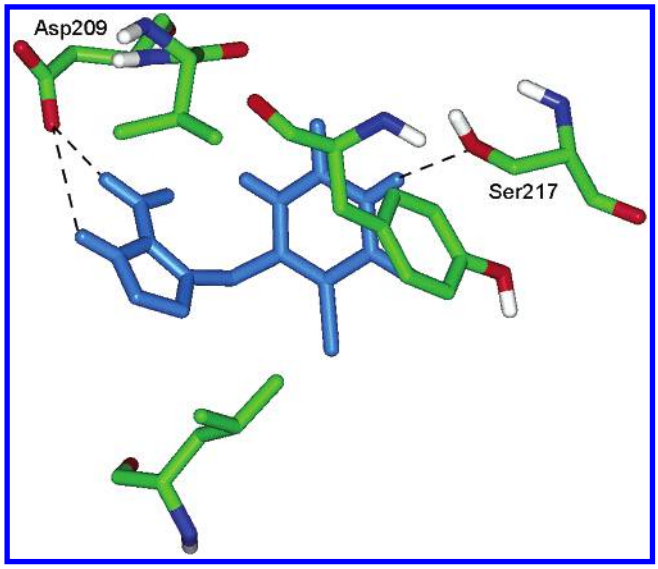
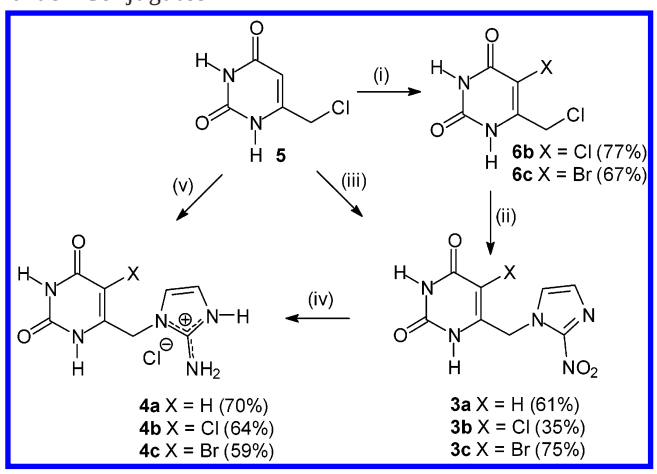


Figure 2. Binding mode of 5-bromo-6-[(2-aminoimidazol-1-yl)methyl]uracil (**4c**) in the active site of human TP.

Scheme 1. Synthesis of Nitro- and Aminoimidazolyl-uracil Conjugates[a]



[a] Reagents and conditions: (i) NCS or NBS, AcOH, 60 °C; (ii) 1-potassio-2-nitroimidazole, DMF, rt, N_2, 24 h; (iii) 1-potassio-2-nitroimidazole, DMF, 75 °C, N_2, 2 h; (iv) $NaBH_4$, Pd/C, MeOH/H_2O, N_2, 24 h; (v) 2-aminoimidazole sulfate, NaOEt, DMF, rt, 26 h.

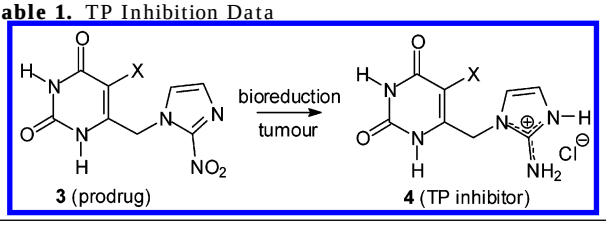
energies for **TPI** and **4c** (X = Br) for the human enzyme were also similar (−47.90 and −45.96 kcal mol^{-1}, respectively). Thus, on the basis of the active site structures of either the *E. coli* or human TP, it was predicted that the enzyme would strongly discriminate the nitro from the amino forms of the appropriate imidazolyl-substituted uracil derivatives (**3** and **4**).

The modeling studies also demonstrated that TP has an affinity for an amino group four bond-lengths from the 6-position of uracil, which can mimic an oxycarbenium ion transition-state. The conformational restriction afforded by a ring system aids the binding of the amino-substituent to active site residues (Figure 2).

Chemical Synthesis. The nitrouracil prodrugs were synthesized from 6-(chloromethyl)uracil (**5**) (Scheme 1). Reaction of **5** with either *N*-chlorosuccinimide (NCS) or *N*-bromosuccinimide (NBS) gave the desired 5-halo-6-(chloromethyl)uracil derivatives (**6b** and **6c**, respectively).[10] The 6-(chloromethyl)uracils (**5**, **6b**, and **6c**) were coupled with 1-potassio-2-nitroimidazole to give the 5-substituted-6-[(2-nitroimidazol-1-yl)methyl]uracil derivatives (**3a**; X = H, **3b**; X = Cl, **3c**; X = Br) in reasonable yields. Reduction of the nitro derivatives with $NaBH_4$/Pd gave the desired 6-[(2-aminoimidazol-1-yl)methyl]uracil conjugates (**4a**; X = H, **4b**; X = Cl, **4c**; X = Br) in good yields. Alternatively, the amino derivatives were synthesized from the appropriate 5-substituted-6-(chloromethyl)uracils by coupling with 2-aminoimidazole. TPI and 6A5BU were synthesized using literature methods.[7,18]

Evaluation as TP Inhibitors. The TP inhibition assay was carried out using a continuous spectrophotometric assay adapted for a 96-well plate Molecular Devices Spectromax instrument at 355 nm with 5-nitro-2′-deoxyuridine (0.13 mM) as the substrate in the presence of the enzyme (45 nM, 25 °C). The IC_{50} values (Table 1) were calculated as the concentration at which 50% reduction of the initial rate (V_0) was attained (see Supporting Information).[19] The inhibition selectivity ratio (ISR) is obtained by dividing the IC_{50} of a nitro compound by those for the corresponding amino compound.

Table 1. TP Inhibition Data



compound	X	IC_{50} (μM)	ISR[a]
TPI	–	0.02 ± 0.002	–
6A5BU	–	1.60 ± 0.17	–
3a	H	1.60 ± 0.08	2.8
4a	H	0.56 ± 0.02	
3b	Cl	21.7 ± 1.2	1033
4b	Cl	0.021 ± 0.014	
3c	Br	24.4 ± 1.0	1284
4c	Br	0.019 ± 0.002	

[a] ISR is the inhibition selectivity ratio (IC_{50} nitro/IC_{50} amino).

The results in Table 1 show that the 5-halo-6-[(2-aminoimidazol-1-yl)methyl]uracils (**4b**; X = Cl **4c**; X = Br) were significantly (100-fold) more potent than 6A5BU, and were as potent as TPI. The very strong inhibition by **4b**, **4c**, and **TPI** is noteworthy, and our preliminary analysis indicates tight-binding inhibition, so that for these compounds with *E. coli* TP, the IC_{50} values are limited by the enzyme concentration. More importantly, in all cases the 2-aminoimidazolyl compounds **4** were markedly more potent than their nitro congeners **3**. For the 5-halo analogues, there was at least a 1000-fold increase in potency for the amino derivatives (**4b**; X = Cl and **4c**; X = Br) compared with their nitro analogues (**3b**; X = Cl and **3c**; X = Br). Thus, under bioreductive conditions, the nitro compounds could act as prodrugs to their amino counterparts. The 5-unsubstituted aminoimidazolyl conjugate (**4a**) showed a marked decrease in potency compared to the 5-halo conjugates. This may be due either to the fact that bulky substituents at the C-5 position results in the imidazolyl ring adopting a favorable conformation in the active site of TP, or to an electronic effect. The presence of the amino group enhances potency further, possibly due to hydrogen bonding with Asp 209 (this interaction is absent for the nitro derivatives) (see Figure 2).

Conclusion. Docking studies of the modeled TP predicted that the binding of the amino derivatives was energetically more favored than that of their corresponding nitro counterparts. This has been confirmed experimentally for the *E. coli* TP enzyme. For the 5-halo-2′-aminoimidazoyl compounds (**4b** and **4c**) there is at least a 1000-fold increase in potency in TP inhibition compared with the nitro analogues (**3b** and **3c**). Thus, this approach can potentially be used to deliver very potent TP inhibitors preferentially into the hypoxic areas of solid tumors.

Acknowledgment. This work was funded, in part, by the MRC, BBSRC, and AICR.

Supporting Information Available: The ^{1}H NMR, ^{13}C NMR, MS, and microanalytical data for all target compounds are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM020964W