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Synthesis and enzymatic evaluation of xanthine oxidase-activated prodrugs based on inhibitors of thymidine phosphorylase

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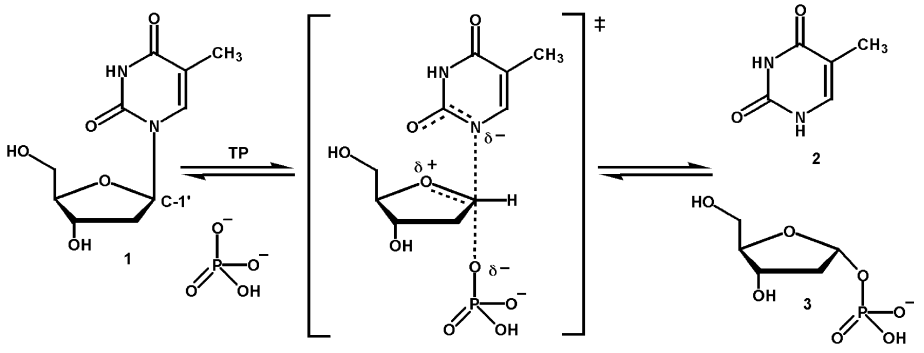
Abstract—A series of xanthine oxidase-activated prodrugs of known inhibitors of thymidine phosphorylase has been designed and synthesised to introduce tumour selectivity. These prodrugs were oxidised by xanthine oxidase at C-2 and/or C-4 of the uracil ring to generate the desired TP inhibitor.

Thymidine phosphorylase (TP, EC 2.4.2.4) catalyses the reversible phosphorolysis of thymidine (**1**) to thymine (**2**) and α-D-2-deoxyribose-1-phosphate (**3**, Scheme 1). The dephosphorylated sugar, D-2-deoxyribose, has been shown to have chemotactic activity in vitro and angiogenic activity in vivo[1,2] TP is elevated in several hypoxic tumours, promoting both angiogenesis and metastasis, and suppressing apoptosis.[3] TP is also known as platelet-derived endothelial cell growth factor (PD-ECGF).[4] TP is distinct from other angiogenic growth factors, since it exerts its actions through its enzymatic activity.

Inhibitors of human TP are of interest in cancer chemotherapy.[5] The 'classical' inhibitor 6-amino-5-bromouracil (**4**, 6A5BU) is typically used as the benchmark for the design of more potent inhibitors.[6] 7-Deazaxanthine (**5**, 7-DX) was the first purine derivative to display inhibition of both *E. coli* TP and angiogenesis in chorioallantoic membrane.[7] One of the most potent inhibitors is 5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl]-uracil hydrochloride (**6**, TPI), which causes a reduction of tumour growth, promotes apoptosis and inhibits angiogenesis.[8] TPI in combination with the anti-tumour agent 2′-deoxy-(5-trifluoromethyl)uridine is currently in phase 1 clinical studies (Fig. 1).[9]

Since TP is expressed at high levels in human blood platelets and normal tissues (e.g., ovaries, salivary glands, brain), there is an advantage in selectively inhibiting TP at the site of the tumour.



Scheme 1. The reaction catalysed by TP and proposed transition state.

Keywords: Thymidine phosphorylase; Xanthine oxidase; Prodrug; Inhibitor.
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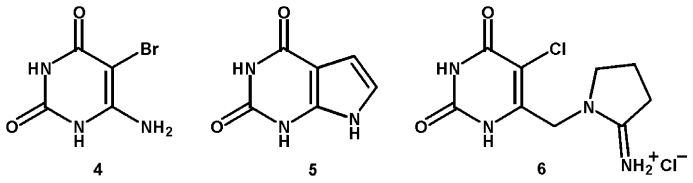
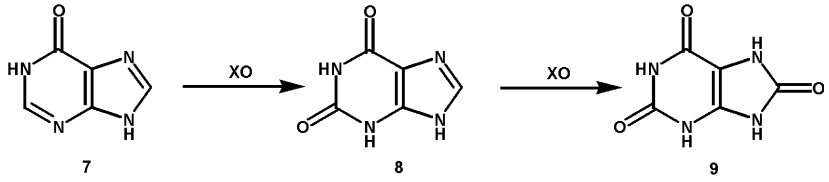
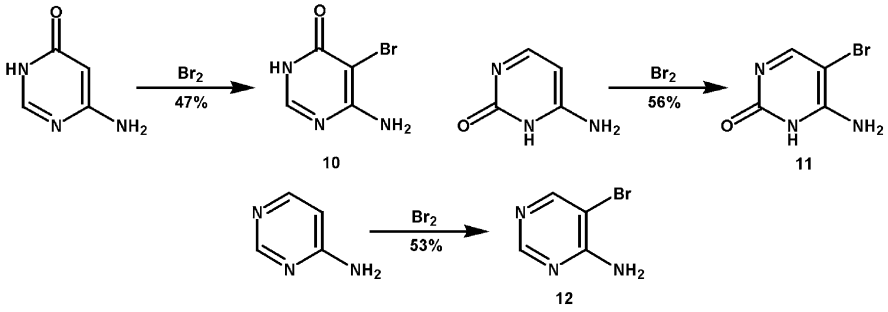


Figure 1. Known TP inhibitors.



Scheme 2. The enzymatic activity of XO.



Scheme 3. Synthesis of XO-activated prodrugs of 6A5BU (**10**–**12**).

Xanthine oxidase (XO, EC 1.1.3.22) catalyses the oxidation of hypoxanthine (**7**) and xanthine (**8**) to uric acid (**9**, Scheme 2). XO-mediated prodrug activation has been used to increase the bioavailability, solubility and selectivity of a number of antivirals.[10,11] XO exhibits broad substrate specificity[12] the known TP inhibitors being uracil-based heterocycles are attractive targets for the XO-activated prodrug strategy. Furthermore, both TP and XO are expressed at high levels in hypoxic tissue,[13,14] breast,[15] colorectal[14,16] and brain tumours.[17,18]

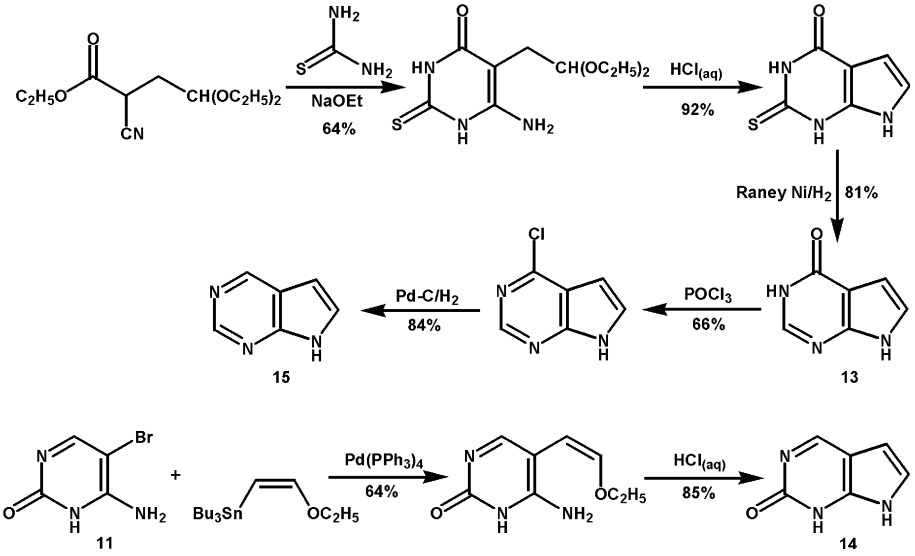
Here, we report the design, synthesis and enzymatic evaluation of a series of potential tumour-selective XO-activated prodrugs of the known TP inhibitors 6A5BU (**4**), 7-DX (**5**) and TPI (**6**). These prodrugs lack the carbonyl substitution at C-2 and/or C-4 of the uracil ring.

The 6-amino-5-bromopyrimidine series of XO-activated prodrugs of 6A5BU (**10**–**12**) was synthesised by electrophilic substitution of the appropriate 6-aminopyrimidine with molecular bromine (Scheme 3).[19]

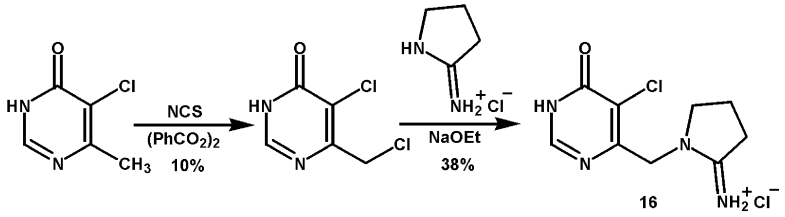
The 7*H*-pyrrolo[2,3-*d*]pyrimidine series of XO-activated prodrugs of 7-DX (**13**–**15**) was synthesised as outlined in Scheme 4. Prodrug **13** was prepared via cyclocondensation, followed by acid-catalysed hydrolysis of the acetal and cyclisation and finally desulfurisation with Raney Nickel.[20] The deoxygenation of **13** via the chlorinated intermediate afforded prodrug **15**.[20] Palladium-catalysed Stille coupling[21] of **11** with *Z*-1-ethoxy-2-(tributylstannyl)ethane followed by deprotection of the resulting enol ether with concomitant cyclisation gave the novel XO prodrug **14**.

The novel XO-activated prodrug of TPI, 5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl]-3*H*-pyrimidin-4-one hydrochloride (**16**, Scheme 5) was synthesised by radical halogenation[22] of 5-chloro-6-methyl-3*H*-pyrimidin-4-one, using *N*-chlorosuccinimide/benzoyl peroxide, with subsequent coupling to 2-iminopyrrolidine hydrochloride (Scheme 5).

The TP inhibition assay, using purified recombinant *E. coli* TP (69% sequence similarity to human TP), was accomplished using a continuous spectrophotometric assay,[23] adapted for a spectrophotometer with a 96-well microplate reader, at 355 nm and 25 °C. The reaction mixture (0.2 mL) contained 0.13 mM 5-nitro-2′-deoxyuridine as the substrate in potassium phosphate buffer (0.1 M, pH 7.4). Reaction was initiated by addition of TP (0.00883 U/0.2 mL). The IC_{50} values for both the TP inhibitors and their XO-activated prodrugs are given in Table 1. As anticipated and required, the parent TP inhibitors (**4**–**6**) were significantly more potent TP inhibitors than their corresponding prodrugs. Indeed, prodrugs **10**–**16** showed no inhibition of *E. coli* TP even at high concentrations, suggesting that C-2 and C-4 carbonyl substitution of the uracil ring moiety is essential for TP inhibition.



Scheme 4. Synthesis of XO-activated prodrugs of 7-DX (**13**–**15**).



Scheme 5. Synthesis of XO-activated prodrug of TPI (**16**).

The kinetic constants (K_m and V_{max}) for the XO-activation of the prodrugs were determined using a continuous spectrophotometric assay.[25] The reaction mixture (3.0 mL) contained potassium phosphate buffer (0.05 M, pH 7.5) and the prodrug substrates (25–150 μM) at 25 °C; reaction was initiated by addition of XO (0.9 U/mL). Prodrug oxidation was monitoring at a wavelength pre-determined for the appropriate TP inhibitor. The XO substrate data are given in Table 1. The 6-amino-5-bromopyrimidine prodrugs **10**–**12** were good substrates for XO and the formation of 6A5BU (**4**) was observed in each case. The 7*H*-pyrrolo[2,3-*d*]-pyrimidines **13**–**15** were also good substrates for XO yielding 7-DX (**5**) as the oxidation product.[10,25] Similarly prodrug **16** was selectively oxidised at C-2 by XO to give TPI (**6**).

Table 1. Inhibition data for *E. coli* TP for the TP inhibitors (**4**–**6**) and their prodrugs (**10**–**16**), and bovine XO-mediated activation data for prodrugs (**10**–**16**)

Compound	*E. coli* TP IC_{50} (μM)	Bovine XO	
		K_m (μM)	V_{max} (mM/min)
4, 6A5BU	1.6	—	—
10	No inhibition at 148	23.8	1.25
11	No inhibition at 137	32.5	1.20
12	No inhibition at 213	44.4	0.98
5, 7-DX	6.5	—	—
13	No inhibition at 207	45.1	0.51
14	No inhibition at 148	29.8	0.48
15	No inhibition at 163	67.2	0.24
6, TPI	0.02[a]	—	—
16	No inhibition at 47.2	64.9	0.51

The data for the kinetic constants represent the mean of five independent measurements.

[a] This is an upper limit for IC_{50} as the inhibition is stoichiometric.[24]

In summary, XO-activated prodrugs of three known TP inhibitors have been investigated. In all cases, the TP inhibitor was a more potent inhibitor of *E. coli* TP, than the corresponding prodrug. All prodrugs were oxidised by XO at C-2 and/or C-4 of the uracil ring resulting in the formation of the appropriate TP. The XO prodrug approach provides an opportunity for the development of tumour-selective inhibitors of TP.

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