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Hypoxia-driven elimination of thiopurines from their nitrobenzyl prodrugs

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Abstract—A novel bioreductive prodrug of 6-thioguanine, 2-amino-6-[2-(4-nitrophenyl)prop-2-ylsulfanyl]-9*H*-purine, containing a gem-dimethyl thioether linkage, was synthesised and compared with its unsubstituted analogue. In A549 whole cell experiments hypoxia selective release of 6-thioguanine was observed with the substituted prodrug only.

Tumour hypoxia is a potentially exploitable factor for the treatment of many solid tumour types.[1] Hypoxia occurs as a result of poor blood supply, and therefore oxygenation, due to the chaotic network of the neovasculature in growing tumours. It is well documented that tumour hypoxia is directly correlated with disease progression and patient survival, with higher levels of hypoxia leading to generally poorer prognoses.[2] Unfortunately, tumour hypoxia protects cells from radiotherapy, photodynamic therapy and some cytotoxic reagents. There is thus a clear need for new agents that can target hypoxic regions and even utilise hypoxia to their advantage.

Many studies have been carried out using bioreductive drug targeting.[3–9] One approach utilises a prodrug which delivers an agent, for example a cytotoxin, via reductive elimination from a non-toxic parent molecule. Reduction and prodrug fragmentation occurs within regions of low oxygen concentration but is absent in healthy, aerobic tissues. This process can be driven by a number of 'reductase' enzymes, including cytochrome P450, xanthine oxidase and NADPH-cytochrome P450 reductase. The prodrug must therefore be a substrate for the enzyme, possess a chemical function capable of being reduced, and fragment upon reduction. There has been much interest in the use of quinoid and nitroaromatic 'triggers' because such compounds possess the necessary functionality for reduction.[7,10] Recently, we reported the synthesis and biological properties of nitrothiophene derived prodrugs of combretastatin A4.[11] Our findings show that inclusion of a gem-dimethyl substituted linkage between the active drug and trigger improves a number of properties including metabolic stability, prodrug fragmentation rate and elimination efficiency.

The antileukaemic drugs 6-thioguanine (6-TG) and 6-mercaptopurine (6-MP) show little clinical activity against solid tumours despite broad in vitro cytotoxicity. Such limitations probably result from their short elimination half-lives, rapid cellular metabolism and relative hydrophilicity, which together would restrict penetration into solid tumour tissue. In humans, catabolism takes place largely via an inactivating sulfur methylation.[12,13] We reasoned that prodrugs with enhanced metabolic stability and increased lipophilicity might be better able to penetrate tumour tissue and deliver the active cytotoxic agents to tumour cells unreached by systemic administration of the parent drugs.

Previously, Kirkpatrick et al. reported the synthesis of 6-(4-nitrobenzyl)thioguanine (**1**).[14] Biological evaluation of this compound in aerobic and hypoxic EMT6 tumour cells failed to show an advantage over 6-thioguanine. They demonstrated, by isolating the reduction product

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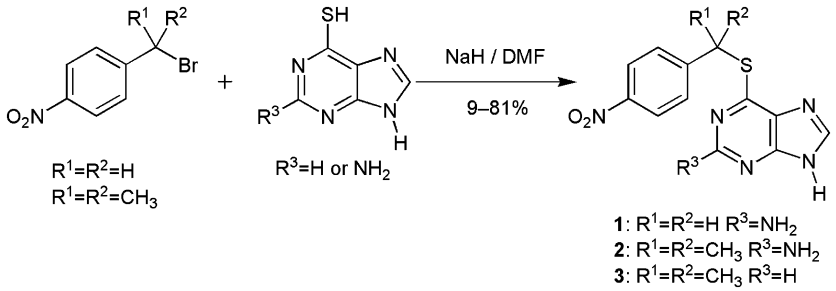

Scheme 1. Synthesis of thiopurine prodrugs.

6-(4-aminobenzyl)thioguanine via chemical hydrogenation, that the thioether linkage was stable to fragmentation. We therefore sought to use our gem-disubstitution technology to improve the delivery of thioguanine to tumours whilst capitalising on the inherent lower toxicity of 6-alkyl analogues.

During our studies with combretastatin A4, we synthesised mono-methyl, gem-dimethyl and unsubstituted analogues for comparison. In this thiopurine investigation, we decided to omit the mono-methyl analogue as the beneficial properties imbued by the gem-dimethyl linker in the combretastatin A4 series, such as better elimination efficiency, lower radical half-life and absence of a chiral centre, are all attractive properties for drug design and biological evaluation. Prodrugs were synthesised in variable yields (9–81%) by nucleophilic substitution of 4-nitrobenzyl bromide or 2-bromo-2-(4-nitrophenyl)propane[15] with 6-thioguanine in DMF using sodium hydride as the base (Scheme 1). The compounds were identified by ^{1}H NMR, mass spectrometry and elemental analysis.

Pulse and steady state γ-radiolysis experiments were used to measure the radical anion half-lives and fragmentation efficiency, respectively (Table 1). Incorporation of the gem-dimethyl linker significantly lowered the radical half-life of the substituted prodrugs **2** and **3** compared to the unsubstituted **1** (half-life not measurable within the timeframe of the experiment). The importance of the gem-dimethyl group for successful drug release is underlined with the sixfold increase in fragmentation efficiency over the des-methyl analogue. These characteristics might be attributed to a drive to release steric strain in the fragmentation transition state combined with stabilisation of the transition state electron density by the alkyl groups.

Following successful radical fragmentation studies, the 6-TG prodrugs were evaluated in A549 whole cell suspension experiments. These were performed under anoxic (N_2) and aerobic conditions to ascertain the relative rates and efficiencies of bioreductive elimination of thiopurines from the prodrugs. It is known that the A549 cell line has an appreciable level of P450 reductase[16,2] and, as a human lung cancer cell line, affords an attractive opportunity for eventual clinical targeting. Table 2 summarises the improved properties of **2**. A tenfold increase in prodrug metabolism under anoxia compared to air is noted for the lead compound whereas a much lower increase is seen for the unsubstituted **1**. Interestingly, the loss of **1** is not matched with production of 6-TG, unlike **2** which demonstrates excellent production under anoxia (Fig. 1). It is evident that the loss of **1** coupled with zero production of 6-TG indicates a metabolic pathway other than reductive elimination may be occurring. This further demonstrates the importance of the gem-dimethyl linker for abrogating unwanted metabolic events. As expected, both prodrugs fail to be metabolised or release 6-TG under oxic conditions.

In conclusion, we have successfully synthesised thiopurine prodrugs and tested our gem-disubstitution technology. Previous work[14] has shown nitrobenzyl conjugated 6-TG prodrugs to be stable under hypoxia, but we have demonstrated that, with our improved dimethyl linker

Table 1. Characterisation of radical fragmentation and drug release efficiency using radiolytic production of radicals

Compound	R^1	R^2	Drug	Radical half-life/ms[a]	% Fragmentation efficiency[b]
1	H	H	6-TG	—[c]	11
2	Me	Me	6-TG	29	67
3	Me	Me	6-MP	18	66

[a] Pulse radiolysis done in N_2O-saturated 50% 2-propanol/water (pH 9.2) using a 6-MeV linear accelerator.

[b] Steady-state γ-radiolysis determined in N_2O-saturated 50% 2-propanol/water (pH 7.4); dose rate, 3.26 Gy min^{-1}; % fragmentation efficiency = G(drug)/G(Me_2COH) × 100.

[c] Not measurable.

Table 2. Metabolism of prodrugs in A549 whole cells

Compound	Rate of loss of prodrug/nM min^{-1}		Rate of 6-TG production/ nM min^{-1}	
	Air	N_2	Air	N_2
1	0	39 ± 6	1.7 ± 0.4	0
2	9.5 ± 2.5	100 ± 6	1.7 ± 0.4	72 ± 4

Compounds **1** or **2** was dissolved in DMSO to a concentration of 625 μmol/L, and 80 μL was added to 10 mL A549 cells in Eagle's MEM (8.19×10^6 cells/mL) to give a final prodrug concentration of 5 μmol/L and incubated at 37 °C. For anoxic experiments, the mixture was degassed with N_2 for 30 min before prodrug addition and then overgassed with N_2 during incubation. Samples (1 mL) were added to 100 mmol/L hydrochloric acid (0.2 mL), mixed and then extracted (via solid-phase extraction) before HPLC analysis.

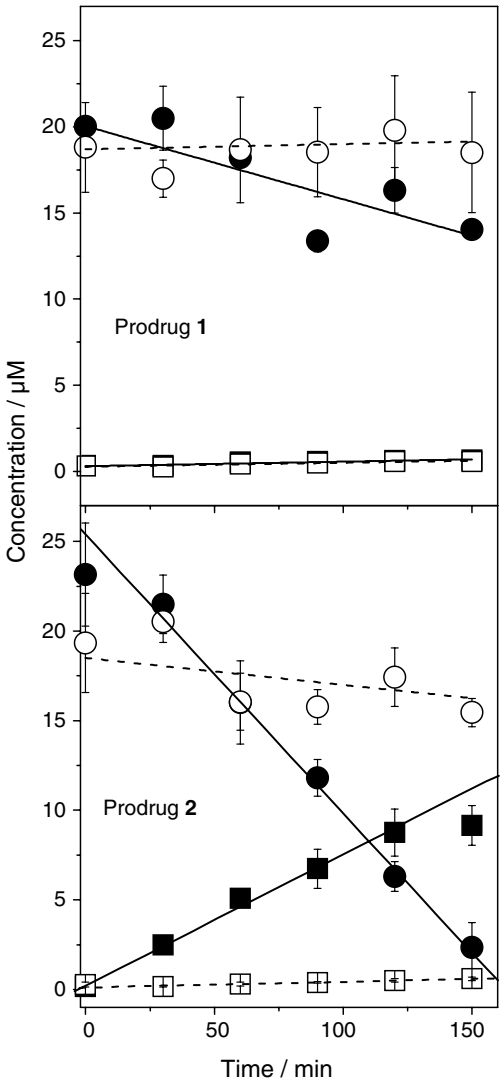

Figure 1. Loss of prodrug under air (○) and N_2 (●), and production of 6-TG under air (□) and N_2 (■).

group incorporated into the prodrug, it is possible to release the active agent using this drug delivery strategy. We are currently evaluating this series of prodrugs in further experiments.

*Synthetic example. 2-Amino-6-[2-(4-nitrophenyl)prop-2-ylsulfanyl]-9H-purine (***2***).* Sodium hydride (160 mg, 4.0 mmol) was added to 6-thioguanine (668 mg, 4.0 mmol) in DMF (15 mL). After 1 h, 2-bromo-2-(4-nitrophenyl)propane (1.08 g, 4.4 mmol) in DMF (5 mL) was added and the reaction was stirred for 24 h. The reaction mixture was partitioned (ethyl acetate and brine), the aqueous phase was extracted (ethyl acetate); the organic phases were combined then washed (water then brine), dried ($MgSO_4$) and evaporated. Flash chromatography, eluting with 50% ethyl acetate/hexane then 100% ethyl acetate and finally 25% methanol/ethyl acetate, afforded an oily, yellow solid. The residue was triturated with acetonitrile and the suspension filtered. The filtrate was concentrated in vacuo then triturated with ether, and afforded an orange powder. This was recrystallized from water/methanol and furnished a white powder (150 mg, 11%); TLC $R_f = 0.19$, ethyl acetate; mp 189–191 °C; ^{1}H NMR (500 MHz, DMSO) δ 8.12 (2H, d, $J = 7.0$, Ar**H**), 7.96 (1H, d, $J = 7.0$, Ar**H**), 6.13 (1H, s, N=C**H**), 2.51 (6H, s, $2 \times C\mathbf{H}_3$) ppm; HPLC-RT 4.08 min (TFA 20–50%); MS (*m*/*z*, %) 330 (M^+, 4%), 167 (100%), 163 (13%), 133 (26%); analysis $C_{14}H_{14}N_6O_2S_1 \cdot 1/2CH_3OH \cdot 1/2H_2O$ calcd C, 49.01; H, 4.79; N, 23.66; found C, 48.88; H, 5.12; N 23.58.

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