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European Journal of Medicinal Chemistry 41 (2006) 330–339
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Original article

Polar, functionalized guanine-*O*6 derivatives resistant to repair by *O*6-alkylguanine–DNA alkyltransferase: implications for the design of DNA-modifying drugs

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Received 30 June 2005; received in revised form 3 November 2005; accepted 28 November 2005
Available online 03 February 2006

Abstract

The protein *O*6-alkylguanine–DNA alkyltransferase (Atase) is responsible for the repair of DNA lesions generated by several clinically important anti-cancer drugs; this is manifest as active resistance in those cancer cell lines proficient in Atase expression. Novel *O*6-substituted guanine analogues have been synthesized, bearing acidic, basic and hydrogen bonding functional groups. In contrast to existing *O*6-modified purine analogues, such as methyl or benzyl, the new compounds were found to resist repair by Atase even when tested at concentrations much higher than *O*6-benzylguanine, a well-established Atase substrate active both in vitro and in vivo. The inactivity of the new purines as covalent substrates for Atase indicates that agents to deliver these groups to DNA would represent a new class of DNA-modifying drug that circumvents Atase-mediated resistance.

Keywords: Purine; *O*6-alkylguanine–DNA alkyltransferase; DNA repair; Drug design

1. Introduction

The biological activity of many clinically-significant, DNA major groove-alkylating drugs, including the methylating agents (e.g. temozolomide and dacarbazine) and chloroethylating agents (e.g. bis(chloroethyl)nitrosourea [BCNU] and chloroethyl(cyclohexyl)nitrosourea), is mediated via the formation of *O*6-alkylguanine adducts. Although these lesions account for only a small percentage of the total DNA adducts formed, they are potentially carcinogenic, mutagenic and cytotoxic [1,2]. Cytotoxicity arises from intervention of the mismatch repair (MMR) enzymes that correct single base mispairs following DNA replication [3]; in the case of *O*6-alkylguanine-modified DNA, cell cycle arrest is triggered through a futile cycle of deoxythymidine excision and replacement, opposite the modified guanine site of the parent strand, that generates long-lived strand breaks [4]. In the absence of MMR, unchecked guanine *O*6-alkylation causes G→A transition mutations in the course of two cycles of DNA replication [5].

The extreme consequences of guanine *O*6-alkylation have driven the evolution of another DNA-repair protein to specifically cleave lesions from guanine-*O*6 positions. *O*6-alkylguanine–DNA alkyltransferase (Atase; EC 2.1.1.63) plays a major role in the cellular defense against alkylating agents: as a component of DNA repair, it participates in a nucleophilic substitution reaction that transfers an alkyl group (e.g. methyl or 2-chloroethyl) from the *O*6-position of guanine in DNA to a cysteine residue in the protein [6]. The roles of the active site amino acid residues determined to be essential for catalysis are outlined in Scheme 1. Briefly, His146, by intervention of a bridging water molecule, acts as the base to deprotonate Cys145, generating a reactive thiolate nucleophile which is able to attack at the *O*6-alkyl carbon of a modified guanine

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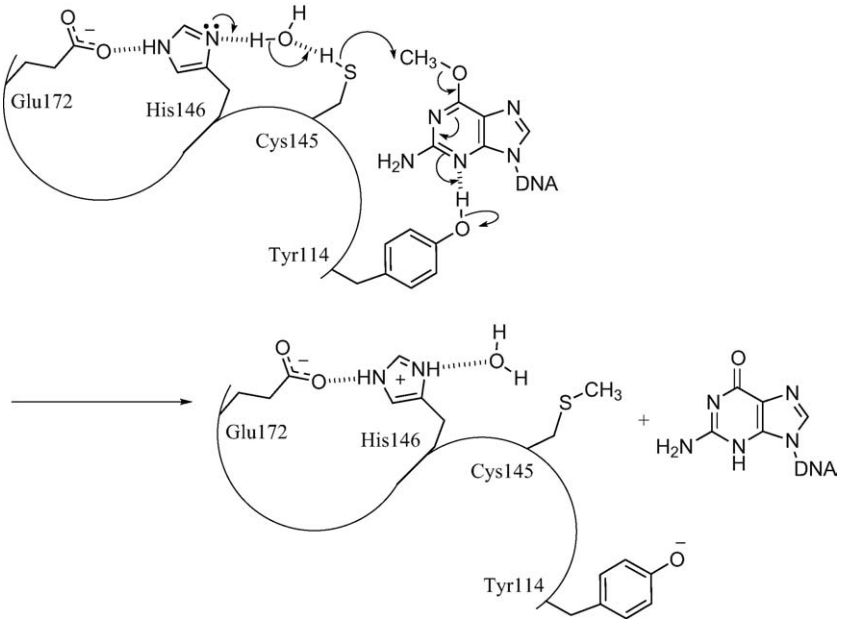
doi:10.1016/j.ejmech.2005.11.007



Scheme 1. Proposed reaction mechanism for hAtase. His146, by intervention of a bridging water molecule, deprotonates Cys145 to facilitate nucleophilic attack at the *O*6-alkyl carbon with simultaneous protonation of *N3* by Tyr114 (adapted from reference 7).

base; guanine-*N3* is simultaneously protonated by Tyr114 [7]. In the course of the repair reaction, Atase is transformed into an *S*-alkylcysteine form. As this process is irreversible, Atase is a "suicide-protein" that cannot formally be classified as an enzyme. The single active site present means that Atase can repair one *O*6-alkyl nucleobase before inactivation, so is consumed stoichiometrically during the repair process. The chemotherapeutic significance of Atase is being the determinant of active resistance to drugs that alkylate in the major groove of double-stranded DNA.

Overall, the response of any cell line (or tumor) to simple alkylating agents depends on the relative activity of MMR and Atase. Low levels of MMR expression result in a phenotype that inherently lacks susceptibility to alkylating drugs (passive resistance) and is likely to accumulate mutations; in contrast, high Atase levels characterize the actively resistant phenotype. A good response to alkylating chemotherapy therefore requires the combination of efficient MMR with low levels of Atase. Clinical strategies to overcome Atase-mediated resistance include divided dose scheduling of alkylating drugs [8–11], or pre-administration of an Atase inactivator such as *O*6-benzylguanine [12–15] or *O*6-(4-bromo)thienylguanine (Patrin 2) [16]. For a recent comprehensive review of the inactivation of Atase in cancer chemotherapy see reference [17].

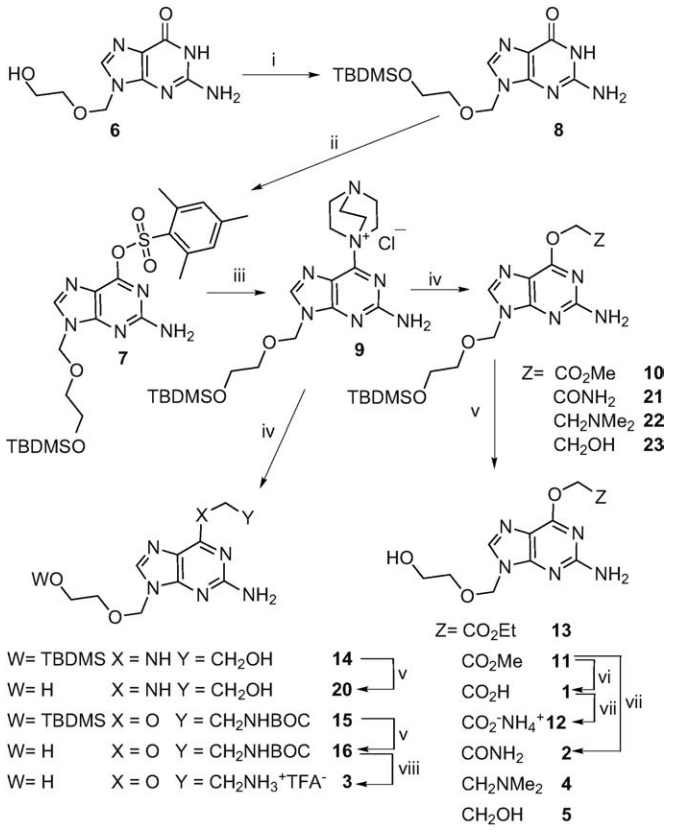
An alternative to Atase inactivation would be drugs that deliver *O*6-guanine modifications to DNA which are not repairable by Atase. Investigation of carcinogens generated during amino acid metabolism has identified *O*6-carboxymethyl-2′-deoxyguanosine-modified DNA (*O*6-CMG-DNA) as an intermediate in mutagenesis related to amino acid metabolism [18–20]. *O*6-CMG-DNA appears to inhibit Atase but without transfer of the *O*6-substituent to the protein active site, in marked contrast to either *O*6-methyl- or *O*6-benzyl-guanine modification [20,21]. Formation of an ionic complex between the carboxymethylpurine and the active site may be postulated in which the H-bonded network essential for generation of the thiolate nucleophile is disrupted, thereby obfuscating the catalytic activity of the protein.

A range of new *O*6-substituted guanines (**1**–**5**) bearing polar, charged, or hydrogen bonding functional groups has been prepared. Complementary molecular models of the target compounds have been constructed and potential interactions with the active site of the protein have been investigated (see Appendix A). Enzyme assays determined the ability of Atase to effect their repair and a new class of DNA lesion, refractory to Atase-mediated repair, has been characterized. Details of the macromolecular interactions that determine the properties of *O*6-CMG-DNA have been highlighted, from which proposals are made for lesions to be delivered by new DNA "alkylating" agents. Such agents would exhibit a spectrum of antitumor activity independent of the mechanisms of active and passive resistance which have hitherto limited application of this class of anticancer drug.

2. Results

2.1. Synthesis

The synthetic strategy developed involved elaboration of the *N9*-derivatized purine, aciclovir **6**. Use of the 6-mesitylenesulfonyl leaving group (**7**) permitted ready introduction of nucleophilic groups to the 6-position of the purine ring (Scheme 2). Protection of the terminal hydroxy group of the *N9* sidechain was essential to direct sulfonylation to the *O6* position. Thus, *t*-butyldimethylsilyl chloride (TBDMS-Cl) was employed to protect the sidechain of aciclovir. The TBDMS-protected aciclovir derivative **8** was obtained in good yield (97%) after recrystallization from MeOH. Qian et al. [22]



Scheme 2. Reagents and conditions. (i) TBDMSCl, imidazole, DMF, 48 h, r.t. (97 %); (ii) 2-mesitylenesulfonyl chloride, DMAP, Et_3N, MeCN, 2 h, r.t., (80 %); (iii) DABCO, THF, 4 h, r.t.; (iv) ROH, DBU, THF, 3 h, r.t. (48–91 %); (v) TBAF, THF, 30 min, r.t., (28–97 %); (vi) 0.1 M NaOH, 2 h, HCl, r.t., (88 %); (vii) NH_3, r.t.; (viii) TFA, CH_2Cl_2, 10 min, r.t., (72 %).

have reported the synthesis of this compound using similar reaction conditions. The mesitylene group was introduced at the *O*6-position by adaptation of a published method [23] to give sulfonate ester intermediate **7** in 80% isolated yield.

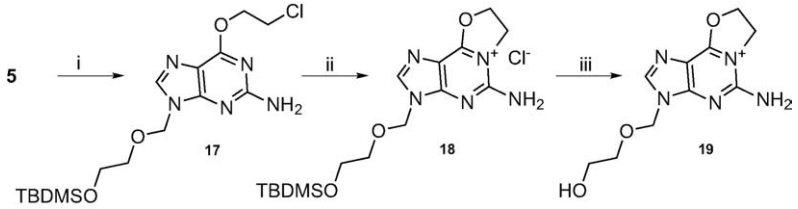
The mesityl derivative **7** and the derived quaternary DABCO salt **9** were key intermediates that gave access to the target compounds; an approach also successfully applied by Lakshman et al. [24] using 2′-deoxyguanosine derivatives for the preparation of *O6*-alkyl and *O6*-aryl ethers as well as *N*6-substituted diaminopurine nucleosides. The carboxymethyl group was introduced as the methyl ester (**10**) by treatment with the glycolate ester anion generated using DBU. Subsequent TBAF deprotection of the TBDMS group gave the target ester **11**. Base hydrolysis yielded the carboxylate sodium salt, which was converted to the free acid **1** by treatment with HCl; neutralization with ammonia gave ammonium salt **12**. Similarly prepared were the ethyl ester **13**, amide **2**, 2-(dimethylamino)-ethyl **4** and 2-hydroxyethyl **5** derivatives.

Synthesis of the *O*6-2-aminoethyl derivative **3**, required protection of the amino group of the ethanolamine reagent since reaction with the unprotected alcohol resulted solely in formation of *N6*-hydroxyethyl derivative **14**. Reaction with *N*-BOC-aminoethanol gave *O*-coupled compound **15**, which was treated first with TBAF to give BOC-intermediate **16** and then with TFA to give the required amine salt **3**. The protected 2-chloroethyl derivative **17** was prepared in a similar manner using 2-chloroethanol (Scheme 3). On warming the oil at 60 °C overnight, intramolecular cyclization resulted in formation of the tricyclic ethano adduct **18**. Indeed, ^{1}H-NMR indicated that conversion of the chloroethyl adduct, although slow, started almost immediately at room temperature. The final ethano salt **19** was obtained after TBAF deprotection.

2.2. Atase assays

The target purines were evaluated in two assays to estimate their activity as suicide and competitive inhibitors of Atase. The Atase assays used measure the transfer of radiolabeled methyl groups from ^{3}H-methylated DNA to protein, the level of tritium detected being proportional to the number of methyl groups transferred to the Cys145-thiol of the protein. The standard Atase assay was developed to measure the activity of suicide inactivators of the protein [25,26]. In this assay, a 45 min pre-incubation of Atase with the test compound is included prior to addition of the [^{3}H]-MeDNA; this provides sufficient time for the protein to recognize the inhibitor and allow it to occupy, and subsequently react with, the active site. With the addition of [^{3}H]-MeDNA and a further 2 h incubation, residual active Atase is allowed to repair ^{3}H-methyl groups. The residual activity of Atase is determined by isolating the protein and comparing the radioactivity counts transferred to the protein against those of a control reaction in the absence of any inhibitor. In this assay, an efficient inhibitor would not allow Atase to react with the [^{3}H]-MeDNA substrate. The established suicide inhibitor *O*6-benzylguanine (0.5 μM) was used as the positive control which showed 28 ± 7% residual Atase activity after 40 min reaction at 37 °C. This result is consistent with data obtained by other investigators [26,27] so allows direct comparisons to be made.

A variation on the standard suicide assay was developed to estimate the potential of the test compounds as competitive inhibitors. The suicide assay was adapted to measure the activity of Atase under virtual steady-state conditions, by eliminating the pre-incubation time and thus give a “snapshot” estimate



Scheme 3. Reagents and conditions. (i) $ClCH_2CH_2OH$, DBU, THF, 3 h, r.t.; (ii) heat 60 °C; (iii) TBAF, THF, 30 min, r.t., (53% over three steps).

of Atase activity in the presence of the inhibitor. For this assay, the inhibitor concentration was fixed at 100 μM and *O*6-benzylguanine (0.5 μM) was again used as a positive control, since there are presently no established competitive inhibitors of Atase.

The results of both assays are presented in the Fig. 1. As expected, *O*6-benzylguanine showed activity in both assays, more so in the suicide assay with a pre-incubation period (28% compared with 64% detectable residual Atase activity). For most test compounds, there was little difference in the Atase activity detected in either of the two distinct assays. In neither assay was potent interaction or reaction with the novel purines detected, despite using concentrations 200-times greater than the positive control compound, *O*6-benzylguanine. This indicates that binding to Atase is weak compared with the interaction between the protein and the second substrate, methylated DNA. Although close to the limits of the experimental error, *O*6-carboxymethyl- (**1**), aminoethyl- (**3**) and carboxamide-substituted (**2**) purines showed some activity as reversible inhibitors of Atase (Fig. 1).

The failure to detect activity for ethano derivative **19** was surprising as this compound was expected to be a suicide, rather than a competitive, inhibitor of Atase; thus mimicking the behavior of chloroethyl-modified guanine lesions generated in DNA by chloroethylating drugs, such as BCNU. However, in this case it seems likely that failure to detect activity was due to rapid hydrolysis under aqueous conditions before the investigational compound was able to encounter its protein target [28], rather than weakness of the purine–protein interaction.

2.3. Purine–Atase interactions in cells

The ability of Atase-binding purines to increase (potentate) the toxicity of DNA alkylating drugs towards Atase-proficient cells is well established [21,29–32]. To assess interactions between the novel purines and Atase in cells a simple potentation assay was performed, using the MCF 7 breast carcinoma cell line, known to express high levels of Atase. BCNU generates *O*6-alkylguanine-DNA adducts that are substrates for Atase [6,33–35], therefore any purine–Atase interactions should translate into an increase in the apparent chemosensitivity to BCNU. On the basis of the cell-free Atase data, the carboxymethyl derivative **1** was selected for evaluation. Since the acid would be ionized at the experimental pH, and anions do not cross cell membranes by passive diffusion, the methyl ester **11** was also included as a more lipophilic prodrug form that may hydrolyse to expose the carboxylate group once inside cells. Cells were exposed to the purine derivatives for 1 h, followed by exposure to BCNU (1 h), data are presented in the Table 1 as relative IC_{50} values [i.e. IC_{50} (BCNU)/IC_{50} (BCNU + purine)].

3. Discussion

In the course of this work, X-ray crystal structures of the complex of Cys145S-mutant Atase with an *O*6-methylguanine-bearing oligonucleotide and of active Atase with a substrate analogue oligonucleotide were published [36]. Signif-

Table 1
Relative in vitro cytotoxicity of BCNU in MCF7 cells (Atase and MMR proficient) in the absence and presence of selected purine derivatives

Test compound (100 μM)	BCNU relative IC_{50}[a]
Control (no additive)	1.0[b]
*O*6-Benzylguanine	1.8
11	1.6
1	1.4

[a] IC_{50}(no additive)/IC_{50}(in presence of test compound).
[b] IC_{50}(BCNU) = 236 μM.

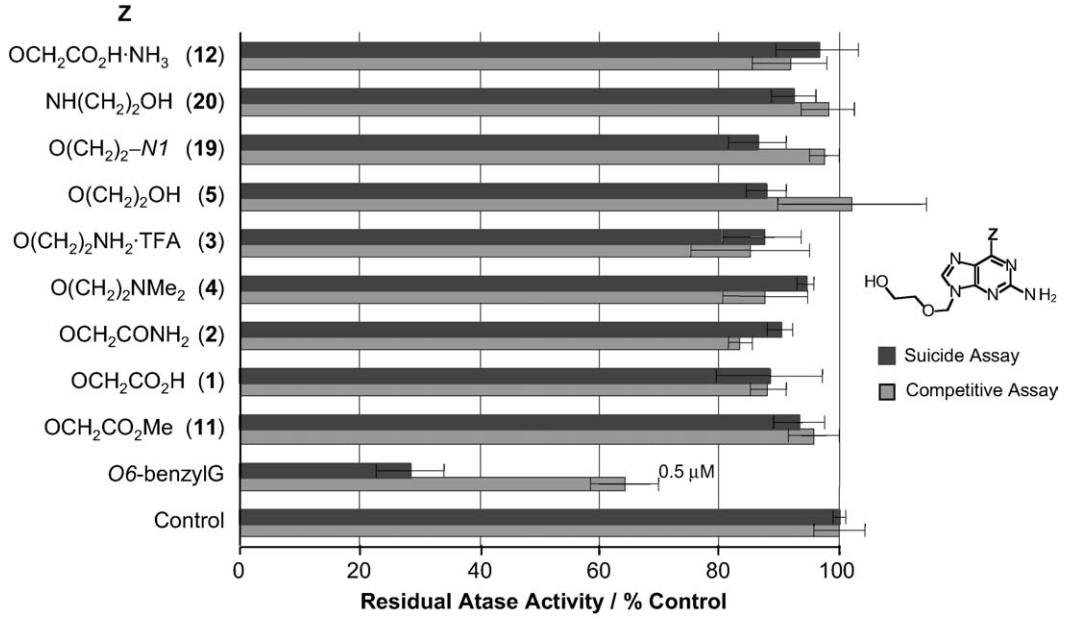


Fig. 1. Alkyltransferase assay data. Plots of the suicide and competitive inhibition assay data for test compounds at 100 μM and the positive control, *O*6-benzylguanine, at 0.5 μM. Data are the mean ± S.D. values of at least three separate determinations.

icantly, the guanine-binding active site structure of the DNA-bound protein was little different from that reported for the free protein (r.m.s. deviation 0.7–0.8 Å) and Cys-*S*-Me or Cys-*S*-benzyl derivatives [7] used as starting structures for this study. The catalytic amino acid residues, previously identified by point mutation studies, and their disposition around the *O*6-modified guanine substrate were confirmed. The proven structural and mechanistic details are entirely consistent with the data and conclusions described herein.

Nine target compounds were synthesized by modification of the 6-oxopurine position via direct mesitylation and DABCO salt formation (Scheme 2). This versatile, convenient and high-yielding synthetic route did not necessitate extensive purification of intermediates or target products. The modest activity of the *O*6-carboxymethylguanine derivative **1** in both of the cell-free Atase assays was surprising. Previous studies on *O*6-CMG-DNA have indicated it to be a potent inactivator of the reaction of Atase with [^{3}H]-Me-DNA [20]; furthermore, if the carboxymethyl group is ^{14}C-radiolabeled, there is no evidence of transfer of the *O*6-substituent group to the Atase protein [20]. Thus it appears that the Atase–*O*6-CMG-DNA interaction is sufficient to divert Atase from reaction with a [^{3}H]-Me-DNA co-substrate and disrupt the catalytic mechanism so that transfer of the *O*6-carboxymethyl group to Cys145 does not take place; i.e. *O*6-CMG-DNA is recognized but not repaired by Atase. In contrast, for simple *O*6-substituted purines (e.g. *O*6-benzylG, Patrin 2 and *O*6-MeG) protein–purine interactions *alone* are sufficient for recognition and catalytic transfer of the *O*6-substituent to Atase Cys145, albeit that the rate of reaction is usually reduced relative to repair of the equivalent lesion borne on a DNA duplex [37]. This provides the basis for the development of drug-like, small-molecule inactivators of Atase.

In the case of *O*6-CMG, it appears that the purine–protein interactions alone are weak and the additional duplex DNA–protein recognition and binding contacts are essential for the formation of a stable complex. Notwithstanding the potential for carboxylate–protein interactions (see Appendix A) the structure of Atase bound to modified DNA shows the *O*6-substituent accommodated within a pocket composed of hydrophobic amino acid sidechains. Thus it appears that potential polar or H-bonding interactions are unable to compensate for the loss of hydrophobic contributions to the binding energy. Subject to steric constraints in the active site, SAR for groups cleaved by Atase have usually been interpreted in terms of the propensity of the cleaved groups to leave during nucleophilic substitution reactions; specifically, to stabilize the incipient carbonium ion transition state [38]. Consideration must additionally be given to hydrophobic factors, which may in part explain the divergence between *O*6-(2-pyridylmethyl)guanine and benzylguanine (Atase IC_{50} = 55 and 0.2 μM, respectively) in the same assay [31]. Similar arguments may also account for the apparent weakness of interaction of the *O*6-methyl aciclovir derivatives **2**–**5** with Atase.

In the in vitro Atase assay, the presence of *O*6-benzylguanine reduced the IC_{50}(BCNU) 1.8-fold (from 236 → 131 μM). A modest potentiation was detected in the presence of the ester **11** (1.6-fold) and the acid **1** (1.4-fold), consistent with the small Atase-inhibitory activity determined for carboxymethyl purine **1** in the cell-free assay. This apparent interaction with the Atase protein must be interpreted with caution since *O*6-carboxymethylguanine has been shown to be a precursor of the suicide substrate *O*6-methylguanine during amino acid metabolism-derived carcinogenesis [19].

4. Conclusions

This study has identified a family of novel and polar purine-*O*6 derivatives, none of which are good substrates for the DNA repair protein Atase, although carboxymethyl derivative **1** may degrade in situ to an Atase-substrate form. The lack of substrate activity may relate to poor hydrophobic interactions, insufficient to drive molecular recognition within the active site binding pocket of Atase. The novel purines are mimics of DNA lesions and so the equivalent modifications on DNA are also be expected to be resistant to repair.

Overall, two conclusions significant to the design of new chemotherapeutic agents directed to guanine-*O*6 can be drawn. Firstly, Atase-mediated drug resistance may be bypassed through the development of agents to deliver the highly polar groups investigated herein to guanine-*O*6 of DNA, rather than simple alkyl groups (methyl, chloroethyl) as used currently. Of particular interest are the *O*6-(2-hydroxyethyl) and *O*6-(2-dimethylaminoethyl) modifications. Furthermore, the possibility then exists for the formation of stable complexes between Atase protein and modified DNA in cells, as apparently the case with *O*6-CMG-DNA [20] — situation that would enhance activity towards Atase-proficient cell lines by a mechanism analogous to the ternary complex formation between HMG-domain proteins and cisplatin-modified DNA, implicated in the biological activity of cisplatin [39]. In this scenario, selective toxicity *towards* Atase-proficient cells would be manifest; moreover, activity would not be dependent on expression of the MMR proteins. On this basis, alkylating chemotherapy against tumor lines hitherto inherently resistant (either actively or passively) to such drugs could be achieved.

5. Experimental procedures

5.1. Synthesis

^{1}H-NMR spectra were acquired at 270.17 MHz and ^{13}C-NMR spectra at 67.80 MHz with Me_4Si as internal standard, using a JEOL GX270 spectrometer. ^{13}C assignments were made using the DEPT135 experiment. Mass spectra were obtained from the EPSRC Mass Spectrometry Service Centre at the University of Wales, Swansea, UK. Elemental analysis data were obtained from the Advanced Chemical and Materials Analysis Unit at the University of Newcastle-upon-Tyne, UK. Melting points (mp) were determined using an Electrothermal IA9200 digital melting point apparatus. Infrared spectra were obtained using a Perkin Elmer (Paragon 1000) FT-IR instrument. TLC was performed on pre-coated plastic plates (silica

gel 60, F_{254}, MERCK) and visualized under UV light or by staining with I_2.

Aciclovir was purchased from CHEFARMA (Athens, Greece). Reagents and solvents were purchased from Aldrich (Gillingham, UK), Lancaster Synthesis (Lancaster, UK) or Avocado (Heysham, UK); other solvents were purchased from Fisher Scientific (Loughborough, UK). *O*6-Benzylguanine was prepared by adaptation of a published procedure [40].

*5.1.1. 2-Amino-9-[2-(tert-butyldimethylsilyloxy)ethoxymethyl]-1,9-dihydropurin-6-one (**8**)*

To a solution of aciclovir **6** (3 g, 13.3 mmol) in DMF (75 ml), imidazole (2.72 g, 40 mmol) and TBDMS (4 g, 26.7 mmol) were added. The mixture was stirred for 48 h at room temperature and then poured into distilled water (150 ml). The precipitate was collected by filtration, dried overnight in a vacuum oven and then recrystallized from methanol to yield **8** as colorless crystals (4.39 g, 97%); m.p. 208–212 °C. NMR data consistent with reference [22]. ^{1}H-NMR (DMSO-d_6) δ 10.58 (s, 1H, NH), 7.75 (s, 1H, 8-H), 6.41 (s, 2H, NH_2), 5.25 (s, 2H, NC$\underline{H}_2$O), 3.60 and 3.45 (2 × t, J = 4.6 Hz, 4H, OC$\underline{H}_2$C$\underline{H}_2$O), 0.75 (s, 9*H*, (C$\underline{H}_3$)$_3$CSi), –0.11 (s, 6H, 2 × C$\underline{H}_3$–Si); ^{13}C-NMR (DMSO-d_6) δ 156.5 (C-2), 154.7 (C-4), 151.2 (C-6), 137.5 (C-8), 116.8 (C-5), 72.5 (N$\underline{C}$H$_2$O), 70.1 (O$\underline{C}$H$_2$CH$_2$OTBDMS), 62.3 (OCH$_2\underline{C}$H$_2$OTBDMS), 26.1 ($\underline{C}$H$_3$CSi), 18.2 (Si–$\underline{C}$(CH$_3$)$_3$), –5.0 (CH$_3$Si). IR (KBr) 3480m (NH), 3280s (NH_2), 3190s (NH_2), 1725s (CO), 1630 s cm^{-1}; MS (FAB): *m*/*z* 340 (10%) [M + H]$^{\bullet+}$; 362 (100%) [M + Na]$^{\bullet+}$. Anal. C, H, N.

5.1.2. 2,4,6-Trimethylbenzenesulfonic acid 2-amino-9-[2-(tert-butyldimethylsilyloxy)ethoxymethyl]-9H-purin-6-yl ester (7)

Protected purine **8** (3.83 g, 11.3 mmol), 2-mesitylenesulfonyl chloride (4.92 g, 22.5 mmol), and DMAP (0.69 g, 5.65 mmol) were suspended in acetonitrile (40 ml). Triethylamine (7.4 ml, 5.3 mmol) was added drop-wise and the suspension stirred at room temperature. After 2 h, the volatile components were removed under reduced pressure. The residue was applied to a column of silica gel and eluted with $CHCl_3$:EtOH (9:1, v/v). Compound **7** was obtained as a white solid (4.67 g, 80%): m.p. 160.3 °C; ^{1}H-NMR (DMSO-d_6) δ 7.85 (s, 1H, 8-H), 7.1 (s, 2H, Ar–H), 5.55 (s, 2H, NC$\underline{H}_2$O), 4.95 (s, 2H, NH_2), 3.59 and 3.75 (2 × t, J = 4.5 Hz, 4H, OC$\underline{H}_2$C$\underline{H}_2$O), 2.78 (s, 6H, 2 × *o*-C$\underline{H}_3$-Ar), 2.32 (s, 3H, *p*-C$\underline{H}_3$–Ar), 0.85 (s, 9*H*, (C$\underline{H}_3$)$_3$CSi), –0.10 (s, 6H, 2 × C$\underline{H}_3$Si); ^{13}C-NMR (DMSO-d_6) δ 160.1 (C-6), 158.2 (C-2) 156.1(C-4), 144.8 (*p*-$\underline{C}$CH$_3$–Ar), 142.4(*o*-$\underline{C}$CH$_3$–Ar), 141.1 (C-8), 137.1 ($\underline{C}$SO$_2$–Ar), 133.5 ($\underline{C}$H–Ar), 117 (C-5), 73.7 (N$\underline{C}$H$_2$O), 71.7(O$\underline{C}$H$_2$CH$_2$OTBDMS), 63.2 (OCH$_2\underline{C}$H$_2$OTBDMS), 26.6 (Ar$\underline{C}$H$_3$), 21.8 (SiC($\underline{C}$H$_3$)$_3$), 19.0 (Si$\underline{C}$(CH$_3$)$_3$), –4.6 ($\underline{C}$H$_3$Si). IR (KBr) 3486s (NH), 3295s (NH_2), 3180s (NH_2), 1640s (NH_2), 1360s (SO), 1170s (SO); MS (FAB): *m*/*z* 522 (100%) [M + H]$^{\bullet+}$; 544 (15%) [M + Na]$^{\bullet+}$; 1043 (5%) (2 × M + H)$^{\bullet+}$; 1065 (5%) (2 × M + Na)$^{\bullet+}$. Anal. C, H, N.

*5.1.3. [2-Amino-9-(2-hydroxyethoxymethyl)-9H-purin-6-yloxy] acetic acid methyl ester (**11**)*

To a solution of **7** (3.9 g, 7.4 mmol) in dry THF (60 ml), DABCO (4.18 g, 37.3 mmol) was added and the mixture stirred at room temperature under N_2. Progress of the reaction was monitored by TLC (ethyl acetate). Disappearance of the starting material and formation of a blue fluorescent spot at the baseline indicated complete formation of the DABCO salt intermediate **9** after 4 h. Methyl glycolate (6.73 g, 74 mmol) and DBU (3.4 g, 22.4 mmol) were added and the mixture stirred at room temperature for another 3 h. Purification by column chromatography eluted with ethyl acetate yielded the TBDMS-protected methyl ester intermediate **10** (2.2 g, 72%) as a white solid: m.p. 133.4 °C; ^{1}H-NMR ($CDCl_3$) δ 7.77 (s, 1H, 8-H), 5.52 (s, 2H, NC$\underline{H}_2$O), 5.04 (s, 2H, *O*6-C$\underline{H}_2$), 4.85 (s, 2H, NH_2), 3.77 (s, 3H, OC$\underline{H}_3$), 3.74 and 3.72 (2 × t, J = 4.5 Hz, 4H, OC$\underline{H}_2$C$\underline{H}_2$O), 0.88 (s, 9*H*, (C$\underline{H}_3$)$_3$CSi), –0.05 (s, 6H, (C$\underline{H}_3$)$_2$Si); ^{13}C-NMR (DMSO-d_6) δ 170.1 ($\underline{C}$O), 160.4 (C-6), 156.3 (C-2), 155.6 (C-4), 140.2 ($\underline{C}$-8), 114.7 ($\underline{C}$-5), 73.4 (N$\underline{C}$H$_2$O), 70.7 (O$\underline{C}$H$_2$CH$_2$OTBDMS), 64.2 (O$\underline{C}$H$_2\underline{C}$H$_2$OTBDMS), 62.1 (*O*6-$\underline{C}$H$_2$), 51.7 (O$\underline{C}$H$_3$), 26.6 (SiC($\underline{C}$H$_3$)$_3$), 19.1 (Si$\underline{C}$(CH$_3$)$_3$), –4.6 ($\underline{C}$H$_3$Si). IR (KBr) 3340s (NH_2), 3210s (NH_2), 1720s (CO), 1645s cm^{-1}; MS (EI): *m*/*z* 250 (100%); 354 (80%); 412.2 (10%) [M + H]$^{\bullet+}$; MS (CI): *m*/*z* 120 (75%); 132 (95%); 159 (50%); 194 (100%); 412 (45%) [M + H]$^{\bullet+}$; HRMS 412.2019; $C_{17}H_{30}SiN_5O_5$ requires 412.2016.

To a solution of **10** (1.75 g, 4.3 mmol) in dry THF (30 ml), TBAF (4.6 ml, 1 M in THF) was added and the solution stirred at room temperature. The reaction was monitored by TLC (ethylacetate) for the loss of **10** and formation of **11**. The solution was evaporated in vacuo and the resulting oil was purified by chromatography on silica gel, eluted with ethylacetate/methanol 95:5. **11** was obtained as a pale orange crystalline solid, recrystallized from methanol (0.91 g, 71%): m.p. 124–126 °C.; ^{1}H-NMR (DMSO-d_6) δ 8.03 (s, 1H, 8-H), 6.5 (s, 2H, NH_2), 5.41 (s, 2H, NC$\underline{H}_2$O), 5.06 (s, 2H, *O*6-C$\underline{H}_2$), 4.65 (t, 1H, J = 4.8 Hz, OCH$_2$CH$_2$O$\underline{H}$), 3.68 (s, 3H, OC$\underline{H}_3$), 3.47 (s, 4H, OC$\underline{H}_2$C$\underline{H}_2$OH); ^{13}C-NMR (DMSO-d_6) δ 169 ($\underline{C}$O), 159.9 (C-6), 159.4 (C-2), 155 (C-4), 140.7 (C-8), 113.4 (C-5), 72.3 (N$\underline{C}$H$_2$O), 70.6 (O$\underline{C}$H$_2$CH$_2$OH), 61.9 (*O*6-$\underline{C}$H$_2$), 60.1 (OCH$_2\underline{C}$H$_2$OH), 52.1 (O$\underline{C}$H$_3$). IR (KBr) 3470 (OH), 3345s (NH_2), 3210s (NH_2), 1720s (CO), 1645s cm^{-1}; MS (FAB): *m*/*z* 133 (37%); 142 (36%); 242 (100%); 298 (10%) [M + H]$^{\bullet+}$; 320 (5%) [M + Na]$^{\bullet+}$. Anal. C, H, N.

*5.1.4. [2-Amino-9-(2-hydroxyethoxymethyl)-9H-purin-6-yloxy] acetic acid ethyl ester (**13**)*

To a solution of **7** (0.38 g, 0.73 mmol) in dry THF (60 ml), DABCO (0.4 g, 3.7 mmol) was added and the mixture stirred at room temperature under N_2. After 4 h, ethyl glycolate (0.76 g, 7.3 mmol) and DBU (0.34 g, 2.2 mmol) were added and the reaction continued at room temperature for another 3 h. The volatile components were removed in vacuo and the residue dissolved in dry THF (5 ml). TBAF (0.6 ml, 1M in THF) was added to the solution and the mixture stirred at room temperature for 30 min. The solvent was removed in vacuo and the

residue purified by chromatography on silica gel, eluted with ethyl acetate-methanol 95:5 v/v. Ester **13** was obtained as a white crystalline solid (0.07 g, 30%). m.p. 133–134 °C; ^{1}H-NMR ($CDCl_3$) δ 7.74 (s, 1H, 8-H), 5.47 (s, 2H, NCH_2O), 5.20 (s, 2H, NH_2), 4.98 (s, 2H, *O*6-CH_2), 4.23 (q, 2H, $J = 3.67$ Hz, OCH_2CH_3), 3.71 and 3.62 (2 × t, 4H, $J = 2.93$ Hz, OCH_2CH_2OH), 1.23 (t, 3H, $J = 7$ Hz, OCH_2CH_3); ^{13}C-NMR ($CDCl_3$) δ 168.4 (CO), 160.2 (C-6), 159.4 (C-2), 154.5 (C-4), 139.9 (C-8), 114.9 (C-5), 72.9 (NCH_2O), 70.6 (OCH_2CH_2OH), 63.0 (OCH_2CH_3), 62.7 (O*6*-CH_2), 61.2 (OCH_2CH_2OH), 14.0 (OCH_2CH_3). MS (FAB): *m/z* 312 (100%) $(M + H)^{\bullet+}$; 334 (55%) $(M + Na)^{\bullet+}$; 623 (10%) $(2M + H)^{\bullet+}$; 645 (10%) $(2M + Na)^{\bullet+}$. Anal. C, H, N.

5.1.5. [2-Amino-9-(2-hydroxyethoxymethyl)-9H-purin-6-yloxy] acetic acid (1)

Ester **11** (0.85 g, 2.7 mmol) was dissolved in aqueous NaOH solution (15 ml, 0.1 M). The solution was stirred at room temperature and the reaction was monitored by TLC. The loss of **11** and formation of a blue fluorescent spot at the baseline indicated the formation of the carboxylate sodium salt. Dilute hydrochloric acid (0.1 M) was added drop-wise until the free acid **1** precipitated from the reaction mixture. The precipitate was collected by filtration, washed with distilled water and dried in a vacuum oven to give **1** (0.71g, 88%) as a white solid: m.p. 213–215 °C; ^{1}H-NMR (DMSO-d_6) δ 12.96 (s, 1H, CO_2H), 8.03 (s, 1H, 8-H), 6.5 (s, 2H, NH_2), 5.43 (s, 2H, NCH_2O), 4.98 (s, 2H, *O*6-CH_2), 4.66 (br s, 1H, CH_2OH), 3.47 (s, 4H, OCH_2CH_2OH); ^{13}C-NMR (DMSO-d_6) δ 169.6 (CO), 159.8 (C-6), 159.5 (C-2), 154.7 (C-4), 140.4 (C-8), 113.4 (C-5), 72.1 (NCH_2O), 70.5, (OCH_2CH_2OH), 59.9 (OCH_2CH_2OH), 61.7 (*O*6-CH_2). IR (KBr) 3380s (OH), 3320s (NH_2), 3210s (NH_2), 1650m (C=O), 1485m cm^{-1}; MS (FAB): *m/z* 107 (100%); 152.1 (55%); 284 (35%) $[M + H]^{\bullet+}$; 306 (37%) $[M + Na]^{\bullet+}$. Anal. C, H, N.

5.1.6. [2-Amino-9-(2-hydroxyethoxymethyl)-9H-purin-6-yloxy] acetic acid ammonium salt (12)

Carboxylic acid **1** (0.1 g, 0.35 mmol) was dissolved in aqueous ammonia solution (15 ml, 35%) and the mixture evaporated in vacuo. The residue was re-dissolved in ethanol and evaporated three times to give salt **12** as a white solid: m.p. 218–219 °C; ^{1}H-NMR (DMSO-d_6) δ 8.00 (s, 1H, 8-H), 6.4 (s, 2H, NH_2), 5.43 (s, 2H, NCH_2O), 4.78 (s, 2H, *O*6-CH_2), 3.47 (br s, 5H, OCH_2CH_2OH; becomes br s, 4H on adding D_2O); ^{13}C-NMR (DMSO-d_6) δ 170.1 (CO), 161.2 (C-6), 160.5 (C-2), 154.2 (C-4), 139.9 (C-8), 114.1 (C-5), 72.6 (NCH_2O), 70.2, (OCH_2CH_2OH), 60.0 (OCH_2CH_2OH), 63.8 (*O*6-CH_2). IR (KBr) 3380 (OH), 3340s (NH_2), 3210s (NH_2), 1620m (C=O), 1485 (C–O) cm^{-1}. MS (ES): *m/z* 282.1 (100%) $(M)^{\bullet+}$. Anal. C, H, N.

5.1.7. 2-[2-Amino-9-(2-hydroxyethoxymethyl)-9H-purin-6-yloxy]acetamide (2)

This was prepared in an analogous series of reactions from mesitylene derivative **7** (1.49 g, 2.85 mmol). Further reaction of the DABCO salt **9** with glycolamide and DBU yielded protected acetamide **21** as a white solid (1.1 g, 88%): m.p. 199.5 °C; ^{1}H-NMR ($CDCl_3$) δ 7.79 (s, 1H, 8-H), 6.62 (br s, 2H, $CONH_2$), 5.54 (s, 2H, OCH_2N), 4.99 (s, 2H,*O*6-CH_2), 4.94 (s, 2H, NH_2), 3.75 and 3.61 (2 × t, $J = 3.7$ Hz, 4H, OCH_2CH_2O), 0.88 (s, 9*H*, $(CH_3)_3CSi$), 0.05 (s, 6H, 2 × CH_3Si); ^{13}C-NMR ($CDCl_3$) δ 170.9 (CO), 160.1 (C-6), 155.8 (C-2), 155.5 (C-4), 140.7 (C-8), 113.8(C-5), 73.6 (NCH_2O), 71.6 ($OCH_2CH_2OTBDMS$), 65.3 ($OCH_2CH_2OTBDMS$), 63.2 (*O*6-CH_2), 26.6 ($SiC(CH_3)_3$), 19.0 ($SiC(CH_3)_3$), –4.6 (CH_3Si). IR (KBr) 3325s (NH_2), 3220s (NH_2), 1690s and 1620m ($CONH_2$), 1585s (C–O) cm^{-1}; MS (FAB): *m/z* 397 (100%) $[M + H]^{\bullet+}$; 419 (20%) $[M + Na]^{\bullet+}$. Anal. C, H, N.

Deprotection of **21** (1.25 g, 3 mmol) was carried as described previously for the formation of the methyl ester (**11**) using TBAF; acetamide **2** was obtained as a white solid (0.83 g, 93%). Alternatively, ester **11** (0.5 g, 1.68 mmol) was dissolved in aqueous ammonia solution (15 ml, 35%). The solution was stirred at room temperature and the reaction was monitored with TLC (ethyl acetate/methanol 3:1 v/v). The product precipitated from the reaction mixture; the precipitate was collected by filtration, washed with distilled water and dried to yield amide **2** (0.49 g, 98%) as a white solid: m.p. 245 °C; ^{1}H-NMR ($CDCl_3$) δ 8.02 (s, 1H, 8-H), 7.39 and 7.23 (2 × br s, 2 × 1H, $CONH_2$), 6.46 (br s, 2H, NH_2), 5.42 (s, 2H, NCH_2O), 4.83 (s, 2H,*O*6-CH_2), 4.65 (t, 1H, $J = 4.0$ Hz, OH), 3.46 (m, 4H, OCH_2CH_2OH); ^{13}C-NMR ($CDCl_3$) δ 169.8 (CO), 160.1 (C-6), 160.2 (C-2), 154.9 (C-4), 140.1 (C-8), 113.2 (C-5), 73.2 (NCH_2O), 70.6 (OCH_2CH_2OH), 62.7 (*O*6-CH_2), 59.6 (OCH_2CH_2OH). IR (KBr) 3450s (OH), 3330s (NH_2), 3220s (NH_2), 1690s and 1620m ($CONH_2$), 1590s cm^{-1}; MS (FAB): *m/z* 283 (50%) $[M + H]^{\bullet+}$; 305 (20%) $[M + Na]^{\bullet+}$. Anal. C, H, N.

5.1.8. 2-[2-Amino-6-(2-dimethylaminoethoxy)purin-9-ylmethoxy]ethanol (4)

This was prepared in an analogous series of reactions from mesitylene derivative **7** (1.49 g, 2.84 mmol). The DABCO salt **9** was further reacted with 2-(dimethylamino)ethanol and DBU to yield protected (dimethylamino)ethylaciclovir **22** as a white solid (0.57 g, 48.2%): m.p. 130 °C; ^{1}H-NMR ($CDCl_3$) δ 7.72 (s, 1H, 8-H), 5.5 (s, 2H, NCH_2O), 4.88 (br s, 2H, NH_2), 4.60 (t, 2H, $J = 6.2$ Hz, *O*6-$CH_2CH_2N(CH_3)_2$), 3.72 and 3.58 (t, 2H, $J = 4.0$ Hz, $OCH_2CH_2OTBDMS$), 2.80 (t, 2H, $J = 6.2$ Hz, *O*6-$CH_2CH_2N(CH_3)_2$), 2.34 (s, 6H, $N(CH_3)_2$), 0.87 (s, 9*H*, $SiC(CH_3)_3$), –0.05 (s, 6H, $(CH_3)_2Si$); ^{13}C-NMR ($CDCl_3$) δ 162.3 (C-6), 160.1 (C-2), 154.9 (C-4), 140.0 (C-8), 116.2 (C-5), 74.1 (NCH_2O), 70.8 ($OCH_2CH_2OTBDMS$), 65.3 (*O*6-$CH_2CH_2N(CH_3)_2$), 63.4 ($OCH_2CH_2OTBDMS$), 58.7 (*O*6-$CH_2CH_2N(CH_3)_2$), 46.9 ($N(CH_3)_2$) 25.8, ($SiC(CH_3)_3$), 17.9 ($SiC(CH_3)_3$), –6.4 (CH_3Si). IR (KBr) 3300s (NH_2), 3200s (NH_2), 1660s cm^{-1}; MS (FAB): *m/z* 411 (100%) $[M + H]^{\bullet+}$; 433 (10%) $[M + Na]^{\bullet+}$; 821 (5%) $[2M + H]^{\bullet+}$; 843 (2%) $[2M + Na]^{\bullet+}$. Anal. C, H, N.

Deprotection of **22** (0.58 g, 1.35 mmol) was carried out in a similar manner as described above using TBAF; dimethylami-

no derivative **4** was obtained as a white solid (0.12 g, 28%): m.p. 132 °C; ^{1}H-NMR (DMSO-d$_6$) δ 7.97 (s, 1H, 8-H), 6.45 (br s, 2H, NH$_2$), 5.4 (s, 2H, NCH$_2$O), 4.68 (br s, 1H, OCH$_2$CH$_2$OH), 4.48 (t, 2H, J = 5.9 Hz, *O*6-CH$_2$CH$_2$N(CH$_3$)$_2$), 3.45 (br s, 4H, OCH$_2$CH$_2$OH), 2.63 (t, 2H, J = 5.9 Hz, *O*6-CH$_2$CH$_2$N(CH$_3$)$_2$; 2.20 (s, 6H, N(CH$_3$)$_2$); ^{13}C-NMR (DMSO-d$_6$) δ 160.6 (C-6), 160.3 (C-2), 154.7 (C-4), 140.1 (C-8), 113.7 (C-5), 72.1 (NCH$_2$O), 70.5 (OCH$_2$CH$_2$OH), 63.5 (*O*6-CH$_2$CH$_2$N(CH$_3$)$_2$), 59.9 (OCH$_2$CH$_2$OH), 57.6 (*O*6-CH$_2$CH$_2$N(CH$_3$)$_2$), 45.5 (N(CH$_3$)$_2$). IR (KBr) 3300s (NH$_2$), 3200s (NH$_2$), 1660 s cm^{-1}. MS (FAB): *m*/*z* 297 [M + H]$^{\bullet+}$. Anal. C, H, N.

*5.1.9. 2-[2-Amino-6-(2-hydroxyethoxy)purin-9-ylmethoxy] ethanol (**5**)*

This was prepared in an analogous series of reactions from mesitylene derivative **7** (0.24 g, 0.46 mmol). The DABCO salt **9** was further reacted with ethanediol and DBU to yield the protected hydroxyethyl derivative **23** as a white solid (0.3 g, 68%): m.p. 134 °C; ^{1}H-NMR (CDCl$_3$) δ 7.77 (s, 1H, 8-H), 5.52 (s, 2H, NCH$_2$O), 4.89 (br s, 2H, NH$_2$), 4.65 (t, 2H, J = 4.4 Hz, *O*6-CH$_2$CH$_2$OH), 4.00 (t, 2H, J = 4.4 Hz, *O*6-CH$_2$CH$_2$OH), 3.75 and 3.59 (2 × t, 4H, J = 4.4 Hz, OCH$_2$CH$_2$OTBDMS), 0.88 (s, 9*H*, SiC(CH$_3$)$_3$), 0.05 (s, 6H, (CH$_3$Si); ^{13}C-NMR (CDCl$_3$) δ 161.9 (C-6), 160.2 (C-2), 155.0 (C-4), 140.4 (C-8), 115.8 (C-5), 73.7 (NCH$_2$O), 71.5 (*O*6-CH$_2$CH$_2$OH), 69.7 (OCH$_2$CH$_2$OTBDMS), 63.2 (*O*6-CH$_2$CH$_2$OH), 62.2 (OCH$_2$CH$_2$OTBDMS), 26.5, (SiC(CH$_3$)$_3$), 19.0 (SiC(CH$_3$)$_3$), –4.5 (CH$_3$Si); MS (low FAB): *m*/*z* 384.3 (100%) [M + H]$^{\bullet+}$; 406.2 (15%) [M + Na]$^{\bullet+}$. Anal. C, H, N.

TBAF deprotection of **23** (0.12 g, 0.31 mmol) gave **5** as a white solid (0.086 g, 97%): m.p. 134 °C; ^{1}H-NMR (CDCl$_3$) δ 7.98 (s, 1H, 8-H), 6.45 (br s, 2H, NH$_2$), 5.41 (s, 2H, NCH$_2$O), 4.88 (t, 1H, J = 5.5 Hz, *O*6-CH$_2$CH$_2$OH), 4.65 (br t, 1H, OCH$_2$CH$_2$OH), 4.41 (t, 2H, J = 5.5 Hz, *O*6-CH$_2$CH$_2$OH), 3.74 (m, 2H, *O*6-CH$_2$CH$_2$OH), 3.45 (br s, 4H, OCH$_2$CH$_2$OH); ^{13}C-NMR (CDCl$_3$) δ 160.5 (C-6), 160.0 (C-2), 154.4 (C-4), 139.9 (C-8), 113.5 (C-5), 72.0 (NCH$_2$O), 70.4 (OCH$_2$CH$_2$OH), 67.5 (*O*6-CH$_2$CH$_2$OH), 59.9 (OCH$_2$CH$_2$OH), 59.3 (*O*6-CH2CH$_2$OH). IR (KBr) 3320s (NH$_2$, OH), 1612m, 1470m (C–O) cm^{-1}; MS (FAB): *m*/*z* 269 (100%) [M + H]$^{\bullet+}$; 291 (30%) [M + Na]$^{\bullet+}$. Anal. C, H, N.

*5.1.10. 2-[2-Amino-9-(2-hydroxyethoxyethyl)-9H-purin-6-ylamino]ethanol (**20**)*

Prepared analogously from mesitylene derivative **7** (1.35 g, 2.6 mmol). The DABCO salt intermediate **9** was further reacted with 2-aminoethanol and DBU to yield protected intermediate **14** as a white solid (1.1 g, 88%): m.p. 129 °C; ^{1}H-NMR (CDCl$_3$) δ 7.63 (s, 1H, 8-H), 6.2 (br s, 1H, *N6*-H), 5.47 (s, 2H, NCH$_2$O), 4.73 (br s, 2H, NH$_2$), 3.86 (t, 2H, J = 4.8 Hz, NHCH$_2$), 3.73 (m, 5H, OCH$_2$CH$_2$OTBDMS, OH), (t, 2H, J = 4.8 Hz, NHCH$_2$CH$_2$OH), 0.88 (s, 9*H*, SiC(CH$_3$)$_3$), 0.05 (s, 6H, (CH$_3$Si)); ^{13}C-NMR (DMSO-d$_6$) δ 160.5 (C-6), 155.0 (C-2), 151.5 (C-4), 137.4 (C-8), 113.0 (C-5), 71.7 (NCH$_2$O), 70.0 (OCH$_2$CH$_2$OTBDMS), 61.9 (NHCH$_2$CH$_2$OH), 60.1 (OCH$_2$CH$_2$OTBDMS), 42.3 (NHCH$_2$CH$_2$OH), 25.8 (SiC(CH$_3$)$_3$), 17.9 (SiC(CH$_3$)$_3$), –5.3 (CH$_3$Si). IR (KBr) 3440s (OH), 3340s (NH$_2$, NH), 3225s (NH$_2$), 1600s, 1455s cm^{-1}; MS (EI): *m*/*z* 255 (75%); 337 (75%); 353 (70%); 382 (100%) [M]$^{\bullet+}$; MS (CI): *m*/*z* 120 (90%); 159 (90%); 177 (100%); 383 (60%) [M + H]$^{\bullet+}$. Anal. C, H, N.

TBAF deprotection of **14** (1.78 g. 4.46 mmol) gave derivative **20** as white plates (1.15 g, 91%): m.p. 167–170 °C; ^{1}H-NMR (DMSO-d$_6$) δ 7.82 (s, 1H, 8-H), 7.03 (br s, 1H, *N6*-H), 5.89 (br s, 2H, NH$_2$), 5.36 (s, 2H, NCH$_2$O), 4.74 (br s, 1H, OH), 4.65 (br s, 1H, OCH$_2$CH$_2$OH), 3.54 (br s, 4H, *N6*-CH$_2$CH$_2$OH), 3.45 (br s, 4H, OCH$_2$CH$_2$OH); ^{13}C-NMR (DMSO-d$_6$) δ 169.8 (CONH$_2$), 160.1 (C-6), 160.2 (C-2), 154.9 (C-4), 140.1 (C-8), 113.2 (C-5), 73.2 (NCH$_2$O), 70.6 (OCH$_2$CH$_2$OH), 62.7 (*N6*-CH$_2$), 59.6 (OCH$_2$CH$_2$OH). IR (KBr) 3450s (OH), 3330s (NH$_2$), 3325s (NH), 3220s (NH$_2$), 1605m, 1610m, 1465m (C–O) cm^{-1}; MS (FAB): *m*/*z* 269 (100%) [M + H]$^{\bullet+}$. Anal. C, H, N.

5.1.11. 2-[2-Amino-6-(2-aminoethoxy)purin-9-ylmethoxy] ethanol (3)

This was prepared in an analogous series of reactions from mesitylene derivative **7** (2.1 g, 4 mmol). The DABCO salt **9** was further reacted with *N*-BOC-aminoethanol and DBU to yield fully-protected intermediate **15** as a colorless oil (91%): ^{1}H-NMR (DMSO-d$_6$) δ: 7.97 (s, 1H, 8-H), 7.02 (br t, 1H, NH-BOC), 6.44 (br s, 2H, NH$_2$), 5.41 (s, 2H, NCH$_2$O), 4.35 (t, 2H, J = 5.5 Hz, *O*6-CH$_2$), 3.62 and 3.51 (2 × t, J = 4.8 Hz, 4H, OCH$_2$CH$_2$OTBDMS), 3.33 (br t, 2H, *O*6-CH$_2$CH$_2$NH-BOC), 1.36 (s, 9*H*, OC(CH$_3$)$_3$), 0.80 (s, 9*H*, (CH$_3$)$_3$CSi), –0.02 (s, 6H, CH$_3$Si); ^{13}C-NMR (DMSO-d$_6$) δ 160.3 (CO), 160.0 (C-6), 155.7 (C-2), 154.5 (C-4), 139.9 (C-8), 113.5 (C-5), 77.8 (OC(CH$_3$)$_3$), 72.1 (NCH$_2$O), 70.2 (OCH$_2$CH$_2$OTBDMS), 64.4 (*O*6-CH$_2$CH$_2$N), 61.9 (OCH$_2$CH$_2$OTBDMS), 40.4 (*O*6-CH$_2$CH$_2$N), 28.2 (OC(CH$_3$)$_3$), 25.8, (SiC(CH$_3$)$_3$), 17.9 (SiC(CH$_3$)$_3$), –6.4 (CH$_3$Si). IR (KBr) 3515s (OH), 3445s (NH), 3300s (NH$_2$), 3190s (NH$_2$), 1710s (CO), 1630s, 1580s cm^{-1}: MS (ES): *m*/*z* 107 (75%); 153 (100%); 191 (80%); 505 (30%) [M + Na]$^{\bullet+}$; 987 (5%) [2M + Na]$^{\bullet+}$. Anal. C, H, N.

TBAF deprotection of **15** (1.4 g, 3.6 mmol) gave BOC-protected amine **16** as a white solid (0.95 g, 98%): m.p. 167–170 °C; ^{1}H-NMR (DMSO-d$_6$) δ 8.0 (s, 1H, 8-H), 7.06 (br t, 1H, NH–BOC), 6.49 (br s, 2H, NH$_2$), 5.42 (s, 2H, NCH$_2$O), 4.70 (br t, 1H, OCH$_2$CH$_2$OH), 4.41 (t, 2H, J = 5.9 Hz, *O*6-CH$_2$CH$_2$NH), 3.47 (br s, 4H, OCH$_2$CH$_2$OH), 3.35 (br t, 2H, *O*6-CH$_2$CH$_2$NH), 1.38 (C(CH$_3$)$_3$); ^{13}C-NMR (DMSO-d$_6$) δ 160.3 (CO), 160.0 (C-6), 155.7 (C-2), 154.5 (C-4), 139.9 (C-8), 113.5 (C-5), 77.8 (C(CH$_3$)$_3$), 72.0 (NCH$_2$O), 70.4 (OCH$_2$CH$_2$OH), 64.4 (*O*6-CH$_2$CH$_2$NH), 59.9 (OCH$_2$CH$_2$OH), 40.4 (*O*6-CH$_2$CH$_2$NH–BOC), 28.2 (C(CH$_3$)$_3$ (BOC)). IR (KBr) 3510s (OH), 3440s (NH), 3300s (NH$_2$), 3200s (NH$_2$), 1715s (CO), 1630s, 1590s, 1470s (C–O) cm^{-1}; MS (FAB): *m*/*z* 165 (30%); 226 (25%); 369 (10%) [M + H]$^{\bullet+}$; 391 (5%) [M + Na]$^{\bullet+}$. Anal. C, H, N.

For BOC deprotection, **16** (0.225 g, 0.58 mmol) was dissolved in TFA (10 ml, 10% in CH$_2$Cl$_2$) and the solution stirred at room temperature for 10 min. The reaction was monitored

by TLC (ethyl acetate/methanol, 95:5) for the loss of **16** and formation of **3**. The reaction volume was reduced by $^2/_3$ and ether added. A white precipitate resulted; the reaction solvents were decanted and ethyl acetate (10 ml) was added. The solid was stirred with ethyl acetate for 30 min and then filtered to collect TFA salt **3** as a white solid (0.12 g, 72%): m.p. 245 °C; ^{1}H-NMR (DMSO-d_6) δ 8.09 ($N\underline{H}_3^+$), 8.04 (s, 1H, 8-H), 6.55 (br s, 2H, NH_2), 5.43 (s, 2H, $NC\underline{H}_2O$), 4.58 (t, 2H, $J = 5.5$ Hz, *O*6-$C\underline{H}_2CH_2NH_3^+$), 3.45 (br s, 4H, $OC\underline{H}_2C\underline{H}_2OH$), 3.29 (br s, 2H, *O*6-$CH_2C\underline{H}_2NH_3^+$); ^{13}C-NMR (DMSO-$d_6$) δ 159.9 (C-6), 159.8 (C-2), 154.7 (C-4), 140.3 (C-8), 113.4 (C-5), 72.1 (NCH_2O), 70.6 (OCH_2CH_2OH), 62.8 (*O*6-$\underline{C}H_2CH_2NH_3^+$), 59.9 ($OCH_2\underline{C}H_2OH$), 38.4 (*O*6-$CH_2\underline{C}H_2NH_3^+$). IR (KBr) 3390s ($NH_2$), 3340s ($NH_2$), 3220s ($NH_2$), 2990s ($NH_2$), 1650s, 1590s, 1470s cm^{-1}; MS (EI): *m*/*z* 269.2 (100%) $[M + H]^{\bullet+}$; MS (CI): 113 (100%) (CF_3CO_2). Anal. C, H, N.

*5.1.12. 5-Amino-3-(2-hydroxyethoxymethyl)-7,8-dihydro-3H-oxazolo[2,3-i]purin-6-ylium chloride (**19**)*

This was prepared in an analogous series of reactions from mesitylene derivative **7** (0.54 g, 1.03 mmol). The DABCO salt **9** was further reacted with 2-chloroethanol and DBU to yield a mixture of 6-(2-chloroethoxy) intermediate **17** and the cyclized product **18** as an oil after flash column chromatography on silica eluted with ethyl acetate. The oil was warmed overnight at 60 °C to complete the conversion. The crude product was analyzed and used without further purification:

17: ^{1}H-NMR ($CDCl_3$) δ 7.75 (s, 1H, 8-H), 5.51 (s, 2H, NC$\underline{H}_2$O), 4.99 (br s, 2H, NH_2), 4.73 (t, 2H, $J = 6.2$ Hz, *O*6-$C\underline{H}_2CH_2Cl$), 3.87 (t, 2H, $J = 6.2$ Hz, *O*6-$CH_2C\underline{H}_2Cl$), 3.73 and 3.57 (2 × t, 4H, 5.1 Hz, $OC\underline{H}_2C\underline{H}_2O$), 0.87 (s, 9*H*, ($C\underline{H}_3$)$_3$CSi), 0.04 (s, 6H, ($C\underline{H}_3$)$_2$Si);

18: ^{1}H-NMR ($CDCl_3$) δ: 7.68 (s, 1H, 8-H), 5.46 (s, 2H, NC$\underline{H}_2$O), 5.12 (br s, 2H, NH_2), 4.41 (t, 2H, $J = 5.5$ Hz, *O*6-$C\underline{H}_2$), 4.00 (t, 2H, $J = 5.5$ Hz, $C\underline{H}_2N^+$), 3.75 and 3.61 (2 × t, 4H, $J = 4.4$ Hz, $OC\underline{H}_2C\underline{H}_2$OTBDMS), 0.89 (s, 9*H*, ($C\underline{H}_3$)$_3$CSi), 0.06 (s, 6H, ($C\underline{H}_3$)$_2$Si); MS (FAB): *m*/*z* 366 (25%) $[M–Cl]^{\bullet+}$, 402 (65%) $[M + H]^{\bullet+}$, 424 (60%) $[M + Na]^{\bullet+}$. Anal. C, H, N.

TBAF deprotection of **18** (0.273 g, 1.22 mmol) was carried out under anhydrous conditions, as described above, to yield **19** as a white crystalline solid (0.1 g, 53%); ^{1}H-NMR (DMSO-d_6) δ 7.88 (br s, 2H, NH_2), 7.81 (s, 1H, 8-H), 5.34 (s, 2H, NC$\underline{H}_2$O), 4.65 (br t, 1H, $OCH_2CH_2O\underline{H}$), 4.04 (t, 2H, $J = 8.6$ Hz, *O*6-$C\underline{H}_2CH_2N^+$), 3.64 (t, 2H, $J = 8.6$ Hz, *O*6-$CH_2C\underline{H}_2N^+$), 3.45 (br s, 4H, $OC\underline{H}_2C\underline{H}_2OH$); ^{13}C-NMR (DMSO-$d_6$) δ 156.8 (C-6), 155.5 (C-2), 151.1 (C-4), 137.8 (C-8), 116.8 (C-5), 72.1 (NCH_2O), 70.4 (OCH_2CH_2OH), 59.9 ($OCH_2\underline{C}H_2OH$), 42.3 (*O*6-$\underline{C}H_2CH_2N^+$), 40.5 (*O*6-$CH_2\underline{C}H_2N^+$). IR (KBr) 3230s br (NH_2, OH), 1680s cm^{-1}; MS (CI): *m*/*z* 252 (80%) $[M – Cl]^{\bullet+}$. Anal. C, H, N.

5.2. Alkyltransferase assays

N-[^{3}H]-methylnitrosourea was purchased from Amersham Biosciences, UK. Calf thymus DNA, ampicillin, isopropyl β-D-thiogalactopyranoside (IPTG), PMSF, polyethyleneimine and dithiothreitol (DTT) were purchased from Sigma (Poole, UK). *E. coli* K12 strain JM101 for the overproduction of the human Atase protein (hAtase) was provided by Dr. P.C.E. Moody (Department of Biochemistry, University of Leicester, UK) with the agreement of Dr. P. Karran (Clare Hall Laboratories, Cancer Research UK). For centrifugation, a Sorval GS_3 centrifuge was used. A Packard (1900CA TRI-CARB) liquid scintillation analyzer and Optiphase Hisafe 3 scintillation cocktail (Perkin Elmer, Beaconsfield, UK) were used for ^{3}H counting. Absorbance measurements were carried out using a Beckman DU-640 UV spectrophotometer.

5.2.1. Preparation of hAtase protein

E. coli K12 strain JM101 were grown in L-broth medium containing ampicillin and crude cell extracts containing hAtase were prepared following published procedures [41,42]. The final hAtase protein stock solution was brought to 15% v/v glycerol, aliquoted and stored at –70 °C; protein content was determined (2.53 mg ml^{-1}) using a Protein Assay Kit (BioRad) with a BSA standard based upon the method of Bradford [43].

5.2.2. Preparation of the [3H]-methyl labeled DNA substrate

[^{3}H]-MeDNA was prepared by adaptation of a published method [44]. Calf thymus DNA (200 mg) was treated with *N*-[^{3}H]-methylnitrosourea (0.2 mCi, 22 mCi mmol^{-1}) in 40 ml buffer (85 mM Tris–Cl, pH 8.0) at r.t. for 2 h. DNA was precipitated with NaCl (final conc. 0.1 M) and 2.5 volumes of ice-cold ethanol, washed three times with 70% ethanol and once with absolute ethanol. The [^{3}H]-MeDNA was dissolved in assay buffer (30 ml, 10 mM Tris–HCl, pH 8.0; 1 mM EDTA pH 8.0) and dialysed against Buffer A (0.1 M NaCl, 0.01 M sodium citrate, 0.01 M potassium phosphate, pH 7.4, 4 °C, 24 h). The DNA solution was incubated at 80 °C for 16 hours and then dialysed sequentially against Buffer B (1 M NaCl, 0.1 M Tris–HCl, 1 mM EDTA, pH 8.0, 4 °C, 24 h), and Buffer C (10 mM Tris–HCl, 1 mM EDTA, pH 8.0, 4 h, 4 °C). The final [^{3}H]-MeDNA stock solution (173 μg ml^{-1}, determined spectrophotometrically at 260 nm) with a specific activity of 41.86 cpm μg^{-1}, was aliquoted and stored at –20 °C.

5.2.3. Suicide inhibition assay

The activity of the newly synthesized compounds was assayed using a modified version of a published method [25]. Assay mixtures (total volume 650 μL) contained 225 μl assay buffer (40 mM DTT, 4 mM EDTA, 200 μM spermidine, 20% glycerol, 200 mM Hepes, 10 μg ml^{-1} BSA), 266 μl Tris–HCl buffer (50 μM Tris–HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT), BSA (50 μl, 10 μg ml^{-1}), 100 μl hAtase stock solution and 9 μl test compound (7.22 mM in DMSO, to give a final concentration of 100 μM in the assay mixture). The assay mixture was pre-incubated at 37 °C for 45 min; reaction was then initiated by addition of [^{3}H]-MeDNA (250 μl) and incubation continued for a further 2 h. The enzyme reaction was terminated by addition of ice-cold $HClO_4$ (300 μl, 1 M), the samples were vortexed rapidly, stored on ice for 30 min and centrifuged (9000 × *g*) for 15 min. The supernatants were discarded and the pellets washed twice with $HClO_4$ (300 μl, 0.25 M) followed by centrifugation (9000 × *g*, 10 min, 4 °C) after

each wash. After removing all traces of the supernatant, the washed pellets were treated with HCl (300 μl, 0.1 M) and the resulting suspension heated at 70 °C for 30 min, with frequent mixing, to hydrolyze residual [^{3}H]-MeDNA. The resulting samples were centrifuged (9000 × *g*, 10 min) and the hydrolysis step repeated on the pellets. Finally, the washed pellets containing the [^{3}H]-Me-Atase were dissolved in warm NaOH (400 μl, 0.1 M) and incubated at 60 °C for 5 min. The resulting mixtures were transferred to scintillation vials, diluted with scintillation (4 ml) cocktail and measured in a scintillation counter. Optimization experiments were undertaken to determine appropriate protein concentration and incubation time for the assay; positive control experiments contained 0.5 μM *O*6-benzylguanine in place of the test compound.

5.2.4. Competitive inhibition assay

All assay mixtures were prepared as described for the suicide inhibition assay but the reaction mixtures were not preincubated. [^{3}H]-MeDNA was added at the same time as the other reagents and incubations were performed at 37 °C for 45 min. The reactions were terminated and worked up as described above.

Acknowledgments

D.P. was supported by a University of Bradford postgraduate studentship; M.C.B. is supported by Cancer Research UK, grant number A2580; T.C.J. was supported by a Yorkshire Cancer Research (YCR) programme grant. The authors thank Dr. P.C.E. Moody (Department of Biochemistry, University of Leicester, UK) and Dr. P. Karran (Clare Hall Laboratories, Cancer Research UK) for a gift of *E. coli* K12 strain JM101 over-expressing hAtase, and Dr. L.J. Adams (YCR Laboratory of Drug Design, University of Bradford, UK) for helpful assistance with molecular modeling.

Appendix A. Supplementary material

Supplementary Material Available: doi:10.1016/j.ejmech.2005.11.007. Molecular models of *O*6-functionalized target purine molecules **1**–**5** with hAtase, tabulated data from the Atase assays.

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