O^6-Alkylguanine-DNA Alkyltransferase Inactivation in Cancer Chemotherapy

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Abstract: The protein *O*^{6}-alkylguanine-DNA alkyltransferase is the basis of an important process for repairing damage to cellular DNA, which renders cells resistant to drugs that alkylate at the *O*^{6}-position of guanine residues. The development of various pseudosubstrates which inactivate this protein is reviewed, from a chemical standpoint. Study of the influence of pseudosubstrate molecular structure on their interaction with the active site cysteine has progressed together with direct investigation of protein structure. Combination therapy using a powerful inactivator with a suitable alkylating agent shows great clinical promise in the treatment of cancer, particularly when some degree of selectivity is possible.

INTRODUCTION

Chemical alteration of vital biological macromolecules by the action of alkylating agents was one of the earliest principles to be successfully exploited in cancer chemotherapy. The lesions produced were often cytotoxic, destroying cancer cells and producing encouraging responses in the treatment of various neoplastic diseases. More recently, the mechanisms by which these drugs exert their effects have become better understood, and in particular the sites of alkylation of DNA have been intensively studied. N-7 of guanine residues is a principal target of the classical nitrogen mustards (*e.g.*, mechlorethamine, melphalan, cyclophosphamide) and sulfonates (busulfan) [1]. O^6 of guanine (**1**) is attacked less frequently but is nevertheless of great importance in the action of nitrosoureas and related methylating agents; cyclophosphamide has also recently been suggested to be significantly involved at this site, probably *via* acrolein, one of its metabolites [2].

A number of repair processes [3] have also been discovered, by which cells circumvent the damage to DNA and effectively provide resistance to the cytotoxic effects. One example is base excision repair (BER) in which certain damaged bases are processed and the resultant single-strand breaks are re-joined, with the enzyme poly(ADP-ribose) polymerase (PARP) playing an important role [4]. Nucleotide excision repair (NER) is biochemically distinct, acting on more distorting lesions in DNA and involving the removal of a section of the DNA strand containing the damage [5]. Lesions caused by alkylation of the *O*^{6}-position of guanine are repaired by the protein *O*^{6}-alkylguanine-DNA alkyltransferase (ATase) [6] which removes the alkyl group in an autoinactivating, stoichiometric process (see below), causing resistance to the cytotoxic, mutagenic and recombinogenic effects of the alkylating agents. Against *O*^{6}-methylating agents like temozolomide ATase activity is the first line of defence, but since the cytotoxic effects are mediated by the mismatch repair system, if this is deficient, the cells are also resistant to temozolomide in a manner overriding any effect of ATase. On the other hand, *O*^{6}-(2-chloroethylating) agents such as carmustine (BCNU) lead to inter-strand cross-links and the resulting cytotoxicity does not involve the mismatch repair process [7].

Suppression of the repair processes, ideally with specificity for malignant over normal cells, maintains the vulnerability of cellular DNA to drugs and can enhance their biological effects. DNA repair inhibition is thus a major goal of research in cancer chemotherapy. Some 9-[(acridin-9-yl)aminoalkyl]adenine derivatives [8] have been identified which inhibit the BER system and potentiate the cytotoxicity of carmustine, and certain benzimidazole-4-carboxamides act strongly against PARP [9], potentiating the cytotoxicity of temozolomide and topotecan; some phthalazinones and related lactams are also good inhibitors of PARP [10]. The marine natural product ecteinascidin 743 which reversibly alkylates N^2 of guanine [11] has recently been shown to exert its anti-tumour effect by incapacitating the NER system [12]. Inactivation of the alkyltransferase ATase is a powerful clinical strategy which has been achieved in a number of ways, and this review addresses the range and synthesis of small molecules which react with the protein as pseudosubstrates, abolishing its activity. A comprehensive review of research on ATase in 1995 emphasised the biochemical aspects [13].

1. EARLY APPROACHES TO INACTIVATION OF ATASE

Attenuation of ATase activity was first approached by prior *O*^{6}-methylation of guanine residues in cellular DNA, in a study of the cytotoxicity of *N*-(2-chloroethyl)-*N*-nitrosoureas (CNUs) [14] . The *O*^{6}-(2-chloroethyl)guanine residues initially formed by these agents are removed before they proceed to form lethal inter-strand cross-links in certain cell lines (Mer$^+$, "methylation repair"). Such proficient cell lines contain ATase, while deficient (Mer$^-$) lines lacking ATase cannot repair the initial guanine-*O*^{6} lesion and cross-links are formed. Pre-treatment of Mer$^+$ cells (human colon carcinoma HT29) by certain methylating agents saturated the repair system and permitted a striking vulnerability to

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subsequently-applied CNU. The agents (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-methyl-*N*-nitrosourea, streptozocin, methyl methanesulfonate) used in this way increased the cytotoxicity to varying degrees, and in due course when the cells had synthesised fresh ATase, they became resistant once more to CNU. However, the doses of these methylating agents necessary to inactivate ATase adequately *in vivo* are already toxic, and the combination with 2-chloroethylating agents results in unacceptable toxicity.

It was then found [15] that direct exposure of the cells to O^6-methylguanine (**2**) as a free base effectively depleted the repair system. This was evidently not due to blocking the synthesis of the protein but was brought about by the O^6-methylguanine reacting with ATase as a pseudosubtrate. Other O^6-alkylguanines (ethyl, propyl, butyl) also showed some activity, while the isopropyl and 2-hydroxyethyl derivatives were much less active. Such pre-treatment of cells with O^6-methylguanine at non-toxic doses enhanced the cytotoxicity of 2-chloroethylating agents [16]. However, the potential clinical usefulness of this finding was negated by the failure to reproduce it *in vivo* (human tumour xenografts in nude mice) even with large doses of this relatively insoluble pseudosubstrate [17]. Subsequent progress was made by replacing the O^6-methyl group by various activated groups, both on an empirical basis and consistent with emerging knowledge of the structure of the ATase protein and its reaction mechanism.

The reaction of the protein with O^6-methylguanine generated in DNA by a radioactive methylating agent takes place at a specific cysteine residue. Labelled *S*-methylcysteine can be identified in the protein hydrolysate in a cell-free experiment [18], and the damaged DNA is returned to the unmodified state; ATase reacts in a similar manner with O^6-methylguanine as a pseudosubstrate. The methyl group is irreversibly transferred in a stoichiometric process to the protein which can no longer react, *i.e.* is autoinactivated. The O^6-methylguanine/thiol reaction is a bimolecular displacement (S_N2 type) [19], with guanine as leaving group, X; the thiol group, especially as anion, is a very good (soft) nucleophile effective in S_N2 substitutions:

$$MeX + (ATase)\text{-}S^- \rightarrow (ATase)\text{-}SMe + X^-$$

Determination of the amino-acid sequence in ATase was first achieved for the *E.coli* ada protein, and the active centre cysteine was located in the unusual sequence Pro. Cys. His, preceded by a very hydrophobic region [20]. The structure of similar proteins from other bacterial, yeast, and mammalian sources showed that this macromolecular family, identified in at least 28 different species, in all cases contains a Pro. Cys. His. Arg sequence at the active site [21]. The crystal structure of the ada protein was determined by Moody and collaborators in 1994 [21], and the more difficult problem of the human protein solved in 2000 [22]. The specific cysteine residue (Cys 145 in the 207 amino-acid human protein, m.w. ~ 22,000) is located in a somewhat buried pocket, considerably hydrophobic in nature, and neighbouring amino-acid residues facilitate the conformational change necessary for the cysteine to carry out nucleophilic attack on the target methyl group as suggested earlier [23, 24]. It has recently been possible [25] to extend the X-ray examinations to crystallised *S*-methylated (and *S*-benzylated) human protein. The schematic representation in Fig. (**1**) of the reaction sequence when ATase removes O^6-methyl groups from alkylated DNA indicates the role of critically-located amino-acid residues. Moreover, certain structural sequences in the otherwise similar proteins of the ATase family influence their individual ability to react with various substrates.

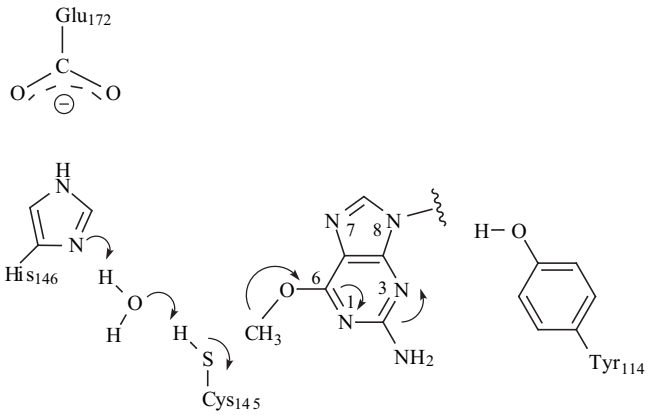


Fig. (1). Possible mode of action of human ATase in removing O^6 -methyl groups from alkylated guanine residues in DNA.

We now consider developments with O^6-methylguanine as starting point. While comparisons made throughout this review are valid, absolute I_{50} values recorded for individual drugs depend on the particular laboratory's assay method, specified in the Tables.

2. ACTIVATION OF O^6-METHYL BY ARYL (O^6-BENZYL GU AND O^6-ARYL•CH_2 GU)

Inactivation of ATase by O^6-alkylguanines decreased rapidly with increasing length of the alkyl chain [15]. O^6-Phenylguanine was inactive [26]. Initially it seemed unlikely

Table 1. ATase-Inactivating Activity [26,28] of O^6-(Substituted Benzyl)guanines (3)

Substituent on O^6-Benzyl, R	$I_{50}(\mu m)^a$
H	0.2
4-F	0.2
4-Cl	0.2
4-Me	0.2
4-Br	0.3
4-Ph	0.3
4-CH_2OH	0.3
4-CHO	0.5
4-$CHMe_2$	0.5
3,5-Me_2	1.0
4-$(CH_2)_3Me$	4.0

[a] I_{50} in Tables 1,8,11 is the concentration of inactivator required to produce 50% reduction in ATase activity in a standard assay after incubation for 30 min in cell-free extracts.

that the activity of O^6-methylguanine could be improved on, but O^6-benzylguanine (**3**; R=H) (I_{50} 0.2 μM) emerged as an inactivator about 2,000 times as effective as O^6-methyl [27,28]. A variety of benzene ring substituents (4-Me,4-Pr^i,4-F, 4-Cl, 4-Br, 4-CHO, 4-CH_2OH, 4-Ph) did not materially alter the activity (I_{50} 0.2-0.5 μM) (Table 1), but a longer alkyl chain did (4-Bu, I_{50} 4.0 μM) [26,28]. Investigation of the influence of substituent position in the ring revealed that *ortho* (2-F, 2-CF_3) gave O^6-benzyl derivatives almost devoid of activity, and the 3-CF_3 (*meta*) compound was also very weak [29]. Fusion of another benzene ring yielded a still very active drug in O^6-(2-naphthylmethyl)guanine(**4a**) , but the 1-naphthyl isomer in which the second ring is effectively an *ortho*-substituent proved almost inactive [29]. Reaction of 2-amino-6-chloropurine with alkoxide in excess alcohol or *tert*-butanol or dioxan gave these O^6-substituted guanines with some difficulty.

These results confirmed our own wide experience (Tables 2 and 3) with ring-substituted and ring-fused O^6-benzylguanines [30,31]. In our assay, O^6-benzylguanine has I_{50} 0.04 μM, while 2-methylbenzyl and 1-naphthylmethyl as O^6-substituents give I_{50} respectively 58 and 95 μM. The effect is evidently steric, determined by the constraints of the ATase active pocket; 2- and 4-methoxybenzyl derivatives, with strong electron-donating groups, have I_{50} 58 and 0.15 μM. O^6-(α-Methylbenzyl)guanine (**5**) (I_{50} >60 μM) further illustrates the significance of steric crowding near the purine ring for pseudosubstrates. No apparent correlation could be established between the degree of ATase inactivation and the electronic nature of the substituent on benzyl. However, the X-ray data for *S*-benzylated human ATase [25] provide a rationale for the observed sequence of activity in *para-, meta-* and *ortho*-substituted benzyl derivatives.

Table 2. ATase-Inactivating Activity [30,31] of O^6-(Substituted Benzyl)guanines (3)

Substituent on O^6-Benzyl, R	Derived Guanine	I_{50} (μm)[a]
4-$MeSO_2$	B.4399	0.018
3-Ph	B.4393 (**26**)	0.019
3,4-OCH_2O	B.4212	0.02
3-Br	B.4355	0.022
4-MeS	B.4398	0.024
4-MeSO	B.4404	0.03
H		0.04
4-I	B.4483	0.045
3-I	B.4403	0.05
3-MeS	B.4415	0.12
4-MeO	B.4411	0.15
4-Br		0.15
3,4-$(MeO)_2$	B.4412	0.23
3,4-$(CH_2)_4$	B.4417	0.3
3-MeO	B.4408	1.1
2-F	B.4284	6
2-I	B.4402	9.5
3,4-OCH_2O[b]	B.4228	50
2-MeO	B.4231	58
2-Me	B.4218	58
α-Me	B.4214 (**5**)	>60

[a]I_{50} in Tables 2,3,5,6,7, 9 and 13 is the concentration of inactivator required to produce 50% reduction in ATase activity after incubation for 1h with purified ATase. [b]S^6-Benzyl in S^6-benzyl-6-thioguanine.

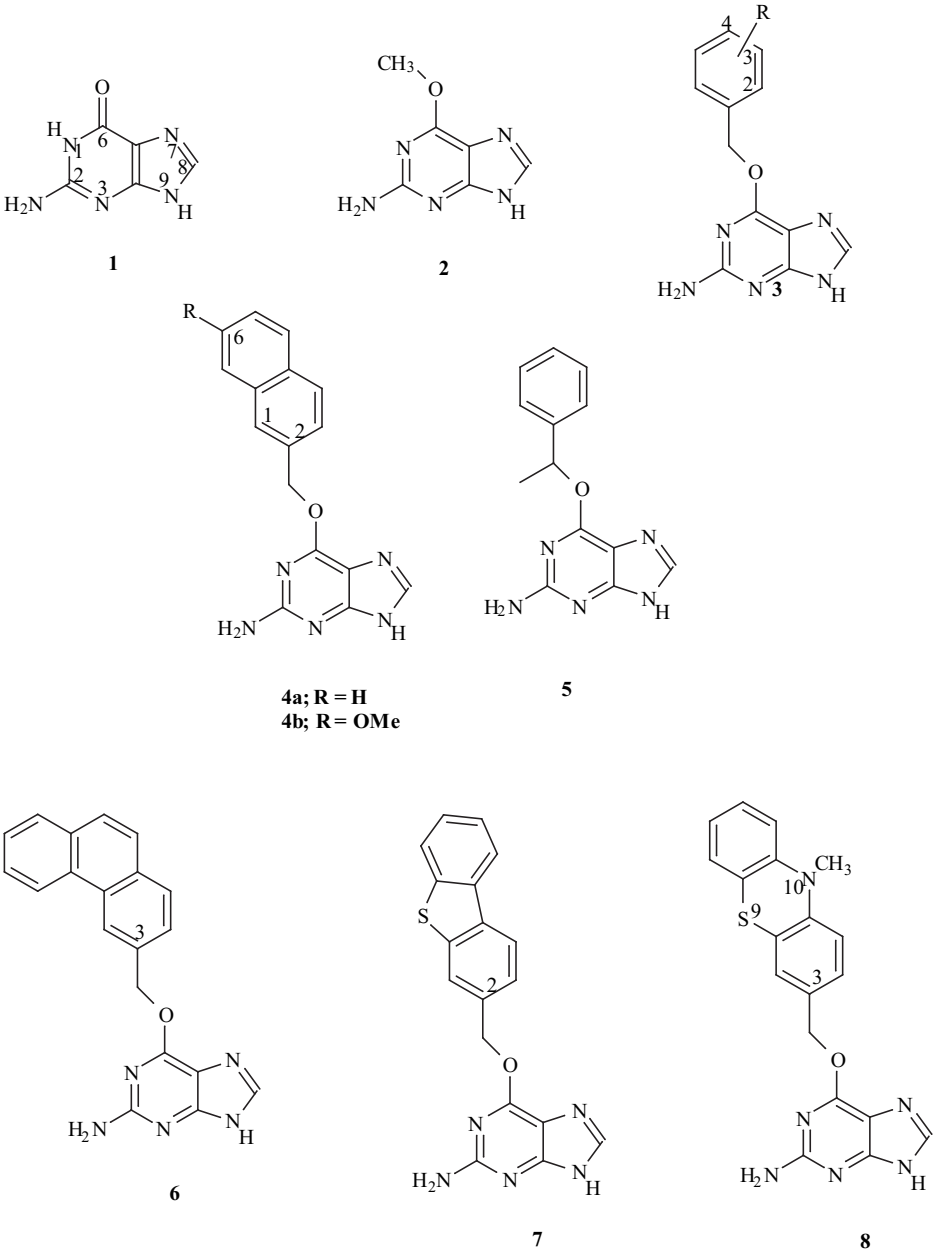
Table 3. ATase-Inactivating Activity [30,31] of O^6-(Polycyclyl)guanines

O^6-Substituent	Derived Guanine	I_{50}(μm)[a]
6-methoxy-2-naphthylmethyl	B.4394 (**4b**)	0.011
3-phenylbenzyl	B.4393 (**26**)	0.019
benzyl (for reference)		0.04
dibenzothien-2-ylmethyl	B.4419 (**7**)	0.075
10-methylphenothiazin-3-ylmethyl	B.4395 (**8**)	0.09
2-naphthylmethyl	B.4213 (**4a**)	0.15
5,6,7,8-tetrahydro-2-naphthylmethyl	B.4417 (**18**)	0.3
3-phenanthrylmethyl	B.4405 (**6**)	0.55
2-phenanthrylmethyl	B.4406	0.72
1-naphthylmethyl	B.4265 (**22**)	95

[a]See Table 2.

Apart from the *ortho*-effect, we found [30, 31] generally high activity with a variety of substituted benzyl compounds. Sulfur substituents fared considerably better than oxygen (4-MeO, 4-MeS, 4-$MeSO_2$ gave I_{50} 0.15, 0.024, 0.018 μM; and 3-MeO, 3-MeS 1.1, 0.12 μM respectively). Bicyclic substitution was actually helpful *e.g.* 3,4-$(MeO)_2$, 3,4-(OCH_2O) (*i.e.,* piperonyl), 2-naphthylmethyl (in **4a**), and 6-methoxy-2-naphthylmethyl (in **4b**) gave I_{50} 0.23, 0.02, 0.15, 0.011 μM. Even tricyclic substituents were acceptable, with 3-phenanthrylmethyl (in **6**) showing I_{50} 0.55 μM. Again sulfur enhanced the activity; the dibenzothien-2-ylmethyl (**7**) and 10-methylphenothiazin-3-ylmethyl (**8**) derivatives had I_{50} 0.075 and 0.09 μM respectively. All these compounds were readily prepared by the action of alkoxide in DMSO at room temperature on the quaternary salt (see Section 4) from trimethylamine and 2-amino-6-chloropurine [19].

Some substituted O^6-benzylguanosines (4-Br, 4-I, 2-I, α-Ph) were prepared [32] in an effort to improve water-solubility. The solubility of the starting peracetylated guanosine in dichloromethane permitted O^6-(2-mesitylenesulfonation) followed by quaternary salt formation using *N*-methylpyrrolidine and displacement by the substituted benzyl alcohol in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU); the corresponding O^6-benzylguanines were not considered in this work.



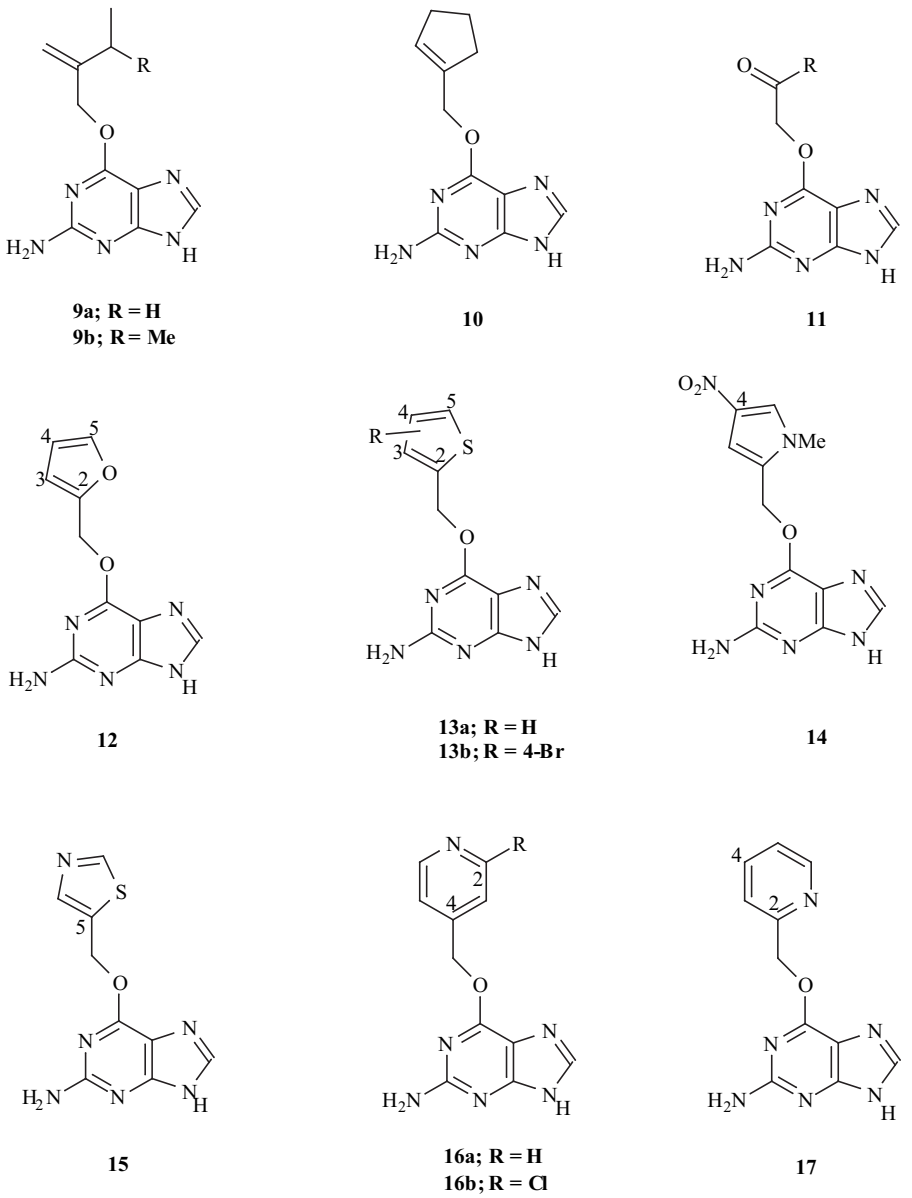
Scheme 1.

However, O^6-(4-fluorobenzyl)guanine labelled with ^{18}F has been prepared from 4-[^{18}F] fluorobenzyl alcohol and the standard trimethylamine quaternary salt, while O^6-(3-iodobenzyl)guanine was stannylated using $(Me_3Sn)_2$ and $(Ph_3P)_2PdCl_2$ and re-iodinated using ^{131}I and H_2O_2 in acetic acid [33]. These compounds radio-labelled in the O^6-substituent are potentially useful tools for the *in vivo* mapping of ATase by positron emission tomography (PET).

O^6-Benzylguanine has undergone clinical trials [34] in combination with carmustine (BCNU). This prototype [35] CNU remains in clinical use, as does lomustine (CCNU) of similar vintage. Ironically, many CNUs with superior properties subsequently designed [36] are still on the shelf because of the cost of clinical studies. In France the sulfone cystemustine [37,32] and the phosphonate ester fotemustine [38] have been used. We have found [39] that the very active 5-fluorouracil/CNU derivative B.4152 is relatively sparing on bone marrow. Elsewhere considerable differences have been observed [40] in the response of various CNUs to the protecting effect of ATase.

3. ACTIVATION OF O^6-METHYL BY VINYL (O^6-ALLYL GU) AND BY CARBONYL (O^6-PHENACYL GU)

The greatly enhanced inactivating power of O^6-benzylguanine *vis-à-vis* O^6-methylguanine paralleled the much greater reactivity of benzyl electrophiles $PhCH_2X$ than of simple alkyl such as MeX in S_N2 displacement reactions, although as noted above this reactivity alone does not govern the anti-ATase effect observed in substituted O^6-benzyl derivatives. However, a further parallel was noted in the intermediate activity of O^6-allylguanine; the I_{50} values



9a; R = H
9b; R = Me

10

11

12

13a; R = H
13b; R = 4-Br

14

15

16a; R = H
16b; R = Cl

17

Scheme 2.

for benzyl, allyl, methyl are respectively 0.2, 20, 350 μM [27], in line with chemical reactivity. Griffin and co-workers [41] explored allyl substitution in considerable detail. They confirmed the level of *O*^{6}-allylguanine activity and while *O*^{6}-(2-ethylallyl)guanine (**9a**) is similar, introduction of what is analogous to an *ortho*-Me in the benzyl series giving *O*^{6}-(2-isopropylallyl)guanine (**9b**) destroys activity (Table 4).

Incorporation of the double bond in a ring as in *O*^{6}-(1-cyclopentenylmethyl)guanine (**10**) raises the level of activity almost to that of *O*^{6}-benzylguanine; like the very active furan and thiophene derivatives in Section 4, this derivative contains an unsaturated 5-membered ring. These compounds were made in moderate to good yields from the alkoxide and 2-amino-6-chloropurine in refluxing excess alcohol or tetrahydrofuran. The quaternary salt from trimethylamine was sometimes used, also the less reactive one from 1,4-diazabicyclo[2.2.2]octane (DABCO) [42].

Table 4. ATase-Inactivating Activity [41] of *O*^{6}-Allylguanine and Related Substituted Guanines

O^6-Substituent	I_{50}(μm)[a] of Derived Guanine
CH_2Ph	0.18
1-cyclopentenylmethyl	0.39 (**10**)
$CH_2CH = CH_2$	8.5
$CH_2C(Et)=CH_2$	16 (**9a**)
CH_2COMe	192
Me	428
$CH_2C(Pr^i) = CH_2$	>1,000 (**9b**)
CH_2COPh	>1,000

[a] I_{50} is the concentration of inactivator required to produce 50% reduction in ATase activity after incubation for 30 min in cell-free extracts.

However attempts to harness the immense S_N2 reactivity of acetonyl or phenacyl electrophiles $RCOCH_2X$ led to O^6-substituted guanines (**11**) with poor or no activity. In these the terminal methylene of the allyl compounds is replaced by the polar oxo-group. To prepare them, ketals of 2-oxoalkan-1-ols yielded the protected O^6-substituted guanines, and selective acid-catalysed cleavage of these ketals was possible without substantial removal of the entire O^6-substituent. It seems that while the hydrophobic pocket at the ATase active site can accommodate a relatively bulky substituent like benzyl (and even phenanthrylmethyl) in the correct configuration for reaction with the Cys thiolate, the polarised carbonyl in the phenacyl group renders it non-accessible.

4. ACTIVATION OF O^6-METHYL BY HETARYL (O^6-(4-BROMOTHENYL)GU AND OTHER HETEROCYCLIC SUBSTITUENTS)

In considering other devices for activating an O^6-alkyl substituent, we turned to heterocyclic groups. The order of reactivity in S_N2 reactions is 3-thienylmethyl <benzyl<thenyl<furfuryl [19], and even though none of the foregoing studies had provided a clear pointer with respect to ATase, we felt that a comparison of O^6-benzylguanine with O^6-furfurylguanine would be worth while and launched a programme of synthesising and testing O^6-(hetarylmethyl)guanines [19,30]. Since the sensitive furan ring of furfuryl alcohol would not survive the harsh conditions of the standard O^6-benzylguanine synthesis from 2-amino-6-chloropurine (benzyl alcohol solvent for 5-10h at 130°C), we adapted a method [43], in which the quaternary salt from trimethylamine and 2-amino-6-chloropurine (giving the much better leaving group $^+NMe_3$) reacted readily with alkoxides in dimethyl sulfoxide at room temperature. This has since become the new standard procedure, and the corresponding DABCO salt [42] was subsequently used; the latter's more convenient synthesis sometimes compensates for its rather lower reactivity. Recently [63b], 4-dimethylaminopyridine has proved a useful catalyst in reaction of the trimethylammonium salt with less reactive alkoxides.

O^6-Furfuryl-, O^6-benzyl- and O^6-thenylguanine proved to have I_{50} respectively 0.08, 0.04, 0.018 μM in our hands, and the last (B.4205, PaTrin 1) was selected for clinical trial by Cancer Research UK. Simple pyrrole alcohols were too sensitive to react as desired with this quaternary salt even under mild conditions, but we did manage to control the reactivity by nitro-substitution, and O^6-(1-methyl-4-nitro-2-pyrrolylmethyl)guanine (**14**) had I_{50} 0.55 μM. Many other O^6-(hetarylmethyl)guanines were then prepared, involving considerable investigation of synthetic sources of the requisite alcohols [19], and several were very effective inactivators of ATase *e.g.* O^6-(5-thiazolylmethyl)- (**15**) and O^6-(2-chloro-4-picolyl)guanine (**16b**) (I_{50} 0.033, 0.04 μM). The influence of sulfur is again evident when thiazole in **15**, and isothiazole, are compared with oxazole (Table 5). O^6-(2-Picolyl)guanine (**17**) had I_{50} 35 μM and this low activity, also noted elsewhere [26,29], was possibly due to an "*ortho*" effect of the nuclear N; we found O^6-(4-picolyl)guanine (**16a**) had I_{50} 0.13 μM.

Evidently "*ortho*" S or O was not disadvantageous, and we explored further the effect of substitution in the thiophene and furan rings. The 4-methylthenyl derivative was almost as effective as the parent thenyl, but the 3-methylthenyl isomer had I_{50} 150 μM - an *ortho* effect as in the benzyl series. Interestingly the 5-methylthenyl isomer was also a relatively poor inactivator (I_{50} 9 μM), probably because 5-methylthenyl gives extremely reactive electrophiles [44], and some of the O^6-substituted guanine in the assay may be lost by hydrolysis. O^6-(5-Methylfurfuryl)guanine too has I_{50} 9.7 μM. A similar explanation may account for the difference between the bicyclic O^6-substituents 5,6,7,8-tetrahydro-2-naphthylmethyl (giving **18**, I_{50} 0.3 μM) and 4,5,6,7-tetrahydro-2-benzo[*b*]thienylmethyl (**19**, I_{50} 185 μM). Otherwise, a wide variety (Table 6) of substituents (Cl, Br, I, CN, N_3) of the thiophene or furan rings gave very powerful inactivators, 11 compounds having I_{50} in the range 0.002-0.009 μM.

Table 5. ATase-Inactivating Activity [19,30] of O^6-(Hetarylmethyl)guanines

Hetarylmethyl	Derived Guanine	$I_{50}(\mu m)^a$
5-thiazolymethyl	B.4275 (**15**)	0.033
2-chloro-4-picolyl	B.4321 (**16b**)	0.04
4-isothiazolylmethyl	B.4354	0.07
4-picolyl	B.4277 (**16a**)	0.13
6-chloro-3-picolyl	B.4319	0.20
5,6-methylenedioxy-3-picolyl	B.4271	0.23
5-bromo-3-picolyl	B.4320	0.25
5-oxazolylmethyl	B.4274	0.35
3-picolyl	B.4211	0.43
1-oxido-3-picolyl	B.4282	1.4
5-(1-methylimidazolyl)methyl	B.4389	8.5
2-picolyl	B.4210 (**17**)	35

[a]See Table 2.

The 4-bromothenyl derivative, B.4280, emerged while PaTrin 1 was still undergoing pre-clinical assessment and superseded it for clinical study, designated PaTrin 2. 4-Bromothenyl alcohol is more stable that the 5-isomer, and reaction with the purine quaternary salt under the standard conditions is straightforward. Selective bromination [45] of thiophene-2-carboxaldehyde in presence of $AlCl_3$ favours the 4-bromoaldehyde which is (like the 5-isomer from uncatalysed reaction) commercially available and readily reduced by $NaBH_4$ [46]. In a study using human melanoma xenografts in mice [47],

PaTrin 2 enhanced the therapeutic index of temozolomide to a greater extent than O^6-benzylguanine. In a Phase I clinical trial [48], at the low dose of 10 mg m^{-2} i.v. or p.o., PaTrin 2 completely inactivated ATase and could be given together with 125 mg m^{-2} of the O^6-methylating agent temozolomide. Phase II trials of the combination are imminent.

Table 6. ATase-Inactivating Activity [19,30] of O^6-(Substituted Thenyl)guanines (13) and Furan and Pyrrole Analogues

Substituent on O^6-Thenyl, R	Derived Guanine	I_{50}(μm)[a]
4-CN	B.4317	0.0028
4-Br	B.4280 (**13b**) (PaTrin 2)	0.0034
5-Cl	B.4281	0.004
5-Br	B.4269	0.0045
5-CN	B.4283	0.005
5-CN[b]	B.4273	0.006
4-N_3	B.4373	0.0063
5-Br[c]	B.4313	0.0065
4-Cl	B.4298	0.008
5-I[c]	B.4357	0.009
4-MeS	B.4356	0.0095
4,5-Cl_2	B.4318	0.015
4-Ph	B.4401 (**25**)	0.015
4-MeO	B.4300	0.0165
4-I	B.4518	0.017
H	B.4205(**13a**) (PaTrin 1)	0.018
5-Br[b]	B.4336	0.025
5-MeSO	B.4294	0.03
H[c]	B.4206	0.03
5-$MeSO_2$	B.4309	0.072
H[b]	B.4203	0.08
5-CO_2Me[b]	B.4229	0.09
4-Me	B.4463	0.095
4-MeSO	B.4377	0.15
H[d]	B.4209	0.15
4-$MeSO_2$	B.4361	0.2
4-NO_2[e]	B.4278 (**14**)	0.55
5-Ph	B.4378 (**24**)	0.75
4-Br[f]	B.4352 (**52a**)	8
5-Me	B.4220	9
5-Me[b]	B.4221	9.7
5-MeS	B.4295	11.5
4-Br[g]	B.4556 (**52b**)	12.5
3-Me	B.4222	150
5-Bu^t [b]	B.4451	220

[a]See Table 2. [b]In O^6-(substituted furfuryl)guanine. [c]In O^6-(substituted 3-thienylmethyl)guanine. [d]In O^6-(3-furylmethyl)guanine. [e]In O^6-(1-methyl-4-nitro-2-pyrrolylmethyl)guanine. [f]On S^6-thenyl in S^6-thenyl-6-thioguanine. [g]On thenyl in 2-amino-6-thenylaminopurine.

Sulfur (and/or halogens) incorporated in the O^6-substituent in general enhances the ATase-inactivating power of guanine derivatives as was already demonstrated in the O^6-benzyl series. This may be a consequence of the hydrophobic nature of the active site pocket of the protein. Elaboration of the thiophene system gives an informative series of polycyclic compounds outlined in Scheme 3 together with analogues and our I_{50} (μM) values [30, and some hitherto unpublished] appended (also Table 7).

Table 7. ATase-Inactivating Activity [30] of Guanines with Thiophene-Containing Polycyclic O^6-Substituents

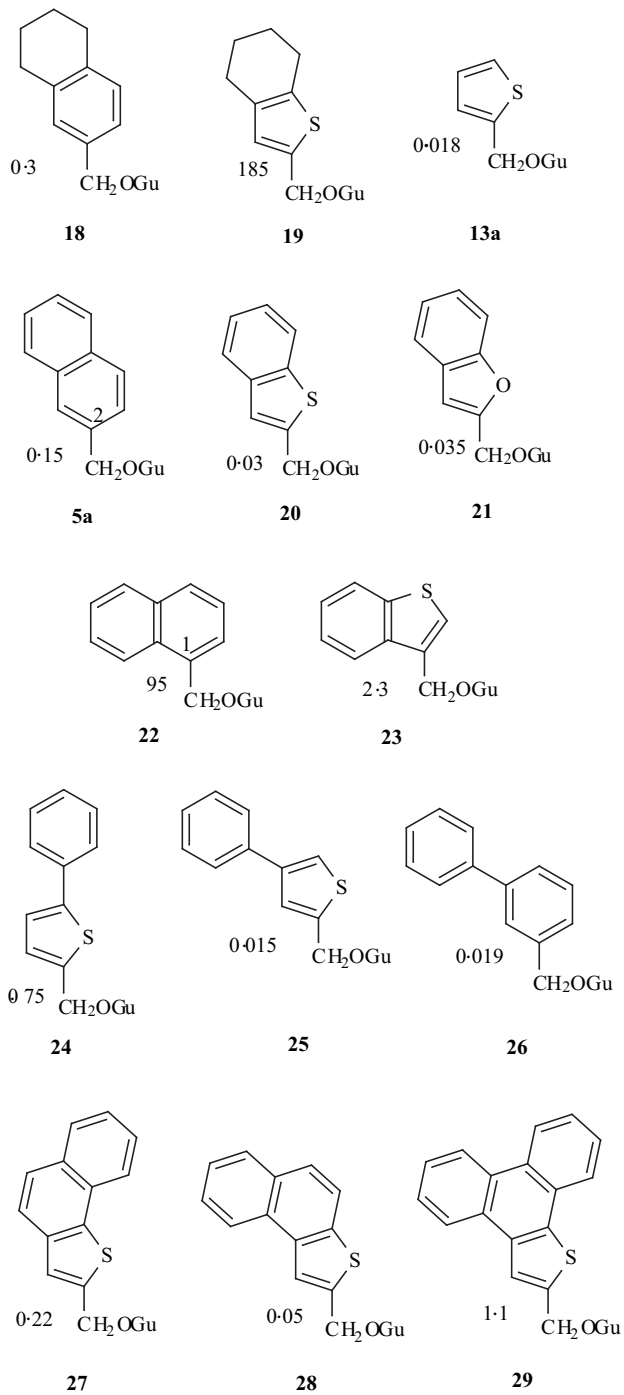
O^6-Substituent	Derived Guanine	I_{50}(μm)[a]
4-phenylthenyl	B.4401 (**25**)	0.015
2-benzo[*b*]thienylmethyl	B.4226 (**20**)	0.03
5-methoxy-2-benzo[*b*]thienylmethyl	B.4436	0.034
2-benzo[*b*]furanylmethyl	B.4266 (**21**)	0.035
2-naphtho[2,1-*b*]thienylmethyl	B.4366 (**28**)	0.05
6-methoxy-2-benzo[*b*]thienylmethyl	B.4438	0.16
5-bromo-2-benzo[*b*]thienylmethyl	B.4416	0.16
2-naphtho[1,2-*b*]thienylmethyl	B.4383 (**27**)	0.22
5-phenylthenyl	B.4378 (**24**)	0.75
2-phenanthro[9,10-*b*]thienylmethyl	B.4407 (**29**)	1.1
3-benzo[*b*]thienylmethyl	B.4413 (**23**)	2.3
4,5,6,7-tetrahydro-2-benzo[*b*]thienylmethyl	B.4409 (**19**)	185

[a] See Table 2.

The benzo[*b*]thiophene derivative (**20**) corresponding to the 2-naphthyl compound (**5a**) has (like the benzo[*b*]furan **21**) high activity. Even the 1-naphthyl analogue (**23**) retains considerable activity despite the "*ortho*" effect. Further enlargement of the O^6-substituent gives O^6-(5-phenylthenyl)guanine (**24**) and its vinylene-bridged tricyclic counterpart (**27**). Geometry apparently hinders their accommodation in the ATase pocket as readily as the corresponding 4-phenylthenyl pair (**25, 28**). But they are still quite active, as is the impressive tetracyclic compound (**29**) These results, together with Kaina's [63] from long-chain 9-polymethylene substituents (Section 5), emphasise the negative effect of the simple *ortho*-substituents in **3** (R=2-Me and 2-OMe) referred to in Section 2.

5. O^6-ARALKYL-9(AND 8)-SUBSTITUTED GUANINES, INCLUDING NUCLEOSIDES

After the identification of O^6-benzylguanine, exploration of the structural features of this compound responsible for the high anti-ATase activity concentrated initially on varying the O^6-substituent, as outlined in Sections 2-4 above. The nuclear N-9 was readily substituted, and early attempts were also made [28] to determine any significance this position of the purine ring might have for activity, actually enhancing it or at least tolerating groups with useful pharmacological activity. Alkylation of O^6-substituted guanines always gives



Scheme 3. I_{50} (mM) values are appended. OGu is 6-substituted guanine.

usually separable mixtures of N-7 and N-9 derivatives, and much effort has been devoted to maximising the proportions at N-9, the normal point of attachment of sugars in nucleosides [49]. Acylation on the other hand gives entirely 9-acetyl (92%) with acetic anhydride in pyridine; the alternative point of attack in this case is at substituent N^2 (by the reagent in toluene) [26].

In the simplest case, the 9- (**30**) and 7-methyl derivatives of O^6-benzylguanine had respectively I_{50} 2.6 and 52 μM [26] (Table 8). Larger N-7 substituents abolished activity, but diverse N-9 substituents were tolerated to some extent, *e.g.* 9-cyanomethyl and 9-(2-hydroxybutyl) both gave I_{50} 13 μM. Further study [26] showed that the presence of polar OH, NHR in the 9-substituent reduced activity while lipophilic groups even as large as steroids had relatively little effect: 9-CH_2CO_2Et and 9-CH_2CO_2(dihydrotestosteron-17-yl) had I_{50} 30 and 4 μM. Interaction with steroid-responsive tumours could concentrate this type of steroidal derivative in tumour cells where it might be expected to cause selective depletion of ATase activity, sparing normal tissue from the effects of an administered alkylating agent.

Table 8. ATase-Inactivating Activity [26,28,60] of 7-, 8- and 9-Substituted Derivatives of O^6-Benzylguanine

Substituent on O^6-Benzylguanine	$I_{50}(\mu m)^a$ of Derived Guanine
8-Br	0.08 (**38c**)
H	0.2
8-oxo	0.3 (**39**)
8-CH_3	0.3 (**38a**)
8-CF_3	0.4 (**38b**)
8-NH_2	0.7 (**38d**)
9-(2'-deoxyriboside)	2 (**33**)
9-CH_3	2.6 (**30**)
9-CH_2OCOBu^t	3.1 (**31**)
9-(CH_2CO_2 dihydrotestosteron-17-yl)	4
9-riboside	11 (**32**)
9-CH_2CN	13
9-$CH_2CH(OH)Et$	13
9-CH_2CO_2Et	30
7-CH_3	52

[a] See Table 1.

O^6-Benzylguanine substituted by 9-$CH_2O.COBu^t$ (**31**) was also prepared (and traces of the 7-isomer [50]), from chloromethyl pivalate, and had I_{50} 3.1 μM in the usual cell-free assay. However, in cell cultures the activity of this formaldehyde derivative was much closer to that of O^6-benzylguanine into which it was converted by esterases, and it or its 8-bromo analogue could be a useful pro-drug [50]. The 9-acetyl derivative gave the O^6-benzylguanine value even in the cell-free medium, being hydrolysed at the pH of the experiment [26].

The first nucleosides to be tested were the known riboside (**32**) and 2'-deoxyriboside(**33**) [28], with I_{50} 11 and 2 μM respectively relative to 0.2μM for O^6-benzylguanine. Further studies on the 2'-deoxyriboside threw light on its metabolism [51] and on the rôle of added DNA in the reaction with ATase [52]. Its superior water-solubility is advantageous but it is unlikely to warrant clinical examination. As indicated above, several halogen-substituted O^6-benzylguanosine and -2'-deoxyguanosine derivatives were made [32], the former proving considerably less potent inactivators than the latter. But even N^2-acetyl-O^6-benzylguanosine (relatively water-soluble) was able to

increase tumour sensitivity to the CNU cystemustine. O^6-Furfuryl-2′-deoxyguanosine was recently prepared as an example of a general method [53].

We made several ribosides (Table 9) with O^6-(hetarylmethyl) substituents [30] while we were still exploring the corresponding guanines and O^6-(2-benzo[*b*]thienylmethyl)guanosine had I_{50} 0.35 μM, about 12 times less effective than the parent base. In general, ribosides were 40-50 times less effective. When PaTrin 2 (I_{50} 0.0034 μM) was discovered, the riboside (**34**), 2'-deoxyriboside (**35**), and arabinoside (**36**) proved to have I_{50} 0.08, 0.095, 0.115 μM respectively.

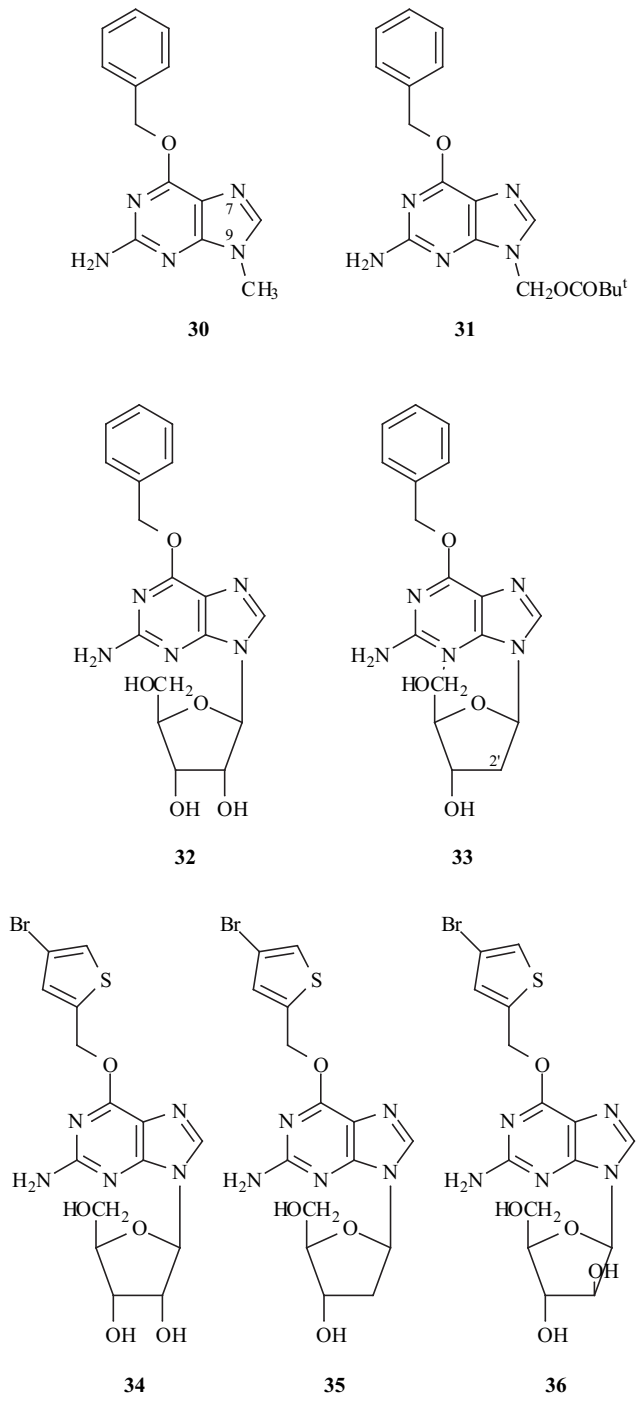
Table 9. ATase-Inactivating Activity of Nucleosides [30,64] of O^6-(4-Bromothenyl)guanine and Related 9-Substituted Derivatives

9-Substituent	Derived Guanine	I_{50}(μm)[a]
riboside	B.4363 (**34**)	0.08
2'-deoxyriboside	B.4379 (**35**)	0.095
arabinoside	B.4368 (**36**)	0.115
$CH_2OCH_2CH_3$	B.4369 (**37b**)	0.28
$CH_2OCH_2CH_2OH$	B.4335 (**37a**)	0.33
riboside[b]	B.4276	0.35
riboside[c]	B.4268	0.75
riboside[d]	B.4279	0.9
$CH_2O(CH_2)_7CH_3$	B.4370	1.2
$CH_2OCH_2CH_2OH$[e]	B.4334	8

[a] See Table 2. [b]On O^6-(2-benzo[*b*]thienylmethyl) guanine (B.4226, **20**). [c]On O^6-piperonylguanine (B.4212). [d]On O^6-thenylguanine (B.4205, **13a**). [e]On O^6-benzylguanine.

Ribosides were made from alkoxide and 2-amino-6-chloropurine riboside. The 9-substituent increased the reactivity at the 6-position of this compound (commercially available), and the desired nucleoside was separated from by-products arising from interference by the sugar hydroxyl groups [54]. Mitsunobu reaction of the alcohol and 2′-deoxyguanosine diacetate yielded the O^6-substituted 2'-deoxyriboside [55], while the arabinoside resulted from condensation of the appropriate chloro-sugar [56] with 2-amino-6-chloropurine followed by displacement with alkoxide as in the case of the riboside. When this last nucleoside acts as a pseudosubstrate for ATase, guanine arabinoside (Ara-G) would be released, but the pharmacokinetics have not so far been investigated. Ara-G has been intensively studied as an anti-tumour and anti-viral agent for almost 40 years [57]. An improved synthesis afforded the opportunity for a very useful review [56], and its water-soluble pro-drug, the O^6-methyl derivative, has been tested clinically in haematological malignancies [58].

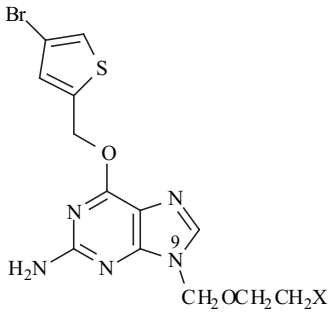
In this connection, we also prepared the O^6-(4-bromothenyl) derivative (**37a**) of the powerful anti-viral agent acyclovir by reaction of the O^6-substituted guanine with $BrCH_2OCH_2CH_2$ OAc [59]. This compound had I_{50} 0.33 μM, considerably more active than the O^6-benzyl analogue (8 μM). The simpler 9-ethoxymethyl derivative



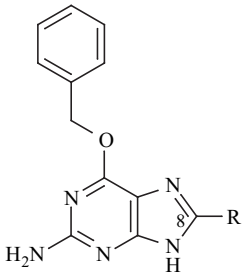
Scheme 4.

(**37b**) of O^6-(4-bromothenyl)guanine had I_{50} 0.28 μM (Table 9).

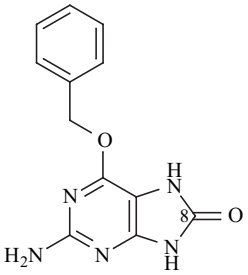
Pegg and co-workers comment that the generally reduced ability of 9-substituted derivatives to interact with ATase does not necessarily have a serious adverse effect on inhibitory activity, and the 9-position is useful in the design of inactivators with improved selectivity or formulation properties [52]. The 8-position would also serve such purposes, but the necessary syntheses are much more difficult and involve construction of the pre-substituted imidazole ring of the purine system. The 8-CH_3 (**38a**) and 8-CF_3 (**38b**) derivatives of O^6-benzylguanine made in this way had I_{50} values approaching that of the parent [60]. So had



37a; X = OH
37b; X = H



38a; R = CH_3
38b; R = CF_3
38c; R = Br
38d; R = NH_2



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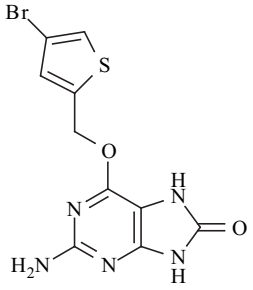

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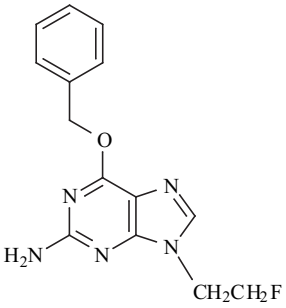

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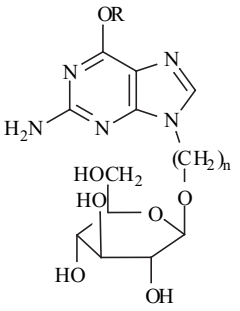
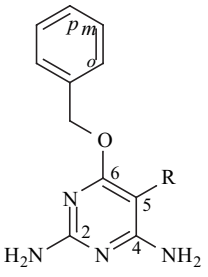
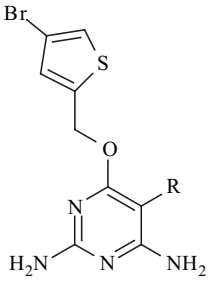


42a; R = Benzyl
42b; R = 4-Bromothenyl



43a; R = H
43b; R = NO
43c; R = NH_2
43d; R = Br
43e; R = NO_2



44a; R = H
44b; R = NO

Scheme 5.

the 8-oxo(**39**) (which incidentally is a principal metabolite of O^6-benzylguanine [61]). The 8-bromo compound (**38c**) was rather more active, the 8-amino (**38d**) rather less [60]. We found that O^6-(4-bromothenyl)-8-oxoguanine (**40**) had I_{50} 0.018 μM [30]; unlike its O^6-benzyl analogue (**39**), this value is substantially lower than that of the parent (PaTrin 2), with implications for the therapeutic index of co-administered temozolomide [47].

The 9-(2-fluoroethyl) derivative (**41**) of O^6-benzylguanine labelled with ^{18}F was prepared [62], but the radio-label needs to be at O^6 in order to image ATase levels. In another direction, a useful and detailed study of non-nucleoside sugar derivatives (**42a,b**) of O^6-benzyl- and O^6-(4-bromothenyl)guanine has recently been carried out by Kaina and co-workers [63a] . Due to anaerobic glycolysis , many tumours have high levels of glucose consumption and enhanced expression of the proteins which transport glucose itself and glucose-containing conjugates. This provides a tumour-targeting mechanism, and a series of 9-$(CH_2)_n$-(β-D-glucosides) (**42**) were prepared *via* ω-bromoalkanols. The optimal I_{50} values (*ca.* 0.03 μM) of *n* were 8-12, while shorter chains were considerably inferior (Table 10). Modelling studies indicated that the latter fitted poorly into the ATase binding groove. In addition to selective uptake by tumour cells, these glucosides have improved water-solubility over the parent O^6-substituted guanines.

Table 10. ATase-Inactivating Activity [63] of 9-Polymethylene(β-D-glucosides) (42a) of O^6-Benzylguanine or (42b) of O^6-(4-Bromothenyl)guanine

n in 42a/b	42a/b	$I_{50}(\mu m)^a$
[O^6-(4-bromothenyl)guanine]		0.009
12	**42b**	0.030
8	**42b**	0.032
6	**42b**	0.45
[O^6-benzylguanine]		0.62
2	**42b**	0.68
2	**42a**	25

[a] I_{50} is the concentration of inactivator required to produce 50% reduction in ATase activity after incubation for 30 min with protein extract.

6. 5-NITROSOPYRIMIDINES RELATED TO O^6-BENZYL GU

In the time-honoured fashion of multi-disciplinary research, available intermediates in chemical synthetic processes may be submitted to biological assay, and it transpired that some monocyclic precursors of O^6-benzylguanine actually inactivated ATase themselves (Table 11). 6-Benzyloxy-2,4-diaminopyrimidine (**43a**) had I_{50} 15 μM, and its 5-nitroso derivative (**43b**) (0.06 μM) was even superior to O^6-benzylguanine [60]. This is reduced to the 2,4,5-triaminopyrimidine (**43c**, the next synthetic stage en route to the purine), which had I_{50} 0.4 μM. Other electron-withdrawing groups at position 5, in compounds prepared from the alkoxide and the appropriate 6-chloride, gave 6-benzyloxy-5-bromo-2,4-diaminopyrimidine (**43d**) and the 5-nitro analogue (**43e**), with respective I_{50} 2 and 0.06 μM. Further exploration showed that 2-amino-6-benzyloxy-5-nitropyrimidine (lacking the 4-NH_2 corresponding to N-9 in the related purine) had I_{50} 0.4 μM, and that the 5-NO_2 or 5-NO could be replaced by nuclear N giving 6-benzyloxy-2,4-diamino-*s*-triazine (I_{50} 4 μM). On the other hand, loss of the 2-NH_2 gave 4-amino-6-benzyloxy-5-nitropyrimidine (I_{50} 28 μM).

Table 11. ATase-Inactivating Activity [60] of 5-Substituted 6-Benzyloxy-2,4-Diaminopyrimidines (43a-e)

5-Substituent	$I_{50}(\mu m)^a$
NO	0.06 (**43b**)
NO_2	0.06 (**43e**)
[O^6-benzylguanine]	0.2
NH_2	0.4 (**43c**)
Br	2 (**43d**)
H	15(**43a**)

[a] See Table 1.

We found that there was not such a marked difference between benzyl and 4-bromothenyl substituents in 5-nitrosopyrimidines as in O^6-substituted guanines [64]. 6-(4-Bromothenyloxy)-2,4-diamino-5-nitrosopyrimidine (**44b**) had I_{50} 0.045 μM (as had the corresponding 2-amino-4-acetamido derivative), although its 4-chlorothenyl analogue was more effective (0.009μM), and the 4-fluorobenzyl showed 0.0175 μM. 2,4-Diamino-5-nitro-6-piperonyloxy-pyrimidine (0.5 μM) was inferior to the 6-benzyloxy compound. The 5-unsubstituted 2,4-diamino-6-(4-bromothenyloxy) (**44a**) and 6-(piperonyloxy)pyrimidines had I_{50} 0.27 and 0.7μM.

Kohda and co-workers [65] studied series of 5-nitro- and 5-nitroso-2,4 -diaminopyrimidines with 6-(fluorobenzyloxy) and 6-(picolyloxy) substituents. They found (Table 12) that *o*-, *m*- and *p*-substituents (2-, 3- and 4-fluorobenzyl) gave a pattern of anti-ATase activity similar to the corresponding O^6-(fluorobenzyl)guanines [29] (Section 2 here), and also that the 6-(2-picolyloxy) derivative was not very active. It was of interest [65] that the 5-nitro compounds when examined in a biomimetic system with the nucleophile thiophenoxide were dearylmethylated like the guanines, but the 5-nitroso compounds, while degraded, underwent a completely different but unidentified reaction possibly involving reduction of the 5-NO. Both the 5-NO and (to a lesser extent) 5-NO_2 series potentiated the cytotoxicity of nimustine (ACNU) towards HeLa S3 cervical tumour cells. This is intriguing, since a metabolic study in mice [66] indicated that the 5-nitroso compound was relatively ineffective in depleting ATase activity *in vivo,* possibly because of rapid cytosolic reduction.

Table 12. ATase-Inactivating Activity [65] of 5,6-Disubstituted 2,4-Diaminopyrimidines

5-Substituent	6-Substituent	$I_{50}(\mu m)^a$
NO	4-$FC_6H_4CH_2O$	0.05
NO	$PhCH_2O$	0.13 (**43b**)
NO_2	4-$FC_6H_4CH_2O$	0.35
NO_2	$PhCH_2O$	1.3 (**43e**)
NO_2	3-$FC_6H_4CH_2O$	2.5
[O^6-benzylguanine]		2.6
NO_2	2-$FC_6H_4CH_2O$	12
H	$PhCH_2O$	170 (**43a**)

[a] I_{50} is the concentration of inactivator required to produce 50% reduction in ATase activity after incubation for 20 min with purified ATase.

7. NON-PURINE BICYCLIC SYSTEMS AND OTHER MODIFICATIONS OF O^6-BENZYL GU

Just as O^6-(4-bromothenyl)guanine 9-(β-D-arabinoside) (**36**) would be expected to release the cytotoxic Ara-G following inactivation of ATase by transferring the 4-bromothenyl group (Section 5), so O^6-(4-bromothenyl)-8-azaguanine (**45b**) should afford 8-azaguanine. Synthesised in 1945 [67] and later found as a natural product (pathocidin) in *Streptomyces* [68], this classical antimetabolite was the first purine antagonist to show anti-tumour activity in mice [69]. Shealy and co-workers [70] prepared the O^6-benzyl derivative (**45a**) as a potential pro-drug activated by hydrolysis, and we

found that the 4-fluoro derivative of this compound and its O^6-(4-bromothenyl) (**45b**) and O^6-(4-chlorothenyl) (**45c**) analogues [64,71] had I_{50} 0.08, 0.045, 0.011 μM respectively in our standard anti-ATase assay. This high activity was confirmed elsewhere [60,63], but the consequence is that the amount of 8-azaguanine released by an adequate dose would be therapeutically insignificant.

The activity of the 5-nitroso derivatives (Section 6) had demonstrated that the imidazole ring of O^6-benzylguanine was not essential for inactivation of ATase, and the activity of these 8-aza compounds is consistent. We extended [64] our search into related bicyclic systems (Table 13), facilitated by the common synthetic intermediate 6-alkoxy-2,4,5-triaminopyrimidine (obtained from the standard but rather temperamental reduction of the 5-nitrosopyrimidine by dithionite, $Na_2S_2O_4$). Reaction with *N*-tosylthionylimine, TsN=SO, in pyridine [72], gave O^6-(4-bromothenyl)-8-thiaguanine (**46a**), while glyoxal trimer and metabisulfite, $Na_2S_2O_5$, [73] afforded O^4-(4-bromothenyl)pterin (**48**). Lead tetraacetate oxidation [74] of the 5-nitroso compound itself gave O^6-(4-bromothenyl)-8-oxaguanine (**46b**).

Table 13. ATase-Inactivating Activity [30,64] of Non-Purine Bicyclic Compounds

Compound	I_{50}(μm)[a]
B.4351 (**46a**)	0.0028
B.4337 (**47**; $ArCH_2$ = 4-bromothenyl)	0.006
B.4343 (**47**; $ArCH_2$ = piperonyl)	0.0065
B.4310 (**47**; $ArCH_2$ = benzyl)	0.009
B.4338 (**47**; $ArCH_2$ = thenyl)	0.01
B.4314 (**45c**)	0.011
B.4296 (**46a**; benzyl for 4-bromothenyl)	0.02
B.4288 (**48**)	0.025
B.4289 (**45b**)	0.045
B.4264 (**48**; benzyl for 4-bromothenyl)	0.07
B.4270 (**45a**; 4-fluorobenzyl for benzyl)	0.08
B.4287 (**46b**)	0.24
B.4232 (**46b**; benzyl for 4-bromothenyl)	0.25
B.4418 (**49**)	200
B.4452 (**50**)	>2,500
B.4426 (**51**)	>2,500

[a] See Table 2.

All these bicyclic analogues of purines were good to outstanding inactivators of ATase. In the 8-thia series, O^6-benzyl and O^6-(4-bromothenyl) gave I_{50} 0.02, 0.0028 μM, while the considerably inferior 8-oxa counterparts (0.25, 0.24 μM respectively) demonstrate yet again the significance of sulfur in pseudosubstrates for the ATase protein. The corresponding figures for the pterin analogues are 0.07 and 0.025 μM. Electronic effects in the various rings influence the reactivity at the "6"- position but as noted above, steric and polar properties are probably of greater importance in determining the most effective substrates for ATase.

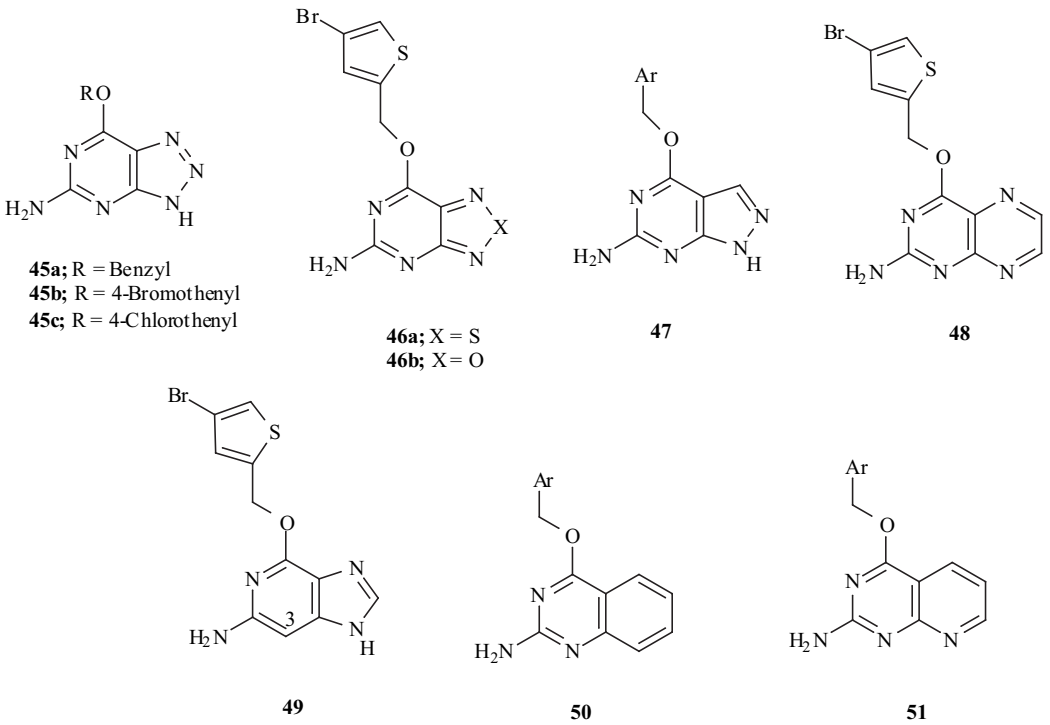
Some earlier attempts to define the molecular features of O^6-alkylguanines necessary for efficient reaction with the protein were made by exchanging each of the purine hetero-atoms in turn for carbon [23]. This gave O^6-methyl-7-deazaguanine (a fused pyrrole), and O^6-methyl-1-deazaguanine and O^6-methyl-3-deazaguanine (containing pyridine rings). Reaction with bacterial ATase (*E. coli* ada) showed that loss of N-1 caused a rate decrease while loss of N-3 or N-7 had very little effect. In an extension of this work to human ATase [75], the rate of reaction of all 3 analogues proved undetectable, indicating the importance of N-1, N-3 and N-7 in the mammalian repair process.

Again turning the spotlight on O^6-(4-bromothenyl), we found that indeed even this substituent gave only I_{50} 200 μM (in **49**) for the 3-deazaguanine system in our usual assay with the human protein [64]. However, the 8-aza-7-deazaguanine series (**47**) (fused pyrazoles) gave, like 8-thiaguanines, consistently excellent results: O^6-benzyl, -piperonyl, -thenyl, and -(4-bromothenyl) showed I_{50} respectively 0.009, 0.0065, 0.01, 0.006 μM. These compounds are made relatively readily from 2-amino-4,6-dihydroxypyrimidine by successive action of Vilsmaier reagents, hydrazine and alkoxide [76], but we have not completed work on the less accessible 7-deazaguanines meant to determine whether these would follow the pattern with 3-deazaguanines indicated by Spratt and co-workers [75].

In the "6-6" compounds with O^4-(4-bromothenyl) pterin as prototype, loss of N from the pyrazine ring obliterated activity, at least for the 5,8-dideaza (**50**, *i.e.* quinazoline, fused benzene) and 5-deaza (**51**, fused pyridine) systems. These were prepared by annulation of the pyrimidine rings onto anthranilate [77] or of the pyridine ring onto 2,6-diaminopyrimidin-4-one [78]. 8-Deazapterins and 9-deazaguanines have not so far been examined.

As well as altering or omitting nuclear purine atoms, and altering or inserting O^6-, 8- and 9-substituents, attempts have been made to define further structural necessities for ATase inactivation by changing the exocyclic O^6 and N^2 atoms. S^6-Benzyl-6-thioguanine was inactive [28], but we found S^6-(4-bromothenyl) (in **52a**) and S^6-piperonyl gave 8 and 50 μM respectively [64]. These were made from the appropriate thiols and the standard quaternary salt in preference to alkylation of 6-thioguanine. Benzylamine and 4-bromothenylamine (from oxime reduction) reacted with 2-amino-6-chloropurine [79] to afford 2-amino-N^6-substituted adenines, respectively inactive and (**52b**) with I_{50} 12.5 μM. The observed activity of these *S*- and *N*-isosteres of O^6-(4-bromothenyl)guanine is mediocre but significant because of the strength of the NCH_2 and SCH_2 bonds relative to OCH_2.

The 2-amino group of O^6-benzylguanine was also altered. Acetic anhydride in an inert solvent (see Section 5) attacked N^2 preferentially, while diazotisation in fluoroboric acid at -20°C [80] substantially retained the O^6-benzyl group and yielded the 2-fluoro analogue which reacted in turn with



45a; R = Benzyl
45b; R = 4-Bromothenyl
45c; R = 4-Chlorothenyl

46a; X = S
46b; X = O

47

48

49

50

51

Scheme 6.

methylamine and with dimethylamine [60]. Exchanging the 2-NH_2 in these ways for 2-NHAc (**53a**), 2-F (**53b**), 2-NHMe (**53c**), 2-NMe_2 (**53d**) gave I_{50} values of 24, 48, 160, 200 μM, and *O*^{6}-benzylhypoxanthine (*i.e.*, 2-H, **53e**) had I_{50} 85 μM [28]. As before, *O*^{6}-(4-bromothenyl) helps somewhat to overcome these deficiencies, with I_{50} values for 2-NHAc (**54a**), 2-F (**54b**), 2-H (**54c**) of 2.6, 1.4, 0.3 μM [64]. Condensation of acetamidine and ethyl cyanoacetate [81] followed by Pfleiderer's one-pot nitrosation/reduction [82] gave 6-amino-5-formamido-2-methylpyrimidin-4-one (**55**), which after cyclisation [83] and activation as the DABCO salt yielded 2-methyl-*O*^{6}-thenylhypoxanthine (**56**), with I_{50} 14 μM.

The work now reviewed illustrates the twin aims of probing in detail the interactions of pseudosubstrates with ATase, and of designing and synthesising still more efficient anti-cancer drugs. Selective inactivation of this important DNA repair system has immense potential in enhancing the clinical performance of some of the most useful agents available after 50 years of chemotherapy - the *O*^{6}-guanine alkylating agents.

The effort expended on developing successful ATase inactivators would certainly justify investigating combinations with other CNUs as indicated in Section 2. The point made earlier [36] remains valid that alkylation of macromolecules is too valuable a tool to be disparaged and neglected because earlier agents or the methods of using them presented clinical problems.

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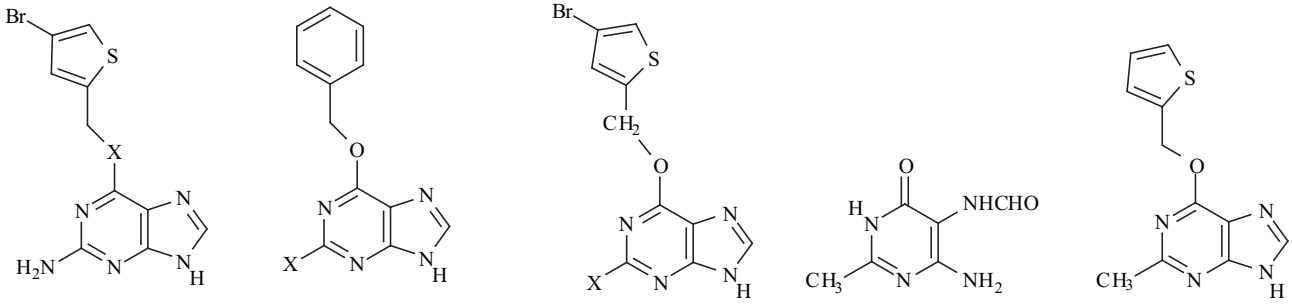

52a; X = S
52b; X = NH

53a; X = $NHCOCH_3$
53b; X = F
53c; X = $NHCH_3$
53d; X = $N(CH_3)_2$
53e; X = H

54a; X = $NHCOCH_3$
54b; X = F
54c; X = H

55

56

Scheme 7.

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