Inactivation of O^6-Alkylguanine-DNA Alkyltransferase. 1. Novel O^6-(Hetarylmethyl)guanines Having Basic Rings in the Side Chain

R. Stanley McElhinney,*,† Dorothy J. Donnelly,† Joan E. McCormick,† Jane Kelly,‡ Amanda J. Watson,‡ Joseph A. Rafferty,‡ Rhoderick H. Elder,‡ Mark R. Middleton,‡ Mark A. Willington,‡ T. Brian H. McMurry,† and Geoffrey P. Margison*,‡

University Chemical Laboratory, Trinity College, Dublin 2, Ireland, and CRC Section of Genome Damage and Repair, Paterson Institute for Cancer Research, Christie Hospital (NHS) Trust, Manchester M20 4BX, United Kingdom

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A number of novel guanine derivatives containing heterocyclic moieties at the O^6-position have been synthesized using a purine quaternary salt which reacts with alkoxides under mild conditions. Initially O^6-substituents were investigated in which the benzene ring of the known agent, O^6-benzylguanine, was replaced by unsubstituted heterocyclic rings. The ability of these agents to inactivate the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase), both as pure recombinant protein and in the human lymphoblastoid cell line Raji, has been compared with that of O^6-benzylguanine. The present paper focuses on O^6-substituents with basic rings, and under standard conditions several of them proved more effective than benzyl for inactivation of both recombinant and Raji ATase. Among the pyridine derivatives, the 2-picolyl compound **7** is not very active in contrast to the 3- and 4-picolyl compounds, and this influenced our choice of isomers of other basic ring systems for study. Since halogen substitution in the thiophene ring considerably increased the activity (**17** versus **6**), similar modifications in the pyridine series were examined. The more polar O^6-substituents in this study are on the whole compatible with the stereochemical requirements of the ATase protein, and their pharmacological properties may be valuable in subsequent in vivo investigations, particularly the thenyl (**6**), 5-thiazolylmethyl (**12**), 5-bromothenyl (**17**), and 2-chloro-4-picolyl (**21**) derivatives.

The cytotoxic effects of the antitumor *N*-(2-chloroethyl)-*N*-nitrosoureas (e.g., BCNU, CCNU, fotemustine) and the related methylating agents (e.g., DTIC, procarbazine, temozolomide) are primarily a consequence of their ability to alkylate DNA at the O^6-position of guanine.[1−3] It is well-established that resistance to these O^6-alkylating chemotherapeutic agents can be mediated by the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase), which removes the alkyl group from the guanine in an autoinactivating, stoichiometric process.[1−3] Tumor cells frequently express high levels of ATase[4,5] and are often resistant to the O^6-alkylating agents used in chemotherapy. There is therefore much current interest in attenuating ATase activity in order to sensitize such resistant cells to killing by these agents.[6]

This was first achieved by prior O^6-methylation of guanine residues in DNA,[7] but it soon transpired that the simple compound O^6-methylguanine[8] effectively inactivated ATase. Numerous other small molecules have subsequently been designed, synthesized, and assayed as inhibitors of the protein both in cell-free extracts and in various cell lines. The most significant agent to emerge was O^6-benzylguanine (**1**), about 2000 times as effective as O^6-methylguanine.[9,10] The structural features of this compound responsible for the high activity have been extensively explored. The variations arose mainly from substitution in the O^6-benzyl nucleus[10−12] and in the 2-, 8-, and 9-positions of the purine ring,[13] as well as O^6-allylic substituents and O^6-(2-oxoalkyl)guanines,[14] and included monocyclic pyrimidines with suitable 5- and 6-substituents.[13]

O^6-Benzylguanine and its analogues inhibit ATase by reacting with a specific cysteine residue (Cys 145 in the human protein).[15] The benzyl group is irreversibly transferred to the cysteine thiol group to form the *S*-benzyl derivative which can no longer react with an O^6-alkylated guanine residue in DNA, leaving such lesions unrepaired. The O^6-benzylguanine/thiol reaction is a bimolecular displacement (S_N2 type), with guanine as leaving group, X; the thiol group, especially as the anion, is a very good (soft) nucleophile (Nuc^-) effective in S_N2 substitutions:

$$RX + Nuc^- \rightarrow RNuc + X^-$$

To design variations of the O^6-benzylguanine molecule which would provide still more effective ATase inactivators, attempts to establish a reactivity sequence for substituted benzyl reagents with thiolate anion are relevant. For some chlorides (X = Cl) and arenethiolates (Nuc = ArS) characteristic U-shaped Hammett ρ plots are observed, with both electron-donating and electron-withdrawing benzylic ring substituents leading to reactivity higher than that of benzyl chloride itself.[16] For a wider range of O^6-benzylguanines (X = guanine), Kohda et al.[12] noted some anomalies and invoked a possible S_N1 mechanism in the case of O^6-(1-naphthylmethyl)guanine. More disappointingly, their observed

* Corresponding authors.
† Trinity College.
‡ Paterson Institute for Cancer Research.

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sequence of reactivity with benzenethiolate was "not obviously related" to the potentiation of ACNU cytotoxicity through ATase depletion. Earlier work[10,11] had also revealed no apparent correlation between the degree of ATase inactivation and the nature of the benzyl substituent.

Evidently other factors are involved in the O^6-benzylguanine/ATase reaction. The available structural data suggest that the reacting Cys residue is located in a pocket deep within the protein,[15] and the pattern of activity of substituted O^6-benzylguanines indicates that this active site must have considerable hydrophobic character. Further evidence for its nature was provided by the inconsistencies in the relationship between ATase-inactivating levels and simple chemical reactivity recorded by Arris et al.[14] In the S_N2 substitution of chlorides by iodide, methyl, allyl, and benzyl chlorides react at roughly similar rates while phenacyl chloride is vastly more reactive. However, O^6-phenacylguanine is devoid of ATase inhibitory activity and O^6-allyl is intermediate between O^6-methyl and O^6-benzyl. A hydrophobic pocket at the active site would accommodate the bulky substituent in the benzyl derivative in the correct configuration to accelerate the reaction with Cys thiolate, while the phenacyl derivative would be rendered ineffective by its polarized carbonyl group.

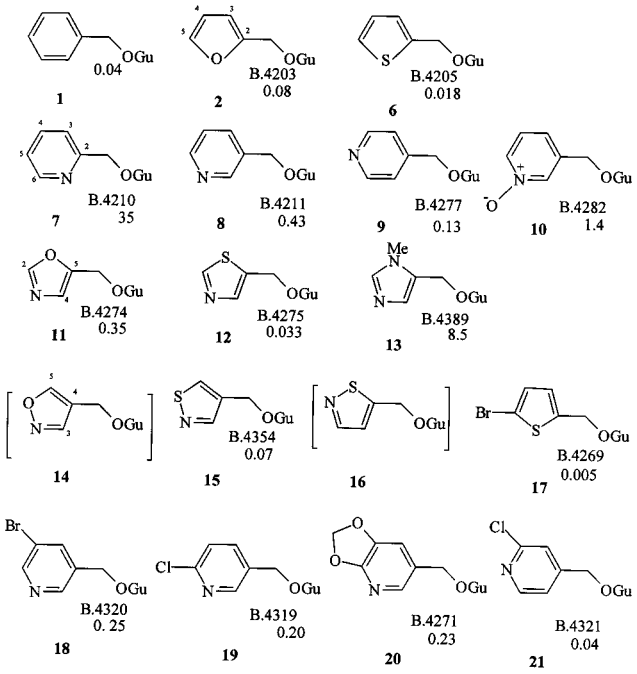
Turning to heterocyclic examples of R other than picolyl,[11,12] it is known that the order of reactivity in S_N2 reactions (with aniline[17] or halogen exchange[18]) is 3-thienylmethyl < benzyl < thenyl < furfuryl. The value of furfuryloxycarbonyl as an *N*-protecting group in some peptide synthetic sequences was advocated since it is cleaved by acid very much more readily[19] than the familiar benzyloxycarbonyl (Z), and the analogous thenyloxycarbonyl derivatives are even more reactive,[20] although these comparisons involve S_N1 reaction mechanisms and transient carbocations stabilized by the heterocyclic ring.

Since none of the foregoing studies provided a clear pointer, we felt that a comparison of O^6-benzylguanine with O^6-furfurylguanine (**2**) and analogues with related hetarylmethyl substituents would at least be instructive, and we therefore embarked on a program of synthesizing and testing such molecules.[21] An account of the influence of ring substituents in the furan- and thiophene-containing side chains is in preparation, and in the present paper we concentrate on some O^6-substituted guanines with pyridine and other basic heterocyclic rings in the side chain. If these types of polar substituents were compatible with the requirements of the protein, they could confer some advantages in terms of solubility and transport in vivo. It transpired that several of these new drugs indeed possessed activity as ATase inactivators, of the same order as O^6-benzylguanine.

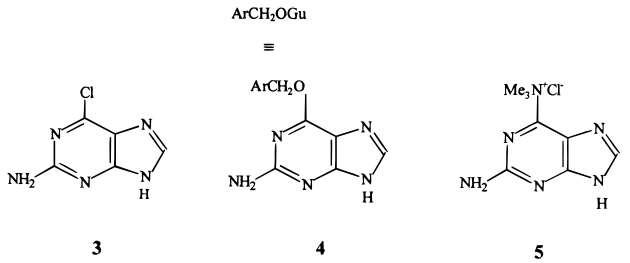
Chemistry

O^6-Benzylguanine (**1**) (Chart 1) has been synthesized in three ways. Displacement by alkoxide of halogen from 2-amino-6-chloropurine (**3**) (in benzyl alcohol solvent for 5−10 h at 130 °C)[22] and annelation of an imidazole ring to 6-(benzyloxy)-2,4,5-triaminopyrimidine at 180 °C[23] both employ very harsh experimental conditions. The O^6-substituted guanines **4** so far

Chart 1. O^6-Substituted Guanines and IC_{50} (μM) Values



described[10−12,14] have usually been made, rather inefficiently, by the former route (sometimes with *tert*-butyl alcohol, dioxane, or tetrahydrofuran as cosolvents) since preparation of a series of 2,4,5-triaminopyrimidines is unattractive. We confirmed that the yields of O^6-benzylguanine from alkoxide and 2-amino-6-chloropurine were poor; the product was difficult to purify, and there was evidence for some N^2-alkylation.[22b,24] Neither approach would have been suitable for synthesis of O^6-furfurylguanine with its sensitive furan ring. However, the third method, outlined without experimental details in a footnote,[25] was much more promising since at room temperature it apparently gave O^6-benzylguanine in 92% yield.



The key reagent was the quaternary salt **5**,[26] possessing the much better leaving group $^+NMe_3$, using dimethyl sulfoxide (DMSO) as solvent. Attempts to prepare or utilize quaternary salts from **3** and triethylamine or pyridine were unsuccessful, but trimethylamine (bp 3 °C, readily available from Fluka as 250-mL pressure tins) provided the salt **5** as described. After some experimentation, we defined standard conditions for displacement by alkoxide usually in good yield with easy workup. Quaternary salts from **3**[27] and its 9-benzyl derivative[28] using 1,4-diazabicyclo[2.2.2]octane (DABCO) were subsequently converted into O^6-aralkylguanines.

Furfuryl and thenyl alcohols are of course available commercially, as are all the picolyl alcohols and pyri-

dine-3-methanol *N*-oxide. Others were synthesized by reduction of the corresponding aldehydes or acid chlorides (using sodium borohydride) or of esters (using lithium aluminum hydride and working up by Fallab's method[29] with carbon dioxide). 5-Bromo- and 6-chloronicotinic acids were commercial samples as was 5-bromothiophene-2-carboxaldehyde, the source of 5-bromothenyl alcohol.[30] Other starting materials, although mentioned relatively infrequently in the literature, were prepared as described.

The nicotinic acids were converted into the acid chlorides using phosphorus oxychloride/pentachloride,[31] and these reagents yielded 2-chloroisonicotinoyl chloride directly from isonicotinic acid *N*-oxide.[32] Reduction of the acid chlorides with sodium borohydride[31,33] afforded respectively 5-bromopyridine-3-methanol,[34] 6-chloropyridine-3-methanol,[31] and 2-chloropyridine-4-methanol.[35]

5,6-(Methylenedioxy)pyridine-3-carboxaldehyde ("5-azapiperonal") required a low-yielding synthetic route[36] in which furfural was transformed by bromine/sulfamic acid/concentrated HCl (fairly efficiently) into 5-bromo-2,3-dihydroxypyridine, and subsequent ring closure with bromochloromethane was followed by reaction with butyllithium and *N*,*N*-dimethylformamide. The alcohol has not been described previously, and indeed few examples of this 1,3-dioxolo[4,5-*b*]pyridine ring system are known.

Thiazole-5-carboxaldehyde, for thiazole-5-methanol,[29] was obtained by deamination, using amyl nitrite, of the 2-amino derivative prepared from bromomalonaldehyde[37] and thiourea.[38] The synthesis of methyl isothiazole-4-carboxylate began from 2-chloro-5-nitrobenzaldehyde, readily converted into 5-nitrobenzisothiazole,[39] followed by several steps including oxidative cleavage of the fused benzene ring.[40] This ester was reduced to isothiazole-4-methanol in the manner applied for the homologous 3-methylisothiazole-4-methanol.[41] Methyl oxazole-5-carboxylate[42] was prepared from dimethyl tartrate, via lead tetraacetate oxidation[43] to methyl glyoxylate, and tosylmethyl isocyanide;[44] oxazole-5-methanol has been described[45] for use in the synthesis of neooxazolomycin. Ethyl isoxazole-4-carboxylate resulted from oximation of (ethoxycarbonyl)malonaldehyde;[46] isoxazole-4-methanol was obtained by reduction, similar to the homologous tertiary alcohol[47] prepared from this ester and methylmagnesium bromide. Methyl 1-methylimidazole-5-carboxylate,[48] from sarcosine methyl ester and Gold's salt, yielded 1-methylimidazole-5-methanol.[49]

Results and Discussion

Typical dose−response curves for the inactivation of recombinant human ATase and ATase activity in Raji cells by three of the inactivators considered are presented in Figure 1. IC_{50} values (Chart 1) were derived using lines of best fit.

The level of activity of O^6-furfurylguanine (**2**), expressed as the effective dose (IC_{50}) required to produce 50% inactivation of ATase in cell-free extracts in phosphate-buffered saline (PBS), was lower (IC_{50} 0.08 μM) than for O^6-benzylguanine (**1**), which, in our hands, has an IC_{50} value of 0.043 ± 0.021 (n = 21) μM. The published[10,14] value for O^6-benzylguanine (0.2 μM)

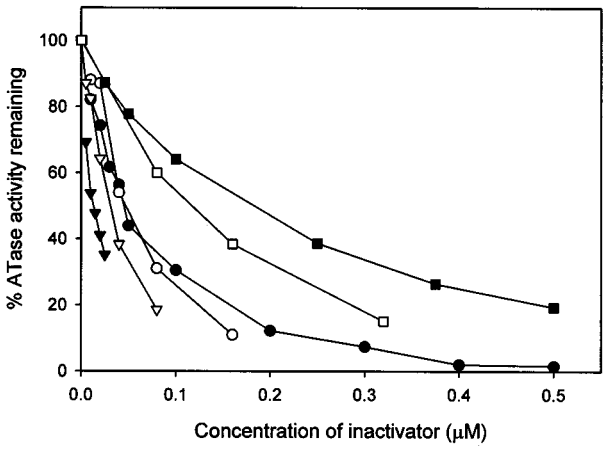


Figure 1. Inactivation of pure recombinant human ATase (closed symbols) or ATase activity in Raji cells (open symbols) by increasing concentrations of the inactivators B.4203 (**2**; squares) B.4205 (**6**; triangles), and B.4321 (**21**; circles). See text for experimental details.

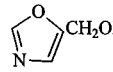
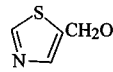
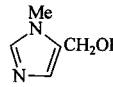
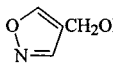
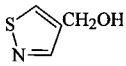
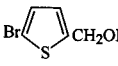
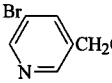
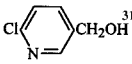
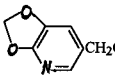
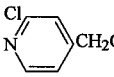
reflects differences in the assay procedures used by the various groups. We found that in Raji cells the IC_{50} values for **1** and **2** are respectively 0.1 and 0.12 μM (Table 3).

O^6-Thenylguanine (**6**) has an IC_{50} of 0.018 and 0.03 μM, respectively, in cell-free extracts and in Raji cells. As anticipated from the reactivity of these hetarylmethyl substituents, **2** and **6** are hydrolyzed to guanine very quickly in acid solutions, much faster than **1**. Even in the assay medium, PBS at pH 8.3, the half-lives ($t_{1/2}$) of **1**, **2**, and **6** are respectively >48h, 23 min, and 40 min. This reactivity will have significant consequences for the behavior of the drugs in vivo.

It proved impossible to make the pyrrole (NH or NMe) analogue of **2** and **6** since addition of sodium hydride to the appropriate alcohols caused polymerization.[50] Although as confirmed later[11,12] synthesis of the pyridine analogue **7** presented no problems, its activity was disappointingly poor (IC_{50} 35 μM, lower even than the 22 μM value we found for O^6-allylguanine). The chemistry of the 2-position in pyridine is somewhat special, while the 3-position is more typical of a benzene ring. As Kohda et al.[12] found, the 3-isomer **8** proved fairly active (IC_{50} 0.41 μM), and perhaps surprisingly the 4-isomer **9** was more active still (IC_{50} 0.13 μM). The increased polarity and water solubility of the *N*-oxide (**10**) of **8** was not advantageous (IC_{50} 1.4 μM).

For investigation of other basic heterocyclic substituents with five-membered rings, we concentrated on the isomers where the alkoxy group was in a 1,3-relationship to the nuclear N because of the poor activity of **7**. Again sulfur proved the most useful heteroatom, with oxazole, thiazole, and *N*-methylimidazole yielding drugs **11**, **12**, and **13** having respective IC_{50} values of 0.35, 0.033, and 8.5 μM. The isoxazole and isothiazole systems provide the option of a 1,4- as well as a 1,3-relationship. While we were unable to prepare the isoxazole **14** since sodium hydride caused decomposition of the appropriate alcohol consistent with the known effect of basic reagents on the isoxazole ring, the isothiazole **15** proved almost as active as the thiazole **12**. The synthesis of the 1,4-related **16** is forbidding since the necessary alcohol requires several steps beyond the already arduous procedure to **15**,[40] and a

Table 1. Spectral Data for Heterocyclic Alcohols

alcohol	precursor (product)	UV λ_{max} (nm)[a]	NMR δ (ppm from TMS)[b]						
			CH_2	OH	H-2	H-3	H-4	H-5	H-6
	$ArCO_2Me$[42] (**11**)		4.49 d (5.7)	5.42 t (5.7)	8.31		7.06		
	ArCHO[38] (**12**)		4.72 dd (5.8, 1.1)	5.61 t (5.8)	9.03 d (0.9)		7.78 dt (0.9,1.1)		
	$ArCO_2Me$[48] (**13**)		4.43	5.04	7.52		6.78		
	$ArCO_2Et$[46]		4.65	1.90 bs		8.41		8.32	
	$ArCO_2Me$[40] (**15**)	248	4.62 d (5.5)	5.35 t (5.5)		8.83		8.54	
	ArCHO (**17**)	246	4.68 d (5.5)	2.45 t (5.5)		6.73 d (3.7)	6.90 d (3.7)		
	ArCOCl[33] (**18**)	272	4.57 d (5.7)	5.50 t (5.7)	8.54 bs		7.96 d (2.0)		8.60 d (2.0)
	ArCOCl[31] (**19**)	268	4.74	1.90 bs	8.38 d (1.8)		7.70 dd (8.2, 1.8)	7.34 d (8.2)	
	ArCHO[36] (**20**)	234, 294	4.59	1.95 bs	7.60 bs		7.07 d (1.8)		
	ArCOCl[32] (**21**)	262,268 sh	4.58 d (5.5)	5.60 t (5.5)		7.44 bs		7.36 dd (4.9, 1.1)	8.36 d (4.9)

[a] UV spectra in MeOH; significant maxima > 240 nm are recorded. [b] Unless otherwise noted, ^1H NMR spectra in $(CD_3)_2SO$ at 300 MHz; *J* values (Hz) in parentheses, otherwise singlets. [c] δ 3.61 (NCH_3). [d] NMR spectrum in $CDCl_3$. [e] NMR spectrum at 80 MHz. [f] δ 6.06 (OCH_2O); mp 82−84 °C, from EtOH. Anal. ($C_7H_7NO_3$) C,H,N.

commercial source of isothiazole-5-carboxaldehyde is no longer available.[51] Of this group, the thiazole **12** is fairly accessible and has properties which make further biological investigation very desirable.

The introduction of halogen to the thiophene nucleus of **6** caused a very significant increase in the inhibitory activity: the 5-bromothenyl derivative **17** has an IC_{50} value of 0.005 μM. In the pyridine series this sort of modification was also helpful. For compounds where N and alkoxy are 1,3-related, 5-bromo substitution (**18**) gave IC_{50} 0.25 μM and 6-chloro (**19**) 0.20 μM. The electron-donating substituent in **20** (5,6-methylenedioxy, giving "azapiperonyl") produced almost identical inhibitory activity (IC_{50} 0.23 μM). Like its parent **9** with N and alkoxy 1,4-related, the 2-chloro derivative **21** was more active (IC_{50} 0.04 μM) and is another candidate for further study as the best of O^6-substituted guanines with a pyridine ring in the side chain.

A few of the compounds were slightly (**8**, **11**, **18**−**20**) or substantially (**7**) more effective in Raji cells than with the recombinant human ATase in vitro; however, the majority of compounds were as effective or slightly less effective in inactivating ATase in Raji cells than in vitro. The reduced effectiveness in Raji cells may be a consequence of a number of factors including binding to serum components, cellular uptake, nuclear translocation, or metabolic processes. Reasons for the increased effectiveness in Raji cells are more obscure: chemical instability does not seem to be an explanation since the relatively short half-lives of some of the compounds did not drastically affect their ability to inactivate ATase in Raji cells.

On the basis of IC_{50} values for the inactivation of pure recombinant human ATase in vitro and ATase activity in Raji cells, and the likelihood that these values would reflect in vivo activity, four compounds (**6**, **12**, **17**, **21**) might, if they displayed no adverse toxicities, be con-

Table 2. Preparation and Characterization of O^6-Substituted Guanines

O^6-substituted guanine	yield, %	mp, °C (dec)	formula[a]	UV λ_{max} (nm)[b]	NMR δ (ppm from TMS)[c] H-9	H-8	NH_2	CH_2	H-2′	H-3′	H-4′	H-5′	H-6′
2, B.4203	85	from 185	$C_{10}H_9N_5O_2 \cdot 0.5H_2O$	243, 285	12.42 bs	7.81	6.32	5.43		6.66 d (3.1)	6.49 dd (3.1, 1.5)	7.71 d (1.5)	
6, B.4205	72	from 160[i]	$C_{10}H_9N_5OS \cdot 0.33H_2O$	243, 284	12.47 bs	7.84	6.30	5.67		7.31 dd (3.5, 1.2)	7.04 dd (5.1, 3.5)	7.56 dd (5.1, 1.2)	
7, B.4210	71	220−221	$C_{11}H_{10}N_6O \cdot 0.5H_2O$	247, 262 269sh, 285	12.47 bs	7.84	6.27	5.58		7.5 m	7.5 m	7.5 m	8.58 dd (4.8, 1.2)
8, B.4211	53	226−228	$C_{11}H_{10}N_6O \cdot 0.5H_2O$	245, 263sh 270sh, 285	12.48 bs	7.87	6.36	5.58	8.80 bs		8.01 dd (7.9, 1.8)	7.49 dd (7.9, 4.8)	8.62 dd (4.8, 1.8)
9, B.4277[d]	72	from 230	$C_{11}H_{10}N_6O$	244, 265sh 286	12.51 bs	7.88	6.34	5.58	8.60 d (5.7)	7.47 d (5.7)		7.47 d (5.7)	8.60 d (5.7)
10, B.4282	54	244−254	$C_{11}H_{10}N_6O_2 \cdot H_2O$	271, 286 [RCH_2OH: 267]	12.52	7.87	6.41	5.48	8.42		7.47 m	7.47 m	8.22 m
11, B.4274[d,e]	32	180−215	$C_9H_8N_6O_2 \cdot 0.25H_2O$	243, 286	12.48 bs	7.85	6.38	5.56	8.45		7.44		
12, B.4275[d,e]	40	190−220	$C_9H_8N_6OS \cdot 0.5H_2O$	244, 286	12.49 bs	7.85	6.42	5.76	9.13		8.14		
13, B.4389[f]	82	from 190[i]	$C_{10}H_{11}N_5O$	241, 284	12.44	7.82	6.28	5.48	7.66		7.11		
15, B.4354	28	from 200	$C_9H_8N_6OS \cdot 0.75H_2O$	244, 284	12.47	7.84	6.41	5.58		8.81		9.22	
17, B.4269[d,e,g]	14	170−180[i]	$C_{10}H_8BrN_5OS$	247, 284	12.47 bs	7.85	6.36	5.62		7.19 d (3.7)	7.16 d (3.7)		
18, B.4320	56	from 220	$C_{11}H_9BrN_6O \cdot 0.5H_2O$	242, 281	12.50	7.86	6.41	5.53	8.73 d (2.2)		8.26 dd (2.2, 1.8)		8.78 d (1.8)
19, B.4319	58	from 215	$C_{11}H_9ClN_6O \cdot 0.5H_2O$	242, 276	12.48	7.87	6.38	5.53	8.64 d (2.4)		8.05 dd (8.2, 2.4)	7.59 d (8.2)	
20, B.4271[d,h]	63	230−240[i]	$C_{12}H_{10}N_6O_3.0.25EtOH$	241, 290	12.46 bs	7.83	6.39	5.40	7.83 d (1.8)		7.48 d(1.8)		
21, B.4321	10	from 234	$C_{11}H_9ClN_6O$	241, 272sh 285	12.56 bs	7.91 bs	6.36	5.58		7.61 bs		7.51 bs	8.44 bs

[a] Anal. C,H,N in all cases except for **7** (N: calcd, 33.47; found, 32.79), **10** (C: calcd, 47.83; found, 47.22), **15** (N: calcd, 32.16; found, 31.53), **17** (N: calcd, 21.47; found, 20.81), and **21** (C, N: calcd, 47.75, 30.37; found, 47.15, 29.32, respectively). [b] UV spectra in MeOH. [c] ^1H NMR spectra in $(CD_3)_2SO$ at 300 MHz; *J* values (Hz) in parentheses, otherwise singlets. [d] $ArCH_2OH$:**5**, 3:1. [e] Product extracted with MeCN. [f] δ 3.68 (NCH_3). [g] 2.5 mL DMF (−10 °C): 1 mmol of **5**: 3 mmol of $ArCH_2OH$. [h] δ 6.18 (OCH_2O). [i] Recrystallized from EtOH.

Table 3. ATase-Inactivating Activity and Stability of O^6-Substituted Guanines

compound	IC_{50} (μM)[a] rhATase	in Raji cells	in vitro half-life (h)[b]
O^6-benzylguanine	0.04	0.1	>48
B.4269 (**17**)	0.005	0.01	1.95
B.4205 (**6**)	0.018	0.03	0.67
B.4275 (**12**)	0.033	0.06	>48
B.4321 (**21**)	0.04	0.05	>48
B.4354 (**15**)	0.07	0.07	>48
B.4203 (**2**)	0.08	0.12	0.38
B.4277 (**9**)	0.13	0.20	>48
B.4319 (**19**)	0.20	0.12	>48
B.4271 (**20**)	0.23	0.09	>48
B.4320 (**18**)	0.25	0.2	>48
B.4274 (**11**)	0.35	0.22	>48
B.4211 (**8**)	0.43	0.22	>48
B.4282 (**10**)	1.4	5.4	>48
B.4389 (**13**)	8.5	21	0.19
B.4210 (**7**)	35	6.0	>48

[a] IC_{50} is the concentration of inactivator required to produce 50% reduction in ATase activity. [b] At 37 °C in phosphate-buffered saline (PBS; pH 7−7.2).

sidered potential clinical alternatives to O^6-benzylguanine.

Experimental Section

UV spectra were measured on a Unicam SP-800 spectrophotometer and NMR spectra (80 MHz) on Bruker WP-80 or (300 MHz) on MSL 300 instruments. Melting points, uncorrected, were determined in capillaries. Column chromatography was performed on Merck silica gel 60 (35−70 mesh ASTM, Art. 7733). Microanalyses of all compounds were within ±0.4% of theory unless otherwise indicated.

Heterocyclic alcohols not commercially available were prepared by literature methods, and UV and NMR spectral characteristics are given in Table 1. The quaternary salt **5** was obtained as described,[26] and O^6-substituted guanines **4** were prepared as outlined[25] for **1**, using the following detailed procedure.

Sodium hydride (60% in oil; 0.8 g, 20 mmol) was added to a solution of alcohol (56 mmol, ca. 5 mL) in DMSO (5 mL) and the mixture stirred at room temperature for 1 h. For solid or higher-molecular-weight alcohols, up to 10 mL of DMSO may be used. 2-Amino-*N*,*N*,*N*-trimethyl-*1H*-purin-6-aminium chloride (**5**) (2.29 g, 10 mmol) was added, and stirring was continued for a further 1 h. The change in UV spectrum was then complete (λ_{max} 312 → 284 nm), and the almost clear solution was treated with acetic acid (1.7 mL). After cooling and dilution with ether (300 mL), the mixture was set aside (2 h) and the solid (A) collected. Trituration with water gave the product. A second fraction can be obtained by evaporation of the ether−DMSO filtrate and trituration of the residue successively with ether and water. Alternatively the product may be extracted from the solid (A) with warm acetonitrile. The recrystallized compounds (usually from MeOH) showed a single spot in TLC (C_6H_6−MeOH, 4:1) and were characterized by analysis and their NMR spectra, as indicated in Table 2. Frequently, they contained solvent of crystallization.

Biological Evaluation. Purification of Recombinant ATases. The cDNA cloning and overexpression of the human ATase has been reported previously.[52] Purification of the recombinant proteins was achieved either by affinity chromatography through a DNA-cellulose column as described[53] or by DEAE-cellulose ion-exchange chromatography. For the latter, the ATase protein was partially purified by ammonium sulfate precipitation (30−60%) and dialyzed against 10 mM

Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 2 mM EDTA, 10% glycerol, before loading onto a DEAE-cellulose column. The ATase was then eluted with a 0−0.1 M NaCl gradient. The purified human ATase protein retained activity for more than 1 year when stored at high concentration at −20 °C in buffer I [50 mM Tris-HCl (pH 8.3)/3 mM DTT/1 mM EDTA] and could be thawed and refrozen several times without substantial loss of activity.

Incubation with Inactivators and ATase Assay. Compounds to be tested were dissolved in DMSO to a final concentration of 10 mM and diluted just before use in buffer I containing 1 mg/mL bovine serum albumin (IBSA). Recombinant ATase was diluted in IBSA and titrated in order that the reaction be conducted under ATase, and not substrate, limiting conditions. In each assay, fixed amounts of ATase (50−60 fmol) were incubated with varying amounts of O^6-benzylguanine, or test compound, in a total volume of 200 μL of IBSA containing 10 μg of calf thymus DNA at 37 °C for 1 h. The ^{3}H-methylated DNA substrate (100 μL containing 4 μg of DNA and 100 fmol of O^6-methylguanine) was added and incubation continued at 37 °C for 1 h, until the reaction was completed. Following acid hydrolysis of the DNA as previously described,[54] the ^{3}H-methylated protein was recovered and quantitated by liquid scintillation counting. Samples were typically assayed in duplicate and experiments repeated several times. IC_{50} is the concentration of inactivator required to produce a 50% reduction in ATase activity.

Cell Treatment and Preparation of Extracts. Raji cells (a human lymphoblastoid cell line derived from a Burkitt's lymphoma) were grown in suspension culture in RPMI medium supplemented with 10% horse serum. Cells were diluted to 10^6/mL in culture medium containing either the appropriate concentration of inactivator or an equivalent volume of vehicle (DMSO). Following incubation at 37 °C for 2 h, the cells were harvested by centrifugation and washed twice with PBS and the resulting cell pellets (between 1 and 2 × 10^7 per pellet) stored at −20 °C. Cell pellets were resuspended in cold (4 °C) buffer I containing 5 μg/mL leupeptin and sonicated for 10 s at 216-μm peak-to-peak amplitude. After the mixture cooled in ice, 0.01 volume of 8.7 mg/mL phenylmethanesulfonyl fluoride in ethanol was added, and the sonicates were centrifuged at 15000*g* for 10 min at 4 °C to pellet cell debris. ATase activity in the supernatant was determined as described above and expressed as the percentage activity remaining based on that present in the untreated controls (usually 350−450 fm/mg).

Stability of Inactivators at 37 °C. Inactivators (10 mM in DMSO) were diluted to 0.01 mM in prewarmed degassed PBS. Samples were immediately transferred to a CARY13 spectrophotometer (cuvette block held at 37 °C) and scanned at an appropriate wavelength (according to the spectral properties of the compound) at 3−10-min intervals for up to 20 h. The results were expressed as percentage absorbance change versus time and $t_{1/2}$ values (half-life) extrapolated from this.

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