Resistance-Modifying Agents. 8.[1] Inhibition of O^6-Alkylguanine-DNA Alkyltransferase by O^6-Alkenyl-, O^6-Cycloalkenyl-, and O^6-(2-Oxoalkyl)guanines and Potentiation of Temozolomide Cytotoxicity in Vitro by O^6-(1-Cyclopentenylmethyl)guanine

Roger J. Griffin,*,† Christine E. Arris,‡ Christine Bleasdale,† F. Thomas Boyle,§ A. Hilary Calvert,‡ Nicola J. Curtin,‡ Christine Dalby,† Sreenivas Kanugula,|| Nicola K. Lembicz,† David R. Newell,‡ Anthony E. Pegg,|| and Bernard T. Golding†

Department of Chemistry, Bedson Building, The University, Newcastle upon Tyne NE1 7RU, U.K.; Cancer Research Unit, Medical School, Framlington Place, Newcastle upon Tyne NE2 4HH, U.K.; AstraZeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K.; and Departments of Cellular and Molecular Physiology and Pharmacology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, P.O. Box 850, Hershey, Pennsylvania 17033

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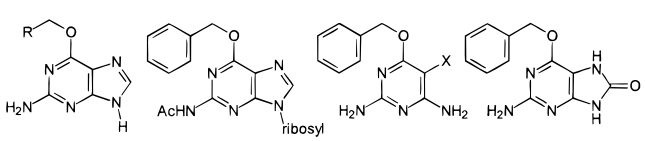
A series of O^6-allyl- and O^6-(2-oxoalkyl)guanines were synthesized and evaluated, in comparison with the corresponding O^6-alkylguanines, as potential inhibitors of the DNA-repair protein O^6-alkylguanine-DNA alkyltransferase (AGT). Simple O^6-alkyl- and O^6-cycloalkylguanines were weak AGT inactivators compared with O^6-allylguanine ($IC_{50} = 8.5 \pm 0.6\ \mu M$) with IC_{50} values ranging from 100 to 1000 μM. The introduction of substituents at C-2 of the allyl group of O^6-allylguanine reduced activity compared with the parent compound, while analogous compounds in the O^6-(2-oxoalkyl)guanine series exhibited very poor activity (150−1000 μM). O^6-Cycloalkenylguanines proved to be excellent AGT inactivators, with 1-cyclobutenylmethylguanine ($IC_{50} = 0.55 \pm 0.02\ \mu M$) and 1-cyclopentenylmethylguanine ($IC_{50} = 0.39 \pm 0.04\ \mu M$) exhibiting potency approaching that of the benchmark AGT inhibitor O^6-benzylguanine ($IC_{50} = 0.18 \pm 0.02\ \mu M$). 1-Cyclopentenylmethylguanine also inactivated AGT in intact HT29 human colorectal carcinoma cells ($IC_{50} = 0.20 \pm 0.07\ \mu M$) and potentiated the cytotoxicity of the monomethylating antitumor agent Temozolomide by approximately 3- and 10-fold, respectively, in the HT29 and Colo205 tumor cell lines. The observation that four mutant AGT enzymes resistant to O^6-benzylguanine also proved strongly cross-resistant to 1-cyclopentenylmethylguanine indicates that the O^6-substituent of each compound makes similar binding interactions within the active site of AGT.

Introduction

The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) acts on DNA in which guanine residues have been converted into potentially mutagenic, carcinogenic, and cytotoxic O^6-alkylguanine adducts following exposure to alkylating agents.[2−5] AGT repairs O^6-alkylguanine lesions by transferring the O^6-alkyl group to an active-site cysteine thiolate in a stoichiometric reaction, which regenerates guanine and produces a chemically stable *S*-alkylcysteine.[5] Importantly, this essentially irreversible process results in the inactivation of AGT, with the restoration of cellular AGT levels requiring de novo resynthesis of the protein.

Tumor sensitivity to chemotherapeutic alkylating agents has been found to correlate inversely with cellular AGT activity, high levels of the protein confering resistance to the cytotoxicity of these drugs.[6,7] Consequently, inactivation and depletion of AGT in tumor cells, either by pretreatment with a methylating agent or by direct administration of an AGT pseudosubstrate, potentiates the cytotoxicity of DNA-damaging agents which function by guanine O^6-alkylation.[5,8,9] Although cellular AGT depletion has been achieved in vitro with the purine free base O^6-methylguanine (MG, **1**)[3,4] this compound is devoid of activity in vivo.[10] In contrast, O^6-benzylguanine (BG, **2**) is a potent inactivator which effects a rapid and pronounced AGT depletion, in vitro and in vivo.[6,11,12] Significant potentiation of methylating and chloroethylating agent cytotoxicity has been observed in both tumor cell lines and mice bearing human tumor xenografts, following pretreatment with BG,[5,6] and clinical trials with BG in combination with BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) are currently in progress.[13,14]



1 R = H
2 R = Ph
3 R = 2-thienyl
7 R = CH_2=CH

4

5 X = NO, NO_2

6

A number of point mutations in the human AGT sequence that render the protein resistant to inactiva-

* To whom correspondence should be addressed. Tel: +44 191 222 8591. Fax: +44 191 222 8591. E-mail: r.j.griffin@ncl.ac.uk.
† Department of Chemistry, Newcastle University.
‡ Cancer Research Unit, Newcastle University.
§ AstraZeneca Pharmaceuticals.
|| Pennsylvania State University College of Medicine.

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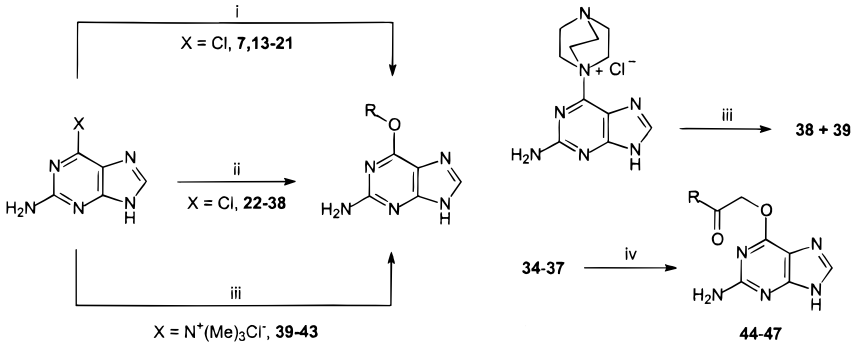
Scheme 1[a]



[a] Reagents: (i) ROH, Na, reflux; (ii) ROH, NaH, THF, reflux; (iii) ROH, NaH, DMSO, 25 °C; (iv) AcOH or CF_3CO_2H, 25 °C.

tion by BG have been reported.[15−18] The recently derived crystal structure of human AGT[19] provides a reasonable explanation for the inability of these mutants to react with BG, because it shows that the altered residues are located in the BG-binding pocket and would tend to exclude BG from this pocket. Although such alterations have not yet been shown to occur in clinical trials of patients treated with BG, these observations raise a serious possibility that the development of resistance to therapy with BG plus an alkylating agent may occur relatively readily. The availability of additional inhibitors that are able to inactive the resistant mutants is therefore highly desirable.

A large number of analogues of BG have been synthesized in order to investigate structure−activity relationships (SARs) for AGT inhibition.[5,20−22] In addition, thienylguanine **3**[23] and guanosine derivatives (e.g. **4**)[24−26] have been investigated for enhanced potency and/or water solubility. Interestingly, a series of 2,4-diamino-4-benzyloxypyrimidines, exemplified by **5**, were also found to be significantly more active than BG and are among the most potent AGT inactivators reported to date.[22,27] Direct administration of O^6-benzyl-8-oxoguanine (**6**), a major metabolite of comparable potency to BG, has also been considered as a means of minimizing polymorphic variations in the metabolism of BG, although **6** is very poorly soluble.[28]

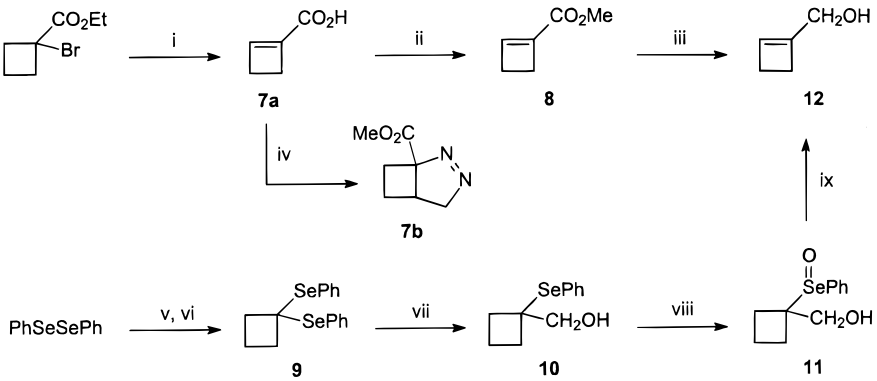
As part of studies to develop novel AGT inactivators, we have previously reported preliminary results with a series of O^6-(2-oxoalkyl)guanines, designed to probe the stereoelectronic and hydrophobic requirements of the AGT-mediated alkyl-transfer reaction.[29] Although these compounds exhibited only weak activity, several of the corresponding O^6-allyl derivatives, prepared for comparative purposes, were relatively potent inactivators of AGT from human HT29 colorectal cells. Indeed, O^6-allylguanine **7**, which has also been reported independently by Moschel et al.,[20] proved to be the most potent AGT inactivator of the series ($IC_{50} = 8.5 \pm 0.6$ μM). While this may be attributed, in part, to stereoelectronic acceleration of the S_N2 substitution reaction by the adjacent alkene, our results with O^6-(2-oxoalkyl)guanines, presented more fully in this paper, and observations by others[5,21] suggest that steric factors are also important. To explore this further, a series of guanines bearing substituted O^6-allyl groups, and also derivatives which incorporate the allylic double bond within a carbocyclic ring, have been synthesized. The latter series of compounds was prepared with the expectation that additional steric and hydrophobic interactions, combined with enhanced S_N2 reactivity arising from conformational restriction of the allylic group, would confer favorable AGT binding and inhibitory activity, respectively. The analogous O^6-alkyl- and O^6-cycloalkylguanines were also synthesized for comparison. In this paper we present the results of these studies. A preliminary account of part of this work has been published previously.[29]

Chemistry

The structures and properties of the compounds evaluated as AGT inactivators are recorded in Table 1, and their syntheses are outlined in Scheme 1. The required O^6-alkylguanines were synthesized from 2-amino-6-chloropurine, either by direct reaction with the appropriate sodium alkoxide, prepared by treating the corresponding alcohol with sodium (**7**, **13**−**21**) or sodium hydride in THF (**22**−**38**),[30,31] or following conversion into the more reactive 2-aminotrimethylpurin-6-ylammonium chloride[32] (**39**−**43**). The target O^6-(2-oxoalkyl)guanines (**44**−**47**) were prepared by acid-catalyzed hydrolysis of the corresponding acetals (**34**−**37**).

Two approaches were investigated for the synthesis of 1-cyclobutenemethanol (**12**), required for the preparation of O^6-(1-cyclobutenylmethyl)guanine **(38)** (Scheme 2). Initial efforts to synthesize **12** by direct $LiAlH_4$ reduction of the unstable 1-cyclobutenecarboxylic acid **(7a)**, prepared by dehydrobromination of commercially available ethyl 1-bromocyclobutanecarboxylate,[33] were unsuccessful, presumably as a consequence of the poor solubility of the lithium salt of the acid in ether. Burger et al.[34] reported a low yield (15%) of **12**, following reduction of methyl 1-cyclobutenecarboxylate (**8**) with $LiAlH_4$. We have attempted to improve this reaction. Esterification of **7a** with *N*,*N*-diisopropyl-*O*-methylisourea[35] or 1 mol equiv of diazomethane afforded the methyl ester **8** in excellent yield, whereas treatment with excess diazomethane gave the pyrazoline ester **7b**, arising from 1,3-dipolar cycloaddition to the reactive cycloalkene. Analysis (NMR and GC) of the reaction mixture resulting from the reaction of **8** with $LiAlH_4$ demonstrated complete consumption of the ester and formation of the required alcohol **12** (57%), together with cyclobutanemethanol (43%) arising from competing 1,4-hydride addition to the cycloalkene. Systematic variation of the reaction conditions employed (nature and quantity of hydride reagent, solvent, and duration of

Scheme 2[a]



[a] Reagents: (i) KOH, PhMe, reflux; (ii) 1 mol equiv CH_2N_2, Et_2O, 0 °C; (iii) $LiAlH_4$, Et_2O, 25 °C; (iv) excess CH_2N_2, Et_2O, 0 °C; (v) $NaBH_4$, EtOH, 25 °C; (vi) cyclobutanone, $ZnCl_2$, CCl_4, 25 °C; (vii) *t*-BuLi, THF, −78 °C, then CH_2O(g); (viii) $NaIO_4$, aq MeOH, 25 °C; (ix) Na_2CO_3, Et_2O, reflux.

reaction) indicated that the reaction time is critical. Thus, conducting the reaction with 0.6 mol equiv of $LiAlH_4$ in ether over 15 min afforded an optimal yield (84%) of **12**. All attempts to remove the residual 1-cyclobutanemethanol by chromatography or fractional distillation resulted in extensive decomposition of **12**, and consequently, the mixture of alcohols was used without further purification. Although the subsequent reaction with 2-amino-6-chloropurine or its trimethylammonio derivative furnished an intractable mixture of products, the use of 'DABCO-purine' (2-amino-6-(azonia-4-azabicyclo[2.2.2-oct-1-yl]purine chloride, Scheme 1)[36] enabled isolation of the target 1-cyclobutenylguanine **38**, albeit in low yield.

The alternative route to **12** utilized a selenoxide fragmentation strategy. Treatment of cyclobutanone with benzeneselenol, freshly prepared by reduction of diphenyldiselenide with $NaBH_4$,[37] afforded 1,1-bis(phenylseleno)cyclobutane **(9)** in excellent yield. Lithiation of **9** and reaction of the resulting α-selenocarbanion with anhydrous methanal gave 1-hydroxymethyl-1-phenylselenocyclobutane **(10)**, which was smoothly converted into the corresponding selenoxide **11** on treatment with sodium periodate in aqueous MeOH.[38] Final thermally induced selenoxide fragmentation of **11** gave the requisite 1-cyclobutenemethanol **(12)** in reasonable yield, after fractional distillation.

Results and Discussion

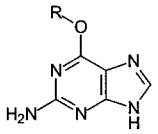
SARs. The concentration of each compound required to inactivate AGT extracted from HT29 human colorectal tumor cells by 50% (IC_{50}) is shown in Table 1. For simple linear O^6-alkylguanines (**13**−**15**, **17**−**19**) homologation to ethyl (**13**) or *n*-propyl (**14**) resulted in a substantial reduction in potency compared with **1**, and the poor activity of **13** is in agreement with the results of Yarosh et al.[2] Interestingly, although the *n*-pentyl homologue **17** was only weakly active, O^6-*n*-butylguanine (**15**) and O^6-*n*-hexylguanine (**18**) were of comparable potency to **1**. Homologation beyond an ethyl group would not be expected to make any significant difference to activity on electronic grounds, suggesting that hydrophobic interactions within the substrate binding site are of importance. This suggestion is consistent with the presence of a deep hydrophobic pocket or cleft adjacent to the active site of AGT, as proposed previously.[21,29]

Although the biological evaluation of compounds of this type was hampered by their very poor solubility, the effects of chain unsaturation and the insertion of a ring system were also investigated. The O^6-hexenylguanine analogues (**22**−**24**) proved less active than the parent O^6-*n*-hexylguanine (**18**). The greater potency of O^6-((*Z*)-3-hexenyl)guanine (**23**) compared with the *E*-stereoisomer (**24**) presumably arises as a result of a more favorable interaction of **24** with the hydrophobic pocket. The introduction of a cycloalkyl substituent at the guanine O^6-position was also detrimental to activity, with the O^6-cyclobutylmethyl (**39**), O^6-cyclopentylmethyl (**40**), and O^6-cyclohexylmethyl (**20**) derivatives all exhibiting IC_{50} values for AGT inactivation of >1 mM. This poor activity presumably reflects the greater steric bulk and reduced flexibility of an alicyclic substituent compared with an open-chain alkyl group and suggests that the hydrophobic pocket giving access to the AGT active site is narrow.

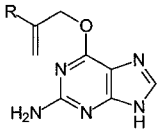
The introduction of an allyl substituent at the guanine O^6-position (**7**) resulted in a marked increase in potency (IC_{50} = 8.5 μM) compared with the corresponding O^6-propyl analogue (**14**) (24% inactivation at 1.0 mM), whereas O^6-propargylguanine (**31**) (IC_{50} = 20 μM) was less potent than **7** as an AGT inactivator. These results are consistent with the observations of Moschel et al.,[20] who reported an IC_{50} value of 20 μM for **7**. For S_N2 substitution reactions a carbon−carbon double bond α to the reaction center elicits a significant rate enhancement, compared to a corresponding saturated group.[39] However, and in keeping with previous reports,[20,23] the reactivities of O^6-alkylguanines as AGT inactivators in our studies did not parallel those reported for a simple chemical system. Thus, the rank order of potency for AGT inactivation is BG (**2**) > **7** ≫ MG (**1**), with BG proving approximately 50- and 1750-fold more active than **7** and **1**, respectively (Table 1). This is in contrast to S_N2 reactions, where benzyl, methyl, and allyl chlorides have been reported to show comparable reactivity toward iodide ion in acetone.[40] These results are consistent with the presence of an active-site hydrophobic pocket, which accommodates the benzyl group of **2** in an orientation so as to provide a stereoelectronic acceleration of the S_N2 reaction with the cysteine thiolate of AGT.[29]

With a view to enhancing interactions with the putative hydrophobic pocket of AGT, a series of substi-

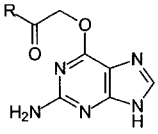
Table 1. Physical Data and Activity of Compounds as AGT Inactivators



A



B



C

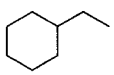
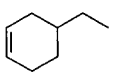
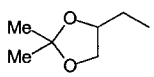
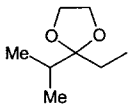
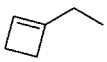
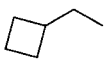
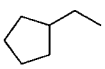
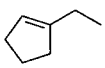
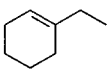
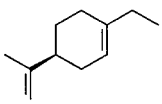
compd	general structure	R	Method[a]	recryst. solvents[b]	yield (%)	formula	mp (°C)	AGT inactivation IC_{50} (μM)[c] in HT29 cell extract and intact cells: Cell extract (% inactivation at 1 mM)	AGT inactivation IC_{50} (μM)[c] in HT29 cell extract and intact cells: Intact cells[d]
MG (1)[e]	A	Me	–	–	–	–	–	428 ± 26	248 ± 48[f]
BG (2)	A	CH_2Ph	–	–	–	–	–	0.18 ± 0.02	0.058 ± 0.007[g]
TG (3)[h]	A	2-thienyl	–	–	–	–	–	0.003 ± 0.002	–
7	B	H	I	*B*	68	$C_8H_9N_5O$	207-208[i]	8.5 ± 0.6	2.7, 5.1
13	A	Et	I	*A*	69	$C_7H_9N_5O$	> 230[j]	>1000 (0%)	–
14	A	*n*-Pr	I	*A*	36	$C_8H_{11}N_5O$	199-201[k]	>1000 (24%)	–
15	A	*n*-Bu	I	*A*	54	$C_9H_{13}N_5O$	176-178[l]	493 ± 85	–
16	A	$CH_2CH_2CH(Me)_2$	I	*A*	56	$C_{10}H_{15}N_5O$	173-174	>1000 (24%)	–
17	A	$(CH_2)_4Me$	I	*A*	71	$C_{10}H_{15}N_5O$	183-185	1006 ± 38	–
18	A	$(CH_2)_5Me$	I	*A*	57	$C_{11}H_{17}N_5O$	207-208	554 ± 151	–
19	A	$(CH_2)_6Me$	I	*A*	54	$C_{12}H_{19}N_5O$	172-175	> 100 (10%)[m]	–
20	A		I	*C*	72	$C_{12}H_{17}N_5O$	198-199	>1000 (0%)	–
21	B	Me	I	*C*	60	$C_{10}H_{12}N_2O_3$	176-178	25 ± 2	13.5, 9.9
22	A	$CH_2=CH(CH_2)_3CH_2$	II	*A*	70	$C_{11}H_{15}N_5O$	203	ND[n] (65%)	–
23	A	*Z*-$EtCH=CHCH_2CH_2$	II	*A*	61	$C_{11}H_{15}N_5O$	201-202	ND (88%)	–
24	A	*E*-$EtCH=CHCH_2CH_2$	II	*A*	61	$C_{11}H_{15}N_5O$	204-205	>1000 (32%)	–
25	A	$EtC\equiv CCH_2CH_2$	II	*A*	65	$C_{11}H_{13}N_5O$	> 203	ND	–
26	A	$PhCH_2CH_2$	II	*G,C*	49	$C_{13}H_{13}N_5O$	206-207	548 ± 26	–
27	B	Et	II	*G,C*	50	$C_{10}H_{13}N_5O$	148-149	16 ± 1	–
28	B	*i*-Pr	II	*G,C*	34	$C_{11}H_{15}N_5O$	170-172	>1000 (22%)	–
29	A	$CH_3CH=C(CH_3)CH_2$	II	*H,C*	73	$C_{10}H_{13}N_5O$	164-165	ND	–
30	B	Ph	II	*G,C*	19	$C_{14}H_{13}N_5O$	83-85	77 ± 2	37.5, 39.6
31	A	$CH\equiv CCH_2$	II	*G,B*	52	$C_8H_7N_5O$	> 230	20 ± 1	11.5, 11.9

Table 1 (Continued)

compd	general structure	R	Method[a]	recryst. solvents[b]	yield (%)	formula	mp (°C)	AGT inactivation IC_{50} (μM)[c] in HT29 cell extract and intact cells: Cell extract (% inactivation at 1 mM)	Intact cells[d]
32	A		II	*H*	95	$C_{12}H_{15}N_5O$	176-177	>1000 (32%)	–
33	A		II	*I*	98	$C_{11}H_{15}N_5O_3$	170-171	>1000 (0%)	–
34	A	$CH_3C(OEt)_2CH_2$	II	*G,E*	87	$C_{12}H_{19}N_5O_3$	158-159	>1000 (0%)	–
35	A	$EtC(OMe)_2CH_2$	II	*G,E*	59	$C_{11}H_{17}N_5O_3$	229-232	>1000 (0%)	–
36	A		II	*G,C*	51	$C_{12}H_{17}N_5O_3$	234-235	ND (54%)	–
37	A	$PhC(OMe)_2CH_2$	II	*G,C*	71	$C_{15}H_{17}N_5O_3$	208-209	>1000 (0%)	–
38	A		II	*G,C*	43	$C_{10}H_{11}N_5O$	150-151	0.55 ± 0.02	–
39	A		II (III)	*A*	63 (64)	$C_{10}H_{13}N_5O$	206-207	>1000 (34%)	–
40	A		II (III)	*H (A)*	82 (62)	$C_{11}H_{15}N_5O$	121	>1000 (0%)	–
41	A		III	*H*	70	$C_{11}H_{13}N_5O$	210	0.39 ± 0.04	0.20 ± 0.07
42	A		III	*E*	71	$C_{12}H_{15}N_5O$	195-197	1.6 ± 0.1	–
43	A		III	*E*	64	$C_{15}H_{19}N_5O$	190-192	2.6 ± 0.42	–
44	C	Me	IV	*B*	50	$C_8H_9N_5O_2$	195-196	192 ± 9	119, 95
45	C	Et	IV	*A*	77	$C_9H_{11}N_5O_2$	195-196	151 ± 3	94.5, 93.2
46	C	*i*-Pr	IV	*F*	69	$C_{10}H_{13}N_5O_2$	> 230[o]	>1000 (31%)	–
47	C	Ph	IV	*E*	35	$C_{13}H_{11}N_5O_2$	> 230[o]	>1000 (0%)	–

[a] See the Experimental Section. [b] Recrystallization solvents: A, H_2O; B, Me_2CO; C, EtOAc−petrol; D, DMF−H_2O; E, MeOH; F, EtOH. Purified by column chromatography on silica: G, eluent = DCM:EtOH (85:15); H, eluent = DCM:MeOH (92:8). I, product crystallized directly from reaction mixture on cooling and was triturated with ether. [c] Concentration of inhibitor required to reduce AGT activity to 50% of control rate. For details of assay see ref 29. [d] Values for 2 independent IC_{50} determinations unless otherwise indicated. [e] O^6-Methylguanine. [f] Mean ± SD of 4 experiments. [g] Mean ± SD of 3 experiments. [h] O^6-Thienylguanine. [i] Lit.[31] mp 205 °C. [j] Lit.[30] mp 293 °C. [k] Lit.[30] mp 208 °C. [l] Lit.[30] mp 175 °C. [m] Determined at 100 μM. [n] ND, IC_{50} value not determined. [o] Decomposes.

tuted O^6-allylguanines (**21**, **27**, **28**, **30**) was synthesized and evaluated. Homologation from allyl (**7**) to methallyl (**21**) resulted in an approximately 3-fold reduction in potency, while a partial restoration of activity was observed with O^6-ethallylguanine (**27**), which was nevertheless some 2-fold less potent than **7**. The introduction

of a phenyl group (**30**) reduced potency approximately 10-fold compared with **7**, whereas an isopropylallyl group at the guanine O^6-position (**28**) resulted in a dramatic loss of potency ($IC_{50} > 1$ mM). Although none of the substituted O^6-allylguanines was as potent as the parent compound **7**, the relative activities of compounds in this series followed a somewhat similar trend to that observed for the simple O^6-alkylguanines (**1**, **13**−**15**, **17**−**19**). This presumably reflects a balance between favorable hydrophobic interactions and unfavorable steric bulk and provides further evidence that the putative hydrophobic pocket of AGT has strict constraints.

In contrast to the O^6-cycloalkylmethylguanine series (**20**, **39**, **40**), incorporation of the allylic double bond into a ring system resulted in a large increase in potency compared with the parent O^6-allylguanine **(7)**. Thus, the potency of O^6-(1-cyclohexenylmethyl)guanine **(42)** was approximately 5-fold that of **7** and at least 1000-fold greater than that of the corresponding cyclohexylmethylguanine **20**. The effect of introducing an allylic double bond was even more dramatic for the 1-cyclobutenylmethyl (**38**) and 1-cyclopentenylmethyl (**41**) derivatives, which were potent AGT inactivators with IC_{50} values of 0.55 ± 0.02 and 0.39 ± 0.04 μM, respectively. The greater activity of the cycloalkenylguanines (**38**, **41**, **42**) relative to **7** may be attributed to several factors. In addition to an increased potential for hydrophobic interactions within the active site of AGT, it is also possible that the cycloalkenyl double bond adopts a more favorable orientation, relative to that of O^6-allylguanine (**7**), for stabilization of the transition state arising from attack on the α-CH_2 by the active-site thiolate. The chemical reactivity of O^6-(1-cyclobutenylmethyl)guanine (**38**) may also be further enhanced by ring strain arising from the 4-carbon ring. However, the fact that O^6-cyclopentenylmethylguanine (**41**) proved the most potent AGT inactivator in the cycloalkenyl series suggests that a subtle combination of stereoelectronic, steric, and lipophilic binding factors is the major determinant of activity. Such factors would account for the reduction in potency observed for the bulkier O^6-cyclohexenylmethylguanine (**42**) and the detrimental effect of introducing an isopropenyl substituent onto the cyclohexenyl ring in **43** ($IC_{50} = 2.6 \pm 0.42$ μM). Consistent with the requirement for an allylic system, transposing the cyclohexenyl double bond to the 3-position (**32**) dramatically reduced potency, confirming that the influence of the activating double bond does not extend to any significant degree beyond the α-carbon atom.[41]

Encouraged by the increased potency of O^6-alkylguanines bearing an α-alkenyl group, we considered alternative structural modifications predicted to enhance the reactivity of the O^6-alkyl group to nucleophilic attack. Classical studies of S_N2 reactions have demonstrated that α-halo ketones are much more reactive to nucleophiles than alkyl or allyl halides. For example, in reactions with iodide in acetone, phenacyl chloride and chloroacetone exhibit reaction rates approximately 100 000 and 35 700 times, respectively, that of *n*-butyl chloride, while benzyl chloride is only some 200-fold more reactive than *n*-butyl.[39] In addition, studies comparing the reactivity of phenacyl bromide and iodomethane with a variety of nucleophiles in acetonitrile have shown that phenacyl bromide is relatively more reactive to charged and polarizable nucleophiles, including thiolates.[42] In light of these observations a series of O^6-(2-oxoalkyl)guanines (**44**−**47**) was synthesized and evaluated for AGT inactivatory activity.

Table 2. Inactivation of Purified Wild-Type and Mutant Forms of AGT by **2** and **41**[a]

	ED_{50} value (μM)			ED_{50} value (μM)	
AGT protein	**2**	**41**	AGT protein	**2**	**41**
wild-type	0.2	0.3	P140K	>1500	>1500
G160R	4.7	13	G156A	60	113
Y158H	620	~1500			

[a] See the Experimental Section.

Comparison of the activities of the 2-oxoalkyl series (**44**−**47**) with the isosteric allyl series (**21**, **27**, **28**, **30**) (Table 1) indicates that a minor structural modification, notably replacing a methylene group by oxygen, has a profound effect upon potency, with the O^6-(2-oxoalkyl)guanines all proving considerably less active than their O^6-allylguanine counterparts. Interestingly, the overall pattern of activity in the 2-oxoalkyl series paralleled that observed in the allyl series, in that homologation from methyl (compare **21** and **44**) to ethyl (compare **27** and **45**) resulted in a slight increase in potency. Similarly, the presence of an isopropyl or phenyl substituent in both the 2-oxoalkyl (**46**, **47**) and allyl (**28**, **30**) series was not conducive to AGT inactivation.

It is evident that the presence of an α-carbonyl group in compounds **44**−**47** confers no significant stereoelectronic benefit, and this may be as a consequence of the polar carbonyl group proving incompatible with the hydrophobic pocket. Alternatively, the preferred binding mode of compounds in this series may be such that the orientation of the carbonyl function does not provide a stereoelectronic acceleration of the cysteine alkylation reaction. The failure of the 2-oxoalkyl substituent to endow the reactivity observed in a simple chemical system serves to illustrate the difficulty of rational design of compounds intended to interact with a complex biological system. With the exception of **36**, which exhibited weak activity, the O^6-(2-oxoalkyl)guanine precursors (**34**−**37**) were all essentially inactive.

Inactivation of Wild-Type and Mutant Forms of AGT by 2 and 41. The results in Table 1 show that **41** is only slightly inferior to **2** in the ability to inactivate wild-type AGT and is therefore a viable potential alternative to O^6-benzylguanine (**2**). The small difference between these agents in this respect is probably insignificant, and questions of pharmacokinetics would determine their relative in vivo potential. However, the results in Table 2 show clearly that **41** does not provide a means to selectively inactivate mutants of AGT resistant to **2**. The four mutants tested represent several sites at which point mutations interfere with the ability of the AGT protein to react with **2**, and in all cases, these changes also impair reaction with **41**. The mutants tested include G160R, which is only moderately resistant but is a naturally occurring, although rare, variant of AGT,[18] and three mutants (G156A, Y158H, and P140K) which are more strongly resistant. It appears from the crystal structure of the protein[19] that the extreme resistance of the P140K mutant to **2** is due to the loss of the proline side chain, which forms a binding

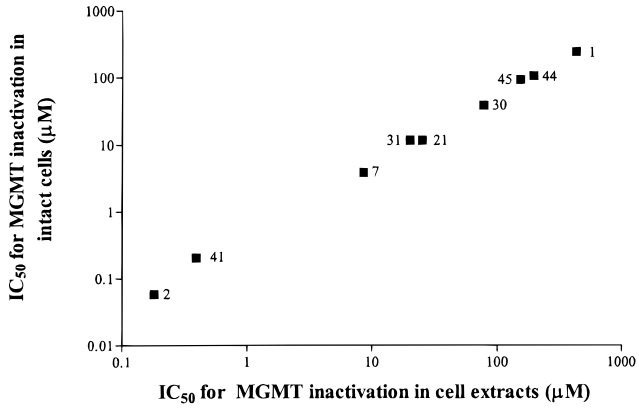


Figure 1. Comparison of AGT inactivation by selected compounds in intact HT29 cells and HT29 extracts. Data are from Table 1. The IC_{50} for MGMT inactivation in intact HT29 cells is the concentration of inactivator required to reduce whole cell MGMT activity by 50%, following incubation of intact cells with the inactivator for 4 h.

surface for the benzyl group and its replacement by the charged lysine side chain which discourages binding. Similarly, the phenyl ring of Y158 appears to take part in an aromatic stacking reaction with the phenyl ring of **2**, which is prevented when Y158 is replaced by histidine. Replacement of G156 by any other residues including the G156A change produces a distortion of the active site that would prevent access of **2**. The G160R change would also be expected to provide some steric interference to the binding of **2**. The cross-resistance of all four mutant AGT proteins to **41** suggests that this compound binds in an analogous fashion to **2** in the active site of AGT and that interactions with the cyclopentenyl ring of **41** are broadly similar to those of the phenyl ring of **2**.

Comparative Inactivation of AGT by Alkylguanines in HT29 Cells and HT29 Cell Extracts. The ability of selected guanine derivatives to inactivate AGT in intact HT29 cells was determined and is shown in Table 1 and Figure 1. There was a strong linear relationship between the potency of individual compounds as AGT inactivators in whole cells and their potency against AGT in cell extracts ($r = 0.998$, $p = <0.00001$). The fact that the relative AGT inactivating potencies of the substrate analogues were the same in whole cells and in cell free extracts suggests that there is no barrier to the entry of the compounds into cells. The finding that the IC_{50} value for AGT inactivation in intact cells is approximately one-half that observed in cell extracts is consistent with similar studies of other AGT-inactivating substrate analogues.[20−22] However, exceptions have been reported, e.g. 8-aza-O^6-benzylguanine and O^6-benzyl-8-bromoguanine[22] and 2-amino-6-benzyloxy-9-(carboxymethyl)purine, sodium salt.[20] These latter compounds are relatively inactive as AGT inactivators in intact cells, suggesting that they have poor membrane permeability.

Potentiation of the Cytotoxicity of Temozolomide by O^6-Cyclopentenylmethylguanine (41) in HT29 and Colo205 Human Colorectal Tumor Cell Lines. The most potent inactivator of AGT identified in these studies was O^6-cyclopentenylmethylguanine (**41**), a compound with an IC_{50} of 0.39 ± 0.34 μM for the depletion of AGT in HT29 cell extracts and 0.204 ±0.065 μM in intact HT29 cells (Table 1). Potentiation of the monomethylating antitumor agent Temozolomide[43] by **41** was investigated, in comparison with **2**, in the HT29 and Colo205 cell lines. These cell lines were selected owing to their similarly high levels of AGT activity (908 ± 76 and 1160 ± 78 fmol/mg cell protein, respectively). Data for the potentiation of Temozolomide cytotoxicity by 10 μM **41** in HT29 cells and Colo205 cells are summarized in Table 3, which shows that there was an approximate 3- and 10-fold increase in Temozolomide activity against HT29 and Colo205 cells, respectively, in the presence of the novel AGT inactivator. Comparative experiments were performed with 10 μM **2**, and these data are also shown in Table 3. Control incubation with 10 μM **2** or **41** alone did not cause any detectable cytotoxicity (data not shown). Examination of the Temozolomide IC_{50} values shown in Table 3 reveals that **41** was less effective at modulating the cytotoxicity of Temozolomide than **2**, in keeping with the greater potency of **2** as an AGT inactivator in cell extracts and whole cells (Table 1 and Figure 1).

Table 3. Comparison of the IC_{50} Values for the Cytotoxicity of Temozolomide Alone and Temozolomide Plus 10 μM O^6-Benzylguanine (**2**) or O^6-Cyclopentenylmethylguanine (**41**)[a]

cell line	Temozolomide	Temozolomide + **2**	Temozolomide + **41**
HT29	951 ± 69[b]	86 ± 8.6	299 ± 38
Colo205	343 ± 38	14 ± 5	36 ± 1

[a] See the Experimental Section. [b] The data represent the IC_{50} for Temozolomide (mean ± SD) from 3 independent experiments.

Conclusions

The clinical value of the AGT inactivator BG (**2**) as a potentiator of methylating and chloroethylating agent cytotoxicity is currently being assessed. However, the likelihood that resistance will arise to this agent necessitates the development of new AGT depletors active against resistant mutants. With a view to identifying such compounds, we have synthesized novel O^6-allyl- and O^6-(2-oxoalkyl)guanines and elucidated SARs for AGT inactivation, in comparison with their O^6-alkylguanine counterparts. As expected, simple O^6-alkyl- and O^6-cycloalkylguanines were weak AGT inactivators. Perhaps more surprisingly, the introduction of substituents onto O^6-allylguanine (**7**) reduced activity compared with the parent compound, while analogous compounds in the O^6-(2-oxoalkyl)guanine series exhibited very poor activity. These results are consistent with the existence of a narrow hydrophobic pocket adjacent to the active site of AGT, which limits the size and polarity of the O^6-substituent and which may accommodate this substituent in an orientation which may impede or assist the cysteine alkylation reaction.

By contrast, O^6-cycloalkenylguanines proved to be excellent AGT inactivators, with 1-cyclobutenylmethylguanine (**38**) and 1-cyclopentenylmethylguanine (**41**) exhibiting potency approaching that of **2** against protein from HT29 colorectal tumor cell extracts. More detailed studies conducted with **41** demonstrated that this compound inactivated AGT in intact HT29 cells and potentiated the cytotoxicity of the monomethylating agent Temozolomide in the HT29 and Colo205 tumor cell lines, to a degree comparable with that of **2**. Unfortunately, the four mutant AGT enzymes resistant

to **2** also proved strongly cross-resistant to **41**, suggesting that the phenyl and cyclopentenyl groups of these two compounds make similar binding interactions within the active site of AGT. In light of these observations, it appears that the development of AGT inactivators active against benzylguanine-resistant mutants will require more dramatic structural modifications to be made to the O^6-alkylguanine pharmacophore.

Experimental Section

Melting points were obtained on a Kofler hot stage apparatus and are uncorrected. Infrared spectra (IR) were recorded as KBr disks on a Nicolet 20 PC Fourier transform spectrometer. Mass spectra were determined on a Kratos MS80 spectrometer in electron impact (EI) mode or fast atom bombardment (FAB) mode using a *m*-nitrobenzyl alcohol matrix. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded at 200 and 50 MHz, respectively, on a Bruker WP 200 spectrometer employing TMS or the solvent as internal standard. Unless indicated otherwise, spectra were recorded in [$^2H_6$]DMSO as solvent, and *J* values are given in hertz (Hz). NH signals appeared as broad singlets (br s) exchangeable with D_2O.

The TLC systems employed Merck 1.05554 aluminum sheets precoated with Kieselgel 60F_{254} (0.2 mm) as the adsorbent and were visualized with UV light at 254 and 365 nm. Column chromatography was conducted under medium pressure on silica (Kieselgel 60, 240−400 mesh). Gas chromatography was performed using a Pye Unicam GCD chromatograph with a Carbowax 1000 (poly(ethylene glycol)) column and Shimadzu Chromatopac CE1B integrator. Elemental analyses were performed in house on a Carlo-Erba Instrumentazione 1106 analyzer or by Butterworth Laboratories, Middlesex, U.K., and are within ±0.4% of theory unless otherwise specified. Reagents were purchased from Aldrich Chemical Co., Gillingham, U.K., and used as received unless otherwise stated. Ethanol and methanol were dried using Mg/I_2 and stored over 4 Å molecular sieves. Diethyl ether and tetrahydrofuran were predried over $CaCl_2$ and distilled from sodium/benzophenone. Petrol (petroleum ether) refers to that fraction in the boiling range 40−60 °C.

Methyl 1-Cyclobutenecarboxylate (8). An ethereal solution of diazomethane (3.32 mmol) was added to 1-cyclobutenecarboxylic acid[34] (0.30 g, 3.06 mmol) in ether (15 mL). After 30 min any residual diazomethane was removed by passing N_2 through the reaction mixture for 1 h, and the solvent was evaporated under reduced pressure to afford **8** in quantitative yield: IR 1731 cm^{-1}; ^{1}H NMR δ 2.60 (2H, m, 3-H),), 2.82 (2H, t, 4-H), 3.81 (3H, s, OCH_3), 7.00 (1H, t, J = 1.3, 2-H); HRMS (EI) *m*/*z* found 112.0523, (M^+) calcd for $C_6H_8O_2$ 112.0524.

Compound **8** was also prepared by treatment of 1-cyclobutenecarboxylic acid (8.0 g, 82 mmol) with *N*,*N*-diisopropyl-*O*-methylisourea[35] (14.3 g, 90 mmol) under N_2 at room temperature for 1 h. After addition of anhydrous ether (25 mL), the reaction mixture was stirred for 48 h, cooled to −15 °C, and filtered. Removal of solvent gave a yellow oil which was purified by chromatography on silica, eluting with petrol: ether (99:1), to afford the ester **8** (7.47 g, 81%) identical to that prepared above.

1,1-Bis(phenylseleno)cyclobutane (9).[37] Cyclobutanone (2.12 g, 30.3 mmol) was added to a solution of benzeneselenol (10.0 g, 63.70 mmol) and zinc chloride (2.19 g, 16.10 mmol) in anhydrous carbon tetrachloride (50 mL), and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was partitioned between ether (90 mL) and aqueous HCl (0.5 M, 30 mL), and the organic layer was washed sequentially with saturated $NaHCO_3$ solution (30 mL) and water (30 mL) and dried ($MgSO_4$). Removal of the solvent gave a yellow oil which was purified by chromatography on silica, employing petrol as eluent, to afford the title compound as a yellow solid (10.44 g, 94%): ^{1}H NMR ($CDCl_3$) δ 1.94 (2H, m, 3-H), 2.30−2.50 (4H, m, 2-H and 4-H), 7.31−7.39 (6H, m, Ph), 7.61−7.72 (4H, m, Ph); MS (EI) 368 (M^+). Anal. ($C_{16}H_{16}Se_2$) C, H.

1-Hydroxymethyl-1-phenylselenocyclobutane (10). A solution of **9** (1.5 g, 3.96 mmol) in anhydrous THF (redistilled from $LiAlH_4$) (14 mL) was stirred at −78 °C under argon (previously rendered oxygen and water free by sequential passage through chromous solution, concentrated sulfuric acid, and solid potassium hydroxide). *tert*-Butyllithium (2.6 mL, 4.0 mmol, 1.54 M in THF) was added over 30 min and the mixture was stirred at −78 °C for a further 6 h. Methanal, generated by heating polyacetal (2.0 g),[44] was passed directly into the reaction mixture and the reaction was allowed to warm to room temperature over 12 h. After removal of solvents under reduced pressure, saturated ammonium chloride solution (50 mL) was added, and the solution was extracted with ether (3 × 30 mL). The combined ether extracts were dried ($MgSO_4$) and the ether removed to yield the product as a brown solid. Purification by chromatography on silica, employing petrol: ether (7:3) as eluent, afforded the title compound as a pale brown oil (0.37 g, 41%): IR 3400, 3100, 2900, 1500 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.00 (2H, m, 3-H), 2.20 (4H, m, 2-H and 4-H), 3.43 (2H, s, CH_2O), 7.10−7.30 (3H, m, Ph), 7.40−7.50 (2H, m, Ph); MS (EI) 242 (MH^+).

1-(Hydroxymethyl)cyclobutyl Phenyl Selenoxide[38] (11). Sodium periodate (2.31 g, 10.82 mmol) was added to a solution of **10** (0.62 g, 2.58 mmol) in 80% aqueous methanol (60 mL). The mixture was stirred for 5 min, the methanol was removed in vacuo, and saturated sodium chloride solution (40 mL) was added. The mixture was extracted with dichloromethane (8 × 40 mL), and the organic layers were combined, washed with water (10 mL), and dried ($MgSO_4$). The solvent was evaporated under reduced pressure to yield the selenoxide as a white solid (0.54 g, 81%): IR 3190, 3046, 2990, 1560 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.78−2.63 (6H, m, cyclobutyl), 3.78 (1H, d, J = 12.5, CH_2O), 4.12 (1H, d, J = 12.5, CH_2O), 7.46−7.49 (3H, m, Ph), 7.65−7.70 (2H, m, Ph); MS (EI) 314 (M^+).

1-Cyclobutenemethanol (12): By Ester Reduction. Lithium aluminum hydride (1.0 M solution in ether) (34.0 mL, 34.0 mmol) was added dropwise to a solution of **8** (6.34 g, 56.6 mmol) in ether (30 mL) at room temperature. After 15 min water (10 mL) was cautiously added to destroy the excess of lithium aluminum hydride, followed by hydrochloric acid (1.5 M, 10 mL). The product was extracted into ether (3 × 20 mL) and dried ($MgSO_4$) and the bulk of the ether removed using a long fractionating column to afford a yellow residue (7.6 g, 83% ether from NMR integrals): IR 3409 cm^{-1}; ^{1}H NMR δ (1-cyclobutenemethanol) 1.40 (1H, t, OH), 2.25 (2H, m, 3-H), 2.35 (2H, t, 4-H), 3.85 (2H, m, CH_2O), 5.75 (1H, m, 2-H); (cyclobutanemethanol) 1.60−2.20 (7H, m, cyclobutyl), 3.59 (2H, m, CH_2O); GLC (100 °C) t_R (min) 20.94 (cyclobutanemethanol, 16%), 30.78 (1-cyclobutenemethanol 84%).

1-Cyclobutenemethanol (12): By Selenoxide Fragmentation. A suspension of **11** (0.60 g, 2.33 mmol) and sodium carbonate (1.24 g, 11.7 mmol) in ether (50 mL) was heated under reflux for 12 h. The bulk of the ether was removed by atmospheric distillation using a long fractionating column, and the residual colorless oil was further purified by vacuum distillation (25 °C; 0.01 mmHg) to afford the title compound (0.152 g 55%): IR 3409 cm^{-1}; ^{1}H NMR ($CDCl_3$) 1.40 (1H, t, J = 7, OH), 2.41 (2H, m, 3-CH_2), 2.51 (2H, t, J = 3, 4-CH_2), 4.01 (2H, m, OCH_2), 5.87 (1H, m, 2-H): MS (EI) 83 (M^+-H).

***O*^{6}-Alkylguanines 7 and 13−21: Method I. General Procedure.** To a stirred solution of sodium (5.0 mol equiv) in the appropriate alcohol (50 mL) was added 2-amino-6-chloropurine (1.0 mol equiv). The mixture was heated to reflux under N_2 until TLC analysis confirmed the absence of starting materials. After cooling, the reaction mixture was neutralized with glacial acetic acid, and the solvent was removed in vacuo. The product was purified by chromatography on silica and/or recrystallization from an appropriate solvent.

***O*^{6}-Allylguanine (7).** Compound **7** was prepared from 2-amino-6-chloropurine (0.96 g, 5.66 mmol), sodium (0.50 g, 22.1 mmol) and allyl alcohol (13.5 mL) according to method I:

IR 3441, 3310, 3185, 2986, 2784 cm^{-1}; ^1H NMR δ 4.94 (2H, d, OCH_2), 5.28 (1H, m, CH=CH_2), 5.42 (1H, m, CH=CH_2), 6.19−6.03 (1H, m, CH=CH_2), 6.27 (2H, s, NH_2), 7.85 (1H, s, 8-H); MS (EI) 191 (M^+).

***O*^{6}-Ethylguanine (13).** Compound **13** was prepared according to method I from 2-amino-6-chloropurine (0.75 g, 4.42 mmol), sodium (0.5 g, 22 mmol) and ethanol (50 mL): IR 3505, 3484, 3432, 3324, 3191, 3110, 2984, 2901, 2705, 2544 cm^{-1}; ^1H NMR δ 1.35 (3H, t, J = 7.1, CH_2CH_3), 4.44 (2H, q, J = 7.1, OCH_2), 6.22 (2H, br s, NH_2), 7.81 (1H, s, 8-H); MS (EI) *m/z* 179 (M^+).

***O*^{6}-*n*-Propylguanine (14).** Compound **14** was prepared from 2-amino-6-chloropurine (0.50 g, 2.95 mmol), sodium (0.35 g, 15.2 mmol) and propan-1-ol (30 mL) according to method I: IR 3490, 3301, 3173, 2975, 2886, 2780, 2539 cm^{-1}; ^1H NMR δ 0.97 (3H, t, J = 7, CH_3), 1.76 (2H, sextet, J = 7, $CH_2CH_2CH_3$), 4.33 (2H, t, J = 7, OCH_2), 6.22 (2H, br s, NH_2), 7.80 (1H, s, 8-H); HRMS (EI) *m/z* found 193.0938, (M^+) calcd for $C_8H_{11}N_5O$ 193.0912.

***O*^{6}-*n*-Butylguanine (15).** Compound **15** was prepared by method I from 2-amino-6-chloropurine (0.50 g, 2.95 mmol), sodium (0.34 g, 15.0 mmol) and butan-1-ol (20 mL): IR 3501, 3374, 3106, 2955, 2874, 2803 cm^{-1}; ^1H NMR δ 0.93 (3H, t, J = 7.2, CH_3), 1.42 (2H, m, CH_2CH$_3$), 1.73 (2H, m, $CH_2CH_2CH_2$), 4.38 (2H, t, J = 6.7, OCH_2), 6.22 (2H, br s, NH_2), 7.80 (1H, s, 8-H); MS (EI) *m/z* 207 (M^+).

***O*^{6}-(3-Methylbutyl)guanine (16).** Compound **16** was synthesized according to method I from 2-amino-6-chloropurine (1.5 g, 8.84 mmol), sodium (1.0 g, 44.2 mmol) and 3-methyl-1-butanol (60 mL): IR 3505, 3310, 3183, 2961, 2919, 2708, 2552 cm^{-1}; ^1H NMR δ 0.93 (6H, d, J = 6.3, CH(CH_3)$_2$), 1.65 (2H, dt, J = 6.7, J = 6.3, CHCH_2CH(CH$_3$)$_2$), 1.74 (1H, septet, J = 6.3, CH(CH$_3$)$_2$), 4.41 (2H, t, J = 6.7, OCH_2), 6.23 (2H, br s, NH_2), 7.79 (1H, s, 8-H); HRMS (EI) *m/z* found 221.1266, (M^+) calcd for $C_{10}H_{15}N_5O$ 221.1255. Anal. ($C_{10}H_{15}N_5O$) C, H, N.

***O*^{6}-*n*-Pentylguanine (17).** Compound **17** was prepared according to method I from 2-amino-6-chloropurine (0.75 g, 4.42 mmol), sodium (0.50 g, 22.1 mmol) and pentan-1-ol (20 mL): IR 3497, 3364, 3107 cm^{-1}; ^1H NMR δ 0.99 (3H, t, CH_2CH_3), 1.47 (4H, m, $CH_2CH_2CH_3$), 1.82 (2H, m, OCH_2CH_2), 4.47 (2H, t, J = 6.7, OCH_2), 6.32 (2H, br s, NH_2), 7.92 (1H, s, 8-H), 12.58 (1H, br s, NH); MS (EI) *m/z* 221 (M^+). Anal. ($C_{10}H_{15}N_5O$) C, H, N.

***O*^{6}-*n*-Hexylguanine (18).** Compound **18** was synthesized following method I, from 2-amino-6-chloropurine (0.75 g, 4.42 mmol), sodium (0.50 g, 22.1 mmol) and hexan-1-ol (20 mL): IR 3499, 3360, 3181 cm^{-1}; ^1H NMR δ 0.99 (3H, t, J = 6.7, CH_2CH_3), 1.42 (6H, m, $(CH_2)_3CH_3$), 1.80 (2H, m, OCH_2CH_2), 4.05 (2H, t, J = 6.6, OCH_2), 6.32 (2H, s, NH_2), 7.92 (1H, s, 8-H), 12.50 (1H, br s, NH); MS (EI) 235 (M^+). Anal. ($C_{11}H_{17}N_5O$) C, H, N.

***O*^{6}-*n*-Heptylguanine (19).** Compound **19** was prepared according to method I from 2-amino-6-chloropurine (0.75 g, 4.42 mmol), sodium (0.50 g, 22.1 mmol) and heptan-1-ol (20 mL): IR 3499, 3300, 3179 cm^{-1}; ^1H NMR δ 0.96 (3H, t, J = 6.4, CH_3), 1,38 (8H, m, $(CH_2)_4CH_3$), 1.84 (2H, m, OCH_2CH_2), 4.47 (2H, t, J = 6.6, OCH_2), 6.33 (2H, s,NH_2), 7.89 (1H, s, 8-H), 12.50 (1H, br s, NH); MS (EI) 249 (M^+). Anal. ($C_{12}H_{19}N_5O$) C, H, N.

***O*^{6}-(Cyclohexylmethyl)guanine (20).** Compound **20** was prepared according to method I from 2-amino-6-chloropurine (0.50 g, 2.95 mmol), sodium (0.5 g, 22 mmol) and cyclohexylmethanol (6 mL): IR 3507, 3301, 3189, 3137, 2924, 2851, 2799 cm^{-1}; ^1H NMR δ 1.06−1.83 (11H, m, C_6H_{11}), 4.21 (2H, d, J = 6.2, OCH_2), 6.24 (2H, s, NH_2), 7.81 (1H, s, 8-H); MS (EI) 247 (M^+). Anal. ($C_{12}H_{17}N_5O$) C, H, N.

***O*^{6}-(2-Methylallyl)guanine (21).** Compound **21** was prepared by method I from 2-amino-6-chloropurine (0.50 g, 2.95 mmol), sodium (0.4 g, 17.4 mmol) and 2-methyl-2-propen-1-ol (10 mL), with the addition of THF (10 mL): IR 3494, 3314, 3185, 2978, 2782 cm^{-1}; ^1H NMR δ 1.78 (3H, s, CH_3), 4.86 (2H, s, OCH_2), 4.93 (1H, s, =CH_2), 5.10 (1H, s, =CH_2), 6.28 (2H, s, NH_2), 7.84 (1H, s, 8-H); HRMS (EI) *m/z* 205.0967, (M^+) calcd for $C_{10}H_{12}N_2O_3$ 205.0972.

***O*^{6}-Alkylguanines 22−38: Method II. General Procedure.** The appropriate alcohol (2.5 mol equiv), dissolved in dry THF (5−10 mL), was added to a solution of sodium hydride (2.0 mol equiv) in dry THF (20 mL) and the mixture was stirred for 30 min. 2-Amino-6-chloropurine (1.0 mol equiv) was added and the mixture was heated to reflux under N_2 until TLC analysis confirmed the absence of starting material. The reaction mixture was neutralized with glacial acetic acid, and the solvent was removed in vacuo. The product was purified by chromatography on silica and/or recrystallization from an appropriate solvent.

***O*^{6}-(5-Hexenyl)guanine (22).** Compound **22** was prepared according to method II from 5-hexen-1-ol (1.47 g, 14.7 mmol), sodium hydride (0.14 g, 5.9 mmol) and 2-amino-6-chloropurine (0.50 g, 2.95 mmol): IR 3483, 3302, 3181 cm^{-1}; ^1H NMR δ 1.60 (2H, m, O(CH$_2$)$_2$CH_2CH$_2$CH=CH_2), 1.87 (2H, m, OCH_2CH_2), 2.22 (2H, q, CH_2CH), 4.49 (2H, t, J = 6.55, OCH_2), 5.1 (2H, m, CH_2=CH), 5.94 (1H, m, CH_2=CH), 6.33 (2H, s, NH_2), 7.93 (1H, s, 8-H), 12.50 (1H, br s, NH); ^{13}C NMR δ (50.3 MHz, DMSO-d_6) 160.1, 138.9, 115.3, 65.6, 33.2, 28.3, 25.1; MS (EI) 233 (M^+). Anal. ($C_{11}H_{15}N_5O$) C, H, N.

***O*^{6}-[(*Z*)-3-Hexenyl]guanine (23).** Compound **23** was prepared from (*Z*)-3-hexen-1-ol (1.47 g, 14.7 mmol) sodium hydride (0.14 g, 5.9 mmol) and 2-amino-6-chloropurine (0.50 g, 2.95 mmol) according to method II: IR 3501, 3399, 3206 cm^{-1}; ^1H NMR δ 1.02 (3H, t, CH_2CH_3), 2.15 (2H, p, CH_2CH_3), 4.48 (2H, t, J = 6.9, OCH_2), 5.57 (2H, m, CH=CH), 6.32 (2H, s, NH_2), 7.92 (1H, s, 8-H), 12.58 (1H, br s, NH); ^{13}C NMR δ (50.3 MHz, DMSO-d_6) 160.0, 138.2, 134.2, 124.8, 65.2, 26.9, 20.5, 14.4; HRMS (EI) *m/z* 233.1271, (M^+) calcd for $C_{11}H_{15}N_5O$ 233.1277.

***O*^{6}-[(*E*)-3-Hexenyl]guanine (24).** Compound **24** was prepared by method II from (*E*)-3-hexen-1-ol (1.47 g, 14.7 mmol) sodium hydride (0.14 g, 5.9 mmol) and 2-amino-6-chloropurine (0.50 g, 2.95 mmol): IR 3500, 3190, 3005 cm^{-1}; ^1H NMR δ 1.03 (3H, t, J = 6.4, CH_3), 2.09 (2H, p, J = 6.9, CH_2CH_3), 2.50−2.60 (m, CH_2CHCH), 5.40−5.70 (2H, m, CH=CH), 6.35 (2H, s, NH_2), 7.91 (1H, s, 8-H), 12.50 (1H, s, NH); MS (EI) 233 (M^+).

***O*^{6}-(3-Hexynyl)guanine (25).** Compound **25** was prepared according to method II from hex-3-yn-1-ol (1.44 g, 14.7 mmol), sodium hydride (0.14 g, 5.9 mmol) and 2-amino-6-chloropurine (0.50 g, 2.95 mmol): IR 3503, 3314, 3284 cm^{-1}; ^1H NMR δ 1.13 (3H, t, J = 7.5, CH_2CH_3), 2.24 (2H, q, J = 7.5, CH_2CH_3), 2.75 (2H, t, J = 7.0, OCH_2CH_2), 4.48 (2H, t, J = 7.0, OCH_2), 6.41 (2H, s, NH_2), 7.98 (1H, s, 8-H); HRMS (EI) *m/z* 231.1123, (M^+) calcd for $C_{11}H_{13}N_5O$ 231.1120.

***O*^{6}-(2-Phenylethyl)guanine (26).** Compound **26** was prepared according to method II from 2-phenylethanol (3.0 g, 25.0 mmol), sodium hydride (0.27 g, 11.0 mmol) and 2-amino-6-chloropurine (0.75 g, 4.42 mmol): IR 3495, 3366, 3127, 2982, 2801 cm^{-1}; ^1H NMR δ 3.08 (2H, t, J = 7.2, CH_2Ph), 4.58 (2H, t, J = 7.2, OCH_2), 6.27 (2H, s, NH_2), 7.22−7.37 (5H, m, Ph), 7.80 (1H, s, 8-H); MS (EI) 255 (M^+). Anal. ($C_{13}H_{13}N_5O$) C, H, N.

***O*^{6}-(2-Ethylallyl)guanine (27).** Compound **27** was prepared by method II from 2-ethylallyl alcohol[45] (1.0 g, 11.6 mmol) sodium hydride (0.60 g, 25 mmol) and 2-amino-6-chloropurine (1.0 g, 5.90 mmol): IR 3465, 3306, 3200, 3137, 2965, 2940, 2915, 2882, 2803, 1630, 1584 cm^{-1}; ^1H NMR δ 1.06 (3H, t, J = 7.4, CH_2CH_3), 2.13 (2H, q, J = 7.4, CH_2CH$_3$), 4.92 (2H, s, OCH_2), 4.96 (1H, s, =CH_2), 5.12 (1H, s, =CH_2), 6.26 (2H, br s, NH_2), 7.83 (1H, s, 8-H); ^{13}C NMR δ 160, 155.5, 146.5, 138.1, 113.8, 110.9, 67.8, 25.8, 12.1; HRMS (EI) *m/z* 219.1121, (M^+) calcd for $C_{10}H_{13}N_5O$ 219.112.

***O*^{6}-(2-Isopropylallyl)guanine (28).** Compound **28** was prepared according to method II from 2-isopropylallyl alcohol[45] (1.77 g, 17.7 mmol), sodium hydride (0.60 g, 25 mmol) and 2-amino-6-chloropurine (1.0 g, 5.90 mmol): IR 3322, 3189, 2963, 2872, 2789 cm^{-1}; ^1H NMR δ 1.07 (6H, d, J = 6.8, CH(CH$_3$)$_2$), 2.39 (1H, septet, J = 6.8, CH(CH$_3$)$_2$), 4.95 (2H, s, OCH_2), 4.97 (1H, d, J = 1, =CH_2), 5.10 (1H, d, J = 1, =CH_2), 6.26 (2H, br s, NH_2), 7.81 (1H, s, 8-H); HRMS (EI) *m/z* 233.1268, (M^+) calcd for $C_{11}H_{15}N_5O$ 233.1260.

***O*^{6}-(2-Methyl-2-butenylmethyl)guanine (29).** Compound **29** was prepared following method II, from 2-methyl-2-buten-

1-ol (2.0 g, 23 mmol),[45] sodium hydride (0.37 g, 15.4 mmol) and 2-amino-6-chloropurine (1.31 g, 7.7 mmol): IR 3500 3350 3200 1610 cm^{-1}; ^1H NMR δ 1.80 (6H, m, 2 × CH_3), 4.95 (2H, s, OCH_2), 5.75 (1H, q, J = 5.5, 3′-H), 6.35 (2H, s, NH_2), 7.90 (1H, s, 8-H), 12.58 (1H, br s, NH); MS (EI) 220 (MH^+). Anal. ($C_{10}H_{13}N_5O$) C, H, N.

***O*⁶-(2-Phenylallyl)guanine (30).** Compound **30** was prepared from 3-hydroxy-2-phenyl-1-propene (820 mg, 6.12 mmol),[46] sodium hydride (0.45 g, 7.90 mmol) and 2-amino-6-chloropurine (0.70 g, 4.13 mmol) according to method II: IR 3484, 3326, 3189, 2787, 1622, 1586 cm^{-1}; ^1H NMR (methanol-d_4) δ 5.65 (2H, s, OCH_2), 5.73 (1H, s, $=CH_2$), 5.82 (1H, s, $=CH_2$), 7.46−7.54 (3H, m, Ph), 7.57−7.75 (2H, m, Ph), 8.02 (1H, s, 8-H); MS (EI) 267 (M^+).

***O*⁶-Propargylguanine (31).** Compound **31** was prepared by method II from propargyl alcohol (3 mL), sodium hydride (0.50 g, 22.0 mmol) and 2-amino-6-chloropurine (1.0 g, 5.90 mmol): IR 3501, 3397, 3173, 2982, 2787, 2124 cm^{-1}; ^1H NMR δ 3.59 (1H, t, J = 2.4, $=CH$), 5.11 (2H, d, J = 2.4, $CH_2C{\equiv}C$), 6.34 (2H, s, NH_2), 6.56 (1H, s, 8-H); HRMS (EI) m/z 189.0644, (M^+) calcd for $C_8H_7N_5O$ 189.0638.

***O*⁶-(Cyclohex-3-en-1-ylmethyl)guanine (32).** Compound **32** was prepared according to method II from cyclohex-3-en-1-ylmethanol (6.16 g, 55 mmol), sodium hydride (0.53 g, 22 mmol) and 2-amino-6-chloropurine (1.87 g, 11 mmol): IR 3340, 3200, 2900, 1620, 1580 cm^{-1}; ^1H NMR δ 1.40 (1H, m, 1′-H), 1.85−2.10 (6H, 3 × m, 2′-H, 5′-H, 6′-H), 4.30 (2H, d, J = 7, OCH_2), 5.70 (2H, s, 3′-H, 4′-H), 6.15 (2H, s, NH_2), 7.76 (1H, s, 8-H), 12.35 (1H, br s, NH); MS (FAB) 246 (MH^+). Anal. ($C_{12}H_{15}N_5O$·0.25MeOH) C, H, N.

***O*⁶-(2,2-Dimethyl-1,3-dioxolan-4-ylmethoxy)guanine (33).** Compound **33** was prepared by method II from 2,2-dimethyl-1,3-dioxolan-4-methanol (12 g, 90.80 mmol), sodium hydride (1.27 g, 10.61 mmol) and 2-amino-6-chloropurine (3.0 g, 17.69 mmol): IR 3413, 3179, 2990 cm^{-1}; ^1H NMR δ 1.31 (3H, s, Me), 1.37 (3H, s, Me), 3.77 (1H, dd, J = 5.4, J = 8.1, OCH_2CHCH_2), 4.11 (1H, dd, J = 5.4, J = 8.1, OCH_2CHCH_2),), 4.39−4.47 (1H, m, OCH_2CH), 4.43 (2H, d, J = 2.4, OCH_2), 6.27 (2H, s, NH_2), 7.85 (1H, s, 8-H); HRMS (EI) m/z 265.1174, (M^+) calcd for $C_{11}H_{15}N_5O_3$ 265.1173.

***O*⁶-(2,2-Diethoxyprop-1-yl)guanine (34).** Compound **34** was prepared according to method II from 2,2-diethoxy-1-propanol (3 mL), sodium hydride (0.35 g, 14.74 mmol) and 2-amino-6-chloropurine (1.0 g, 2.95 mmol): IR 3465, 3314, 2923 cm^{-1}; ^1H NMR δ 1.09 (3H, t, J = 7.1, OCH_2CH_3), 1.37 (3H, s, CH_3), 3.48 (1H, q, J = 7.1, OCH_2CH_3), 3.48 (1H, q, J = 7.1, OCH_2CH_3), 4.39 (2H, s, OCH_2), 6.29 (2H, s, NH_2), 7.83 (1H, s, 8-H); MS (EI) 281 (M^+). Anal. ($C_{12}H_{19}N_5O_3$) C, H, N.

***O*⁶-(2,2-Dimethoxybut-1-yl)guanine (35).** Compound **35** was prepared according to method II from 2,2-dimethoxy-1-butanol[47] (0.94 g, 7 mmol), sodium hydride (0.48 g, 20 mmol) and 2-amino-6-chloropurine (1.0 g, 5.9 mmol): IR 3480, 3434, 3318, 3206, 3104, 2973, 2840, 2795 (cm^{-1}); ^1H NMR δ 0.82 (3H, t, J = 7.6, CH_2CH_3), 1.71 (2H, q, J = 7.6, CH_2CH_3), 3.14 (6H, s, OCH_3), 4.38 (2H, s, OCH_2), 6.29 (2H, s, NH_2), 7.82 (1H, s, 8-H); MS (EI) 267 (M^+) Anal. ($C_{11}H_{17}N_5O_3$) C, H, N.

***O*⁶-(3-Methyl-2-oxobut-1-yl)guanine Ethylene Acetal (36).** Compound **36** was prepared according to method II from 1-hydroxy-3-methylbutan-2-one ethylene acetal[48] (0.95 g, 6.48 mmol), sodium hydride (0.26 g, 11 mmol) and 2-amino-6-chloropurine (0.73 g, 4.32 mmol): IR 3459, 3343, 3223, 3133, 2980, 2878, 2799 cm^{-1}; ^1H NMR δ 0.94 (6H, d, J = 6.9, $CH(CH_3)_2$), 2.11 (1H, septet, J = 6.9, $CH(CH_3)_2$), 3.86−4.09 (4H, m, OCH_2CH_2O), 4.42 (2H, s, OCH_2), 6.27 (2H, s, NH_2), 7.83 (1H, s, 8-H); MS (EI) 279 (M^+) Anal. ($C_{12}H_{17}N_5O_3$) C, H, N.

***O*⁶-(2,2-Dimethoxy-2-phenylethyl)guanine (37).** Compound **37** was prepared by method II from 2,2-dimethoxy-2-phenylethanol[48] (0.68 g, 3.74 mmol), sodium hydride (0.23 g, 9.6 mmol) and 2-amino-6-chloropurine (0.32 g, 1.87 mmol): IR 3497, 3438, 3310, 3206, 2946, 2832, 1626 cm^{-1}; ^1H NMR δ 3.18 (6H, s, OCH_3), 4.68 (2H, s, CH_2), 6.29 (2H, br s, NH_2), 7.34−7.38 (3H, m, Ph), 7.53−7.57 (2H, m, Ph), 7.77 (1H, s, 8-H); MS (EI) 315 (M^+). Anal. ($C_{15}H_{17}N_5O_3$) C, H, N.

***O*⁶(1-Cyclobutenylmethyl)guanine (38).** Compound **38** was synthesized according to method II from 1-cyclobutenemethanol **12** (0.09 g, 1.07 mmol), sodium hydride (0.10 g, 2.5 mmol) and 2-amino-6-chloropurine (0.12 g, 0.71 mmol): ^1H NMR (CD_3OD) δ 2.48 (2H, m, 3′-H), 2.68 (2H, t, 4′-H), 4.98 (2H, s, CH_2O), 6.03 (1H, s, 2′-H), 7.98 (1H, s, 8-H); HRMS (EI) m/z 217.0975, (M^+) calcd for $C_{10}H_{11}N_5O$ 217.0987.

Compound **38** was also prepared as follows: A mixture of **12** and cyclobutylmethanol (84:16) (0.84 g, 10 mmol) was added to sodium hydride (0.082 g, 3.41 mmol) in DMSO (0.3 mL) and the mixture was stirred for 1 h. 2-Amino-6-(azonia-4-azabicyclo[2.2.2-oct-1-yl]purine chloride (DABCO-purine; 0.50 g, 1.72 mmol)[36] was added and the reaction mixture was stirred for a further 12 h at room temperature. After addition of acetic acid (0.06 mL), the mixture was adsorbed onto neutral alumina and purified by chromatography on neutral alumina, employing 7% MeOH in CH_2Cl_2 as eluent, affording **38** as a white solid (0.05 g, 13%).

***O*⁶-Alkylguanines 39−43: Method III. General Procedure.** A mixture of the appropriate alcohol (5.5 mol equiv) and sodium hydride (2.0 mol equiv) in anhydrous DMSO was stirred for 1 h, and 2-aminotrimethylpurin-6-ylammonium chloride[32] (1.0 mol equiv) was added. The reaction mixture was stirred under N_2 at room temperature until TLC analysis confirmed the absence of starting material. The reaction mixture was neutralized with glacial acetic acid, and the solvent was evaporated under reduced pressure. The product was purified by chromatography on silica and/or recrystallization from an appropriate solvent.

***O*⁶-(Cyclobutylmethyl)guanine (39).** Compound **39** was prepared following method III, from cyclobutanemethanol (5.00 g, 58 mmol), sodium hydride (0.51 g, 21 mmol) and 2-aminotrimethylpurin-6-ylammonium chloride (2.41 g, 11 mmol) in DMSO (6 mL): IR 3469, 3333, 3164, 2977, 1671, 1629 cm^{-1}; ^1H NMR δ 1.8−2.3 (6H, m, 2′-CH_2, 3′-CH_2, 4′-CH_2), 2.85 (1H, m, (1′−H), 4.47 (2H, d, J = 7.0, OCH_2), 6.31 (2H, s, NH_2), 7.89 (1H, s, 8-H), 12.50 (1H, br s, NH); MS (EI) 219 (M^+). Anal. ($C_{10}H_{13}N_5O$) C, H, N.

Compound **39** was also prepared according to method II from cyclobutanemethanol (1.2 g, 14.7 mmol), sodium hydride (1.4 g, 5.9 mmol), and 2-amino-6-chloropurine (0.50 g, 2.95 mmol).

***O*⁶-(Cyclopentylmethyl)guanine (40).** Compound **40** was prepared according to method III from cyclopentanemethanol (0.20 g, 1.99 mmol), sodium hydride (17 mg, 0.71 mmol) and 2-aminotrimethylpurin-6-ylammonium chloride (2.41 g, 11 mmol) in DMSO (0.4 mL): IR 3481, 3352, 3204, 2957, 1626, 1581 cm^{-1}; ^1H NMR δ 1.2−2.0 (8H, m, 2′-CH_2, 3′-CH_2, 4′-CH_2, 5′-CH_2), 2.47 (1H, m, 1′H), 4.38 (2H, d, J = 7, OCH_2), 6.35 (2H, s, NH_2), 7.93 (1H, s, 8-H), 12.50 (1H, s, NH); MS (EI) 233 (M^+). Anal. ($C_{11}H_{15}N_5O$·0.5H_2O) C, H, N.

Compound **40** was also prepared according to method II from cyclopentanemethanol (1.45 g, 14.5 mmol), sodium hydride (139 mg, 5.8 mmol) and 2-amino-6-chloropurine (0.50 g, 2.9 mmol).

***O*⁶-(1-Cyclopentenylmethyl)guanine (41).** Compound **41** was prepared by method III from 1-cyclopentenemethanol[49] (6.57 g, 67 mmol), sodium hydride (0.57 g, 24 mmol) and 2-aminotrimethylpurin-6-ylammonium chloride (2.74 g, 12 mmol) in DMSO (6 mL): IR 3460, 3300, 1280, 1150 cm^{-1}; ^1H NMR δ 1.90 (2H, q, J = 7.4, 4′-CH_2), 2.35 (4H, m, 3′-CH_2, 5′-CH_2), 5.00 (2H, s, OCH_2), 5.75 (1H, s, 2′−H), 6.15 (2H, s, NH_2), 7.75 (1H, s, 8-H), 12.35 (1H, br s, NH); MS (FAB) 232 (MH^+). Anal. ($C_{11}H_{13}N_5O$·0.4EtOH) C, H, N.

***O*⁶-(1-Cyclohexenylmethyl)guanine (42).** Compound **42** was prepared according to method III from 1-cyclohexenemethanol[50] (2.04 g, 18.2 mmol), sodium hydride (0.16 g, 6.6 mmol) and 2-aminotrimethylpurin-6-ylammonium chloride (0.75 g, 3.3 mmol) in DMSO (6 mL): IR 3457, 3295, 3186, 2931, 1698, 1631 cm^{-1}; ^1H NMR δ 1.68−2.14 (8H, 2 × m, 3′-CH_2 and 6′-CH_2, 4′-CH_2, 5′-CH_2), 4.88 (2H, s, OCH_2), 5.93 (1H, s, 2′-H), 6.33 (2H, s, NH_2), 7.92 (1H, s, 8-H), 12.50 (1H, br s, NH); MS (EI) 245 (M^+). Anal. ($C_{12}H_{15}N_5O$·0.85H_2O) C, H, N.

***O*^{6}-[(*S*)-(4-Isopropenylcyclohex-1-enyl)methyl]guanine (43).** Compound **43** was synthesized according to method III from (*S*)-perillyl alcohol (3.66 g, 24 mmol), sodium hydride (0.21 g, 8.8 mmol) and 2-aminotrimethylpurin-6-ylammonium chloride (1.00 g, 4.4 mmol) in DMSO (6 mL): IR 3460, 3404, 3315, 3205, 2964, 1626, 1584 cm^{-1}; ^{1}H NMR δ 1.60 (1H, m, 4′-H), 1.82 (3H, s, CH_3), 1.9−2.2 (6H, m, 3′-CH_2, 5′-CH_2, 6′-CH_2), 4.82 (2H, s, OCH_2), 4.91 (2H, s, CH_2=), 5.96 (1H, s, 2′-H), 6.35 (2H, s, NH_2), 7.93 (1H, s, 8-H), 12.50 (1H, br s, NH); MS (EI) 285 (M^+). Anal. ($C_{15}H_{19}N_5O$·0.2MeOH) C, H, N.

***O*^{6}-(2-Oxo-2-alkyl)guanines 44−47: Method IV. General Procedure.** A solution of the appropriate *O*^{6}-(2-oxo-2-alkyl)guanine acetal in aqueous acetic or trifluoroacetic acid (5−20 mL) was stirred at ambient temperature until TLC analysis confirmed the absence of starting material. Solvents were removed under reduced pressure and the product was purified by chromatography on silica and/or recrystallization from the appropriate solvent.

***O*^{6}-(2-Oxopropyl)guanine (44).** Compound **44** was prepared according to method IV from *O*^{6}-(2,2-diethoxypropyl)-guanine **(34)** (0.50 g, 1.78 mmol) by treatment with aqueous acetic acid (1 M, 12 mL): IR 3355, 3119, 2780, 1734 cm^{-1}; ^{1}H NMR δ 2.18 (3H, s, $COCH_3$), 5.07 (2H, s, OCH_2), 6.27 (2H, s, NH_2), 7.90 (1H, s, 8-H); HRMS (EI) *m*/*z* 207.0765, (M^+) calcd for $C_8H_9N_5O_2$ 207.0756). Anal. ($C_8H_9N_5O_2$·0.25AcOH·0.8H_2O) C, H, N.

***O*^{6}-(2-Oxobutyl)guanine (45).** Compound **45** was prepared from *O*^{6}-(2,2-dimethoxybutyl)guanine **(35)** (0.08 g, 0.3 mmol) and 50% aqueous acetic acid (5 mL) according to method IV: IR 3488, 3380, 3322, 3110, 2982, 2942, 2782, 1732 cm^{-1}; ^{1}H NMR δ 0.98 (3H, t, J = 7.3, CH_2CH_3), 2.54 (2H, q, J = 7.3, CH_2CH_3), 5.06 (2H, s, OCH_2), 6.22 (2H, s, NH_2), 7.86 (1H, s, 8-H); HRMS (EI) *m*/*z* 221.0937, (M^+) calcd for $C_9H_{11}N_5O_2$ 221.0962.

***O*^{6}-(3-Methyl-2-oxobutyl)guanine, TFA Salt (46).** Compound **46** was prepared by method IV from *O*^{6}-(3-methyl-2-oxobutyl)guanine ethylene acetal **(36)** (0.2 g, 0.72 mmol) and 80% aqueous trifluoroacetic acid (10 mL): IR 3854, 3501, 3320, 3183, 2977, 2940, 2791, 1732 cm^{-1}; ^{1}H NMR δ 1.09 (6H, d, J = 6.9, $CH(CH_3)_2$), 2.80 (1H, septet, J = 6.9, $CH(CH_3)_2$), 5.26 (2H, s, OCH_2), 6.62 (2H, s, NH_2), 8.23 (1H, s, 8-H); HRMS (EI) *m*/*z* 235.1063, (M^+) calcd for $C_{10}H_{13}N_5O_2$ 235.1057.

***O*^{6}-(2-Oxo-2-phenylethyl)guanine (47).** Compound **47** was prepared by method IV from *O*^{6}-(2,2-dimethoxy-2-phenylethyl)guanine **(37)** (0.09 g, 0.29 mmol) and aqueous acetic acid (3 M, 20 mL): IR 4390, 3391, 3061, 2973, 2924, 1690 cm^{-1}; ^{1}H NMR δ 5.90 (2H, s, CH_2), 6.18 (2H, br s, NH_2), 7.55−7.71 (3H, m, Ph), 7.87 (1H, s, 8-H), 8.00−8.04 (2H, m, Ph); MS (EI) 270 (MH^+). Anal. ($C_{13}H_{11}N_5O_2$) C, H, N.

Inactivation of AGT in HT29 Cell Extracts. The inactivation of AGT in HT29 human colorectal tumor cell extracts by guanine derivatives, and the calculation of IC_{50} concentrations, was performed as previously described.[29]

Inactivation of Purified Wild-Type and Mutant AGT. Recombinant wild-type and mutant AGT proteins P140K, Y158H, G160R, and G156A were produced in *Escherichia coli* and purified as previously described by immobilized metal affinity chromatography.[15,51] (The amino acid sequence at the amino terminus of the protein was changed from M- to M-GS-$(H)_6$GS- to allow this purification.) The purified AGT protein was incubated for 30 min at 37 °C with the concentrations of **2** or **41** indicated in 0.5 mL of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol and 50 μg of hemocyanin in a total volume of 0.5 mL. The residual AGT activity was then determined by a further 30-min incubation with a methylated DNA substrate.[16] The results were expressed as the percentage of the AGT activity lost in response to the inhibitor and an ED_{50} value obtained from a plot of this value against the amount of inhibitor added. Due to solubility considerations, the maximum inhibitor concentration used was 1.2 mM.

Depletion of AGT in Intact HT29 Cells by AGT Inactivators. All inhibitors were dissolved in 100% dry DMSO at 100 or 1000 times the final desired concentration to allow dilution in medium to give a final concentration of 1% or 0.1% DMSO. HT29 cells were seeded at 5×10^6 cells in 7-cm radius Petri dishes in EMEM (Gibco-GBL Life Technologies Ltd., Paisley, Scotland) medium supplemented with 10% (v/v) fetal calf serum (FCS; Globepharm, Esher, U.K.). After 3 days the growth medium was replaced with serum free EMEM containing 1% DMSO ± AGT inactivators for 4 h. Cells were harvested by trypsination, resuspended in serum-containing EMEM, and centrifuged for 5 min at 1750*g* at 4 °C. The pellet was washed twice with EMEM and resuspended in EMEM, and the cells were disaggregated by passing through a 25 G 5/8 hypodermic needle. Viable cell number was determined by trypan blue excluding haemocytometer counts. 10^7 Cells were pelleted by centrifugation, snap-frozen in liquid nitrogen and stored at −80 °C. Extracts were prepared by resuspending cell pellets in 3 mL of extraction buffer (50 mM Tris-HCl, pH 8.3, 0.5 mM EDTA disodium salt, 1 mM dithiothreitol, 200 mM NaCl) and homogenizing with a T25 Ultra-Turrax homogenizer (SH Scientific, Northumberland, U.K.) fitted with an 8-mm head for 15 s at 8000 rpm, 3 × 5 s, each separated by a 30-s period during which the sample was allowed to cool on ice. The homogenizer head was then rinsed using 2 mL of extraction buffer, which was added to the cell homogenate, and the total volume was centrifugated for 5 min at 1750*g*, 4 °C. The supernatant liquid was snap-frozen in liquid nitrogen and stored at −80 °C. The AGT activity of the extracts thus obtained was subsequently determined by the AGT assay described previously,[29] and from these data the concentration of AGT inactivator required to deplete whole HT29 cell AGT activity by 50% was calculated.

Effect of AGT Inactivators on Temozolomide Cytotoxicity in HT29 and Colo205 Human Tumor Cells. Potentiation of Temozolomide activity by **2** and **41** was determined by means of a colony-forming assay. Cells were seeded in 6-well dishes (Nunc) in EMEM + 10% FCS, and after 72 h exponentially growing cultures were exposed to medium supplemented with 10 μM **2** or **41**, in a final concentration of 0.1% DMSO (v/v). After a 1-h preincubation with the AGT inactivator, varying concentrations of Temozolomide dissolved in DMSO were added to give a final concentration range of 0−1000 μM in 1% DMSO (v/v), control cells being treated with 1% v/v DMSO only. After incubation at 37 °C for 4 h the cells were harvested by trypsinisation, resuspended in 2 mL of EMEM, disaggregated and counted as described above. A known number of HT29 cells was transferred into 10-cm plastic Petri dishes containing EMEM. Colo205 cells have poor cloning efficiency on plastic and thus were dispensed into tissue culture tubes containing 0.15% (w/v) agarose (Seakem ME; FMC Bioproducts, Rockland, ME) in EMEM. The AGT inactivators were also present throughout the cloning period at a concentration of 10 μM (0.1% v/v DMSO final concentration). After approximately 2 weeks adherent HT29 colonies were fixed with acetic acid:methanol (3:1 v/v), stained with 0.4% crystal violet (*N*-hexamethyl-*p*-rosaniline; Sigma) (w/v) for 1 min, rinsed with water, air-dried and counted by eye. Colonies of Colo205 cells were visualized by adding 1 mL 0.5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma), incubating at 37 °C overnight and counting by eye. The percentage cloning efficiencies for untreated HT29 and Colo205 cells were 62 ± 11% (n = 6) and 50 ± 15% (n = 6), respectively. From the cloning efficiencies relative to control the % cell survival at each concentration of Temozolomide (±10 μM AGT inactivator) was calculated. The Temozolomide concentration associated with 50% inhibition of colony formation was determined from a point-to-point curve plot using GraphPad PRISM software.

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