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Antitumor Imidazo[5,1-*d*]-1,2,3,5-tetrazines: Compounds Modified at the 3-Position overcome Resistance in Human Glioblastoma Cell Lines‡

David Cousin,[†] Jihong Zhang,[§] Marc G. Hummersone,[†] Charles S. Matthews,[§] Mark Frigerio[†], Tracey D. Bradshaw,*[,§] and Malcolm F. G. Stevens*[,§]

Pharminox Ltd., Biocity, Pennyfoot St., Nottingham NG1 1GF, U.K. and School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, U.K.

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*To whom correspondence should be addressed: tracey.bradshaw@nottingham.ac.uk tel. 44(0)115 951 3419; or malcolm.stevens@nottingham.ac.uk tel. 44(0)115 951 3404.

†Pharminox Ltd. §University of Nottingham.

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Abstract

Synthetic routes to 3-substituted imidazo[5,1-*d*]-1,2,3,5-tetrazines structurally related to temozolomide were explored. Interaction of 4-diazoimidazole-5-carboxamide with an isocyanate afforded high product yields when the isocyanate was available in acceptable purity. Alternatively, alkylation of the nor-temozolomide anion afforded high yields of new imidazotetrazines.

Several compounds, evaluated against a panel containing matched MGMT+/– glioma cell lines, showed equal inhibitory activity irrespective of MGMT status; the N3-propargyl-imidazotetrazine (**10m**) was prioritised as an alternative to temozolomide able to bypass drug-resistance mechanisms. In Taq polymerase assays **10m**, like temozolomide and its ring-opened counterpart MTIC, alkylated DNA at clusters of three and five guanine residues; covalent modification of N-7 sites of guanine were detected in piperidine cleavage assays. Compound (**10m**) did not cross-link DNA but induced double-strand breaks evidenced by γ-H2AX detection. Propargyl-substituted imidazotriazene (**13g**), showed comparable activity to **10m** indicating that ring-opening of the bicyclic nucleus of novel imidazotetrazine is probably required for activity.

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Introduction

Originally synthesized in 1980 the anticancer drug temozolomide (**1**: TMZ) is the first successful antitumor agent based on the bicyclic imidazo[5,1-*d*]-1,2,3,5-tetrazine skeleton, and its chemistry was first described in 1984.[1] Although originally envisaged as a competitor to the anti-melanoma drug DTIC,[2] it has proven to be an unlikely commercial success story: at a molecular weight of only 194 Da the agent is one of the most lucrative anticancer agents in the current clinical armamentarium on a dollar sales/Da basis. Its robust pharmaceutical properties, oral bioavailability, ability to pass the blood-brain barrier and suitability for out-patient use have contributed to the drug being the standard of care, in combination with radiotherapy, for the treatment of glioblastoma multiforme (GBM); these properties also commend the drug as a suitable partner in clinical trials of drug combinations with novel agents.[3] Unlike the precursor 3-(2-chloroethyl) analog mitozolomide (**2**: MTZ), which induced profound myelosupression in clinical trials, **1** has an acceptable safety profile:[3] arguably superior to that of many `molecularly-targeted` agents. TMZ deserves a higher status than that commonly accorded to 'cytotoxic agents' which are routinely vilified by the modern drug discovery community. Surprisingly, in the years since its original synthesis in 1980 and launch in 1999, no mechanistic 'me-too' has entered the market. This may be attributed to the unique chemical structure of **1** where every atom contributes to the efficient delivery and activity of the molecule.[3] A recent review has highlighted the difficulties in developing second-generation analogs of TMZ.[4] The closest approach has been revealed in the recent work of Wheelhouse and his colleagues who recruited the imidazotetrazine prodrug scaffold to carry a substituted 3-(β-aminoethyl) residue which acted as a precursor of an aziridinium warhead. [5] TMZ is a chemically-activated prodrug, being hydrolytically ring-opened to the monomethyltriazene (**3**: MTIC). Protolytic fragmentation of **3** then generates the methyldiazonium cation (**4**) with liberation of 5-aminoimidazole-4-carboxamide (**5:**

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AIC).[6] The reactive intermediate (**4**) methylates genomic DNA especially at guanine residues (G) in runs of guanines;[7] the significant antitumor event is associated with methylation at guanine O-6 sites in DNA. 6-Methoxyguanine-DNA lesions (**6**) are repaired by capture of the methyl group by a cysteine thiolate residue of methylguanine-DNA methyltransferase (MGMT) and, in the absence of repair, 6-methoxyguanine sites mispair (in the Watson-Crick sense) with thymine residues. These loci are processed by the mismatch repair (MMR) system to trigger futile cycles of thymine insertion adfacent to the methylated guanine base; this eventually leads to DNA strand breaks, engagement of the apoptotic or autophagic mechanisms and cell death.[8, 9] The recognition that epigenetic silencing of the *MGMT* gene by *hyper*methylation at C-5 sites of cytosine residues in GC-rich gene promoter sequences can be measured in tumor samples in clinical practice, has allowed patients to be selected with tumors more likely to respond to the drug.[10,11] Conversely, tumors with a *hypo*methylated ('switched on') *MGMT* gene, or with deficiency in MMR, are constituitively resistant to TMZ and patients with such tumor profiles have limited treatment options; in addition, acquired resistance pathways have been identified which restrict the clinical utility of the drug. [10,11,12]

Figure 1. Structures of the antitumour imidazotetrazines temozolomide (**1**) and mitozolomide (**2**), methylation of DNA at guanine residues and repair of methylated DNA by MGMT.

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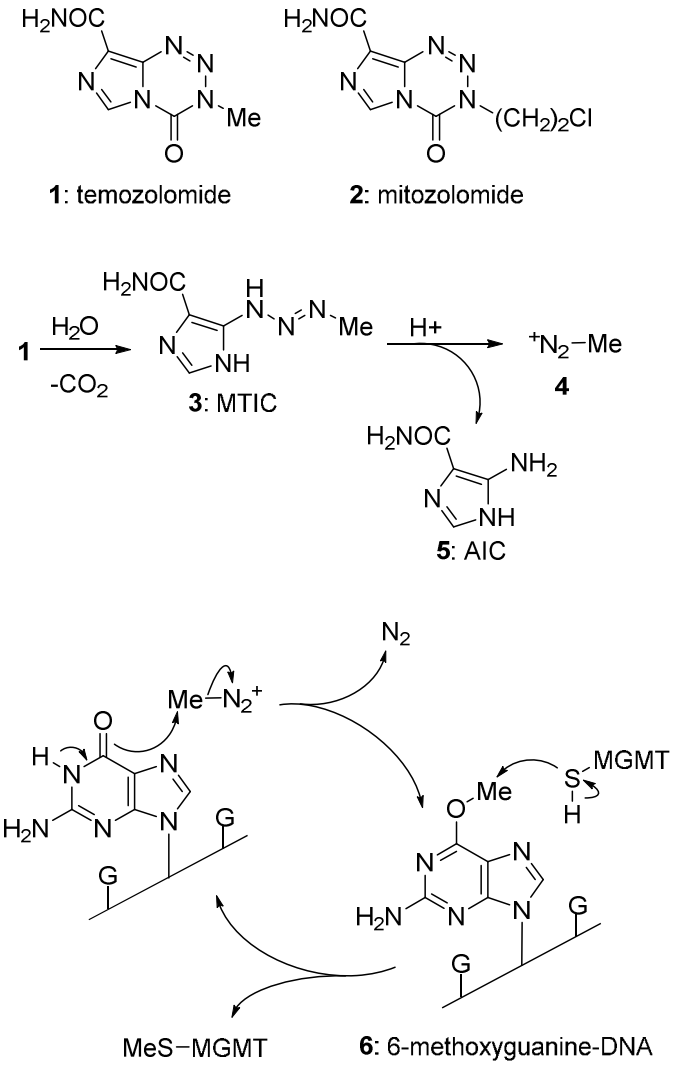

In a previous publication we have shown that conjugating the 8-carboxamide substituent in imidazotetrazines with DNA major and minor groove-binding motifs was not rewarded with enhanced biological activity in in vitro systems.[13] Accordingly, we initiated a new research program with an audacious biological end-point. In a nutshell we asked the following question: is it possible to develop safe new imidazotetrazine derivatives which intrude novel alkyl lesions into DNA at the guanine O-6 position which are non-repairable by MGMT and unaffected by the MMR status of the tumors? Potentially, such moieties might be

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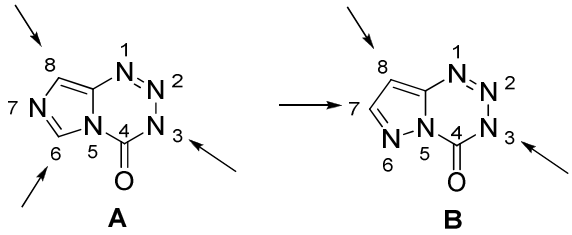
more efficacious than TMZ against the brain tumors for which the drug is currently approved, but also extend the spectrum of action to tumor types currently inherently resistant to agents of this class by virtue of their relatively abundant MGMT levels or MMR deficiency.

In many respects TMZ is a model prodrug, most notably because of the clean chemical activation process from **1**→**3** to active alkylating species (**4**).[6] In our design considerations we have chosen to retain the imidazotetrazine scaffold which brings the desirable physico-chemical and pharmaceutical attributes mentioned earlier: but this imperative limits the structural modifications which can be considered to only three sites. (For the numbering system in imidazo[5,1-*d*]-1,2,3,5-tetrazines see Figure 2A). Although the corresponding pyrazolo[5,1-*d*]-1,2,3,5-tetrazine core (Figure 2B) would be predicted to have a similar ring-opening chemistry, earlier research efforts showed that adopting this related heterocyclic template did not bring any particular benefits.[14,15]

Figure 2. Potential sites of chemical modification of imidazo- (**A**) and pyrazolo[5,1,*d*]-1,2,3,5-tetrazines (**B**).



In this report we focus on the synthesis and biological properties of compounds retaining an H atom at C-6 and a carboxamide moiety at C-8, and differing from TMZ only in variations at N-3 (Figure 2A). Several N-3 modified compounds prepared, but inadequately tested, in the original work[1, 2] have been re-synthesized and evaluated with new compounds in a primary *in vitro* cell panel containing matched MGMT+/– human glioma cell lines. Certain compounds showing equal activity against cells irrespective of their MGMT status

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were subjected to a broader in vitro screen and further pharmacological evaluation. Making the assumption that the O-6 position of guanine residues in DNA is still the locus of required covalent modification by novel agents, we decided to synthesize and evaluate a broad range of novel structural types, but specifically *excluding* agents with DNA cross-linking potential, like the ill-fated MTZ.[16,17]

Chemistry

Synthetic routes to three types of imidazo[5,1-*d*]-1,2,3,5-tetrazines (**10-12**) differing in the nature of the 3-substituent were explored. The most widely applicable chemistry to access a broad range of compounds was the Stone synthesis[1](Synthetic Method 1: see Experimental Section) starting with AIC (**5**) which was converted to 4-diazoimidazole-5-carboxamide (**7**) and reacted with the appropriate isocyanate in DMSO (Scheme 1). Yields of imidazotetrazines were near quantitative with pure isocyanates from commercial sources, when available. We have previously used this route to synthesize **1**, **2**, **10a-d**: to access other compounds, isocyanates were prepared by classical routes including treatment of primary amines with phosgene (or a phosgene equivalent), or the Curtius rearrangement of acyl azides, prepared either from acid chlorides and sodium azide or directly from carboxylic acids and diphenylphosphoryl azide. Many isocyanates required for this project prepared by the above routes were used in an impure state and, because of their instability, it was rarely possible to improve their purity by vacuum distillation. Accordingly, the modest to low yields of imidazotetrazines reported (Table 1) underestimate the intrinsic efficiency of the Stone synthesis. We have recently reported the synthesis of the (formerly) elusive 3-hydroxymethyl-imidazotetrazinone (**8**) and **9** ('nor-temozolomide').[18] Although the anion generated from **9** can be alkylated with methyl iodide to generate **1** in 70% yield, this alternative approach to substituted 3-alkylimidazotetrazines (Synthetic Method 2) generally

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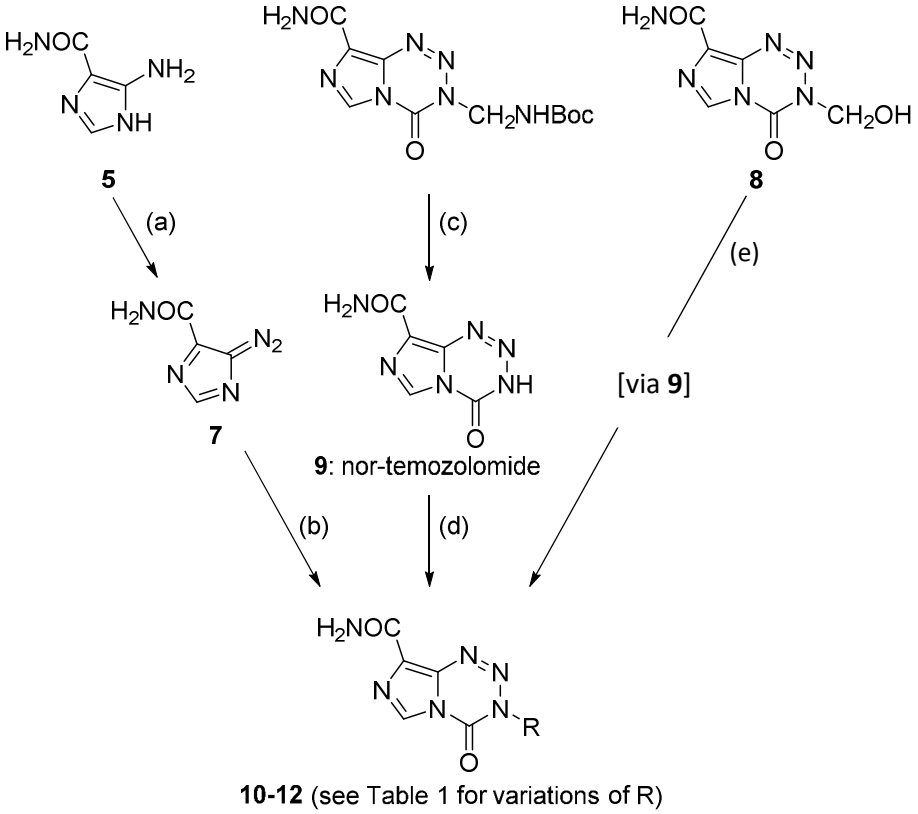
affords modest yields and is restricted in its scope.[18] Nevertheless, the utility of such routes to efficiently synthesize ^{11}C TMZ is noteworthy [19, 20].

Scheme 1. Synthetic Method 1 (**5** → **7** → Products) and Synthetic Method 2 (**8** → **9**→ Products) for the preparation of 3-substituted imidazotetrazines (**10-12**).

[a] Reagents and conditions: a) $NaNO_2$, 1M HCl, H_2O, b) RNCO, DMSO, c) 3N HCl, d) i) NaH, DMF, ii) RX, e) DBU, RX, MeCN.



Examples of new 3-alkylimidazotetrazines and related congeners (**10**) prepared mainly by Synthetic Method 1 were those from isopropyl, cyclopropyl, cyclopentyl, cyclohexyl, (cyclopentyl)methyl and (cyclohexyl)methyl isocyanates (**10e-j**), respectively. Alternatively, the 3-(cyclopropyl)methyl-substituted imidazotetrazine (**10k**) could be prepared from **8** and (cyclopropyl)methyl bromide in the presence of DBU in acetonitrile

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(Synthetic Method 2). Efforts to synthesize compounds suitable for biological evaluation with 3-(fluoromethyl), 3-(bromomethyl), 3-(iodomethyl), 3-(2-fluoroethyl), 3-(2,2-difluoroethyl), or 3-(2-iodoethyl) substituents, and several fluorinated 3-cyclopropyl and cyclopropylmethyl variants, foundered because we were unable to prepare the required isocyanates in adequate purity for application in Synthetic Method 1. More rewarding were the syntheses of imidazotetrazines with a 3-allyl (**10l**) and 3-propargyl substituent (**10m**). Syntheses of the substituted 3-propargylimidazotetrazines (**10n**) and (**10o**) were obtained from 3-hydroxymethyl-imidazotetrazinone (**8**)[18] and 1-bromo-2-butyne or 3-bromo-1-(trimethylsilyl)-1-propyne, respectively, in the presence of DBU in acetonitrile.

Table 1. Synthetic Methods and Yields of 3-Alkylimidazotetrazines (**10**), Imidazotetrazine Carboxylic Acids, Esters, and Amides (**11**), 3-Alkylimidazotetrazines Bearing Additional Heteroatoms (**12**), and Imidazotriazenes (**13**).

Compound	R	Synthetic Method [a]	Yield (%)
10a	CH_2Cl	1[b]	59
10b	CD_3	1 (2)[c]	20 (75)
10c	CH_2CH_3	1[d]	60
10d	CH_2CF_3	1	66
10e	$CH(CH_3)_2$	1	29
10f	$CH(CH_2)_2$	1 (2)	7 (12)
10g	$CH(CH_2)_4$	1	31
10h	$CH(CH_2)_5$	1	18
10i	$CH_2CH(CH_2)_4$	1	3
10j	$CH_2CH(CH_2)_5$	1	15

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10k	$CH_2CH(CH_2)_2$	2	12
10l	$CH_2CH{=}CH_2$	1[d]	100
10m	$CH_2C{\equiv}CH$	1 (2)[c]	3 (30)
10n	$CH_2C{\equiv}CCH_3$	*e*	28
10o	$CH_2C{\equiv}CSiCH_3$	*e*	11
11a	$CH_2CO_2CH_3$	2	36
11b	CH_2CO_2Et	1[b] (2)[c]	82 (13)
11c	$CH_2CO_2C(CH_3)_3$	1	71
11d	(*R*)-$CH(CH_3)CO_2CH_3$	1	30
11e	(*S*)-$C(CH_3)(CH_2OH)CO_2CH_3$	1	34
11f	$(CH_2)_2CO_2Et$	1	75
11g	CH_2CO_2H	*b*	80
11h	$(CH_2)_2CO_2H$	*e*	47
11i	CH_2CONH_2	3	45
11j	$CH_2CONHCH_3$	3	31
11k	$CH_2CON(CH_3)_2$	3	8
11l	$CH_2CON(CH_2CH_2)_2O$	3	33
11m	$(CH_2)_2CONH_2$	3	24
12a	CH_2OCH_3	1[b]	31
12b	CH_2OCH_2Ph	1	34
12c	CH_2OAc	2	12
12d	$CH_2OCOC(CH_3)_3$	2	14
12e	CH_2SCH_3	1	2
12f	CH_2SOCH_3	*e*	98
12g	$CH_2SO_2CH_3$	1	68

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12h	$(CH_2)_2OSi(CH_3)_2C(CH_3)_3$	*e*	7
12i	$(CH_2)_2OH$	*e*	45
12j	$(CH_2)_2SCH_3$	1	26
12k	$(CH_2)_2SO_2CH_3$	1	95
13a	$CH(CH_2)_2$	4	54
13b	$CH_2CH(CH_2)_2$	4	97
13c	CH_2CF_3	4	69
13d	CH_2CHF_2	4	69
13e	$(CH_2)_2OH$	4	70
13f	$(CH_2)_2OCH_3$	4	97
13g	$CH_2C{\equiv}CH$	4	93
13h	$CH_2C{\equiv}N$	4	93
13i	$CH_2CO_2CH_3$	4	97
13j	CH_2CO_2Et	4	90

[a] See Experimental Section for details of Synthetic Methods 1-4. [b] Ref.21 [c] Ref.18

[d] Ref.2 [e] See Experimental Section.

The series of acetic and propionic acid esters (**11a-11f**) were prepared efficiently from the diazoimidazole (**7**) and the appropriate commercially-available isocyanates by Synthetic Method 1. Alternatively esters (**11a**, **11b**) can be prepared from **9** and methyl bromoacetate or ethyl iodoacetate, respectively, albeit in low yields by Synthetic Method 2.[18] Hydrolysis of the ethyl esters (**11b, 11f**) in 5 M-hydrochloric acid at 40-45 ^{o}C afforded the corresponding carboxylic acids (**11g, 11h**), respectively. These acids were then converted into the series of amides (**11i-11m**) *via* their mixed anhydrides, formed with isopropyl chloroformate and *N*-methylmorpholine, followed by treatment with the appropriate amine (Synthetic Method 3) (Scheme 2).

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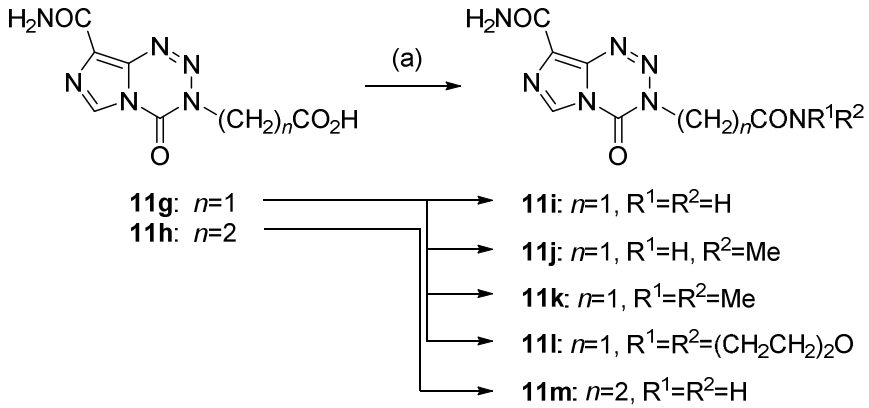
Scheme 2. Synthetic Method 3 for the conversion of acetic and propionic acid derivatives of imidazotetrazines to amides.

[a] Reagents and conditions: a) *i*-BuOCOCl, NMM, DMF, ii) $R^1R^2NH_2$.



Imidazotetrazines with additional O, S and N hetero atoms in the 3-alkyl group (**12**) fell into two categories depending on the length of the carbon linker (1-2 atoms) between the imidazotetrazine nucleus and the additional heteroatom; most of the compounds (Table 1) were prepared by modifications of Synthetic Methods 1 or 2. For other agents requiring different approaches, details are given in the Experimental Section. We have shown previously that the 3-hydroxymethyl-imidazotetrazine (**8**) can be prepared from nor-temozolomide (**9**) and formaldehyde (79%).[18] The bicycle with a 3-(2-methoxymethyl) (MOM) substituent (**12a**) has been prepared previously by Synthetic Method 1[21] and the benzyloxymethyl analog (**12b**) was prepared in the present work by the same route. Two examples of esters (**12c**, **12d**) which are potential prodrug forms of the hydroxymethyl-imidazotetrazine (**8**) were prepared for biological evaluation by Synthetic Method 2 utilizing bromomethyl acetate or chloromethyl pivaloate as alkylating agent, respectively, although

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yields were poor.[18] The thioether variant (**12e**) of the MOM-substituted imidazotetrazine (above) was prepared by Synthetic Method 1. Oxidation of this thioether with periodic acid in acetonitrile gave the sufoxide (**12f**) (46%), but a quantitative yield was obtained utilizing ozone as oxidant in a DCM-MeOH mixture at -78 ^{o}C. The corresponding sulfone (**12g**) was prepared by the isocyanate route (68%) (Table 1). The prototypic example of a compound with a 2-carbon linker 3-(2-hydroxyethyl)-imidazotetrazine (**12i**) was recovered (45%) by deprotection of the *t*-butyldimethylsilyl-protected derivative (**12h**) with THF in acetic acid at 25 ^{o}C. The silyl precursor (**12h**) was itself prepared in meagre yield by Synthetic Method 1. The methylthioethyl-imidazotetrazine (**12j**) and its related sulfone (**12k**) were prepared by Method 1 in 26 and 95% yields, respectively.

Certain examples of 1-alkyl-3-(4-carbamoylimidazol-5-yl)triazenes **(13)** related in structure to MTIC (**3**), which were required for pharmacological comparisons with their ring-closed imidazotetrazine counterparts, were prepared by interaction of the diazoimidazole (**7**) and commercially available amines (10 mol. equiv.) in EtOAc at 25 ^{o}C (Synthetic Method 4) (Scheme 3). Unoptimized yields are recorded in Table 1 and these samples were used for biological studies without further purification. Previous experience with this synthetic method has shown that > 95% purity is achieved.[2, 22] Because of the availability of 2,2-difluoroethylamine and aminoacetonitrile it was possible to prepare samples of triazenes (**13d**, **13h**); the corresponding imidazotetrazines bearing a 3-(2,2-difluoroethyl) or 3-cyanomethyl group, respectively, were not accessible by Synthetic Method 1 because of the unavailability of the required isocyanates. The triazenes (**13i**, **13j**) were prepared from **7** and methyl or ethyl glycinate hydrochloride salts in the presence of triethylamine (10 mol. equiv.).

Scheme 3. Synthetic Method 4 for the preparation of 1-alkyl-3-(4-carbamoylimidazol-5-yl)triazenes (**13**).

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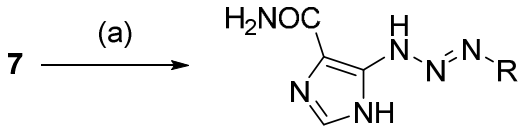
[a] Reagents and conditions: a) RNH_2, EtOAc, 25 °C.



13 (see Table 1 for variations of R)

Biological Results and Discussion

Growth inhibitory assays on glioma (GBM) cell lines. All compounds listed in Table 1 were shown to have > 95% purity by LCMS before biological evaluation [23] (see Experimental Section). It should be pointed out that, despite its clinical utility, TMZ yielded only low growth inhibitory potency against the NCI 60 human cell line panel; assays were performed after 48 h exposure of cells to TMZ, GI_{50} values > 100 μM in all lines. Compounds were evaluated in a primary screen consisting of four GBM-derived cell lines: two sensitive lines (SNB19V and U373V) gave GI_{50} values of 45.6 and 72.9 μM, respectively, for TMZ; and two MGMT-transfected lines (SNB19M and U373M) which demonstrated approximately 11- and 5-fold resistance to TMZ compared to their vector only counterparts with GI_{50} values of 526 and 395 μM, respectively. GI_{50} values of other compounds (Table 2) are the mean of at least three separate determinations, whereas for the marker compound TMZ, n > 50. MTZ (**2**) was more than 3-fold more potent than TMZ against the sensitive vector-only (V) pair of cell lines, and 10-fold more potent against the MGMT-transfected (M) lines than TMZ, yet the same trend persists; MGMT-transfected cells were > 2-fold more resistant to MTZ than vector control cells.

Consistent with one of our objectives – to discover novel compounds which might be active against glioma cell lines irrespective of their MGMT proficiency – compounds

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selected for further scrutiny were required to have two activity characteristics: (i) they were at least equiactive with, or preferably more potent than TMZ against the V lines; and crucially (ii) maintained similar potency against the M lines. GI_{50} values of compounds with these desirable qualities are listed in Table 2, together with values for some reference agents: compounds which failed to pass muster on these two criteria were included in Table 2 only if results were valuable in assessing SAR in the series, or not listed and deemed to be 'deprioritized' at this stage.

The 3-chloromethyl analog (**10a**) of TMZ showed the type of desirable 'flatline' profile which was sought: the agent had higher potency (lower GI_{50} values) against the V lines than TMZ and activity was maintained against the M lines. Results of representative derivatives with lipophilic 3-substituents (Table 2) demonstrate the practicability of the glioma panel to select/deprioritize compounds. Thus compounds with cyclopropyl (**10f**), cyclopropyl-methyl (**10k**) and allyl groups (**10l**) at N-3 had low potency against all four lines; similarly, other compounds with lipophilic 3-alkyl groups listed in Table 1, revealed profiles which excluded them for further consideration at this stage (data not shown). In contrast three compounds with propargylic groups (**10m-o**) were more potent than TMZ and equiactive against V and M cell lines.

Of the acetic acid derivatives (Table 2) only the methyl (**11a**) and ethyl esters (**11b**) had profiles consistent with greater activity than TMZ in V and M cell lines. The pivaloyl ester (**11c**) and two α-substituted methyl acetates (**11d, 11e**) had markedly less inhibitory effects (data not shown). Interestingly the homologous ethyl propionate (**11f**) was less active than the corresponding ethyl acetate (**11b**). Notably also the acetic acid (**11g**) lacked the required activity profile for further consideration despite being more active than TMZ against both U373 cell lines. The homologous propionic acid (**11h**) was of low interest showing GI_{50}

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values > 250 μM against all four cell lines (data not shown). The primary acetamide (**11i**) had higher potency than the secondary and tertiary acetamides (**11j-l**). Consistent with the results in the ester series, in the corresponding amides extension of the methylene chain to a two-carbon ethylene chain led to a major loss of *in vitro* potency in the propionamide (**11m**) (Table 2).

The 3-hydroxymethyl-imidazotetrazine (**8**), although showing equivalent activity against the SNB19 V and M cell lines, proved to be too unstable for reliable bio-evaluation. However, a seam of potentially useful activity extends through its more stable ethers and esters with the MOM ether (**12a**), benzyloxymethyl (**12b**) derivatives, and two potential prodrug forms of **8**, the acetate (**12c**) and pivaloate esters (**12d**), being noteworthy for potent inhibitory activity against both SNB19 and U373 V and M cell lines (Table 2). Interestingly the thio-MOM analog (**12e**) was less active than its MOM counterpart, especially against the SNB19 lines. Potency was restored to both V and M lines when the sulfide was oxidized to sulfoxide (**12f**) or sulfone (**12g**). 3-(2-Hydroxyethyl)-imidazotetrazine (**12i**), a homolog of **8**, was only inhibitory against the four cell lines at concentrations in the 200-400 μM range (Table 2); other examples where the linker between the heteroatom and the bicycle was a two-carbon fragment gave derivatives (**12j-k**) which were deprioritized on the activity characteristics (i) and (ii) defined earlier (data not shown).

Table 2. *In Vitro* Growth Inhibitory Activity of 3-Substituted Imidazotetrazines (**10-12**) and Related Triazenes (**13**) Against Human-derived Glioblastoma Cell Lines.[a]

Compound	Mean GI_{50} (μM)[b]			
	SNB19V	SNB19M	U373V	U373M
1 (TMZ)	45.6	526	72.9	395
2 (MTZ)	10.0	68.4	22.0	40.3

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3 (MTIC)	48.5	466	93.7	398
8	46.2	42.1	-	-
10a	34.6	31.0	29.3	24.4
10f	428	>500	306	360
10k	286	223	386	149
10l	258	239	141	199
10m	35.6	37.8	37.6	36.1
10n	39.6	37.6	36.5	33.0
10o	39.1	39.5	30.3	30.7
11a	47.5	49.0	32.1	46.4
11b	52.4	61.1	63.2	55.6
11f	367	255	129	143
11g	265	296	69.5	140
11i	38.0	95.3	65.4	72.3
11j	65.8	246	260	263
11k	67.7	73.4	85.1	92.5
11l	62.3	102	243	132
11m	321	398	138	355
12a	30.8	37.9	26.0	33.1
12b	52.2	48.5	16.5	28.8
12c	48.3	40.4	11.8	39.4
12d	45.0	28.0	13.2	28.9
12e	107	151	59.0	56.5
12f	26.8	20.1	13.7	14.4
12g	13.8	18.3	20.2	7.5

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12i	204	417	288	322
13a	>500	>500	>500	>500
13b	>500	>500	>500	>500
13c	68.8	131	209	423
13d	68.8	70.3	205	248
13e	441	>500	383	336
13f	>500	>500	>500	>500
13g	39.1	33.9	32.0	35.6
13h	111	271	106	242
13i	47.7	49.7	32.6	46.4
13j	33.6	37.4	30.3	23.4

[a] SNB19V, U373V vector controls; SNB19M, U373M MGMT-transfected. [b] Results are means of ≥ 3 experiments (n = 4 per experiment). Where mean values < 500 μM, SEM values ranged between 3.08% and 21.9% of given mean values.

Despite the fact that the ring-opened 1-alkyl-3-(4-carbamoylimidazol-5-yl)triazene series of compounds (**13**) were less stable than their bicyclic imidazotetrazine counterparts, analysis of the inhibitory activities of triazenes tentatively supported the hypothesis that the established chemical activation pathway **1**→**3** to methyldiazonium ion (**4**), previously thoroughly established for TMZ (Figure 1),[6] is also operating for the new triazenes. Significantly, MTIC (**3**) shows an almost identical profile across the V (sensitive) and M (resistant) cell lines to that of TMZ (Table 2); moreover, where direct comparisons between compounds were possible, potencies of new triazenes (**13**) usually paralleled those of the imidazotetrazines thereby validating the potential of the screening panel to identify compounds with discriminating activities depending on the MGMT repair status of the cell lines. Thus triazenes (**13a**, **13b**) with small lipophilic cyclopropyl and cyclopropylmethyl groups were of

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low potency across the screening panel, reproducing the behaviour of the corresponding cyclic forms (**10f**, **10k**); and triazenes substituted with hydroxyethyl (**13e**) and methoxyethyl (**13f**) groups had low potency across the cell panel. Significantly, the propargyl-substituted triazene (**13g**), like the corresponding imidazotetrazine (**10m**), had activity superior to TMZ irrespective of the V or M status of the glioma cell lines; replacement of the alkyne group of **13g** with a nitrile functionality in the acetonitrile (**13h**) had a profound dyschemotherapeutic effect.

Of notable significance were the biological profiles of the two glycine esters (**13i**, **13j**) in the triazene series which exactly mirrored the activities of the imidazotetrazine glycine esters (**11a**, **11b**). This observation is fully consistent with the hypothesis that the new imidazotetrazines require ring-opening before exerting their inhibitory activities. We propose that hydrolytic ring-opening of **11b** first generates the corresponding triazene (**13k**). Our earlier predictions on tautomeric preferences in monoalkyltriazenes such as MTIC (**3**)[22] and more extensive NMR studies by Vaughan and his colleagues on aryltriazenes bearing H-bonding substituents *ortho* to the triazene linker,[24] predict that the preferred tautomer of **13k** is stabilized by an intramolecular H-bond which then steers the subsequent decomposition pathway (Scheme 4). Protolytic fragmentation of **13k** generates AIC (**5**) and a reactive cationic species (**14**) which (presumably) can alkylate DNA. Because of the strongly acidic nature of protons flanked by two EWGs as in **14**, an alternative scenario involves ionization of a proton from **14** leading to the formation of the (relatively) stable ethyl diazoacetate (**15**) which also has the potential to alkylate DNA by a neutral carbene mechanism. However the latter compound, which is commercially available, showed no activity against the four glioma cell lines (GI_{50} > 800 μM) and is unlikely to be involved in the bioactivity of the imidazotetrazine (**11b**) and its ring-opened partner, the triazene (**13k**).The same argument probably explains the inactivity of the cyanomethyltriazene (**13h**).

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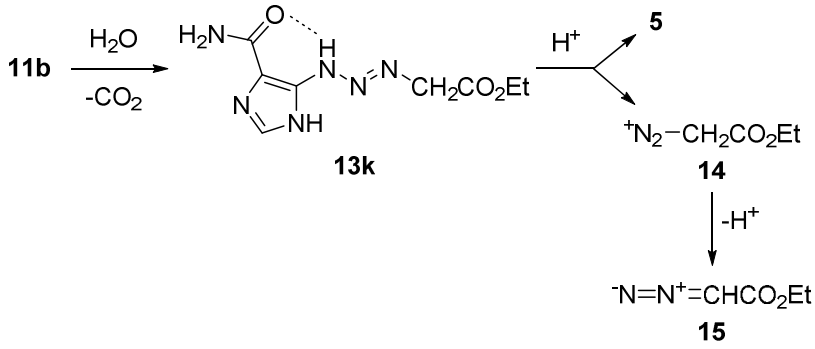
Scheme 4. Decomposition of triazenes bearing glycinate groups.



Although the sulfone (**12g**) was identified as one of the most potent agents overall in the glioma cell panel (above), this agent was not selected for further biological evaluation because of its instability. Instead, the propargyl-substituted imidazotetrazine (**10m**) and the corresponding ring-opened triazene (**13g**) were investigated to determine how they compared with TMZ and MTIC in a broader spectrum of tumor cell lines and, crucially, the specificity of their interaction with DNA.

In previous work we have shown that variant glioma cell lines SNB19VR and U373VR, demonstrating acquired resistance to TMZ can be derived from SNB19V and U373V respectively [25] and used as tools to explore cross resistance between analogs, including **10m** and TMZ. U373VR re-expressed MGMT, but loss of MSH6 (a protein key to functional MMR) contributed to TMZ tolerance (and resistance) in SNB19VR cells. Whereas TMZ gave GI_{50} values of 280.2 μM and 288.8 μM in SNB19VR and U373VR cells respectively, **10m** revealed GI_{50} values < 50 μM against these cells. [9] Thus, resistance to TMZ is not only conferred by direct repair of *O*6-MeG by MGMT but, in the absence of MGMT, tolerance to lesions arises when *O*6-MeG-T mispairs cannot be processed, a consequence of MMR deficiency. Inherited defects in MMR genes *MSH6* and *MLH1* are

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common in colorectal carcinoma (CRC).[26] CRC cell lines HCT116 (*hMLH1 mut*) and DLD1 (*hMSH6 mut*), insensitive to TMZ (GI_{50} values > 500 μM) were > 10-fold more sensitive to imidazotetrazine (**10m**). The ability of novel imidazotetrazines to retain activity in MMR deficient cells may thwart emergence of a hypermutation phenotype conferred by the absence of MMR and promoted by TMZ treatment of glioma clinically.[27, 28] However, it is likely that (as yet unknown) mechanisms of resistance to these agents might emerge.

In U373VR, HCT116 and DLD1 cells the primary *O*6-MeG lesion can be repaired by MGMT, any unrepaired *O*6-MeG lesions will be tolerated in MMR deficient HCT116, DLD1 and SNB19VR cells. In addition, more efficient repair of N-7 or N-3 methylated purines may occur in SNB19VR cells through enhanced transcription of the BER protein *NTHL1*. Indeed, PARP inhibition sensitized SNB19VR cells to TMZ >3-fold,[8] whereas PARP inhibition potentiated the activity of **10m** < 2-fold (unpublished).

Our hypothesis underpinning the search for novel biologically-active imidazotetrazines reasons that cytotoxicity is a consequence of DNA alkylation which cannot be repaired by MGMT. To examine DNA sequence selective alkylation by imidazotetrazine **10m** and the corresponding triazene **13g**, Taq polymerase stop assays were performed. Vehicle-treated DNA showed very few sites of early termination. Compounds **10m** and **13g** at 100 μM heavily alkylated DNA at clusters of three and five guanines (Fig. 3A). Alkylation damage intensity increased with increasing concentrations of agents (1-1000 μM). Triazene **13g** showed quantitatively greater binding at equimolar doses consistent with the assumption that the triazene is an activated form of prodrug **10m**. These dominant polymerase halt sites at runs of guanines are also major binding sites for TMZ and the corresponding triazene MTIC.[7] The same region of plasmid pBR322 was used in piperidine cleavage assays to confirm covalent modifications at N-7 guanine positions (Fig. 3B). The gel reveals the same clusters

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of guanines as predominant sites of agent-DNA reaction, with runs of contiguous guanines being the most reactive to analogs (**10m**) and (**13g**); DNA alkylation data (pattern, intensity, N-7 guanine modification) are comparable to those of TMZ and MTIC.[7]

Imidazotetrazine **10m** was investigated for its ability to crosslink DNA in the linear pBR322 plasmid. The control, native pBR322 plasmid, migrates as a DNA band corresponding to double stranded (DS) DNA and the control denatured pBR322 plasmid migrates as a higher mobility DNA band coresponding to single stranded (SS) DNA. The DNA cross-linking agent cisplatin served as a positive control. Neither TMZ nor **10m** at ≤ 1000 μM formed inter-strand cross-links after incubation for 2 h (Fig. 3C), or 40 h (not shown).

Figure 3.

DNA adducts resulting in formation of DSBs induce rapid phosphorylation of Ser 139 at the carboxy terminus of histone H2AX (γH2AX).[29] Qualitative techniques including Western blot and immunohistochemistry allowed detection of γH2AX following treatment of GBM cells with TMZ, **10m** and **13g**. A method to quantify γH2AX by flow cytometry was subsequently developed. Results following exposure of U373V and U373M cells to **10m** and **13g** are shown (Fig. 3D). Whereas TMZ and MTIC were able to evoke DNA double strand breaks in U373V cells only, **10m** and the ring-opened triazene (**13g**) enhanced γH2AX expression in both vector control and MGMT-transfected U373 cells, albeit to a lesser extent in U373M cells. Nevertheless, compared to DMSO controls, ≥2-fold enhanced γH2AX levels were evident in U373M cells treated with both compounds for 72 h.

Conclusions

Our primary goal at the outset of this work was to identify novel imidazotetrazines which, unlike the clinically-used agent TMZ, display growth inhibitory activities against

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glioma cell lines irrespective of their MGMT status. In the case of 3-substituted modifications of TMZ described herein this objective has been realized and several compounds with desirable attributes are listed in Table 2. The propargyl-substituted imidazotetrazine (**10m**), selected as an exemplar compound, shows a 'flat' inhibitory profile with GI_{50} values at < 40 μM against SNB 19V and M, and U373V and M cell lines: in contrast the profile of TMZ is 'spiky' with both the M cell lines proving to be resistant to the drug. Significantly, the propargyl-substituted imidazotriazene (**13g)** shows a strikingly similar 'flat' profile (Table 2) to that of the related imidazotetrazine prodrug (**10m**) and in other examples where direct comparisons between imidazotetrazines and their related triazenes were examined, both species showed parallel inhibitory activities: this implies that ring-opening, like that shown in the TMZ to MTIC transformation,[7] is also a requirement for bioactivity in this new series. We will report detailed stability studies on **10m** and **13g** in a future publication.

We have shown previously that, whereas TMZ gave GI_{50} values of 280.2 μM and 288.8 μM in cells lines with acquired resistance to the drug (SNB19VR and U373VR), compound **10m** showed GI_{50} values < 50 μM against these cells.[25] In CRC cell lines HCT116 (*hMLH1 mut*) and DLD1 (*hMSH6 mut*), insensitive to TMZ (IC_{50} values > 500 μM), were > 10-fold more sensitive to imidazotetrazine (**10m**). This result provides encouragement that new imidazotetrazines could be developed which will impede evolution of a hypermutation phenotype and may possess broader spectrum antitumor activity compared to TMZ.

Taq polymerase stop assays confirmed that **10m** and **13g**, like TMZ and MTIC, alkylated plasmid pBR322 DNA at runs of three and five guanines (Figure 3A). Similarly, piperidine cleavage assays confirmed that both these agents induced covalent modifications at N-7 guanine positions of the plasmid DNA (Figure 3B) with runs of contiguous guanines being the prime targets. As might be expected for a putative monofunctional alkylating agent,

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imidazotetrazine **10m**, like TMZ, did not crosslink DNA in a linear pBR322 plasmid. That alkylation/modification of guanine led to DNA DSBs in cells following their exposure to **10m** (and ring opened **13g**) can be concluded by detection of γH2AX foci. In contrast to TMZ, **10m** was able to evoke DNA double strand breaks in GBM cells irrespective of MGMT status, therefore overcoming MGMT repair.

Finally, although the screening cascade has identified compounds with intriguing *in vitro* properties, this is only the start of the process to select a clinical candidate. Going forward we are hoping to select a novel imidazotetrazine with desirable 'temozolomide-like' physicochemical characteristics and acceptable PK properties, to allow for oral administration against *in vivo* glioma xenograft models; these models will include TMZ-sensitive tumors and tumors with intrinsic and acquired resistance to TMZ.

Experimental Section

Chemistry. All compounds were purified by crystallization or flash chromatography on Merck silica gel 60. In general imidazotetrazines and imidazotriazenes (Table 1) decomposed vigorously in their melting ranges, which were highly variable depending on the rate of heating and were not a reliable means to characterise compounds.

Synthetic Method 1. A substituted isocyanate (4.2 mmol) was added dropwise to a stirred suspension of 4-diazoimidazole-5-carboxamide (**7**) (0.5 g, 3.65 mmol) in dry DMSO (5 mL) at 25 ^{o}C under nitrogen. After 24 h the mixture was poured onto ice and the product was extracted into DCM (3 x 25 mL). The organic fraction was washed with water and dried (Na_2SO_4), and solvent removed by vacuum evaporation. The solid residue was triturated with EtOAc and the imidazotetrazine was collected and dried in vacuo at 25 ^{o}C. Selected examples are the following: **8-Carbamoyl-3-(*i*-propyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (10e)** prepared from 4-diazoimidazole-5-carboxamide (**7**) and isopropyl isocyanate was

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isolated as a beige solid (29%); LCMS (ES^+) m/z 223 $(M+H)^+$; ^{1}H NMR (400 MHz, δ_6-DMSO) d 8.77 (1H, s, H-6), 7.77 (2H, brs, 2 x NH), 5.03 (1H, m, CH), 1.49 (6H, d, 2 x CH_3); **8-Carbamoyl-3-propargylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (10m)** from **7** and propargyl isocyanate was isolated (3%) as a white solid; LCMS (ES^+) m/z 219 $(M+H)^+$; ^{1}H NMR (400 MHz, δ_6-DMSO) d 8.86 (1H, s, H-6), 7.82 (1H, brs, NH), 7.70 (1H, brs, NH), 5.14 (2H, d, CH_2), 3.52 (1H, t, CH). The product was identical to the same imidazotetrazine (30%) prepared from nor-temozolomide (**9**) and propargyl bromide.[18] Other imidazotetrazines prepared by this method are listed in Table 1 with full spectroscopic characteristics (LCMS and ^{1}H NMR) detailed in the patent literature.[23]

Synthetic Method 2. Details of the synthesis of selected 3-alkyl imidazotetrazines prepared either (a) from the direct alkylation of nor-temozolomide (**9**) (for compounds **10b**, **10m**, **12c**), or (b) (for compounds **10m, 11a**, **11b**) following the in situ generation of **9** from the precursor 3-hydroxymethyl imidazotetrazine (**8**) have been described in recent work.[18] Other compounds prepared from **8** by this method are listed in Table 1. Full spectroscopic characteristics (LCMS and ^{1}H NMR) of compounds are detailed in the patent literature.[23]

8-Carbamoyl-3-(but-2-ynyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (10n). To a solution of **8** in acetonitrile (0.17 M concentration of substrate in solvent) at 0 ^{o}C was added 1-bromo-2-butyne (4 mol. equiv.) and DBU (1.4 mol. equiv.) and the mixture was stirred for 0.5 h. The mixture was acidified (1 M HCl) and products extracted into EtOAc. Combined organic extracts were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The oily residue was triturated with Et_2O to furnish **10n** (28%); ^{1}H NMR (400 MHz, δ_6-DMSO) d 8.84 (1H, s, H-6), 7.82 (1H, brs, NH), 7.70 (1H, brs, NH), 5.08 (2H, q, J = 2.4 Hz, CH_2), 1.84 (3H, t, J = 2.4 Hz, CH_3).

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8-Carbamoyl-3-(trimethylsilylprop-2-ynyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (10o). Similarly prepared as above (11%) from **8** and 3-bromo-1-(trimethylsilyl)-1-propyne; ^{1}H NMR (400 MHz, δ_6-DMSO) d 8.87 (1H, s, H-6), 7.86 (1H, brs, NH), 7.72 (1H, brs, NH), 5.18 (2H, s, CH_2), 0.16 (9H, s, 3 x CH_3).

Synthetic Method 3. Typical conditions were the following: a solution of *i*-propyl chloroformate (1M in toluene; 1.55 mL, 1.155 mmol) and *N*-methylmorpholine (0.127 mL, 1.55 mmol) were added to a stirred solution of either the imidazotetrazine acetic acid (**11g**) or propionic acid (**11h**) (1.05 mmol) in DMF (1.34 mL) under nitrogen at -10 to -15 ^{o}C. To the stirred mixture was added either ammonia (0.5 M solution in 1,4-dioxane; 0.396 mmol) and triethylamine (0.198 mmol) (for compounds **11i** or **11m**), or the appropriate amine (for compounds **11j-11l**). The reaction mixture was stirred (1 h) and allowed to warm to 25 ^{o}C (24 h). Et_2O (5 mL) was added and the resulting precipitate was washed with acetonitrile and DCM and finally more Et_2O. The white product was purified by flash chromatography using acetonitrile/DCM (9:1) as eluting solvent. A specific example is the following: **8-Carbamoyl-3-(carbamoyl)methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (11i)** prepared from imidazotetrazine (**11g**) and ammonia was isolated as a white solid (45%); LCMS (ES^+) m/z 238 $(M+H)^+$; ^{1}H NMR (400 MHz, δ_6-DMSO) δ 8.88 (1H, s, H-6), 7.84 (1H, brs, NH), 7.72 (1H, brs, NH), 7.70 (1H, brs, NH), 7.44 (1H, brs, NH), 4.88 (2H, s, CH_2). Other imidazotetrazines (**11j-11m**) prepared by this Method are listed in Table 1. Full spectroscopic characteristics (LCMS and ^{1}H NMR) of compounds are detailed in the patent literature.[23]

Synthetic Method 4. A mixture of 4-diazoimidazo-5-carboxamide (**7**) and the appropriate amine (1 mol. equiv.) were stirred in dry EtOAc at 25 ^{o}C for 4 h. The imidazotriazenes (**13a-h**) were collected and washed with dry EtOAc. Compounds prepared by this method are listed in Table 1 (with yields). **13i** and **13j** were prepared from the hydrochloride salts of methyl and ethyl glycinate, respectively, in the presence of

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triethylamine (1 mol. equiv.). (Note: imidazotriazenes are unstable in polar solvents and were stored at 0-4 ^{o}C. Their melting points vary according to the rate of heating and are often accompanied by vigorous decomposition).

3-(8-Carbamoyl-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-3-yl)propanoic acid (11h). A suspension of the ester **(11f)** 0.5 g, 1.784 mmol) in 5 M hydrochloric acid (2.4 mL) was heated at 45 ^{o}C for 16 h. The product was collected, washed with water followed by acetone, and crystallized from an acetonitrile/water mixture to furnish the propanoic acid (47%) as an off-white solid; LCMS (ES^+) m/z 281 $(M+H)^+$; ^{1}H NMR (400 MHz, δ_6-DMSO) δ 12.5 (1H, s, OH), 8.80 (1H, s, H-6), 7.75 (1H, brs, NH), 7.65 (1H, brs, NH), 4.45 (2H, t, CH_2), 2.75 (2H, t, CH_2).

8-Carbamoyl-3-(methylsulfinylmethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (12f). (i) The 3-(methylthiomethyl)imidazotetrazine **(12e)** prepared according to Synthetic Method 1 (0.05 g) in DCM/MeOH (1:1 mixture, 5 mL) was cooled to -78 ^{o}C and ozone was gently bubbled through the solution until saturated. The mixture was gassed with nitrogen, allowed to warm to 25 ^{o}C and stirred with excess dimethyl sulphide for 0.5 h. The precipitate was collected, washed with Et_2O and furnished the pure title compound (98%) as a white solid**;** LCMS (ES^+) m/z 257 $(M+H)^+$; ^{1}H NMR (400 MHz, δ_6-DMSO) δ 8.92 (1H, s, H-6), 7.89 (1H, brs, NH), 7.74 (1H, brs, NH), 5.59 (1H, d, J = 13.2 Hz), 5.47 (1H, d, J = 13.2 Hz), 2.77 (3H, s, CH_3). (ii) Oxidation of **12e** (0.17 mmol) in acetonitrile (1 mL) containing $FeCl_3$ (0.005 mmol) with periodic acid (0.18 mmol) at 25 ^{o}C for 1.5 h gave the same product (45%).

3-{2-(*t*-Butyldimethylsilyloxy)ethyl}-8-carbamoylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (12h). Triphosgene (1.675 g, 5.65 mmol.) was added portionwise to a stirred mixture of 2-(*t*-butyldimethylsilyloxy)ethylamine (3.0 g, 17.11 mmol.) in DCM(25 mL) and saturated sodium hydrogen carbonate solution (25 mL) at 0 ^{o}C. After 1.5 h the organic layer

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was washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to give an oil which was purified by vacuum distillation. 2-(*t*-Butyldimethylsilyloxy)ethyl isocyanate distilled at 124 ^oC as a colorless oil which was used without further purification. The crude isocyanate was reacted with 4-diazoimidazole-5-carboxamide (**7**) according to the conditions of Synthetic Method 1 to afford **12i** as a white solid 7%; LCMS (ES^+) m/z 339 $(M+H)^+$; ^1H NMR (400 MHz, δ_6-DMSO) δ 8.87 (1H, s, H-6), 7.83 (1H, brs, NH), 7.70 (1H, brs, NH), 4.41 (2H, t, CH_2), 3.98 (2H, t, CH_2), 0.78 (9H, s, 3 x CH_3), -0.03 (6H, s, 2 x CH_3).

8-Carbamoyl-3-(2-hydroxyethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (12i). A solution of **12h** (0.05 g, 0.148mmol.) in THF:AcOH:H_2O (5 mL of a 1:1:1 mixture) was stirred a 25 ^oC. After 3 h the reaction mixture was concentrated in vacuo and the residue purified by flash column chromatography (SiO_2, gradient 0-100% acetonitrile in DCM) to furnish the **12h** as a white solid (45%); LCMS (ES^+) m/z 225 $(M+H)^+$; ^1H NMR (400 MHz, δ_6-DMSO) δ 8.84 (1H, s, H-6), 7.81 (1H, brs, NH), 7.68 (1H, brs, NH), 4.85 (1H, t, OH), 4.34 (2H, t, CH_2), 3.79 (2H, q, CH_2O).

Biology. Stock solutions of TMZ and analogs were prepared (100 mM in DMSO) and aliquots stored at -20 ^oC, protected from light. SNB19V, SNB19M, U373V and U373M cells were gifts from Schering-Plough Research Institute; HCT116 carcinoma cell lines were purchased from the European Cell Culture Collection (ECCC, UK); DLD1 cells were a gift from Dr. Srinivasan Madhusudan, University of Nottingham, U.K. Cells were maintained in RPMI 1640 nutrient medium supplemented with 10% fetal bovine serum (FBS), subcultivated twice weekly and incubated at 37 ^oC in an atmosphere containing 5% CO_2. SNB19VR and U373VR, variant cell lines derived from SNB19V and U373V respectively[8,25] were routinely maintained in medium additionally supplemented with 100 μM TMZ. All cell lines were verified as being mycoplasma free.

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Anti-proliferative assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays were performed as reported previously.[8,25] Compound concentrations (μM) required to inhibit 50 % cell growth (GI_{50}) were calculated using non-linear regression analysis.

TAQ polymerase Stop Assay. The sequence specificity of covalent DNA modification by **1** (TMZ), **10m**, **13g** on the top strand of pBR322 plasmid was identified by Taq polymerase stop assays.[13,30] Linearized plasmid pBR322 DNA (0.5μg) was treated with compounds at concentrations 1, 10, 100 and 1000 μM for 2 h at 37 ^{o}C and amplified with the primer 5′-TATGC-GACTCCTGCATTAGG-3′, which binds to the top strand pBR322 sequence region 622-641. The primer was 5′-end labeled with [γ^{32}P] ATP using T4 polynucleotide kinase. After primer extension, samples were precipitated with ethanol and sodium acetate, washed (70% ethanol) and lyophilized. Samples were resuspended in 6 μL formamide loading buffer, separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Piperidine Cleavage Assay. A modification of the Maxam and Gilbert sequencing technique was used to probe N-7 alkylation on the same region of plasmid pBR322 as examined in the Taq polymerase assay.[31] The 209-bp probe subjected to modification was amplified by PCR and defined by the top primer used in the Taq polymerase stop assay and a second primer, 5′-GCGC-CTATATCGCCGACATC-3′, which binds to the bottom strand of the plasmid at positions 435-452. Experimental conditions employed were as described by Hartley et al.[30] After amplification, the resulting PCR product was detected by 1% TAE agarose gel electrophoresis, isolated and purified using the Bio101 GENECLEAN Π Kit according to the manufacturer's protocol. Following precipitation and lyophilization, dried DNA pellets were treated with 10% piperidine (100 μL) at 90 ^{o}C for 15 min to produce strand

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breaks specifically at guanine N-7 alkylation sites. The lyophilized DNA fragments were then dissolved in formamide dye solution, separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

DNA Crosslink Assay. BamHI linearized and dephosphorylated pBR322 plasmid DNA was 5'-end labeled with [γ^{32}P]-ATP (40 μCi, Amersham) and T4 polynucleotide kinase (10 units); 125 ng 5′end-labeled DNA was used at each reaction. Reactions were performed with compound in 25 mM triethanol-amine / 1 mM EDTA, pH 7.2 at 37 ^{o}C for 2 h. Samples were precipitated, denatured and subjected to agarose gel (0.8%) electrophoresis at 30 V for 16 h and visualized by autoradiography.

Quantification of γH2AX by Flow Cytometry. U373 (V and M) cells were seeded in 6 cm Petri dishes (2-4 x 10^5). After 24 h, medium was aspirated and replaced with fresh nutrient medium containing 100 μM **1** (TMZ), **5 (**MTIC), **10m** or **13g,** prepared from 50 mM stock solutions in DMSO. Control samples received vehicle alone. Following 24 h or 72 h exposure, floating and attached cells were pooled for each sample, washed twice in PBS, pelleted by centrifugation and fixed in 1% MeOH-free formaldehyde in PBS (500 μL; 5 min; 25 ^{o}C). Cells were permeabilized by addition of 0.4% Triton-X-100 in PBS (500 μL; 1 min), pelleted and re-suspended in 1 mL 1% FBS in PBS. After 30 min (25 ^{o}C), cells were re-suspended in 200 μL 1^{o}Ab solution (1:5000 anti-γH2AX Millipore 05-636; 1.5 h, 25 ^{o}C). Cells were incubated in goat anti-mouse Alexafluor 488 2^{o} Ab solution for 1 h in the dark at 25 ^{o}C (200 μL; 1 μg/mL; Molecular Probes A10684). PBS (1 mL) was added to each sample before analysis by flow cytometry (Beckman Coulter EPICS-XL).

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Conflicts of Interest. MFGS and TDB were consultants to, and MFGS was a shareholder of, Pharminox Ltd.

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Figure Legends.

Figure 3

A) Autoradiogram of denaturing sequencing gel (Taq polymerase stop assay) showing alkylation sites induced by imidazotetrazine **10m** and imidazotriazene **13g**. Lane a, control, unmodified BamHI fragment of pBR322 DNA; lane b, cisplatin (1 μM); lane c, TMZ (1000 μM); lanes d-g, analog **10m** (1 μM, 10 μM, 100 μM, 1000 μM); lanes h-k, analog **13g** (1 μM, 10 μM, 100 μM, 1000 μM). Arrows, indicate the positions and sequence of the binding sites on the plasmid DNA. Drug-DNA incubations were for 2 h at 37 ºC

B) Autoradiogram of denaturing sequencing gel (piperidine cleavage assay) showing N-7 alkylation sites by TMZ and imidazotetrazine **10m** and imidazotriazene **13g**. Lane a, control, unmodified BamHI fragment of pBR322 DNA; lanes b-c, G + A sequencing to provide a sequence reference (DNA depurinated with formic acid); lane d, TMZ (500 μM); lanes e-g, **10m** (10 μM, 100 μM, 1000 μM; lanes h-j, **13g** (10 μM, 100 μM, 1000 μM). Arrows, indicate positions and sequence of the binding sites on the plasmid DNA. Drug-DNA incubations were for 2 h at 37 ºC.

C) Autoradiograph of a neutral agarose gel. Cisplatin, TMZ and imidazotetrazine **10m** were incubated with linear ^{32}P -end labelled pBR322 DNA for 2 h at 37 ºC. Denaturation was performed under alkaline conditions. C1, native DNA control; C2, denatured DNA control; DS, double stranded DNA; SS, single stranded DNA.

D) U373V and M cells were exposed to TMZ, MTIC, **10m, 13g** (100 μM) or vehicle control for 72 h. Following incubation with anti-γH2AX 1^{o}Ab and goat anti-mouse Alexafluor 488

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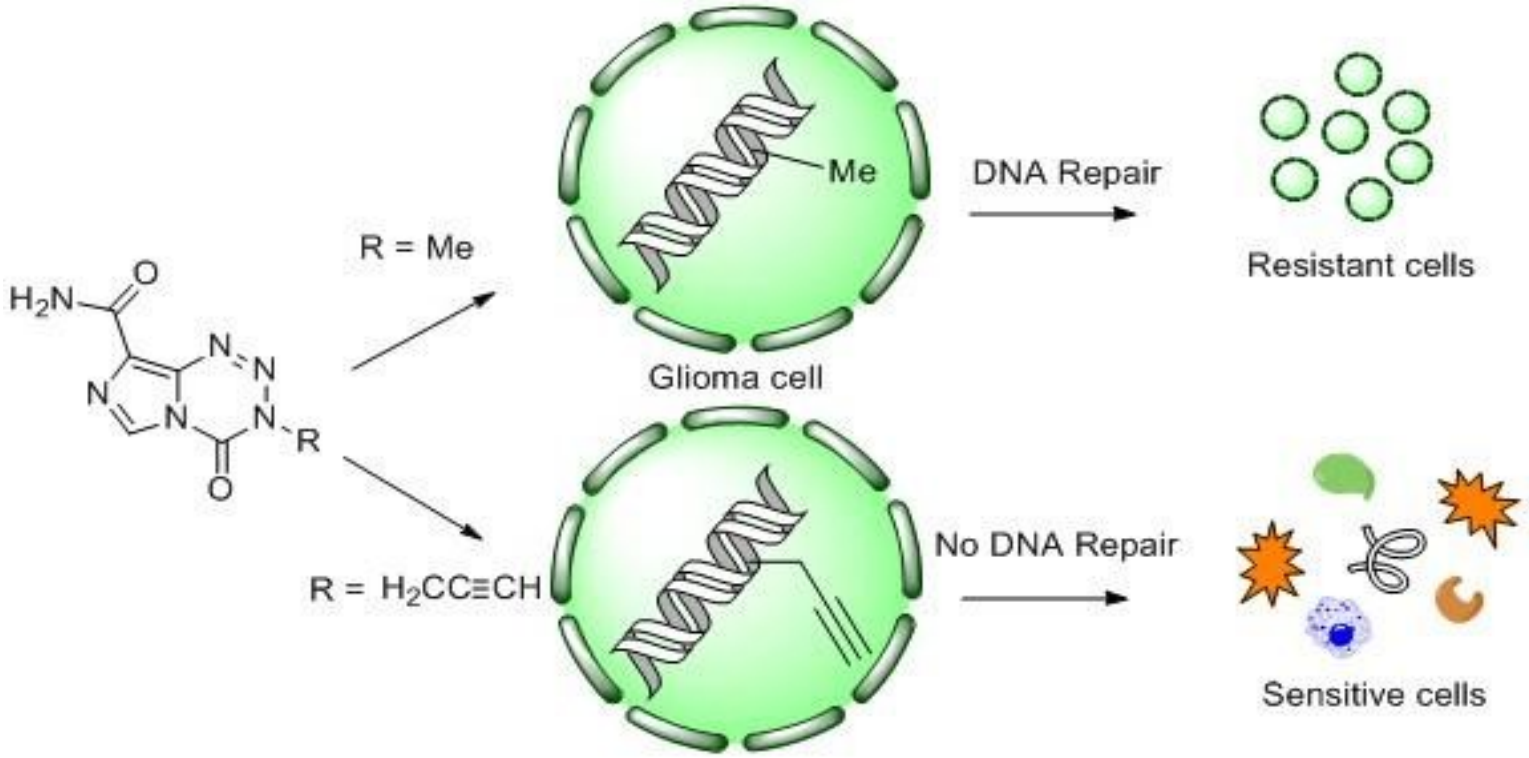
2°Ab, γH2AX expression was analyzed by flow cytometry; 20,000 events were analyzed per sample (n = 3). Values are means ± SD of 3 independent experiments.



Imidazotetrazines substituted at the N-3 position overcome resistance or tolerance to temozolomide conferred, respectively, by MGMT or DNA MMR defects.