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RESEARCH ARTICLE

Synthesis and growth-inhibitory activities of imidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamides related to the anti-tumour drug temozolomide, with appended silicon, benzyl and heteromethyl groups at the 3-position†‡

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A series of 3-(benzyl-substituted)-imidazo[5,1-*d*]-1,2,3,5-tetrazines (**13**) and related derivatives with 3-heteromethyl groups has been synthesised and screened for growth-inhibitory activity *in vitro* against two pairs of glioma cell lines with temozolomide-sensitive and -resistant phenotypes dependent on the absence/presence of the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT). In general the compounds had low inhibitory activity with GI_{50} values > 50 μM against both sets of cell lines. Two silicon-containing derivatives, the TMS-methylimidazotetrazine (**9**) and the SEM-analogue (**10**), showed interesting differences: compound (**9**) had a profile very similar to that of temozolomide with the MGMT+ cell lines being 5 to 10-fold more resistant than MGMT– isogenic partners; the SEM-substituted compound (**10**) showed potency across all cell lines irrespective of their MGMT status.

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† *Part 44 in the series 'Antitumour Imidazotetrazines'. Part 43 is ref.12.*

‡ Electronic supplementary information (ESI) available: experimental details, crystallographic data; NMR spectroscopic studies. See **xxxxxxxxxxxx**

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Introduction

The anticancer drug temozolomide (**1**, TMZ) was originally synthesised in 1980 and its properties were first described in 1984.[1] The chemical and biological properties of the extensive family of anticancer imidazotetrazines have been extensively reviewed over the years, most recently in 2014.[2,3]

TMZ is a small molecule of MW only 194 Da but has a constellation of desirable pharmaceutical properties which led to the drug being approved, in combination with radiotherapy, for the treatment of glioblastoma multiforme (GBM). Contributing to its commercial and clinical success is the benefit of the drug being administered orally in an out-patients setting. Surprisingly, in the nearly two decades since its marketing launch, no mechanistic 'me-toos' have entered the market in this niche clinical application. It is the ambitious objective of our present research to develop a new agent with the pharmaceutical virtues of TMZ, but which overcomes known resistance mechanisms inhibiting its broader use.

It is well established that TMZ is a prodrug that is converted chemically into the triazene 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (**2**: MTIC), which is a precursor of a fugitive methylating species, the methyldiazonium ion (**3**) and 5-aminoimidazole-4-carboxamide (**4**: AIC) (Scheme 1).[4,5] Methylation of DNA (**5**) at runs of guanine residues generates *inter alia* 6-methoxyguanine-DNA lesions (**6**) that can be repaired, restoring the integrity of DNA by removal of the methyl group by the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT): tumours where the *MGMT* gene is epigenetically silenced respond better to the drug than those where the *MGMT* gene is switched on.[6,7] Although repair of methylated DNA by MGMT represents the major pathway by which tumours derive resistance to TMZ, other mechanisms, both constitutive and acquired, such as mismatch repair (MMR) deficiency, further compromise the clinical utility of the drug,[8-10] and long term survival from this most malevolent of diseases is rare. Surprisingly, a case has been made recently to propose a wider role for TMZ in the era of precision medicine.[11]

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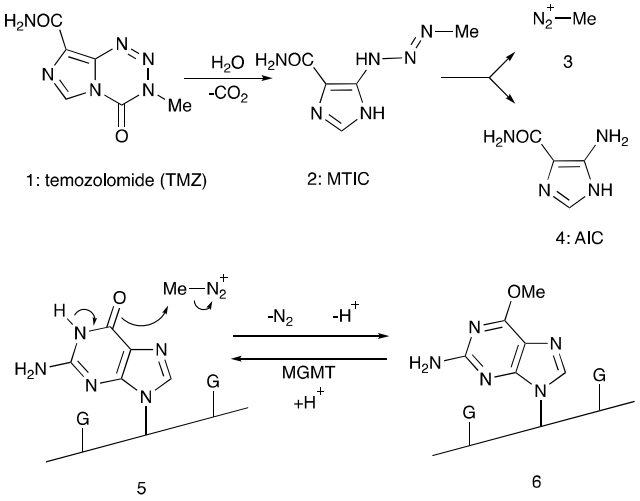

Scheme 1. Structures of temozolomide (**1**) and MTIC (**2**), the formation of O^6-methylguanine residues (**6**) in DNA at runs of guanines (G) and repair of methylated DNA by O^6-methyl-DNA methyltransferase (MGMT).

The versatile Stone synthesis of imidazotetrazines originally employed to synthesise TMZ involves the reaction of 5-diazoimidazole-4-carboxamide (**7**), prepared by nitrosation of AIC (**4**), and an isocyanate and can be exploited to furnish a range of related compounds (Scheme 2).[1] We have recently reported on our efforts to identify new imidazotetrazines,[12] mainly prepared by this method, which can introduce novel alkyl lesions into the O-6 position of guanine residues in DNA which cannot be repaired by MGMT and are unaffected by the MMR status of tumour cells. Of the 50 compounds described, the imidazotetrazine with a 3-propargyl substituent (**8**), for example, was equiactive against two sensitive GBM-derived cell lines (SNB19V and U373V) and two MGMT-transfected lines (SNB19M and U373M). A closer examination of the properties of **8** in a Taq polymerase assay showed that this propargyl-imidazotetrazine alkylated plasmid pBR322 DNA at runs of three to five guanines. In addition, a piperidine cleavage assay confirmed that **8** covalently modified N-7 sites in plasmid DNA in runs of contiguous guanines. These preferential locations are also targeted by TMZ.[13]

Herein we report new investigations on compounds that can be considered as lipophilic modifications of the propargyl substituent. These include two silicon-containing imidazotetrazines that have had important roles in past synthetic work and are now shown to have interesting differences in their activities on GBM cells *in vitro*, as well as a series of 3-benzylimidazotetrazines that, in general, have low growth inhibitory activity.

Chemistry

The TMS-methylimidazotetrazine (**9**), formed from **7** and TMS-methyl isocyanate has been converted into **1** with TBAF and acetic acid in acetonitrile at room temperature in 78% yield (Scheme 2),[14] providing the first practicable route to the prodrug **1** avoiding use of the

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volatile methyl isocyanate. In an alternative approach the imidazotetrazine (**10**) formed from **7** and TMS-ethyloxymethyl isocyanate (SEM isocyanate) could be deprotected by use of excess boron trifluoride in chloroform to provide the 3-hydroxymethylimidazotetrazine (**11**) in 95% yield. In turn **11** could be transformed to TMZ (**1**) with DBU and methyl iodide in acetonitrile in a modest 22% yield *via* an intermediate nor-temozolomide (**12**) generated *in situ*.[15] The method has been adopted recently, by others, to synthesise the [^{11}C]-methyl variant of temozolomide.[16] The benzyl-imidazotetrazine (**13a**) has been synthesised previously (36%) from **7** and benzyl isocyanate in DMSO[14] or from nor-temozolomide (**12**) and benzyl alcohol under Mitsunobu conditions (25%).[15] The syntheses of three other imidazotetrazines (**13e**,[14] **13i**,[17] and **13t**[14]) have been reported previously, but the compounds were not subjected to pharmacological investigation against the discriminating four cell line panel.

In the present work, reaction of **7** and phenethyl isocyanate afforded the homologue (**13b**) (Scheme 2). Methoxy-substituted benzyl isocyanates were converted into imidazotetrazines (**13c-13h**) in yields that reflect the variable purities of the different isocyanates: commercially-available isocyanates of high purity, gave yields of imidazotetrazines ranging from 80-90% in the Stone synthesis, whereas less pure isocyanates afforded yields of products in the 30-50% range. Attempts to improve the quality of isocyanates by vacuum distillation were thwarted by their decomposition under these conditions. Isocyanates derived from 5-membered heterocycles, generally prepared by Curtius rearrangement of the corresponding acyl azides,[17,18] were cyclised with **7** to afford the series (**13i-13m**) where the heteromethyl moieties could be considered as benzylic surrogates, with the heterocycle offering additional non-covalent bonding opportunities. Synthesis of the (1,2,3-triazol-4-yl)methylimidazotetrazine (**13n**) required a different approach: this was prepared (42%) from **8** and TMS-azide in an aqueous *t*-butanol medium containing sodium ascorbate and copper(II) sulfate. Commercially available α-substituted benzyl isocyanates were cyclised with **7** to afford the imidazotetrazines (**13o**-**13t**) in moderate to good yields.

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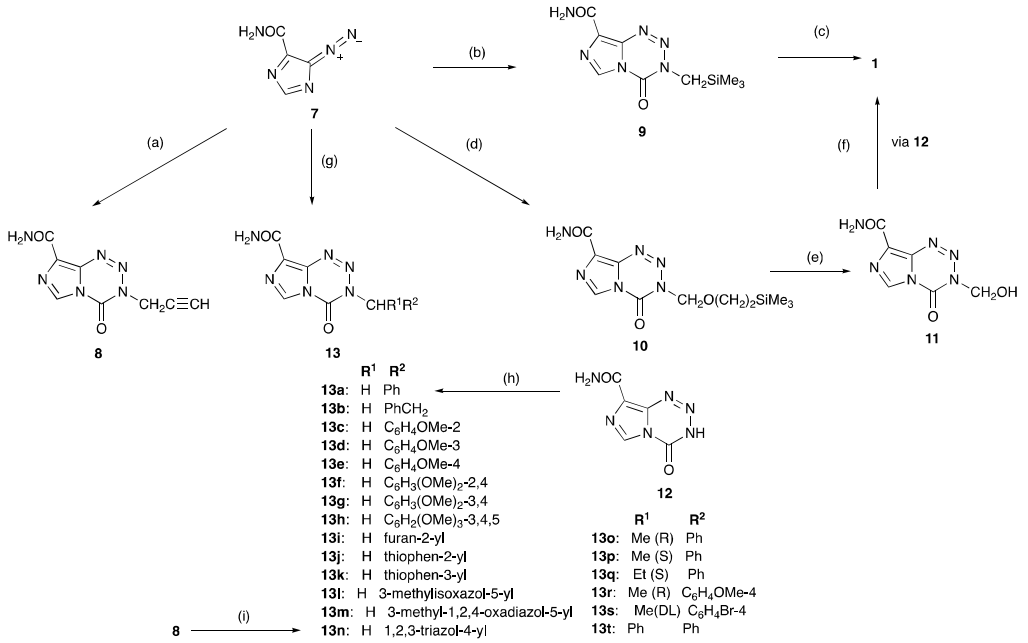
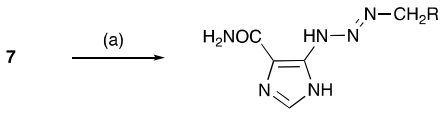


Scheme 2. Synthetic routes to 3-substituted imidazotetrazines. Reagents and Conditions: a) $HC{\equiv}CCH_2NCO$, DMSO, 25 °C; b) $TMSCH_2NCO$, DMSO, 25 °C; c) TBAF, AcOH, MeCN, 25 °C; d) SEM-NCO, DMSO, 25 °C; e) BF_3, $CHCl_3$, 0 °C; f) DBU, MeI, MeCN, 25 °C; g) R^1R^2CHNCO, DMSO, 25 °C) BnOH, PS-PPh_3, DIAD, DMF; i) TMS-N_3, Na ascorbate, Cu(II)SO_4, *t*-BuOH, H_2O, 25 °C.

On the assumption that the new imidazotetrazines would, like TMZ and **8**, act as prodrugs and undergo ring-opening to their respective imidazotriazenes **2**[1,4] and **14a**,[12] respectively, a small series of new triazenes (**14b-14g**) was prepared by coupling **7** and the appropriate amines (Scheme 3) for biological evaluation in comparison with their ring-closed counterparts. Yields of triazenes were in the range 75-98%.



14a: R = C≡CH
14b: Ph
14c: $PhCH_2$
14d: furan-2-ylmethyl
14e: furan-3-ylmethyl
14f: thiophen-2-ylmethyl
14g: thiophen-3-ylmethyl

Scheme 3. Synthesis of 1-substituted-3-(4-carbamoylimidazol-5-yl)triazenes (**14**). Reagents and Conditions: a) RCH_2NH_2, EtOAc, 25 °C.

Crystallography

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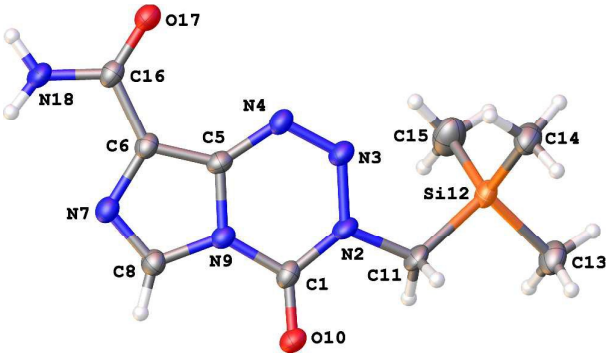
The influence of coplanarity of the 8-carboxamide group with the bicyclic nucleus of imidazotetrazines in ordering crystal packing has previously been reported for mitozolomide[19,20] The ability of the carboxamide group to make multiple H-bonded structures has led to the characterisation of several polymorphs and co-crystals of temozolomide.[21] Similar influences dominate crystal packing in the TMS-methylimidazotetrazine (**9**) and its SEM analogue (**10**) (Figures 1 and 2). In contrast to the TMS-derivative (**9**), the SEM derivative (**10**) shows intermolecular H-bonding between the planes of stacked imidazotetrazines. These H-bonds arise from an out-of-plane torsion in the carboxamide that allows it to access the side-chain SEM oxygen of imidazotetrazines in the plane below. These may account for increased stability in the solid phase, evident as lesser water solubility observed qualitatively when handling.

(A)



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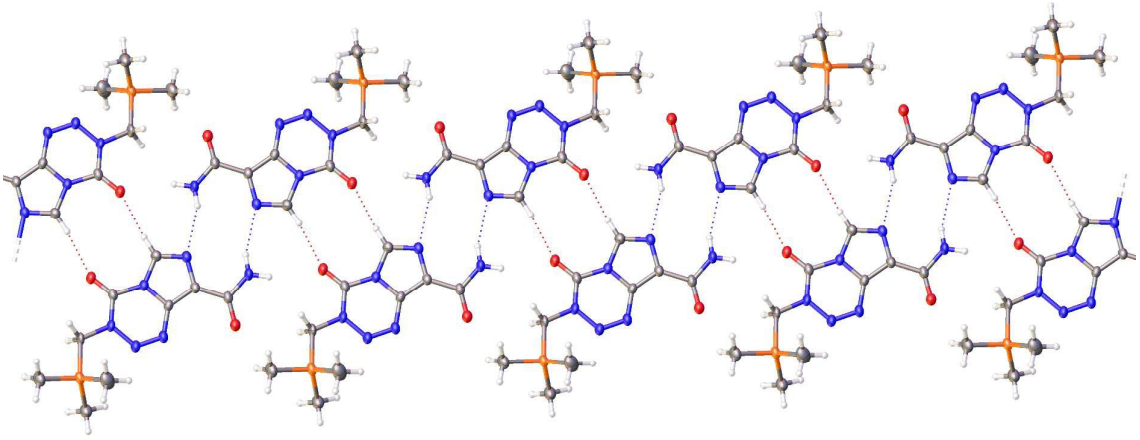

Figure 1 (A) X-Ray crystal structure of 3-trimethylsilylmethylimidazotetrazine (**9**); (B) crystal packing through hydrogen bonding.

(A)

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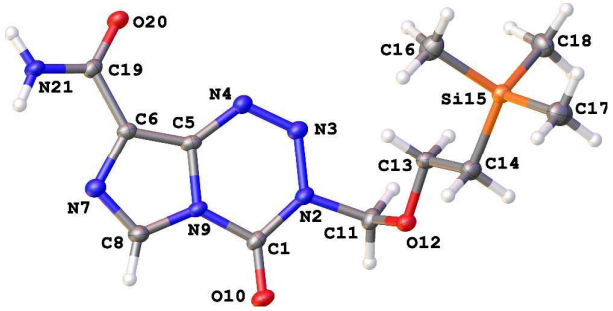

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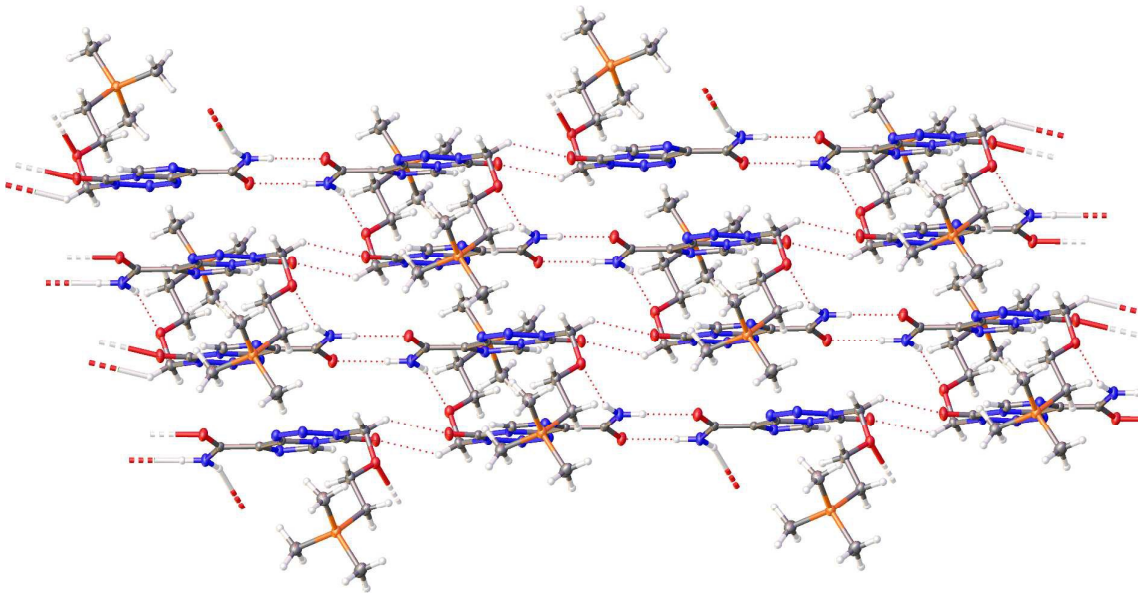

Figure 2 (A) X-Ray crystal structure of 3-(2-trimethylsilylethoxy)methylimidazotetrazine (**10**); (B) crystal packing through hydrogen bonding.

^{1}H NMR spectroscopic studies

Kinetics experiments observed by ^{1}H NMR spectroscopy proved invaluable in identifying methyldiazonium ion as an intermediate in the prodrug activation chemistry of TMZ.[4,5] The aqueous chemistry of the new silyl-imidazotetrazines (**9**) and (**10**) was investigated by similar methods using a neutral phosphate buffer in D_2O, pD = 7.0 (equivalent to pH = 7.4).[4] Data for compound **9** are shown in Figure 3 and exhibit significant parallels to TMZ. In the aromatic region, exponential loss of the 6-H signal (δ_H = 8.67) is accompanied by concomitant evolution of the final heterocyclic product of the reaction, AIC (**4**) 2-H at δ_H = 7.34 (for confirmation, see Figure S3, ESI). The silane region showed a similarly clean conversion of the TMS substituent of **9** δ_H = 0.22) to final product (δ_H = 0.16) presumed to be trimethylsilanol.

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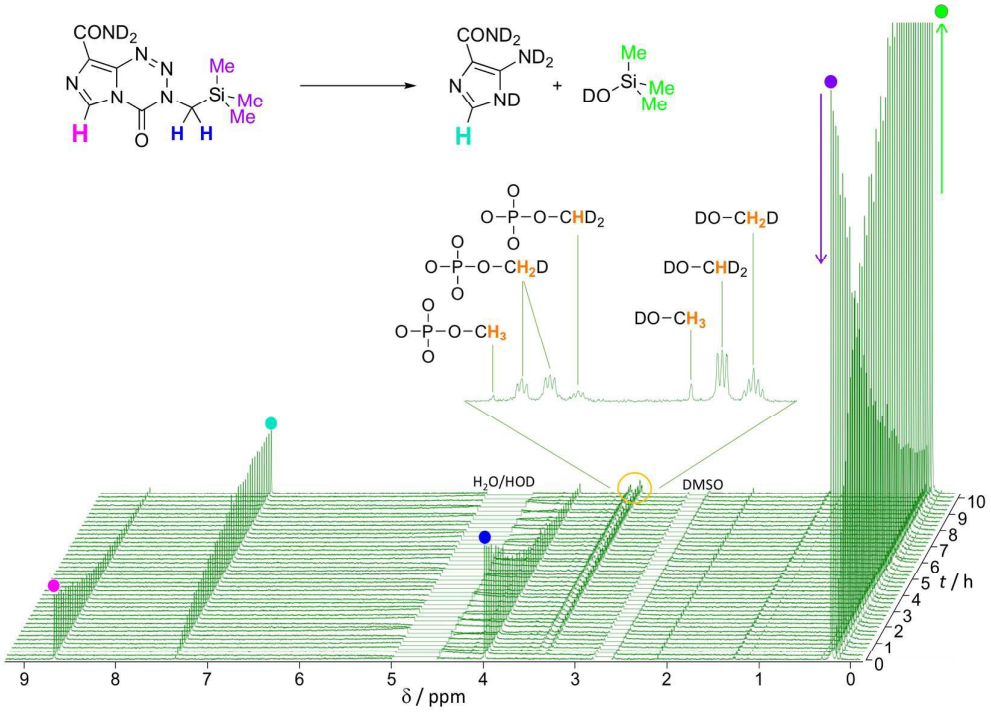
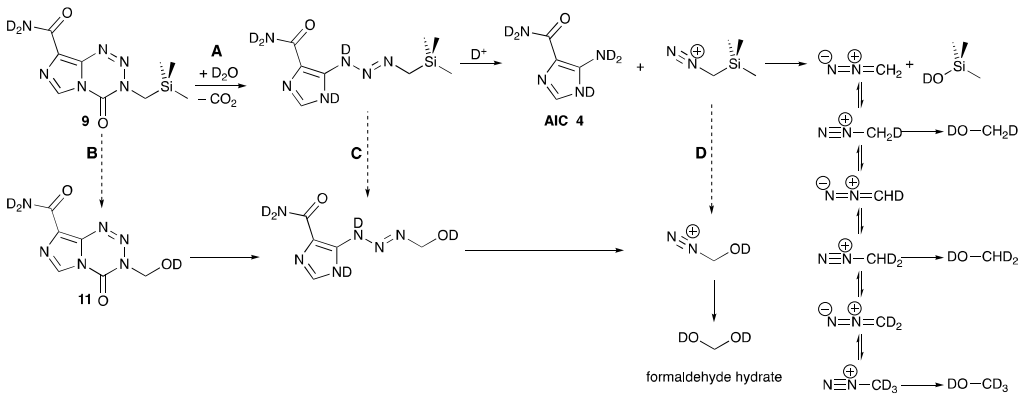


Figure 3. Reaction of TMS-methylimidazotetrazine (**9**) in deuterated phosphate buffer.

The signal for the methylene resonance of **9** (δ_H = 3.99) disappeared exponentially with time and the products derived from this part of the starting molecule were very informative. Two products were detected, methanol (δ_H = 3.36) and methyl phosphate (δ_H = 3.45), each in predominantly deuterated methyl forms (Figure 3, inset); there was no evidence of formaldehyde production. The product ratios and deuterium incorporation data were closely analogous to those obtained from TMZ (Figure S4, ESI).[4] Unlike TMZ, however, which ultimately transfers an intact methyl group to a nucleophile, any methylation products from **9** necessarily derive a proton or deuteron from solvent.



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Scheme 4 Reaction pathways of TMS-methylimidazotetrazine (**9**) in deuterated phosphate buffer (pD = 7.00). Solid arrows indicate a reaction pathway consistent with the NMR spectroscopic data. Dotted arrows show theoretical alternative routes for which no evidence was obtained.

A reaction pathway consistent with these observations is summarised in Scheme 4, route A. The imidazotetrazine undergoes hydrolytic ring-opening to the triazene which fragments to the diazonium ion. The diazonium ion loses the TMS group to water to liberate diazomethane that is deuterated by solvent to methyl diazonium and methylates a proximal nucleophile. There is no evidence to support alternative pathways (B–D) that would involve premature hydrolysis of the TMS group as these would necessarily lead to formaldehyde (in the hydrate form) as product (ESI). Whilst the silane group of compound **9** renders it more lipophilic than TMZ, the current study implies that *in vitro* biological responses are likely to be similar as they are elicited through similar chemical routes.

The aqueous behaviour of the SEM-substituted imidazotetrazine (**10**) was similarly investigated (see Figure 4). The signal-to-noise ratio was determined by the poor water solubility of the imidazotetrazine and the need to identify the multiplet structures in the aliphatic products. All of the signals from **10** were clearly visible and clean conversion to the products AIC, formaldehyde hydrate and trimethylsilylethanol was evident, (see also Figure S5, ESI) without any accumulation of intermediates, Scheme 5, route A, is consistent with this result.

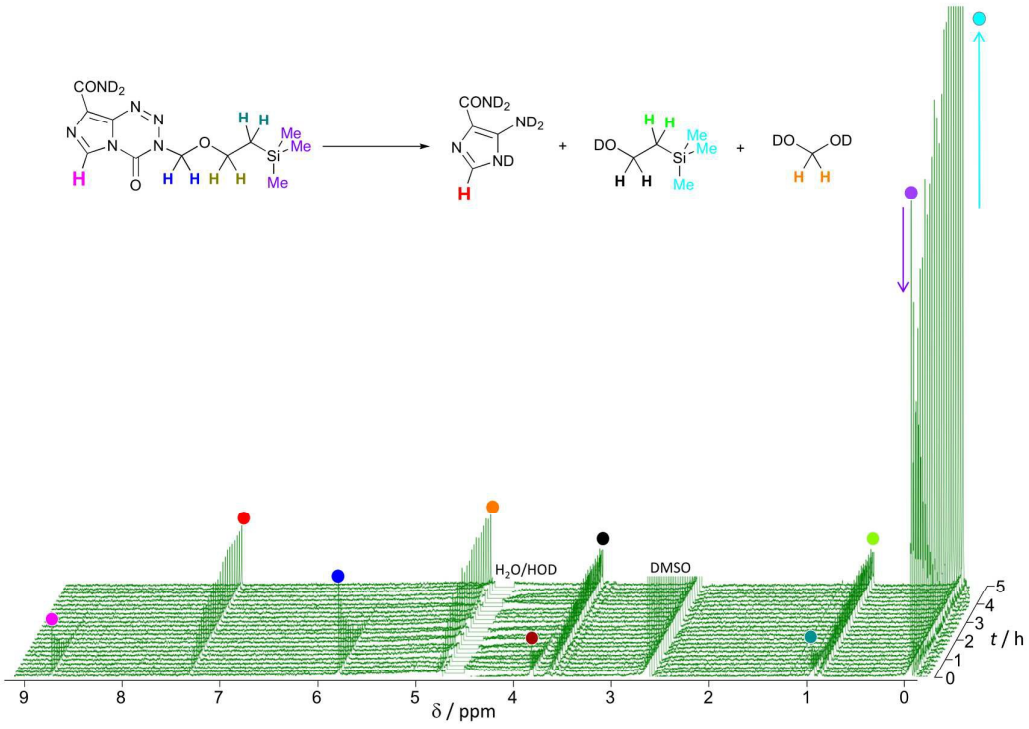

Figure 4 Reaction of SEM-imidazotetrazine (**10**) in deuterated phosphate buffer (pD = 7.00).

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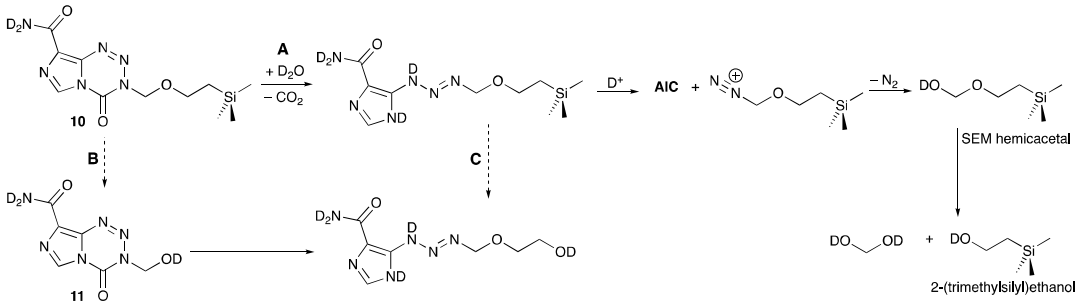
It was notable that product peaks were already evident in the first increment of the kinetic run, just a few minutes after initiation of the reaction. Decay of **10** below the detection limit of the experiment was complete within 3 h, in contrast to persistence beyond 10 h for the TMS derivative (**9**) (Figure 3) or TMZ.[4,5] Assignment of the silyl product as trimethylsilyethanol was based on the small chemical shift changes in the two multiplets of the ethylene unit during reaction. The second order multiplet structure of the starting material was preserved in the products and the methylene adjacent to the silane shifted from 0.98 to 0.89 ppm. This result shows that reaction of **10** is by hydrolysis of the imidazotetrazine and then loss of formaldehyde rather than fragmentation starting at the silane terminus of the molecule. There was no evidence of early-stage loss of the SEM group (e.g. Scheme 5 routes B, C). This result suggests that **10** would be unlikely to have activity as a biological alkylating agent *per se*.



Scheme 5 Proposed reaction pathway for decomposition of the SEM-imidazotetrazine (**10**).

Biological results and discussion

Growth inhibitory assays on glioma (GBM) cell lines

Prior to biological evaluation, all compounds were assessed to have >95% purity by LCMS.[18] Compounds were evaluated against four GBM-derived cell lines: two lines sensitive to TMZ (SNB19V and U373V) gave GI_{50} values of 35.7 and 68.0 μM, respectively; two lines transfected with *MGMT* (SNB19M and U373M) showed approximately 13-fold and 5-fold resistance to TMZ with GI_{50} values of 470 and 369 μM, respectively (Table 1). The pattern of growth inhibition values for the corresponding ring-opened triazene MTIC (**2**) is consistent with the known prodrug character of TMZ.[4] and these results are in accord with previously published work on these two compounds.[12] Also we reported[12] that the 3-propargyl-imidazotetrazine (**8**) and its ring-opened triazene derivative (**14a**) showed growth-inhibitory potency superior to TMZ across all four cell lines, confirming that these variants overcome the principal resistance mechanism involving expression of MGMT: these results are included in Table 1 for comparison purposes.

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In interpreting the results of tumour cell *in vitro* growth inhibition assays of the new compounds revealed in the present work, it is important to bear in mind that, despite its clinical success, TMZ has a very low order of potency against the NCI60 cell panel; typically IC_{50} values are > 100 μM across all cell lines. In keeping with our objective to discover new imidazotetrazines active against GBM tumours irrespective of their MGMT status, to warrant further interest compounds were required to be approximately equiactive with, or more potent than, TMZ against the "V" lines and maintain potency against the "M" lines – i.e. to have a 'flat' activity profile, as exemplified by the marker compound (**8**). Consequently, new compounds with a GI_{50} value > 50 μM against the temozolomide-sensitive SNB19V cell line were, in the absence of other unusual selectivities against the cell panel, de-prioritised and their inhibitory activities recorded at the default value (Table 1). As predicted by the NMR study, the profile of the TMS-methylimidazotetrazine (**9**) against the four cell lines is strikingly similar in its 'spikey' nature to that of TMZ itself (Table 1) with sub 50 μM potency only against the SNB19V cell line, and those lines with transfected *MGMT* (M) being approximately 10- and 5-fold resistant to the compound, compared with their vector (V) only counterparts. On the other hand the 3-SEM compound (**10**) showed surprising potency against all four cell lines, notably against the more resistant U373 V and M lines. The NMR study suggests that this non-selective growth-inhibitory activity may be due to liberation of cytotoxic formaldehyde within cells.

Disappointingly all the 3-benzylimidazotetrazines (**13a-13h**) showed GI_{50} values greater than the cut-off value and in most cases were only inhibitory in the range 100-200 μM (data not shown). Of the heteromethyl compounds (**13i-13n**) the (furan-2-yl)methyl derivative (**13i**) was the most inhibitory, but did not achieve the threshold GI_{50} value, and gave and gave a disappointingly featureless profile in the NCI60 screen, reminiscent of TMZ (Figure S6, ESI). Bucking the trend, both enantiomers of the α–methylbenzylimidazotetrazines (**13o** and **13p**) showed good and equivalent potency against both SNB19V and M lines although they were less active against the U373 pair. Other α-substituted benzylimidazotetrazines (**13q-t**) were uninteresting with GI_{50} values > 50 μM. With the exception of propargyltriazene (**14a**), already mentioned, the series of related triazenes (**14b-14g**) were deprioritised with GI_{50} values in the 100-200 μM range in most cases. Again, the (furan-2-yl)methyltriazene (**14d**), the ring-opened counterpart of imidazotetrazine **(13i),** was the most active albeit exceeding the required threshold GI_{50} value of < 50 μM.

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Table 1 In vitro growth inhibitory activities of 3-substituted imidazotetrazines and related triazenes against human-derived glioblastoma cell lines [a]

	Mean GI_{50} (μM) [b]			
Compound (s)	SNB19V	SNB19M	U373V	U373M
1, TMZ	35.7 ± 12	470 ± 88.7	68.0 ± 32	369 ± 86.1
2	48.5 ± 17.9	472 ± 88.7	93.7 ± 39.7	398 ± 33.7
8	35.6 ± 14.2	37.8 ± 11.7	37.6 ± 11.4	36.1 ± 9.6
9	28.4 ± 5.9	302 ± 69.5	45.6 ± 14.5	249.5 ± 72.1
10	29.9 ± 5.3	27.5 ± 1.4	25.3 ± 3.0	24.2 ± 2.7
13a-13n	>50	>50	>50	>50
13o	32.7 ± 1.3	49.7 ± 5.2	>50	>50
13p	34.7 ± 0.8	45.3 ± 6.0	>50	>50
13q-13t	>50	>50	>50	>50
14a	39.1 ± 5.3	33.9 ± 1.5	32.0 ± 6.9	35.6 ± 1.2
14b-14g	>50	>50	>50	>50

[a] Glioma lines: SNB19V and U373V (vector transfection only, MGMT activity 4 and 56 fmol/mg MGMT, respectively); SNB19M and U373M (stable MGMT transfection 649 and 648 fmol/mg, respectively). [b] GI_{50} values were determined by a 7-day MTT assay and where GI_{50} values are < 50 μM are shown as the mean ±SD of ≥ 3 independent experiments (n=4-8 per experiment). All compounds were dissolved in DMSO at stock concentration 100 mM, stored at –20 °C and diluted in culture medium immediately prior to use.

Conclusions

The 3-benzylimidazotetrazine series does not reveal useful inhibitory activity against the glioblastoma cell panel or a characteristic profile like the methyl analogue TMZ notable for enhanced activity against the V cell lines and resistance towards the MGMT-expressing M lines.[12] A possible reason for this observation could be a chemical inability of the compounds (or their ring-opened forms) to transfer a benzyl fragment to DNA or, alternatively, that an O^6-benzylguanine intrusion, if indeed formed, is not perceived to be a cytotoxic lesion by the cell. NMR Spectroscopic investigation (Figures S7, 8, ESI) implied that there may be more than one pathway for reaction of derivative **13a** in phosphate buffer and showed that benzyl group transfer to solvent (benzyl alcohol) was not the major reaction product. Moreover, investigation of tautomerism in the triazene showed that the tautomer present in DMSO solution was not that from which a diazonium ion could be released (Figure S9, ESI). The failure to generate O^6-benzylguanine-DNA seems the likely reason for inactivity as it is known that O^6-benzylguanine is a substrate for MGMT, and had DNA benzylation occurred, it should have been reflected in activity against MGMT– cell lines.[22] Despite its overall potency against the four cell line panel, the SEM-substituted imidazotetrazine (**10**), which outperforms that of the lead propargylimidazotetrazine (**8**) as reported in the present work, has pharmaceutical liabilities in terms of stability and potential vulnerability to metabolic degradation. The NMR study confirms that this compound does not fragment by a TMZ-like alkylation mechanism. Tentatively we suggest that its overall inhibitory properties may be explained by its liberating cytotoxic formaldehyde within cells.

In future publications we will report on the extensive series of compounds we have synthesised and evaluated with a propargyl substituent at N-3 and modifications of the 8-

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carboxamide group in an effort to select a candidate which overcomes known mechanisms by which GBM tumours mount resistance towards the prototype compound TMZ. Additionally, by fine-tuning the pharmaceutical properties of future drug candidates by varying the nature of the substituent at C-8, we are seeking to identify a new agent for a proof-of-principle *in vivo* evaluation in a mouse xenograft model implanted with GBM tumours sensitive and resistant to TMZ.

Experimental section

Chemistry

Most imidazotetrazines and all imidazotriazenes decomposed in their melting ranges which varied according to the rate of heating. Melting points were not a reliable means to assess purity. Compounds were purified by crystallisation or flash chromatography on Merck silica gel 60. Compounds **9**,[14] **10**,[14] **13a**,[14] **13e**,[1] **13i** [17] and **13t**[14] have been reported previously.

General method for the synthesis of 3-substituted imidazotetrazines (13)

To a stirred suspension of 4-diazoimidazole-5-carboxamide (**7**) (0.5 g, 3.65 mmol) in dry DMSO (5 mL) was added dropwise a substituted isocyanate (4.2 mmol) at 25 °C under nitrogen. After 24 h the mixture was poured into ice and the imidazotetrazine extracted in DCM (3 × 25 mL). The organic fraction was washed with water, dried (Na_2SO_4) and solvent removed by vacuum evaporation. The product was triturated with EtOAc, collected by filtration and dried *in vacuo* at 25 °C. A selected example was the 3-benzylimidazotetrazine **13a**, formed from **7** and benzyl isocyanate, isolated as a grey solid (48%), m.p. 188 °C (decomp.) with physical characteristics (IR, ^{1}H and ^{13}C NMR) identical to an authentic sample.[14]

General method for the synthesis of imidazotriazenes (14)

A mixture of 4-diazoimidazo-5-carboxamide (**7**) and an amine (1 mol. equiv.) was stirred at 25 °C for 4 h in dry EtOAc. Products **14 a-g** were collected and washed with dry EtOAc. (Note: imidazotriazenes are unstable in polar solvents and were stored at 0–4 °C. Their melting points vary according to the rate of heating and are often accompanied by vigorous effervescence.) If pure when analysed by highfield NMR, compounds were used in biological evaluations without further purification.

Experimental details and characterization data for imidazotetrazines **13** and imidazotriazenes **14** are given in the ESI.

Crystallography

Details of X-ray crystallography are given in the ESI.

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NMR Kinetics

^{1}H NMR kinetic experiments were performed on a Jeol ECA600 instrument observing ^{1}H at 600.1723 MHz. Data were processed using Delta NMR software (5.0.5, Jeol Resonance Ltd). Phosphate buffer (200 mM in D_2O) was prepared by mixing solutions of NaH_2PO_4 (0.138 g in 5 mL D_2O) and Na_2HPO_4 (0.178 g in 5 mL D_2O) with monitoring by a recently calibrated pH meter until a reading of pD=7.000 was obtained. Imidazotetrazines were dissolved in d_6-DMSO (10 mM).

In a typical NMR experiment, buffer (0.7 mL) was placed in an NMR tube, equilibrated at 37 °C in the probe for 10 min and the magnet shimmed. The tube was ejected, imidazotetrazine stock (200 µL) added, mixed thoroughly and returned to the spectrometer. Shims were adjusted and spectra recorded every 15 min for 12–18 h. Spectra were acquired with 64 transients, sweep width 15 ppm and digitised to 65 K data points. The residual H_2O/HOD peak was presaturated during the relaxation delay (5 s). Data were zero-filled once and an exponential window function (0.3–1.0 Hz) was applied during Fourier transformation. Artefacts due to water suppression were removed digitally. At the end of reaction, a further high resolution spectrum was acquired and peaks identified by spiking with small samples of authentic materials (AIC, HCHO, Figures S3, S5, ESI).

Anti-proliferative assays

HCT 116 cells were purchased from the American Type Tissue collection

Stock solutions of TMZ and other compounds listed in Table 1 were prepared in DMSO (100 mM) and aliquots stored at –20 °C, protected from light. SNB19V, SNB19M, U373V and U373M were originally obtained from Schering-Plough Research Institute, USA and were verified as being mycoplasma free. Cells were maintained in RPMI 1640 nutrient medium supplemented with 10% foetal bovine serum (FBS), sub-cultivated twice weekly and incubated at 37 °C in an atmosphere containing 5% CO_2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed as reported previously.[8,9] Compound concentration required to inhibit 50% growth (GI_{50} / µM) were calculated using non-linear regression analysis.

Conflicts of interest

DC, MGH, JZ and HSS were former employees of Pharminox Ltd; TDB and MFGS were consultants to, and MFGS a shareholder of, Pharminox Ltd.

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The synthesis and biological evaluation of imidazotetrazines substituted at N-3 is described.