European Journal of Medicinal Chemistry 257 (2023) 115507

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: www.elsevier.com/locate/ejmech

Research paper

Discovery of new imidazotetrazinones with potential to overcome tumor resistance

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ARTICLE INFO

Keywords:
Nitrogen heterocycles
Imidazotetrazinones
Tumor growth inhibition of glioblastoma
DMPK studies

ABSTRACT

We describe the design, organic synthesis, and characterization, including X-ray crystallography, of a series of novel analogues of the clinically used antitumor agent temozolomide, together with their *in vitro* biological evaluation. The work has resulted in the discovery of a new series of anticancer imidazotetrazines that offer the potential to overcome the resistance mounted by tumors against temozolomide. The rationally designed compounds that incorporate a propargyl alkylating moiety and a thiazole ring as isosteric replacement for a carboxamide, are readily synthesized (gram-scale), exhibit defined solid-state structures, and enhanced growth-inhibitory activity against human tumor cell lines, including MGMT-expressing and MMR-deficient lines, molecular features that confer tumor resistance. The cell proliferation data were confirmed by clonogenic cell survival assays, and DNA flow cytometry analysis was undertaken to determine the effects of new analogues on cell cycle progression. Detailed ^{1}H NMR spectroscopic studies showed that the new agents are stable in solution, and confirmed their mechanism of action. The propargyl and thiazole substituents significantly improve potency and physicochemical, drug metabolism and permeability properties, suggesting that the thiazole **13** should be prioritized for further preclinical evaluation.

1. Introduction

The importance of DNA repair was recognized by the award of the 2015 Nobel Prize for Chemistry to Lindahl, Sancar and Modrich who elucidated the mechanisms of base excision, nucleotide excision and mismatch repair respectively. Not only do these mechanisms play a crucial role in safeguarding genetic information, they also have profound influence on both the development of cancer and its treatment [1]. Many cancer deaths are a result of failure of therapy due to tumor resistance, and since many clinically used chemotherapeutic agents target DNA, a major factor in resistance is DNA repair. This is particularly true in the case of the DNA methylating agent temozolomide (TMZ, **1a**).

Originally prepared in the 1980s by one of us (MFGS), TMZ **1a** is a less toxic analogue of mitozolomide **1b**, a compound that entered clinical trial but was withdrawn due to severe and unpredictable toxicity [2,3]. TMZ was subsequently marketed in 1999 as first line treatment for glioblastoma multiforme (GBM), the most prevalent and aggressive primary brain tumor in adults, where median survival is generally less than one year from the time of diagnosis and the 5-year survival rate is only ~6% [4]. TMZ is a small, nitrogen-rich, hydrophilic heterocycle of the imidazotetrazine class [5,6], and has low molecular weight (194 Da) and clogP (−1.97). It is acid stable, orally bioavailable and able to cross the blood-brain barrier (BBB), and these robust pharmaceutical properties and freedom from metabolic liabilities have significantly contributed to its clinical success, allowing convenient oral delivery in an out-patient setting.

TMZ **1a** is a prodrug that is converted into a reactive DNA-alkylating agent via the unstable triazene, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide **2** (MTIC) that undergoes proteolysis to liberate 5-aminoimidazole-4-carboxamide (AIC, **3**) and a fugitive methyldiazonium cation **4** that methylates DNA at O-6, N-3 and N-7 in sequences of guanine residues (Scheme 1a) [7–9]. The functional integrity of DNA can be restored if the tumor-damaging 6-methoxyguanine DNA lesions **5** so formed are repaired by removal of the methyl group by a cysteine residue in the DNA repair protein O^{6}-methylguanine-DNA

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https://doi.org/10.1016/j.ejmech.2023.115507
Received 13 April 2023; Received in revised form 17 May 2023; Accepted 18 May 2023
Available online 21 May 2023
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methyltransferase (MGMT). In response to TMZ treatment, clinical outcomes against tumors where the *MGMT* gene is epigenetically silenced are more positive than those where the *MGMT* gene is switched on [10,11]. Other resistance pathways in GBM, particularly DNA mismatch repair (MMR) deficiency, where O^6-methylguanine is tolerated, and ATP-binding cassette-mediated (P-glycoprotein) BBB efflux (<20% brain distribution for TMZ is achieved), may further compromise the clinical utility of TMZ [12–14]. In addition, the half life of TMZ (<2 h at physiological pH) limits efficacy. Hence long term survival from this most malevolent of diseases is rare; median survival with standard of care treatment (surgery, radiotherapy and TMZ) is 16 months [15].

It is surprising that, despite being developed 40 years ago, TMZ remains the only drug of its class to have reached the market, despite continued efforts to develop analogues [16]. Hence there remains an urgent need to circumvent the clinical resistance mounted against TMZ by tumors, while maintaining, or improving, potency and physicochemical properties (low MW, clogP, and polar surface area) suitable for oral administration. Additionally, a broader spectrum of activity than TMZ itself would be desirable, particularly in MMR deficient tumors. We now report the rational design and chemical synthesis of such a series of imidazotetrazines that meets these stringent requirements, together with a study of their solid-state structures, solution stability, physicochemical properties and *in vitro* evaluation. Our data suggest that an analogue of TMZ worthy of detailed pre-clinical evaluation has been identified.

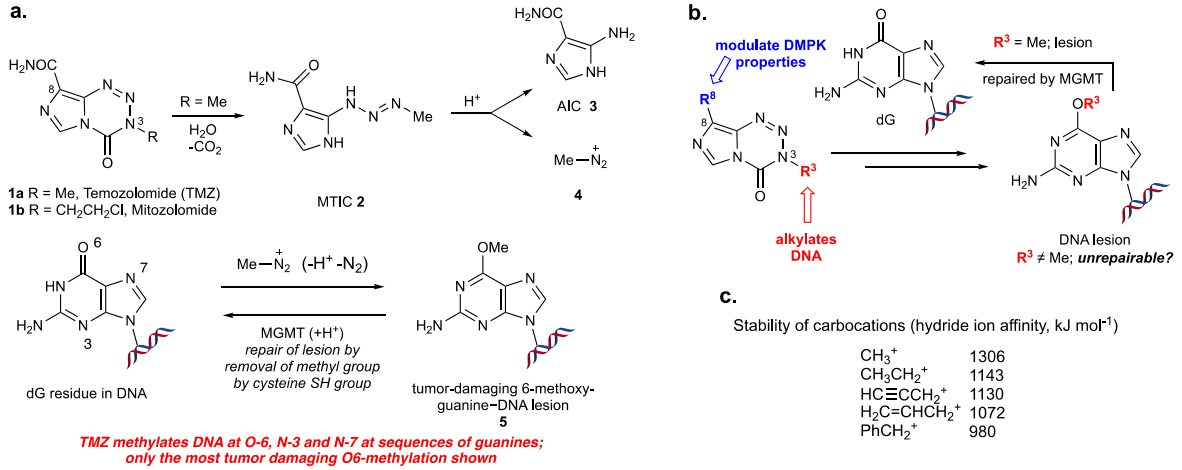
2. Results and discussion

Compound design and rationale. Consideration of the structure of TMZ reveals two positions for chemical manipulation, N-3 and C-8, although it is the group at N-3 that is critical for biological activity. Therefore to overcome tumor resistance to TMZ, we reasoned that an alternative to the N-3-methyl group capable of producing a cytotoxic lesion on DNA, but, crucially, unrepairable by MGMT was needed (Scheme 1b). A key consideration is the mechanism of action of TMZ that leads to a methyldiazonium cation, the precursor of the methyl carbocation (Scheme 1a), an extremely reactive alkylating species. Recognizing the need to retain carbocation reactivity without steric encumbrance, we considered precursors for a range of primary carbocations, RCH_2^+. A common measure of carbocation stability, albeit in the gas phase, is hydride ion affinity (HIA), defined as ΔH^o for $RH \rightarrow R^+ + H^-$, i.e. the heterolytic bond dissociation energy for hydride. The HIA values for exemplar primary carbocations are shown in Scheme 1c [17], where it can be seen that, as expected, the methyl carbocation is the least stable/most reactive, whereas the benzyl carbocation is much more stable, and too unreactive to be considered. On this basis, we were attracted to the propargyl group, whose carbocation is substantially less stable than the allyl cation, a direct result of the inductive electron withdrawing effect of the *sp*-hybridized alkyne, and comparable in stability to a simple ethyl group [17].

Supporting evidence for the choice of the propargyl group, that has the added benefit of being sterically undemanding, comes from the preliminary evaluation of a number of analogues across a panel of two TMZ-sensitive GBM-derived cell lines and two resistant *MGMT*-transfected lines [18,19]. Thus the N-3-propargyl imidazotetrazine **7** was potently inhibitory, whereas the allyl and benzyl analogues were considerably less active. Significantly the data suggest that **7**, through its ring-opened triazene derivative **8**, propargylates plasmid pBR322 DNA at runs of three to five guanines, that cannot be repaired by MGMT and is unaffected by the cellular MMR status [18]. These observations give confidence to the conclusion that mechanism of action of *N*-propargyl imidazotetrazines is similar to the *N*-methyl derivatives such as TMZ.

Although analysis of carbocation stability and preliminary biological data led us to select propargyl as a prioritized substituent at N-3, we recognized that such a group is not a common motif in medicinal chemistry as it is considered a metabolic liability. However, encouraged by the clinical success of the *N*-propargyl containing monoamine oxidase-B inhibitors selegiline and rasagiline, we turned our attention to the C-8 substituent, which has no function in the mechanism of action of TMZ, but can influence the stability of the intact 3-methylimidazotetrazine prodrug to generate more effective levels of methylating species, as tentatively explained by others [20]. Indeed, in earlier work we have shown that inclusion of an imidazole-2-yl substituent at C-8 can partly thwart MGMT- and MMR-mediated resistance [21]. Hence, we elected to make an isosteric replacement of the polar C-8 carboxamide with a monocyclic heterocyclic fragment [22], specifically the thiazole moiety, an established tactic in medicinal chemistry to lower the polar surface area (PSA) [23,24], with a new series of 8-thiazolyl-imidazotetrazines, for comparison with TMZ **1a** and its 8-carbamoyl-3-propargyl congener **7**.

Chemical synthesis. The 3-propargylimidazotetrazinone **7** was prepared by the Stone synthesis [25] from 5-diazoimidazole-4-carboxamide **6** and propargyl isocyanate in DMSO at 25 °C in an optimized 67% yield. This reaction could be accomplished on a 10 g scale, with high-purity product liberated from the reaction mixture on addition of water. Attempts to convert carboxamide **7** into thioamide **9** using Lawesson's reagent were unsuccessful with unreacted starting material recovered. However, reacting **7** with phosphorus pentasulfide in dichloromethane



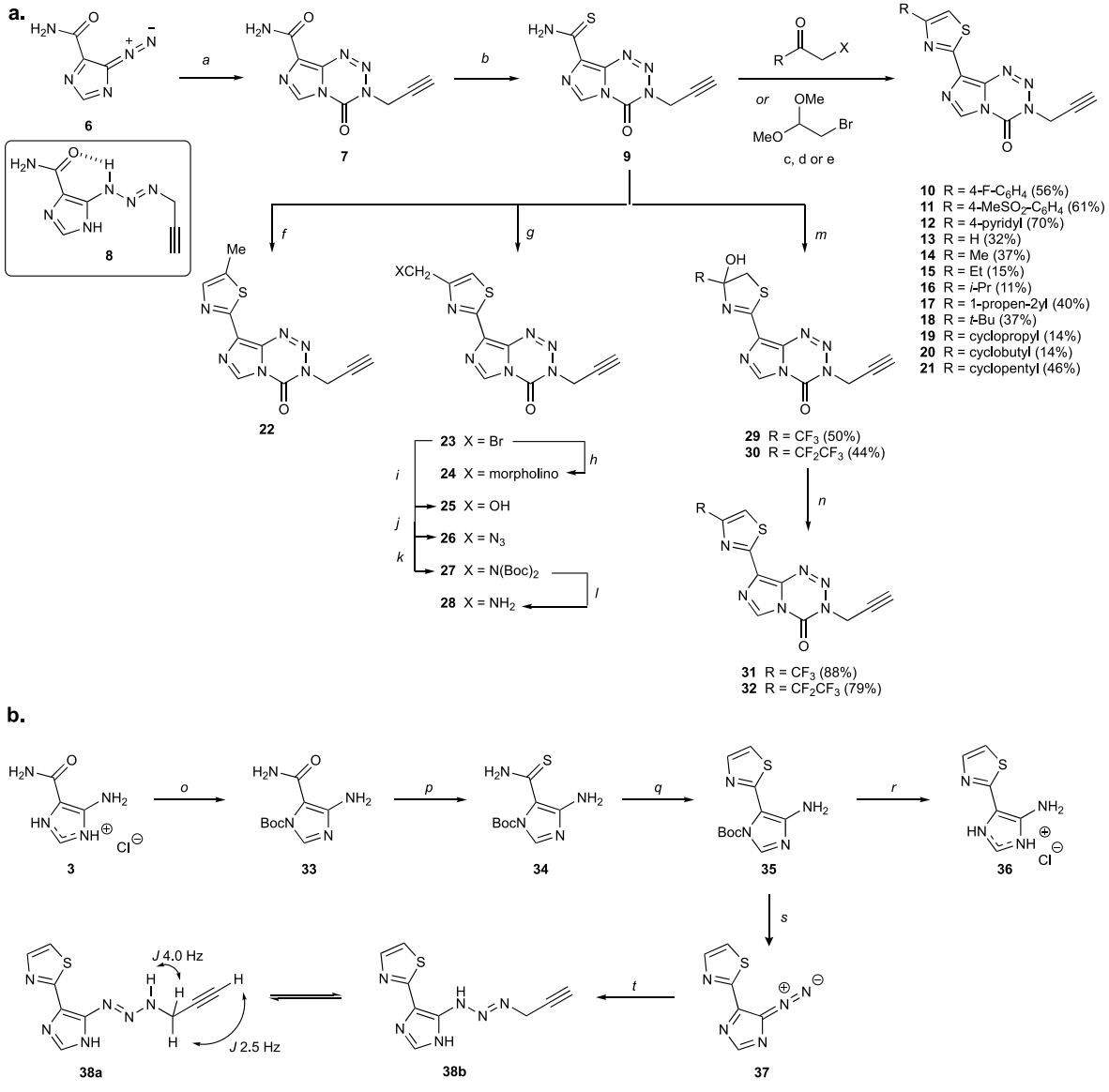
Scheme 1. **a**. Methylation of guanine residues of DNA by TMZ **1a**, and repair of the tumor-damaging 6-methoxyguanine DNA lesion **5** by the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT). **b**. Imidazotetrazine structure indicating role of N-3 and C-8 substituents. **c**. Alkylating ability of R^3-group as indicated by carbocation stability.

containing hexamethyldisiloxane [26] gave thioamide **9** in 84% yield after chromatography. In trial experiments, standard Hantzsch cyclizations [27] of **9** with representative aryl- and pyridyl-substituted bromomethylketones afforded the (thiazol-2-yl)-substituted imidazotetrazines **10**–**12** in moderate yields (56–70%) (Scheme 2a). However, to meet our target molecular weight of <300 Da, we focused on thiazoles bearing small substituents. Synthesis of the unsubstituted thiazole **13** was atypical in that thioamide **9** was reacted with bromoacetaldehyde dimethyl acetal under acidic conditions in acetone at 50 °C to liberate the free aldehyde. α-Chloroacetone was employed to furnish the methyl analogue **14** whereas synthesis of congeners **15**–**21** employed α-bromoketones. Overall these Hantzsch syntheses with less electrophilic haloketones proceeded in only low to moderate yields (11–46%). A single example of a 5-substituted thiazole **22** was furnished from thiocarboxamide **9** and 2-bromopropionaldehyde (Scheme 2a).

Reaction between **9** and 1,3-dibromoacetone afforded the 4-bromomethylthiazole **23**, which proved to be a useful substrate for further elaboration, and gave rise to a series of compounds **24**–**28** as shown in Scheme 2a, although aminomethyl **28** proved to be too unstable and unsuitable for further study.

Conditions to obtain thiazoles substituted at the 4-position with fluoroalkyl groups required modification as Hantzsch reactions between **9** and 3-bromo-1,1,1-trifluoroacetone or 1-bromo-4,4,5,5,5-pentafluorobutan-2-one produced the stable covalent hydrates **29** and **30**, that required dehydrating conditions (trifluoroacetic anhydride/triethylamine) to give the desired thiazoles **31** and **32** in 88 and 79% yields, respectively (Scheme 2a).

Given the importance of the ring-opened MTIC **2** in the mechanism of action of TMZ **1a**, and based on the promising activity of the thiazole analogue **13** (*q.v.*), it was imperative to access the corresponding



Scheme 2. a. Hantzsch synthesis of 8-(thiazol-2-yl)imidazotetrazines. *Reagents and conditions*: (a) HC ≡ $CCH_2N{=}C{=}O$, DMSO, 25 °C, 24 h, (67%); (b) P_4S_{10}, $(Me_3Si)_2O$, CH_2Cl_2, reflux, 16 h, (84%); (c) $RCOCH_2Br$, MeCN, 25 °C, 17–66 h, (11–70%); (d) $(MeO)_2CHCH_2Br$, HCl, dioxan, acetone, 50 °C, 5 h, 32%; (e) $RCOCH_2Cl$, MeCN, 25 °C, 48 h, (37%); (f) MeCHBrCHO, acetone, reflux, 16 h, (25%); (g) 1,3-dibromoacetone, MeCN, 25 °C, 17 h (40%); (h) morpholine, Et_3N, THF, 25 °C, 2 h (73%); (i) $AgOCOCF_3$, wet DMF, 25 °C, 6 h (60%); (j) NaN_3, DMF, 50 °C, 18 h (22%); (k), NaH, $HN(Boc)_2$, MeCN, 25 °C, 19 h (65%); (l) 4 M HCl in dioxan, 25 °C (impure); (m) 1-bromo-1,1,1-trifluoropropanone (for **29**) or 1-bromo-3,3,4,4,4-pentafluorobutan-2-one (for **30**), MeCN, 50 °C, 4 h; (n) $(CF_3CO)_2O$, Et_3N, MeCN, 0 °C, 2 h. **b.** Synthesis of 5-(3-propargyltriazen-1-yl)-4-(thiazol-2-yl)imidazole. *Reagents and conditions*: (o) Boc_2O, Et_3N, CH_2Cl_2, 25 °C, 15 h (98%); (p) Lawesson's reagent, THF, reflux, 15 h (67%); (q) $BrCH_2CHO$, K_2CO_3, DMF, 60 °C, 2 h, then Boc_2O, 2 h (30%); (r) 4 M HCl in dioxan, 2 h (quant); (s) aq HCl (2 M), 10 min, then aq $NaNO_2$, 0 °C, 30 min, then 25 °C, 20 min (quant); (t) propargylamine, EtOAc, 25 °C, 1.5 h (50%).

propargyl-thiazolyl triazene **38**. However, attempts to effect a controlled ring-opening of the imidazotetrazinone **13** to the triazene in aqueous $NaHCO_3$, an approach which had been successful in the efficient conversion of TMZ **1a** to MTIC **2** [25], led to a purple solution in which no triazene could be detected (LC-MS). Instead, the route outlined in Scheme 2b was employed and gave the required triazene **38** in a highly pure state (>99% by LC-MS). The propargyl CH_2 group occurs as a doublet of doublets in the ^{1}H NMR spectrum with coupling to both the terminal acetylenic proton ($J = 2.5$ Hz) and the NH proton ($J = 4.0$ Hz) indicating that the propargylamino-tautomer **38a** is preferred over the propargylazo-tautomer **38b** in DMSO. This is in contrast to carboxamide containing triazenes such as **2** and **8** that exist as the intramolecular H-bonded alkylazo-tautomers as indicated for triazene **2** in Scheme 1a [25].

Solid-state structures. The study of crystal structures of potential drug candidate molecules by X-ray crystallography not only provides structural confirmation but also the detail of their solid-state structures. This includes valuable information about the packing of molecules in the crystal lattice, and the intermolecular forces that determine such packing, factors that will likely influence the solubility of the molecule in aqueous media. The propargylimidazotetrazines tend to be crystalline solids and therefore amenable to crystallographic study, hence providing structural confirmation for 3-propargylimidazotetrazine **7** and the 8-thiazolylimidazotetrazines **13** and **14** (Supplementary Data Figures S1-S3). The studies also reveal that the 3-propargylimidazotetrazine carboxamide **7** displays the characteristic network of intermolecular H-bonds linking carboxamide groups of four molecules (Fig. 1a) that are a feature of published X-ray structures of 8-carbamoyl-imidazotetrazines [28,29]. This templating promotes the propargyl groups to stack in an ordered ladder-like arrangement. In addition, the presence of a hydrogen bond between the alkyne-H and the C-4 carbonyl oxygen (2.3 Å) was observed (Fig. 1b). The loss of the dominating effect of a carboxamide group was evident in the crystal structure of the thiazole derivative **14**, although the ordering of the propargyl groups remains (Fig. 1c). The alkyne hydrogen makes close intermolecular contacts to a thiazole sulfur (3.17 Å) and to N-7 (2.79 Å). The center-center distance of the stacked tetrazine rings is 4.64 Å.

Thiazole and propargyl substituents increase stability of imidazotetrazinones in solution: NMR spectroscopic studies. Determination of the half-life of drug candidates at different pHs aids in the understanding how they will behave in the body. ^{1}H NMR spectroscopy was ideal for this study as it would monitor the decomposition/stability, while also allowing the possible identification of decomposition products.

As reported previously [7,8], the decomposition of TMZ **1a** in aqueous solution is very simple with only two major products AIC **3** and methanol identified at completion. Scrutiny of ^{1}H NMR spectra of 3-propargylimidazotetrazine **7** showed that decomposition in phosphate buffer at pH 7.4 was considerably more complex (Supplementary Data Figure S4). Disappearance of signals for **7** were replaced with signals for the ring-opened triazene **8** and for minor amounts of AIC **3** and propargyl alcohol and the generation of several uncharacterized propargylated imidazole species. Surprisingly, the triazene **8** had far superior stability compared to that of MTIC **2**, reaching a maximum concentration after 2–3 h and displaying a $t_{1/2}$ of 4.5 h.

Introduction of the thiazole moiety to the C-8 position of 3-propargylimidazotetrazines significantly increased stability of these analogues compared to the carboxamide **7**. However, decompositions of the thiazole derivatives **13** and **14** at pH 7.4 and 5.5 showed no build-up of signals for the respective triazenes, suggesting that either the imidazotetrazines are indeed very stable, and do not readily break down, or that the triazene intermediates were less stable (i.e. more reactive) than triazene **8**. Spectra recording the degradation of the thiazoles **13** and **14**

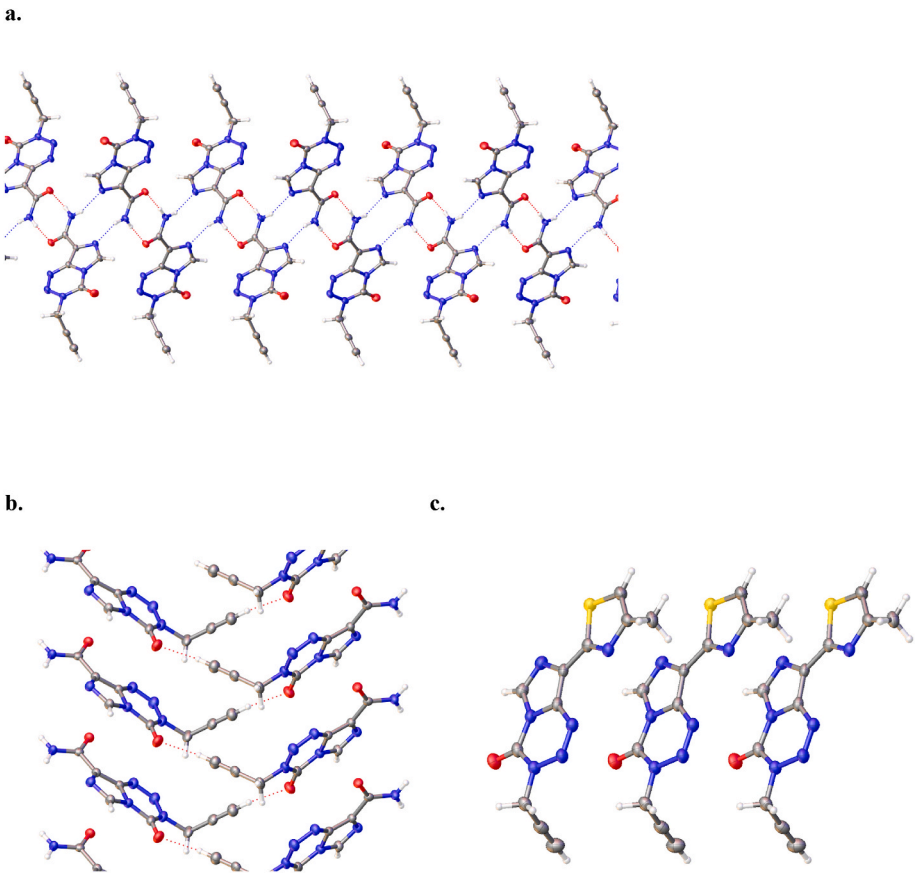


Fig. 1. X-Ray crystal structures of 3-propargylimidazotetrazinones, showing **a**, the network of hydrogen bonds in the 8-carboxamide **7** (CCDC 2035205), **b**, the ordering of the propargyl groups in the carboxamide **7** and **c**, the thiazole **14** (CCDC 2035208).

were complex indicating the formation of multiple propargylated imidazoles (Supplementary Data Figures S5, S6). Application of HRMS at the end-point of the decomposition of **14** in water containing D_2O showed, *inter alia*, major peak masses at *m/z* 181.0543 and 220.0762 corresponding to the thiazole-substituted aminoimidazole **39** and a (deuterated) propargylated aminoimidazole **40** (or multiple isomers thereof) respectively (Fig. 2). Following identification of these fragments, re-scrutiny of the NMR spectra identified several peak patterns in common. For example, in the spectrum (in H_2O) of the 4-methylthiazole **14** (Supplementary Data Figure S7), there were two double doublets consistent with a diastereotopic CH_2 of the propargyl group attached to a carbon chiral center. We propose, tentatively, that the peak *m/z* 220 is produced by the 4-amino-4-(deuterated) propargylimidazole **41**. There are reports in the literature of the synthesis of C-4 alkylated imidazoles of a similar substitution pattern [30,31].

Thus, the above studies imply that, like propargyl-carboxamide **7**, a compound known to generate a DNA-damaging alkylating species [18], the proposed degradation of propargyl-thiazole **14** (and by extension analogue **13**) proceeds via a triazene intermediate that cleaves to liberate an alkylating species, the propargyl cation, and an aminoimidazole as we had previously found for the 8-carboxamido compound **7** [18].

Thiazole and propargyl substituents improve in vitro potency of imidazotetrazinones including against TMZ resistant cell lines. All the novel 3-propargylimidazotetrazines (>95% purity by LC-MS (Supplementary Data) were screened *in vitro* against human derived glioblastoma (U373) and colorectal (HCT 116) cell lines and their activity determined by the colorimetric MTT assay. HCT 116 colorectal tumors are MGMT+ and MMR deficient and hence are a good *in vitro* model for resistance to TMZ conferred by O^6-methylguanine removal or tolerance. The full set of data is shown in Table 1. In line with previously published results [5,12,13], the human GBM U373V (vector control) line was relatively sensitive to TMZ **1a** and gave a GI_{50} value of 51.9 μM whereas the isogenic line (U373 M) transfected with *MGMT* showed an approximately 6-fold resistance to TMZ with a GI_{50} value of 302 μM. 3-Propargylimidazotetrazine **7** showed inhibitory activity against both the U373V and U373 M and MMR-deficient HCT 116 cell lines (GI_{50} ~30 μM), confirming earlier work that this agent overcomes the principle resistance mechanisms involving expression of MGMT and MMR loss [18,22,32], All the 8-thiazolyl-imidazotetrazines showed enhanced potency compared to both TMZ **1a** and **7** against both U373 and HCT 116 cell lines. However, within this rich seam of activity, there was a general trend towards improved potency in compounds with smaller substituents at the 4-position of the thiazole ring especially against the TMZ-resistant, MGMT-expressing U373 M cell line. Thus 4-alkyl-substituted thiazoles were more inhibitory than 4-aryl thiazoles, and within the 4-alkyl derivatives, the potency decreased as the size of the alkyl group increased through the series Me < Et < $MeCH{=}CH_2$ < *i*-Pr < *t*-Bu. Replacement of the methyl and ethyl substituents with their perfluorinated analogues CF_3 and CF_2CF_3 had a deleterious effect on potency. We also note that the position of methyl substitution in the thiazole ring is not critical because both the 4-methylthiazole **14** and its 5-methylthiazole isomer **22** were approximately equiactive across the three cell lines (Table 1). The unsubstituted thiazole **13** and its 4-methyl analogue **14** were the most inhibitory compounds overall and were appreciably more active than **1a** against the human colon cancer HCT 116 cell line, thereby extending the spectrum of activity, and were therefore prioritized.

The prioritized thiazolyl-substituted 3-propargylimidazotetrazines **13** and **14** were also tested against the normal fetal lung fibroblast cell line MRC-5 and showed GI_{50} values in a narrow range of 25–40 μM. Importantly, compound **13** demonstrated a 'selectivity' (GI_{50} for MRC-5/GI_{50} for HCT 116) of >5, superior to that of 1.4 for TMZ.

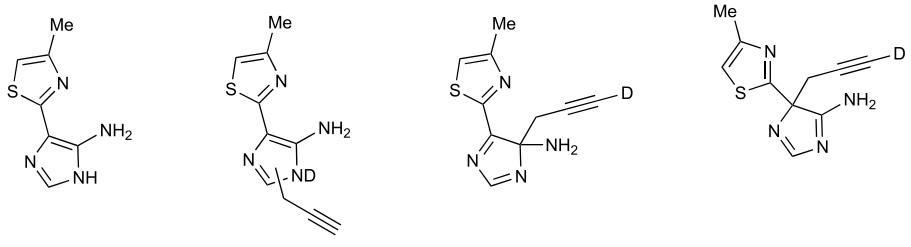
Significantly, the inhibitory activity of the triazene **38**, the putative active form of prodrug **13**, closely matched that of **13**, whereas the thiazole-substituted aminoimidazole, tested as the imidazolium chloride salt **36**, one of the final products of the activation pathway, was of low potency and unlikely to play any biological role.

Corroborative clonogenic assay data were obtained consolidating the thesis that the prioritized C-8 thiazole analogues overcome MGMT repair and MMR deficiency. Assays were conducted to compare the ability of U373V, U373 M and HCT 116 cells to survive brief challenge to test agents, retain proliferative capacity and form progeny colonies. TMZ **1** inhibited only U373V colony formation (at 20 and 50 μM) (Fig. 3); strikingly, compound **13** (≥10 μM) virtually eradicated clonogenic survival in the 3 cell lines tested (Fig. 3).

DNA flow cytometry analysis was undertaken to determine the effects of new thiazole analogues on cell cycle progression in isogenic U373V, U373 M (MGMT-transfected) GBM and HCT 116 (MMR-deficient) colorectal carcinoma cell lines. Cells were treated with TMZ **1** (100 μM), **7** (100 μM), and thiazole **13** (20 μM), and cell cycle was determined after 72 and 120 h exposure. Untreated cells were also analysed at each time point as a control. Representative profiles following 120 h exposure are shown in Fig. 4. Following exposure of cells to TMZ **1**, significant perturbation (G2/M accumulation) was observed in U373V populations only (Fig. 4b), consistent with accumulation of O^6-methylguanine lesions. In addition, some pre-G1 events were detected in U373V cells, consistent with a small apoptotic population (9% compared to 1% in control). It has been identified that GBM is largely resistant to apoptosis [33]. Evasion of apoptosis is a cancer hallmark described initially in the seminal paper of 2000 by Hanahan and Weinberg [34]; imidazotetrazines have been shown to exert autophagic cell death [13], yet the observation of some pre-G events indicates a level of apoptotic activity. Neither U373 M nor HCT 116 incurred cell cycle perturbation following treatment with TMZ, consistent with repair or tolerance of O^6-methylguanine lesions respectively.

The *N*-3-propargyl analogue **7** evoked substantial S and G2/M accumulation in both U373V and M GBM cells (33% G2/M U373 M, compared to ~15% control), again some sub-G1 (apoptotic) events were evident. These observations support the thesis that the DNA damage inflicted by propargylation at runs of guanine [18] remains unrepaired by MGMT. In HCT 116 populations exposed to **7**, cell cycle profile integrity was lost completely, most (96% at 120 h, compared with ~5% control) events appear in sub-G1 suggesting intolerance to propargyl lesions and catastrophic apoptosis.

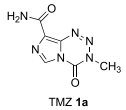
In MMR-deficient HCT 116, as exemplified by thiazole **13** in Fig. 4d,



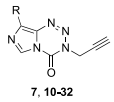
39 **40** **41** **42**

Fig. 2. Possible structures of decomposition products of 3-propargyl-8-(4-methylthiazol-2-yl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one 14.

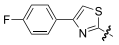
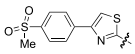
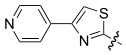
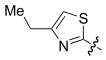
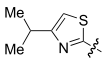
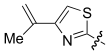
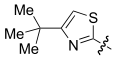
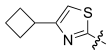
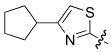
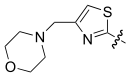
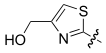
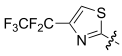
Table 1
In vitro growth inhibitory activity of N-3-propargyl-C-8 substituted thiazolyl imidazotetrazines and reference compounds against human derived glioblastoma and colorectal cell lines as determined by 7-day MTT assay.



TMZ **1a**



7, **10-32**

Compound number	R	GI_{50} (μM)		
		U373V [a]	U373 M [b]	HCT 116 [c]
TMZ 1a	–	51.9 ± 7.4	302 ± 56	291 ± 4.9
7		30.0 ± 2.3	26.6 ± 4.1	28.1 ± 0.64
10		27.9 ± 2.0	19.7 ± 2.6	27.6 ± 4.7
11		22.0 ± 3.2	14.7 ± 7.6	37.0 ± 5.2
12		23.5 ± 4.0	25.1 ± 0.91	29.0 ± 3.2
13		3.59 ± 1.4	4.09 ± 0.27	5.35 ± 2.1
14		8.45 ± 0.40	7.44 ± 0.78	7.04 ± 0.02
15		23.5 ± 2.3	9.67 ± 1.4	14.8 ± 8.8
16		23.6 ± 0.64	19.0 ± 6.3	25.4 ± 0.14
17		18.7 ± 0.64	14.2 ± 7.8	16.7 ± 8.8
18		27.3 ± 2.4	19.2 ± 5.0	25.1 ± 1.7
19		27.1 ± 0.15	25.8 ± 3.7	27.7 ± 2.1
20		24.2 ± 4.0	25.5 ± 1.6	20.3 ± 3.0
21		23.7 ± 4.8	11.8 ± 4.9	19.2 ± 4.5
22		6.84 ± 2.1	3.99 ± 1.36	4.60 ± 0.28
24		23.9 ± 5.7	10.2 ± 3.1	12.2 ± 5.6
25		24.4 ± 3.0	14.8 ± 0.49	17.8 ± 0.92
31		28.2 ± 1.6	25.4 ± 0.66	21.5 ± 1.2
32		27.1 ± 0.15	25.8 ± 3.7	27.7 ± 2.1
36	–	>100	89.3 ± 18	97.0 ± 2.9
38	–	7.51 ± 0.72	5.81 ± 2.9	5.97 ± 0.02

[a] U373V glioblastoma vector control.
[b] U373 M glioblastoma MGMT-transfected.
[c] HCT 116 colorectal MMR deficient. Results are expressed as the mean of >3 experiments (n = 5 per experiment) ± SD of >3 independent experiments. Compounds were made up to a 50 or 10 mM stock solution in DMSO (stored at −20 °C) and diluted in culture medium prior to treatment of cells.

apoptosis was again triggered (>60% events in the pre-G1 population at 120 h, compared to ~5% control). In U373 M, despite MGMT expression, G2/M accumulation (21%) and decreased G1 (43% compared to 66% control) events suggest cells are not able to pass through the G2/M checkpoint, possibly due to recognition of DNA damage (alkylation). In addition, small sub-G1 populations were evident, consistent with lethal DNA damage accumulation.

In summary, TMZ exerted no influence on the cell cycle of TMZ-resistant cell lines HCT 116 and U373 M. All N-3 propargyl analogues induced G2/M accumulation in GBM cell lines after 120 h, although this was more pronounced in U373 M. MMR-deficient HCT 116 cells displayed evidence of high levels of apoptosis after treatment with the N-3 propargyl analogues. These data strongly suggest that propargyl and methyl lesions are processed by cancer cells distinctly, and propargylation is neither tolerated in MMR-deficient cells, nor removed by MGMT.

Thiazole and propargyl substituents improve physicochemical and drug metabolism and pharmacokinetic (DMPK) properties of imidazotetrazinones. We also evaluated the physicochemical and DMPK properties of the prioritized 8-thiazolylimidazotetrazines **13** and **14**. Examination of physicochemical properties suggests that the new imidazotetrazines might, like TMZ, achieve blood brain barrier (BBB) penetration. They have molecular weights in the range 250–275 Da, with clogP <1.0. Clinically used drugs that penetrate the BBB have a mean molecular weight of 310 (compared with 377 for all marketed orally available compounds), and a mean clogP of 2.5 [35], suggesting that the thiazolylimidazotetrazinones are well within the required range. Additionally, polar surface area (PSA in $Å^2$) has been used to predict efficient BBB passage, overcoming ATP-binding cassette (ABC) proteins, including P-glycoprotein (P-gp), expressed on BBB endothelia and glioma cells [36], and is a measure of the molecular surface arising from polar atoms [37]. The upper 'limit' for PSA is considered to be 90 $Å^2$ and compounds **13** and **14** are within this range (Table 2) [38], although it should be noted that the value for TMZ (108 $Å^2$), which is known by PET studies to access brain tumors in clinical studies [39], exceeds this 'limit'.

In the present study, the half-life ($t_{½}$) of TMZ as measured by ^{1}H NMR in phosphate buffer was 92 min at pH 7.4 and > 100 h at pH 5.5. Comparative $t_{½}$ values for other compounds under study are shown in Table 2 with compound **13** having the highest stability at both pH 7.4 ($t_{1/2}$ 180 min) and 5.5. Chemical stability studies were also undertaken in phosphate buffered solutions at various pH monitored by LC-MS. These results confirm the stability at acidic pH values suggesting that the thiazoles **13** and **14** could be suitable for oral delivery.

The compounds are much less stable in both human and rat plasma with TMZ **1a** possessing the longest half-life at 19.8 min in human plasma. While **13** and **14** have short half-lives, triazene **38** was monitored to have a half-life of 14.6 min. The apparent elimination half-life of TMZ in patients has been shown to be 1.8–1.9 h [40], while *in vitro* values in human plasma are reported as only 15 min [41], suggesting TMZ does not act in the same manner *in vitro* and *in vivo* possibly due to absorption rates into the bloodstream. Although the blood plasma stability profiles of the C-8 thiazole analogues are shorter than TMZ, NMR studies at both pH7.4 and 5.5 confirm that compounds **13** and **14** would be suitable for oral delivery.

Drug metabolism studies were carried out in human and rat liver microsomes to mimic phase I metabolism. The half-life of the drug was determined in the presence and absence of NADPH and the intrinsic clearance rate Cl_{int} calculated. Both TMZ and **7** showed a moderate intrinsic clearance rate both in the presence and absence of NADPH, indicating that they are most likely not metabolized in the liver by cytochrome P450 enzymes, although the data for TMZ was markedly different in human and rat microsomes. Of the thiazole-containing analogues, **14** had very high clearance rates (97.8 μL/min/mg) in NADPH+ experiments. In the absence of NADPH, the intrinsic clearance rate for **14** dropped significantly, suggesting that the compound is most likely hydroxylated by cytochrome P450 enzymes probably on the methyl group.

The parallel artificial membrane permeability assay (PAMPA) simulates passive diffusion across a membrane, reminiscent of the

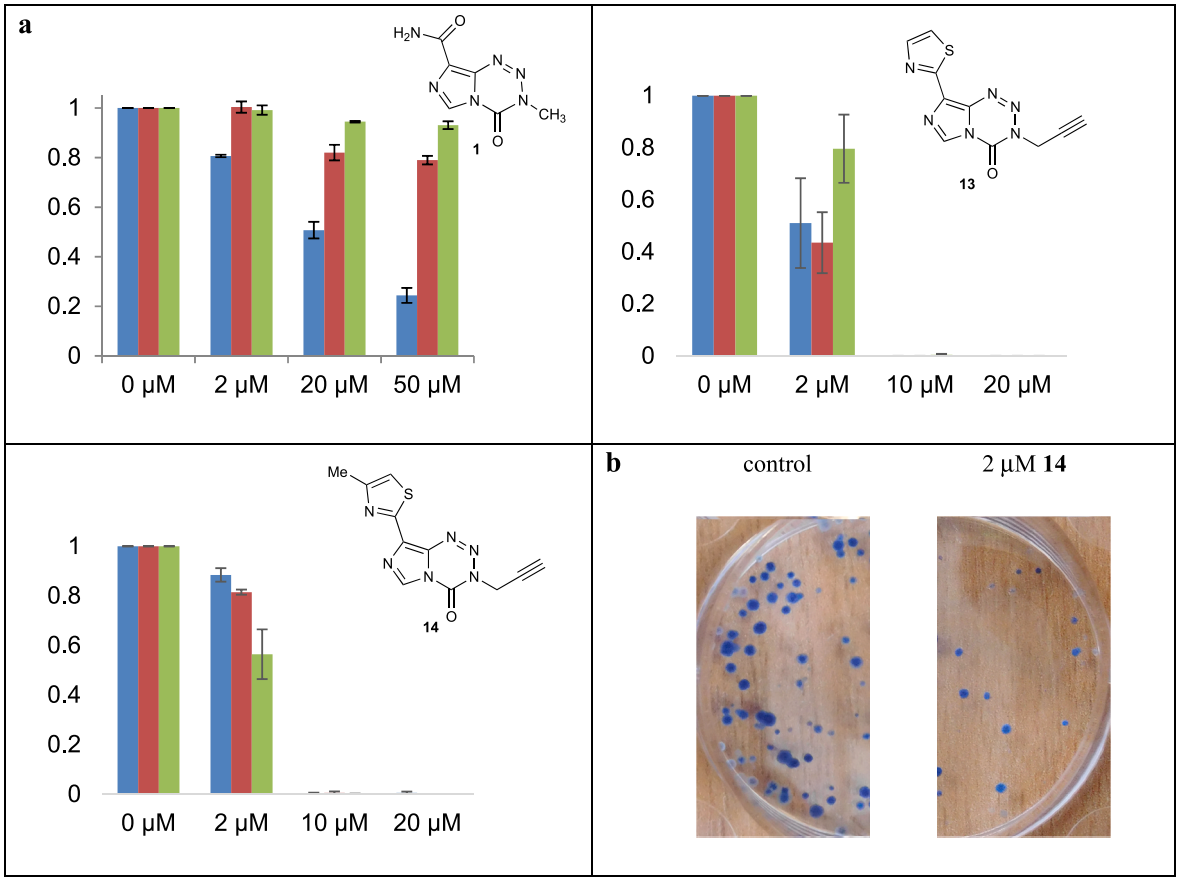


Fig. 3. Cell survival assays for TMZ **1** and thiazoles **13** and **14**. **a.** Cell survival ratios as determined from clonogenic assays in U373V (blue), U373 M (red) and HCT 116 (green) for TMZ **1** and thiazoles **13** and **14** with plating efficiencies of between 15 and 64%. Values are means ± SD of 2–3 individual experiments ($n = 3$ per experiment). Cells were seeded (400 per well) and exposed to test agent for 24 h; thereafter plates were incubated (37 °C; 5% CO_2) until colonies in control wells contained ≥50 cells. Colonies were fixed and stained as detailed in the Experimental Section. **b.** Representative control HCT 116 colonies and those formed following exposure of cells to 2 μM thiazole **14** are shown.

gastrointestinal tract and is used to determine oral availability. After incubation for 6 h, the permeability (P_e) and percentage recovery were determined. P_e values below 1.5×10^{-6} cm s^{-1} indicate poor permeability. Whilst TMZ has very poor permeability, the thiazoles **13** and **14** have excellent permeability at 5 and 6 times that of TMZ (Table 2).

To determine the ability of our compounds to pass through the BBB and if they are P-gp substrates, further permeability studies were undertaken in Madin-Darby canine kidney (MDCK) isogenic cell lines: wild-type (WT) and following stable transfection with the multidrug resistance-1 gene (MDR1). MDCK MDR1 cells, cultured as a monolayer, possess tight junctions between cells, replicating the BBB. The data show that not only is TMZ less likely to permeate the BBB, but it is also actively pumped out of the brain back into the blood by P-gp. The thiazole compounds **13** and **14** have far higher permeability (2–6 times TMZ) and are not P-gp substrates, therefore unlikely to be removed from the brain by this ABC protein pump.

To summarize these results, the thiazole-containing analogues showed superior *in vitro* DMPK properties over TMZ. Not only do **13** and **14** have improved permeability properties, but they also possess the desired chemical stability profiles required for orally available drugs. The difference between these two compounds was in the microsomal metabolic stability assay where it was observed that **14** undergoes phase 1 metabolism by cytochrome P450 enzymes, most likely by hydroxylation in the liver. Therefore, thiazole **13** was selected as the lead compound for further evaluation.

Conclusions. In conclusion we have revealed new work that has opened an innovative front in the imidazotetrazine story, offering the potential to counter the ability of GBM and other tumors to mount resistance towards the clinically used agent temozolomide. Rational consideration of the mechanism of action resulted in replacing the substituents at the 3-methyl and 8-carboxamide positions of TMZ with a propargyl group and a non-acidic thiazole residue, respectively, and is rewarded by enhanced growth-inhibitory activity of new compounds against three human tumor cell lines. Notably, this enhanced activity is observed against MGMT-expressing GBM and MMR-deficient colorectal carcinoma lines, molecular features that confer clinical resistance to TMZ. Growth inhibitory and cytotoxic activity of C-8 thiazole-substituted imidazotetrazines (irrespective of MGMT expression or MMR proficiency) was confirmed by MTT and clonogenic assays, and corroborated by cell cycle perturbation consistent with DNA damage. Detailed ^{1}H NMR spectroscopic studies have confirmed that the new imidazotetrazines share a mechanism of action common to compounds of this class, undergoing ring-opening to active triazene counterparts.

Additionally, a suite of DMPK analyses including blood plasma and microsomal stability, PAMPA permeability have determined that thiazole **13** should be prioritized for *in vivo* proof of principle xenograft studies against tumor models expressing common clinical mechanisms of resistance to TMZ, i.e. MMR-deficiency and/or MGMT expression in order to confirm its status as an improved analogue of TMZ.

3. Experimental section

3.1. General experimental details

Commercially available reagents were used throughout without further purification. Anhydrous acetonitrile, methanol, acetone, ether,

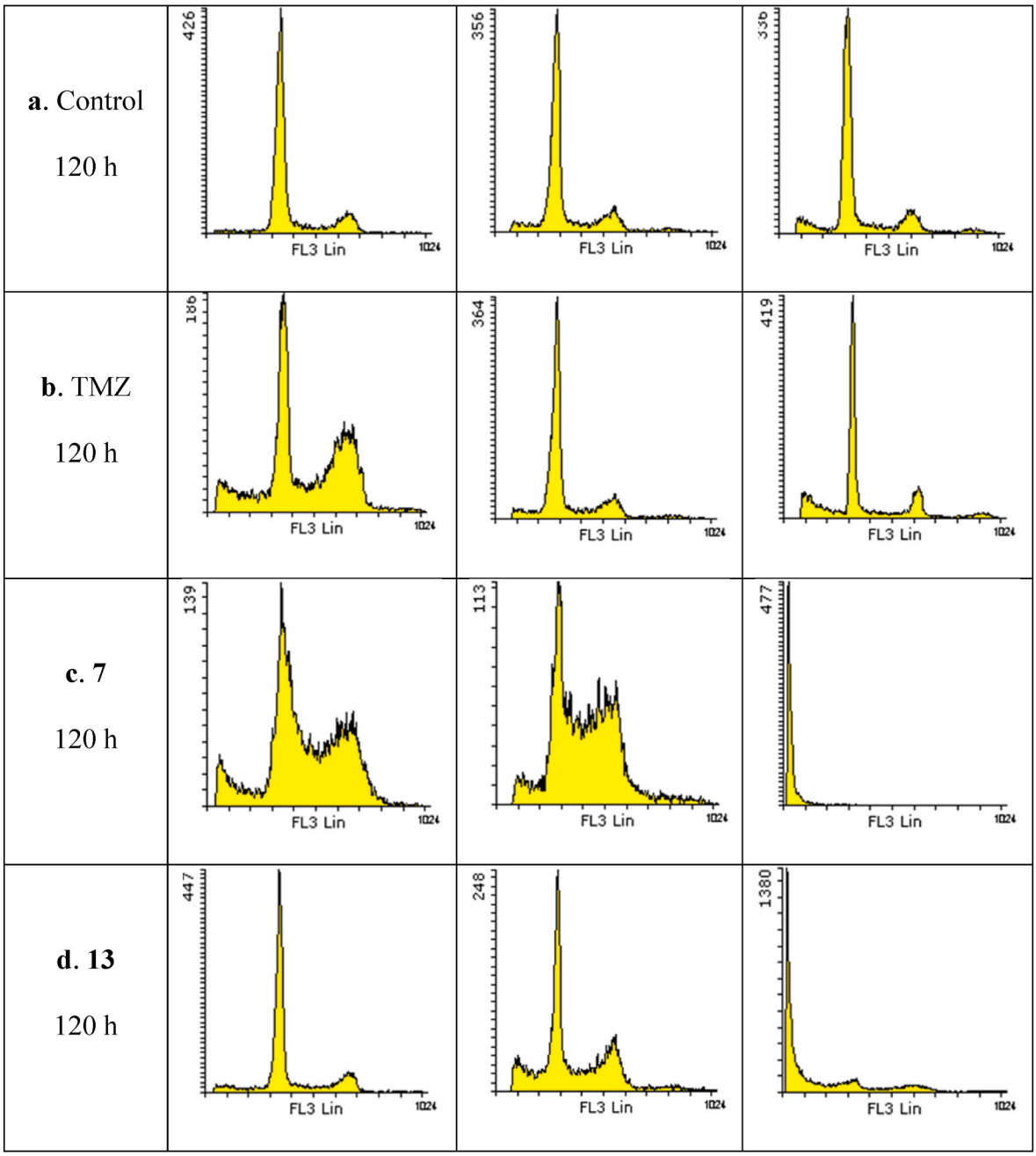


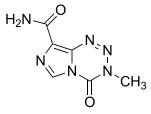
Fig. 4. Representative DNA histograms of U373V, U373 M and HCT 116 cells to show the effect on cell cycle progression after 120 h in **a**) control cells; **b**) TMZ **1**; **c**) **7**; **d**) **13**. DNA was stained with PI and the DNA content was determined by flow cytometry. X axis: DNA content, Y axis: cell count.

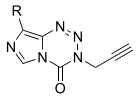
1,4-dioxane, DMF and DMSO were purchased from Acros Organics and used as supplied. Dichloromethane was distilled from calcium hydride under nitrogen and THF was distilled from benzophenone ketyl under nitrogen. Triethylamine was distilled from calcium hydride and stored under an argon atmosphere. Ether refers to diethyl ether, and light petroleum refers to the fraction with bp 40–60 °C. Reactions were carried out under an argon atmosphere if anhydrous conditions were required with oven-dried or flame-dried glassware. Thin layer chromatography was carried out using Merck aluminum-backed plates coated with silica gel 60 F_{254}. Visualization was under UV light at 360 nm and chemical staining including basic aqueous potassium permanganate solution, acidic ninhydrin *t*-butanol solution or acidic *p*-anisaldehyde solution. Column chromatography was carried out using Aldrich technical grade 60 Å 230–400 mesh silica gel, with the eluent indicated.

^{1}H and ^{13}C NMR spectra for compound characterization were recorded using a range of 400 and 500 MHz instruments (^{1}H frequencies, ^{13}C frequencies 100 and 125 MHz); ^{19}F NMR spectra were recorded using a 400 MHz instrument (^{19}F frequency 75 MHz); Bruker AV(III)500, Bruker AV(III)400, Bruker AV(III)400hd, Bruker AV400 and Bruker DPX400 at the given frequencies. Chemical shifts are quoted in parts per million (ppm), coupling constants, *J*, are quoted in Hz and all spectra were referenced using deuterated solvents. Multiplicity of peaks reported with the following abbreviations: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; p, pentet; h, heptet; and m, multiplet. MestReNova was used to reference and process NMR spectra. DEPT 90 and 135 experiments were used to assign ^{13}C, and complex structure elucidation was aided by COSY, HSQC and HMBC experiments where required.

High resolution mass spectra were recorded on a Bruker microTOF mass spectrometer with electrospray ionization (ESI). ATR solid phase

Table 2
Tumor cell growth inhibitory activity, physicochemical, and drug metabolism and pharmacokinetic (DMPK) properties of N-3-propargylimidazotetrazinones.


TMZ **1a**


7, 13, 14

In vitro Growth inhibitory activity against human cancer cell lines as determined by 7-day MTT assay

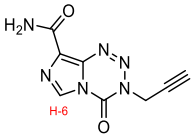
	–			
Cell line	**1a**	**7**	**13**	**14**
U373V[a] GI_{50} (μM)	51.9 ± 7.4	30.0 ± 2.3	3.59 ± 1.4	8.45 ± 0.40
U373 M[b] GI_{50} (μM)	302 ± 56	26.6 ± 4.1	4.09 ± 0.27	7.44 ± 0.78
HCT 116[c] GI_{50} (μM)	291 ± 4.9	28.1 ± 0.64	5.35 ± 2.1	7.04 ± 0.02
Physicochemical properties				
	–			
Property	**1a**	**7**	**13**	**14**
MW (Da)	194	218	258	272
clogP [d]	−1.97	−0.95	0.82	1.18
tPSA (Å^2)[e]	108	108	78	78
NMR t ½ pH7.4 (min)[f]	92	49	180	88
NMR t ½ pH5.5 (h)[f]	>100	–	64	7.2
LC-MS t ½ pH7.4 (min)[g]	148.0 ± 15.2	115.4 ± 4.3	157.7 ± 48.0	168.7 ± 22.6
LC-MS t ½ pH6.8 (min)[g]	241.3 ± 30.3	231.8 ± 36.7	327.6 ± 111.7	214.5 ± 5.7
LC-MS t ½ pH5.0 (min)[g]	>480	>480	>480	>480
DMPK properties				
	–			
Assay[h]	**1a**	**7**	**13**	**14**
t ½ human plasma (min)	19.8 ± 0.48	14.5 ± 1.4	3.34 ± 0.35	2.97 ± 0.35
t ½ rat plasma (min)	17.9 ± 1.2	25.9 ± 24.0	2.66 ± 0.06	3.54 ± 0.78
Human liver microsomes + NADPH; Cl_{int} (μL/min/mg)	27.3	18.1 ± 0.75	44.0 ± 3.3	97.8 ± 0.005
t ½ (min)	50.9	77.8 ± 3.2	31.6 ± 2.4	14.2 ± 0.00
Human liver microsomes–NADPH; Cl_{int} (μL/min/mg)	23.0	25.2 ± 2.0	16.4 ± 6.7	18.6 ± 4.1
t ½ (min)	60.3	55.1 ± 4.3	92.1 ± 37.5	76.3 ± 16.8
Rat liver microsomes + NADPH; Cl_{int} (μL/min/mg)	<1	35.5 ± 4.3	37.2 ± 6.1	68.5 ± 8.7
t ½ (min)	>480	39.3 ± 4.8	37.8 ± 6.2	20.4 ± 2.6
Rat liver microsomes–NADPH; Cl_{int} (μL/min/mg)	<1	29.2 ± 6.5	17.5 ± 1.3	14.7 ± 0.34
t ½ (min)	>480	48.7 ± 10.8	79.2 ± 5.9	94.3 ± 2.2
PAMPA permeability P_e (10^{-6} cm s^{-1})	0.63	1.38 ± 0.01	3.18 ± 0.64	3.68 ± 0.51
PAMPA % recovery	65.3	6.9 ± 0.79	17.5 ± 2.3	18.5 ± 2.2
MDCK-MDR1/WT; net efflux	3.21	0.93	1.11	1.16

[a] U373V glioblastoma vector control.
[b] U373 M glioblastoma MGMT-transfected.
[c] HCT 116 colorectal MMR deficient. Results are expressed as the mean of >3 experiments (n = 5 per experiment) ± SD of >3 independent experiments. Compounds prepared as 50 or 10 mM stock solutions in DMSO (stored in aliquots at −20 °C) and diluted in culture medium prior to treatment of cells.
[d] Calculated using Stardrop
[e] Calculated using Molinspiration (www.molinspiration.com).
[f] Determined by ^{1}H NMR spectroscopy in phosphate buffers.
[g] Determined by LC-MS in phosphate buffers (mean of two independent experiments.
[h] DMPK assays (with appropriate control compounds) were conducted as contract research at Sygnature Discovery using their published protocols.

IR spectra were recorded using a Bruker Alpha series FT-IR spectrometer over the range 4000 to 600 cm^{-1}. UV spectra were recorded in the range of 200–500 nm on a PerkinElmer Lambda 25 spectrophotometer. Melting points were measured on an Electrothermal IA9200 apparatus.

All compounds are >95% pure by HPLC, using either a Bruker Impact Ultimate 3000 UHPLC or Agilent 1200 Infinity Series HPLC. HPLC traces for representative compounds that have *in vitro* data are shown in the Supplementary Data.

3.1.1. *Optimized synthesis of 8-carbamoyl-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (7)*



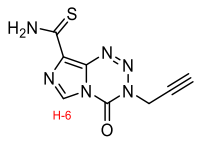
(a) A solution of sodium nitrite (5.0 g, 72 mmol) in water (116 mL) was added over 20 min to a stirred solution of 5-aminoimidazole-4-carboxamide hydrochloride (10.0 g, 66 mmol) in hydrochloric acid (1 M; 200 mL) at 0 °C and the mixture was left to stir for 5 min in the dark. The precipitate formed was collected under vacuum, washed with cold water, ethanol and ether and dried under vacuum to give the 5-diazoimidazole-4-carboxamide (**6**) as pale yellow crystals (6.65 g, 79%); mp 198–200 °C (lit. [42], mp 205–210 °C); ^{1}H NMR (400 MHz; DMSO-d_6) δ 7.99 (1H, bs, NH), 7.80 (1H, bs, NH), 7.61 (1H, s, H-2); ^{13}C NMR (100 MHz; DMSO-d_6) δ 161.3 (C), 155.4 (CH), 149.7 (C), 102.14 (C); HRMS found 138.0410, $[C_4H_3N_5O + H]^+$ requires 138.0411.

(b) Triphosgene (15.4 g, 52 mmol) was added to a biphasic mixture of propargylamine (11.6 mL, 182 mmol) in dichloromethane (200 mL) and sodium hydrogen carbonate (sat. aq.; 200 mL) in an ice bath. The reaction mixture was stirred vigorously for 25 min and extracted with dichloromethane (2 × 150 mL), dried ($MgSO_4$) and concentrated at under vacuum in an ice bath to approximately 10 mL. The recovered propargyl isocyanate was used in the next step without further purification (4.3 g, 47%); ^{1}H NMR (400 MHz; $CDCl_3$) δ 4.00 (2 H, d, J = = 2.5, CH_2), 2.42 (1 H, t, J = = 2.5, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 125.8 (C), 78.2 (C), 72.9 (CH), 32.5 (CH_2).

(c) Propargyl isocyanate (4.3 g, 53.0 mmol) was added dropwise to a suspension of diazoimidazole (**6**) (5.6 g, 41.0 mmol) in dry DMSO (25 mL) at room temperature in the dark under argon. The reaction mixture was left to stir for 24 h, poured onto ice and allowed to warm to room temperature. The resulting precipitate was collected by vacuum filtration and dried under vacuum. The crude product was purified by flash column chromatography (0–2% methanol in dichloromethane) to give the 3-propargylimidazotetrazine (**7**) as a cream colored solid (4.27 g, 67%); mp 163 °C; λ_{max} (EtOH)/nm 324 (log ε 2.90); ν_{max} (ATR)/cm^{-1} 3296, 3264, 3124, 2989, 2793, 2129, 1736, 1690, 1634, 1580, 1449, 1412, 1362, 1341, 1303, 1282, 1256, 1210, 1177, 1118, 1071, 1037; ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.86 (1 H, s, CH, H-6), 7.82 (1 H, br s, NH), 7.70 (1 H, br s, NH), 5.13 (2 H, d, J = = 2.5, CH_2), 3.52 (1 H, t, J = = 2.5, CH); ^{13}C NMR (100 MHz; DMSO-d_6) δ 161.4 (C), 138.5 (C), 134.2 (C), 131.3 (C), 129.2 (CH), 77.3 (C), 76.5 (CH), 38.5 (CH_2); m/z (ESI) 241 ($[M+Na]^+$, 100%), 219

([M+Na]$^+$, 1%); HRMS found 241.0448, [$C_8H_6N_6O_2$+Na]$^+$ requires 241.0444. ^{1}H NMR data matched literature values [43].

3.1.2. 3-Propargyl-8-thiocarbamoylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (9)

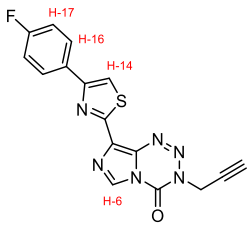


Phosphorus pentasulfide (1.27 g, 2.86 mmol), compound (**7**) (780 mg, 3.58 mmol) and hexamethyldisiloxane (1.7 mL, 7.87 mmol) were stirred in dry dichloromethane (36 mL) at reflux for 16 h under argon. The reaction mixture was poured onto a flash column and purified (10% acetonitrile in dichloromethane) to give the 8-thiocarbamoylimidazotetrazine (**9**) as an orange solid (896 mg, 84%); mp 133 °C; λ_{max} (EtOH)/nm 265 (log ε 3.92), 330 (log ε 3.89); ν_{max} (ATR)/cm^{-1} 3370, 3252, 3172, 3114, 2794, 2361, 2342, 2130, 1744, 1605, 1543, 1479, 1453, 1416, 1389, 1351, 1301, 1271, 1247, 1204, 1160, 1075, 1038, 1001; ^{1}H NMR (400 MHz; DMSO-d_6) δ 9.98 (1 H, br s, NH), 9.48 (1 H, br s, NH), 8.85 (1 H, s, CH, H-6), 5.13 (2 H, d, J = = 2.5, CH_2), 3.53 (1 H, t, J = = 2.5, CH); ^{13}C NMR (100 MHz; DMSO-d_6): δ 187.8 (C), 138.5 (C), 134.7 (C), 134.1 (C), 128.6 (CH), 77.3 (C), 76.5 (CH), 38.5 (CH_2); *m/z* (ESI) 257 ([M+Na]$^+$, 100%), 491 ([2 M + Na]$^+$, 30%); HRMS found 257.0223, [$C_8H_6N_6OS$ + Na]$^+$ requires 257.0216. ^{1}H NMR data matched literature values [22].

3.1.3. General method for the Hantzsch synthesis of 3-propargyl-8-(thiazol-2-yl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-ones

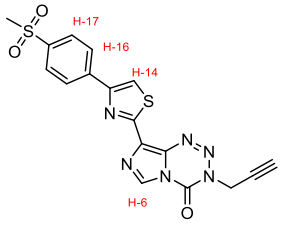
To a solution of thioamide (**9**) (typically 200–400 mg) in dry acetonitrile (15–25 mL) was added a bifunctional electrophile, normally a halomethyl ketone (1.05 mol equiv.) unless otherwise specified, and the reaction mixture was stirred for the indicated time at room temperature under argon. The reaction mixture was concentrated under vacuum, dissolved in EtOAc (20 mL), washed with saturated aqueous sodium hydrogen carbonate (15–25 mL), dried ($MgSO_4$) and concentrated under vacuum. When required, the product was further purified by flash column chromatography (solvent mixture specified). The following compounds were prepared using this method.

3.1.4. 8-[4-(4-Fluorophenyl)-thiazol-2-yl]-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (10)



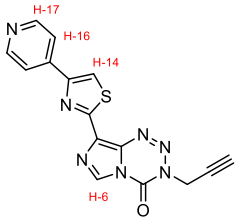
From (**9**) and 2-bromo-1-(4-fluorophenyl)ethan-1-one (66 h at room temp.), the product (**16**) was isolated as a green-yellow solid (56%); mp 236 °C; λ_{max} (EtOH)/nm 258 (log ε 3.98), 355 (log ε 3.59); ν_{max} (ATR)/cm^{-1} 3277, 3225, 3165, 3076, 3031, 3003, 2948, 2868, 2761, 2699, 2608, 1884, 1702, 1655, 1559, 1535, 1490, 1465, 1443, 1413, 1376, 1335, 1311, 1249, 1154, 1130, 1045; ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.94 (1 H, s, CH, H-6), 8.30 (1 H, s, CH, thiazole H-5), 8.14–8.09 (2 H, m, CH, Aryl CH), 7.36–7.30 (2 H, m, CH, Aryl CH), 5.15 (2 H, d, J = = 2.5, CH_2), 3.54 (1 H, t, J = = 2.5, CH); ^{13}C NMR (100 MHz; DMSO-d_6) δ 162.1 (CF, d, J = = 245.3), 158.8 (C), 154.9 (C), 138.6 (C), 131.7 (C), 131.4 (C), 130.7 (CH), 130.4 (C), 128.3 (CH, d, J = = 8.2), 115.9 (CH), 115.8 (CH, d, J = 21.3), 77.4 (C), 76.5 (CH), 38.5 (CH_2); ^{19}F NMR (376 MHz; $CDCl_3$) δ −113.50 (s, CF); *m/z* (ESI) 727 ([2 M + Na]$^+$, 100%), 375 ([M+Na]$^+$, 56%), 353 ([M+H]$^+$, 16%); HRMS found 353.0616, [$C_{16}H_9FN_6OS$ + H]$^+$ requires 353.0615.

3.1.5. 8-[4-(4-Methylsulfonylphenyl)-thiazol-2-yl]-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (11)



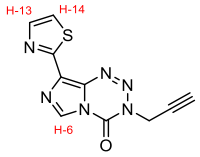
From (9) and 2-bromo-1-[4-(methylsulfonyl)phenyl]-1-ethanone (66 h at room temp) the product (**11**) was isolated as a yellow solid (61%) after chromatography (2–10% acetonitrile in dichloromethane); mp 178–181 °C; λ_{max} (EtOH)/nm 251 (log ε 3.00), 274 (log ε 3.02), 370 (log ε 2.75); ν_{max} (ATR)/cm^{-1} 3294, 3266, 3243, 3134, 3109, 1749, 1729. 1597, 1503, 1451, 1411, 1378, 1349, 1294, 1234, 1185, 1145, 1110, 1077, 1058, 1017; ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.98 (1 H, s, CH, H-6), 8.61 (1 H, s, CH, thiazole H-5), 8.34 (2 H, d, J = 8.5, CH, Aryl CH), 8.06 (2 H, d, J = 8.5, CH, Aryl CH), 5.16 (2 H, d, J = 2.5, CH_2), 3.55 (1 H, t, J = 2.5, CH), 3.27 (3 H, s, CH_3); ^{13}C NMR (100 MHz; DMSO-d_6) δ 159.3 (C), 154.1 (C), 140.1 (C), 138.6 (C), 138.2 (C), 131.6 (C), 131.4 (C), 130.8 (CH), 127.8 (CH), 126.8 (CH), 119.2 (CH), 77.4 (C), 76.5 (CH), 43.6 (CH_3), 38.5 (CH_2); *m/z* (ESI) 435 ([M+Na]$^+$, 100%), 413 ([M+H]$^+$, 42%); HRMS found 413.0500, [$C_{17}H_{12}N_6O_3S_2$+H]$^+$ requires 413.0485.

3.1.6. 3-Propargyl-8-[4-(pyridin-4-yl)-thiazol-2-yl]imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (12)



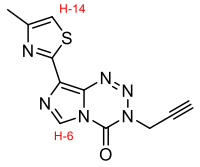
From (9) and 4-(bromoacetyl)pyridine hydrobromide (18 h at room temp.), the imidazotetrazine (**12**) was isolated as a green-yellow solid (70%) after chromatography (dichloromethane); mp 255 °C; λ_{max} (EtOH)/nm 268 (log ε 3.28), 362 (log ε 3.24); ν_{max} (ATR)/cm^{-1} 3381, 3288, 3191, 3085, 2562, 2362, 1757, 1653, 1632, 1607, 1595, 1522, 1512, 1454, 1358, 1333, 1281, 1244, 1230, 1210, 1172, 1109, 1082, 1048, 1021; ^{1}H NMR (400 MHz; DMSO-d_6) δ 9.08 (1 H, s, CH, H-6), 9.01 (1 H, s, thiazole H-5), 8.96 (2 H, d, J = 6.7, Aryl CH), 8.51 (2 H, d, J = 6.7, Aryl CH), 5.17 (2 H, d, J = 2.5, CH_2), 3.56 (1 H, t, J = 2.5, CH); ^{13}C NMR (100 MHz; DMSO-d_6) δ 160.3 (C), 151.2 (C), 146.6 (C), 144.5 (CH), 138.5 (C), 131.9 (C), 131.0 (CH), 130.8 (C), 125.1 (CH), 122.4 (CH), 77.3 (C), 76.6 (CH), 38.9 (CH_2); *m/z* (ESI) 336 ([M+H]$^+$, 61%), 358 ([M+Na]$^+$, 27%); HRMS found 336.0656, [$C_{15}H_9N_7OS$ + H]$^+$ requires 336.0662.

3.1.7. 3-Propargyl-8-(thiazol-2-yl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (13)



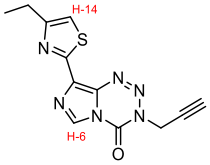
To (9) (300 mg) in dry acetone (3 mL) was added 2-bromo-1,1-dimethoxyethane (0.3 mL), a solution of HCl in 1,4-dioxane (4 M, 1.6 mL) and the mixture was stirred (5 h at 50 °C). The thiazolylimidazotetrazine (**13**) was isolated (32%) after chromatography (0–0.5% MeOH in dichloromethane) as an orange solid, mp 153 °C; λ_{max} (EtOH)/nm 268 (log ε 2.58), 360 (log ε 2.80); ν_{max} (ATR)/cm^{-1} 3225, 3134, 3123, 3092, 2972, 2956, 2922, 2852, 2115, 1727, 1503, 1452, 1427, 1396, 1373, 1344, 1302, 1274, 1233, 1143, 1118, 1069, 1056, 1014; ^{1}H NMR (400 MHz; CD_3CN) δ 8.49 (1 H, s, CH, H-6), 8.00 (1 H, d, J = 3.2, CH, thiazole H-4), 7.66 (1 H, d, J = 3.2, CH, thiazole H-5), 5.09 (2 H, d, J = 2.5, CH_2), 2.73 (1 H, t, J = 2.5, CH); ^{13}C NMR (100 MHz; CD_3CN) δ 160.0 (C), 145.7 (CH), 139.8 (C), 134.1 (C), 132.2 (C), 130.8 (CH), 122.4 (CH), 77.5 (C), 75.1 (CH), 39.4 (CH_2); *m/z* (ESI) 539 ([2 M + Na]$^+$, 100%), 281 ([M+H]$^+$, 65%), 259 ([M+H]$^+$, 51%); HRMS found 259.0397, [$C_{10}H_6N_6OS$ + H]$^+$ requires 259.0397. LC-MS purity = 95%.

3.1.8. 8-(4-Methylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (14)



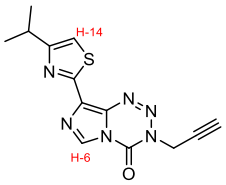
From (**9**) and 1-chloroacetone (48 h at room temp.), the methylthiazole (**14**) was isolated after chromatography (0–1% *i*-propanol in dichloromethane) as a green solid (37%); mp 168 °C; λ_{max} (EtOH)/nm 269 (log ε 4.70), 366 (log ε 4.90); ν_{max} (ATR)/cm^{-1} 3281, 3104, 2970, 2929, 2361, 2342, 2138, 1980, 1736, 1655, 1560, 1511, 1446, 1372, 1350, 1305, 1263, 1233, 1173, 1076, 1019; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.47 (1 H, s, CH, H-6), 7.08 (1 H, s, CH, thiazole H-5), 5.14 (2 H, d, J = 2.5, CH_2), 2.59 (3 H, s, CH_3), 2.43 (1 H, t, J = 2.5, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 157.5 (C), 155.6 (C), 138.5 (C), 134.7 (C), 130.6 (C), 129.7 (CH), 116.4 (CH), 76.0 (C), 74.5 (CH), 39.0 (CH_2), 17.5 (CH_3); *m/z* (ESI) 273 ([M+H]$^+$, 100%), 295 ([M+Na]$^+$, 14%); HRMS found 295.0374, [$C_{11}H_8N_6OS$ + Na]$^+$ requires 295.0373). LC-MS purity = 98%.

3.1.9. 8-(4-Ethylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (15)



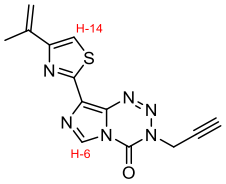
From (**9**) and 1-bromobutan-2-one (64 h at room temp.), the ethylthiazole (**15**) was isolated after chromatography (0–10% acetonitrile in dichloromethane) as a green-yellow solid (74 mg, 15%); mp 74–76 °C; λ_{max} (EtOH)/nm 270 (log ε 2.84), 366 (log ε 2.74); ν_{max} (ATR)/cm^{-1} 3285, 3124, 2970, 2934, 2128, 1740, 1508, 1448, 1372, 1348, 1307, 1266, 1230, 1076, 1020, 1003; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.48 (1 H, s, CH, H-6), 7.11 (1 H, t, J = 1.0, CH, thiazole H-5), 5.14 (2 H, d, J = 2.5, CH_2), 2.98 (2 H, dq, J = 7.5, 1.0, CH_2), 2.44 (1 H, t, J = 2.5, CH), 1.37 (3 H, t, J = 7.5, CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) δ 161.8 (C), 157.3 (C), 138.5 (C), 134.8 (C), 130.7 (C), 129.7 (CH), 115.1 (CH), 76.0 (CH), 74.5 (C), 39.0 (CH_2), 25.1 (CH_2), 13.5 (CH_3); *m/z* (ESI) 309 ([M+Na]$^+$, 100%), 287 ([M+H]$^+$, 43%); HRMS found 309.0529, [$C_{12}H_{10}N_6OS$ + Na]$^+$ requires 309.0529. LC-MS purity = 97%.

3.1.10. 3-Propargyl-8-(4-isopropylthiazol-2-yl)-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (16)



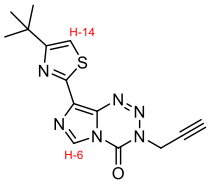
Isolated (11%) after chromatography (dichloromethane), from (9) and 1-bromo-3-methylbutan-2-one (17 h at room temp.), compound (16) had mp 120–122 °C; λ_{max} (EtOH)/nm 268 (log ε 3.97), 366 (log ε 4.17); ν_{max} (ATR)/cm^{-1} 3280, 3133, 2992, 2962, 2926, 2869, 1742, 1508, 1446, 1410, 1375, 1346, 1309, 1290, 1259, 1229, 1179, 1168, 1106, 1074, 1058; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.48 (1 H, s, CH, H-6), 7.10 (1 H, s, thiazole H-5), 5.14 (2 H, d, J = 2.5, CH_2), 3.29 (1 H, h, J = 6.9, CH), 2.44 (1 H, t, J = 2.5, CH), 1.39 (6 H, d, J = 6.9, 2 x CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) δ 166.4 (C), 157.1 (C), 138.6 (C), 135.0 (C), 130.8 (C), 129.7 (CH), 114.0 (CH), 76.0 (C), 74.5 (CH), 39.0 (CH_2), 31.1 (CH), 22.6 (CH_3); *m/z* (ESI) 301 ([M+H]$^+$), 100%); HRMS found 301.0866, [$C_{13}H_{12}N_6OS$ + H]$^+$ requires 301.0866). LC-MS purity = 98%.

3.1.11. 3-Propargyl-8-[4-(1-propen-2-yl)thiazol-2-yl]-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (17)



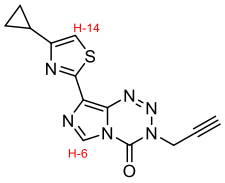
From (9) and 1-bromo-3-hydroxy-3-methylbutan-2-one (17 h at room temp.) After purification by chromatography (dichloromethane) the imidazotetrazine (**17**) was isolated (40%) as a yellow solid, mp 132–133 °C; λ_{max} (EtOH)/nm 228 (log ε 4.45), 254 (log ε 4.28), 372 (log ε 4.26); ν_{max} (ATR)/cm^{-1} 3279, 3125, 3100, 2981, 2949, 2922, 2851, 2127, 1742, 1661, 1626, 1572, 1530, 1500, 1475, 1446, 1423, 1376, 1350, 1307, 1263, 1233, 1105, 1071, 1014; ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.46 (1 H, s, CH, H-6), 7.30 (1 H, s, CH, thiazole H-5), 6.12 (1 H, dd, J = 1.1, 2.0, 1.1, CH), 5.27 (1 H, dd, J = 2.0, 1.1, CH), 5.13 (2 H, d, J = 2.5, CH_2), 2.44 (1 H, t, J = 2.5, CH), 2.18 (3 H, t, J = 1.1, CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) δ 158.8 (C), 157.4 (C), 138.5 (C), 136.5 (C), 134.5 (C), 130.9 (C), 129.7 (CH), 115.7 (CH_2), 115.6 (CH), 76.0 (C), 74.5 (CH), 38.9 (CH_2), 20.7 (CH_3); *m/z* (ESI) 321 ([M+Na]$^+$, 100%), 299 ([M+H]$^+$, 77%); HRMS found 321.0527, [$C_{13}H_{10}N_6OS$ + Na]$^+$ requires 321.0529. LC-MS purity = 99%.

3.1.12. 8-(4-tert-Butylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (18)



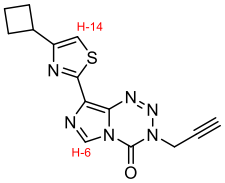
From (9) and 1-bromopinacolone, this imidazotetrazine (**18**) was purified by chromatography (2% acetonitrile in dichloromethane) and isolated as a yellow solid (37%); mp 122–128 °C; λ_{max} (EtOH)/nm 268 (log ε 2.69), 365 (log ε 2.78); ν_{max} (ATR)/cm^{-1} 3287, 3125, 2961, 2867, 2134, 1743, 1500, 1452, 1349, 1307, 1267, 1230, 1075; ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.90 (1 H, s, CH, H-6), 7.49 (1 H, s, CH, thiazole H-5), 5.13 (2 H, d, J = 2.5, CH_2), 2.53 (1 H, t, J = 2.5, CH), 1.37 (9 H, s, CH_3); ^{13}C NMR (100 MHz; DMSO-d_6) δ 169.2 (C), 156.7 (C), 138.6 (C), 135.2 (C), 130.9 (C), 129.7 (CH), 113.4 (CH), 76.0 (C), 74.5 (CH), 38.5 (CH_2), 35.2 (C), 30.3 (CH_3); *m/z* (ESI) 315 ([M+H]$^+$), 100%), 337 ([M+Na]$^+$, 94%); HRMS found 315.1023, [$C_{14}H_{14}N_6OS$ + H]$^+$ requires 315.1023.

3.1.13. 8-(4-Cyclopropylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (19)



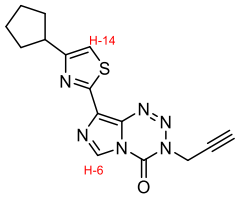
From (9) and 2-bromo-1-cyclopropylethan-1-one (17 h at room temp.). After chromatography (0–4% MeOH in dichloromethane) the cyclopropylthiazole (**19**) was isolated (14%) as a green solid, mp 118 °C, following crystallization from dichloromethane-light petroleum mixture); λ_{max} (EtOH)/nm 271 (log ε 4.75), 373 (log ε 4.83); ν_{max} (ATR)/cm^{-1} 3293, 3117, 2981, 2361, 2342, 2128, 1737, 1578, 1542, 1510, 1445, 1369, 1346, 1305, 1265, 1230, 1172, 1074, 1032, 1022; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.46 (1 H, s, CH, H-6), 7.00 (1 H, s, CH, thiazole H-5), 5.13 (2 H, d, J = 2.5, CH_2), 2.44 (1 H, t, J = 2.5, CH), 2.20 (1 H, quint, J = 6.6, CH), 1.00 (4 H, d, J = 6.6, 2 x CH_2); ^{13}C NMR (100 MHz; $CDCl_3$) δ 161.8 (C), 157.1 (C), 138.6 (C), 134.8 (C), 130.7 (C), 129.7 (CH), 113.4 (CH), 76.0 (C), 74.5 (CH), 38.9 (CH_2), 12.5 (CH), 8.7 (CH_2); *m/z* (ESI) 321 ([M+Na]$^+$, 100%), 299 ([M+H]$^+$, 89%), 619 ([2 M + H]$^+$, 53%); HRMS found 321.0527, [$C_{13}H_{10}N_6OS$ + Na]$^+$ requires 321.0529. LC-MS purity = 100%.

3.1.14. 8-(4-Cyclobutylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (20)



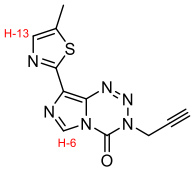
From (9) and and 2-bromo-1-cyclobutylethan-1-one (17 h at room temp.). After chromatography (0–1% MeOH in dichloromethane) and crystallization (dichloromethane-light petroleum) the cyclobutylthiazole (**20**) formed yellow needles (14%), mp 105 °C; λ_{max} (EtOH)/nm 289 (log ε 4.05), 368 (log ε 3.96); ν_{max} (ATR)/cm^{-1} 3239, 3114, 2982, 2936, 2859, 1726, 1666, 1575, 1504, 1457, 1443, 1433, 1372, 1353, 1285, 1249, 1229, 1170, 1116, 1090, 1028, 1018; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.48 (1 H, s, CH, H-6), 7.14 (1 H, s, CH, thiazole H-5), 5.14 (2 H, d, J = 2.5, CH_2), 3.88–3.79 (1 H, p, J = 8.8, CH), 2.44 (1 H, t, J = 2.5, CH), 2.42–2.31 (4 H, m, 2 x CH_2), 2.09–2.02 (1 H, m, CH), 1.95–1.93 (1 H, m, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 164.1 (C), 157.3 (C), 138.6 (C), 135.0 (C), 130.8 (C), 129.7 (CH), 114.8 (CH), 76.0 (C), 74.5 (CH), 39.0 (CH_2), 37.0 (CH), 29.3 (CH_2), 18.7 (CH_2); *m/z* (ESI) 313 ([M+H]$^+$, 100%), 335 ([M+Na]$^+$, 89%); HRMS found 335.0689, [$C_{14}H_{12}N_6OS$ + Na]$^+$ requires 335.0686. LC-MS purity = 98%.

3.1.15. 8-(4-Cyclopentylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (21)



From (9) and 2-bromo-1-cyclopentylethan-1-one (17 h at room temp.). The reaction mixture was evaporated to dryness and purified by flash column chromatography (0–2% MeOH in dichloromethane) to give product (**21**) as a yellow solid (46%), mp 132–133 °C after crystallization (dichloromethane-light petroleum); λ_{max} (EtOH)/nm 272 (log ε 4.00), 366 (log ε 4.10); ν_{max} (ATR)/cm^{-1} 3243, 3122, 2963, 2941, 2911, 2885, 2866, 2126, 1736, 1502, 1442, 1412, 1373, 1339, 1307, 1295, 1264, 1234, 1106, 1073, 1014; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.48 (1 H, s, CH, H-6), 7.11 (1 H, s, CH, thiazole H-5), 5.15 (2 H, d, J = 2.5, CH_2), 3.43–3.35 (1 H, p, J = 7.9, CH), 2.44 (1 H, t, J = 2.5, CH), 2.20–2.14 (2 H, m, 2 x CH), 1.84–1.78 (4 H, m, 2 x CH_2), 1.74–1.70 (2 H, m, 2 x CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 164.4 (C), 157.1 (C), 138.6 (C), 135.0 (C), 130.8 (C), 129.7 (CH), 114.5 (CH), 76.0 (C), 74.5 (CH), 42.4 (CH), 39.0 (CH_2), 33.4 (CH_2), 25.4 (CH_2); *m/z* (ESI) 327 ([M+H]$^+$, 100%); HRMS found 349.0840, [$C_{15}H_{14}N_6OS$ + Na]$^+$ requires 349.0842. LC-MS purity = 98%.

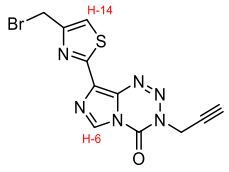
3.1.16. 8-(5-Methylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (22)



Prepared, by Hantzsch synthesis (see above), from (**9**) (135 mg, 0.62 mmol) in dry acetone (6 mL) and 2-bromopropionaldehyde (150 mg, 1.23 mmol) heated for 16 h at reflux under argon, the reaction mixture was evaporated to dryness and purified by flash column chromatography (0–2% MeOH in dichloromethane) to give the 5-methylthiazole (**22**) as a bright yellow solid (42 mg, 25%); mp 167 °C; λ_{max} (EtOH)/nm 239 (log ε 1.83), 366 (log ε 2.01); ν_{max} (ATR)/cm^{-1} 3240, 3127, 3071, 2126, 1736, 1521, 1485, 1436, 1413, 1378, 1345, 1307, 1266, 1237, 1170, 1138, 1115, 1072, 1034, 1017; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.46 (1 H, s, CH, H-6), 7.74 (1 H, q, J = 1.1, CH, thiazole H-4), 5.14 (2

H, d, $J = 2.5$, CH_2), 2.58 (3 H, d, $J = 1.1$, CH_3), 2.44 (1 H, t, $J = 2.5$, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 157.0 (C), 143.4 (CH), 138.6 (C), 136.8 (C), 134.8 (C), 130.4 (C), 129.6 (CH), 76.0 (C), 74.5 (CH), 39.1 (CH_2), 12.2 (CH_3); *m/z* (ESI) 567 ($[2\ M + Na]^+$, 100%), 273 ($[M+H]^+$, 63%), 295 ($[M+Na]^+$, 31%); HRMS found 295.0377, $[C_{11}H_8N_3OS + Na]^+$ requires 295.0373. LC-MS purity = 99%.

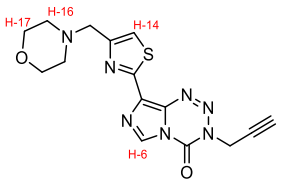
3.1.17. 8-(4-Bromomethylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (23)



From (9) and 1,3-dibromopropan-2-one in CH3CN 17 h at room temp.). The reaction mixture was evaporated to dryness and purified by flash column chromatography (0–1% MeOH in dichloromethane) to give product (**23**) as a yellow solid (40%), mp 157–160 °C; λ_{max} (EtOH)/nm 263 (log ε 4.74), 362 (log ε 4.95); ν_{max} (ATR)/cm^{-1} 3245, 3118, 3100, 2924, 2127, 1723, 1579, 1551, 1507, 1458, 1439, 1426, 1372, 1354, 1286, 1252, 1230, 1213, 1156, 1120, 1085, 1033, 1020; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.50 (1 H, s, CH, H-6), 7.53 (1 H, s, CH, thiazole H-5), 5.16 (2 H, d, $J = 2.5$, CH_2), 4.73 (2 H, s, CH_2), 2.45 (1 H, t, $J = 2.5$, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 158.7 (C), 154.8 (C), 138.4 (C), 134.0 (C), 131.0 (C), 129.8 (CH), 120.4 (CH), 75.9 (C), 74.6 (CH), 39.1 (CH_2), 26.9 (CH_2); *m/z* (ESI) 352/350 ($[M+H]^+$, 100%), 374/372 ($[M+Na]^+$, 23%), 727/725 ($[2\ M + Na]^+$, 24%); HRMS found, 350.9658 $[C_{11}H_8BrN_6OS + H]^+$ requires 350.9658.

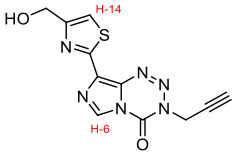
The following compounds were prepared from bromide (**23**).

3.1.18. 8-(4-Morpholinomethylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (24)



To a solution of 8-(4-bromomethylthiazol-2-yl)-3-propargylimidazo [5,1-*d*]-1,2,3,5-tetrazinone (**23**) (54 mg, 0.15 mmol) in dry THF (0.6 mL) was added morpholine (0.03 mL, 0.31 mmol) and triethylamine (0.04 mL, 0.31 mmol) and the reaction mixture was stirred for 2 h at room temperature under argon. The reaction mixture was evaporated to dryness and purified by flash column chromatography (0–4% MeOH in dichloromethane) to give the morpholinomethylthiazole (**24**) as a yellow solid (40 mg, 73%); mp 143-4 °C; λ_{max} (EtOH)/nm 273 (log ε 4.00), 364 (log ε 4.09); ν_{max} (ATR)/cm^{-1} 3226, 3127, 3106, 2977, 2967, 2885, 2853, 2799, 2124, 1737, 1651, 1515, 1446, 1437, 1351, 1328, 1310, 1292, 1281, 1258, 1236, 1201, 1139, 1131, 1105, 1081, 1068, 1035, 1005; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.49 (1 H, s, CH, H-6), 7.40 (1 H, s, CH, thiazole H-5), 5.15 (2 H, d, $J = 2.5$, CH_2), 3.85 (2 H, s, CH_2), 3.75 (4 H, t, $J = 4.7$, 2 x CH_2), 2.61 (4 H, t, $J = 4.7$, 2 x CH_2), 2.44 (1 H, t, $J = 2.5$, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 157.9 (C), 156.1 (C), 138.5 (C), 134.5 (C), 130.8 (C), 129.8 (CH), 118.8 (CH), 75.9 (C), 74.6 (CH), 67.1 (CH_2), 58.8 (CH_2), 53.8 (CH_2), 39.1 (CH_2); *m/z* (ESI) 358 ($[M+H]^+$, 100%); HRMS found 358.1086, $[C_{15}H_{15}N_7O_2S + H]^+$ requires 358.1081. LC-MS purity = 97%.

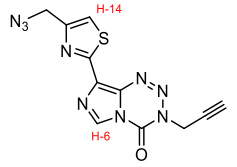
3.1.19. 8-(4-Hydroxymethylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (25)



To a solution of the bromomethylthiazole (**23**) (144 mg, 0.41 mmol) in wet DMF (1.0 mL) under argon was added silver trifluoroacetate (136 mg, 0.62 mmol) and the reaction mixture was stirred (7 h at room temp.), then diluted with ether (5 mL) and washed with hydrochloric acid (1 M; 3 × 5 mL). The aqueous layer was extracted with dichloromethane (3 × 5 mL), washed with brine (20 mL), dried over $MgSO_4$ and concentrated under vacuum and purified by flash column chromatography (0–3% methanol in dichloromethane) to give the hydroxymethythiazole (**25**) as a green solid (70 mg, 60%); mp 135–137 °C; λ_{max} (EtOH)/nm 273 (log ε 3.91), 365 (log ε 4.02); ν_{max} (ATR)/cm^{-1} 3413, 3279, 3262, 3125, 3093, 2977, 2947, 1736, 1519, 1508, 1450, 1434, 1370, 1353, 1301, 1251, 1229, 1170, 1151, 1076, 1048; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.47 (1 H, s, CH, H-6), 7.40 (1 H, s, CH, thiazole H–5H), 5.12 (2 H, d, $J = 2.5$, CH_2), 4.90 (2 H, d, $J = 0.9$, CH_2O), 3.65 (1 H, br s, OH), 2.45 (1 H, t, $J = 2.5$, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 158.9 (C), 158.6 (C), 138.4 (C), 134.0 (C), 130.8 (C), 129.8 (CH), 117.4 (CH), 75.9 (C), 74.6 (CH), 61.0 (CH_2), 39.1 (CH_2); *m/z* (ESI) 311 (M^++Na^+, 100%), 289 ($[M+H]^+$, 63%), 599 ($[2\ M + H]^+$, 35%); HRMS found 311.0321, $[C_{11}H_8N_6O_2S + Na]^+$ requires 311.0322. LC-MS purity = 96%.

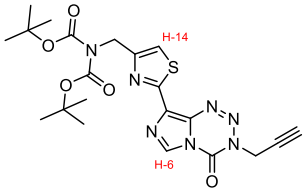
If dry DMF is used in the reaction after 2 h a quantitative yield of the intermediate trifluoroacetate can be isolated; this can be converted into the hydroxymethythiazole (25) in 95% EtOH at 25 °C after 12 h.

3.1.20. 8-(4-Azidomethylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (26)



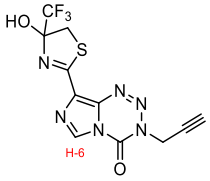
Sodium azide (186 mg, 2.86 mmol) was added to a stirred solution of bromomethylthiazole (**23**) (500 mg, 1.43 mmol) in dry DMF (2.30 mL) under argon and the mixture was stirred at 50 °C for 18 h. Organic products were partitioned between EtOAc (50 ml) and water (5 × 50 mL) and the organic layer dried ($MgSO_4$) and concentrated under reduced pressure. The resulting solid was purified by flash column chromatography (0–3% MeOH in dichloromethane) to give the azido compound (**26**) as a pale yellow solid (89 mg, 22%); mp 114 °C; λ_{max} (EtOH)/nm 268 (log ε 2.64), 360 (log ε 2.89); ν_{max} (ATR)/cm^{-1} 3293, 3128, 3103, 2990, 2935, 2103 (N_3), 1722, 1515, 1488, 1477, 1436, 1421, 1373, 1346, 1296, 1235, 1175, 1142, 1117, 1074, 1017; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.49 (1 H, s, CH, H-6), 7.46 (1 H, s, CH, thiazole H-5), 5.15 (2 H, d, $J = 2.5$, CH_2), 4.07 (2 H, s, CH_2), 2.45 (1 H, t, $J = 2.5$, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 159.0 (C), 153.7 (C), 138.4 (C), 134.0 (C), 131.0 (C), 129.8 (CH), 118.7 (CH), 75.9 (C), 74.6 (CH), 50.6 (CH_2), 39.1 (CH_2); *m/z* (ESI) 314 ($[M+H]^+$, 100%), 336 ($[M+Na]^+$, 67%), 649 ($[2\ M + Na]^+$, 48%); HRMS found, 314.0567 $[C_{11}H_7N_9OS + H]^+$ requires 314.0567.

3.1.21. 8-[4-(N,N-tert-Butoxycarbonylamino)methylthiazol-2-yl]-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (27)



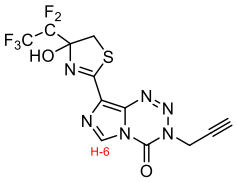
Sodium hydride (60% w/w, 35 mg, 0.13 mmol) was added to a stirred solution of di-*tert*-butyl-imino dicarboxylate (30 mg, 0.14 mmol) in dry acetonitrile (1.26 mL) under argon and allowed to stir for 20 min. The bromomethylthiazole (**23**) (44 mg, 0.13 mmol) was added and the mixture was stirred (19 h) and then concentrated to dryness. The residue was partitioned between dichloromethane (5 mL) and water (10 mL) and dichloromethane fractions (4 × 5 mL) were dried ($MgSO_4$) and concentrated under reduced pressure. The resulting solid was purified by flash column chromatography (0–1% MeOH in dichloromethane) to give the imidazotetrazine (**27**) as a pale yellow solid (40 mg, 65%); mp 132 °C; λ_{max} (EtOH)/nm 269 (log ε 1.82), 361 (log ε 1.89); ν_{max} (ATR)/cm^{-1} 3280, 3119, 2979, 2927, 2854, 1743, 1696, 1513, 1450, 1391, 1368, 1349, 1308, 1258, 1229, 1143, 1113, 1078, 1036; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.48 (1 H, s, CH, H-6), 7.23 (1 H, s, CH, thiazole H–5H), 5.15 (2 H, d, J = 2.5, CH_2), 5.10 (2 H, d, J = 1.0, CH_2), 2.45 (1 H, t, J = 2.5, CH), 1.48 (18 H, s, 6 x CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) δ 158.0 (C), 157.0 (C), 152.4 (C), 138.6 (C), 134.6 (C), 129.7 (CH), 116.1 (CH), 110.1 (C), 83.0 (C), 76.0 (C), 74.5 (CH), 47.0 (CH_2), 39.0 (CH_2), 28.2 (CH_3); *m/z* (ESI) 510 ([M+Na]$^+$, 100%), 997 ([2 M + Na]$^+$, 47%), 488 ([M+H]$^+$, 27%); HRMS found 488.1714, [$C_{21}H_{25}N_7O_5S$ + H]$^+$ requires 488.1711. De-protection of **27** with 4M − HCl in 1,4-dioxane at 25 °C for 0.5 h afforded an impure sample of 8-[(4-aminomethyl)thiazol-2-yl]-3-propargylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (**28**).

3.1.22. 8-[4-Hydroxy-4-(trifluoromethyl)-5,5-dihydrothiazol-2-yl]-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (29)



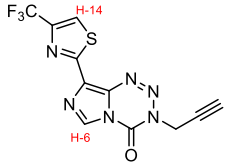
To a solution of (**9**) (450 mg, 1.92 mmol) in dry acetonitrile (12 mL) was added 3-bromo-1,1,1-trifluoroacetone (404 mg, 2.11 mmol) and the reaction mixture was stirred for 4 h at 50 °C under argon. The reaction mixture was evaporated to dryness and purified by flash column chromatography (0–4% MeOH in dichloromethane) to give the title compound (**29**) as a beige solid (328 mg, 50%); mp 162–163 °C; ν_{max} (ATR)/cm^{-1} 3264, 3133, 2969, 2926, 2133, 1723, 1578, 1524, 1495, 1459, 1440, 1369, 1350, 1296, 1279, 1228, 1162, 1122, 1074, 1018; ^{1}H NMR (400 MHz; CD_3CN) δ 8.54 (1 H, s, CH, H-6), 5.23 (1 H, s, OH), 5.14 (2 H, d, J = 2.5, CH_2), 3.80 (1 H, d, J = 13.0, CH), 3.50 (1 H, d, J = 13.0, CH), 2.77 (1 H, t, J = 2.5, CH); ^{13}C NMR (100 MHz; CD_3CN) δ 168.3 (C), 139.4 (C), 134.9 (C), 131.5 (C), 130.7 (CH), 129.1–120.6 (CF_3, q, J = 277.5), 107.5–106.6 (C, q, J = 30.1), 77.1 (C), 75.4 (CH), 39.9 (CH_2), 38.4 (CH_2); ^{19}F NMR (376 MHz; CD_3CN) δ −82.71 (s, CF_3); *m/z* (ESI) 345 ([M+H]$^+$, 100%); HRMS found 345.0381, [$C_{11}H_7F_3N_6O_2S$ + H]$^+$ requires 345.0376.

3.1.23. 8-[4-Hydroxy-4-(pentafluoroethyl)-5,5-dihydrothiazol-2-yl]-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (30)



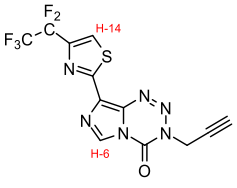
Prepared, from (9) (115 mg, 0.49 mmol) and 1-bromo-4,4,5,5,5-pentafluorobutan-2-one (0.08 mL, 0.54 mmol) and purified (as above), the pentafluoroethyl compound (30) was isolated as a red solid (84 mg, 44%); mp 108–110 °C; λ_{max} (EtOH)/nm 328 (log ε 3.73); ν_{max} (ATR)/cm^{-1} 3302, 3132, 2359, 2130, 1748, 1606, 1556, 1455, 1422, 1378, 1343, 1310, 1273, 1216, 1188, 1157, 1074; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.59 (1 H, s, CH, H-6), 5.15 (2 H, d, J = 2.5, CH_2), 5.07 (1 H, br s, OH), 3.84 (1 H, d, J = 12.8, CH), 3.55 (1 H, d, J = 12.8, CH), 2.46 (1 H, t, J = 2.5, CH); ^{13}C NMR (100 MHz; CD_3CN) δ 167.3 (C), 137.9 (C), 133.4 (C), 131.5 (C), 130.1 (CH), 123.7–114.4 (CF_3, qt, J = 35.0, 273.0), 115.7–109.5 (CF_2, tq, J = 35.0, 273.0), 107.2–106.7 (C, t, J = 24.5), 75.5 (C), 75.0 (CH), 39.4 (CH_2), 38.3 (CH_2); ^{19}F NMR (376 MHz; $CDCl_3$) δ −78.87 (3 F, s, CF_3), −123.82 (2 F, s, CF_2); *m/z* (ESI) 811 ([2 M + Na]$^+$, 100%); HRMS found 395.0350, [$C_{12}H_7F_5N_6O_2S$ + H]$^+$ requires 395.0344.

3.1.24. 3-Propargyl-8-(4-trifluoromethylthiazol-2-yl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (31)



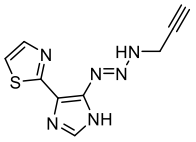
To a solution of (**29**) (108 mg, 0.31 mmol) and dry triethylamine (0.065 mL, 0.47 mmol) in dry acetonitrile (1.5 mL) in an ice bath was added trifluoroacetic anhydride (0.065 mL, 0.47 mmol) under argon. The reaction mixture was allowed to warm to room temperature and left to stir for 2 h. The reaction mixture was evaporated to remove solvents and purified by column chromatography (0–3% acetonitrile in dichloromethane) to give (**31**) as a beige crystalline solid (89 mg, 88%); mp 133 °C; λ_{max} (EtOH)/nm 264 (log ε 3.32), 352 (log ε 3.77); ν_{max} (ATR)/cm^{-1} 3264, 3133, 1723, 1578, 1524, 1495, 1459, 1440, 1369, 1350, 1296, 1279, 1228, 1162, 1122, 1074, 1018; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.53 (1 H, s, CH, H-6), 7.94 (1 H, s, CH, thiazole H-5), 5.17 (2 H, d, J = 2.5, CH_2), 2.46 (1 H, t, J = 2.5, CH); ^{13}C NMR (100 MHz; $CDCl_3$): δ 160.4 (C), 147.4–146.3 (C, q, J = 37.0), 138.2 (CH), 133.0 (C), 131.4 (C), 129.9 (CH), 124.4–116.3 (CF_3, q, J = 269.0), 122.8 (CH), 75.8 (C), 74.7 (CH), 39.2 (CH_2); ^{19}F NMR (376 MHz; CD_3CN) δ −64.44 (s, CF_3); *m/z* (ESI) 349 ([M+Na]$^+$, 100%); HRMS found 349.0088, [$C_{11}H_5F_3N_6OS$ + Na]$^+$ requires 349.0090. LC-MS purity = 98%.

3.1.25. 8-(4-(Pentafluoroethylthiazol-2-yl)-3-propargylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (32)



Prepared (as above) from (**30**) in a mixture of dry triethylamine, and trifluoroacetic anhydride in dry acetonitrile the pentafluoroethylthiazole (**32**) was obtained as a colorless crystalline solid (79%); mp 106 °C; λ_{max} (EtOH)/nm 264 (log ε 3.78), 351 (log ε 4.05); ν_{max} (ATR)/cm^{-1} 3295, 3122, 2938, 2360, 2342, 2245, 2132, 1743, 1581, 1581, 1560, 1496, 1452, 1420, 1371, 1348, 1304, 1267, 1192, 1143, 1095, 1075, 1021; ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.51 (1 H, s, CH, H-6), 7.98 (1 H, s, CH, thiazole H–5H), 5.17 (2 H, d, J = 2.5, CH_2), 2.46 (1 H, t, J = 2.5, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 160.4 (C), 146.2–145.6 (C, t, J = 28.5), 138.2 (C), 133.1 (C), 131.5 (C), 129.9 (CH), 124.2 (CH, t, J = 4.5), 120.6–112.1 (CF_3, qt, J = 38.5, 270), 113.5–107.3 (CF_2, tq, J = 38.5, 270), 75.8 (C), 74.7 (CH), 39.2 (CH_2); ^{19}F NMR (376 MHz; $CDCl_3$) δ −83.57 (3 F, t, J = 2.3, CF_3), −112.79 (2 F, q, J = 2.3, CF_2); *m/z* (ESI) 775 ([2 M + Na]$^+$, 100%); HRMS found 399.0061, [$C_{12}H_5F_5N_6OS$ + Na]$^+$ requires 399.0058. LC-MS purity = 99%.

3.1.26. Synthesis of 5-(3-propargyltriazen-1-yl)-4-(thiazol-2-yl)imidazole (38)



(a) 5-Aminoimidazole-4-carboxamide (**3**) hydrochloride salt (1.0 g, 6.15 mmol) was stirred in dichloromethane (61.5 mL) and triethylamine (2.14 mL, 15.4 mmol) was added, followed by di-*tert*-butyl dicarbonate (7.4 g, 33.8 mmol). The reaction mixture was stirred at room temperature for 15 h, concentrated under reduced pressure and the resulting solid was purified by flash column chromatography (0–2% MeOH in dichloromethane) to give the Boc-protected imidazole (**33**) as a colorless solid (1.36 g, 98%); mp 144-6 °C; ν_{max} (ATR)/cm^{-1} 3458, 3419, 3337, 3182, 3150, 2985, 1750, 1663, 1615, 1537, 1503, 1469, 1447, 1396, 1374, 1318, 1293, 1259, 1248, 1212, 1183, 1160, 1136, 1082; ^{1}H NMR (400 MHz; $CDCl_3$) δ 7.35 (1 H, s, CH), 6.44 (1 H, br s, NH), 6.30 (2 H, br s, NH_2), 5.35 (1 H, br s, NH), 1.62 (9 H, s, 3 x CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) δ 166.8 (C), 148.2 (C), 143.9 (C), 127.4 (CH), 111.5 (C), 86.8 (C), 28.1 (CH_3); *m/z* (ESI) 475 ([2 M + Na]$^+$, 100%), 249 ([M+Na]$^+$, 61%), 227 ([M+H]$^+$, 11%); HRMS found, 249.0956 [$C_9H_{14}N_4O_3$+Na]$^+$ requires 249.0958.

(b) Compound (**33**) (1.13 g, 5.00 mmol) and Lawesson's reagent (1.01 g, 2.5 mmol) was stirred in dry THF (50 mL) at reflux under argon for 15 h. The reaction mixture was concentrated under reduced pressure and purified by flash column chromatography (0–4% MeOH in dichloromethane) to give the Boc-protected thiocarboxamide (**34**) as a pale yellow solid (757 mg, 67%); mp 146–147 °C; ν_{max} (ATR)/cm^{-1} 3370, 3272, 3177, 3139, 2982, 1750, 1650, 1612, 1537, 1505, 1479, 1455, 1416, 1390, 1368, 1355, 1298, 1258, 1226, 1155, 1129; ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.78 (1 H, br s, NH), 7.36 (1 H, s, CH), 6.89 (1 H, br s, NH), 1.60 (9 H, s, 3 x CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) δ 186.9 (C), 148.0 (C), 146.7 (C), 127.2 (CH), 116.1 (C), 87.4 (C), 28.0 (CH_3); *m/z* (ESI) 265 ([M+Na]$^+$, 100%), 507 ([2 M + Na]$^+$, 62%), 243 ([M+H]$^+$, 18%); HRMS, found 243.0915, [$C_9H_{14}N_4O_2S$ + H]$^+$ requires 243.0910.

(c) Bromoacetaldehyde (488 mg, 4 mmol) was added dropwise to a stirred solution of the thiocarboxamide (**34**) (484 mg, 2 mmol) and potassium carbonate (1.38 g, 10 mmol) in DMF (10 mL). The reaction mixture was heated to 60 °C for 2 h under argon then cooled to room temperature. Di-*tert*-butyl dicarbonate (436 mg, 2 mmol) was added and stirred for 2 h at room temperature. The reaction mixture was diluted with ether (20 mL) and water (20 mL) and organic products extracted into ether (3 × 20 mL). The combined organic extracts were washed with water (3 × 100 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (2:8 ether:pentane) to give the Boc-protected imidazole (**35**) as a colorless solid (160 mg, 30%); mp 121 °C; ν_{max} (ATR)/cm^{-1} 34129, 1339, 1312, 1294, 1253, 1210, 1160, 1138, 1123, 1065, 1033, 3292, 3152, 3114, 3081, 3004, 2975, 2936, 2871, 1738, 1614, 1542, 1503, 1473, 1458, 1424, 1394, 136; ^{1}H NMR (400 MHz; $CDCl_3$) δ 7.69 (1 H, d, J = 3.3, CH), 7.50 (1 H, s, CH), 7.06 (1 H, d, J = 3.3, CH), 6.31 (2 H, br s, NH_2), 1.61 (9 H, s, 3 x CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) δ 164.6 (C), 148.3 (C), 142.5 (CH), 137.1 (C), 128.6 (CH), 114.9 (C), 114.3 (CH), 86.5 (C), 28.1 (CH_3); *m/z* (ESI) 167 ([M-Boc + H]$^+$, 100%), 267 ([M+H]$^+$, 47%), 555 ([2 M + Na]$^+$, 13%); HRMS found, 289.0727 [$C_{11}H_{14}N_4O_2S$ + Na]$^+$ requires 289.0730. Starting material (60%) was also recovered (290 mg, 1.2 mmol).

(d) A solution of HCl in 1,4-dioxane (4 M, 2 mL) was added to the thiazole (**35**) (30 mg, 0.11 mmol) under argon and allowed to stir for 2 h. The resulting precipitate was filtered, washed with ether and left to dry under vacuum to give 4-amino-5-(thiazol-2-yl)-1*H*-imidazole hydrochloride salt (36) as a pale brown solid (18 mg, 100%); mp 195 °C; ν_{max} (ATR)/cm^{-1} 3234, 3154, 3103, 3041, 2994, 2924, 2851, 2752, 2647, 2577, 2350, 2137, 1824, 1705, 1614, 1583, 1547, 1482, 1455, 1385, 1346, 1332, 1279, 1236, 1165, 1136, 1104, 1072, 1035, 1007; ^{1}H NMR (400 MHz; CD_3OD) δ 8.50 (1 H, s, CH), 7.87 (1 H, d, J = 3.3, CH), 7.54 (1 H, d, J = 3.3, CH); ^{13}C NMR (100 MHz; CD_3OD) δ 157.6 (C), 143.3 (CH), 140.1 (C), 130.2 (CH), 118.2 (CH), 106.7 (C); *m/z* (ESI) 167 ([M+H]$^+$, 66%), 189 ([M+Na]$^+$, 7%); HRMS found, 167.0392 [$C_6H_6N_4S$ + H]$^+$ requires 167.0386.

(e) The Boc-protected imidazole (36) (125 mg, 0.47 mmol) was stirred in hydrochloric acid (2 M; 1 mL) for 10 min protected from light. The reaction mixture was cooled in an ice bath and sodium nitrite (52 mg, 0.75 mmol) in water (1.5 mL) was added dropwise then left to stir for 30 min in the ice bath followed by 20 min at room temperature. The resulting orange solution was extracted with ethyl acetate (4 × 15 mL), dried over $MgSO_4$ and concentrated under vacuum to give 4-diazo-5-(thiazol-2-yl)-4*H*-imidazole (37) (83 mg, 100%) which was used without further purification; mp 120 °C; ν_{max} (ATR)/cm^{-1} 3086, 2923, 2865, 2133 (diazo), 1740, 1697, 1637, 1545, 1518, 1483, 1460, 1441, 1371, 1328, 1272, 1241, 1133, 1058, 1033, 1011; ^{1}H NMR (400 MHz; $CDCl_3$) δ 7.95 (1 H, d, J = 3.1, CH), 7.62 (1 H, s, CH), 7.53 (1 H, d, J = 3.1, CH); ^{13}C NMR (100 MHz; $CDCl_3$) δ 158.7 (C), 155.9 (C), 150.9 (CH), 144.4 (CH), 121.6 (CH), 99.0 (C); *m/z* (ESI) 178 ([M+H]$^+$, 14%); HRMS found, 178.0184 [$C_6H_3N_5S$ + H]$^+$ requires 178.0182.

Alternatively, the diazoimidazole (**37**) could be prepared (100%) from the aminoimidazolium salt (**36**) by nitrosation under similar conditions.

(f) 4-Diazo-5-(thiazol-2-yl)-4H-imidazole (37) (83 mg, 0.48 mmol) was stirred in dry EtOAc (2 mL) under argon, protected from light. Propargylamine (40 μL, 0.54 mmol) was added dropwise and the mixture was left to stir for 1.5 h. The resulting precipitate was washed with ether dried under vacuum to give 5-(3-propargyltriazen-1-yl)-4-(thiazol-2-yl)imidazole (38) as a beige solid (56 mg; 50%); mp 86 °C (WARNING: explosive decomposition); ν_{max} (ATR)/cm^{-1} 3287, 3226, 3119, 3008, 2927, 2856, 1581, 1483, 1397, 1349, 1331, 1310, 1246, 1164, 1097, 1060, 1003; ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.98 (1 H, br s, NH), 8.17 (1 H, br s, NH), 8.09 (1 H, d, J = 3.3, CH), 7.70 (1 H, s, CH), 7.68 (1 H, d, J = 3.3, CH), 4.34 (2 H, dd, 2.5, 4.0, CH_2), 3.25 (1 H, t, J, 2.5, CH); *m/z* (ESI) 233 ([M+H]$^+$, 100%); HRMS found 233.0612, [$C_9H_8N_6S$ + H]$^+$ requires 233.0604. LC-MS purity = 96%.

4. Biological studies

4.1. Anti-proliferative assays

Stock solutions of TMZ and other compounds listed in Table 1 were prepared in DMSO (10 or 50 mM) and aliquots stored at −20 °C, protected from light. U373V and U373 M were originally obtained from Schering-Plough Research Institute, USA; HCT 116 and MRC5 were sourced from the American Type Tissue Culture (ATTC) collection. All cell lines were verified as being mycoplasma free. HCT 116 and MRC-5 cells were maintained in RPMI 1640 nutrient medium supplemented with 10% fetal bovine serum (FBS), U373V and U373 M cultures were additionally supplemented with 1% NEAA, 50 μg/mL gentamicin and 400 μg/mL geneticin. Cells were sub-cultivated twice weekly and incubated at 37 °C in an atmosphere containing 5% CO_2. Cells were passaged <25 times before being discarded, to minimize phenotypic drift.

MTT Assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed as reported previously [12,13]. Briefly, cells were seeded into 96-well plates (6.5×10^2/well). Following overnight incubation, the vehicle control, TMZ and analogues were added to the wells (n = 4–8; final concentrations 0.5–1000 μM). MTT assays were performed at the time of agent addition and following 6 days of incubation (37 °C, 5% CO_2). MTT was added to the wells (50 μL; 0.4 mg/mL). The plates were reincubated (4 h) allowing MTT metabolism by viable cells to formazan crystals. Medium and unconverted MTT were aspirated and DMSO (150 μL) was added to each well ensuring formazan solubilization. Absorbance was read on an Anthos Labtec Systems plate reader (550 nm) and data transferred to a computer using Deltasoft 3™ software. Compound concentrations required to inhibit 50% growth (GI_{50}/μM) were calculated using non-linear regression analysis.

Clonogenic survival assays. Exponentially growing cells were seeded in triplicate at a density of 400 cells/well in a 6 well plate and allowed to attach overnight in an incubator (37 °C; 5% CO_2.). They were then exposed to increasing concentrations of TMZ or analogues (0, 2, 20 and 50 μM) for 24 h, after which time, medium containing test agents were removed and replaced with medium alone. After 14 days (or once colonies in control wells contained ≥50 cells), the plates were rinsed with PBS (2 × 1 mL), fixed with pre-chilled methanol (1 mL) for 20 min, stained with 0.5% methylene blue in 1:1 methanol/water (v/v) (1 mL) for 10 min, washed in distilled water and allowed to air dry. Cell colonies were counted and expressed proportionally compared to control colony formation.

Cell cycle. Cell cycle analysis of U373V, U373 M and HCT 116 cell lines was undertaken by flow cytometry measurement of cellular DNA content. Exponentially growing cells were seeded in 6 well plates at densities of 1×10^5 cells/well (72 h; U373 V/M), 5×10^4 cells/well (120 h; U373 V/M), 2.5×10^4 cells/well (72 h; HCT 116) or 1.5×10^4 cells/well (120 h; HCT 116) in 1 mL of culture medium. Cells were allowed to attach overnight in an incubator at 37 °C. Medium was aspirated and replaced with 2 mL of medium containing test agents, to achieve final concentrations of 100 μM TMZ **1**, 100 μM **7**, 20 μM **13** and 20 μM **14**, diluted in medium from DMSO stock solutions immediately prior to addition. The cells were incubated for the appropriate time (72 or 120 h) at 37 °C, after which time, the medium from each well was transferred into individual FACS tubes and attached cells were trypsinized, collected and added to the corresponding FACS tube. Each sample was centrifuged (1200 rpm, 4 °C, 5 min) and the medium aspirated, leaving the pellet intact. The cell pellet was resuspended and washed with PBS (0.5 mL), then centrifuged (1200 rpm, 4 °C, 5 min) and medium aspirated. The cell pellet was once more resuspended in 0.3 mL of hypotonic fluorochrome solution (50 μg/mL PI, 0.1 mg/mL ribonuclease A, 0.1% v/v Triton X-100 and 0.1% w/v sodium citrate in d.H_2O) and stored overnight at 4 °C in the dark. Cell cycle analysis was undertaken on a Beckman Coulter FC500 Flow Cytometer and data analysis was carried out using Flowing Software 2.

DMPK assays. These were conducted under contract at Sygnature Discovery using their published protocols with appropriate control compounds.

Author contributions

M.F.G.S., C.J.M. and T.D.B. conceived the project, designed experiments and analysed data; H.S.S. designed and conducted the experiments and analysed the data. W.L. and H.E.L.W. carried out X-ray crystallography and NMR spectroscopy experiments respectively. M.F. G.S., C.J.M., T.D.B. and H.S.S. contributed to preparation of the manuscript.

Declaration of competing interest

The authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

We thank Dr Stephen P. Argent for help with the crystallography figures. Dr Magda Foreiter provided the crystals of compound **7**. We thank Dr Vincenzo Cilibrasi (Charnwood Molecular) for the clogP calculations.

Appendix A. Supplementary data

Supplementary data to this article, including X-ray data, copies of NMR spectra, spectroscopic studies of compound decomposition, and LC-MS analyses of selected compounds can be found online at https://doi.org/10.1016/j.ejmech.2023.115507. Crystallography data: the X-ray crystallographic coordinates for compounds **7**, **13** and **14** have been deposited at the Cambridge Crystallographic Data Centre (CCDC) with accession codes 2035205, 2035207 and 2035208 respectively. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures/.

References

[1] A.S. Rodrigues, B.C. Gomes, C. Martins, M. Gromicho, N.G. Oliveira, P.S. Guerreiro, J. Rueff, DNA repair and resistance to cancer therapy, in: C. Chen (Ed.), New Research Directions in DNA Repair, IntechOpen, London, 2013.
[2] E.S. Newlands, G. Blackledge, J.A. Slack, C. Goddard, C.J. Brindley, L. Holden, M.F. G. Stevens, Phase I clinical trial of mitozolomide, Cancer Treat Rep. 69 (1985) 801–805.
[3] J.H. Schornagel, G. Simonetti, R. Dubbelman, W.W.t.B. Huinink, J.G. McVie, Phase I study of mitozolomide on a once daily for 5 days schedule, Cancer Chemother. Pharmacol. 26 (1990) 237–238.
[4] R. Stupp, W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J.B. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S. K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J.G. Cairncross, E. Eisenhauer, R. O. Mirimanoff, Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, N. Engl. J. Med. 352 (2005) 987–996.
[5] M.F.G. Stevens, Temozolomide: from cytotoxic to molecularly targeted agent, in: S. Neidle (Ed.), Cancer Drug Design and Discovery, second ed., Elsevier Science, San Diego, 2014, pp. 145–164.
[6] C.L. Moody, R.T. Wheelhouse, The medicinal chemistry of imidazotetrazine prodrugs, Pharmaceuticals 7 (2014) 797–838.
[7] R.T. Wheelhouse, M.F.G. Stevens, Decomposition of the antitumor drug temozolomide in deuteriated phosphate buffer - methyl-group transfer is accompanied by deuterium-exchange, Chem. Commun. (1993) 1177–1178.
[8] B.J. Denny, R.T. Wheelhouse, M.F.G. Stevens, L.L.H. Tsang, J.A. Slack, NMR and molecular modeling investigation of the mechanism of activation of the antitumor drug temozolomide and its interaction with DNA, Biochemistry 33 (1994) 9045–9051.

[9] J.A. Hickman, M.F.G. Stevens, N.W. Gibson, S.P. Langdon, C. Fizames, F. Lavelle, G. Atassi, E. Lunt, R.M. Tilson, Antitumor imidazotetrazines. 6. Experimental antitumor-activity against murine tumor-model systems of 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*] -1,2,3,5-tetrazin-4(3H)-one(mitozolomide), a novel broad-spectrum agent, Cancer Res. 45 (1985) 3008–3013.

[10] M.E. Hegi, A.C. Diserens, S. Godard, P.Y. Dietrich, L. Regli, S. Ostermann, P. Otten, G. Van Melle, N. de Tribolet, R. Stupp, Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide, Clin. Cancer Res. 10 (2004) 1871–1874.

[11] M.E. Hegi, A. Diserens, T. Gorlia, M. Hamou, N. de Tribolet, M. Weller, J.M. Kros, J.A. Hainfellner, W. Mason, L. Mariani, J.E.C. Bromberg, P. Hau, R.O. Mirimanoff, J.G. Cairncross, R.C. Janzer, R. Stupp, *MGMT* gene silencing and benefit from temozolomide in glioblastoma, N. Engl. J. Med. 352 (2005) 997–1003.

[12] J. Zhang, M.F.G. Stevens, T.D. Bradshaw, Temozolomide: mechanisms of action, repair and resistance, Curr. Mol. Pharmacol. 5 (2012) 102–114.

[13] J.H. Zhang, M. Hummersone, C.S. Matthews, M.F.G. Stevens, T.D. Bradshaw, N3-substituted temozolomide analogs overcome methylguanine-DNA methyltransferase and mismatch repair precipitating apoptotic and autophagic cancer cell death, Oncology 88 (2015) 28–48.

[14] Z.D. Nagel, G.J. Kitange, S.K. Gupta, B.A. Joughin, I.A. Chaim, P. Mazzucato, D. A. Lauffenburger, J.N. Sarkaria, L.D. Samson, DNA repair capacity in multiple pathways predicts chemoresistance in glioblastoma multiforme, Cancer Res. 77 (2017) 198–206.

[15] A. Brodbelt, D. Greenberg, T. Winters, M. Williams, S. Vernon, V.P. Collins, Glioblastoma in england: 2007–2011, Eur. J. Cancer 51 (2015) 533–542.

[16] Despite the clinical failure of the N-3 chloroethyl compound MTZ 1b almost 40 years ago, a very recent report argues that the fluoroethyl analogue is active against TMZ resistant GBM tumors K. Lin, S.E. Gueble, R.K. Sundaram, E. D. Huseman, R.S. Bindra, S.B. Herzon, Mechanism-based design of agents that selectively target drug-resistant glioma, Science 77 (2022) 502–511.

[17] E.V. Anslyn, D.A. Dougherty, Modern Physical Organic Chemistry, University Science Books, Sausalito, CA, 2006.

[18] D. Cousin, J.H. Zhang, M.G. Hummersone, C.S. Matthews, M. Frigerio, T. D. Bradshaw, M.F.G. Stevens, Antitumor imidazo[5,1-*d*]-1,2,3,5-tetrazines: compounds modified at the 3-position overcome resistance in human glioblastoma cell lines, Med. Chem. Commun. 7 (2016) 2332–2343.

[19] When this manuscript was in preparation, others have rediscovered the benefits of a propargyl substituent on N-3 of the imidazotetrazine ring R.L. Svec, S.A. McKee, M.R. Berry, A.M. Kelly, T.M. Fan, P.J. Hergenrother, Novel imidazotetrazine evades known resistance mechanisms and is effective against temozolomide-resistant brain cancer in cell culture, ACS Chem. Biol. 17 (2022) 299–313.

[20] R.L. Svec, L. Furiassi, C.G. Skibinski, T.M. Fan, G.J. Riggins, P.J. Hergenrother, Tunable stability of imidazotetrazines leads to a potent compound for glioblastoma, ACS Chem. Biol. 13 (2018) 3206–3216.

[21] Z.K. Yang, D.P. Wei, X.L. Dai, M.F.G. Stevens, T.D. Bradshaw, Y. Luo, J.H. Zhang, C8-substituted imidazotetrazine analogs overcome temozolomide resistance by inducing DNA adducts and DNA damage, Front. Oncol. 9 (2019) 485.

[22] M.G. Hummersone, M.F.G. Stevens, D. Cousin, 3-substituted-8-substituted-3H-imidazo[5,1-d][1,2,3,5]tetrazin-4-one compounds and their use, US Patent 9 (2010), 024,018 B2.

[23] F. Cohen, M.F.T. Koehler, P. Bergeron, L.O. Elliott, J.A. Flygare, M.C. Franklin, L. Gazzard, S.F. Keteltas, K. Lau, C.Q. Ly, V. Tsui, W.J. Fairbrother, Antagonists of inhibitor of apoptosis proteins based on thiazole amide isosteres, Bioorg. Med. Chem. Lett. 20 (2010) 2229–2233.

[24] S. Kumari, A.V. Carmona, A.K. Tiwari, P.C. Trippier, Amide bond bioisosteres: strategies, synthesis, and successes, J. Med. Chem. 63 (2020) 12290–12358.

[25] M.F.G. Stevens, J.A. Hickman, R. Stone, N.W. Gibson, G.U. Baig, E. Lunt, C. G. Newton, Antitumor imidazotetrazines. 1. Synthesis and chemistry of 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one, a novel broad-spectrum antitumor agent, J. Med. Chem. 27 (1984) 196–201.

[26] T.J. Curphey, Thionation with the reagent combination of phosphorus pentasulfide and hexamethyldisiloxane, J. Org. Chem. 67 (2002) 6461–6473.

[27] N.L. Snyder, C.J. Boisvert, Hantzsch synthesis, in: J.J. Li (Ed.), Name Reactions in Heterocyclic Chemistry Ii, John Wiley & Sons, Inc., Hoboken, N. J., 2011, pp. 591–644.

[28] P.R. Lowe, C.H. Schwalbe, M.F.G. Stevens, Antitumour imidazotetrazines. 5. Crystal and molecular-structure of 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (mitozolomide), J. Chem. Soc., Perkin Trans. 2 (1985) 357–361.

[29] P.R. Lowe, C.E. Sansom, C.H. Schwalbe, M.F.G. Stevens, A.S. Clark, Antitumor imidazotetrazines. 25. Crystal-structure of 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (temozolomide) and structural comparisons with the related drugs mitozolomide and DTIC, J. Med. Chem. 35 (1992) 3377–3382.

[30] K.H. Buchel, Chlorinated imidazole derivatives and a process for preparing them, US Patent 3.997 552 (1975).

[31] J. Atzrodt, R. Beckert, H. Gorls, Reduction of 4H-imidazoles - synthesis and reactivity of 4,5-diaminoimidazoles bearing a tetraaminoethene substructure, Heterocycles 51 (1999) 763–783.

[32] D. Cousin, M.G. Hummersone, T.D. Bradshaw, J. Zhang, C.J. Moody, M.B. Foreiter, H.S. Summers, W. Lewis, R.T. Wheelhouse, M.F.G. Stevens, 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, Med. Chem. Commun. 9 (2018) 545–553.

[33] S. Fulda, Cell death-based treatment of glioblastoma, Cell Death Dis. 9 (2018) 121.

[34] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (2000) 57–70.

[35] P.D. Leeson, A.M. Davis, Time-related differences in the physical property profiles of oral drugs, J. Med. Chem. 47 (2004) 6338–6348.

[36] M.C. de Gooijer, N.A. de Vries, T. Buckle, L.C.M. Buil, J.H. Beijnen, W. Boogerd, O. van Tellingen, Improved brain penetration and antitumor efficacy of temozolomide by inhibition of ABCB1 and ABCG2, Neoplasia 20 (2018) 710–720.

[37] H. Pajouhesh, G.R. Lenz, Medicinal chemical properties of successful central nervous system drugs, NeuroRx 2 (2005) 541–553.

[38] tPSA values were calculated using Molinspiration, using contributions from O and N only, although others advocate the inclusion of “slightly polar” fragments containing S and P Ertl, P.; Rohde, B.; Selzer, P. Fast calculation of molecular polar surface area as a sum of fragment-based contributions and its application to the prediction of drug transport properties. J. Med. Chem. 2000, 43, 3714-3717. (www.molinspiration.com).

[39] E.S. Newlands, M.F.G. Stevens, S.R. Wedge, R.T. Wheelhouse, C. Brock, Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials, Cancer Treat Rev. 23 (1997) 35–61.

[40] H.K. Kim, C.-C. Lin, D. Parker, J. Veals, J. Lim, P. Likhari, P. Statkevich, A. Marco, A.A. Nomeir, High-performance liquid chromatographic determination and stability of 5-(3-methyltriazen-1-yl)-imidazo-4-carboxamide, the biologically active product of the antitumour agent temozolomide, in human plasma, J. Chromatogr. B Biomed. Sci. Appl. 703 (1987) 225–233.

[41] H. Kim, P. Likhari, D. Parker, P. Statkevich, A. Marco, C.-C. Lin, A.A. Nomeir, High-performance liquid chromatographic analysis and stability of anti-tumour agent temozolomide in human plasma, J. Pharm. Biomed. Anal. 24 (2001) 461–468.

[42] Y.F. Shealy, J.A. Montgomery, R.F. Struck, L.B. Holum, Synthesis of potential anticancer agents. 29. 5-Diazoimidazole-4-carboxamide and 5-diazo-v-triazole-4-carboxamide, J. Org. Chem. 26 (1961) 2396–2401.

[43] M.F.G. Stevens, D. Cousin, S. Jennings, A.J. McCarroll, J.G. Williams, M. G. Hummersone, J. Zhang, Preparation of Oxodihydroimidazotetrazinecaboxylic Acid Derivatives for Use as Antiproliferative Agents. WO2009/077741 A2, 2009.