Synthesis and Quantitative Structure–Activity Relationship of Imidazotetrazine Prodrugs with Activity Independent of *O*6-Methylguanine-DNA-methyltransferase, DNA Mismatch Repair and p53.

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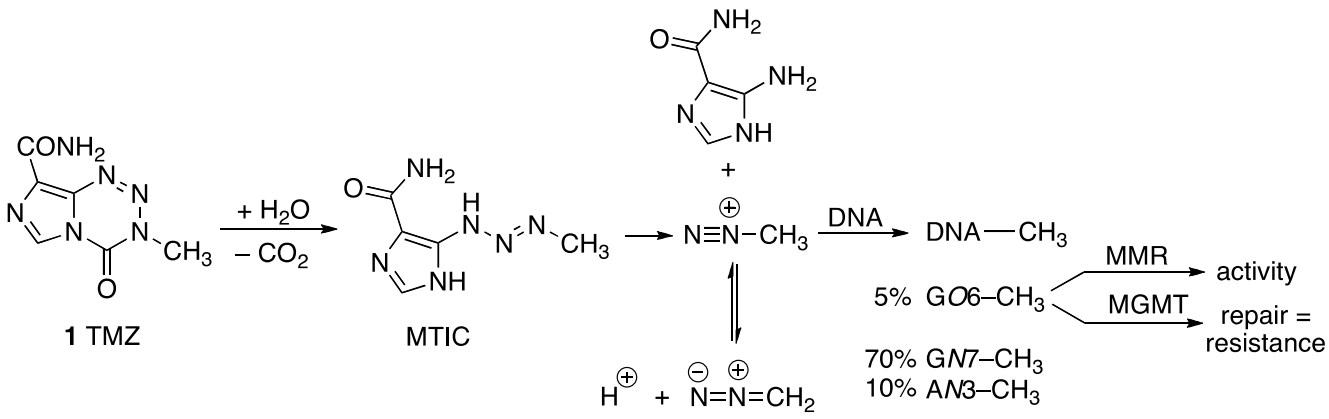
Abstract

The antitumor prodrug Temozolomide is compromised by its dependence for activity on DNA mismatch repair (MMR) and the repair of the chemosensitive DNA lesion, *O*6-methylguanine (*O*6-MeG), by *O*6-methylguanine-DNA-methyltransferase (EC 2.1.1.63, MGMT). Tumor response is also dependent on wild-type p53. Novel 3-(2-anilinoethyl)-substituted imidazotetrazines are reported that have activity independent of MGMT, MMR and p53. This is achieved through a switch of mechanism so that bioactivity derives from imidazotetrazine-generated arylaziridinium ions that principally modify guanine-*N*7 sites on DNA. Mono- and bi-functional analogs are reported and a quantitative structure-activity relationship (QSAR) study identified the *p*-tolyl-substituted bi-functional congener as optimized for potency, MGMT-independence and MMR-independence. NCI60 data show the tumor cell response is distinct from other imidazotetrazines and DNA-guanine-*N*7 active agents such as nitrogen mustards and cisplatin. The new imidazotetrazine compounds are promising agents for further development and their improved *in vitro* activity validates the principles on which they were designed.

Introduction

The imidazotetrazine prodrug temozolomide **1** (TMZ), a DNA methylating agent, in combination with radiotherapy, is now first line treatment for glioblastoma multiforme (GBM) in North America and Europe. However, intrinsic and acquired resistance significantly limits the ultimate efficacy of therapy. In particular, tumor re-growth up to two years following TMZ treatment is aggressive and resistant to further TMZ therapy.[1-3] This paper reports new compounds of the TMZ class that have activity independent of the two principal constraints on the ability of a tumor to respond to TMZ therapy: MGMT activity which directly repairs TMZ G*O*6-methylation of DNA, and MMR which enables the tumor response to TMZ therapy.[2]

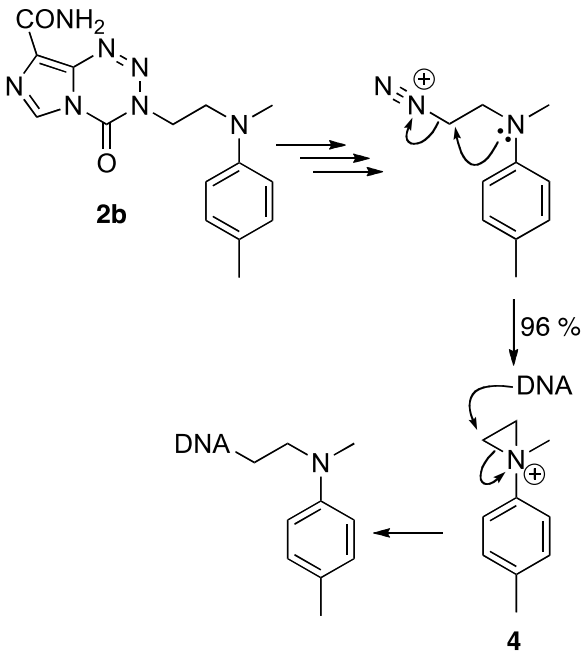


Scheme 1. The mechanism of action of temozolomide **1**.

At neutral pH, TMZ is relatively unstable ($t_{1/2}$ = 1.24 h, pH 7.4)[4] and undergoes hydrolytic ring-cleavage to the open chain triazene 5-(3-methyltriazen-1-yl)- imidazole-4-carboxamide (MTIC, $t_{1/2}$ = 8 min, pH 7.4),[5] which then fragments to the highly reactive electrophile, methyldiazonium ($t_{1/2}$ = 0.39 sec, pH 7.4).[6-8] Methyldiazonium reacts with nucleophilic groups on DNA, resulting in DNA methylation, Scheme 1. Approximately 70% of the methyl groups transferred to DNA appear at *N*7-guanine, 10 % at *N*3-adenine, and 5 % at *O*6-guanine sites. The *N*-methylation products are readily repaired by the base-excision repair pathways so are not major contributors to chemosensitivity.[9] In contrast, *O*6-MeG lesions are reversed by direct removal by the MGMT protein. The MGMT gene is silenced by promoter methylation in approximately 35% of GBM.[1] In these tumors, persistent *O*6-MeG lesions form wobble base-pairs with thymidine during replication; *O*6-MeG=T pairings result in futile cycles of mismatch repair, leading to stalled replication forks, DNA double-strand breaks and ultimately cell death.[1] Low levels of MMR expression (or mutations leading to non-functional MMR) lead to a tolerant phenotype that cannot respond to TMZ. Consistent with these mechanisms of *O*6-MeG processing and repair, high-level expression of the MGMT protein is a major mechanism of inherent TMZ resistance in primary tumors. Down regulation or mutations of the MMR pathway or up-regulation of MGMT expression are important causes of acquired TMZ resistance in GBM.[2] Herein we report the synthesis, pre-clinical activity *in vitro* and QSAR of new imidazotetrazines that have activity independent of MMR and MGMT.

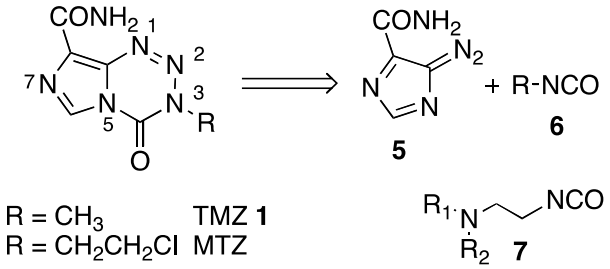
Compound Design

The imidazotetrazine bicycle is a prodrug of alkyldiazonium ions which are liberated by pH-dependent hydrolysis.[6] Careful control of these reactive intermediates, in particular the suppression of competing side reactions, such as hydrolysis, elimination or re-arrangement,[10-12] is essential in the design of effective new agents.[13, 14] Furthermore, of the methyl groups transferred from TMZ to DNA, only a small fraction becomes the therapeutically-beneficial *O*6-MeG lesion: we sought to achieve therapeutic benefit though generation of *N*7-G-adducts, the major products of reaction of TMZ with DNA, thereby making more efficient use of the imidazotetrazine prodrug. We have designed novel series of mono- (**2a–i**) and bi-functional (**3a–f**) imidazotetrazines in which a 3-(2-anilinoethyl) group was substituted for the 3-methyl group of TMZ. The new compounds are efficient precursors of aziridinium ions, **4**, Scheme 2. This strategy provides for effective control of the reactivity of incipient diazonium ions using a neighboring group participation mechanism not available to TMZ. Moreover, aziridinium ions are reactive intermediates of proven clinical utility, being closely related to those generated by nitrogen mustard drugs, and are established as working through *N*7-guanine adducts.[15] An additional feature of the drug design is the aniline *para*-substituent "X" of derivatives **2** and **3** that can be optimized to fine-tune pharmacological activity. This group affects the electron density at the aniline nitrogen by resonance or inductive effects and thereby controls the basicity and nucleophilicity of this site: i.e. the propensity either to protonate or to form an aziridinium ion. In addition, the bi-functional molecules would be expected to generate DNA cross-links, that would not be processed by MMR or MGMT and so avert those constraints on activity; previous studies have demonstrated that polar[16] and bulky[17] G*O*6 adducts cannot be processed by MGMT.



Scheme 2. Aziridinium ion formation by imidazotetrazine **2b.**[15]

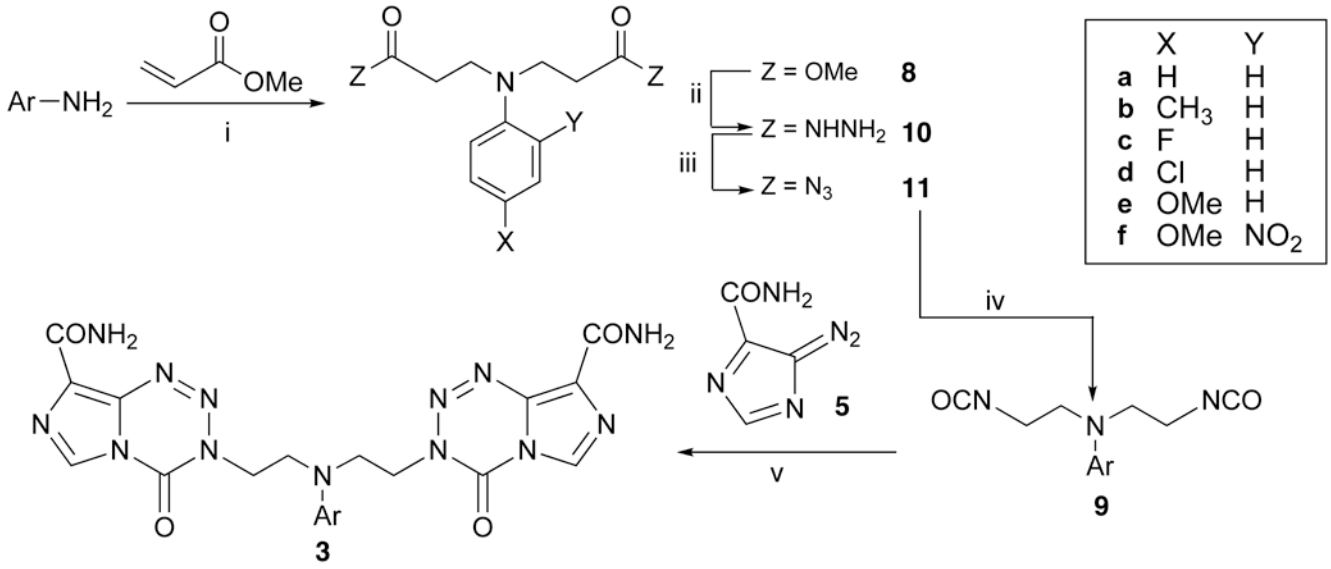
Synthesis



Scheme 3.

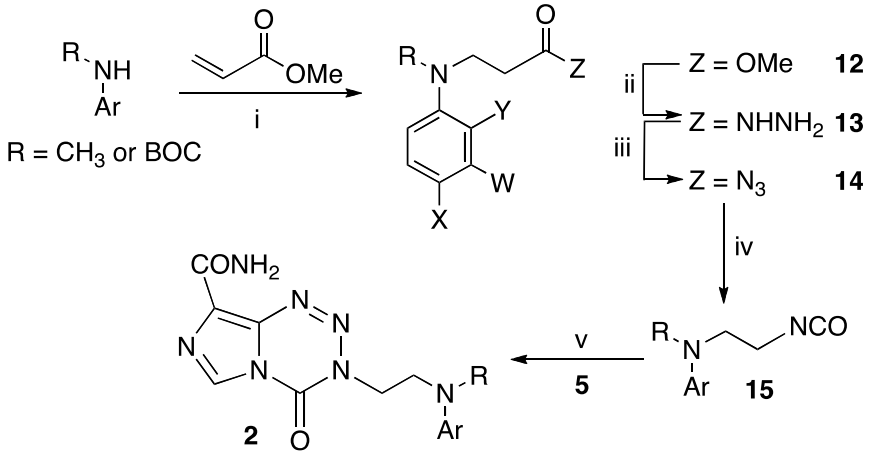
The new imidazotetrazines were prepared by variants of the established TMZ synthesis where a diazoimidazole **5** is reacted with an isocyanate **6** to yield the imidazotetrazine, Scheme 3.[18] The target compounds all included an aminoethyl group in the 3-position. The requisite β-aminoisocyanate precursors **7** are inherently unstable compounds as they bear incompatible functional groups. In the two aniline series, the anilines were sufficiently deactivated to allow isolation of the isocyanates without

recourse to protecting group strategies. The formation of the relatively fragile tetrazine ring was usually planned to be the last step in the synthesis.



Scheme 4. Route to bis-imidazotetrazines **3**. *Reagents and conditions:* (i) CuCl, AcOH, Δ, 76–98%; (ii) $NH_2NH_2 \cdot xH_2O$, IPA, Δ, 64–99%; (iii) $NaNO_2$, AcOH, H_2O, CH_2Cl_2, 0 °C; (iv) PhMe, Δ, [>95%]; (v) DMSO, RT, 2 d, 6–60% (over two steps)

To prepare the dimers **3a–f**, Cu(I)-catalyzed Aza-Michael conjugate addition of methyl acrylate to the requisite aniline furnished the diesters **8** in good (60–98%) yields, Scheme 4. Conversion to isocyanates **9** was achieved through the intermediate hydrazides **10** and azides **11** followed by Curtius rearrangement. For the electron rich anilines (X=CH_3, OMe), hydrazide formation had to be performed under very mildly acidic (0.17 M AcOH) conditions to avoid nitration of the aniline ring (**10e** → **11f** → **3f**). This is an attractive route as the bis-hydrazides are usually solids and easily purified while the Curtius rearrangement produces pure isocyanates directly, free from contaminating by-products.



	R	W	X	Y
a	CH_3	H	H	H
b	CH_3	H	CH_3	H
c	CH_3	H	F	H
d	CH_3	H	Cl	H
e	CH_3	H	NO_2	H
f	BOC	H	Cl	H
g	H	H	Cl	H
h	CH_3	NO_2	CH_3	H
i	CH_3	H	OMe	NO_2
j	CH_3	H	OMe	H

Scheme 5 Route to mono-imidazotetrazines **2**. *Reagents and conditions:* (i) CuCl, AcOH, Δ, 58–97%; (ii) $NH_2NH_2{\cdot}xH_2O$ / IPA, Δ, 44–98%; (iii) $NaNO_2$, AcOH, H_2O, CH_2Cl_2, 0 °C; (iv) PhMe, Δ, [>95%]; (v) DMSO, RT, 6–51% over two steps.

The mono-imidazotetrazine analogs **2a–i** were similarly prepared from *N*-methyl anilines, Scheme 5. In one example, the secondary aniline version **2g** (R=H, X=Cl) was achieved using a BOC protection strategy that exploited the relative acid stability of the imidazotetrazine ring. Carefully controlled diazotization (**12f** → **13f** → **14f**) yielded BOC-protected isocyanatoethylaniline **15f**; tetrazine ring closure followed by final treatment with TFA gave secondary aniline **2g**. As a similar yield was obtained omitting the BOC group, the protection was shown to be redundant. The *p*-nitro derivative **2e** was accessed from hydrazide **13a** by simultaneous diazotization and nitration under more vigorous conditions (10 M HCl); the mixture of regioisomers was carried through to the final step where the *p*-NO_2 isomer, tetrazine **2e**, was isolated by flash column chromatography.

***In vitro* QSAR: influence of MGMT and MMR**

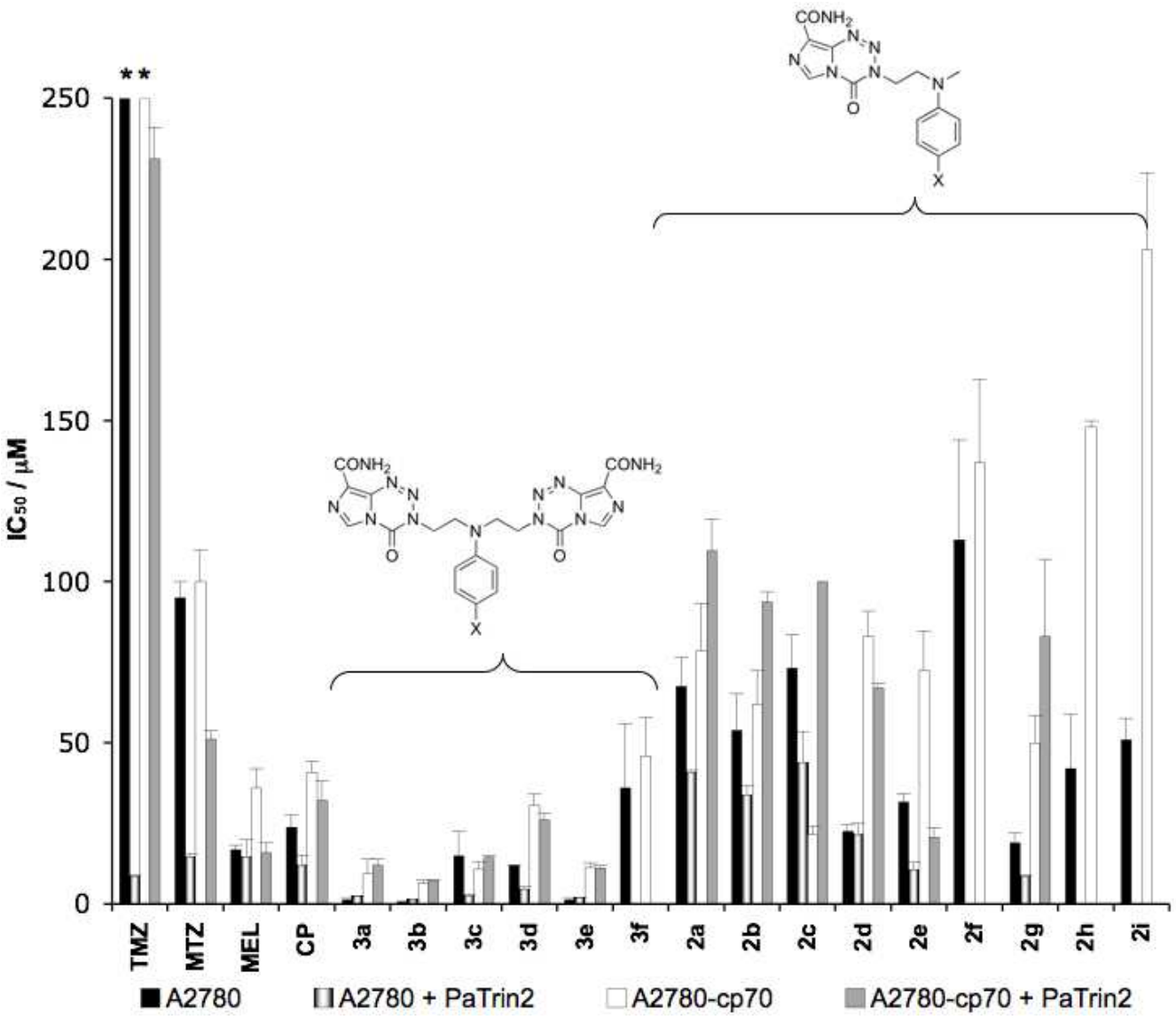


Figure 1 . *In vitro* chemosensitivity (IC_{50} / μM) of new imidazotetrazine compounds against A2780 (MMR+) and A2780-cp70 (MMR–) cell lines in the absence (MGMT+) and presence (MGMT–) of the MGMT inactivator PaTrin2 (10 μM). All data are the mean of ≥3 determinations, error bars are the SD; t-test analysis is presented in Table S3. * IC_{50} >250 μM. MEL, melphalan; CP, cisplatin.

Screening of all new compounds for chemosensitizing efficacy was undertaken following a protocol by Margisson *et al* that used the MGMT and MMR-proficient ovarian carcinoma cell line A2780, and its MMR-deficient derivative A2780-cp70, Figure 1.[19] To assess dependence of cytotoxicity on MGMT function, each cell line was further treated with the MGMT inactivator PaTrin2.[20] The data are presented graphically in Figure 1 and fully tabulated in Table S1. As expected, sensitivity to TMZ and mitozolomide (MTZ) was highly dependent on both lack of MGMT activity and presence of wild-type MMR capacity. For TMZ, the IC_{50} was >250 μM in both A2780 and A2780-Cp70 cell lines, compared with IC_{50} = 8.5 and 231 μM respectively for the same cell lines co-treated with PaTrin2. In contrast, the novel imidazotetrazines were significantly more potent, with IC_{50} values in the A2780 wild-type line ranging from 22–73 μM for the mono-functional (**2a–i**) and 1–15 μM for the bi-functional (**3a–f**) compounds. Dependence on MMR can be examined by comparing the shaded bars with the grey bars (i.e. MMR+/MMR– with MGMT inactivated in both cases). For temozolomide the IC_{50} was >27–fold lower in the MMR-proficient cell line. For the new bi-functional agents **3**, this ratio was reduced to 5–5.8-fold and for the mono-functional agents **2**, 2.8–10-fold. The extent of MGMT-mediated resistance can be assessed by comparing the shaded and the black bars (i.e. MGMT–/MGMT+ with MMR competent in both cases). Here, temozolomide was >30-fold more potent when MGMT was inactivated whilst for the new agents, this ratio was 0.5–5-fold for the bi-functional and 1.1–2.9-fold for the mono-functional agents. Importantly, in the absence of MMR, all compounds showed activity greater than temozolomide irrespective of the MGMT status of the cells (white bars and grey bars) showing that MMR-dependent toxicity and MGMT-mediated resistance are now only minor determinants of the chemosensitizing effect.

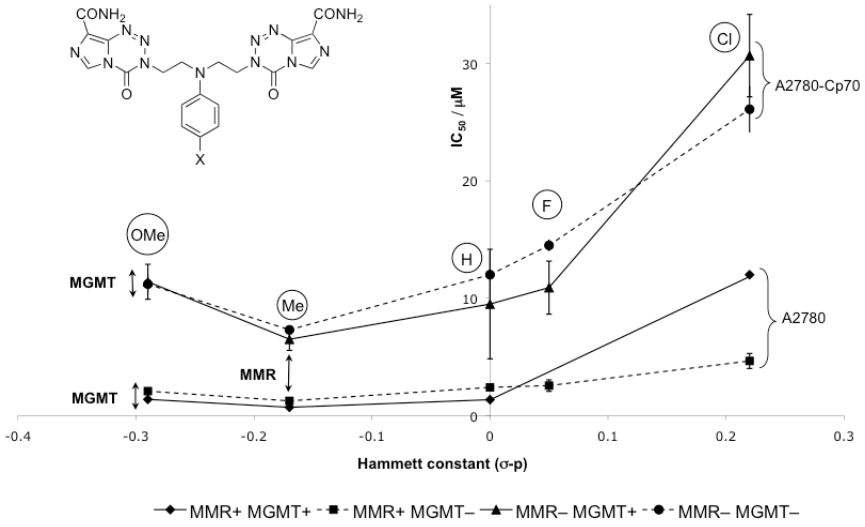
The similarity of response of examples **2d** and **2g** indicate that the *N*-methyl aniline is not essential to activity as the secondary and tertiary aniline compounds are equipotent (see Figure 1 and Table S1). Retention of the BOC group in analog **2f** significantly reduced potency, presumably by impeding the aziridine/aziridinium formation step. Nitration in the aniline ring, whether as the sole or as an additional

substituent, resulted in reduced potency. This is consistent with electron withdrawal reducing nucleophilicity of the aniline and hence the propensity for aziridinium ion formation, (for examples, compare the compound pairs **3e**/**3f**, **2a**/**2e**, and **2b**/**2h**).

To probe the structure-activity relationship and examine MGMT and MMR dependence in more detail, the results for dimeric compounds **3a-e** were presented in Hammett plots, Figure 2. The Hammett constant σ-*p* is a structural parameter that measures the electron donating or withdrawing effect of the substituent "X" on the aniline ring. The plot of IC_{50} against σ-*p* is informative in several respects, Figure 2A. For each cell type, the graph shows a pair of lines, the dotted line being MGMT-inactivated data (i.e. PaTrin 2 present); the difference between the solid and dotted lines shows the effect of MGMT on the cellular response. For most analogs this separation is of the same order as the error bars on the data, so in effect is zero. This is a clear demonstration that these new compounds have activity independent of MGMT function and is consistent with a mechanism of action independent of guanine-*O*6 alkylation (or involving a guanine-*O*6 adduct that cannot be repaired by MGMT). The separation between the two pairs of lines is the MMR effect which ranges from about 2–5-fold on the IC_{50}, which is greatly reduced from the >27-fold effect measured for TMZ.

p-Methyl-substituted analog **3b** lies on a minimum in the graph so is optimally potent, with a modestly electron-donating substituent on the aniline ring, Figure 2A. The data for all analogues except **3e** (X = OMe) lie on exponential curves (see the semi-log plot, Figure 2B). The anomalous data for **3e** are likely due to enhancement of the basicity of the aniline nitrogen lone pair leading to appreciable protonation at the pH of the experiment, and thereby a reduced propensity to act as a nucleophile in the aziridinium ion-forming reaction. These data clearly demonstrate the direct effect of electron density at the aniline nitrogen in determining the *in vitro* activity of the compounds, so provide further evidence that the aziridinium ion mechanism occurs in cells.

A



B

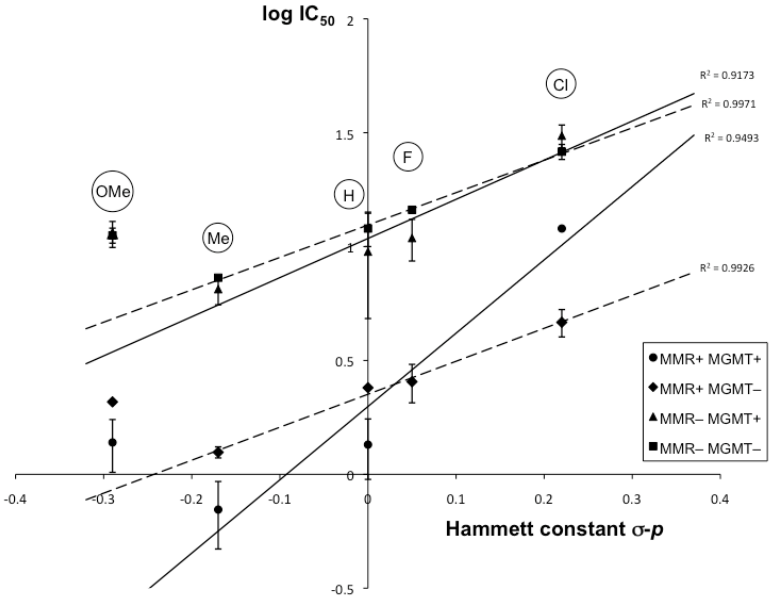


Figure 2. (A, upper panel) Hammett plot of the IC_{50} data for the dimers **3a–e**, in each case the MGMT– data are shown in dotted lines; the substituents X are shown in circles. (B, lower panel) QSAR Semi-log plot of

log IC_{50} vs. Hammett constant (σ-p) for the dimeric imidazotetrazines **3a–e**. Regression lines were fitted omitting the data for OMe derivative **3e**.

NCI60 Panel Data

Compounds **2d**, and **3c-e** were selected for full screening in the NCI 60 cell line panel. Mean graph data are presented in Figures S1–6 and are summarized in Table 1. These data show that the NCEs have pharmacological activity distinct from TMZ. TMZ is essentially inactive against the panel ($GI_{50} > 10^{-4}$ M for 57/60 cell lines, Figure S1). In contrast, the new agents exhibit strong patterns of discrimination over a 2–3-log range of GI_{50} (Table 1 and Figures S2–5); this is important as it shows that the new compounds possess a degree of selectivity and are not uniformly cytotoxic against cells grown in culture. These data compare favorably with the logGI_{50} mean and range data for established agents CHB, MEL, CP, BCNU and CCNU; this shows there is good reason to suppose that the new compounds may have an acceptable therapeutic index and be no more systemically toxic *in vivo* than these other clinically useful agents.

	Log GI_{50} Mean (SD)	**Log GI_{50} range**
2d	–4.69 (0.58)	2.05
3c	–5.11 (0.69)	2.81
3d	–5.07 (0.52)	1.72
3e	–4.83 (0.46)	1.80
MEL	–4.54 (0.55)	1.88
CHB	–4.21 (0.56)	1.64
CP	–5.40 (1.10)	2.38
BCNU	–4.12 (0.82)	1.86
CCNU	–4.53 (0.48)	2.22

Table 1 Summary of NCI60 statistics for compounds **2d**, **3c–e**, and established prodrugs for comparison.

Particular sensitivity is evident in the leukemia, CNS and ovarian sub-panels. In the CNS sub-panel, the pattern of activity is similar to MTZ and CP, with particular sensitivity in the glioblastoma lines SF295 and SNB75. In the ovarian sub panel, particular sensitivity is seen in the OVACR-3 and SK-OV-3 lines. Here, in

contrast to the CNS activity, the new compounds show improvement over CP (see Figure S6) this is a particularly interesting result given the wide clinical use of CP against ovarian adenocarcinoma and the fact that TMZ lacks clinical activity against ovarian cancer.[21]

COMPARE analysis of mean graph data can be valuable in discerning molecular mechanisms of action. The similarity in patterns of response is assessed by the Pearson rank correlation coefficient, P: values > 0.7 are considered highly significant, values 0.6–0.7 less so.[22, 23] Matrix COMPARE was valuable in confirming the novelty of mechanism of action of the new agents. In this method of data handling, the new compounds were compared with standard agents selected for similarities of chemical structure or predicted mechanism of action. Agents selected were other imidazotetrazines (TMZ, MTZ), nitrogen mustard cross linkers (chlorambucil CHB, melphalan MEL), nitrosoureas (1,3-Bis(2-chloroethyl)-1-nitrosourea BCNU, *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea CCNU) and CP, Table 2. Correlations amongst the four new imidazotetrazine compounds examined were all strong ($0.92 \leq P \geq 0.77$) and, interestingly, there was no clear discrimination between the mono- and bi-functional agents: for monofunctional **2d**, $0.83 \leq P \geq 0.77$ and in particular $P = 0.79$ with the equivalent *p*-Cl dimer **3d**. Gratifyingly, the new compounds showed only moderate correlations ($0.52 \leq P \geq 0.38$) with the established imidazotetrazines TMZ and MTZ, so are distinct as new members of this compound class. The absence of correlation with MTZ is significant as this compound, exhibits a characteristic pattern of differential activity across the panel, unlike TMZ.

The putative DNA lesion of the bifunctional agents **3** is a five-atom crosslink, related in structure to those formed by the nitrogen mustard prodrugs; however, no drugs of this class showed strong correlations $0.59 \leq P \geq 0.29$. Notably, there was no similarity to the nitrosoureas which are also diazonium ion precursors, $0.45 \leq P \geq 0.05$. The new compounds all showed strong correlations with dacarbazine (DTIC, highest dose = 1 μM). This observation is not easy to interpret, as this prodrug requires hepatic

oxidative demethylation in order to release its active electrophile, so it is somewhat surprising that DTIC scored so highly against the new imidazotetrazines. Part of the reason for this may lie in the two relatively featureless datasets of DTIC [mean log GI_{50}(SD) = –4.30(0.28), range = 0.99, high conc. = 10^{4} M; mean log GI_{50}(SD) = –3.66(0.548), range = 2.55, high conc. = 10^{-3} M] and a shared sensitivity in the leukemia sub-panel. Simple COMPARE using DTIC GI_{50} (high conc. = 10^{-3} M) data as seed in the Standard Agents database yields no correlations P>0.537.

	2d	**3c**	**3d**	**3e**	**TMZ**	**MTZ**	**DTIC**	**MEL**	**CHB**	**BCNU**	**CCNU**	**CP**
2d	1	0.83	0.79	0.77	0.50	0.42	0.62	0.35	0.33	0.25	0.08	0.27
3c	0.83	1	0.92	0.92	0.52	0.35	0.72	0.4	0.33	0.32	0.06	0.32
3d	0.79	0.92	1	0.81	0.46	0.38	0.64	0.36	0.29	0.27	0.05	0.35
3e	0.77	0.92	0.81	1	0.51	0.46	0.71	0.59	0.53	0.45	0.10	0.42

Table 2. Results of Matrix COMPARE (P values) for NCEs **2d**, **3c**-**e** with selected standard agents.

Simple COMPARE analysis was performed with the Standard Agents and Molecular Targets databases using the GI_{50} data as seed, Table 3. The Standard Agents data again identified similarity with DTIC, as noted above. In addition, modest correlations with 6MP and 6TG appeared for all four compounds, which is presumably linked to residual MMR-dependence,[24, 25] a property that is shared with MTZ and DTIC. With the exception of compound **3e**, correlations in the Molecular Targets database were not strong. Notable amongst the correlations of analog **3e** are the lymphocyte and leukocyte related proteins LCP1, LRMP, RASSF5 and LAIR1 which may account for the high sensitivity of the leukemia sub-panel to these new compounds. Also intriguing are the correlations with the RAS-related proteins ARHGAP4 and RASSF5 and the moderate inverse correlation with RAB1A, a RAS superfamily GTPase.[26]

All of the NCI cell lines are characterized for expression of MGMT and components of MMR.[27] Plots of protein expression vs. log GI_{50} were prepared to probe correlation or inverse correlation between GI_{50} and

expression of MGMT, hMLH1 (the core MMR protein) or MSH6 (the MMR domain that recognizes *O*6-alkylG-DNA damage), see Figures S6-8. For each of the compounds **2d**, **3c–e**, the graphs showed scatters with no connection between the protein expression levels and GI_{50}: a result consistent with the independence of activity from MGMT and MMR found in the A2780 cell line screen.

	Standard Agents Database			Molecular Targets Database	
	P[a] DTIC[b]	P 6TG	P 6MP	Strong positive correlations[c] (P)	Strongest negative correlation (P)
2d	0.669	0.563	0.571	U2AF1 (0.61)	RAB1A (–0.611)
3c	0.751	0.543	0.524	RCSD1 (0.686) HCLS1 (0.683)	RAB1A (–0.636)
3d	0.717	0.515	0.527	WAS (0.526)	RAB1A (–0.526)
3e	0.727	0.573	0.515	HCLS1, WAS, NCKAP1L, LCP1, LRMP, ARHGAP4, RASSF5, CD53, LAIR1 (>0.7 for all)	K1AA1191 (–0.64) RAB1A (–0.5136)
TMZ					
MTZ	0.326	0.517	0.418		

[a]P, Pearson rank correlation coefficient; [b]in all cases the highest ranking correlation was with DTIC; [c]examples from the 10 top correlations, P values in parentheses. 6TG, 6-thioguanine; 6MP, 6-mercaptopurine; DTIC, dacarbazine.

Table 3. Selected results from NCI60 screen and COMPARE analysis using the log GI_{50} data as seed in the Standard Agents and Molecular Targets databases.

Further *in vitro* evaluation

The new compounds were further assessed in selected drug-resistant cell lines (Tables 4 and 5). HCT116 is a human colon carcinoma cell line that is deficient in both MGMT and MMR, in consequence it is insensitive to both TMZ and CP.[28-30] Both mono- and bi-functional agent families showed considerably more potent activity than TMZ and MTZ in this cell line and, in further contrast, activity was retained in the p53-deficient mutant, Table 5. TMZ activity is known to be p53 dependent.[31] Compounds were also evaluated against an isogenic pair of SNB19 GBM cell lines competent in MMR that either expressed MGMT, SNB19(MGMT), or were negative for MGMT, SNB19(vector). This pair of cell lines highlights the improvements the new bi-functional agents offer over TMZ. The MGMT-proficient line being remarkably insensitive to TMZ (IC_{50} is unattainable) and the new bi-functional agents showing equivalent activity against the two lines (IC_{50} 6-12 μM) with increased potency against both SNB19(vector) and SNB19 (MGMT). A2058 is an aggressive melanoma line that is TMZ-sensitive and even so, the new bi-functional agents show a 10-fold increase in potency.

	A2058	**HCT116**	**SNB19(vector)**	**SNB19(MGMT)**
2g	>100	20.0	77.0	>100
3c	5.5	12.5	11.0	12.0
3e	2.5	5.5	6.6	7.0
TMZ	35.5	>250	37.0	>250

Table 4. IC_{50} (μM) for new compounds in selected TMZ-resistant cell lines using the Cyquant Assay (n=2).

	HCT116+/+[a]	HCT116−/− [a]	Ratio +/+: −/−
2d	55 (8.2)	35.8 (7.1)	1.5
2g	13.2 (14.1)	9.5 (4.8)	1.4
3a	5.5 (1.2)	4.8 (2.1)	1.1
3b	6.2 (n=2)	4.4 (2.7)	1.4
3c	28 (8.1)	17 (5.3)	1.6
3e	7.6 (2.1)	9.9 (n=2)	0.8
MTZ	60 (11.2)	17.9 (8.9)	3.4
CP	11.8 (n=2)	1.9 (2.5)	6.2

Table 5. p53 Independence of chemosensitivity of HCT116 cell lines proficient and deficient in p53. IC_{50}, μM (SD). [a] IC_{50} (μM)

Discussion and Conclusions

The aims of generating new imidazotetrazine prodrugs with activity independent of MGMT and MMR have been achieved through a rationally-designed switch in molecular mechanism. This complements other work, where alternative G*O*6-lesion dependent strategies have been pursued.[29] Two new families of mono- and bi-functional 3-anilinoethyl imidazotetrazines have been prepared. These were designed to shift the mechanism of action from dependence on G*O*6-DNA modification to G*N*7-modification. This was achieved through trapping the diazonium electrophile released on hydrolysis of the imidazotetrazine prodrug as an aziridinium ion. The mono-functional agent **2b** is efficiently converted to an aziridinium ion (Scheme 2) and **2b** and **3b** are both DNA G*N7*-alkylators.[15] It is proposed, on the basis of analogy with reactive intermediates generated by nitrogen mustard and similar prodrugs, that biological activity is likely associated with this lesion. However it is not yet possible to distinguish definitively between a mechanism of action deriving from G*N*7-adduct formation and one proceeding from G*O*6-adducts that are resistant to MGMT-mediated repair.

Screening in A2780 and A2780-cp70 cell lines showed that the new compounds are more potent than TMZ. The mono-functional agents are approximately equipotent with MTZ and the bi-functional agents more active. This activity is independent of MGMT activity as determined by use of the MGMT inactivator PaTrin2. Dependence of the IC_{50} on MMR is greatly reduced from approximately 30-fold for TMZ to 2–5-fold for the new bi-functional agents. The Hammett plot, Figure 2, for the bi-functional agents demonstrates the critical rôle of the *para*-substituent in directing the reactivity and bioactivity of the compounds, consistent with the aziridinium ion mechanism occurring *in vitro* and corroborating the design principles. The data identified the *p*-toluidine derivative **3b** as the optimal analog for MGMT and MMR independence and potency.

In the NCI60 screen, the new agents were not uniformly cytotoxic and showed distinct and individual patterns of response over a near 3-log range of GI_{50}. The patterns of biological response to the new agents were distinct from standard agents drawn from the imidazotetrazine, nitrogen mustard and nitrosourea families which, by chemical consideration, may be predicted to share similar mechanisms of action. For all compounds evaluated in the full 60 cell line screen, there was neither correlation with MLH1 or MSH6, nor inverse correlation with MGMT protein expression levels. Correlations in the Molecular Targets database suggest there may be a specific molecular mechanism that accounts for the sensitivity of the leukemia sub-panel to the new agents and, moreover, that activity could be linked to RAS family oncogenes.

Collectively, the *in vitro* data show that the initial aims of improved potency and MGMT and MMR independence have been achieved. The switch of chemical mechanism has resulted in new agents that are distinct from existing imidazotetrazines, nitrogen mustards and nitrosoureas. This implies that an increased diversity of tumor types may now be able to respond to drugs of the imidazotetrazine class. NCI60 panel data identified CP-resistant ovarian carcinoma cell lines as responsive to the new agents. Chemosensitivity in selected drug-resistant colon carcinoma and glioma cell lines (HCT116 and SNB19) showed that there is activity against TMZ- and CP-resistant tumor lines of these types, and

moreover, that the HCT116 response is independent of p53 status. The ability of cells to repair damage by the new agents is linked to the ATR and FANC pathways for the bi-functional and ATM and FANC pathways for mono-functional agents.[32] Mutation in one of these genes would hypersensitize a tumour to these new agents.

Overall these findings validate our understanding of the underlying chemistry of imidazotetrazine prodrugs. The members of our compound library are distinctive new agents, worthy of further development and application against a more diverse range of tumor types than TMZ.

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Experimental

Synthesis

Reagents were purchased from Sigma-Aldrich, Alfa Aesar and Fluka, solvents from Fisher Scientific. TLC was performed on highly-purified silica gel plates with UV indicator (silica gel F_{245}), manufactured by Merck and visualized under UV light (366 or 254 nm) or stained with iodine. Melting points were determined with an Electrothermal IA9200 digital melting point apparatus. Infrared data were obtained using a Perkin Elmer (Paragon 1000) FT-IR Spectrophotometer. NMR spectra were acquired on a JEOL GX270 Delta, or where indicated ECA600, spectrometers observing ^{1}H at 270.05 and 600.17 MHz and ^{13}C at 67.80 and 150.91 MHz respectively. ^{13}C assignments were made with the aid of the DEPT135 experiment. Mass spectra were obtained from the EPSRC National Mass Spectrometry Service Centre at the University of Swansea, UK, and the Analytical Centre at the University of Bradford using a Micromass Quattro Ultima mass spectrometer. Elemental analyses were obtained from the Advanced Chemical and Material Analysis Unit at the University of Newcastle upon Tyne, UK. All compounds entering biological evaluation were dried *in vacuo* for 3–4 days ≤35 ℃ ; ≥95% purity was adjudged by combustion analysis and hplc, solvation is indicated where appropriate; corresponding analytical hplc chromatograms and highfield ^{1}H NMR spectra are shown in ESI.

Compounds **2b** and **3b** were prepared as described in reference 15.

Methyl 3-(4-chlorophenylamino)propanoate 12g

General Method A: Single Conjugate Addition to Anilines.

4-Chloroaniline (20.0 g, 157 mmol) was mixed with methyl acrylate (54.0 g, 627 mmol, 4 eq), cuprous chloride (1.56 g, 15.68 mmol, 0.1 eq) and AcOH (30 mL) and heated under reflux at 160 ℃ for 2 h. The solvents were removed by evaporation and the residue partitioned between chloroform (500 mL) and water (500 mL). The organic layer was dried ($MgSO_4$) in the presence of charcoal and filtered through celite. The solvents were removed leaving an oily residue. Petroleum

ether (200 mL) was added and heated to reflux. The solvent was decanted and the product precipitated upon standing. The solid was collected by filtration, washed with petroleum ether and dried to give the ester (23.7 g, 71 %): ^{1}H NMR ($CDCl_3$) 7.11 (d, *J* = 8.8 Hz, 2H, 2-H & 6-H), 6.55 (d, *J* = 8.8 Hz, 2H, 3-H & 5-H), 3.68 (OCH_3), 3.41 (t, *J* = 7.2 Hz, 2H, CH_2N), 2.61 (t, *J* = 7.2 Hz 2H, CH_2CO); ^{13}C NMR ($CDCl_3$) 172.8 (C=O), 146.1 (C-1), 129.2 (C-2 & C-6), 122.5 (C-4), 114.3 (C-3 & C-5), 51.9 (OCH_3), 39.7 (CH_2N), 33.6 ($\underline{C}H_2CO$); MS (ES): m/z 214.1 $(M+H)^{\bullet+}$; IR (film) 3400, 1725, 1600, 1500, 1435 cm^{-1}.

Methyl 3-(methyl(phenyl)amino)propanoate 12a[15, 33]

Prepared by General Method A. Ester **12a** was obtained as a colorless oil (13.3 g, 74 %). ^{1}H NMR ($CDCl_3$) 7.16 (t, 2H, *J* = 8.6 Hz, 3-H & 5-H), 6.64 (m, 3H, 2-H, 4-H & 6-H), 3.58 (m, 5H, CH_2N & CH_3N), 2.85 (s, 3H, OCH_3), 2.49 (t, *J* = 7.3 Hz, 2H, CH_2CO) ^{13}C NMR ($CDCl_3$) 172.8 (C=O), 148.6 (C-1), 129.4 (C-3 & C-5), 116.9 (C-4), 112.6 (C-2 & C-6), 51.8 (OCH_3), 48.7 (NCH_2), 38.3 (NCH_3), 31.6 ($\underline{C}H_2CO$); MS (ES): m/z 194.1$(M+H)^{\bullet+}$; ν_{max} (film) 1725s (C=O), 1175m (C-O) cm^{-1}

Methyl 3-((4-fluorophenyl)(methyl)amino)propanoate 12c

Prepared according to General Method A. Ester **12c** was obtained as an orange oil (2.22 g, 75%). ^{1}H NMR ($CDCl_3$) 6.92 (m, 2H, 3-H & 5-H), 6.66 (m, 2H, m, 2-H & 6-H), 3.65 (s, 3H, OCH_3), 3.60 (t, *J* = 7.3 Hz, 2H, NCH_2), 2.86 (NCH_3), 2.52 (t, *J* = 7.3 Hz, 2H, CH_2CO); ^{13}C NMR ($CDCl_3$) 172.8 (C=O), 155.7 (d, $^{1}J_{CF}$ = 235 Hz, C-4), 145.5 (C-1), 115.7 (d, $^{2}J_{CF}$ = 22 Hz, C-3 & C-5), 114.1 (d, $^{3}J_{CF}$ = 7 Hz, C-2 & C-6), 51.8 (CH_3O), 49.5 (CH_2N), 38.7 (CH_3N), 31.5 ($\underline{C}H_2CO$); MS (ES): m/z 212.1 $(M+H)^{\bullet+}$; IR (KBr) 3025m (Ar C-H), 2950m (C-H), 1725s (C=O), 1525m (Ar C=C), 1175m (C-O) cm^{-1}.

Methyl 3-((4-chlorophenyl)(methyl)amino)propanoate 12d

Prepared according to General Method A. Ester **12d** was obtained as a yellow oil (9.1 g, 54%). ^{1}H NMR ($CDCl_3$) 7.15 (d, *J* = 8.9 Hz, 2H, 3-H & 5-H), 6.63 (d, *J* = 8.9 Hz, 2H, 2-H & 6-H), 3.63 (s,

3H, OCH_3), 3.62 (t, *J* = 7.2 Hz, 2H, NCH_2), 2.89 (s, 3H, NCH_3), 2.54 (t, *J* = 7.2 Hz, 2H, CH_2CO); ^{13}C NMR ($CDCl_3$) 172.6 (C=O), 147.2 (C-1), 129.1 (C-3 & C-5), 121.7 (C-4), 113.9 (C-2 & C-6), 51.9 (OCH_3), 48.8 (NCH_2), 38.4 (NCH_3), 31.5 ($\underline{C}H_2CO$); MS (ES): m/z 228.1 $(M+H)^{\bullet+}$; IR (KBr) 3025m (Ar C-H), 2950m (C-H), 1725s (C=O), 1525m (Ar C=C), 1175m (C-O) cm^{-1}.

Methyl 3-((4-methoxyphenyl)(methyl)amino)propanoate 12e[33]

According to General Method A. Ester **12e** was obtained as a colorless oil (4.72 g, 58%): ^1H NMR ($CDCl_3$) 6.76 (d, 2H, *J* = 9.0 Hz, 3-H & 5-H), 6.67 (d, 2H, *J* = 9.0 Hz, 2-H & 6-H), 3.68 (s, 3H, CH_3O-Ar), 3.59 (s, 3H, CH_3OCO), 3.51 (t, *J* = 7.3 Hz, 2H, CH_2N), 2.78 (s, 3H, CH_3N), 2.46 (t, *J* = 7.3 Hz, 2H, CH_2CO); ^{13}C NMR ($CDCl_3$) 172.4 (C=O), 151.6 (C-4), 143.1 (C-1), 114.6 & 114.3 (C-3, C-5 & C2, C6), 55.3 (CH_3O-Ar), 51.2 (CH_3OCO), 49.4 (NCH_2), 38.4 (CH_3N), 30.9 ($\underline{C}H_2CO$); MS (ES): m/z 224.1 $(M+H)^{\bullet+}$; IR (film) 1725s (C=O), 1175m (C-O) cm^{-1}.

Methyl 3-(*tert*-butoxycarbonyl-(4-chlorophenyl)amino)propanoate 12f

Ester **12g** (2.27 g, 10.6 mmol) was mixed with di-*tert*-butyl carbonate (9.3 g, 42.6 mmol, 4 eq) in the absence of solvent. The mixture was heated at 100 ℃ for 18 h. The mixture was partitioned between H_2O and petroleum ether. The organic phase was washed with H_2O (5 x 50 mL), dried and evaporated to give ester **4h** as an oil (3.2 g, 97%). ^1H NMR ($CDCl_3$) 7.29 (d, *J* = 8.8 Hz, 2H, 2-H & 6-H), 7.11 (d, *J* = 8.8 Hz, 2H, 3-H & 5-H), 3.91 (t, *J* = 7.2 Hz, 2H, NCH_2), 3.53 (OCH_3), 2.57 (t, *J* = 7.2 Hz, 2H, CH_2CO), 1.41 (s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$) 172.3 (OC=O), 154.4 (NC=O), 141.0 (C-1), 130.9 (C-4), 129.1 (C-2 & C-6), 113.7 (C-3 & C-5), 79.8 ($\underline{C}(CH_3)_3$), 51.8 (OCH_3), 48.2 (NCH_2), 32.8 ($\underline{C}H_2CO$), 28.4 (CH_3); MS (ES): m/z 314.1 $(M+H)^{\bullet+}$; IR (film) 1725s (C=O), 1175m (C-O) cm^{-1}.

3-(4-Chlorophenylamino)propanehydrazide 13g[34, 35]

General Method B: Preparation of monohydrazides.

Ester **12g** (3.7 g, 11.8 mmol, 1 eq) was mixed with hydrazine hydrate (5.9 g, 118 mmol, 10 eq) in propan-2-ol (10 mL) for 48h at RT. The volatiles were removed with evaporation to leave hydrazide **13g** as colorless oil (3.5 g, 95%). ^{1}H NMR (DMSO-d_6) 9.03 (br s, 1H, N<u>H</u>NH$_2$), 7.05 (d, 2H, *J* = 8.9 Hz, 2-H & 6-H), 6.53 (d, 2H, *J* = 8.8 Hz, 3-H & 5-H), 5.76 (br s, 1H, NH-aniline), 4.28 (s, br, 2H, NH$_2$), 3.19 (t, *J* = 7.2 Hz, 2H, NCH$_2$), 2.41 (t, *J* = 7.2 Hz, 2H, CH$_2$CO); ^{13}C NMR (DMSO-d_6) 170.5 (C=O), 148.0 (C-1), 129.1 (C-3 & C-5), 119.4 (C-4), 113.9 (C-2 & C-6), 39.9 (CH$_2$N), 33.7 (<u>C</u>H$_2$CO); MS (ES): m/z 214.1 (M+H)$^{\bullet+}$; IR (KBr) 3300s (NH), 3050m (Ar C-H), 1650s (CONH) cm^{-1}.

3-(Methyl(phenyl)amino)propanehydrazide 13a

Prepared according to General Method B. Hydrazide **13a** was obtained as a colorless oil (12.7 g, 98%). ^{1}H NMR (CDCl$_3$) 7.94 (br s, 1H, NH), 7.20 (t, 2H, *J* = 7.8 Hz, 3-H & 5-H), 6.69 (m, 3H, 2-H, 4-H, 6-H), 3.62 (br, 4H, CH$_2$N & NH$_2$), 2.86 (s, 3H, CH$_3$N), 2.33 (t, *J* = 6.8 Hz, 2H, CH$_2$CO); ^{13}C NMR (CDCl$_3$) 172.5 (C=O), 148.7 (C-1), 129.4 (C-3 & C-5), 117.1 (C-4), 112.8 (C-2 & C-6), 49.2 (NCH$_2$), 38.6 (NCH$_3$), 31.9 (<u>C</u>H$_2$CO); MS (ES): m/z 194.1 (M+H)$^{\bullet+}$; IR (KBr) 3300s (NH), 3050m (Ar C-H), 1650s (CONH) cm^{-1}.

3-((4-Fluorophenyl)(methyl)amino)propanehydrazide 13c

Prepared according to General Method B. Hydrazide **13c** was obtained as a yellow oil (2.16 g, 97%): ^{1}H NMR (CDCl$_3$) 7.47 (br s, 1H, NH), 6.91 (m, 2H, 3-H & 5-H), 6.66 (m, 2H, 2-H & 6-H), 3.56 (t, *J* = 6.8 Hz, 2H, CH$_2$N), 3.5 (br s, 2H, NH$_2$), 2.83 (s, 3H, NCH$_3$), 2.34 (t, *J* = 6.8 Hz, 2H, CH$_2$CO); ^{13}C NMR (CDCl$_3$) 172.4 (C=O), 156.0 (d, $^{1}J_{CF}$ = 237 Hz, C-4), 145.6 (C-1), 115.8 (d, $^{2}J_{CF}$ = 22 Hz, C-3 & C-5), 114.8 (d, $^{3}J_{CF}$ = 8 Hz, C-2 & C-6), 50.1 (CH$_2$N), 39.3 (CH$_3$N), 31.8 (<u>C</u>H$_2$CO); MS (EI): m/z 212.1 (M+H)$^{\bullet+}$; IR (KBr) 3300m (NH), 3050m (Ar C-H), 1650s (CONH), 1525m (Ar C=C) cm^{-1}.

3-((4-Chlorophenyl)(methyl)amino)propanehydrazide 13d

Prepared according to General Method B. Hydrazide **13d** was obtained as white solid (4.0 g, 44%): ^{1}H NMR (CDCl$_3$) 7.15 (d, 2H, *J* = 8.9 Hz, 3-H & 5-H), 7.03 (s, 1H, NH), 6.62 (d, 2H, *J* = 8.9

Hz, 2-H & 6-H), 3.86 (br s, 2H, NH_2), 3.63 (t, *J* = 6.8 Hz, 2H, CH_2N), 2.88 (s, 3H, NCH_3), 2.35 (t, *J* = 6.8 Hz, 2H, CH_2CO); ^{13}C NMR ($CDCl_3$) 172.2 (C=O), 147.3 (C-1), 129.2 (C-3 & C-5), 122.1 (C-4), 114.0 (C-2 & C-6), 49.3 (NCH_2), 38.8 (NCH_3), 31.8 ($\underline{C}H_2CO$); MS (ES): m/z 226.9 $(M+H)^{\bullet+}$; IR (KBr) 3300m (NH), 3050m (Ar C-H), 1650s (CONH), 1525m (Ar C=C) cm^{-1}.

3-((4-Methoxyphenyl)(methyl)amino)propanehydrazide 13e

Prepared according to General Method B. Hydrazide **13e** was obtained as an oil which crystallized upon standing (4.5 g, 95%): ^1H NMR ($CDCl_3$) 7.63 (br s, 1H, NH), 6.80 (d, 2H, *J* = 9.2 Hz, 3-H & 5-H), 6.75 (d, 2H, *J* = 9.2 Hz, 2-H & 6-H), 3.72 (s, 3H, CH_3O-Ar), 3.47 (t, *J* = 6.6 Hz, 2H, CH_2N), 3.3 (br s, 2H, NH_2), 2.79 (s, 3H, NCH_3), 2.33 (t, *J* = 6.6 Hz, 2H, CH_2CO); ^{13}C NMR ($CDCl_3$) 172.7 (C=O), 152.9 (C-4), 143.7 (C-1), 116.5 (C-2 & C-6), 114.9 (C-3 & C-5), 55.8 (CH_3O-Ar), 50.7 (CH_2N), 40.0 (NCH_3), 31.9 ($\underline{C}H_2CO$); MS (ES): m/z 224.1 $(M+H)^{\bullet+}$; IR (KBr) 3300s (NH), 3050m (Ar C-H), 1650s (CONH) cm^{-1}.

***tert*-Butyl-4-chlorophenyl(3-hydrazinyl-3-oxopropyl)carbamate 13f**

Prepared according to General Method B. Hydrazide **13f** was obtained as a colorless oil (3.5 g, 95%). ^1H NMR ($CDCl_3$) 7.76 (br s, 1H, NH), 7.22 (d, 2H, *J* = 7.8 Hz, 2-H & 6-H), 7.04 (d, 2H, *J* = 7.8 Hz, 3-H & 5-H), 3.86 (t, *J* = 6.8 Hz, 2H, NCH_2), 3.65 (s, br, 2H, NH_2), 2.41 (t, *J* = 6.8 Hz, 2H, CH_2CO), 1.41 (s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$) 173.6 (C=O), 154.3 (NC=O), 141.8 (C-1), 131.1 (C-4), 129.2 (C-2 & C-6), 114.1 (C-3 & C-5), 79.8 ($\underline{C}(CH_3)_3$), 48.7 (NCH_2), 34.2 ($\underline{C}H_2CO$), 20.3 ($ArCH_3$); MS (ES): m/z 314.1 $(M+H)^{\bullet+}$; IR (KBr) 3300s (NH), 3050m (Ar C-H), 1650s (CONH) cm^{-1}.

3-(2-(4-Chlorophenylamino)ethyl)-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide 2g

General Method C: Preparation of mono-imidazotetrazines

Hydrazide **13g** (1.0 g, 4.68 mmol) was dissolved in dichloromethane (20 mL). Water (20 mL) was added followed by HCl (2.5 mL, 37%). The mixture was cooled in an ice/$CaCl_2$ bath and a solution of $NaNO_2$ (0.39 g, 56.2 mmol, 1.2 eq) in water (10 mL) was added with strong agitation at below –5 ℃. The ice bath was removed and dichloromethane (20 mL) was added. The reaction mixture was stirred for 40 min and the organic layer was separated, dried over $MgSO_4$ and evaporated to give the crude azide **14g** as an oil, identified by IR. The oil was diluted with toluene (100 mL) and heated under reflux for 1 h under N_2. The volatile components were removed to give the crude isocyanate **15g** as an oil. Diethylether (150 mL) was added and the mixture heated with strong agitation. The hot ether solution was decanted leaving an oily residue of impurities behind. The ether layer was evaporated to leave pure isocyanate as pale yellow oil (IR ν_{max} 2260s). The isocyanate (0.1 g, 0.48 mmol) was mixed with diazo-IC **5**[18] (0.07 g, 0.48 mmol, 1 eq) in dry DMSO (0.3 mL) under N_2 at RT in the absence of light for 24 h. The reaction was quenched with water (10 mL) and the resultant solid collected by filtration and washed with copious amounts of water to leave imidazotetrazine **2g** as a brown solid (0.16 g, 29%): m.p. 160–161 ℃; ^{1}H NMR (DMSO-d_6, 600 MHz) 8.84 (s, 1H, imidazole CH), 7.82 & 7.70 (2 × br s, 2H, $CONH_2$), 7.62 (d, *J* = 8.8 Hz, 2H, 3-H & 5-H), 7.54 (d, *J* = 8.8 Hz, 2H, 2-H & 6-H), 4.52 (t, *J* = 6.0 Hz, 2H, NCH_2), 4.42 (t, *J* = 6.0 Hz, 2H, CH_2CO), 3.3 (br s, NH-aniline & H_2O); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.8 (C=O amide), 140.0 (C-1), 139.4 (C=O tetrazine), 134.4 (Cq tetrazine), 132.7 (C-4), 131.8 (Cq imidazole), 130.0 (C-3 & C-5), 129.5 (C-H imidazole), 122.3 (C-2 & C-6), 45.9 (CH_2N-aniline), 42.0 (CH_2N-tetrazine); MS (ES): m/z 334.1 $(M+H)^{\bullet+}$; IR (KBr) 3450m & 3300 ($CONH_2$), 1750s (C=O), 1650s ($CONH_2$), 1600m & 1500s (Ar-H) cm^{-1}. Anal. $C_{20}H_{19}N_{13}O_4 \cdot 0.25H_2O$, CHN.

3-(2-(Methyl(phenyl)amino)ethyl)-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide 2a

Prepared according to General Method C. The product was purified by flash column chromatography (2% MeOH/$CHCl_3$) and imidazotetrazine **2a** was obtained as a white solid (0.34 g, 21%): m.p. 186–187 ℃. ^{1}H NMR (DMSO-d_6, 600 MHz) 8.77 (s, 1H, imidazole CH), 7.79 & 7,68 (2

× br s, 2H, $CONH_2$), 7.06 (t, *J* = 8.6 Hz, 2H, 3-H & 5-H), 6.68 (d, *J* = 8.6 Hz, 2H, 2-H & 6-H), 6.50 (t, *J* = 8.6 Hz, 1H, 4-H), 4.46 (t, *J* = 6.4 Hz, 2H, NCH_2), 3.80 (t, *J* = 6.4 Hz, 2H, CH_2CO), 2.91 (s, 3H, NCH_3); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.9 ($CONH_2$), 148.9 (C-1), 139.6 (C=O tetrazine), 134.7 (Cq tetrazine), 131.3 (Cq imidazole), 129.4 (C-3 & C-5), 129.2 (C-H imidazole), 116.6 (C-4), 112.4 (C-2 & C-6), 50.3 (CH_2N-aniline), 46.4 (CH_2N-tetrazine), 38.3 (CH_3); MS (ES): m/z 314.2 $(M+H)^{\bullet+}$; 627.4 $(2M+H)^{\bullet+}$; IR [illegible] (1750s (C=O), 1675s ($CONH_2$), 1600m (Ar-H) cm^{-1}.

Anal. $C_{14}H_{15}N_7O_2$, CHN.

3-(2-((4-Chlorophenyl)(methyl)amino)ethyl)-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide 2d

Prepared by General Method C. The product was precipitated with water, collected by filtration and the crude solid dried and suspended in chloroform (20 mL). The impurities were removed by filtration and the filtrate evaporated to yield imidazotetrazine **2d** as a yellow solid (0.084 g, 25%): m.p. 154–155 ℃ ^{1}H NMR (DMSO-d_6, 600 MHz) 8.76 (s, 1H, imidazole CH), 7.75 & 7.65 (2 × br s, 2H, $CONH_2$), 7.07 (d, *J* = 8.8 Hz, 2H, 3-H & 5-H), 6.66 (d, *J* = 8.8 Hz, 2H, 2-H & 6-H), 4.43 (t, *J* = 6.6 Hz, 2H, CH_2N), 3.77 (t, *J* = 6.6 Hz, 2H, CH_2CO), 2.87 (s, 3H, NCH_3); ^{13}C NMR (151 MHz, DMSO-d_6) 161.9 ($CONH_2$), 147.8 (C-1), 139.6 (C=O tetrazine), 134.7 (Cq tetrazine), 131.4 (Cq imidazole), 129.2 (C-H imidazole), 129.1 (C-3 & C-5), 120.3 (C-4), 113.9 (C-2 & C-6), 50.4 (CH_2N-aniline), 46.3 (CH_2N-tetrazine), 38.5 (CH_3); MS (ES): m/z 348.2 $(M+H)^{\bullet+}$; 695.3 $(2M+H)^{\bullet+}$; 717.3 $(2M+Na)^{\bullet+}$; IR (KBr) 3450m ($CONH_2$), 1750s (C=O), 1675s ($CONH_2$), 1600m & 1500s (Ar-H) cm^{-1}.

Anal. $C_{14}H_{14}ClN_7O_2 \cdot 0.5\ H_2O$, CHN.

***tert*-Butyl 2-(8-carbamoyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-3(4H)-yl)ethyl(4-chlorophenyl) carbamate 2f**

Prepared according to General Method C. The product was purified by flash column chromatography (10% AcOH/$CHCl_3$) and imidazotetrazine **2f** was obtained as a white solid (0.15 g, 51%): m.p. 177–178 ℃; ^{1}H NMR (DMSO-d_6, 600 MHz) 8.91 (s, 1H, imidazole CH), 7.83 & 7.70 (2

× br s, 2H, $CONH_2$), 7.40 (d, *J* = 8.8 Hz, 2H, 3-H & 5-H), 7.32 (d, *J* = 8.8 Hz, 2H, 2-H & 6-H), 4.40 (t, *J* = 6.7 Hz, 2H, NCH_2), 4.04 (t, *J* = 6.5 Hz, 2H, CH_2CO), 1.11 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.8 ($CONH_2$), 154.3 (N(CO)O), 140.8 (C-1), 139.6 (CO tetrazine), 134.6 (Cq tetrazine), 131.4 (Cq imidazole), 131.0 (C-4), 129.4 (C-H imidazole), 129.2 & 129.1 (C-3 & C-5,C-2 & C-6), 80.74 ($\underline{C}(CH_3)_3$), 48.3 & 48.0 (CH_2N-tetrazine & CH_2N-aniline), 28.0 (CH_3); MS (ES): m/z 434.3 $(M+H)^{\bullet+}$; 456.2 $(M + Na)^{\bullet+}$; IR (KBr) 3450m ($CONH_2$), 3150m ($CONH_2$), 1750s (C=O), 1700s ($CONH_2$), 1600m & 1500s (Ar-H) cm^{-1}. Anal. $C_{18}H_{20}ClN_7O_4 \cdot {}^1/_3\ H_2O$, CHN.

3-(2-(Methyl(4-methyl-3-nitrophenyl)amino)ethyl)-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide 2h

Prepared according to General Method C from hydrazide **13b**. On completion of the reaction, the mixture was quenched with propan-2-ol (10 mL) and the solid collected by filtration and washed with propan-2-ol then methanol to leave imidazotetrazine **2h** as an orange solid (0.43 g, 24%): m.p. 133–134 ℃. ^1H NMR (DMSO-d_6, 600 MHz) 8.76 (s, 1H, imidazole CH), 7.77 & 7,66 (2 × br s, 2H, $CONH_2$), 7.38 (d, *J* = 2.0 Hz, 1H, 2-H), 7.20 (d, *J* = 8.4 Hz, 1H, 5-H), 7.14 (br d, *J* = 8.4 Hz, 1H, 6-H), 4.44 (t, *J* = 6.2 Hz, 2H, CH_2N-aniline), 3.45 (t, *J* = 6.2 Hz, 2H, CH_2N-tetrazine), 2.75 (s, 3H, NCH_3), 2.12 (s, 3H, $ArCH_3$); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.9 ($CONH_2$), 143.0 (C-3), 142.5 (C-1), 139.4 (C=O tetrazine), 134.6 (Cq tetrazine), 134.3 (C-2), 131.4 (Cq imidazole), 131.3 (C-4), 129.2 (C-H imidazole), 125.3 (C-6), 121.8 (C-5), 53.4 (CH_2N-aniline), 46.4 (CH_2N-tetrazine), 40.7 (CH_3N), 20.0 ($ArCH_3$); MS (ES): m/z 373.2 $(M+H)^{\bullet+}$; 395.2 $(M + Na)^{\bullet+}$. Anal. $C_{15}H_{16}N_8O_4 \cdot 0.15\ H_2O$, CHN.

3-(2-((4-Methoxy-2-nitrophenyl)(methyl)amino)ethyl)-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide 2i

Prepared according to General Method C from **13e**. The product was purified by flash column chromatography (10% AcOH/$CHCl_3$). The solid was washed with 50% MeOH/H_2O and imidazotetrazine **2i** was obtained as a purplish red solid (0.21 g, 26%): m.p. 166–168 ℃. ^1H NMR

(DMSO-d_6, 600 MHz) 8.80 (s, 1H, imidazole CH), 7.79 & 7.67 (2 × br s, 2H, $CONH_2$), 7.36 (d, *J* = 8.9 Hz, 1H, 6-H), 7.18 (d, *J* = 3.0 Hz, 1H, 3-H), 7.07 (dd, *J* = 3.0, 8.9 Hz, 1H, 5-H), 4.42 (t, *J* = 6.0 Hz, 2H, CH_2N-aniline), 3.68 (s, 3H, OCH_3) 3.35 (t, *J* = 6.0 Hz, 2H, CH_2N-tetrazine), 2.70 (s, 3H, NCH_3); ^{13}C NMR (DMSO-d_6, 151 MHz) 162.0 ($CONH_2$, 155.2 (C-4), 145.8 (C-2), 139.4 (C=O tetrazine), 138.6 (C-1), 134.7 (Cq tetrazine), 131.3 (Cq imidazole), 129.2 (C-H imidazole), 125.1 (C-6), 119.9 (C-3), 108.9 (C-5), 56.4 (CH_3O), 54.5 (CH_2N-aniline), 46.6 (CH_2N-tetrazine), 42.0 (NCH_3); MS (ES): m/z 389.2 $(M+H)^{\bullet+}$; 411.2 $(M + Na)^{\bullet+}$; IR (KBr) 3425m ($CONH_2$), 3150m ($CONH_2$), 1750s (C=O), 1700s ($CONH_2$), 1600m & 1525s (Ar-H) cm^{-1}. Anal. $C_{15}H_{16}N_8O_5 \cdot 0.1\ H_2O$, CHN.

Modified Procedure for tetrazine 2c

3-(2-(*N*-(4-Fluorophenyl)-*N*-methylamino)ethyl)-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 2c

3-(*N*-(4-Fluorophenyl)-*N*-methylamino)propanoyl azide 14c

Hydrazide **13c** (0.25 g, 1.2 mmol) was dissolved in a mixture of AcOH (0.66M 15 mL) and DCM (15 mL), the mixture was then stirred on a $CaCl_2$-ice bath at 0 ºC, a solution of $NaNO_2$ (0.49 g, 7.1 mmol) in H_2O (25 mL) was added dropwise keeping the exothermic reaction at 0–5 ºC. A further amount of DCM (20 mL) was added, the DCM layer separated, washed with H_2O (20 mL) dried over $MgSO_4$, then filtered. Formation of azide **14c** was confirmed by IR. ^{1}H NMR ($CDCl_3$, 600 MHz): 6.93 (t, 2H, $J_{HH} = {}^3J_{HF}$ = 9.1 Hz, 3',5'-H), 6.66 (dd, 2H, J_{HH} = 9.1 Hz, $^4J_{HF}$ = 4.3 Hz, 2',6'-H), 3.60 (t, 2H, *J* = 7.1 Hz, 3-H), 2.86 (s, 3H, NCH_3), 2.55 (t, 2H, *J* = 7.1 Hz, 2-H); ^{13}C NMR ($CDCl_3$, 151 MHz) 179.2 (C-1), 155.3 (d, $^1J_{CF}$ = 237 Hz, C-4'), 145.2 (C-1'), 115.8 (d, $^2J_{CF}$ = 21.7 Hz, C-3',5'), 114.3 (d, $^3J_{CF}$ = 7.2 Hz, C-2',6'), 49.4 (C-3), 38.9 (NCH_3), 34.2 (C-2); IR (liq. film) 2914w (CH st), 2137s (CON_3), 1711s (CO), 1511m (C=C), 815s (*p*-di-substituted aromatic ring) cm^{-1}.

2-(*N*-(4-Fluorophenyl)-*N*-methylamino)ethyl isocyanate 15c

The anhydrous DCM solution of azide **14c** was stirred under nitrogen at RT overnight. Isocyanate **15c** formation was confirmed by IR and ^{1}H NMR. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature). The isocyanate was collected as a yellow oil (0.2 g, 77%). ^{1}H NMR ($CDCl_3$, 600 MHz) 6.95 (t, 2H, J_{HH} = $^{3}J_{HF}$ = 9.1 Hz, 3',5'-H), 6.69 (dd, 2H, J_{HH} = 9.1 Hz, $^{4}J_{HF}$ = 4.3 Hz, 2',6'-H), 3.43 (m, 4H, 1,2-H), 2.94 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 151 MHz) 157.2 (NCO), 155.3 (d, $^{1}J_{CF}$ = 237 Hz, C-4'), 145.2 (C-1'), 115.8 (d, $^{2}J_{CF}$ = 21.7 Hz, C-3',5'), 114.4 (d, $^{3}J_{CF}$ = 7.2 Hz, C-2',6'), 54.5 (C-3), 40.9 (C-2), 39.6 (NCH_3); IR (liq film) 3057w (Ar-CH st), 2917w (CH st), 2270s (NCO), 1512m (C=C), 1228m, 816s (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES) m/z 195 (100%) $(M+H)^{+}$.

3-(2-(*N*-(4-Fluorophenyl)-*N*-methylamino)ethyl)-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 2c

Isocyanate **15c** (0.2 g, 1.03 mmol) was diluted with DMSO (1.5 mL) under nitrogen then added to a suspension of diazo-IC **5** (0.14 g, 1.03 mmol) in DMSO (1.5 mL), the mixture was stirred at rt protected from light for 48 h. The ^{1}H NMR spectrum showed product **2c** formation. The reaction mixture was suspended in H_2O (30 mL) and filtered, the residue was washed with copious amounts of H_2O until the washings came through colorless and then washed with Et_2O. The solid was purified by flash column chromatography, 10% AcOH in $CHCl_3$ was used for the elution; the fractions containing the product were evaporated to give an orange solid. The solid was then re-dissolved in $CHCl_3$ and filtered, the $CHCl_3$ evaporated and the residue re-suspended in Et_2O and then collected by filtration to give **2c** (0.057 g, 17%), m.p. 172–173 ºC . ^{1}H NMR (DMSO-d_6, 600 MHz): 8.77 (s, 1H, 6-H), 7.75 & 7.65 (2 × br s, 2H, $CONH_2$), 6.91 (t, 2H, J_{HH} = J_{HF} = 9.1 Hz, 3',5'-H), 6.69 (dd, 2H, J_{HH} = 9.1 Hz, J_{HF} = 4.3 Hz, 2',6'-H), 3.45 (t, 2H, J = 6.4 Hz, 1-H), 3.77 (t, 2H, J = 6.4 Hz, 2-H), 2.90 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 151 MHz) 161.89 ($CONH_2$), 155.0 (d, $^{1}J_{CF}$ = 235.5 Hz, C-4'), 145.9 (C-1'), 139.5 (C-4), 134.7 (C-8a), 131.3 (C-8), 129.2 (C-6), 115.7 (d, $^{2}J_{CF}$ = 21.7 Hz, C-3',5'), 113.6 (d, $^{3}J_{CF}$ = 7.2 Hz, C-2',6'), 50.9 (C-2), 46.3 (C-1) 38.7 (NCH_3); IR (KBr) 3439s (NH), 3117m (Ar-CH st), 2914w (CH st), 1749s (C(4)O), 1682s ($CONH_2$), 1515 s, 1458m (C=C),

820m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES): m/z 331.9 (20%) $(M+H)^+$, 353.9 (10%) $(M+Na)^+$, 195.0 (100%) $(C_{10}H_{11}FN_2O+H)^+$. Anal. $C_{14}H_{14}FN_7O_2 \cdot 0.75H_2O$, CHN.

Modified Procedure for tetrazine 2e

3-(*N*-Methyl-*N*-(4-nitrophenyl)amino)propanoyl azide 14e and

3(*N*-methyl-*N*-(2-nitrophenyl)amino)propanoyl azide

Hydrazide **13a** (0.3g, 1.6 mmol) was dissolved in a mixture of DCM (10 mL) and HCl (10 mL, 14.8%), the solution was stirred at 0 ºC on a $CaCl_2$-ice bath, $NaNO_2$ (0.66 g, 9.6 mmol) solution in H_2O (10 mL) was added gradually, keeping the exothermic reaction between 0–5 ºC. A further portion of DCM (15 mL) was added, the DCM layer was separated, washed with two portions of H_2O (20 mL) dried over $MgSO_4$, then filtered. Formation of the azide **14e** was confirmed by IR and ^{1}H NMR which also showed the formation of small amount of the *ortho*-nitrated azide in ratio 1:5. Azide **14e** ^{1}H NMR ($CDCl_3$, 600 MHz) 8.12 (½AB, 2H, *J* = 9.5 Hz, 3',5'-H), 6.61 (½AB, 2H, *J* = 9.5 Hz, 2',6'-H), 3.77 (t, 2H, *J* = 7.0 Hz, 3-H), 3.10 (s, 3H, NCH_3) 2.62 (t, 2H, *J* = 7.0 Hz, 2-H); ^{13}C NMR ($CDCl_3$) δ : 178.5 (CON_3), 152.8 (C-4'), 133.2 (C-1'), 126.4 (C-3',5'), 110.6 (C-2',6'), 48.0 (C-3), 39.1 (NCH_3), 34.4 (C-2); IR (liq film) 2918w (CH st), 2140s (N_3), 1711s (CO), 1597s (NO_2 st as), 1518 s (C=C), 1311s (NO_2 st) cm^{-1}. ***o*-NO_2-Azide:** ^{1}H NMR ($CDCl_3$, 600 MHz): 7.72 (d, 1H, *J* = 8.9 Hz, 5'-H), 7.42 (t, 1H, *J* = 8.9 Hz, 4'-H), 7.21 (d, 1H, , *J* = 8.9 Hz, 2'-H), 6.96 (t, 1H, *J* = 8.9 Hz, 3'-H), 3.46 (t, 2H, *J* = 7.1 Hz, 3-H), 2.80 (s, 3H, NCH_3) 2.62 (t, 2H, *J* = 7.1 Hz, 2-H).

2-[(4-Nitrophenyl)methylamino]ethylisocyanate 15e

The anhydrous DCM solution of the crude azide **14e** was stirred under nitrogen at RT overnight. Isocyanate formation was confirmed by IR. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature) and the crude isocyanate **15e** collected as a yellow oil (0.27 g, 90 %). ^{1}H NMR ($CDCl_3$, 600 MHz) 8.15 (½AB, 2H, *J* = 9.4 Hz, 3',5'-H), 6.67 (½AB, 2H, *J* = 9.4 Hz, 2',6'-H), 3.66 (t, 2H, *J* = 6.2 Hz, 2-H), 3.57 (t, 2H, *J* = 6.2 Hz, 1-

H), 3.15 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$, 151 MHz)153.1 (NCO), 145.2 (C-4'), 138.1 (C-1'), 126.4 (C-3',5'), 110.7 (C-2',6'), 52.6 (C-2), 40.7 (C-1) , 39.5 (NCH_3); IR (liq film) 2919w (CH st), 2270s (NCO), 1597s (NO_2 st as), 1517 s (C=C), 1311s (NO_2 st) cm^{-1}. **Isocyanate *ortho*-nitro isomer:** ^1H NMR ($CDCl_3$, 600 MHz) 7.74 (d, 1H, *J* = 8.4 Hz, 5'-H), 7.45 (t, 1H, , *J* = 8.4 Hz, 4'-H), 7.18 (d, 1H, , *J* = 8.4 Hz, 2'-H), 6.99 (t, 1H, *J* = 8.9 Hz, 3'-H), 3.48 (t, 2H, *J* = 6.0 Hz, 3-H), 2.90 (s, 3H, NCH_3), 3.31 (t, 2H, *J* = 7.1 Hz, 2-H).

3-(2-*N*-Methyl-*N*-(4-nitrophenyl)-*N*-methylamino)ethyl)-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 2e and

3-(2-(*N*-methyl-*N*-(2-nitrophenyl)-*N*-methylamino)ethyl)-4-oxo-3H,4H-imidazo[5,1d][1,2,3,5]tetrazine-8-carboxamide

The crude mixture of the isocyanate **15e** (0.27 g, 1.22 mmol) was diluted with DMSO (1.5 mL) under N_2 then added to a suspension of diazo-IC **5** (0.17 g, 1.22 mmol) in DMSO (1.5 mL), the mixture was stirred at RT protected from light for 48 h. The reaction mixture was then suspended in water (30 mL) and filtered. The solid on the filter was washed with copious amounts of H_2O until the washings came through colorless, then with Et_2O. The dry solid was purified by flash column chromatography eluted with a gradient 5–20% AcOH in $CHCl_3$. ^1H NMR showed impurities from silica, so the imidazotetrazinone solid was dissolved in DMF, filtered and the imidazotetrazinone precipitated as a yellow solid by H_2O addition. The solid was collected by filtration, washed with copious amounts of water and then dried to give **2e** (0.025g, 6 %), m.p. 179–180 ºC. ^1H NMR (DMSO-d_6, 600 MHz)8.81 (s, 1H, 6-H), 8.00 (½AB, 2H, *J* = 9.2 Hz, 3',5'-H), 7.77 & 7.67 (2 × br s, 2H, $CONH_2$), 6.82 (½AB, 2H, *J* = 9.2 Hz, 2',6'-H), 4.52 (t, 2H, *J* = 6.3 Hz, 1-H), 3.96 (t, 2H, *J* = 6.3 Hz, 2-H), 3.07 (s, 3H, NCH_3); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.9 ($CONH_2$), 154.0 (C-4'), 139.7 (C-4), 136.6 (C-1'), 134.7 (C-8a), 131.6 (C-8), 129.5 (C-6), 126.3 (C-3',5'), 111.4 (C-2',6'), 50.3 (C-2), 46.4 (C-1) 39.2 (NCH_3); IR (KBr) 3443m (NH), 2919w (CH st), 1749s (C(4)O), 1684s (CONH), 1597s (NO_2 st as), 1457m (C=C), 1316s (NO_2 st) cm^{-1}; MS(ES): m/z 359.1(80%) $(M+H)^+$, 381.1(100%) $(M+Na)^+$; Anal. $C_{14}H_{14}N_8O_4 \cdot 0.6\ AcOH \cdot 0.2\ CHCl_3$, CHN.

***meta*-NO_2 isomer:** ^{1}H NMR (DMSO-d_6, 600 MHz) 8.83 (s, 1H, 6-H), 7.83 & 7.71 (2 × br s, 2H, $CONH_2$), 7.65 (d, 1H, *J* = 9.1 Hz, 5'-H), 7.46 (t, 1H, *J* = 9.1 Hz, 4'-H), 7.31 (t, 1H, *J* = 9.1 Hz, 2'-H), 6.92 (d, 1H, *J* = 9.1 Hz, 3'-H), 4.52 (t, 2H, *J* = 6.1 Hz, 1-H), 3.59 (t, 2H, *J* = 6.1 Hz, 2-H), 2.83 (s, 3H, NCH_3).

Dimethyl 3,3'-(4-chlorophenylazanediyl)dipropanoate 8d[36]

General Method D: Double Conjugate addition to anilines.

4-Chloroaniline (10 g, 78.4 mmol) was mixed with methyl acrylate (67.5 g, 784 mmol, 10 eq), cuprous chloride (1.24 g, 12.5 mmol, 0.16 eq) and AcOH (100 mL) and heated under reflux at 140 ℃ for 48 h. The reaction mixture was allowed to reach RT and water (300 mL) was added with strong agitation. The batch was allowed to stand in the fridge overnight and the water layer was decanted leaving the oil behind. The oil was washed with more water (2 × 300 mL), diluted with diethylether (200 mL), washed with water (300 mL), dried over $MgSO_4$ and evaporated to give diester **8d** as a light brown oil (19.0 g, 88%). ^{1}H NMR ($CDCl_3$) 7.17 (d, *J* = 8.8 Hz, 2H, 3-H & 5-H), 6.68 (d, *J* = 8.8 Hz, 2H, 2-H & 6-H), 3.65 (s, 6H, 2 x CH_3), 3.61 (t, *J* = 7.2 Hz, 4H, 2 x CH_2N), 2.57 (t, *J* = 7.2 Hz, 4H, 2 x CH_2CO); ^{13}C NMR ($CDCl_3$) 172.3 (C=O), 145.0 (C-1), 129.4 (C-3 & C-5), 129.3 (C-4), 114.1 (C-2 & C-6), 51.9 (CH_3), 47.2 (NCH_2), 32.1 ($\underline{C}H_2CO$); MS (ES): m/z 300.1 $(M+H)^{\bullet+}$; IR (film) 1725s (C=O), 1175m (C

Dimethyl 3,3'-(phenylazanediyl)dipropanoate 8a[37]

Prepared according to General method D. Diester **8a** was obtained as an orange oil (18.8 g, 67%). ^{1}H NMR ($CDCl_3$) 7.16 (m, 2H, 3-H & 5-H), 6.62 (m, 3H, 2-H, 4-H, 6-H), 3.60 (m, 10H, 2 x CH_2N-aniline & 2 x OCH_3), 2.52 (t, *J* = 7.1 Hz, 4H, 2 x CH_2CO); ^{13}C NMR ($CDCl_3$) 172.6 (C=O), 146.7 (C-1), 129.6 (C-3 & C-5), 117.2 (C-4), 112.6 (C-2 & C-6), 51.8 (CH_3), 47.0 (NCH_2), 32.3 ($\underline{C}H_2CO$); MS (ES): m/z 266.1 $(M+H)^{\bullet+}$; IR (film) 1725s (C=O), 1175m (C-O) cm^{-1},

Dimethyl 3,3'-(4-fluorophenylazanediyl)dipropanoate 8c[37] Prepared according to General Method D. Diester **8c** was obtained as a yellow oil (0.99 g, 75%). ^{1}H NMR ($CDCl_3$) 6.92 (m, 2H, 3-H & 5-H), 6.74 (m, 2H, m, 2-H & 6-H), 3.64 (s, 6H, 2 x CH_3), 3.56 (t, *J* = 7.2 Hz, 4H, 2 x CH_2N), 2.52 (t, *J* = 7.2 Hz, 4H, 2 x CH_2CO); ^{13}C NMR ($CDCl_3$) 172.6 (C=O), 156.0 (d, $^{1}J_{CF}$ = 237 Hz, C-4), 143.5 (C-1), 115.9 (d, $^{2}J_{CF}$ = 22 Hz, C-3 & C-5), 114.9 (d, $^{3}J_{CF}$ = 7 Hz, C-2 & C-6), 51.8 (CH_3), 47.7 (CH_2N), 32.3 ($\underline{C}H_2CO$); ^{19}F NMR ($CDCl_3$) –127.62; MS (ES): m/z 284.0 $(M+H)^{\bullet+}$; IR □ (KBr) 3025 (Ar C-H), 2950m (C-H), 1725s (C=O), 1525m (Ar C=C), 1175m (C-O), cm^{-1}.

Dimethyl 3,3'-(4-methoxyphenylazanediyl)dipropanoate 8e[37]

Prepared according to General Method D. Diester **8e** was obtained as an orange oil (21.7 g, 90%). ^{1}H NMR ($CDCl_3$) 6.84 (d, 2H, *J* = 8.1 Hz, 3-H & 5-H), 6.74 (d, 2H, *J* = 8.1 Hz, 2-H & 6-H), 3.75 (s, 3H, CH_3), 3.64 (s, 6H, 2 x CH_3), 3.53 (t, *J* = 7.1 Hz, 4H, 2 x NCH_2), 2.51 (t, *J* = 7.1 Hz, 4H, 2 x CH_2CO); ^{13}C NMR ($CDCl_3$) 175.4 (C=O), 152.9 (C-4), 141.0 (C-1), 121.4 (C-3 & C-5), 114.9 (C-2 & C-6), 55.8 (CH_3), 51.9 (CH_3), 50.5 (NCH_2), 33.5 ($\underline{C}H_2CO$); MS (ES): m/z 296.1 $(M+H)^{\bullet+}$; IR □ (film) 1725s (C=O), 1175m (C-O) cm^{-1}.

3,3'-(4-Chlorophenylazanediyl)dipropanehydrazide 10d

General Method E: Preparation of bis-hydrazides

Diester **8d** (19 g, 63.4 mmol) was mixed with hydrazine hydrate (12.6 g, 0.25 mol, 4 eq) in propan-2-ol (60 mL) for 48 h at RT. The resulting solid was collected by filtration, washed with propan-2-ol and dried *in vacuo* to give hydrazide **10d** as white solid (18.8 g, 99%): ^{1}H NMR (DMSO-d_6) 9.03 (s, 2H, 2 x NH), 7.15 (d, 2H, *J* = 9.1 Hz, 3-H & 5-H), 6.66 (d, 2H, *J* = 9.1 Hz, 2-H & 6-H), 4.18 (br s, 4H, 2 x NH_2), 3.46 (t, *J* = 7.2 Hz, 4H, 2 x NCH_2), 2.23 (t, *J* = 7.2 Hz, 4H, 2 x CH_2CO); ^{13}C NMR (DMSO-d_6) 170.4 (C=O), 146.4 (C-1), 129.3 (C-3 & C-5), 119.8 (C-4), 113.8 (C-2 & C-6), 47.4 (NCH_2), 31.9 ($\underline{C}H_2CO$); MS (ES): m/z 300.1 $(M+H)^{\bullet+}$; IR □ (KBr) 3300 (Ar C-H), 1650s (CONH) cm^{-1}.

3,3'-(Phenylazanediyl)dipropanehydrazide 10a[38]

Prepared according to General Method E. Hydrazide **10a** was obtained as a white solid (6.3 g, 72%). ^{1}H NMR (DMSO) 9.04 (s, 2H, 2 x NH), 7.15 (t, 2H, *J* = 7.4 Hz, 3-H & 5-H), 6.66 (d, 2H, *J* = 7.4 Hz, 2-H & 6-H), 6.58(t, 2H, *J* = 7.4 Hz, 4-H), 4.18 (br s, 4H, 2 x NH_2), 3.48 (t, *J* = 7.2 Hz, 4H, 2 x CH_2N), 2.25 (t, *J* = 7.2 Hz, 4H, 2 x CH_2CO); ^{13}C NMR (DMSO) 170.5 (C=O), 147.6 (C-1), 129.7 (C-3 & C-5), 116.2 (C-4), 112.4 (C-2 & C-6), 47.4 (NCH_2), 32.1 ($\underline{C}H_2CO$); MS (ES): m/z 266.1 $(M+H)^{\bullet+}$; IR [illegible] cm^{-1}.

3,3'-(4-Fluorophenylazanediyl)dipropanehydrazide 10c

Prepared according to General Method E. Hydrazide **10c** was obtained as a white solid (0.63 g, 64%): m.p. 131.4 °C. ^{1}H NMR (DMSO-d_6) 8.91 (br s, 2H, 2 x NH), 6.88 (m, 2H, 3-H & 5-H), 6.56 (m, 2H, 2-H & 6-H), 4.07 (br s, 4H, 2 x NH_2), 3.33 (t, *J* = 7.2 Hz, 4H, 2 x CH_2N), 2.11 (t, *J* = 7.2 Hz, 4H, 2 x CH_2CO); ^{13}C NMR (DMSO-d_6) 170.5 (C=O), 154.8 (d, $^{1}J_{CF}$ = 228 Hz, C-4), 144.5 (C-1), 116.0 (d, $^{2}J_{CF}$ = 22 Hz, C-3 & C-5), 113.7 (d, $^{3}J_{CF}$ = 7 Hz, C-2 & C-6), 47.8 (CH_2N), 31.9 ($\underline{C}H_2CO$); ^{19}F NMR ($CDCl_3$) –129.53; MS (EI): m/z 283.0 $(M+H)^{\bullet+}$; ν_{max} (KBr) 3300m (NH), 3050m (Ar C-H), 1650s (CONH), 1525m (Ar C=C) cm^{-1}

3,3'-(4-Methoxyphenylazanediyl)dipropanehydrazide 10e

Prepared according to General Method E. Hydrazide **10e** was obtained as a white solid (20.5 g, 95%): ^{1}H NMR ($CDCl_3$) 7.39 (br s, 2H, 2 x NH), 6.85 (d, 2H, *J* = 8.1 Hz, 3-H & 5-H), 6.80 (d, 2H, *J* = 8.1 Hz, 2-H & 6-H), 4.10 (br s, 4H, 2 x NH_2), 3.75 (s, 3H, CH_3), 3.32 (t, *J* = 7.1 Hz, 4H, 2 x CH_2N), 2.28 (t, *J* = 7.1 Hz, 4H, 2 x CH_2CO); ^{13}C NMR ($CDCl_3$) 173.4 (C=O), 155.0 (C-4), 142.4 (C-1), 121.4 (C-3 & C-5), 114.7 (C-2 & C-6), 55.6 (CH_3), 50.7 (NCH_2), 33.3 ($\underline{C}H_2CO$); MS (ES): m/z 296.1 $(M+H)^{\bullet+}$; ν_{max} (KBr) 3300s (NH), 3050m (Ar C-H), 1650s (CONH) cm^{-1}.

3,3'-(2,2'-(4-Chlorophenylazanediyl)bis(ethane-2,1-diyl))bis-(4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide) 3d

General Method F: Preparation of bis-imidazotetrazines

Hydrazide **10d** (1.0 g, 3.18 mmol, 1 eq) was dissolved in dichloromethane (10 mL). Water (10 mL) was added followed by HCl (2.5 mL, 37 %). The mixture was cooled in an ice/$CaCl_2$ bath and a solution of $NaNO_2$ (0.57 g, 8.26 mmol, 2.6 eq) in water (10 mL) was added with strong agitation below 5 ℃. After the addition, the reaction was allowed to reach RT and stirred overnight. The organic layer was separated, dried over $MgSO_4$ and evaporated to give the azide **11d** as a crude oil, identified by IR. The oil was diluted with toluene (100 mL) and heated under reflux for 2 h under N_2. The volatile components were removed to give the crude isocyanate **9d** as an oil. Diethylether (150 mL) was added and the mixture heated with strong agitation. The hot solution was decanted leaving a residue of oily impurities behind. The ether was evaporated to leave pure isocyanate as pale yellow oil (IR ν_{max} 2260s). The isocyanate (0.29g, 1.03 mmol) was mixed with diazo-IC **5** (0.3 g, 2.17 mmol, 2.1 eq) in dry DMSO (0.1 mL) under N_2 at RT in the absence of light for 24 h. The bis-imidazotetrazine was purified by flash column chromatography eluting with $CHCl_3$:AcOH (1:1) to give imidazotetrazine **3d** as a light brown solid (0.1 g, 6%): m.p. 143-144 ℃. ^{1}H NMR (DMSO-d_6, 600 MHz) 8.79 (s, 2H, imidazole CH), 7.77 & 7.66 (2 × br s, 4H, 2 × $CONH_2$), 7.09 (d, *J* = 8.9 Hz, 2H, 3-H & 5-H), 6.80 (d, *J* = 8.9 Hz, 2H, 2-H & 6-H), 4.42 (t, *J* = 6.6 Hz, 4H, 2 x NCH_2 aniline), 3.78 (t, *J* = 6.6 Hz, 4H, 2 x CH_2N-tetrazine); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.9 (C=O amide), 146.2 (C-1), 140.0 (C=O tetrazine), 134.7 (Cq tetrazine), 131.4 (Cq imidazole), 2 x 129.3 (C-3 & C-5, C-H imidazole), 120.9 (C-4), 114.2 (C-2 & C-6), 49.1 (CH_2N-aniline), 46.4 (CH_2N-tetrazine); MS (ES): m/z 540.2 $(M+H)^{\bullet+}$; 562.3 $(M + Na)^{\bullet+}$; IR (KBr) 3450m ($CONH_2$), 1750s (C=O), 1675s ($CONH_2$), 1600m & 1500s (Ar-H) cm^{-1}. Anal. $C_{20}H_{18}ClN_{13}O_4 \cdot 0.85$ AcOH, CHN.

3,3'-(2,2'-(Phenylazanediyl)bis(ethane-2,1-diyl))bis-(4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide) 3a

Prepared according to General Method F. The product was purified by flash column chromatography (5% AcOH/CH_3CN) and imidazotetrazine **3a** was obtained as a yellow solid (0.38 g, 60%): m.p. 194–195 ℃; ^{1}H NMR (DMSO-d_6, 600 MHz) 8.79 (s, 2H, imidazole CH), 7.77 & 7.66 (2 × br s, 4H, 2 × $CONH_2$), 7.06 (t, *J* = 7.5 Hz, 2H, 3-H & 5-H), 6.80 (d, *J* = 7.5 Hz, 2H, 2-H & 6-H), 6.51 (t, *J* = 7.5 Hz, 1H, 4-H), 4.44 (t, *J* = 6.9 Hz, 4H, 2 x CH_2N-aniline), 3.79 (t, *J* = 6.9 Hz, 4H, 2 x CH_2N-tetrazine); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.9 (C=O amide), 147.2 (C-1), 139.7 (C=O tetrazine), 134.7 (Cq tetrazine), 131.3 (Cq imidazole), 129.6 & 129.3 (C-3 & C-5, C-H imidazole), 117.1 (C-4), 112.5 (C-2 & C-6), 48.8 (CH_2N-aniline), 46.6 (CH_2N-tetrazine); MS (ES): m/z 506.3 $(M+H)^{\bullet+}$; ν_{max} (KBr) 3450m & 3150m ($CONH_2$), 1740s (C=O), 1675s ($CONH_2$), 1600m & 1510s (Ar-H) cm^{-1}. Anal. $C_{20}H_{19}N_{13}O_4 \cdot H_2O \cdot 0.8$AcOH, CHN.

3,3'-(2,2'-(4-Fuorophenylazanediyl)bis(ethane-2,1-diyl))bis-(4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide) 3c

Prepared according to General Method F. Imidazotetrazine **3c** was obtained as dark yellow solid (0.04 g, 42%): m.p. 290–291 ℃; ^{1}H NMR (DMSO-d_6, 600 MHz) 8.78 (s, 2H, imidazole CH), 7.77 & 7.66 (2 × br s, 4H, 2 × $CONH_2$), 6.91 (m, 2H, 3-H & 5-H), 6.80 (m, 2H, 2-H & 6-H), 4.41 (t, *J* = 6.7 Hz, 4H, 2 x CH_2N-aniline), 3.76 (t, *J* = 6.7 Hz, 4H, 2 x CH_2N-tetrazine); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.9 (C=O amide), 155.0 (d, $^1J_{CF}$ = 243 Hz, C-4), 144.1 (C-1), 139.7 (C=O tetrazine), 134.7 (Cq tetrazine), 131.3 (Cq imidazole), 129.3 (C-H imidazole), 116.0 (d, $^2J_{CF}$ = 23.1 Hz, C-3 & C-5), 114.1 (d, $^3J_{CF}$ = 7.2 Hz, C-2 & C-6), 49.4 (CH_2N-aniline), 46.5 (CH_2N-tetrazine); MS (ES): m/z 524.3 $(M+H)^{\bullet+}$; 546.2 $(M + Na)^{\bullet+}$; IR (KBr) 3450m ($CONH_2$), 1725s (C=O), 1675s ($CONH_2$), 1600m & 1510s (Ar-H) cm^{-1}. Anal. $C_{20}H_{18}FN_{13}O_4 \cdot 0.35\ H_2O \cdot 0.3$ AcOH requires, CHN.

3,3'-(2,2'-(4-Methoxy-2-nitrophenylazanediyl)bis(ethane-2,1-diyl))bis-(4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide) 3f

Prepared according to General Method F. The product was purified by flash column chromatography (5% AcOH/CH_3CN) and imidazotetrazine **3f** was obtained as an orange/yellow

solid (0.21 g, 26%): m.p. 139–140 ℃. ^{1}H NMR (DMSO-d_6, 600 MHz) 8.72 (s, 2H, imidazole CH), 7.77 & 7.67 (2 × br s, 4H, 2 × $CONH_2$), 7.49 (d, *J* = 9.2 Hz, 1H, 6-H), 7.16 (d, *J* = 3.0 Hz, 1H, 3-H), 7.04 (dd, *J* = 9.2, 3.0 Hz, 1H, 5-H), 4.32 (t, *J* = 6.2 Hz, 4H, 2 x CH_2N-aniline), 3.67 (s, 3H, OCH_3), 3.47 (t, *J* = 6.2 Hz, 4H, 2 x CH_2N-tetrazine); ^{13}C NMR (DMSO-d_6, 151 MHz) 161.9 (C=O amide), 156.3 (C-4), 147.8 (C-2), 139.5 (C=O tetrazine), 135.6 (C-1), 134.8 (Cq tetrazine), 131.3 (Cq imidazole), 129.1 (C-H imidazole), 126.9 (C-5), 119.4 (C-6), 109.1 (C-3), 56.4 (CH_3O), 52.1 (CH_2N-aniline), 46.9 (CH_2N-tetrazine); MS (ES): m/z 581.3 $(M+H)^{\bullet+}$; 603.1 $(M + Na)^{\bullet+}$; IR (KBr) 3450m ($CONH_2$), 1750s (C=O), 1675s ($CONH_2$), 1600m & 1525s (Ar-H) cm^{-1}. Anal. $C_{21}H_{20}N_{14}O_7 \cdot 1.4\ H_2O \cdot 0.4$ AcOH, CHN.

3,3'-(2,2'-(4-Methoxyphenylazanediyl)bis(ethane-2,1-diyl))bis-(4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide) 3e

Prepared by a variation on General Method F using AcOH (0.17 M) in place of HCl. The product was purified by flash column chromatography (10% AcOH/$CHCl_3$) and **3e** was obtained as red solid (0.2 g, 11%): m.p. 178-179 ℃. ^{1}H NMR (DMSO-d_6, 600 MHz) 8.75 (s, 2H, imidazole CH), 7.76 & 7,65 (2 × br s, 4H, 2 × $CONH_2$), 6.73 (d, *J* = 8.1 Hz, 2H, 3-H & 5-H), 6.62 (d, *J* = 8.1 Hz, 2H, 2-H & 6-H), 4.39 (t, *J* = 6.6 Hz, 4H, 2 x CH_2N-aniline), 3.72 (t, *J* = 6.6 Hz, 4H, 2 x CH_2N-tetrazine), 3.53(s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 151 MHz) 162.0 (C=O amide), 152.0 (C-4), 141.5 (C-1), 139.6 (C=O tetrazine), 134.7 (Cq tetrazine), 131.2 (Cq imidazole), 129.2 (C-H imidazole), 115.2 & 114.9 (C-2 & C-6, C-3 & C-5), 55.6 (CH_3), 49.6 (CH_2N-aniline), 46.9 (CH_2N-tetrazine); MS (ES): m/z 536.3 $(M+H)^{\bullet+}$; 558.3 $(M + Na)^{\bullet+}$; IR (KBr) 3450m ($CONH_2$), 1750s (C=O), 1675s ($CONH_2$), 1600m & 1500s (Ar-H) cm^{-1}. Anal. $C_{21}H_{21}N_{13}O_5$, CHN.

***In vitro* Chemosensitivty**

Cells used were A2780 (human ovarian carcinoma, from European Collection of Cell Cultures), the MMR-deficient derivative A2780-Cp70 (gift of Professor G Margisson, University of Manchester, UK). Isogenic HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ came from Bert Vogelstein.[39] Cells

were plated into 96-well culture plates at 1 x 10^3 cells per well and incubated over night at 37 ℃ in a CO_2 enriched (5%) atmosphere to enable cells to adhere to the plate. Culture medium was removed and replaced with fresh medium containing test compound at concentrations ranging from 0 (controls) to 250 µM. Following 5 days incubation at 37 ˚C, cell survival was determined using the MTT assay. All TMZ-related compounds were dissolved in DMSO, and the final concentration of DMSO in the culture plates was <0.1% (v/v). PaTrin2 was used as an inhibitor of MGMT and cells were incubated with test compounds in the presence or absence of 10 µM PaTrin2

NCI Data Handling

Standard COMPARE and matrix COMPARE were run using the NCI database Data Build Date: 2012-04-28. GI_{50} data were selected and where multiple datasets were available, those averaged from the larger number of individual experiments were used.

Supporting Information Available: including tabulated IC_{50} data and statistical analysis for Figures 1 and 2; NCI mean graphs and COMPARE results for compounds **2d**, **3c**–**e**; HPLC analysis of new tetrazines **2**, **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations Used. ATM, ataxia telangiectasia mutated serine/threonine protein kinase; ATR, ataxia telangiectasia and Rad3-related protein; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea; CHB, chlorambucil; CP, cisplatin; DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; FANC, Fanconi's anemia; GBM, glioblastoma multiforme; MEL, melphalan; MGMT, *O*6-Methylguanine-DNA-methyltransferase; MMR, DNA Mismatch repair; 6MP, 6-

mercaptopurine; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide; MTZ, mitozolomide; QSAR, quantitative structure-activity relationship; 6TG, 6-thioguanione; TMZ, temozolomide.

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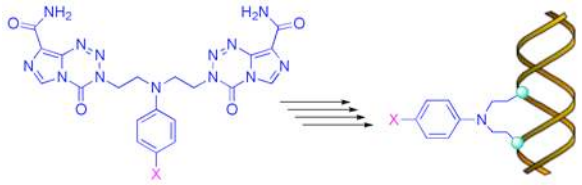

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