Antitumor Imidazotetrazines. 41.[1] Conjugation of the Antitumor Agents Mitozolomide and Temozolomide to Peptides and Lexitropsins Bearing DNA Major and Minor Groove-Binding Structural Motifs

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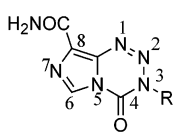
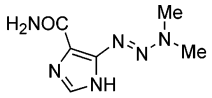
Carboxylic acids derived from the amido groups of the antitumor agents mitozolomide and temozolomide have been conjugated to simple amino acids and peptides by carbodiimide coupling. Solid-state peptide synthesis has been applied to link the acids to DNA major groove-binding peptidic motifs known to adopt α-helical conformations. Attachment of the acids to pyrrole and imidazole polyamidic lexitropsins gave a series of potential DNA minor groove-binding ligands. In vitro biological evaluation of a limited number of these novel conjugates failed to demonstrate any enhanced growth-inhibitory activity compared to the unconjugated drugs; sites of alkylation at tracts of multiple guanines were also unaffected. Attachment of additional residues at C-8 of the imidazotetrazines did not perturb the chemistry of activation of the bicyclic nucleus, and biological sequelae can be rationalized by invoking the liberation of a common, diffusible, reactive chemical intermediate, the methanediazonium ion.

Introduction

Since the first paper in this series describing the chemical and biological properties of 3-(2-chloroethyl)-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(*H*)-one **1** (azolastone; subsequently known as mitozolomide) was published in 1984,[2] the 'Antitumor Imidazotetrazines' project has been piloted from bench to clinic and thence, through regulatory approval, to culminate with the marketing of temozolomide **2** in Europe (Temodal) in 1998 and the U.S. (Temodar) in 1999.



1 R = $(CH_2)_2Cl$ mitozolomide
2 R = Me temozolomide



3 DTIC

The ultimate success of the endeavor was imperiled at several stages in the developmental pathway. Despite the fact that mitozolomide could cure many of the mouse in vivo tumor models available in the early 1980s in a single dose,[3] clinical trials in humans were terminated when the drug was shown to elicit profound, prolonged, and irreversible bone-marrow toxicity, notably thrombocytopenia,[4] probably because of its ability to generate a bifunctional DNA cross-linking reactive species.[5] Mitozolomide thus joined the extensive list of investigational agents which might have cured cancer if only the patient had not succumbed in the meantime. Subsequently, a tentative case was accepted by the Cancer Research Campaign UK for considering the monofunctional methylating analogue temozolomide as a potential clinical alternative to DTIC **3**.[6] Phase 1 trials of temozolomide using a monthly single dose schedule failed to demonstrate clinical activity and the drug might well have been abandoned at that point. However, preclinical antitumor evaluation of temozolomide in mouse models had predicted that the drug would be schedule dependent,[6] and it was only when a daily (× 5) schedule was investigated that responses were seen in patients with brain tumors, malignant melanoma, and a case of mycosis fungoides.[7]

When a vesicant pall of methyl isocyanate from a runaway reactor in Bhopal, India, in 1987 engulfed a densely occupied neighborhood, the environmental and political fallout again threatened the very survival of the project. The original synthesis of temozolomide involved stirring methyl isocyanate and solid 5-diazoimidazole-4-carboxamide **4** (Scheme 1) in ethyl acetate at 25 °C; even on a 10 g scale the process took >30 days to reach completion. (Nowadays this synthesis might be considered a perfect example of 'atom economy', albeit not an environmentally friendly one.) Considering the potentially explosive nature of diazo compounds it was not considered prudent to accelerate the reaction by grinding or ultrasonic reduction of crystalline **4** to a fine powder. Similarly, given the notorious history of the pariah molecule methyl isocyanate,[8] any suggestions to confine the reaction contents within a sealed container, and heat it, might be considered cavalier, if not criminally negligent, especially in a crowded university laboratory in downtown Birmingham, UK. Finally chemists at Aston Molecules Ltd discovered the trick of using a mixed ethyl acetate/DMSO solvent system which allowed for clinical grade temozolomide to be prepared in 250 g batches in 3 days. Despite the discovery of several alternative syntheses of temozolo-

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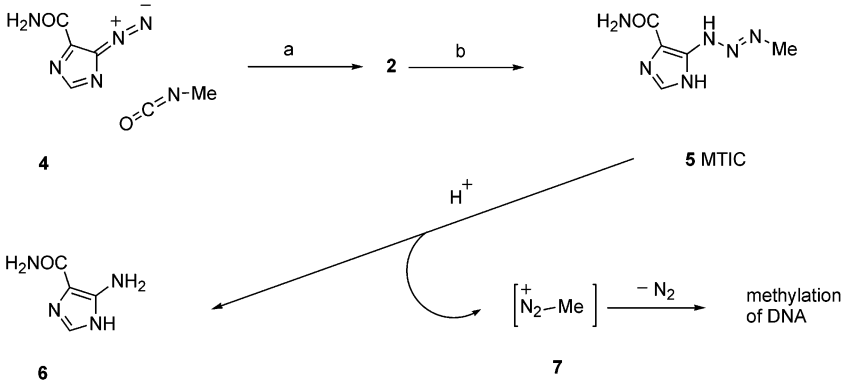
Scheme 1[a]



[a] Reagents and conditions: (a) EtOAc-DMSO, 25 °C; (b) H_2O, pH 7.4, $-CO_2$.

mide, some of which avoid the use of the diazoimidazole carboxamide and methyl isocyanate altogether,[9] the drug is still manufactured by the original route discovered by graduate pharmacy student Robert Stone in 1980.[10]

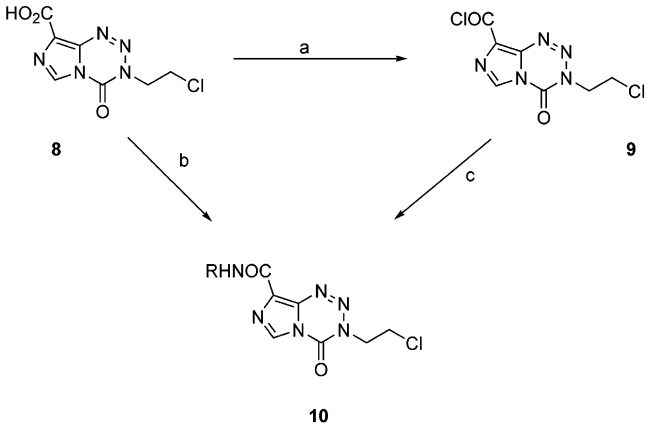
Temozolomide is a prodrug and serves as a molecular drug-delivery device to transport a methylating agent to guanine sequences in the major groove of DNA.[11] The mechanism of activation involves entirely different chemistry to the synthetic process: thus the tetrazinone ring is cleaved hydrolytically at physiological pH to give the unstable monomethyl triazene MTIC **5**, which then undergoes proteolysis to liberate 5-aminoimidazole-4-carboxamide **6** and the fugitive methylating methanediazonium reactive species **7** (Scheme 1).[12] The relative abundance of covalently modified DNA in brain tumors relative to normal tissue, as evidenced by spectacular positron emission tomography imaging using temozolomide with a ^{11}C label at the methyl group,[1] may possibly be attributable to the slightly different pH environments of normal or malignant brain tissues,[13] coupled with differential capacities to repair the methylation lesions by O^6-alkyl-DNA alkyltransferase (Atase) or other repair processes.[14]

In past efforts to develop imidazotetrazines with greater selectivity for specific DNA sequences, and possibly different antitumor profiles, we have conjugated their 8-carboxylic acid derivatives to H-bonding heterocycles, spermidine,[11] and peptidic DNA minor groove-recognition moieties such as the octapeptide SPKKSPKK, a component of histone proteins:[15] these efforts did not lead to any differences in the locus of methylation of the DNA which still occurred at guanine residues in guanine-rich tracts in the major groove. In this final paper in the series describing new chemistry of imidazotetrazines we return to this strategy and report the results of our work to harness mitozolomide and temozolomide to simple amino acids and peptides, polypeptides with known DNA major groove-recognition motifs, and polyamidic 'lexitropsins' directed to the minor groove.

Chemistry

Conjugation of Imidazotetrazines to Amino Acids and Peptides. In previous work we have shown that the 8-carboxylic acid derivative **8** derived by hydrolysis of mitozolomide can be converted to the acid chloride **9** and then reacted with *tert*-butylamine or

Scheme 2[a]



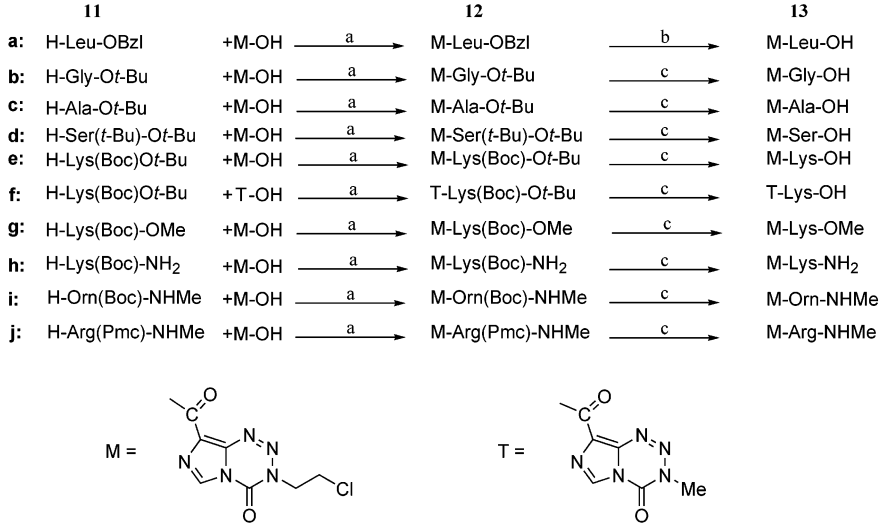
a: R = *t*-Bu
b: Gly-OEt
c: Gly-NH_2
d: Ala-OMe
e: Ser-OMe
f: Met-OMe
g: Trp-OMe
h: Ala-Val-OMe
i: Ala-Val-Ser-OMe

[a] Reagents and conditions: (a) $SOCl_2$, DMF, 80 °C; (b) RNH_2 or $RNH_3^+Cl^-$,DCC or Morpho-CDl, HOBt, DMF or MeCN, 0 °C; (c) RNH_2, THF.

glycine ethyl ester to afford the amides **10a**,**b**.[16] To adapt this route for linking **8** through the carboxylic acid group to more elaborate peptides, the use of carbodiimide coupling reagents was investigated. The amides **10a**–**c** were isolated (45–50% yields) employing dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as coupling auxiliary, but the use of 1-cyclohexyl-3-(2-morpholino)carbodiimide metho-*p*-toluenesulfonate (Morpho-CDI) gave superior products free of urea impurities. Using the latter reagent **8** was coupled successfully to a range of amino acid methyl esters to furnish amino acid derivatives **10d**–**g** and the dipeptide **10h** (50–75% yields) (Scheme 2). Less successful was the synthesis of the tripeptide methyl ester **10i** (6%), and no products were isolated from **8** and the methyl esters of L-histidine and L-proline.

Peptides with DNA major groove-recognition capability often include amino acids with basic side chains which form ionic contacts with backbone phosphate residues.[17] To prepare imidazotetrazine–peptide con-

Scheme 3[a]



[a] Reagents and conditions: (a) PyBOP, HOBt, TEM or DIPEA, MeCN or DCN; (b) H_2, IM-HCl, DCM; (c) TFA, TES/TIS.

Table 1. Conjugates of Mitozolomide-8-acid, Temozolomide-8-acid, and Peptidic Motifs

compd	structure of conjugate[a]	yield (%)	origin of peptidic motif
14a	M−Thr-Val-Gly-OH	50	CAP (helix)
14b	M−Thr-Val-Gly-Arg-OH	57	CAP (helix)
14c	M−Thr-Val-Ser-Arg-OH	53	LAC I/GAL R (helix)
14d	M−Val-Ser-Thr-Lys-Tyr-OH	31	Hin (helix)
14e	M−Arg-Lys-Gln-Val-Ala-OH	58	Hin (helix)
14f	M−Tyr-Glu-Thr-Val-Ser-Arg-Val-Val-Asn-OH	59	LAC I (helix)
14g	M−Arg-Glu-Thr-Val-Gly-Arg-NH_2	30	CAP (helix)
14h	M−Lys-Ser-Gln-Ile-Ser-Arg-NH_2	40	λcII (helix)
14i	M−Arg-Ser-Asp-Glu-Leu-Thr-Arg-His-NH_2	47	zinc finger (GCG)
14j	M−Arg-Ser-Asp-His-Leu-Ser-Arg-His-NH_2	22	zinc finger (GGG)
14k[b]	T−Ile-Ala-Thr-Ile-Thr-Arg-Gly-Ser-Asn-NH_2	36	Trp R (helix)

[a] For definition of M and T see Scheme 3. [b] See ref 15.

jugates where the amino acid carried an additional basic side-chain substituent (e.g., Lys, Orn, Arg) which might autocatalyze opening of the tetrazinone ring, an appropriate protecting group strategy was required. Suitable protected amino acid precursors **11a**−**h** were available commercially: *tert*-butyloxycarbonyl (Boc) protected H-Orn(Boc)-NHMe **11i** and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) protected H-Arg(Pmc)-NHMe **11j** were prepared from Z-Orn(Boc)-OH and Z-Arg(Pmc)-OH by the method of Bodanszky[18] followed by hydrogenolytic removal of the Z group. To prepare the conjugates **12a**−**j** (Scheme 3) typically a solution of the imidazotetrazine-8-acid derived from mitozolomide or temozolomide in acetonitrile or DCM at 25 °C was activated by benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in the presence of HOBt and a tertiary base triethylamine (TEA) or *N*,*N*-diisopropylethylamine (DIPEA); the protected amino acid was then added and interaction monitored to completion by TLC. The organic layer was extracted with 1 M HCl and the organic layer was concentrated in vacuo and purified by flash chromatography.

The extreme resilience of imidazotetrazines to pH values < 7.0 and instability at >7.4 dictated that only acidic deprotection of derivatives **12a**−**j** could be considered.[12,15] In a pilot experiment M-Leu-OBzl **12a** was successfully debenzylated by hydrogenation in DCM/1 M HCl to yield the corresponding acid M-Leu-OH **13a** (97%) (Scheme 3). However this approach did not work with a Z-protected lysine M-Lys(Z)-OBzl where hydrogenation under a range of acidic conditions all gave multiple products with incomplete removal of the Z group. Consequently, only Boc/*t*-Bu protecting group combinations were generally employed. Successful deprotection of the mitozolomide conjugates of *tert*-butyl esters **12b**−**d** to the corresponding acids **13b**−**d** was achieved using TFA and the *tert*-butyl carbocation scavenger mixture triethylsilane (TES)/triisopropylsilane (TIS). This tactic could also be used to effect double deprotection of M-Lys(Boc)-O*t*-Bu **12e** and its temozolomide equivalent **12f** to the acids **13e**,**f** respectively, and conversion of Boc-protected carboxylic derivatives **12g**−**i** to **13g**−**i**; the Pmc-protected arginine derivative **12j** was also converted to the arginine conjugate **13j** using the same acid deprotection strategy.

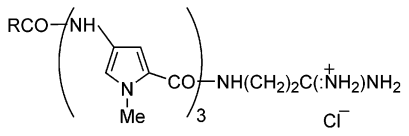
Conjugation of Imidazotetrazines to α-Helical Peptides. The initial intention to use standard solid-phase peptide synthesis (SPPS) to prepare imidazotetrazines conjugated to peptides bearing helix-turn-helix motifs was not realized since it proved practicable to prepare and purify only small (<10-mer) drug−peptide conjugates on solubility grounds. Efforts therefore focused on the synthesis of conjugates with peptides exemplifying short α-helical DNA-recognition sequences (Table 1). NovaSyn KA100 resin was employed for the synthesis of conjugates **14a**−**f** with a free carboxyl terminus, whereas conjugates with C-terminal acids capped as amides **14g**,**h** were assembled on NovaSyn PR500. Amino acids were Fmoc protected and side chains modified with Boc, *t*-Bu, Pmc, or trityl (Trt)

groups as appropriate. Mitozolomide-8-acid **8** was attached in the last step, and the conjugates were cleaved from the resin and deprotected with TFA/H_2O/TES/TIS. Conjugates were purified by HPLC, lyophilized, dried under vacuo, and characterized by HRMS. Yields of purified conjugates were in the range 30−60%.

As the covalent interaction between imidazotetrazines and DNA is primarily at oligoguanine sequences,[11,12] the α-helix motifs from GCG-[19] and GGG-directed [20] zinc fingers were an attractive prospect to tether to mitozolomide-8-acid **8** by SSPS to generate conjugates with potential for enhanced interaction at target sequences. The octapeptides **14i**,**j** (Table 1) were isolated in 47 and 22% yields, respectively, from NovaSyn PR 500 resin.

Hydrolysis of Mitozolomide−Peptide Conjugates. We have shown previously that modifications at the 8-carbamoyl group and N-3 positions do not perturb the ring-opening chemistry of the imidazotetrazine nucleus.[15,21] Stabilities of mitozolomide **1** and three amino acid/peptide conjugates **13g**, **14h**, and **14j** in phosphate buffer at pH 7.4 were measured by UV spectroscopy, monitoring the decrease of the imidazotetrazine absorption band at 325−330 nm. No major differences in stability were evident at this pH (Table 2). Moreover, the close similarity in the absorption curves of mitozolomide and the conjugates following decomposition, with the emergence of a band at 270 nm attributable to 5-aminoimidazole-4-carboxamide **6** (data not shown) indicates that the overall mechanism of activation of these compounds is identical.

Conjugation of Imidazotetrazines to Lexitropsins. Molecular approaches to exert control over gene expression by drug intervention is a burgeoning subject in anticancer drug discovery[22] but the exquisite molecular interactions between DNA sequence-specific agents designed to achieve transcriptional regulation has yet to be translated into clinical breakthroughs. The base recognition rules underlying the binding of DNA minor groove-binding hairpin polyamides ('lexitropsins') based on the natural product distamycin **15a** have been deciphered elegantly by Dervan and colleagues.[23] A−T or T−A base pairs are recognized by pyrrole/pyrrole motifs, G−C and C−G base pairs by imidazole/pyrrole and pyrrole/imidazole motifs, respectively. Tallimustine **15b**, a conjugate of benzoic acid mustard and distamycin, alkylates the N-3 position of adenine (*A*) in a concensus sequence 5′-TTTPu*A*.[24] The bicyclic imidazotetrazinone structure is itself a 'modified' amino acid with a carboxylic acid function at C-8 and a quasi N-terminal substituent at N-3; moreover, the tetrazinone 4-carbonyl substituent is a potential H-bond acceptor. Thus, conjugates of mitozolomide and temozolomide with polypyrrole and polyimidazole modules could extend the noncovalent DNA recognition reading frame of this novel type of lexitropsin.



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a: R = H distamycin

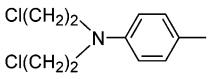
b: R =  tallimustine

Table 2. Hydrolysis of Imidazotetrazines[a]

	25 °C		37 °C	
compd	$t_{1/2}$ (min)	$k \times 10^{-3}$ (min^{-1})	$t_{1/2}$ (min)	$k \times 10^{-3}$ (min^{-1})
1	265	2.62	61.6	1.12
13g	238	2.91	53.7	1.29
14h	205	3.38	45.4	1.53
14j	227	3.05	48.9	1.51

[a] 0.1 M Phosphate buffer, pH 7.4.

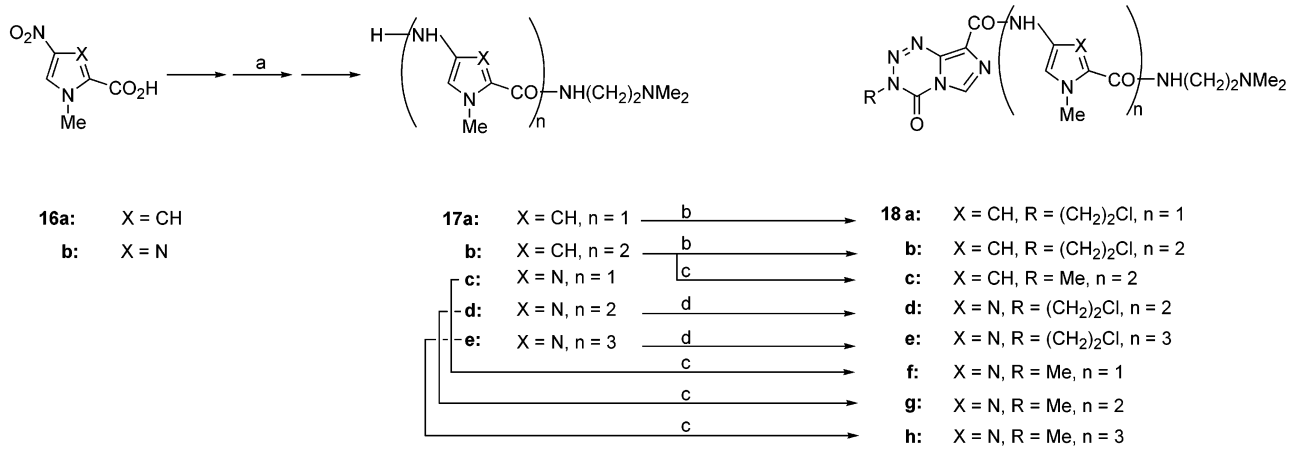
The starting materials for the synthesis of imidazotetrazine−lexitropsin conjugates (**18**) were the nitropyrrole and nitroimidazole carboxylic acids **16a**,**b** which were converted to the aminopyrroles **17a**,**b** and aminoimidazoles **17c**−**e** by literature processes (Scheme 4).[25,26] These were coupled immediately with either the carboxylic acids derived from mitozolomide or temozolomide using PyBOP as coupling reagent, or by direct interaction with the corresponding imidazotetrazine 8-acid chlorides without base. The imidazotetrazine−lexitropsin conjugates were generally unstable hygroscopic solids and were isolated with difficulty; no single coupling method was applicable in all cases and yields overall were low. The conjugates were surprisingly water-soluble and separation from polar contaminants proved to be troublesome. Structures of imidazotetrazine−lexitropsins were assigned on the basis of ^1H, ^{13}C NMR, and MS in the absence of reliable microanalytical data. The ^1H NMR spectrum of conjugate **18b** shows the 2 × NH signals at δ 10.65 and 9.97 (exchangeable with D_2O), the downfield imidazotetrazine C-6 proton at δ 9.00, and four pyrrolic protons in the range δ 6.87−7.37.

Biological Results and Discussion

To assess the comparative in vitro activities of mitozolomide and the series of simple amino acid conjugates **10a**−**h**, compounds were evaluated against the mouse TLX5 lymphoma (Table 3) which, in previous work, we have shown to be particularly sensitive to agents of the imidazotetrazine class.[2,3] IC_{50} values extended over a 30-fold dose range with the most potent compound overall being the alanine methyl ester **10d** (IC_{50} 1.5 μM), marginally more potent than mitozolomide (IC_{50} 2.5 μM). However, the amino acid moiety was not essential to potency since the *tert*-butyl amide **10a** was of equivalent cytotoxicity (IC_{50} 2.0 μM). Although the glycine conjugates **10b**,**c** and methionine derivative **10f** were considerably less cytotoxic than mitozolomide or temozolomide no obvious S/A correlation is apparent.

To explore the possibility that new conjugates might initiate DNA lesions less susceptible to repair processes than those produced by the unconjugated mitozolomide they were also tested against the human *Xeroderma pigmentosum* fibroblast cell line HMGZip 1a (Mer^-) with levels of Atase < 2 fmol mg^{-1} protein (Table 4).[27] In this model mitozolomide **1** was the most potent agent (IC_{50} 20 μM) whereas the polypeptide conjugates **14a**−**c** were the least potent. We have noted previously that increasing molecular weight has a dyschemotherapeutic effect in the imidazotetrazine series.[16,21] There were no

Scheme 4[a]



[a] Reagents and conditions: (a) See refs 25 and 26; (b) **8**, PyBOP, TEA, dry DMF, 0 °C; (c) **9** (Me for CH_2CH_2Cl, TEA, in DCM, 0 °C; (d) **9**, TEA, in DCM, 0 °C.

Table 3. in Vitro Cytotoxicity of Imidazotetrazines against Mouse TLX5 Lymphoma Cells

compd	IC_{50} (μM)[a]
1 (mitozolomide)	2.5
2 (temozolomide)	5.0[b]
8	15.8
10a	2.0
10b	25
10c	34
10d	1.5
10e	5.0
10f	50
10g	3.7
10h	6.9

[a] Mean of two determinations. [b] Reference 11.

Table 4. In Vitro Cytotoxicity of Imidazotetrazines against Human Xeroderma Pigmentosum Fibroblast Cells HMGZip **1a** (Mer^-) and HMGhAT **2b** (Mer^+)

	IC_{50} (μM)[a]	
compd	HMGZip **1a**	HMGhAT **2b**
1 (mitozolomide)	20	30
13a	57	-
13c	198	-
13e	42	-
13g	68	-
14a	437	>500
14b	288	>500
14c	351	>500

[a] Mean of 2 determinations.

significant differences in the activities of these compounds in the human Atase c-DNA transfected HMGhAT 2b (Mer^+) cell line (Atase levels > 800 fmol mg^{-1} protein) and on this limited evidence it is unlikely that the new conjugates would exhibit enhanced clinical activity in inherently resistant (Mer^+) human tumors.

In the NCI in vitro cell panel[28] the imidazotetrazines mitozolomide and temozolomide display mean GI_{50} values > 100 μM with only marginal evidence of selectivity toward cells of CNS origin (eg for mitozolomide against SF-268, SNB-75; GI_{50} values 10−50 μM). This performance alone would not have been sufficient to guarantee interest in further screening for either imidazotetrazine, let alone selection for clinical trial. Disappointingly, all the mitozolomide−peptide conjugates and imidazotetrazine pyrrole/imidazole lexitropsins also gave mean GI_{50} values in the 50−100 μM range

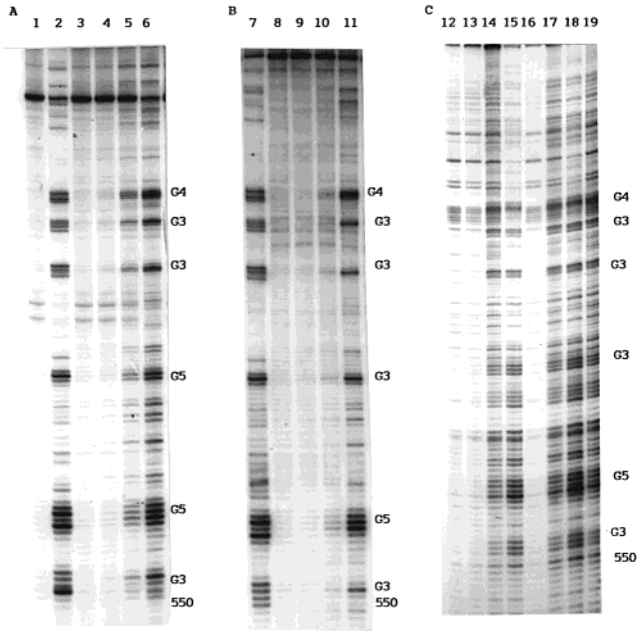


Figure 1. Autoradiogram of 6% denaturing sequencing gel showing blocks to *Taq* polymerase by (A) cisplatin and temozolomide **2**; (B) cisplatin and mitozolomide **1**; and (C) the mitozolomide conjugates **14g** and **14j**. Lane 1, control unmodified *Bam*HI-*Sal*I fragment of pB*R*322 DNA; lane 2, cisplatin (1 μM); lanes 3−6, temozolomide (1 μM, 10 μM, 100 μM and 1 mM); lane 7, cisplatin (1 μM); lanes 8−11, mitozolomide (1 μM, 10 μM, 100 μM and 1 mM); lanes 12−15, conjugate **14g** (1 μM, 10 μM, 100 μM, and 1 mM); lanes 16−19, conjugate **14j** (1 μM, 10 μM, 100 μM and 1 mM). G_3−G_5 represent runs of three to five guanines.

(data not shown). Clearly the imidazotetrazines as a class, irrespective of the nature of the substituent conjugated at the 8-carbamoyl group, have low overall growth-inhibitory activity against human tumor cells in vitro, at least in a 2-day drug exposure assay.

Sites of covalent DNA modification by representative conjugates formed by linking mitozolomide **1** to peptidic motifs which recognize the DNA major groove **14g**,**j** were identified following a 2 h incubation of drug with *Bam*HI linearized pB*R*322 DNA. The primer extension procedure utilizes multiple polymerization cycles with the thermostable DNA polymerase from *Thermus aquaticus*.[29] After annealing of a 5′-end-labeled primer,

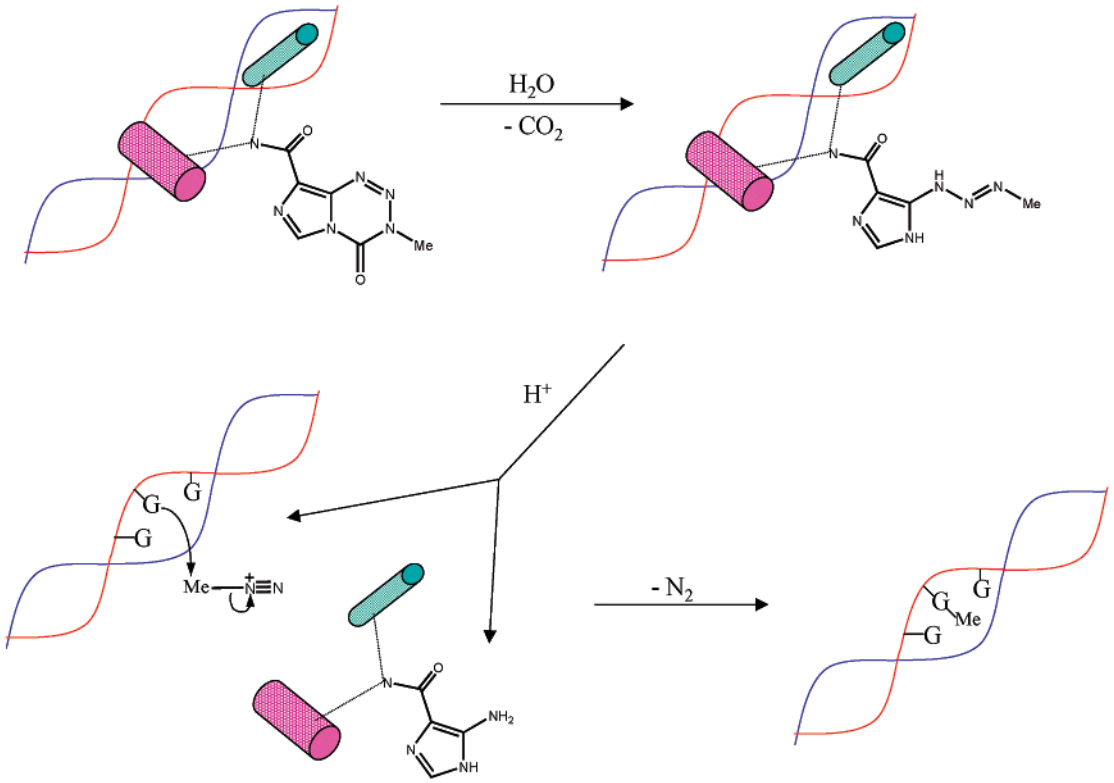


Figure 2. Interaction of temozolomide conjugates with DNA and methylation of guanine residues, showing minor groove-binding motif (green) and major groove motif (magenta).

complimentary to bases 621−640 of the *Bam*HI-*Sal*I fragment of pBR 322 (for sequence see Experimental Section), extension with *Taq* DNA polymerase produced a full-length fragment of 263 bp. Covalent alkylation of the DNA by the conjugates serves to block progression of the polymerase, causing cessation of chain elongation. Products of linear amplification were run on sequencing gels to reveal sites of covalent modification. The pB*R*322 base sequence positions indicated in autoradiograms (Figure 1) indicate the termination by *Taq* polymerase one nucleotide before a modified base, and the positions of runs of three or more guanine residues are highlighted.

Cisplatin, temozolomide, and mitozolomide are qualitatively and quantitatively similar in their alkylation patterns (Figure 1A and 1B). Although this method does not identify the position(s) of covalent modification within individual DNA bases, clearly both imidazotetrazines selectively alkylate guanine residues within runs of guanine tracts. Also, we have shown previously that the blocks to the polymerase elicited by temozolomide conjugate **14k** (which bears a helix binding motif of the *trp* repressor protein) are qualitatively and quantitatively comparable to the alkylation patterns displayed by cisplatin and the monofunctional alkylating agent temozolomide **2**.[15] A broadly similar profile was shown by the mitozolomide conjugates **14g** and **14j** although conjugate **14j** which bears a zinc finger motif recognizing the GGG triplet is approximately 10-fold more reactive at multiple guanine sequences (Figure 1C; compare band densities at equimolar concentrations in lanes 13/17 and 14/18).

Conclusion

The imidazotetrazines mitozolomide and temozolomide have been conjugated though the 8-carboxylic acid group to a range of amino acids and peptides and moieties known to adopt a DNA major groove-binding α-helical motif. The inherent acid stability of the imidazotetrazine allows for simultaneous removal of protecting groups and cleavage of conjugates from resin. In an effort to redirect the covalent interaction of imidazotetrazines from the DNA major groove locus of the unconjugated drugs[11−12,14−15,27] to the minor groove, mitozolomide and temozolomide have also been tethered, with difficulty, to form a series of unstable pyrrole and imidazole polyamides.

The imidazotetrazines as a class have quite unremarkable in vitro potencies against human tumor cell lines; mean GI_{50} values in the NCI 60 cell panel being > 100 μM. We have shown previously that intact imidazotetrazine−peptide conjugates definitely bind to DNA as evidenced by thermal denaturation, fluorescence quenching, and circular dichroism studies.[15] Disappointingly, the new conjugates did not show any enhanced potency or cell line selectivity in a spectrum of in vitro evaluations.

Moreover, conjugates **14g**,**j** exhibited the same specificity for DNA alkylations in guanine-rich tracts in DNA as the major groove interactive agents cisplatin and temozolomide.

Attachment of peptides at C-8 has little effect on the chemical stability of imidazotetrazines and this corroborates our earlier experience on the consequences of other derivatizations at the 8-carbamoyl group of mitozolomide and temozolomide.[11,15] The initial activating ring-opening reaction, involving nucleophilic addition at

C-4 of the 1,2,3,5-tetrazinone ring[2,6,12] is not affected by bulky moieties at C-8 (although it is sensitive to bulky groups at C-6).[30] However, irrespective of the nature of the targeting group conjugated at C-8 (to the DNA major or minor grooves) the final step in the activation process releases an electrophilic methanediazonium reactive species[12,15] with sufficient stability to be able to diffuse to, and methylate, the most nucleophilic sites available—guanine N-7 and O^6 positions—within runs of guanine residues in the *major* groove (Figure 2).

Following the demise of mitozolomide, the close analogue temozolomide progressed from bench to market where its oral bioavailability, relative freedom from toxicity, and acceptability on an out-patient basis have contributed to its use in areas beyond the brain tumor types for which it is specifically licensed. At a molecular weight of only 194 Da temozolomide is a compact, cute, and competitive molecule.

Many hundreds of analogues have been prepared in the 20-plus year history of this project, and only a comprehensive in vivo evaluation of these would determine whether any might be superior to temozolomide. The additional evidence gleaned from this work confirms that structural modification at C-8 in imidazotetrazines is unlikely to be productive. If a third-generation imidazotetrazine were to be considered, new biological insights into the fundamental nature of the alkylation process itself, and its repair, will be required. These might suggest mechanism-based alternatives to β-chloroethyl (mitozolomide) or methyl (temozolomide) groups at N-3 or rationally underpin the design of bis(imidazotetrazine)s conjoined with smarter linker units.

Experimental Section

General. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 250 instrument. IR spectra (KBr disks) were recorded on a Mattson Instruments Galaxy Series FTIR 2020 instrument. Mass spectra were recorded on either a Bio-ion 20 plasma desorption instrument or a VG Autospec. Fast atom bombardment (FAB) mass spectra were recorded on a VG Autospec. Peptides were synthesized on a Novabiochem Novasyn Gem Peptide Synthesizer with monitoring using a Novasyn Prism UV/vis spectrophotometer. Analytical TLC was performed using Merck Kieselgel 60 F_{254} plates and flash chromatography was carried out on Kieselgel 60 mesh grade 24; compounds were visualized with UV irradiation. HPLC was performed with a Beckman System Gold HPLC instrument with a Programmable Solvent Module 125 and a Programmable Detector Module 166, using Hichchrom KR 100-5C8-2900 4.5 × 250 mm and Hichchrom KR 100-5C8-25031 10 × 250 mm columns.

3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carboxylic acid **8** (88.5%) was prepared from mitozolomide **1** by treatment with sodium nitrite in sulfuric acid.[16] The unstable 3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl chloride **9** was prepared from compound **8** and thionyl chloride/DMF[16] and was used without further purification.

Conjugation of Imidazotetrazines to *tert*-Butylamine, Amino Acids, and Peptides. 8-(*N*-*tert*-Butylcarbamoyl)-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (10a). A solution of carboxylic acid **(8)** (1.0 g, 4.0 mmol) and HOBt (0.54 g, 4.0 mmol) in dry DMF (30 mL) was stirred at 0 °C and a solution of DCC (0.87 g, 4.2 mmol) in dry DMF (5 mL) was added. The mixture was stirred at 0 °C (1 h), treated with *tert*-butylamine (0.29 g, 4.0 mmol), stirred for a further 1 h at 0 °C followed by 1 h at 25 °C, and then mixed with 1 M HCl to give a colorless solid. Purification by flash chromatography (EtOAc as eluent) gave **10a** (49%), identical (mp, TLC, IR, and ^{1}H NMR) to an authentic sample.[16]

***N*-[3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl]glycine Ethyl Ester (10b).** A mixture of **8** (0.66 g, 2.5 mmol), glycine ethyl ester hydrochloride (0.36 g, 2.5 mmol), TEA (0.253 g, 2.5 mmol), HOBt (0.34 g, 2.5 mmol), and Morpho-CDI (1.27 g, 3.0 mmol) in acetonitrile (50 mL) was stirred at 0 °C for 6 h, followed by 48 h at 25 °C. Solvent was removed in vacuo, and the residue was partitioned between EtOAc and 1 M HCl. The organic layer was collected, dried (Na_2SO_4), and subjected to flash chromatography (EtOAc–petroleum ether, 4:1, as eluent). The white solid (**10b**) (35%) was identical (mp, TLC, IR, and ^{1}H NMR) to an authentic sample.[16] Similarly prepared were the following:

***N*-[3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl]glycinamide (10c).** Prepared from **8** and glycinamide hydrochloride, the glycinamide (**10c**) (44%) had mp 146–148 °C (decomp): UV (EtOH) λ_{max} 329 nm; IR 3619 (NH), 3473 (NH), 3390 br (NH), 3150 br (NH), 1737 (C=O), 1665 (NH–C=O), 1639 (NH–C=O) cm^{-1}; δ_H (DMSO-d_6) 8.91 (1H, s, H-6), 8.46 (1H, t, J = 5.4 Hz, CONH), 7.46 (1H, br s, CONH), 7.13 (1H, br s, CONH), 4.63 (2H, t, J = 6.0 Hz, CH_2Cl), 4.02 (2H, t, J = 6.0 Hz, NCH_2), 3.89 (2H, d, J = 5.5 Hz, CH_2). Anal. ($C_9H_{10}ClN_7O_3 \cdot H_2O$) C, H, N.

***N*-[3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl]-L-alanine Methyl Ester (10d).** Prepared from **8** and L-alanine methyl ester hydrochloride, the ester (**10d**) (67%) had mp 106–108 °C (decomp): UV (EtOH) λ_{max} 328 nm; IR 3392 (NH), 3112 (NH), 1746 (C=O), 1675 (NHC=O) cm^{-1}; δ_H (DMSO-d_6) 8.93 (1H, s, H-6), 8.80 (1H, d, J = 7.4 Hz, CONH), 4.65 (2H, t, J = 6.0 Hz, CH_2Cl), 4.57 (1H, dq, J = 7.2, 7.4 Hz, α-CH), 4.03 (2H, t, J = 6.0 Hz, NCH_2), 3.66 (3H, s, CO_2CH_3), 1.43 (3H, d, J = 7.2 Hz, Ala CH_3). Anal. ($C_{11}H_{13}ClN_6O_4$) C, H, N.

***N*-[3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl]-L-serine Methyl Ester (10e).** Prepared from **8** and L-serine methyl ester hydrochloride, the ester (**10e**) (30%) had mp 119–121 °C (decomp): UV (EtOH) λ_{max} 328 nm; IR 3550–3250 br (OH), 3470 (NH), 3402 (NH), 1743 (C=O), 1656 (NHC=O) cm^{-1}; δ_H (DMSO-d_6) 8.94 (1H, s, H-6), 8.41 (1H, d, J = 7.5 Hz, CONH), 5.31 (1H, t, J = 5.6 Hz, OH), 4.62 (3H, m, CH_2Cl and α-CH), 4.03 (2H, t, J = 6.0 Hz, NCH_2), 3.93 (2H, m, Ser CH_2), 3.67 (3H, s, CO_2CH_3). Anal. ($C_{11}H_{13}ClN_6O_5$) C, H, N.

***N*-[3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl]-L-methionine Methyl Ester (10f).** Prepared from **8** and L-methionine methyl ester hydrochloride, the ester (**10f**) (52%) had mp 93–95 °C (decomp): UV (EtOH) λ_{max} 326 nm; IR 3390 br (NH), 3127 (NH), 1739 (C=O), 1675 (NHC=O) cm^{-1}; δ_H ($CDCl_3$) 8.94 (1H, s, H-6), 7.91 (1H, d, J = 8.0 Hz, CONH), 4.98 (1H, m, α-CH), 4.74 (2H, m, J = 6.0 Hz, CH_2Cl), 3.98 (2H, t, J = 6.0 Hz, NCH_2), 3.79 (3H, s, CO_2CH_3), 2.56 (2H, m, CH_2S), 2.31 (1H, m, Met β-CH), 2.15 (1H, m, Met β-CH), 2.09 (3H, s, SCH_3). Anal. ($C_{13}H_{17}ClN_6O_4S$) C, H, N.

***N*-[3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl]-L-tryptophan Methyl Ester (10g).** Prepared from **8** and L-tryptophan methyl ester hydrochloride, the yellow ester (**10g**) (74%) had mp 181–183 °C (decomp): UV (DMF) λ_{max} 328 nm; IR 3407 (indole NH), 3345 br (NH), 3133 (NH), 1751 (C=O), 1737 (C=O), 1663 (NHC=O) cm^{-1}; δ_H (DMSO-d_6) 10.96 (1H, br s, Indole NH), 8.89 (1H, s, H-6), 8.53 (1H, d, J = 7.8 Hz, CONH), 7.51–6.93 (5H, m, Indole CH), 4.84 (1H, dq, J = 7.8 Hz, α-CH), 4.63 (2H, m, J = 6.0 Hz, CH_2Cl), 4.01 (2H, t, J = 6.0 Hz, NCH_2), 3.65 (3H, s, CO_2CH_3), 3.36 (2H, m, β-CH_2). Anal. ($C_{19}H_{18}ClN_7O_4$) C, H, N.

***N*-[3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl]-L-alanyl-L-valyl Methyl Ester (10h).** A mixture of acid **8** (0.66 g, 2.5 mmol), L-alanyl-L-valyl methyl ester (0.84 g, 2.5 mmol) (prepared by hydrogenation of *Z*-L-alanyl-L-valine methyl ester in EtOH over a 10% Pd–C catalyst), HOBt (0.34 g, 2.5 mmol), Morpho-CDI (1.27 g, 3.0 mmol), and acetonitrile (50 mL) was stirred at 0 °C in the dark for 6 h and then at 25 °C (48 h). The product was isolated (as

above for **10b**) and purified by flash chromatography (EtOAc as eluent). The white alanyl-valyl methyl ester (**10h**) (48%) had mp 75−77 °C (decomp): UV (EtOH) λ_{max} 330 nm; IR 3420−3230 br (NH), 3107 br (NH), 1743 (C=O), 1662 (NH−C=O) cm^{-1}; δ_H (DMSO-d_6) 8.43 (1H, s, H-6), 7.84 (1H, d, J = 7.0 Hz, CONH), 6.65 (1H, d, J = 7.0 Hz, CONH), 4.80 (1H, m, α-CH), 4.76 (2H, m, J = 6.0 Hz, CH_2Cl), 4.54 (1H, dd, J = 4.8 Hz, 8.7, α-CH), 3.99 (2H, t, J = 6.0 Hz, NCH_2), 3.76 (3H, s, CO_2CH_3), 2.19 (1H, m, Val CH), 1.54 (3H, d, J = 6.9, Ala CH_3), 0.92 (3H, d, J = 6.8 Hz, Val CH_3), 0.89 (3H, d, J = 6.8 Hz, Val CH_3). Anal. ($C_{16}H_{22}ClN_7O_5$) C, H, N.

***N*-[3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-carbonyl]-L-alanyl-L-valyl-L-serine Methyl Ester (10i).** Prepared (as above), from **8** and L-alanyl-L-valyl-L-serine methyl ester, this tripeptide (**10i**) (6%) had mp 211−212 °C (decomp): UV (EtOH) λ_{max} 329 nm; IR 3600−3150 br (OH and NH), 1741 (C=O), 1641 (NH−C=O) cm^{-1}; δ_H (DMSO-d_6) 8.93 (1H, s, H-6), 8.45 (1H, d, J = 7.7 Hz, CONH), 8.35 (1H, d, J = 7.2 Hz, CONH), 8.08 (1H, d, J = 9.0 Hz, CONH), 5.06 (1H, t, J = 5.9 Hz, Ser OH), 4.63 (3H, m, CH_2Cl and α-CH), 4.30 (2H, m, 2 × α-CH), 4.02 (2H, t, J = 6.0 Hz, NCH_2), 3.70 (2H, m, Ser CH_2), 3.68 (3H, s, CO_2CH_3), 1.95 (1H, m, Val CH), 1.35 (3H, d, J = 6.9 Hz, Ala CH_3), 0.87 (6H, t, J = 7.1 Hz, 2 × Val CH_3). Anal. ($C_{19}H_{27}ClN_8O_7$) C, H, N.

3,4-Dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylic acid. To a solution of temozolomide (**2**) (24.1 g, 0.12 mol) in concentrated sulfuric acid (240 mL) was added a solution of sodium nitrite (30.0 g, 0.35 mol) in water (240 mL) in an ice-bath at a sufficient rate to keep the solution below 15 °C. The mixture was stirred for a further 2 h and quenched with excess ice−water and the white solid collected and washed with water/acetone. The acid (44%) had mp 177 °C (decomp); IR 3491, 1765 (C=O), 1707 (C=O), 1557, 1462, 1360, 1242, 1186, 1049, 943 cm^{-1}; δ_H (DMSO-d_6) 8.81 (1H, s, H-6), 3.87 (3H, s, CH_3); δ_C (DMSO-d_6) 162.0, 139.3, 136.6, 129.3, 128.0, 36.5 (CH_3); m/z (EI) 195 (M^+). Anal. ($C_6H_5N_5O_3$) C, H, N.

To form the corresponding acid chloride, the carboxylic acid (1.21 g) was refluxed in thionyl chloride (12 mL) and DMF (0.75 mL) for 2.5 h. The solution was vacuum evaporated with dry toluene (3 × 10 mL) to give 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl chloride as an unstable orange solid which was used for reactions (below) without further purification.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-leucine Benzyl Ester (12a).** To a slurry of the carboxylic acid **8** (1.0 g, 4.12 mmol) in dichloromethane (20 mL) were added PyBOP (2.2 g, 4.12 mmol) and H-Leu-OBzl *p*-tosylate (1.78 g, 4.52 mmol). Triethylamine (1.04 g, 10.3 mmol) was added, and the reaction was stirred overnight at room temperature. The crude product, in dichloromethane, was extracted with 1 M HCl (2 × 20 mL) and water (2 × 20 mL). The organic layer was separated and subjected to flash chromatography (hexane:ethyl acetate as eluent; gradient of 2:1, increasing to 3:2). The solvent was removed in vacuo, and the residue was triturated with diethyl ether. The colorless solid (**12a**) (63%) had mp 96−97 °C (decomp); IR 3374 (NH), 1736 (C=O, RO−C=O), 1672 and 1530 (NH−C=O), 1582 (benzene ring) cm^{-1}; δ_H (DMSO-d_6) 8.94 (1H, s, imidazole H), 8.82 (1H, d, J = 8.2 Hz, CONH), 7.35 (5H, m, ArH), 5.16 (2H, s, CO_2CH_2), 4.65 (2H, t, J = 6.0 Hz, CH_2Cl), 4.63 (1H, m, α-CH), 4.03 (2H, t, J = 6.0 Hz, NCH_2), 1.93 (1H, m, CH), 1.66 (2H, m, CH_2), 0.90 (6H, t, J = 6.0 Hz, 2 × CH_3); δ_C (DMSO-d_6) 172.81, 160.61, 139.89, 136.83, 135.10, 131.14, 130.17, 129.28, 128.60, 128.59, 66.87, 51.21, 50.87, 42.33, 25.30, 23.73, 22.02. MS (FAB) m/z 447/449 (M + H^+). Anal. ($C_{20}H_{23}ClN_6O_4$) C, H, N.

Similarly prepared were the following:

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-glycine *tert*-Butyl Ester (12b).** Prepared from **8** and L-glycine *tert*-butyl ester hydrochloride, the ester (**12b**) (19%) had mp 125 °C (decomp); IR 3410 (NH), 1750 (C=O, RO−C=O), 1692 (NH−C=O) cm^{-1}; δ_H (DMSO-d_6) 8.93 (1H, s, imidazole H), 8.75 (1H, t, J = 6.05 Hz, CONH), 4.65 (2H, t, J = 6.0 Hz, CH_2Cl), 4.03 (2H, t, J = 6.0 Hz, NCH_2), 3.94 (2H, d, J = 6.1 Hz, CH_2), 1.43 (9H, s, $(CH_3)_3$); δ_C (DMSO-d_6) 169.13, 160.24, 139.51, 134.55, 130.82, 126.76, 81.23, 50.49, 41.90, 28.21. MS (FAB) m/z 357/359 (M + H^+). Anal. ($C_{13}H_{17}ClN_6O_4$) C, H, N.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-alanine *tert*-Butyl Ester (12c).** Prepared from **8** and L-alanine *tert*-butyl ester hydrochloride, the ester (**12c**) (72%) had mp 50−51 °C; IR 3391 and 3127 (NH), 1746 (C=O, RO−C=O), 1676 (NH−C=O) cm^{-1}; δ_H ($CDCl_3$) 8.93 (1H, s, imidazole H), 8.60 (1H, d, J = 7.6 Hz, CONH), 4.64 (2H, t, J = 6.1 Hz, CH_2Cl), 4.41 (1H, m, α−CH), 4.03 (2H, t, J = 6.1 Hz, NCH_2), 1.41 (3H, d, CH_3), 1.42 (9H, s, $(CH_3)_3$); δ_C (DMSO-d_6) 172.25, 160.09, 139.89, 135.00, 131.21, 130.12, 81.59, 50.88, 49.18, 42.31, 28.48, 17.96. HRMS (FAB) m/z 371.0995 (M + H^+), $C_{14}H_{20}ClN_6O_4$ requires 371.1234.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-*O*-*tert*-butyl-L-serine *tert*-Butyl Ester (12d).** Prepared from **8** and *O*-*tert*-butyl-L-serine *tert*-butyl ester hydrochloride, the ester (**12d**) (69%) had mp 101 °C (decomp); IR 3426, 3425 (NH), 1746 (C=O, RO−C=O), 1676 (NH−C=O) cm^{-1}; δ_H (DMSO-d_6) 8.94 (1H, s, imidazole H), 8.08 (1H, d, J = 8.7 Hz, CONH), 4.65 (2H, t, J = 6.0 Hz, CH_2Cl), 4.60 (1H, m, α-CH), 4.03 (2H, t, J = 6.0 Hz, NCH_2), 3.78 (2H, dd, CH_2), 1.44 (9H, s, $CO_2C(CH_3)_3$), 1.14 (9H, s, $OC(CH_3)_3$); δ_C (DMSO-d_6) 169.74, 159.79, 139.82, 135.06, 130.59, 130.35, 82.04, 73.69, 62.61, 53.69, 50.98, 42.25, 28.50, 27.98. MS (FAB) m/z 442/444 (M + H^+). Anal. ($C_{18}H_{27}ClN_6O_5$) C, H, N.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-*N*-ϵ-*tert*-butoxycarbonyl-L-lysine *tert*-Butyl Ester (12e).** Prepared from **8** and *N*-ϵ-*tert*-butoxycarbonyl-L-lysine *tert*-butyl ester hydrochloride, the ester (**12e**) (88%) had mp 60−61 °C (decomp); IR 3388 (NH), 2976, 2930 (CH_2, CH_3), 1750 (C=O, RO−C=O), 1705 (NH−CO_2), 1678 (NH−C=O) cm^{-1}; δ_H ($CDCl_3$) 8.93 (1H, s, imidazole H), 8.46 (1H, d, J = 7.4 Hz, CON*H*CH), 6.80 (1H, t, J = 5.7 Hz, CO_2NH), 4.65 (2H, t, J = 6.0 Hz, CH_2Cl), 4.37 (1H, m, α-CH), 4.03 (2H, t, J = 6.0 Hz, NCH_2), 2.89 (2H, q, J = 5.8 Hz, CH_2), 1.78 (2H, m, CH_2), 1.43 (9H, s, $NHCO_2C(CH_3)_3$), 1.35 (9H, s, $CHCO_2C(CH_3)_3$), 1.4 (4H, m, 2 × CH_2); δ_C ($CDCl_3$) 171.08, 158.87, 155.92, 138.80, 132.17, 128.32, 82.47, 77.22, 52.47, 50.18, 40.69, 40.41, 32.51, 29.66, 28.40, 28.03, 22.46. MS (FAB) m/z 528/530 (M + H^+). Anal. ($C_{22}H_{34}ClN_7O_6$) C, H.

***N*-α-{3-Methyl-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-*N*-ϵ-*tert*-butoxycarbonyl-L-lysine *tert*-Butyl Ester (12f).** Prepared from 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylic acid and *N*-ϵ-*tert*-butoxycarbonyl-L-lysine *tert*-butyl ester hydrochloride, the ester (**12f**) (61%) had mp 108 °C (decomp); IR 3393, 3353 and 3133 (NH), 1746 (C=O, RO−C=O), 1694 (NH−CO_2), 1663 (NH−C=O) cm^{-1}; δ_H (DMSO-d_6) 8.88 (1H, s, imidazole H), 8.40 (1H, d, J = 7.75 Hz, CONH), 6.80 (1H, t, J = 5.3 Hz, CO_2NH), 4.37 (1H, m, J = 6.7 Hz, α-CH), 3.88 (3H, s, NCH_3), 2.88 (2H, m, CH_2), 1.83 (2H, m, CH_2), 1.43 (9H, s, $NHCO_2C(CH_3)_3$), 1.36 (9H, s, $CHCO_2C(CH_3)_3$), 1.4−1.5 (4H, m, 2 × CH_2); δ_C (DMSO-d_6) 171.48, 160.07, 156.07, 139.67, 135.23, 130.15, 129.10, 81.46, 77.83, 53.04, 40.11, 36.74, 31.14, 29.60, 28.76, 28.17, 23.24. MS (FAB) m/z 480 (M + H^+). Anal. ($C_{21}H_{33}N_7O_6$) C, H, N.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8- carbonyl}-*N*-ϵ-*tert*-butoxycarbonyl-L-lysine Methyl Ester (12g).** Prepared from **8** and *N*-ϵ-*tert*-butoxycarbonyl-L-lysine methyl ester hydrochloride, the ester (**12g**) (64%) had mp 85−86 °C (decomp); IR 3379 (NH), 2866 (OCH_3), 1744 (C=O, RO−C=O), 1680 (NH−C=O) cm^{-1}; δ_H (DMSO-d_6) 8.94 (1H, s, imidazole H), 8.70 (1H, d, J = 7.8 Hz, CON*H*CH), 6.80 (1H, t, J = 5.7 Hz, CO_2NH), 4.65 (2H, t, J = 6.0 Hz, CH_2Cl), 4.49 (1H, m, α-CH), 4.03 (2H, t, J = 6.0 Hz, NCH_2), 3.66 (3H, s, CO_2CH_3), 2.88 (2H, q, J = 6.1 Hz, CH_2), 1.88 (2H, m, CH_2), 1.35 (9H, s, $(CH_3)_3$), 1.31 (4H, m, 2 × CH_2); δ_C (DMSO-d_6) 173.08, 160.51, 156.43, 139.91, 135.10, 131.09, 130.18, 78.20, 52.86, 52.80, 50.88, 42.74, 40.44, 34.05, 29.94,

29.11, 23.7. MS (FAB) *m/z* 486/488 (M + H^+). Anal. ($C_{19}H_{28}ClN_7O_6$) C, H, N.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-*N*-ϵ-*tert*-butoxycarbonyl-L-lysine Amide (12h).** Prepared from **8** and *N*-ϵ-*tert*-butoxycarbonyl-L-lysine amide hydrochloride, the hygroscopic amide (**12h**) (35%) had IR 3368 (NH), 1751 (C═O), 1686 and 1514 (NH−C═O) cm^{-1}; δ_H (DMSO-d_6) 8.91 (1H, s, imidazole H), 8.20 (1H, d, *J* = 8.0 Hz, CON*H*CH), 7.60 (1H, s, CONH), 7.23 (1H, s, CONH), 6.78 (1H, br s, CON*H*CH_2), 4.64 (2H, t, *J* = 6.0 Hz, CH_2Cl), 4.48 (1H, m, α-CH), 4.02 (2H, t, *J* = 6.0 Hz, NCH_2), 2.76 (2H, m, CH_2), 1.72 (2H, m, CH_2), 1.33 (9H, s, $(CH_3)_3$), 1.30 (4H, m, 2 × CH_2); δ_C (DMSO-d_6) 173.82, 159.64, 156.40, 139.89, 134.87, 131.24, 130.19, 78.18, 52.92, 50.90, 42.34, 40.60, 33.19, 30.17, 29.11, 23.22. MS (FAB) *m/z* 471 (M + H^+).

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-*N*-δ-*tert*-butoxycarbonyl-L-ornithine Methyl Amide (12i).** Prepared from **8** and *N*-ϵ-*tert*-butoxycarbonyl-L-ornithine methyl amide, the amide (**12i**) (71%) had mp 138−140 °C (decomp); IR 3331 (NH), 1761 (C═O), 1682 and 1557 (NH−CO_2), 1651 and 1541 (NH−C═O) cm^{-1}; δ_H (DMSO-d_6) 8.93 (1H, s, imidazole H), 8.23 (1H, d, *J* = 8.1 Hz, CONH), 8.09 (1H, d, *J* = 4.7 Hz, N*H*CH_3), 6.83 (1H, m, CO_2NH), 4.65 (2H, t, *J* = 6.0 Hz, CH_2Cl), 4.49 (1H, m, α-CH), 4.03 (2H, t, *J* = 6.0 Hz, NCH_2), 2.90 (2H, m, CH_2), 2.62 (3H, d, *J* = 4.5 Hz, NHC*H*$_3$),1.67 (2H, m, CH_2), 1.35 (9H, s, $(CH_3)_3$), 1.3−1.5 (2H, m, CH_2); δ_C (DMSO-d_6) 172.18, 159.72, 156.44, 139.88, 134.90, 131.17, 130.21, 78.27, 52.90, 50.92, 42.34, 40.39, 31.02, 29.12, 26.77, 26.41. MS (FAB) *m/z* 471 (M + H^+). Anal. ($C_{18}H_{27}ClN_8O_5$) C, H, N.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-N^G-2,2,5,7,8-pentamethylchroman-6-sulfonyl-L-arginine Methyl Amide (12j).** Prepared from **8** and N^G-2,2,5,7,8-pentamethylchroman-6-sulfonyl-L-arginine methyl amide, the amide (**12j**) (52%) had mp 126−128 °C (decomp); IR 3437 (NH), 3339 (C═NH), 1751 (C═O), 1655 (NH−C═O), 1551 (benzene ring) cm^{-1}; δ_H ($CDCl_3$) 8.95 (1H, s, imidazole H), 8.31 (1H, d, *J* = 8.45 Hz, NH), 7.44 (1H, s, NH), 6.25 (2H, s, 2 × NH), 6.10 (1H, s, NH), 4.84 (1H, m, α-CH), 4.78 (2H, t, *J* = 6.0 Hz, CH_2Cl), 4.01 (2H, t, *J* = 6.0 Hz, NCH_2), 3.52 (2H, m, CH_2), 2.87 (3H, d, *J* = 4.6 Hz, NHC*H*$_3$), 2.66 (2H, t, *J* = 6.8 Hz, Pmc CH_2), 2.60 (3H, s, CH_3), 2.58 (3H, s, CH_3), 2.13 (3H, s, CH_3), 1.83 (2H, t, *J* = 6.8 Hz, Pmc CH_2), 1.8−2.1 (2H, m, CH_2), 1.5−1.8 (2H, m, CH_2), 1.33 (6H, s, 2 × CH_3); δ_C (DMSO-d_6) 172.08, 159.75, 156.81, 153.21, 139.87, 135.41, 135.35, 134.95, 134.90, 131.13, 130.22, 123.53, 118.61, 74.32, 52.82, 50.93, 33.00, 31.00, 27.30, 26.40, 21.62, 19.02, 17.94, 12.77. MS (FAB) *m/z* 679/681 (M + H^+).

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-leucine (13a).** A solution of **12a** (300 mg, 0.67 mmol) in dichloromethane (20 mL) was hydrogenated over 10% Pd/C at atmospheric pressure. HCl (1 M, 5 mL) was added, and the catalyst was removed by filtration. The solvent was removed in vacuo. The pale pink, hygroscopic conjugate (**13a**) (97%) had IR 3385 and 3131 (NH), 1736 (C═O, RO−C═O), 1672 and 1530 (NH−C═O), 1582 (benzene ring) cm^{-1}; δ_H (DMSO-d_6) 8.93 (1H, s, imidazole H), 8.53 (1H, d, *J* = 6.1 Hz, CONH), 4.65 (2H, t, *J* = 6.0 Hz, CH_2Cl), 4.52 (1H, m, α-CH), 4.03 (2H, t, *J* = 6.0 Hz, NCH_2), 1.85 (1H, m), 1.62 (2H, m, CH_2), 0.91 (6H, t, *J* = 5.8 Hz, 2 × CH_3); δ_C (DMSO-d_6) 173.85, 159.67, 139.24, 134.33, 130.60, 129.48, 50.39, 50.23, 41.66, 39.86, 24.73, 23.14, 21.50. HRMS (FAB) *m/z* 357.1004 (M + H^+), $C_{13}H_{18}ClN_6O_4$ requires 357.1078.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-glycine (13b).** A solution of **12b** (0.3 g, 0.84 mmol) in 1:1 dichloromethane and aqueous TFA (8.03 M) was maintained at <8 °C (7 days). The solution was triturated with diethyl ether. The cream, hygroscopic conjugate (**13b**) (88%) had IR 3624 (OH), 3470 and 3302 (NH), 3000 (H-bonded OH), 1736 (C═O), 1699 (HO−C═O), 1663 (NH−C═O) cm^{-1}; δ_H (DMSO-d_6) 13 (1H, br s, OH), 8.96 (1H, s, imidazole H), 8.74 (1H, t, *J* = 5.75 Hz, CONH), 4.67 (2H, t, *J* = 6.0 Hz, CH_2Cl), 4.06 (2H, t, *J* = 6.0 Hz, NCH_2), 4.00 (2H, d, *J* = 6.0 Hz, CH_2); δ_C (DMSO-d_6) 171.85, 160.60, 139.93, 134.96, 131.24, 130.18, 50.88, 42.31, 41.64. HRMS (FAB) *m/z* 301.0455 (M + H^+), $C_9H_{10}ClN_6O_4$ requires 301.0452.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-alanine (13c).** A solution of **12c** (152 mg, 0.62 mmol) in TFA:water:TES/TIS, 95:2.5:2.5 (20 mL) was maintained at room temperature for 2 h. The solvent was removed in vacuo and the residue triturated with diethyl ether. The beige solid (**13c**) (68%) had mp 155 °C (decomp); IR 3385 (NH), 3100−2500 (OH), 1744 (C═O), 1734 (HO−C═O), 1630 and 1535 (NH−C═O) cm^{-1}; δ_H (DMSO-d_6) 12.8 (1H, br s, OH), 8.93 (1H, s, imidazole H), 8.57 (1H, d, *J* = 7.5 Hz, CONH), 4.65 (2H, t, *J* = 6.0 Hz, CH_2Cl), 4.46 (1H, m, α-CH), 4.03 (2H, t, *J* = 6.0 Hz, NCH_2), 1.45 (3H, d, *J* = 7.2 Hz, CH_3); δ_C (DMSO-d_6) 174.55, 159.98, 139.90, 134.98, 131.23, 130.13, 50.87, 48.41, 42.31, 18.09. MS (FAB) *m/z* 315/317 3:1 (M + H^+).

***N*-α-{3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-serine (13d).** A solution of **12d** (130 mg, 0.29 mmol) in TFA:water:TES/TIS, 95:2.5:2.5 (20 mL), was maintained at room temperature for 2 h. The solvent was removed in vacuo and the residue triturated with diethyl ether. The precipitate was filtered, washed with ether (10 mL), dissolved in water (5 mL) and lyophilized. The beige hygroscopic conjugate (**13d**) (41%) had IR 3515, 3366 (NH), 2900−3100 (OH), 1740 (C═O), 1672 (C═O), 1647 (NH−C═O) cm^{-1}; δ_H (DMSO-d_6) 12.5 (1H, br s, OH), 8.94 (1H, s, imidazole H), 8.28 (1H, d, *J* = 8.0 Hz, CONH), 5.25 (1H, br s, OH), 4.64 (2H, t, *J* = 6.0 Hz, CH_2Cl), 4.52 (1H, m, α-CH), 4.03 (2H, t, *J* = 6.0 Hz, NCH_2), 3.84 (2H, dd, CH_2); δ_C (DMSO-d_6) 172.49, 160.02, 139.90, 135.00, 130.92, 130.26, 62.03, 55.24, 50.95, 42.32. HRMS (FAB) *m/z* 331.0567 (M + H^+), $C_{10}H_{11}ClN_6O_5$ requires 331.0558.

Similarly prepared were the following:

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8 -carbonyl}-L-lysine Trifluoroacetic Acid Salt (13e).** Prepared by $C_{10}H_{12}ClN_6O_5$ deprotection of **12e**, the hygroscopic conjugate (**13e**) (63%) had IR 3391 (NH), 1755 (C═O), 1672 (HO−C═O, NH−C═O), 1202 (CF) cm^{-1}; δ_H (DMSO-d_6) 8.95 (1H, s, imidazole H), 8.55 (1H, d, *J* = 8.0 Hz, CONH), 7.64 (3H, br s, $^+NH_3$), 4.65 (2H, t, *J* = 6.0 Hz, CH_2-Cl), 4.47 (1H, q, *J* = 7.25, α-CH), 4.04 (2H, t, *J* = 6.0 Hz, NCH_2), 2.7 (2H, m, CH_2), 1.9 (2H, m, CH_2), 1.5 (2H, m, CH_2), 1.4 (2H, m, CH_2); δ_C (DMSO-d_6) 173.98, 160.38, 139.88, 134.97, 131.14, 130.16, 52.52, 50.90, 42.34, 39.42, 31.07, 27.44, 23.28. MS (FAB) *m/z* 372/374 3:1 (M + H^+).

***N*-α-{3-Methyl-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-lysine Trifluoroacetic Acid Salt (13f).** Prepared by deprotection of **12f**, the hygroscopic conjugate (**13f**) (42%) had IR 3399 (NH), 2900−3200 ($^+NH_3$, OH), 1751 (C═O), 1672 (HO−C═O, NH−C═O), 1202 (CF) cm^{-1}; δ_H (DMSO-d_6) 8.90 (1H, s, imidazole H), 8.48 (1H, d, *J* = 8.0 Hz, CONH), 7.69 (3H, br s, $^+NH_3$), 4.48 (1H, q, *J* = 7.25 Hz, α-CH), 3.89 (3H, s, CH_3), 2.79 (2H, m, CH_2), 1.89 (2H, m, CH_2), 1.56 (2H, m, CH_2), 1.41(2H, m, CH_2); δ_C (DMSO-d_6) 173.70, 160.18, 139.70, 135.23, 130.19, 129.13, 52.17, 39.12, 36.76, 30.84, 27.16, 22.98. HRMS (FAB) *m/z* 324.1456 (M + H^+), $C_{12}H_{18}N_7O_4$ requires 324.1420.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-lysine Methyl Ester (13g).** Prepared by deprotection of **12g**, the hygroscopic conjugate (**13g**) (50%) had IR 3391 (NH), 2800−3100 ($^+NH_3$), 2872 (OCH_3), 1751 (C═O, RO−C═O), 1686 (NH−C═O), 1204 (CF) cm^{-1}; δ_H (DMSO-d_6) 8.95 (1H, s, imidazole H), 8.76 (1H, d, *J* = 7.75 Hz, CONH), 7.8 (3H, br s, $^+NH_3$), 4.65 (2H, t, *J* = 6.0 Hz, CH_2Cl), 4.52 (1H, m, α-CH), 4.03 (2H, t, *J* = 6.0 Hz, NCH_2), 3.66 (3H, s, OCH_3), 2.77 (2H, t, *J* = 7.25 Hz, CH_2), 1.9 (2H, m, CH_2), 1.5 (2H, m, CH_2), 1.4 (2H, m, CH_2); δ_C (DMSO-d_6) 172.58, 160.20, 139.49, 136.69, 130.63, 129.70, 52.52, 52.19, 50.51, 41.96, 39.51, 30.51, 27.00, 22.92. HRMS (FAB) *m/z* 386.1284 (M + H^+), $C_{14}H_{21}ClN_7O_4$ requires 386.1344.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-lysine Amide Trifluoroacetic Acid Salt (13h).** Prepared by deprotection of **12h**, the hygroscopic conjugate (**13h**) (45%) had IR 3399 (NH),

3000−3200 ($^+NH_3$), 1757 (C═O), 1676 and 1524 (NH−C═O), 1204 (CF) cm^{-1}; δ_H (DMSO-d_6) 8.95 (1H, s, imidazole H), 8.27 (1H, d, J = 8.1 Hz, CON*H*CH), 7.63 (4H, br s, NH_3^+ and CONH), 7.28 (1H, s, NH), 4.66 (2H, t, J = 6.0 Hz, CH_2Cl), 4.50 (1H, m, α-CH), 4.04 (2H, t, J = 6.0 Hz, NCH_2), 2.78 (2H, m, CH_2), 1.77 (2H, m, CH_2), 1.55 (2H, m, CH_2), 1.34 (2H, m, CH_2); δ_C (DMSO-d_6) 173.72, 159.79, 139.88, 135.05, 131.21, 130.22, 52.76, 50.93, 42.34, 39.55, 32.71, 27.64, 22.88. HRMS (FAB) m/z 371.1327 (M + H$^+$), $C_{13}H_{20}ClN_8O_3$ requires 371.1347.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-ornithine Methyl Amide Trifluoroacetic Acid Salt (13i).** Prepared by deprotection of **12i**, the residue from trituration with diethyl ether was subjected to HPLC (25% MeOH, 0.06% aqueous TFA eluent). The hygroscopic conjugate (**13i**) (36%) had IR 3378 (NH), 2900−3100 ($^+NH_3$), 1755 (C═O), 1674 (NH−C═O), 1580 (CO_2^-), 1202 (CF) cm^{-1}; δ_H (DMSO-d_6) 8.96 (1H, s, imidazole H), 8.30 (1H, d, J = 8.25 Hz, CONH), 8.23 (1H, m, N*H*CH$_3$), 7.79 (3H, br s, $^+NH_3$), 4.66 (2H, t, J = 6.0 Hz, CH_2Cl), 4.53 (1H, m, α-CH), 4.04 (2H, t, J = 6.0 Hz, NCH_2), 2.79 (2H, m, CH_2), 2.63 (3H, d, J = 4.5 Hz, NHC*H*$_3$), 1.7 (2H, m, CH_2), 1.5 (2H, m, CH_2); δ_C (D_2O) 173.83, 162.27, 139.92, 135.03, 130.18, 129.62, 53.70, 51.11, 41.59, 39.21, 28.47, 26.24, 23.67. HRMS (FAB) m/z 371.1311 (M + H$^+$), $C_{13}H_{20}ClN_8O_3$ requires 371.1347.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-arginine methyl amide, trifluoroacetic acid salt (13j).** Prepared by deprotection of **12j**, the residue from trituration with diethyl ether was subjected to HPLC (25% acetonitrile, 0.06% aqueous TFA eluent). The hygroscopic conjugate (**13j**) (53%) had IR 3366 (NH, C═NH), 1753 (C═O), 1655 (NH−C═O) cm^{-1}; δ_H (DMSO-d_6) 8.95 (1H, s, imidazole H), 8.30 (1H, d, J = 8.25 Hz, CONH), 8.15 (1H, m, N*H*CH$_3$), 7.49 (1H, s, NH), 7.0 (4H, br s, NH and $^+NH_3$), 4.65 (2H, t, J = 5.8 Hz, CH_2Cl), 4.53 (1H, m, α-CH), 4.04 (2H, t, J = 5.8 Hz, NCH_2), 3.12 (2H, m, CH_2), 2.63 (3H, d, J = 4.5 Hz, NHC*H*$_3$), 1.74 (2H, m, CH_2), 1.47 (2H, m, CH_2); δ_C (D_2O) 171.93, 160.09, 154.90, 137.76, 132.85, 127.94, 127.43, 51.76, 48.93, 39.40, 38.62, 26.42, 24.04, 22.56. HRMS (FAB) m/z 413.1599 (M + H$^+$), $C_{14}H_{22}ClN_{10}O_3$ requires 413.1565.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Thr-L-Val-L-Glycine (14a).** Fmoc-Gly-NovaSyn KA resin (1 g, 0.1 mmol) was soaked in DMF (30 min) and loaded into the column of a Novasyn Gem peptide synthesizer. The resin was washed with DMF (5 min, 3.5 mL/min) and Fmoc-deprotected using 20% piperidine in DMF (8 min, 3.5 mL/min). The resin was washed again with DMF (15 min, 3.5 mL/min). A solution of Fmoc-Val-OH (136 mg, 0.4 mmol), PyBOP (208 mg, 0.4 mmol), and DIPEA (0.14 mL, 0.8 mmol) in DMF was added through the loading port (2 mL/min) and recirculated (3.5 mL/min, 1 h). The system was then flushed with DMF (6 min, 3.5 mL/min), and the resin was Kaiser tested. The cycle was repeated to add Fmoc-valine (136 mg, 0.4 mmol), Fmoc-Thr(*t*Bu)-OH (159 mg, 0.04 mmol), and **8** (97 mg, 0.04 mmol). The resin−peptide-conjugate was isolated by filtration and washed with DMF and diethyl ether. The resin−peptide-conjugate (1 g as resin) was agitated in a solution of TFA:H_2O:TES/TIS (95:2.5:2.5) (20 mL) at 30 °C (2−3 h). The resin was removed by filtration and the filtrate concentrated in vacuo. The residue was triturated with diethyl ether and collected by filtration. The peptide-conjugate was purified by HPLC (acetonitrile gradient 30−40%, 0.06% aqueous TFA, UV analysis 210 nm), and the solvent was removed in vacuo. The lyophilized conjugate (maintained at −18 °C) (**14a**) (50%) had MS (FAB) m/z 501.2 (M + H$^+$), $C_{18}H_{26}ClN_8O_7$ requires 501.2.

Similarly prepared were the following:

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Thr-L-Val-L-Gly-L-arginine Trifluoroacetic Acid Salt (14b).** Prepared from Fmoc-Arg(Pmc)-NovaSyn KA100 resin (1 g, 0.09 mmol) and purified by HPLC (acetonitrile gradient 25−35%, 0.06% aqueous TFA) as a lyophilized conjugate (**14b**) (57%). HRMS (FAB) m/z 657.2616 (M + H$^+$), $C_{24}H_{38}ClN_{12}O_8$ requires 657.2624.

***N*-α-{3-(2-chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Thr-L-Val-L-Ser-L-arginine trifluoroacetic acid salt (14c).** Prepared from Fmoc-Arg(Pmc)-NovaSyn KA100 resin (1 g, 0.09 mmol) and purified by HPLC (acetonitrile gradient 20−30%, 0.06% aqueous TFA) as a lyophilized conjugate (**14c**) (53%). HRMS (FAB) m/z 687.2717 (M + H$^+$), $C_{24}H_{40}ClN_{12}O_9$ requires 687.2730.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Val-L-Ser-L-Thr-L-Leu-L-tyrosine (14d).** Prepared from Fmoc-Tyr(*t*Bu)-NovaSyn KA100 resin (1 g, 0.1 mmol) and purified by HPLC (acetonitrile gradient 20−30%, 0.06% aqueous TFA) as a lyophilized conjugate (**14d**) (31%). MS (FAB) m/z 808.7 (M + 2H$^+$).

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Arg-L-Lys-L-Gln-L-Val-L-alanine Trifluoroacetic Acid Salt (14e).** Prepared from Fmoc-Ala-NovaSyn KA100 resin (1 g, 0.09 mmol) and purified by HPLC (acetonitrile gradient 15−30%, 0.06% aqueous TFA) to yield a lyophilized conjugate (**14e**) (58%). MS (FAB) m/z 826.4 (M + H$^+$), $C_{32}H_{53}ClN_{15}O_9$ requires 826.4.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Tyr-L-Glu-L-Thr-L-Val-L-Ser-L-Arg-L-Val-L-Val-L-asparagine Trifluoroacetic Acid Salt (14f).** The peptide conjugate was only obtained in crude form.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Arg-L-Glu-L-Thr-L-Val-L-Gly-L-arginine Amide Trifluoroacetic Acid Salt (14g).** Prepared on NovaSyn PR500 resin (0.5 g, 0.16 mmol), with TBTU and HOBt, and purified by HPLC (methanol gradient 20−30%, 0.06% aqueous TFA, UV analysis 310 nm) as a lyophilized conjugate (**14g**) (30%). HRMS (FAB) m/z 941.4307 (M + H$^+$), $C_{35}H_{58}ClN_{18}O_{11}$ requires 941.4221.

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Lys-L-Ser-L-Gln-L-Ile-L-Ser-L-arginine Amide Trifluoroacetic Acid Salt (14h).** Prepared on NovaSyn PR500 resin (0.5 g, 0.16 mmol), with TBTU and HOBt, and purified by HPLC (methanol isocratic 35%, 0.06% aqueous TFA) as the lyophilized conjugate (**14h**) (40%). MS (FAB) m/z 943 (M + H$^+$).

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Arg-L-Ser-L-Asp-L-Glu-L-Leu-Thr-L-Arg-L-histidine Amide Trifluoroacetic Acid Salt (14i).** Prepared on NovaSyn TGR resin (330 mg, 0.08 mmol) with PyBOP and HOBt and purified by HPLC (methanol isocratic 30%, 0.06% aqueous TFA) as a lyophilized conjugate (**14i**) (47%). MS (FAB) m/z 1239.4 (M + 2H$^+$).

***N*-α-{3-(2-Chloroethyl)-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl}-L-Arg-L-Ser-L-Asp-L-His-L-Leu-L-Ser-L-Arg-L-histidine Amide Trifluoroacetic Acid Salt (14j).** Prepared on NovaSyn PR500 resin (0.5 g, 0.16 mmol) with TBTU and HOBt and purified by HPLC (methanol gradient 30−34%, 0.06% aqueous TFA) as a lyophilized conjugate (**14j**) (22%). MS (FAB) m/z 1231.6 (M + H$^+$), $C_{47}H_{72}ClN_{24}O_{14}$ requires 1231.5.

***N*-α-(3-Methyl-3,4-dihydro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl)-L-Ile-L-Ala-L-Thr-L-Ile-L-Thr-L-Arg-L-Gly-L-Ser-asparagine Amide Trifluoroacetic Acid Salt (14k).** This conjugate was prepared by a published method.[15]

3-(2-Chloroethyl)-*N*-{5-[2-(dimethylamino)ethylcarbamoyl]-1-methyl-pyrrol-3-yl}-3,4-dihydro-4-oxoimidazo-[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide 18a. A solution of 5-[2-(dimethylamino)ethylcarbamoyl]-1-methyl-3-nitropyrrole (0.1 g, 0.4 mmol)[25] in MeOH (5 mL) was hydrogenated over 10% Pd/C at 25 °C and atmospheric pressure for 3 h. The catalyst was removed by filtration, and the filtrate was vacuum evaporated and then extracted with DCM (3 × 5 mL) to give 3-amino-5-[2-(dimethylamino)ethylcarbamoyl]-1-methylpyrrole **17a** as an unstable yellow oil. To a solution of the oil in dry DMF (5 mL) at 0 °C under nitrogen were added mitozolomide-8-acid **8** (0.09 g, 0.4 mmol), PyBOP (0.20 g, 0.4 mmol), and TEA (0.13 mL, 0.9 mmol), dropwise. The mixture was stirred at 25 °C for 12 h, and solvent was removed in vacuo. The residue was triturated with diethyl ether/acetonitrile, and

the orange conjugate **18a** (31%) had mp 184−187 °C (decomp); IR 3449 br (NH), 3391, 3133, 1755 (C═O), 1627, 1580, 1460, 1252 cm^{-1}; δ_H (DMSO-d_6) 10.65 (1H, s, NH), 9.0 (1H, s, imidazotetrazine H-6), 8.23 (1H, s, N*H*CH_2), 7.35 (1H, d, J = 0.1 Hz, pyrrole C−H), 7.15 (1H, d, J = 0.1 Hz, pyrrole C−H), 4.67 (2H, t, J = 6.0 Hz, CH_2Cl), 4.05 (2H, t, J = 6.0 Hz, NCH_2), 3.85 (3H, s, CH_3), 3.47 (2H, m, NHC*H*$_2$), 3.15 (2H, m, C*H*$_2$-NMe_2), 2.80 (6H, s, 2 × CH_3); δ_C (DMSO-d_6) 162.5, 157.5, 140.0, 136.0, 132.5, 130.0, 123.4, 122.5, 119.0, 105.9, 57.5, 52.0, 43.7, 42.4, 37.0, 35.0; MS (EI) m/z 435 (M + 100%).

3-(2-Chloroethyl)-*N*-(5-{5-[2-(dimethylamino)ethylcarbamoyl]-1-methyl-pyrrol-3-ylcarbamoyl}-1-methylpyrrol-3-yl)-3,4-dihydro-4-oxoimidazo[5,1-*d*][1,2,3,5]-tetrazine-8-carboxamide 18b. Compound **17b** (prepared by hydrogenation of the 3-nitro precursor)[25] was coupled with mitozolomide 8-acid **8** in the presence of PyBOP/TEA (see above). The conjugate **18b** (13%) had mp > 300 °C (decomp); IR 3389br (NH), 1738(C═O), 1640 (C═O), 1580, 1528, 1437, 1404, 1265, 847 cm^{-1}; δ_H (DMSO-d_6) 10.65 (1H, s, NH), 9.97 (1H, s, NH), 9.00 (1H, s, imidazotetrazine H-6), 7.95 (1H, s, N*H*CH_2), 7.37 (1H, d, J = 0.1 Hz, pyrrole C−H), 7.20 (2H, d, 2 × pyrrole C−H), 6.87 (1H, d, J = 0.1 Hz, pyrrole C−H), 4.65 (2H, t, J = 6.0 Hz, CH_2Cl), 4.05 (2H, t, J = 6.0, NCH_2), 3.90 (3H, s, CH_3), 3.83 (3H, s, CH_3), 3.37 (4H, m, NHC*H*$_2$ and C*H*$_2$$NMe_2$), 2.30 (6H, s, 2 × CH_3); MS (EI) m/z 557 (M^+, 100%).

***N*-(5-{5-[2-(Dimethylamino)ethylcarbamoyl]-1-methylpyrrol-3-ylcarbamoyl}-1-methylpyrrol-3-yl)-3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-[1,2,3,5]tetrazine-8-carboxamide 18c.** 3,4-Dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl chloride, prepared from 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylic acid (0.045 g) (see above), was mixed with dry DCM (5.0 mL) and TEA (50 μL). To this was added a solution of **17b** (prepared by hydrogenation of the 3-nitropyrrole precursor) (0.1 g)][25] in dry DCM (5.0 mL) at 0 °C. The precipitated temozolomide conjugate **18c** (87%) had mp 114−116 °C (decomp); IR 3343br (NH), 1655 (C═O), 1561, 1404, 1263, 1206, 1099, 775 cm^{-1}; δ_H (DMSO-d_6) 10.53 (1H, s, NH), 9.99 (1H, s, NH), 8.92 (1H, s, imidazotetrazine H-6), 8.24 (1H, t, J = 6.5 Hz, N*H*CH_2), 7.38 (1H, d, J = 0.1 Hz, pyrrole C−H), 7.23 (1H, d, J = 0.1 Hz, pyrrole C−H), 7.21 (1H, d, J = 0.1 Hz, pyrrole C−H), 6.97 (1H, d, J = 0.1 Hz, pyrrole C−H), 3.88 (3H, s, CH_3), 3.87 (3H, s, CH_3), 3.83 (3H, s, CH_3), 3.52 (2H, m, NHC*H*$_2$), 3.12 (2H, t, C*H*$_2$-NMe_2), 2.75 (6H, s, 2 × CH_3); δ_C (DMSO-d_6) 161.6, 158.4, 156.8, 139.2, 135.0, 134.8, 130.5, 128.4, 123.0, 122.3, 122.2, 121.5, 118.9, 118.3, 105.1, 104.8, 56.4, 42.7, 36.2, 36.1, 36.0, 34.2; MS (CI) m/z 509 (M^+, 73%).

3-(2-Chloroethyl)-*N*-(2-{2-[2-(dimethylamino)ethylcarbamoyl]-1-methyl-imidazol-4-ylcarbamoyl}-1-methylimidazol-4-yl)-3,4-dihydro-4-oxoimidazo[5,1-*d*][1,2,3,5]-tetrazine-8-carboxamide 18d. The diimidazole carboxamide **17d** in DCM (5.0 mL), prepared by hydrogenation of the 4-nitroimidazole precursor,[26] was coupled with the acid chloride **9**, prepared from mitozolomide 8-acid **8** (0.066 g) in DCM (5.0 mL) containing TEA (42 μL) at 0 °C. The conjugate **18d** (0.13 g, 78%) had mp 230−235 °C (decomp); IR 3399 br (NH), 1746 (C═O), 1663 (C═O), 1543, 1460, 1246, 1078, 793 cm^{-1}; δ_H (DMSO-d_6) 10.35 (1H, s, NH), 10.03 (1H, br s, NH), 9.60 (1H, s, NH), 8.97 (1H, s, imidazotetrazine H-6), 8.61 (1H, t, J = 6.5 Hz, N*H*CH_2), 7.74 (1H, s, imidazole C−H), 7.55 (1H, s, imidazole C−H), 4.66 (2H, t, J = 6.5 Hz, CH_2Cl), 4.04 (2H, t, J = 6.5 Hz, NCH_2), 4.03 (3H, s, CH_3), 3.96 (3H, s, CH_3), 3.57 (2H, t, C*H*$_2$$NMe_2$), 3.23 (2H, q, NHC*H*$_2$), 2.79 (6H, s, 2 × CH_3); δ_C (DMSO-d_6) 158.9, 156.7, 155.2, 138.8, 135.2, 134.5, 134.4, 134.0, 133.2, 129.8, 129.5, 115.8, 115.5, 114.0, 55.7, 50.1, 42.3, 41.4, 35.1, 35.0, 33.8; MS (EI) m/z 559 (M^+, 100%).

3-(2-Chloroethyl)-*N*-[2-(2-{2-[2-(dimethylamino)ethylcarbamoyl]-1-methylimidazol-4-ylcarbamoyl}-1-methylimidazol-4-ylcarbamoyl)-1-methylimidazol-4-yl]-3,4-dihydro-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide 18e. Similarly prepared to the above, from the triimidazole carboxamide **17e**[26] and acid chloride **9**, the conjugate **18e** (23%) had mp 235−241 °C (decomp); IR 3401 br (NH), 1751 (C═O), 1665 (C═O), 1528, 1260, 1101, 795 cm^{-1}; δ_H (DMSO-d_6) 10.27 (1H, s, NH), 10.02 (1H, s, NH), 9.66 (1H, s, NH), 9.00 (1H, s, imidazotetrazine H-6), 8.37 (1H, t, J = 6.5 Hz, N*H*CH_2), 7.77 (1H, s, imidazole C−H), 7.67 (1H, s, imidazole C−H), 7.56 (1H, s, imidazole C−H), 4.68 (2H, t, J = 6.5 Hz, CH_2Cl), 4.06 (2H, t, J = 6.5 Hz, NCH_2), 4.05 (3H, s, CH_3), 4.02 (3H, s, CH_3), 3.97 (3H, s, CH_3), 3.48 (2H, t, C*H*$_2$-NMe_2), 2.89 (2H, br s, NHC*H*$_2$), 2.50 (6H, s, 2 × CH_3); MS (EI) m/z 682 (M^+, 30%).

***N*-{2-[2-(Dimethylamino)ethylcarbamoyl]-1-methylimidazol-4-yl}-3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-[1,2,3,5]tetrazine-8-carboxamide 18f.** Prepared, as above, from **17c**[26] and 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl chloride, the imidazole-carboxamide **18f** (19%) had mp 172−176 °C; IR 3391 br (NH), 1742 (C═O), 1655 (C═O), 1543, 1474, 1099, 793 cm^{-1}; δ_H (DMSO-d_6) 10.04 (1H, s, NH), 8.91 (1H, s, imidazotetrazine H-6), 8.22 (1H, br s, N*H*CH_2), 7.62 (1H, d, imidazole C−H), 3.99 (3H, s, CH_3), 3.90 (3H, s, CH_3), 3.37 (2H, q, J = 6.9 Hz, NHC*H*$_2$), 3.09 (2H, t, J = 6.9 Hz, C*H*$_2$$NMe_2$), 2.50 (6H, d, J = 1.8 Hz, 2 × CH_3); δ_C (DMSO-d_6) 163.6, 161.8, 144.1, 140.1, 140.0, 139.3, 134.3, 119.4, 70.0, 49.5, 41.5, 40.7, 40.3, 20.3; MS (EI) m/z 376 (M^+, 92%).

***N*-(2-{2-[2-(Dimethylamino)ethylcarbamoyl]-1-methylimidazol-4-ylcarbamoyl}-1-methylimidazol-4-yl)-3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide 18g.** Prepared, as above, from **17d**[26] and 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl chloride, the diimidazole-carboxamide **18g** (16%) had mp 174−177 °C; IR 3424 br (NH), 1751 (C═O), 1663 (C═O), 1543, 1458, 1107, 785 cm^{-1}; δ_H (DMSO-d_6) 10.26 (1H, s, NH), 10.00 (1H, br s, NH), 9.62 (1H, s, NH), 8.91 (1H, s, imidazotetrazine H-6), 8.61 (1H, t, J = 6.5 Hz, N*H*CH_2), 7.72 (1H, s, imidazole C−H), 7.55 (1H, s, imidazole C−H), 4.03 (3H, s, CH_3), 3.95 (3H, s, CH_3), 3.88 (3H, s, CH_3), 3.58 (2H, t, J = 6.5 Hz, C*H*$_2$$NMe_2$), 3.23 (2H, m, NHC*H*$_2$), 2.80 (6H, d, J = 1.8 Hz, 2 × CH_3); δ_C (DMSO-d_6) 159.0, 156.9, 155.3, 139.0, 135.1, 134.6, 134.0, 133.2, 129.2, 129.0, 115.5, 114.1, 55.8, 50.3, 42.4, 36.4, 35.3, 35.2, 33.9; MS (EI) m/z 511 (M^+, 100%).

***N*-[2-(2-{2-[2-(Dimethylamino)ethylcarbamoyl]-1-methylimidazol-4-ylcarbamoyl}-1-methylimidazol-4-ylcarbamoyl)-1-methylimidazol-4-yl]-3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide 18h.** Prepared, as above, from **17e**[26] and 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl chloride, the triimidazole-carboxamide **18h** (12%) had mp >300 °C (decomp); IR 3413 br (NH), 1753 (C═O), 1665 (C═O), 1543, 1476, 1126, 795 cm^{-1}; δ_H (DMSO-d_6) 10.13 (1H, s, NH), 9.86 (1H, br s, NH), 9.55 (1H, s, NH), 8.92 (1H, s, imidazotetrazine H-6), 8.57 (1H, t, J = 5.5 Hz, N*H*CH_2), 7.73 (1H, s, imidazole C−H), 7.65 (1H, s, imidazole C−H), 7.55 (1H, s, imidazole C−H), 4.03 (3H, s, CH_3), 4.01 (3H, s, CH_3), 3.96 (3H, s, CH_3), 3.57 (2H, m, NHC*H*$_2$), 3.21 (2H, t, C*H*$_2$$NMe_2$), 2.79 (6H, s, 2 × CH_3); MS (EI) m/z 634 (M^+, 100%).

Hydrolysis of Mitozolomide−Peptide Conjugates. Stability studies were performed by UV spectroscopy according to the method of Denny et al.[12] Briefly, an aliquot of an aqueous 1 mg/mL drug stock solution was added to a 1 cm cuvette containing 0.1 M phosphate buffer (pH 7.4) to generate a final drug concentration of 50−80 μM. UV monitoring was performed at 25 or 37 °C with data points recorded at 5 min intervals at the λ_{max} for each compound. Plots of log[drug] versus time over several half-lives indicated that the reactions followed pseudo first-order kinetics.

In Vitro Growth Inhibition of Mouse TLX5 Lymphoma Cells. This bioassay was conducted according to a published procedure.[11] Briefly, TLX5 lymphoma cells were maintained in exponential growth phase at 2 × 10^4 cells/mL in RPMI 1640 supplemented with 15% fetal calf serum. Aliquots (2 mL) of the cells were plated out into six wells of multiwell dishes and treated with 10 μL of the required drug solutions in DMSO, with three replicates for each drug solution. Control incubates were composed of cells treated with 10 μL of DMSO. After being incubated for 72 h at 37 °C in an atmosphere of 5%CO_2, the cells were counted using a Coulter Laboratories ZM

counter. Results were plotted as percent of control population growth (data not shown), from which IC_{50} values (μM) were calculated.

In Vitro Growth Inhibition of Human Xeroderma Pigmentosum Cells. Human *xeroderma pigmentosum* fibroblast cell line HMGZip 1a (Mer^-) and HMGhAT 2b (Mer^+) cell lines were grown in MEM and 1000 cells/well were plated and assayed according to the method of Baer et al.[27]

NCI In Vitro Cytotoxicity Assays. The cytotoxicity of test agents was assessed in a panel of 60 cell lines using a sulforhodamine B assay.[28] Briefly, cell lines were inoculated into a series of 96-well microtiter plates, with varying seeding densities depending on the growth characteristics of the particular cell lines. Following a 24 h drug-free incubation, test agents were added routinely at five 10-fold dilutions with a maximum concentration of 10^{-4} M. After 2 days of drug exposure, the change in protein stain optical density allowed the inhibition of cell growth to be analyzed.

Sites of Covalent DNA Modification. Sites of covalent DNA modification were identified by the *Taq* polymerase method following a 2 h incubation of *Bam*HI linearized pB*R*322 DNA with test and control agents according to published methods.[15,29] The nucleotide sequence of pB*R*322 bases 376−650 corresponding to the *Bam*HI-*Sal*I fragment:

5′-GATCC(380) TCTACGCCGG ACGCATCGTG GCCG-GCATCA CCGGCGCCAC AGGTGCGGTT GCTGGCGCCT ATATCGCCGA CATCACCGAT GGGGAAGATC GGGCTCGC-CA CTTCGGGCTC ATGAGCGCTT GTTTCGGCGT GGG-TATGGTG GCAGGCCCGT GGCCGGGGGA CTGTTGGGCG CCATCTCCTT GCATGCACCA TTCCTTGCGG CGGCGGT-GCT CAACGGCCTC AACCTACTAC TGGGCTGCTT CCTAAT-GCAG GAGTCGCATA AGGGAGAGCG(650)

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