Antitumor Imidazotetrazines. 32. Synthesis of Novel Imidazotetrazinones and Related Bicyclic Heterocycles To Probe the Mode of Action of the Antitumor Drug Temozolomide

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Received November 23, 1994©

A series of new imidazo[5,1-d]-1,2,3,5-tetrazinones with additional hydrogen-bonding or ionic substituents at the 8-carboxamide position of the antitumor drugs temozolomide (1) and mitozolomide (2) has been prepared. None of these compounds were significantly more cytotoxic in vitro against the mouse TLX5 lymphoma than the lead structures. Molecular modeling techniques have been used to design benzo- and pyrazolo[4,3-d]-1,2,3-triazinones bearing carboxamide groups in appropriate positions which are isosteric with temozolomide and mitozolomide but which cannot ring open to alkylating species. As predicted, these compounds have no inhibitory properties against human GM892A or Raji cell lines in vitro. Temozolomide and the spermidine-temozolomide conjugate 28 preferentially methylate guanines within guanine-rich sequences in DNA, but no experimental evidence has been found to support the hypothesis that such regions are involved in catalyzing the ring opening of the imidazotetrazinone prodrugs to their active forms.

Introduction

The chemical mechanism underlying the antitumor properties of temozolomide (1) and mitozolomide (2) (Figure 1) is reasonably clear. Temozolomide is a prodrug which undergoes base-catalyzed hydrolytic ring opening followed by loss of a molecule of carbon dioxide to generate 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC, 4); this further fragments to the methylidiazonium species 6 as the proximal DNA-methylating agent (Scheme 1). Of the atoms making up the temozolomide ring, N-2 and N-3 are lost as a molecule of nitrogen; C-4 is processed to a molecule of carbon dioxide; and N-1 and all the imidazole ring atoms are eliminated as 5-aminoimidazole-4-carboxamide (5, AIC). Thus temozolomide can be considered as a small molecular weight drug-delivery device able to transfer an electrophilic methyl group to vulnerable sites within tumor cells. However, we have not yet found a convincing explanation for the biological inactivity of the 3-ethylimidazotetrazine 3 (ethazolastone), which has an identical ring-opening chemistry to temozolomide and mitozolomide. More tentatively, we proposed that runs of guanine residues represent an accessible nucleophilic and basic microenvironment in DNA which would facilitate sequence-selective conversion of the prodrug temozolomide to MTIC, possibly by an 'activated' water molecule in the major groove of DNA. This proposal was supported by molecular modeling studies which indicated that temozolomide could make a productive hydrogen-bonded association with DNA in which the role of the carboxamide group may be crucial in orienting the drug molecule at guanine-cytosine sites. Structure-activity relationships (SAR) in the imidazotetrazine series, as well as the clinical properties of temozolomide itself, can be rationalized in terms of this model.

The fact that the unstable species MTIC preferentially methylates the N-7 residue of the middle guanine in runs of guanines in isolated DNA® rather confuses the picture. It is clear, however, that the antitumor properties of temozolomide correlate with methylation at O-6 guanine sites, since tumor lines expressing high levels of the O-alkyl-DNA alkyltransferase repair protein are refractory to treatment by temozolomide.©
In this paper we give details of molecular modeling, synthesis, cytotoxicity, and sequence-selective DNA binding of compounds of two types designed to examine the validity of the overall working hypothesis. These are imidazotetrazinones bearing hydrogen-bonding and polyamine functionalities attached to the 8-carboxamide residue which could extend the DNA sequence tract recognized by this class of agent and bicyclic heterocycles bearing carboxamide groups which, although sterically and electronically related to temozolomide and mitozolomide, are unable to ring open to alkylating species. We envisaged that these molecular probes (see Figure 2 for structures) might compete for imidazotetrazine recognition sites on DNA, if such loci exist, and that this competition might antagonize the activity of temozolomide in a susceptible cell line.

### Results and Discussion

**Molecular Modeling.** Data for the electrostatic similarities and calculated atomic charges at C-4 of the tetrazinone or triazinone rings are shown in Table 1. Based solely on shape, all compounds might be expected to mimic closely temozolomide and mitozolomide; whereas in terms of electrostatic potentials, only the pyrazolotetrazinones 11 and 12 (HPSI of 0.933 and 0.919, respectively) were predicted to be similar to the lead structures. However, since active imidazotetrazinones only expose their cytotoxicity after ring opening at C-4, calculation of the electron density at this site was a crucial factor in predicting activity. In this respect the pyrazolotetrazinones 13 and 14 have a theoretical partial charge at C-4 equivalent to the active imidazotetrazinones, and indeed compound 14 displays anti-tumor activity in mouse tumor models in vivo on par with mitozolomide (2).14

### Table 1. Similarities of Shape, Electrostatic Potentials, and Atomic Charges at C-4 for Imidazo- and Pyrazolo[5,1-d]-1,2,3,5-tetrazinones and Benzo- and Pyrazolo-1,2,3-triazinones

<table>
<thead>
<tr>
<th>compd</th>
<th>shape&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPSI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>atomic charge at C-4</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.963</td>
<td>0.644</td>
<td>0.325</td>
</tr>
<tr>
<td>2</td>
<td>0.966</td>
<td>0.789</td>
<td>0.327</td>
</tr>
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<td>7</td>
<td>0.970</td>
<td>0.791</td>
<td>0.325</td>
</tr>
<tr>
<td>8</td>
<td>0.999</td>
<td>0.933</td>
<td>0.319</td>
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<tr>
<td>9</td>
<td>0.997</td>
<td>0.819</td>
<td>0.313</td>
</tr>
<tr>
<td>10</td>
<td>0.995</td>
<td>0.831</td>
<td>0.411</td>
</tr>
<tr>
<td>20</td>
<td>0.963</td>
<td>0.644</td>
<td>0.325</td>
</tr>
<tr>
<td>21</td>
<td>0.966</td>
<td>0.789</td>
<td>0.327</td>
</tr>
<tr>
<td>22</td>
<td>0.970</td>
<td>0.791</td>
<td>0.325</td>
</tr>
<tr>
<td>23</td>
<td>0.999</td>
<td>0.933</td>
<td>0.319</td>
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<td>0.313</td>
</tr>
<tr>
<td>25</td>
<td>0.995</td>
<td>0.831</td>
<td>0.411</td>
</tr>
</tbody>
</table>

<sup>a</sup> Compounds 7, 9, 11, and 13 are compared with temozolomide (value 1) and compounds 8, 10, 12, and 14 with mitozolomide (value 1).

**Chemistry.** We have synthesized a series of substituted 8-carboxanilides, 23–26, bearing an additional carboxamide group (Figure 3) to explore the biological outcome of increasing hydrogen-bonding potential at the 8-position. We have also synthesized imidazotetrazinones conjugated to spermidine through an 8-(3-aminopropyl)carboxamide linkage. The second objective of the present work was to synthesize nonalkylating analogues which might compete with active imidazotetrazinones for putative DNA binding sites: our efforts have focused on bicyclic heterocycles lacking the bridgehead nitrogen atom, i.e., the benzo-1,2,3-triazinones 9 and 10 and pyrazolo[4,3-d]-1,2,3-triazinones 11 and 12.

Figure 2. Structural analogues of antitumor imidazotetrazinones.

Figure 3. Structures of imidazotetrazinones conjugated to substituted anilines and spermidine in the 8-position.
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In an earlier paper\textsuperscript{14} we showed that 5-nitroimidazole-4-carboxylic acid (15) did not form the isolatable acid chloride 16 when treated with thionyl chloride or phosphorus pentachloride: instead, the previously reported tricyclic lactam 17 was formed (Scheme 2).\textsuperscript{15} This lactam ring opened in the presence of primary and secondary amines to give poor yields of substituted 5-nitroimidazole-4-carboxamides which were subsequently transformed to imidazotetrazines.\textsuperscript{14,16} In the present work we intended to exploit reactions of 17 with more complex amines but found that newly synthesized samples were contaminated with significant amounts of the chloro nitro lactam 18 and the dichloro lactam 19. The crude products showed a molecular ion for compound 17 at 278 m/z and major peaks at 267/269 and 256/258/260 m/z for the monochloro (18) and dichloro (19) lactams, respectively. These impurities presumably account for the low yields recorded previously. Efforts to improve the yield of the dinitro lactam 17 by conducting the thionyl chloridelphosphorus pentachloride to give the amides 30 and 31 and cyclized to the benzotriazinones\textsuperscript{32-37}. These impurities presumably account for the low yields recorded previously. Efforts to improve the yield of the dinitro lactam 17 by conducting the thionyl chloride/phosphorus pentachloride cyclizations under milder conditions, or by separating it from the mixture, were not successful.

As an alternative approach, mitozolomide (2) was converted to the carboxylic acid 20 and then to the acid chloride 21.\textsuperscript{16} Reaction of the acid chloride with aniline and a series of carbamoyl-substituted anilines afforded the anilides 22–26 in poor yields. The pyrimidinyl-anilinoimidazotetrazine 27 was synthesized in 28% yield from the acid chloride 21 and 2-amino-6-(4-aminophenyl)pyrimidin-4-one and crystallized as a solvate with 1-methylpyrrolidin-2-one. The spermidine conjugates 28 and 29 of temozolomide and mitozolomide were prepared by reacting the appropriate imidazotetrazine acid chlorides with \(\text{N}^\text{1},\text{N}^\text{8}\text{-bis(tert-butoxycarbonyl)}-\text{N}^\text{9}.\) (3-aminopropyl)spermidine followed by deprotection and isolation of the trihydrochloride salts: the hygroscopic nature of the salts dictated characterization by high-field NMR and accurate-mass FAB mass spectrometry.

Syntheses of 7- or 8-carbamoyl-1,2,3-triazinazoles 36 and 37 were accomplished in three high-yielding steps starting from the nitrobenzenedicarboxylic acids 31 and 30 (Scheme 3). These were converted to the amides 32 and 33 with thionyl chloride and ammonia, catalytically reduced to the amines 34 and 35, and cyclized to the benzotriazinones 36 and 37.

Selective alkylation at the 3-position of the benzotriazinone substrates 36 and 37 did not seem a practical proposition on the basis of earlier experiences,\textsuperscript{17} and a modified strategy was necessary to incorporate a range of substituents at this site (Scheme 4). Thus, the dicarboxylic acids 30 and 31 were firstly hydrogenated to give the amines 38 and 39 and converted to the isatoic anhydrides 40 and 41 with triphosgene. Selective ring opening at C-4 yielded the substituted amides 42–49 which were cyclized to the benzotriazinonecarboxylic acids 50–57 by nitrosation. The carboxylic acids were converted with thionyl chloride and ammonia to give the diamides 58–66 and a series of benzotriazinones, bearing carboxamide groups at the C-7 (7, 58, 59) or C-8 positions (9, 10, 60–62). During the ammonolysis step, the methyl ester group of compound 56 was converted to an amide in 61.

An alternative route to the 7-carbamoyl-3-methylbenzo-1,2,3-triazinazone 7 started with the substituted methyl anthranilate 63 which was diazotized and coupled with methylamine or aniline to afford the triazenes 64 and 65 in 70% and 80% yields, respectively (Scheme 5). The \(^1\text{H}\text{NMR spectrum of the methyltriazine 64 in CDCl}_3 showed the N-methyl resonance as a singlet at \(\delta 3.60\), suggesting that it exists as the methylaze tautomeric form,\textsuperscript{18} probably preferred because of stabilization by intramolecular hydrogen bonding.\textsuperscript{10} Cyclization of the triazenes in boiling 95% ethanol afforded the benzotriazinones 66 and 67 in 75% yields, and ammonolysis of the ester 66 yielded the carboxamide 7, identical to the sample prepared by the isatoic anhydride route. Incorporation of 2% piperidine in the cyclization of the phenyltriazene 65 in ethanol led to ester exchange and isolation of the ethyl ester 68 in 66% yield. Similarly, treatment of triazene 65 with 70% aqueous methanol containing 2% piperidine led to the isolation of the benzotriazinonecarboxylic acid 52.

We were unable to synthesize the carbamoylpyrazolotriazinone 11. In the closest attempt, the pyrazole dimethyl ester 69 was converted to the diazopyrazole 70 and immediately reacted with excess anhydrous methylamine in ethyl acetate solution; the product was the methylcarboxamide 72 (5%), probably formed via an intermediate (methyltriazetyl)pyrazole, 71 (Scheme 6). Finally, the 3-benzylpyrazolotriazinone 74 was synthesized in 85% yield by reacting the diazopyrazole 73 with benzyl isocyanate.

Hydrolysis of Mitozolomide. The rate of hydrolysis of imidazotetrazines is strongly influenced by pH.\textsuperscript{2,5} Initial experiments were conducted in which hydrolytic degradation of mitozolomide 2 was examined in different solutions at pH 7.4 at 37 °C which contained the
following: (a) phosphate buffer (0.1 M) which acted as a positive control in the measurement of the rate of pH dependent hydrolysis \( k_{pH} \), (b) a solution of d(G)-d(C) homopolymer in phosphate buffer, (c) an equimolar solution of guanosine-5'-monophosphate and cytidine-5'-monophosphate in phosphate buffer to measure any catalytic influence of individual components of G-C DNA, and (d) a solution of d(A)-d(T) homopolymer in phosphate buffer which acted as a control to monitor whether any observed catalytic effect was confined to G-C-rich DNA or DNA as a whole.

Mitozolomide was incubated with two concentrations of the oligonucleotide solutions, an equimolar concentration and a 10 mole excess of oligonucleotide. Disappearance of mitozolomide was monitored by HPLC, and hydrolysis was found to follow pseudo-first-order kinetics: the rate constants \( k \) were obtained from plots of log(mitozolomide peak area) versus time that were linear to three half-lives (data not shown). Values for \( k \) and calculated half-lives \( (t_{1/2}) \) are shown in Table 2. There was no significant difference between the \( t_{1/2} \) values in phosphate buffer (A) at pH 7.4 alone or in buffer containing G-C or A-T oligonucleotides or component G and C nucleotides.

In order to examine the possibility that the pH dependent hydrolysis in the presence of the phosphate nucleophile might dominate any effects associated with the presence of the oligonucleotide (i.e., \( k_{pH} > k_{G-C} \)), decompositions were also conducted in phosphate buffer at pH 6.5 and sodium cacodylate buffer at pH 7.4 and 6.5. Again there was no difference in the \( t_{1/2} \) values in buffered solution alone and in the presence of oligonucleotides (Table 3). However there was a moderate increase in the rate constants for the degradation of mitozolomide in cacodylate buffer compared with those in phosphate buffer suggesting that the large mole excesses of nucleophiles in the buffers may be masking any effects due to the interactions of the drug with oligonucleotides in these experiments.

**In Vitro Cytotoxicity of Imidazotetrazinones and Related Compounds.** The cytotoxicities of several compounds prepared in this work were tested...
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Table 2. First-Order Rate Constants (k) and Half-Lives (t_{1/2}) for the Hydrolysis of Mitozolomide (2) in Buffers at Different pH Values at 37 °C in the Presence of Oligonucleotides and Nucleotides

<table>
<thead>
<tr>
<th>incubation mixture</th>
<th>buffer</th>
<th>pH</th>
<th>rate constant (k \times 10^2 \text{ min}^{-1})^a</th>
<th>mean rate constant (\times 10^3 \text{ min}^{-1})</th>
<th>half-life, t_{1/2} (\text{min})^b</th>
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</thead>
<tbody>
<tr>
<td>control^c</td>
<td>A</td>
<td>7.4</td>
<td>9.74</td>
<td>9.69 ± 0.18</td>
<td>71.5</td>
</tr>
<tr>
<td>d(G)-d(C) (1:1)^f</td>
<td>A</td>
<td>7.4</td>
<td>9.39</td>
<td>9.40 ± 0.28</td>
<td>73.3</td>
</tr>
<tr>
<td>d(G)-d(C) (10:1)^f</td>
<td>A</td>
<td>7.4</td>
<td>8.68</td>
<td>9.11 ± 0.43</td>
<td>76.1</td>
</tr>
<tr>
<td>5’-GMP/5’-CMP (1:1)</td>
<td>A</td>
<td>7.4</td>
<td>9.79</td>
<td>9.79 ± 0.07</td>
<td>70.8</td>
</tr>
<tr>
<td>d(A)-d(T) (1:1)^f</td>
<td>A</td>
<td>7.4</td>
<td>8.56</td>
<td>9.15 ± 0.20</td>
<td>75.8</td>
</tr>
<tr>
<td>d(A)-d(T) (10:1)^f</td>
<td>A</td>
<td>7.4</td>
<td>9.23</td>
<td>9.32 ± 0.09</td>
<td>74.4</td>
</tr>
<tr>
<td>control^c</td>
<td>B</td>
<td>6.5</td>
<td>1.52</td>
<td>1.52 ± 0.02</td>
<td>450.0</td>
</tr>
<tr>
<td>d(G)-d(C) (10:1)^f</td>
<td>A</td>
<td>6.5</td>
<td>1.54</td>
<td>1.54 ± 0.01</td>
<td>450.0</td>
</tr>
<tr>
<td>d(A)-d(T) (10:1)^f</td>
<td>B</td>
<td>6.5</td>
<td>1.55</td>
<td>1.55 ± 0.01</td>
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<tr>
<td>control^c</td>
<td>D</td>
<td>6.5</td>
<td>2.01</td>
<td>2.01 ± 0.03</td>
<td>341.5</td>
</tr>
<tr>
<td>d(G)-d(C) (10:1)^f</td>
<td>D</td>
<td>6.5</td>
<td>2.02</td>
<td>2.02 ± 0.03</td>
<td>346.6</td>
</tr>
</tbody>
</table>

^a Calculated from plots of log( ([mitozolomide] peak area)] versus time with a minimum of 10 data points. ^b Calculated from the mean rate constant using the equation t_{1/2} = 0.693/k. ^c Buffer solution only. Buffers: A, phosphate buffer (0.1 M) at pH 7.4; B, phosphate buffer (0.1 M) at pH 6.5; C, sodium cacodylate buffer (0.1 M) at pH 7.4; D, sodium cacodylate buffer (0.1 M) at pH 6.5. ^f Equimolar ratio of mitozolomide to nucleotide bases in the oligonucleotide. ^a Ten-fold molar excess of nucleotide bases in the oligonucleotide compared to mitozolomide. ^f Represents equimolar ratios of guanosine-5'-monophosphate to cytidine-5'-monophosphate.

Table 3. In Vitro Cytotoxicity of Imidazo-[5,1-d]-1,2,3,5-tetrazinones and Benzo-1,2,3-triazinones against Mouse TLX5 Lymphoma Cells

<table>
<thead>
<tr>
<th>compd</th>
<th>IC50 (µM)^a</th>
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<tbody>
<tr>
<td>1</td>
<td>5.0</td>
</tr>
<tr>
<td>2 (mitozolomide)</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>380</td>
</tr>
<tr>
<td>8</td>
<td>350</td>
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<tr>
<td>9</td>
<td>&gt;500</td>
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<td>225</td>
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<td>22</td>
<td>15.8</td>
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<tr>
<td>33</td>
<td>&gt;500</td>
</tr>
<tr>
<td>34</td>
<td>&gt;500</td>
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</table>

^a Results are the mean of two separate determinations.

vitro against the mouse TLX5 lymphoma, a cell line which has proved valuable in evaluating SAR in imidazotetrazines. The reference compounds were temozolomide (1) and mitozolomide (2) which gave IC50 values of 5 and 2.5 µM, respectively. As predicted from the modeling studies, representative 1,2,3-benzotriazinones 7–10, 37, and 58–62 were generally at least 100-fold less cytotoxic than mitozolomide and had IC50 values in the range 100–>500 µM (Table 3). The carboxanilide 22 was equipotent with mitozolomide but attachment of an additional carboxamide residue to the anilide fragment 23–25 reduced activity approximately 10-fold; incorporation of two carboxamide groups furnished a compound, 26, which was extremely insoluble and inactive. Activity was only partially restored in the substituted aminopyrindinone-conjugated mitozolomide 27 (IC50 = 25 µM) despite the potential of this compound to undergo Watson–Crick type hydrogen bonding to guanine and cytosine residues in single-stranded DNA or Hoogsteen bonding to duplex DNA.

Drug cytotoxicity has also been determined in two cell lines differing in their capacity to effect repair of O6-

alkyguanine lesions, the O6-alkyguanine-DNA alkyltransferase deficient (Mer−) human lymphoblastoid cell line GM892A and the repair proficient (Mer+) Raji cell line originally obtained from a patient with Burkitt's lymphoma. IC50 values determined from growth inhibition assays are recorded in Table 4. For the reference imidazotetrazinones, the order of potency was mitozolomide > temozolomide > ethazolastone. The Mer− cell line was 23- and 8-fold more sensitive to temozolomide and mitozolomide, respectively, than the Mer+ Raji line, whereas ethazolastone showed only a 2-fold differential between the two cell lines. MTIC (4), the intermediate generated upon ring opening of temozolomide, also showed high potency and discriminating activity, as did the mutagenic agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG); the related methylating agent N-methyl-N'-nitrosourea (MNU) was less potent but still showed a 7.5-fold greater activity against the Mer− cell line. These data can be explained by the fact that all three agents are themselves progenitors of a common methyl transfer agent, the methyl diazonium cation 6, which can alkylate a variety of sites on DNA including O6- and N-7 of guanine.

Puzzlingly, the antimalanoma drug 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide (DTIC) also displayed moderate potency against the GM892A cells and 9-fold selectivity.
in favor of the Mer cells despite the accepted view that this agent requires prior metabolic demethylation to exert a cytotoxic action.\textsuperscript{23} Possibly the GM892A cells can effect this transformation even \textit{in vitro}.

In our hands the 3-methylpyrazolotetrazinone 13 showed impressive \textit{in vitro} potency and selectivity, with a 50-fold differential toxicity toward the Mer\textsuperscript{-} cell line. The latter result contrasts with the reported inactivity of this methylpyrazolo-tetrazinone against mouse tumors \textit{in vivo}\textsuperscript{24} whereas the 3-(2-chloroethyl) analogue 14 is very active.\textsuperscript{14} The spermidine—temozolomide conjugate 28 had low cytotoxicity against both GM892A and Raji cells (IC\textsubscript{50} > 500 \(\mu\)M).

As expected, none of the new 1,2,3-triazinones (7, 9, and 27) showed useful activity against either cell line with IC\textsubscript{50} values > 500 \(\mu\)M (Table 4). Competition assays were performed to assess the ability of these stable compounds to antagonize the cytotoxic action of temozolomide. Preincubation of GM892A cells for 2 h with the 7- or 8-carbamoylbenzo-1,2,3-triazinone 7 or 9 did not inhibit the cytotoxic properties of temozolomide (data not shown) suggesting that these compounds do not compete with the drug for uptake into cells or for binding sites in DNA.

**DNA Footprinting Studies.** Various DNA footprinting techniques were employed in attempts to determine potential binding sites for temozolomide, but these studies were not successful. Briefly, a homogeneous sample of a labeled 160-base pair Tyr T DNA fragment\textsuperscript{25} was digested by DNAase I in the presence and absence of drugs, and the fragments were visualized by autoradiography following electrophoresis. Whereas the model drug echinomycin, at 100 \(\mu\)M, showed DNA binding sites as distinct gaps near positions 35, 55, 75, and 100 corresponding to sections where the DNA remained uncut by DNAase I, there were no observable differences from solvent controls for DNA digested in the presence of temozolomide (1) or MTIC (4) at 4 or 37 °C with drug concentrations in the range 20 \(\mu\)M to 1 mM (data not shown). Similarly, the benztiazainones 7 and 9 showed no impediment to DNAase I-mediated DNA cleavage.

Further footprinting studies were performed using a modification of the Maxam and Gilbert sequencing technique to examine the ability of drugs to protect DNA from alkylation.\textsuperscript{26} Temozolomide did not protect guanine residues from dimethyl sulfate methylation, nor did temozolomide or MTIC preserve guanine residues from diethyl pyrocarbonate ethylation (data not shown). Thus, if an imidazotetrazinone specific receptor site did exist, these footprinting techniques appeared unable to aid clarification of such interactions.

**Sequence Specificity of Covalent Modification of DNA.** In order to examine the DNA sequence selectivities of temozolomide (1), the pyrazolotetrazinone isostere 13 and the spermidine—temozolomide conjugate 28, a modification of the standard Maxam and Gilbert sequencing technique was used to examine guanine N-7 alkylation. 5'-End-labeled \textit{BamHI-SalI} DNA fragments were incubated with drug at 37 °C for 2 h, and the DNA was cleaved with piperidine to produce breaks at sites of guanine N-7 alkylation. The patterns of fragments obtained are shown (Figure 4). Results are compared with those from two related compounds devoid of antitumor activity—ethazolastone (3) and the 3-benzylpyrazolotetrazinone 74.

Bands predominated at sites corresponding to runs of three or more contiguous guanine bases indicating temozolomide, the pyrazole analogue 13, and the spermidine conjugate 28 do preferentially alkylate these sites. Ethazolastone (3) and the benzylpyrazolotetrazinone 74 were less discerning. In addition, qualitative examination of the level of fragment cleavage following alkylation indicates ethazolastone to have very low reactivity (Figure 4A, lanes a, b) by comparison with temozolomide (lanes c-g), a factor which may contribute to the lack of antitumor activity of the ethyl congener. It was anticipated that conjugation with spermidine would provide temozolomide with enhanced DNA-directed activity, and a significant increase in DNA reactivity was indeed detected (Figure 4A, lanes h–m). However, initial studies indicated that \textit{in vitro} antitumor activity may not parallel DNA reactivity since at 500 \(\mu\)M the percent control population growth in GM892A and Raji cells was 52.5% and 85%, respectively. Cytotoxicity was not improved by depletion of cellular polyamine levels, using difluoromethylornithine (0.5 mM, 72 h) prior to exposure of these cell lines to the conjugate. Hence, cellular delivery of 28 would appear to be hampered by the necessity for a spermidine uptake mechanism, although this in turn may allow the targeting of tumor cells possessing such an uptake system.\textsuperscript{27} Quantitative comparison between the 3-methylpyrazolotetrazinone 13 (Figure 4B, lane c), the 3-benzylpyrazolotetrazinone 74 (lane d), and temozolomide (lane e) shows that even a 10-fold higher concentration of the benzyl compound 74 did not elicit equivalent levels of alkylation as the two methyl compounds.

The piperidine cleavage assay is confined to the detection of guanine N-7 alkylations, and so sequence selectivity was also examined using a primer extension procedure utilizing multiple cycles of polymerization with the thermostable DNA polymerase from \textit{Thermus aquaticus}. Following annealing of a 5'-end-labeled primer to template DNA, extension with \textit{Taq} DNA polymerase produced a full length fragment of 263 bp in a solvent control. The ability of temozolomide alkylations to block the progress of the polymerase, causing premature termination of chain elongation, is presented in Figure 5. Unmodified DNA showed very few sites of early termination, whereas with temozolomide-alkylated DNA termination occurred at guanine bases preferentially in the nucleophilic microenvironment of clustered, rather than isolated, guanines. This confirmed the results of the piperidine cleavage assay and indicated that the major lesion produced by this agent is at the guanine N-7 position.

**Conclusions**

Earlier structural and bioactivity studies on antitumor imidazotetrazinones conducted with the responsive mouse TLX5 lymphoma demonstrated the importance of the hydrogen-bonding property of the 8-carboxamide substituent.\textsuperscript{2,4,14,18} However, 8-carboxanilides 23–27 designed to potentially augment bonding at this site were less active than the prototype agents temozolomide (1) and mitozolomide (2) against this tumor \textit{in vitro}.

Molecular modeling approaches were employed to design bicyclic isosteres of temozolomide and mitozolo-
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A

Figure 4. Sites of guanine N-7 alkylation produced in the 276-bp BamHI-SalI fragment of pBR322, 5'-labeled at the BamHI site. (A) Lane (a) solvent control; (b) formic acid purine marker lane; (c) 250 μM 3-methylpyrazolotetrazinone 13; (d) 2.5 mM 3-benzylpyrazolotetrazinone 74; (e) 250 μM temozolomide. Numbers refer to the base sequence (see the supplementary material) for which sites of three or more contiguous guanine residues are marked.

Temozolomide and the spermidine conjugate both preferentially methylated guanine-rich sequences in a BamHI-SalI DNA fragment as determined by piperidine cleavage experiments and induced blocks to Taq DNA polymerase action at similar sites. The same sites were also methylated by MTIC (data not shown). Investigations with mitozolomide, its corresponding (chloroethyl)-triazene, and a series of other triazenes9 have indicated that the imidazocarboxamide group has some influence in determining the fine details of reactivity with DNA although selectivity is mainly a property of the DNA sequence and not the drug. Presentation of the alkylating agent either as imidazotetrazine prodrug or open-chain triazene has no discernable affect on the products of the reaction. Furthermore, the SAR5 and modeling studies5 make no proposals for the interaction of temozolomide with DNA which could not also apply to MTIC.

The evidence presented here suggests that the conversion from imidazotetrazinone prodrug to drug occurs in free solution under the influence of local pH30 rather than in the major groove catalyzed by a target DNA sequence as previously proposed. This theory is in

mide based on benzo- and pyrazolo-1,2,3-triazinones, which cannot ring open to alkylation species; these compounds were not active (IC50 > 500 μM) against human GM892A (Mer-) and Raji (Mer+) cell lines in vitro, nor did they protect these cell lines from subsequent challenge by temozolomide. The lack of potency of the spermidine conjugates 28 and 29 can be attributed to the lack of a polyamine uptake system in these cells.

The rate of ring opening of mitozolomide was significantly influenced by pH (t1/2 = 1.25 h at pH 7.4 and 7.5 h at pH 6.5 in phosphate buffer) and the nature of the buffer (phosphate or cacodylate); furthermore, no rate enhancements were observed in the presence of d(G)-d(C) oligonucleotides. This could be due to weak association of the drug with DNA, the consequence being that the reaction in bulk solution dominated any effects due to association with DNA. Further evidence for this weak association came from the failure of footprinting techniques to detect noncovalent associations between temozolomide and DNA. Recent NMR studies (unpublished) further support this hypothesis.
accord with the observed half-life of temozolomide in phosphate buffer (pH 7.4 at 37 °C) being comparable with its mean plasma half-life in patients.6 The enhanced clinical activity of temozolomide over the other prodrug of MTIC (DTIC) must therefore be due to more effective generation of unstable MTIC inside cell nuclei.

**Experimental Section**

**Molecular Modeling.** The published crystal structures of temozolomide14 and mitozolomide15 were the starting templates for the construction of structures of carbamoyl-substituted benzo-1,2,3-triazinones and related heterocycles (Figure 2) using the modeling package Chem-X.21 The structures were then optimized using the ab initio molecular orbital package GAMESS28 with the 321-G basis set. The similarities of the analogues were measured with the similarity program ASP.28 For the electrostatic similarities, the structures were optimized with the Hodgkin potential similarity algorithm.13

**Synthesis.** All new compounds were characterized by elemental microanalysis (C, H, and N values) and mass spectrometry (recorded on a V.G. Micromass 12B instrument at 70 eV; source temperature 250—300 °C). FAB and high-resolution FAB mass spectra were run at the EPSRC Mass Spectrometry Service Centre, University of Wales, Swansea. UV spectra were recorded on a Cecil CE 5095 spectrometer in 95% ethanol solutions. IR spectra were determined on a Mattson 2020 Galaxy Series FT spectrometer or a Perkin Elmer 1310 infrared spectrometer as either Nujol mulls or KBr discs. NMR spectra were recorded in DMSO-d6 (unless otherwise indicated) on a Jeol GX 500, Bruker ARX 400, or Bruker ARX 250 spectrometers. J values are recorded in hertz (Hz). TLC systems for routine monitoring of reaction mixtures and confirming the homogeneity of analytical samples employed Kieselgel 60F254 (0.25 mm) with either CHCl3 or CH2Cl2—2% ethanol as developing solvents. Sorbfil silica gel C 60-H (40—60 μm) was used for flash chromatography.

**Synthesis of Imidazo[1,2,3,5-tetrazinones.** 1,6-Dinitro-5H,10H-diimidazo[1,5-a:1',5'-pyrazine-5,10-dione (17). A mixture of 5-nitroimidazo-4-carboxylic acid (15)β (0.6 g), anhydrous toluene (8 mL), and thionyl chloride (1.3 mL) was refluxed (3 h) and evaporated to yield an oily residue which was triturated in toluene–petroleum ether (60—80 °C, 1:1, 10 mL). The brown solid (0.43 g) had mp 247—249 °C (lit.22 mp 249—251 °C) and IR 1750 (C=O), 1540 (NO2) cm−1 but was shown by mass spectrometry to be a mixture of 17 (M+ 278), 18 (M+ 267/269, 3:1), and 19 (M+ 256/258/260, 9:6:1).

3-(2-Chloroethyl)-N-phenyl-4-oxoimidazo[5,1-d]1,2,3,5-tetrazine-8-carboxamide (22). This compound was prepared in 95% yield from the 8-carboxyl chloride 21 and aniline by the method of Horspool.

Similarly prepared from 21 and the appropriate aminobenzamides were the following.

3-(2-Chloroethyl)-N-(2-carbamoylphenyl)-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxamide (23): from 2-aminobenzamide (15%; mp 179—181 °C dec; IR 3525—3100 br (NH), 1742, 1685, 1642 (C=O) cm−1. Anal. (C14H12ClN7O3) C, H, N.

3-(2-Chloroethyl)-N-(3-carboxamidobenzyl)-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxamide (24): from 3-aminoacetanilide (21%; mp 250—252 °C dec; IR 3435, 3368, 3187 br (NH), 1740, 1661 (C=O) cm−1. Anal. (C11H12ClN6O2) C, H, N.

3-(2-Chloroethyl)-N-(4-carboxamidobenzyl)-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxamide (25): from 4-aminobenzamide (24%; mp 249—250 °C dec; IR 3378 br, 3168 br (NH), 1734, 1677, 1655 (C=O) cm−1. Anal. (C12H12ClN5O4) C, H, N.

3-(2-Chloroethyl)-N-(2,4-dihydroxy-4-oxopyrimidin-5-yl)phenyl-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxamide (26): from 2-amino-5-carboxamidobenzamidinamide (32%); crystallized from 1-methylpyrrolidin-2-one; mp 292—295 °C dec; IR 3192, 3000, 1655 (C=O) cm−1. Anal. (C13H14ClN5O4) C, H, N.

3-(2-Chloroethyl)-N-[4-(2-amino-3,4-dihydro-4-oxopyrimidin-5-yl)phenyl]-4-oxoimidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxamide (27): from 2-amino-5-carboxamidobenzamidinamide (28%); crystallized from a mixture of 1-methylpyrrolidin-2-one and ether; mp >320 °C dec; IR 3500—3000 br (NH), 1746, 1690, 1654 (C=O) cm−1. Anal. (C16H15ClN5O4·0.5C3H5NO) C, H, N.

**Spermidine–Temozolomide (28) and Mitozolomide (29) Conjugates.** Temozolomide-8-carbonyl chloride23 was dissolved in dry dichloromethane (10 mL) under nitrogen and cooled to −40 °C. A solution of N,N'-bis[tert-butoxycarbonyl]-N-(3-aminopropl) spermidine21 (0.94 g) and triethylamine (0.26 g) in dichloromethane (10 mL) was added to the acid chloride solution at −40 °C over 5 min with rapid stirring. The solution was stirred at −40 °C for 2 h and then allowed to warm to room temperature overnight. The solution was recooled to −40 °C, TFA (1.5 g) added, and the solution allowed to warm to room temperature. Solvent and excess TFA were removed by evaporation in vacuo, and the orange residue was dissolved in 1 M HCl (100 mL). The acid solution was shaken with chloroform (10 mL) to remove a small oily fraction, and the aqueous layer was loaded onto a Dowex 50 X2-200 ion exchange column. The column was washed with 1 M HCl (400 mL), 1.25 M HCl (100 mL), 1.5 M HCl (450 mL), and 1.8 M HCl (450 mL). Evaporation of the 1.5 and 1.8 M HCl eluents yielded a pink foam of the spermidine–temozolomide conjugate 28 as a hygroscopic trihydrochloride salt (30%).18 1H NMR (500 MHz, hexamethylenimine) and 13C NMR (125 MHz, hexamethylenimine) spectra were recorded in D2O with 1H and 13C labels. The 1H NMR spectrum showed the expected signals for the spermidine–temozolomide conjugate 28.

**Figure 5**. Sequence specificity of covalent adduct formation measured by a Taq DNA polymerase stop assay. Bands indicate the sites of termination for extension of a 5'-end-labeled primer complementary to bases 621—640 of the BamHI-SstI fragment of pBR322 (bases 375—650): (a) control unmodified fragment and (b) 1 mM temozolomide phosphate buffer (pH 7.4).
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MS assignments by DEPT135, HETCOR) 6 160.7 (CONH), 139.8 (CH3), 24.7 (C-7"), 23.9 (C-2'), 21.8 (C-2), 20.4 (C-6); FAB MS m/z 280,252 (M + H+), C16H30N9O2 requires 380.252. Similarly prepared, from mitozolomide-8-carboxyl chloride (21), was the spemidine–mitozolomide conjugate 29 as a hydroscopic trihydrochloride salt (74%); 1H NMR (400 MHz) δ 9.46 (1H, br s, N(4")H), 8.82 (1H, s, H-6), 8.53 (1H, t, J = 5.8, CONH), 7.50 (1H, br, N(1")H), 6.19 (1H, br, N(3")H), 4.64 (2H, t, J = 6.1, CH2CH2Cl), 4.04 (2H, t, J = 6.1, CH2CH2Cl), 3.43 (2H, qnt, J = 6.3, 3.5") 3.25 (2H, m, 3", H-3), 3.10 (4H, m, 5", H-1"), 2.93 (2H, m, 1", H-1") 2.83 (2H, m, 5", H-5"), 2.14 (2H, m, 2"H), 2.06 (2H, m, 2") 1.85 (2H, qnt, J = 7.7, 6") 1.58 (2H, qnt, J = 7.3, 7") (C-4) NMR (62.9 MHz) δ 160.0 (CONH), 139.2 (C-4), 134.1 (C-8a), 131.4 (C-6), 128.4 (C-6), 51.6 (C-1"), 50.5 (C-5"), 50.2 (CH2CH2Cl), 49.5 (C-3), 41.6 (CH2CH2Cl), 38.3 (C-8b), 36.6 (C-3"), 36.5 (C-1"), 24.4 (C-7"), 23.6 (C-2''), 21.6 (C-2''), 20.3 (C-6"); FAB MS m/z 428,230 (M + H+), C16H30N9O2 requires 428,229.

Synthesis of Benzo-1,2,3-triazinones by Nitrosation of o-Aminobenzamides. 2-Nitrobenzene-1,3-dicarboxamide (32). A mixture of the 1,3-dicarboxylic acid 30 (1.68 g) thionyl chloride (10 mL), and DMF (0.1 mL) was refluxed for 12 h. The solution was evaporated and the residue triturated with toluene (10 mL) and re-evaporated. The resulting solid was recrystallized from water as colorless needles: mp 288-290 °C (lit. 33 mp 278-280 °C). Anal. (C8H5N2O4) C, H, N.

2-Amino-benzene-1,3-dicarboxamide (34). A mixture of the 1,3-dicarboxylic acid 30 (0.25 g) crystallized from aqueous ethanol as white plates (from aqueous ethanol); MS (EI) m/z 190 (M+). Anal. (C7H5NO3) C, H, N.

2-Amino-3-(N-phenylcarbamoyl)benzoic acid (46): Similarly prepared from 2-amino-3-(N-phenylcarbamoyl)benzoic acid (33): mp 275-277 °C dec; IR 3220-2600 br (OH), 1703 (C=O) cm-1; MS (EI) m/z 208 (M+). Anal. (C11H9N2O4) C, H, N.

3-Amino-4-(N-methylcarbamoyl)benzoic acid (47): with aniline in dioxane; mp 248 °C (from aqueous ethanol); MS (EI) m/z 256 (M+). Anal. (C7H8N2O3) C, H, N.

2-Amino-3-(N-ethylcarbamoyl)benzoic acid (44): with 75% aqueous ethylamine; mp 233-235 °C (from water); MS (EI) m/z 208 (M+). Anal. (C7H8N2O3) C, H, N.

2-Amino-3-(N-methylthio)benzamide (45): with 2-chloroethylamine (prepared by the addition of triethylamine in ethanol to a solution of 2-chloroethylhydrochloride in water); mp 207 (M+). Anal. (C10H12N2O3) C, H, N.

3-Amino-4-(N-ethylthio)benzamide (46): with aniline in dioxane; mp 231-233 °C (from aqueous ethanol); MS (EI) m/z 256 (M+). Anal. (C7H8N2O3) C, H, N.

3-Amino-4-(N-phenylthio)benzamide (47): with aniline in dioxane; mp 195-197 °C; IR 3250-2500 br (OH), 1703 (C=O) cm-1; MS (EI) m/z 219 (M+). Anal. (C11H8N2O3) C, H, N.

2-Amino-3-(N-(2-chloroethyl)carbamoyl)benzoic acid (48): with 2-chloroethylamine (prepared by the addition of triethylamine in ethanol to a solution of 2-chloroethylhydrochloride in water); mp 200-202 °C (from aqueous acetone); MS (EI) m/z 242,244 (M+). Anal. (C9H11ClN3O4) C, H, N.

2-Amino-3-(N-ethylcarbamoyl)benzoic acid (49): with glycine methyl ester (synthesized by the addition of triethylamine in ethanol to a solution of glycine methyl ester hydrochloride in water); mp 192-193 °C (from water); MS (EI) m/z 252 (M+). Anal. (C9H10N2O3) C, H, N.

2-Amino-3-(N-phenylcarbamoyl)benzoic acid (50): The N-methylamide 47 (0.6 g) and anhydrous sodium carbonate (0.18 g) were warmed in water (15 mL) until all the methylamide had dissolved. The solution was cooled to room temperature and sodium nitrite (0.27 g) added. The solution was poured into 0.5 M hydrochloric acid at 0 °C, and the mixture was stirred at 0 °C for 1 h. Acidification of the mixture to pH 2 liberated the benzotriazine 50, which crystallized from aqueous ethanalam as colorless needles (81%): mp 245-247 °C dec; IR 3220-2600 cm-1; MS (EI) m/z 208 (M+). Anal. (C7H8N3O3) C, H, N.

2-Amino-3-(N-methylthio)benzamide (51): from the N-methylamide 47 in 75% yield as white crystals; mp 200-201 °C (from water); MS (EI) m/z 208 (M+). Anal. (C7H8N2O3) C, H, N.

2-Amino-3-(N-ethylthio)benzamide (52): from the N-ethylamide 48 in 65% yield; with aniline in dioxane; mp 231-233 °C (from aqueous ethanol); MS (EI) m/z 256 (M+). Anal. (C7H8N2O3) C, H, N.

2-Amino-3-(N-phenylthio)benzamide (53): from the N-phenylamide 49 in 75% yield; mp 200-201 °C (from water); MS (EI) m/z 208 (M+). Anal. (C7H8N2O3) C, H, N.

2-Amino-3-(N-(2-chloroethyl)thio)benzamide (54): from the N-(2-chloroethyl)amide 48 in 59% yield; mp 232-235 °C dec; IR 3220-2500 br (OH), 1692 (C=O) cm-1; m/z 205 (M+). Anal. (C7H8N2O3) C, H, N.

2-Amino-3-(N-ethylthio)benzamide (55): from the N-ethylamide 49 in 69% yield as colorless plates from aqueous ethanol; mp 237-239 °C dec; IR 3250-2400 br (OH), 1694 (C=O) cm-1. Anal. (C7H8N2O3) C, H, N.

2-Amino-3-(N-methylthio)benzamide (56): from the N-methylamide 47 in 75% yield as white plates from aqueous ethanol; mp 232-235 °C dec; IR 3220-2500 br (OH), 1692 (C=O) cm-1. Anal. (C7H8N2O3) C, H, N.
thionyl chloride (5 mL), and DMF (0.1 mL) was refluxed (3 h). Removal of excess thionyl chloride gave a residue which was distilled in vacuo to obtain the 1,2,3-triazine-7-carboxamide (63) from the carboxylic acid (8) in 51% yield as colorless plates from aqueous ethanol, mp 260–262 °C; IR: 3441, 3204 (NH), 3127 (CH), 1746, 1690 (C=O) cm⁻¹; MS (EI) m/z 204 (M⁺). Anal. C₁₁H₁₁N₅O₃ C, H, N.

Sodium acetate-buffered solution at 0 °C. The triazene 64 was refluxed in methanol containing 2% piperidine for 3 h. The colored solution was concentrated in vacuo and extracted with chloroform (3 × 10 mL). The organic extracts were dried (anhydrous sodium sulfate) and evaporated to give a yellow oil which solidified when triturated with hexane.

Methyl 3,4-Dihydro-4-oxo-3-methylbenzo-1,2,3-triazine-7-carboxylate (65) (0.1 g) was stirred with benzyl isocyanate (0.28 mL) in dry DMSO (10 mL) at 25 °C for 12 h. The solid was collected. Purification of the crude product by flash chromatography using ethyl acetate as eluent gave the pyrazolotetrazinone (0.29 g) typical to the aforementioned sample.

Hydrolysis of Mitozolomide in the Presence of Nucleic Acids and Nucleotides. Synthetic nucleic acid oligomers were purchased from Sigma Chemical Co., Ltd., Poole, U.K., and HPLC grade solvents were obtained from Pisons, Loughborough, U.K. HPLC analyses were performed using a Waters 600E gradient solvent delivery system fitted with a Merck 250 Lichrosorb RP select B column and a Lichrocart reversed-phase C-18 endcapped guard column and monitored at 325 nm. Hydrolyses were performed in glass screw-capped vials in a final volume of 0.8 mL and monitored for approximately three half-lives. Aliquots (10 μL) were removed from the incubation mixture, injected directly onto the column using a Waters WISP 710B automatic injection sampler, and eluted with a mobile phase of acetic acid (0.5%) in water/methanol (70:30) at a flow rate of 1 mL/min for 10 min. Typically, mitozolomide had a retention time of 4.8 min.

In Vitro Growth Inhibition. (a) Mouse TLX5 Lymphoma Cells. TLX5 cells were maintained in exponential growth phase at 2 × 10⁶ cells/mL in RPMI 1640 supplemented with 15% fetal calf serum. Aliquots (2 mL) of the cells were plated out into six well plates and treated with 10 μL of the required drug solutions in DMSO, with three replicates for each drug concentration. The control incubates were composed of cells treated with 10 μL of DMSO. After being incubated at 37 °C for 48 h, the supernatant was removed and 5% CO₂, the cells were counted using a Coulter Laboratories ZM counter. Results were plotted as percent of control population growth (data not shown), from which the IC₅₀ values (μM) were calculated.

(b) Human Raji and GM892A Cells. Raji and GM892A cells were routinely cultured in RPMI 1640 supplemented with 10% fetal calf serum and 1% L-glutamine. Cells were seeded at 0.8 × 10⁶ cells/mL in Nunc 24-well plates and treated with a range of drug concentrations. Drugs were
dissolved in DMSO and added so that the DMSO concentration did not exceed 0.5%. A minimum of three cell populations were treated for each drug concentration, and cell numbers were duplicate-counted following a 72 h incubation period. The growth of the treated cell population was compared to the growth of a solvent-treated control cell population, after subtraction of the seed number from the final cell counts. IC₅₀ values (µM) were calculated as above.

Effect of Pre-exposure to GM892 Cells by Benzo-1,2,3-triazinones on the Cytotoxicity of Temozolomide. GM892A cells were seeded at 0.5 × 10⁶ cells/mL and treated with a range of concentrations of potential antagonists as described above. Following incubation for 2 h at 37 °C, the cells were centrifuged, washed with PBS, resuspended in media, and then aliquoted into Nunc 24-well plates before being challenged with temozolomide. Cells were incubated for 3–4 days at 37 °C. The ability of temozolomide to inhibit growth of cells pretreated with the inactive benzo-1,2,3-triazinones was compared with the inhibition elicited by temozolomide alone in solvent-treated controls.

Determination of Guanine N-7 Alkylation Sites in Defined DNA Sequences. (a) DNA Fragment Isolation and Labeling. The basic procedure was as described by Hartley et al. Allowment of pBR322 DNA was labeled by standard methods and a 276-bp BamHI-SalI fragment was isolated following further cleavage with Sall. A 346-bp HindIII-BamHI fragment of pBR322 DNA 5’-labeled at the HindIII restriction site was prepared by the same method following initial cutting by HindIII.

(b) Fragment Alkylation and Piperdine Cleavage. The DNA fragment (5000 cpm/sample) was alkylated in 25 mM triethanolamine HCl–1 mM EDTA at pH 7.2 at 37 °C for 2 h using a range of concentrations of drug in a final reaction volume of 50 µL (drugs were dissolved in DMSO to give a 20% DMSO in the final reaction mixture). Following ethanol precipitation and washing, the alkylated DNA was treated with 100 µL of a fresh solution of 10% piperidine at 90 °C for 15 min to produce strand breaks specifically at the sites of guanine N-7 alkylation. The DNA fragments were then dissolved in formamide dye solution, heated at 90 °C for 2 min, and separated using electrophoresis on a 20 cm 6% denaturing polyacrylamide gel run at 3000 V for 30 min; samples were then blotted on to nitrocellulose paper and ethanolextracted. DNA fragments were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography as described for the piperidine cleavage method.

Acknowledgment. This study was supported by the Cancer Research Campaign, U.K. We thank Ms. H. Hussey for conducting the TLX5 lymphoma in vitro cytotoxicity tests and Mr. K. Farnell for technical support of the synthetic work.

Supplementary Material Available: Base sequence of the BamHI-SalI fragment of pBR322 DNA (1 page). Ordering information is given on any current masthead page.

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ASP; Oxford Molecular Ltd.: Oxford, U.K.


