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A Strategy for Imidazotetrazine Prodrugs with Anti-cancer Activity Independent of MGMT and MMR.

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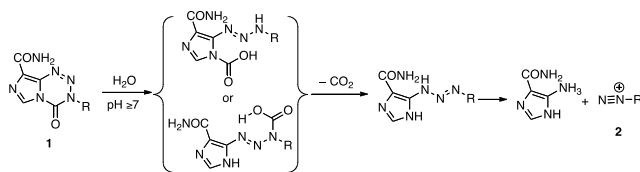
KEYWORDS imidazotetrazine · aziridinium · temozolomide · cancer · ¹³C NMR · DNA

Supporting Information Placeholder

ABSTRACT: The imidazotetrazine ring is an acid-stable precursor and prodrug of highly-reactive alkyl diazonium ions. We have shown that this reactivity can be managed productively in an aqueous system for the generation of aziridinium ions with 96% efficiency. The new compounds are potent DNA alkylators and have antitumor activity independent of the *O*6-methylguanine-DNA methyltransferase and DNA mismatch repair constraints that limit the use of temozolomide.

Imidazotetrazine **1** is a fascinating heterocycle, on account of both its Chemistry and the therapeutic applications of its derivatives. It forms the core of the anti-cancer prodrug Temozolomide¹ (**1** R = CH₃; TMZ). Although TMZ has achieved “blockbuster” status,² it remains the lone member of its drug class because of the constraints placed on tumor response to the prodrug by a requirement for DNA mismatch repair (MMR) and resistance mediated through *O*6-alkylguanine-DNA alkyltransferase (MGMT). Herein we report a strategy that achieves remarkably-effective control of the reactive intermediates generated by the imidazotetrazine ring in an aqueous environment, in this example to generate aziridinium ions. This design achieves compounds that react with DNA but exhibit chemosensitivity independently of MMR and MGMT.

Scheme 1. Release of electrophiles on hydrolysis of the imidazotetrazine ring.^a



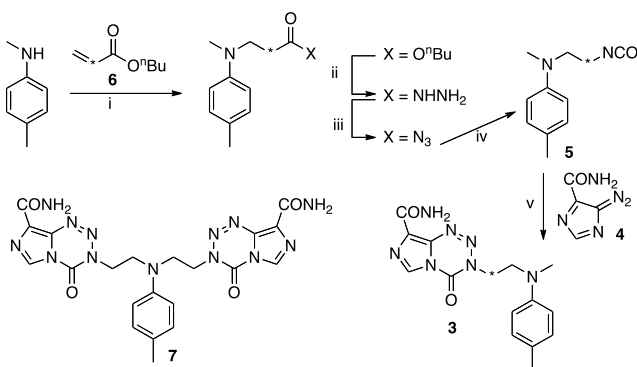
^aFor TMZ, R=CH₃, MTZ, R=CH₂CH₂Cl.

At neutral or alkaline pH, imidazotetrazines **1** (e.g. TMZ) act as a source of diazonium ions **2**, with few exceptions ring-opening as shown in Scheme 1.³ Methyl diazonium is released from TMZ and methylates DNA; lethal interaction of this adduct with MMR causes cell death. There are two kinetic parameters that determine the effectiveness of imidazotetrazine

prodrugs. The first is the rate of addition of water (or hydroxide ion) to initiate the ring-opening reaction. This is slow at low pH, so confers acid stability and the convenience of oral dosing.⁴ The pH-dependence of this reaction also influences distribution of the prodrug around the body: hydrolysis kinetics at pH 7.4 match closely the uptake rate (peak plasma concentration after 30 min) and metabolic half life ($t_{1/2}$ = 1.29 h) in patients.^{1,5} The other significant kinetic parameter is the reactivity of the latent electrophile. Hydrolysis of methyl diazonium in a purely chemical system has $t_{1/2}$ = 0.39 s.⁶ Again, a sub-optimal value detracts from clinical effectiveness: ethyldiazonium eliminates or reacts with water before it is able to locate a reactive nucleic acid target site,⁷ while longer-lived intermediary electrophiles such as chloronium achieve clinical efficacy.⁸ For TMZ, these clinically-useful, if not formally optimized, pharmacokinetic properties were achieved serendipitously.

In the design of TMZ analogues with altered spectra of activity, we reasoned that a neighboring group participation (NGP)-based mechanism could be employed to control the incipient alkyl diazonium ions. This would serve the dual functions of directing reactivity and delivering an alternative form of damage to DNA. Since the response of tumors to TMZ is determined by the interaction of covalently modified DNA with DNA repair systems,⁹ altering the electrophile would necessarily alter the tumor response. In these respects, the potential of the imidazotetrazine as an acid-stable precursor of aziridines or aziridinium ions was explored. These are reactive intermediates with proven clinical utility, being found widely in, or generated by, synthetic and natural product anti-tumor drugs and prodrugs. Furthermore, *O*6-aminoethylguanine is known to be refractory to cleavage by MGMT.¹⁰

Scheme 2.^a



^aReagents and conditions: (i) CuCl, AcOH, Δ , 12 h, 41%; (ii) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, EtOH, rt, 12 h 89%; (iii) NaNO_2 , AcOH, $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$, 0–2 °C; (iv) CH_2Cl_2 , rt, 48 h; (v) DMSO, rt, 48 h, 14% (over steps iii–v).

The synthesis of an aziridinium-precursor imidazotetrazine **3** is presented in Scheme 2. Imidazotetrazine ring closure is achieved by reaction of diazoazole **4** with an isocyanate **5**.^{11, 12} This ring closure was placed toward the end of the synthesis to avoid complications arising from reactivity of the imidazotetrazine itself. Preparation of these derivatives posed a further synthetic challenge as β -aminoisocyanates were required. Reactivity of the amine group was controlled by use of anilines, rather than a protecting group strategy: β -anilinoisocyanates **5** were stable and isolable. For mechanistic studies, a ¹³C-labeled version was prepared using enriched ¹³C(2)-acrylate **6** (the site of the label is marked * in the figure and schemes), the label was estimated as 75 atom% ¹³CH₂ by ¹H NMR. The analogous bisimidazotetrazine **7** was similarly prepared from *p*-toluidine.

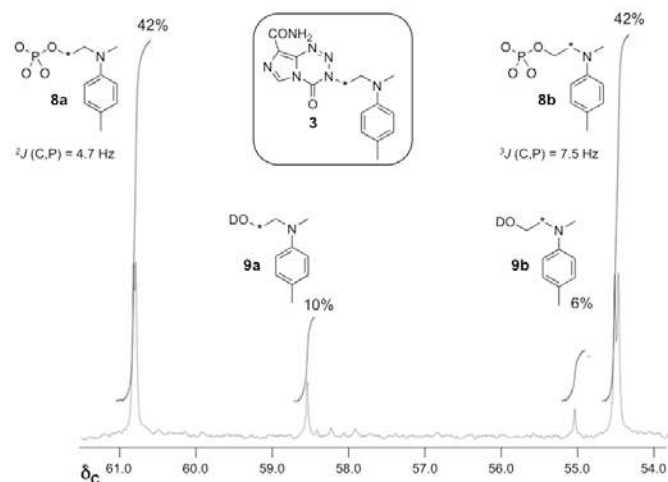


Figure 1. Gated decoupled ¹³C NMR spectrum showing identification and quantitation of the final products of ¹³C-labeled imidazotetrazine **3** reaction in phosphate buffer, pD 7.8. Resonances were assigned on the basis of ²J_{C,P} coupling constants¹² and spiking with authentic, unlabelled alcohol **9**.¹³

The prodrug activation mechanism was investigated by reaction of ¹³C-labeled **3** in phosphate buffer. Final products were identified and quantitated using gated decoupled ¹³C NMR spectroscopy, Figure 1. Products arose from reaction of the latent electrophiles with both nucleophiles in the system,

the D₂O solvent and phosphate of the buffer. In the phosphate products **8a,b**, the label was detected equally in the two methylene positions; the alcohol products **9a,b**, showed a small excess of label retained in the starting position. A mechanism that accounts for these observations is presented in Scheme 3. It is proposed that diazonium ion **10** is formed as in Scheme 1. Subsequent reaction is either by intramolecular displacement of N₂ by the aniline or direct hydrolysis. The evidence of the NMR spectrum is that only ca. 4% direct hydrolysis occurs, with the majority of material forming aziridinium ion **11**. This renders the two methylene positions equivalent, so equally susceptible to attack by nucleophiles, in consequence of which the label is scrambled in the products. The cleanness of this result is gratifying as previous reports on 2-aminoethyl diazonium ions derived from triazolines and alkylaminotriazenes showed only about 50% ring closure to aziridines, with pathways of diazonium elimination and direct hydrolysis contributing significantly to the detected products.^{13–15} The phosphate ester/alcohol product ratio reflects the relative nucleophilicities of phosphate and D₂O.

Scheme 3. Fate of the ¹³C Label. Approximately 96 % conversion to the aziridinium ion **11** and 4 % direct hydrolysis of the diazonium ion occur. Final products show complete scrambling (1:1 mixtures) of the label confirming the intermediacy of a discrete aziridinium ion **11**.

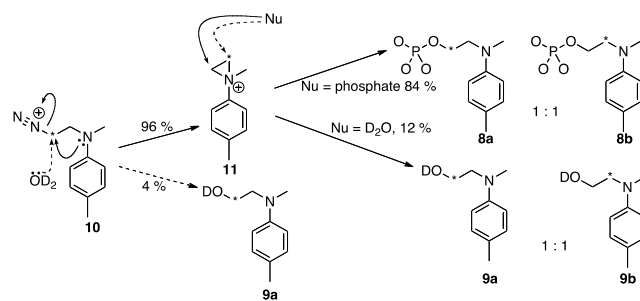


Table 1. pH Dependence of imidazotetrazine hydrolysis kinetics at 37 °C.

	pH = 4.0		pH = 7.4		pH = 8.0	
	<i>k'</i> [a]	<i>t</i> _{1/2} [b]	<i>k'</i> [a]	<i>t</i> _{1/2} [b]	<i>k'</i> [a]	<i>t</i> _{1/2} [b]
TMZ	0.100 ±0.001	253.0	14.0 ±0.0	1.38	46.2 ±0.0	0.42
3	0.500 ±0.003	41.5	15.2 ±0.3	1.27	49.5 ±0.0	0.39
7	0.400 ±0.006	48.5	14.4 ±0.7	1.33	45.1 ±1.0	0.43

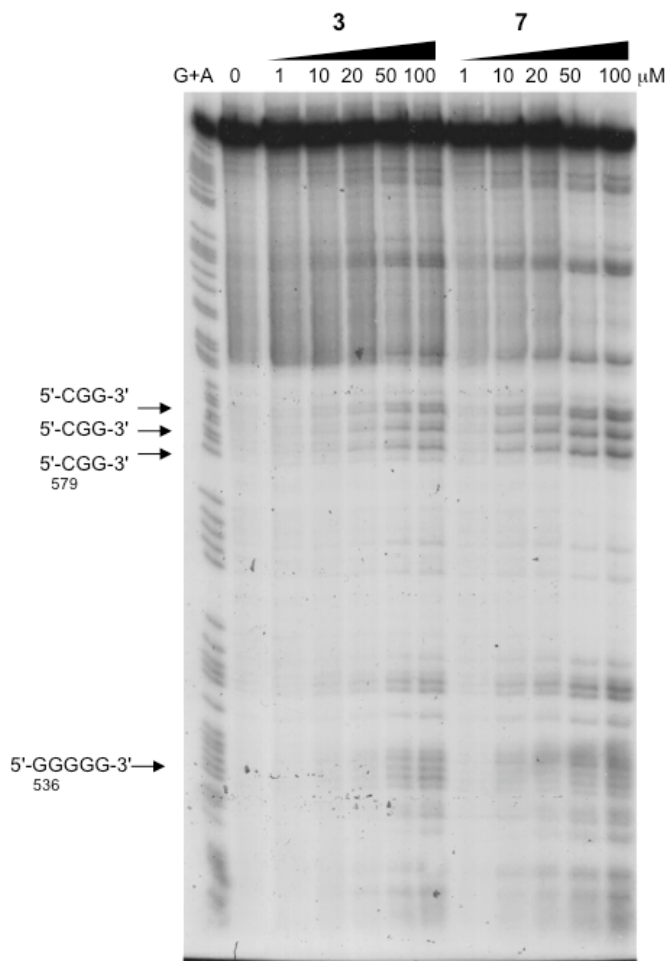
[a] *k'* pseudo first order rate constant, × 10⁻⁵ s⁻¹, data are mean ±SD of 3 independent determinations. [b] *t*_{1/2} half life, h. Citrate-phosphate buffers.

The hydrolysis kinetics (pseudo-first order disappearance of imidazotetrazine, λ_{max} = 330 nm) were measured for compounds **3**, **7** and TMZ (Table 1). For all compounds, the half life was much longer under acidic than neutral or basic conditions, although compounds **3** and **7** show a subtly increased

reactivity under acidic conditions compared with TMZ. Even so, the $t_{1/2}$ values measured at pH 4.0 remain much longer than the uptake and elimination rates of orally-administered TMZ. It is interesting to note that the site of structural variation in imidazotetrazines **3** and **7**, compared with TMZ, is distant from and not conjugated with the hydrolytic reaction centre, C-4. This change in acid reactivity demonstrates a curious complexity of the imidazotetrazine system.

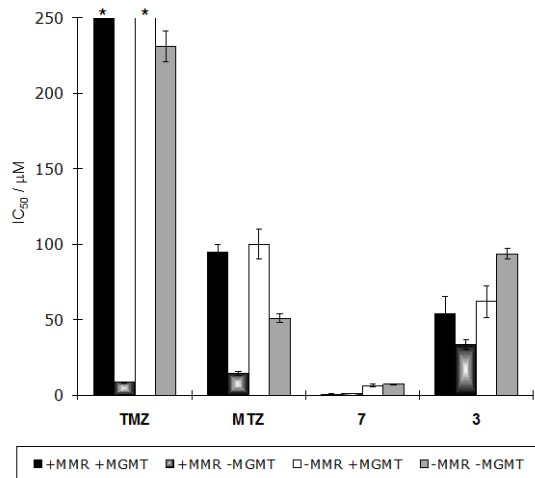
The interaction of compounds **3** and **7** with DNA and the sequence specificity of covalent DNA modification were assessed by a combination of cleavage-based and polymerase stop assays. The piperidine cleavage assay indicated that the compounds alkylate identical GN7 sites at guanine-rich sequences, with the bifunctional agent **7** being more reactive than the monofunctional **3** (Figure 2). The same sites of reaction were identified using the polymerase stop assay which detects all covalent adducts (see Figure S9; supporting information). Additionally it was shown that compound **3** produced the same GN7 alkylation pattern and identical sites of covalent bonding as the nitrogen mustard drug melphalan (Figures S8 and S9; supporting information). The reaction of TMZ with the same DNA sequence has previously been investigated by these methods and shows similar patterns of sequence selectivity. {Clark, 1995 #169} {Arrowsmith, 2000 #165}

Figure 2. DNA reaction by the piperidine cleavage method showing GN7 alkylation on the upper strand of pBR322 DNA modified by compounds **3** and **7**. Arrows indicate the position and sequence context of the alkylated guanines.



In vitro anti-cancer activity of compounds **3** and **7** was assessed in a pair of MMR proficient and deficient cell lines: A2780 and A2780-cp70,¹⁶ Figure 3. Compounds **3** and **7** were more active than TMZ and MTZ and their relative potency correlates with their different DNA reactivity (Figure 2). For TMZ the IC_{50} was >27-fold lower in the MMR-proficient cell line (compare the shaded and grey bars). This ratio was reduced to 5.8-fold for new bifunctional agent **7** and 2.8-fold for mono-functional **3**. The extent of MGMT-mediated resistance can be assessed by comparing the black and the shaded bars (i.e. MGMT⁻/MGMT⁺ with MMR proficient). TMZ was >30-fold more potent in the absence of MGMT whilst the new agents were equipotent. Moreover, in the absence of MMR, all compounds showed activity greater than TMZ, irrespective of the MGMT status of the cells, showing that MMR-dependent activity and MGMT-mediated resistance are now only minor determinants of the antitumor effect.

Figure 3. *In vitro* chemosensitivity (IC_{50} / μ M) of selected compounds against A2780 (MMR⁺) and A2780-cp70 (MMR⁻) cell lines in the absence (MGMT⁺) and presence (MGMT⁻) of the MGMT inactivator PaTrin2 (10 μ M). All data are the mean of ≥ 3 determinations, error bars are the SD. * IC_{50} >250 μ M.



Overall, remarkable control of the reactive intermediates generated from imidazotetrazines has been achieved with 96% of an alkylidiazonium ion being directed to aziridinium ring closure under aqueous conditions. In reaction with DNA, compound **7** has sequence selectivity and reactivity similar to melphalan. Moreover, the pH-dependence of the hydrolysis kinetics mirrors the parent TMZ, so the new compounds have the potential for oral bioavailability. The new compounds disclosed herein have potent anti-cancer activity that is independent of the two principal constraints on TMZ activity – MMR and MGMT.

ASSOCIATED CONTENT

Supporting Information. Full experimental details and example data are provided in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

TMZ, temozolomide; MTZ, mitozolomide; MGMT, O⁶-alkylguanine-DNA transferase; MMR, DNA mismatch repair.

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