

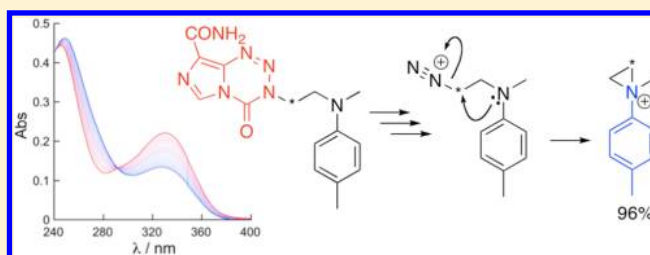
## Strategy for Imidazotetrazine Prodrugs with Anticancer Activity Independent of MGMT and MMR

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## S Supporting Information

**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 O6-methylguanine-DNA methyltransferase and DNA mismatch repair constraints that limit the use of Temozolomide.

**KEYWORDS:** Imidazotetrazine, aziridinium, Temozolomide, cancer, <sup>13</sup>C NMR, DNA



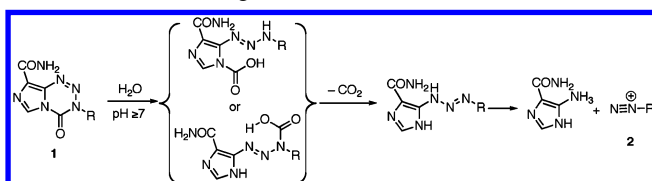
Imidazotetrazine **1** is a fascinating heterocycle, on account of both its chemistry and the therapeutic applications of its derivatives. It forms the core of the anticancer prodrug Temozolomide<sup>1</sup> (**1**, R = CH<sub>3</sub>; TMZ). Although TMZ has achieved “blockbuster” status,<sup>2</sup> 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 O6-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.

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.<sup>3</sup> 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 and, thus, confers acid stability and the convenience of oral dosing.<sup>4</sup> 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*<sub>1/2</sub> = 1.29 h) in patients.<sup>1,5</sup> The other significant kinetic parameter is the reactivity of the latent electrophile. Hydrolysis of methyl diazonium in a purely chemical system has *t*<sub>1/2</sub> = 0.39 s.<sup>6</sup> Again, a suboptimal value detracts from clinical effectiveness: ethyldiazonium eliminates or reacts with water before it is able to locate a reactive nucleic acid target site,<sup>7</sup> while longer-lived intermediary electrophiles such as chloronium achieve clinical efficacy.<sup>8</sup> 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,<sup>9</sup> altering the electrophile would necessarily alter the tumor response. In these respects, the

**Scheme 1. Release of Electrophiles on Hydrolysis of the Imidazotetrazine Ring<sup>a</sup>**



<sup>a</sup>For TMZ, R = CH<sub>3</sub>; MTZ, R = CH<sub>2</sub>CH<sub>2</sub>Cl.

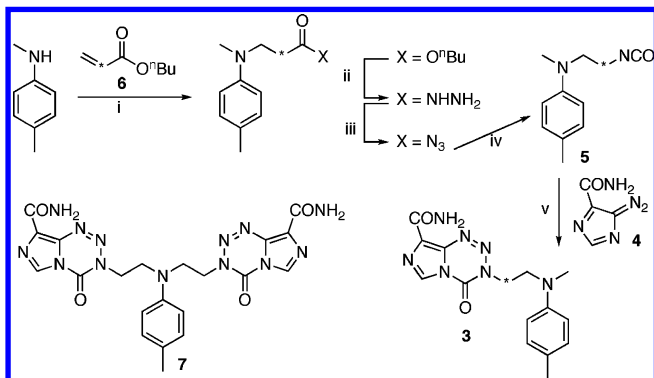
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potential of the imidazotetrazine as an acid-stable precursor of aziridines or aziridinium ions was explored. These are reactive intermediates with proven clinical utility, being found widely in, or generated by, synthetic and natural product antitumor drugs and prodrugs. Furthermore, O6-aminoethylguanine is known to be refractory to cleavage by MGMT.<sup>10</sup>

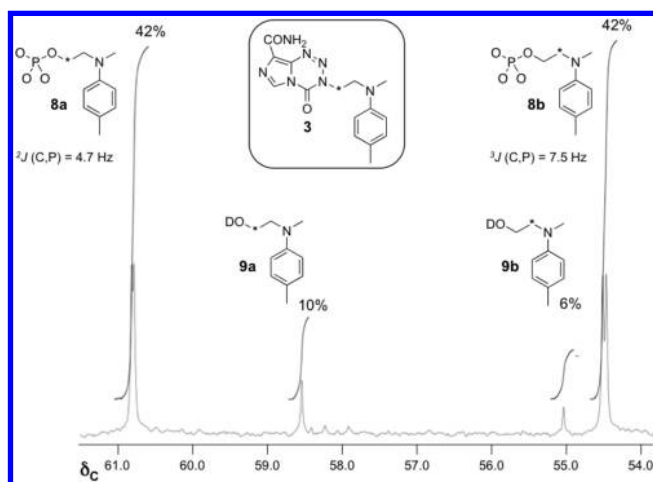
The synthesis of an aziridinium-precursor imidazotetrazine **3** is presented in Scheme 2. Imidazotetrazine ring closure is

Scheme 2<sup>a</sup>

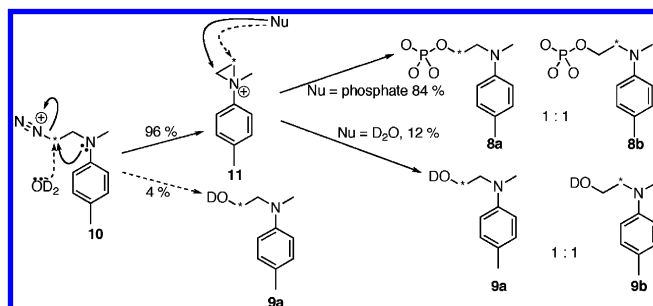
<sup>a</sup>Reagents and conditions: (i) CuCl, AcOH,  $\Delta$ , 12 h, 41%; (ii)  $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$ , EtOH, rt, 12 h 89%; (iii)  $\text{NaNO}_2$ , AcOH,  $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$ , 0–2 °C; (iv)  $\text{CH}_2\text{Cl}_2$ , rt, 48 h; (v) DMSO, rt, 48 h, 14% (over steps iii–v).

achieved by reaction of diazoazole **4** with an isocyanate **5**.<sup>11,12</sup> 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  $\beta$ -aminoisocyanates were required. The reactivity of the amine group was controlled by use of anilines, rather than a protecting group strategy:  $\beta$ -anilinoisocyanates **5** were stable and isolable. For mechanistic studies, a  $^{13}\text{C}$ -labeled version was prepared using enriched  $^{13}\text{C}(2)$ -acrylate **6** (the site of the label is marked \* in the figure and schemes); the label was estimated as 75 atom %  $^{13}\text{CH}_2$  by  $^1\text{H}$  NMR. The analogous bisimidazotetrazine **7** was similarly prepared from *p*-toluidine.

The prodrug activation mechanism was investigated by reaction of  $^{13}\text{C}$ -labeled **3** in phosphate buffer. Final products were identified and quantitated using gated decoupled  $^{13}\text{C}$  NMR spectroscopy (Figure 1). Products arose from reaction of the latent electrophiles with both nucleophiles in the system: the  $\text{D}_2\text{O}$  solvent and the 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 via intramolecular displacement of  $\text{N}_2$  by the aniline or via 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, and thus, equally susceptible to attack by nucleophiles, as a 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



**Figure 1.** Gated decoupled  $^{13}\text{C}$  NMR spectrum showing identification and quantitation of the final products of the  $^{13}\text{C}$ -labeled imidazotetrazine **3** reaction in phosphate buffer, pH 7.8. Resonances were assigned on the basis of  $^nJ_{\text{C,P}}$  coupling constants<sup>13</sup> and spiking with authentic, unlabeled alcohol **9**.<sup>14</sup>

Scheme 3. Fate of the  $^{13}\text{C}$  Label<sup>a</sup>

<sup>a</sup>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**.

elimination and direct hydrolysis contributing significantly to the detected products.<sup>15–17</sup> The phosphate ester/alcohol product ratio reflects the relative nucleophilicities of phosphate and  $\text{D}_2\text{O}$ .

The hydrolysis kinetics (pseudo-first-order disappearance of imidazotetrazine,  $\lambda_{\text{max}} = 330 \text{ nm}$ ) were measured for compounds **3** and **7** and TMZ (Table 1). For all compounds, the half-life measured under acidic conditions was much longer than that determined at neutral or basic pH, although compounds **3** and **7** showed increased reactivity under acidic conditions as compared with TMZ.<sup>1,5</sup> 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.<sup>1,5</sup> 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 center, C-4. This change in acid reactivity demonstrates a curious complexity of the imidazotetrazine system.

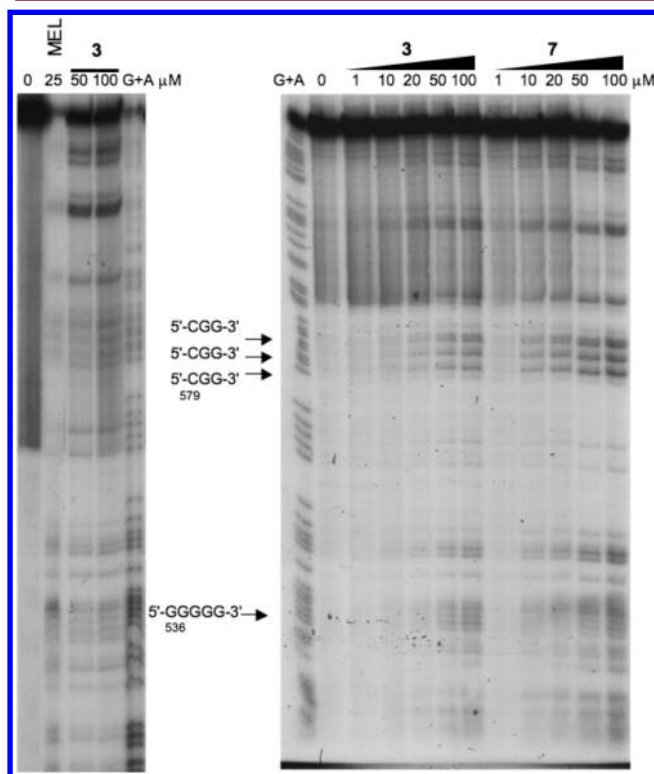
The reaction of compounds **3** and **7** and melphalan with DNA, and the sequence specificity of covalent DNA modification were assessed by a combination of cleavage-based and polymerase stop assays. The piperidine cleavage assay indicated that the new compounds alkylate identical GN7 sites at guanine-rich sequences to melphalan, with the

Table 1. pH Dependence of Imidazotetrazine Hydrolysis Kinetics at 37 °C

	pH = 4.0		pH = 7.4		pH = 8.0	
	$k'^a$	$t_{1/2}^b$	$k'^a$	$t_{1/2}^b$	$k'^a$	$t_{1/2}^b$
TMZ	0.100 ± 0.001	253.0	14.0 ± 0.0	1.38	46.2 ± 0.2	0.42
3	0.500 ± 0.003	41.5	15.2 ± 0.3	1.27	49.5 ± 0.4	0.39
7	0.400 ± 0.006	48.5	14.4 ± 0.7	1.33	45.1 ± 1.5	0.43

<sup>a</sup> $k'$  pseudo-first-order rate constant,  $\times 10^{-5} \text{ s}^{-1}$ ; data are mean  $\pm$  SD of three independent determinations. <sup>b</sup> $t_{1/2}$  half-life, h. Citrate-phosphate buffers.

bifunctional agent 7 being more reactive than the monofunctional 3 (Figure 2). The same sites of reaction were identified

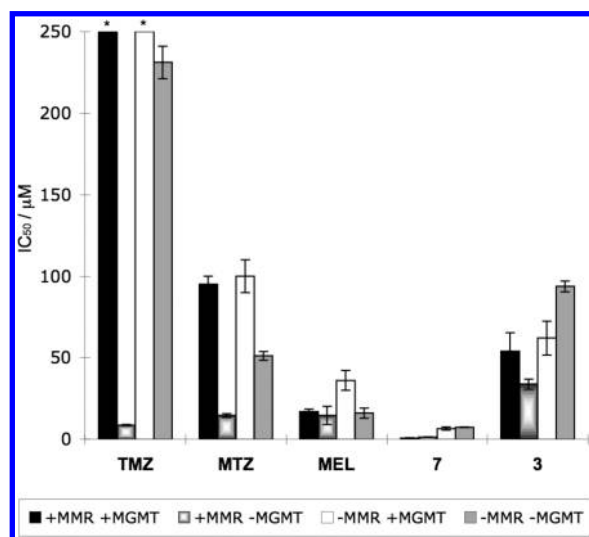


**Figure 2.** DNA reaction by the piperidine cleavage method showing GN7 alkylation on the upper strand of pBR322 DNA modified by compounds 3 and 7 and melphalan (MEL). Arrows indicate the position and sequence context of the alkylated guanines.

using the polymerase stop assay, which detects all covalent adducts (see Figure S8; Supporting Information). The reaction of TMZ with the same DNA sequence has previously been investigated by these methods<sup>19,20</sup> and also shows a similar pattern of sequence selectivity (see Figure S9).

The *in vitro* anticancer activity of compounds 3 and 7 was assessed in a pair of MMR proficient and deficient cell lines: A2780 ovarian carcinoma and its MMR-deficient variant A2780-cp70<sup>18</sup> (Figure 3). MGMT was inactivated using the benzylguanine analog PaTrin2. Compound 7 was found to be more active than TMZ, MTZ, and MEL; compound 3 was approximately equipotent with MTZ. The relative potencies correlate with their different DNA reactivities (Figure 2).

The extent of MGMT-mediated resistance can be assessed by comparing the shaded and the black bars (i.e., MGMT−/MGMT+ with MMR proficient). TMZ was >30-fold more potent in the absence of MGMT; in contrast, both of the new agents 3 and 7 showed equivalent potency in the absence and presence MGMT. MMR dependence can be assessed by



**Figure 3.** *In vitro* chemosensitivity ( $IC_{50}/\mu\text{M}$ ) 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  $\mu\text{M}$ ). All data are the mean of  $\geq 3$  determinations; error bars are the SD; \* $IC_{50} > 250 \mu\text{M}$ .

comparing the shaded and gray bars. For TMZ the  $IC_{50}$  was >27-fold lower in the MMR-proficient cell line. This ratio was reduced to 5.8-fold for new bifunctional agent 7 and 2.8-fold for monofunctional 3. Moreover, in the absence of MMR (white and gray bars), all compounds showed activity considerably greater than that of 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.

The activity of the new compounds was further investigated in a panel of cell lines representing major tumor types (colon, NSCL, pancreatic, and TMZ-resistant glioma); see Table S2 of the Supporting Information. All cell lines were unresponsive toward TMZ ( $IC_{50} > 250 \mu\text{M}$ ); the positive control MEL elicited a range of responses ( $IC_{50}$  11–3  $\mu\text{M}$ ). Both new compounds showed similar patterns of chemosensitivity, with the bifunctional agent 7 having potency and selectivity similar to that of MEL; the monofunctional agent 3 was 7–30-fold less active.

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 that of melphalan. Moreover, the pH-dependence of the hydrolysis kinetics mirrors that of the parent TMZ, so the new compounds have the potential for oral bioavailability. The new compounds disclosed herein have potent anticancer activity that is independent of the two principal constraints on TMZ

activity (MMR and MGMT), and they may offer versatile alternatives to TMZ and the nitrogen mustard drugs.

## ■ 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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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

TMZ, Temozolomide; MTZ, mitozolomide; MGMT, O<sup>6</sup>-methylguanine-DNA methyltransferase; MMR, DNA mismatch repair

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