Toward Hypoxia-Selective DNA-Alkylating Agents Built by Grafting Nitrogen Mustards onto the Bioreductively Activated, Hypoxia-Selective DNA-Oxidizing Agent 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (Tirapazamine)

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Supporting Information

ABSTRACT: Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide) is a heterocyclic di-N-oxide that undergoes enzymatic deoxygenation selectively in the oxygen-poor (hypoxic) cells found in solid tumors to generate a mono-N-oxide metabolite. This work explored the idea that the electronic changes resulting from the metabolic deoxygenation of tirapazamine analogues might be exploited to activate a DNA-alkylating species selectively in hypoxic tissue. Toward this end, tirapazamine analogues bearing nitrogen mustard units were prepared. In the case of the tirapazamine analogue 18a bearing a nitrogen mustard unit at the 6-position, it was found that removal of the 4-oxide from the parent di-N-oxide to generate the mono-N-oxide analogue 17a did indeed cause a substantial increase in reactivity of the mustard unit, as measured by hydrolysis rates and DNA-alkylation yields. Hammett sigma values were measured to quantitatively assess the magnitude of the electronic changes induced by metabolic deoxygenation of the 3-amino-1,2,4-benzotriazine 1,4-dioxide heterocycle. The results provide evidence that the 1,2,4-benzotriazine 1,4-dioxide unit can serve as an oxygen-sensing prodrug platform for the selective unmasking of bioactive agents in hypoxic cells.

INTRODUCTION

The nitrogen mustard mechlorethamine (1), developed in the 1940s, was the first cancer chemotherapeutic agent.1,2 Analogues such as chlorambucil (2), melphalan (3), bendamustine (4), estramustine (5), uramustine (6), cyclophosphamide (7), and ifosfamide (8) see widespread clinical use today.3,4 Nitrogen mustards generate aziridinium ions that alkylate DNA at a variety of positions including N7-guanosine, N3-adenosine, N1-adenosine, and N3-cytosine.3,5–12 The predominant site of DNA alkylation by nitrogen mustards is the N7-atom of guanine residues.5,8,9

Nitrogen mustards cause serious side effects that arise from the alkylation of DNA and other biomolecules in nonmalignant tissue.4 Consequently, there have been many efforts to develop "targeted" nitrogen mustards with improved potency and selectivity against cancer cells.13–21 One promising approach for the design of cancer-cell-selective mustards exploits selective enzymatic reduction of nitroaryl compounds in the oxygen-starved (hypoxic) cells found in solid tumors.22–32 One-electron reductases such as NADPH:cytochrome P450 reductase, cytochrome b5 reductase, xanthine oxidase, and aldehyde oxidase can convert nitroaryl compounds to nitroso, hydroxylamino, and amino metabolites selectively under hypoxic conditions (Scheme 1).22–32 In normal tissue, O2 blocks production of reduced metabolites via oxidation of the radical anion intermediates involved in this process (reverse reactions, Scheme 1).28,33,34 The initial nitro-to-nitroso conversion typically is a key oxygen-sensitive step in the bioreduction of nitroaryl compounds,26–28 but there is also evidence that the hydroxylamino-to-aniline step can be inhibited by O2.22

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It is well established that aziridinium ion formation by N-aryl nitrogen mustards is suppressed by electron-withdrawing substituents and favored by electron-releasing substituents on the aromatic ring. Thus, hypoxia-selective conversion of the electron-withdrawing nitro substituent (Hammett $\sigma = 0.78$) to the electron-donating hydroxylamino (Hammett $\sigma = -0.34$) or amino substituents (Hammett $\sigma = -0.66$) constitutes an "electronic switch" that can transform a deactivated N-aryl nitrogen mustard into an activated nitrogen mustard selectively in tumor tissue (Scheme 2). Two anticancer drug candidates, TH-302 and PR-104, that employ this design principle are currently undergoing phase I and II clinical trials.

In the pursuit of clinically useful hypoxia-selective DNA cross-linking agents, it may be important to explore the utility of oxygen-sensing units other than the nitroaryl motif. In this regard, the 1,2,4-benzotriazine 1,4-dioxide scaffold deserves consideration. In terms of both basic and clinical research, the compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, 9) may be the best-characterized hypoxia-selective antitumor agent. On the basis of its potent hypoxia-selective cytotoxicity in preclinical testing, this compound was examined in a wide variety of phase I, II, and III clinical trials. In early clinical trials, the drug showed promise that was not realized in subsequent studies. The disappointing clinical performance of 9 may stem, in part, from failure to stratify patients on the basis of the hypoxic character of their tumors. In addition, pharmacokinetic models suggested that 9 may be metabolized in a small zone of hypoxic tumor cells, leaving a significant fraction of neighboring cancer cells untouched by the drug. Second-generation analogues of 9 are in development.

Rational use of the 1,2,4-benzotriazine 1,4-dioxides as components in the design of hypoxia-selective DNA-alkylating agents rests upon an existing understanding of the mechanisms by which these agents selectively kill hypoxic cells. Intracellular one-electron reductases convert 9 to an oxygen-sensitive radical intermediate 10 (Scheme 3). We have presented evidence that, under hypoxic conditions, the protonated drug radical 11 fragments to release the DNA-damaging agent, hydroxyl radical, though other mechanisms also have been considered. The deoxygenation product, 1,2,4-benzotriazine 1-oxide 12, is the major metabolite generated by hypoxic metabolism of 9 and other 1,2,4-benzotriazine 1,4-dioxides similarly are converted to the 1-oxide products. Compound 9 is not extensively metabolized to the mono-N-oxide 12 by oxygen-insensitive pathways involving two-electron reductases such as DT-diaphorase. The mono-N-oxide metabolite of tirapazamine is not cytotoxic on its own, although it does display useful oxygen mimetic (radiosensitizing) properties that potentiate the DNA strand-cleaving properties of hydroxyl radical.

We envisioned that electronic changes resulting from the deoxygenation of tirapazamine analogues might be exploited to activate DNA-alkylating functional groups selectively in hypoxic tissue. Indeed, the charge distribution in the mono-N-oxide metabolite 12 is quite different than that of the parent di-N-oxide 9. We describe the design, synthesis, and characterization of hypoxia-selective DNA-alkylating agents constructed by grafting nitrogen mustard units onto the 1,2,4-benzotriazine 1,4-dioxide scaffold (Scheme 4).
RESULTS AND DISCUSSION

Synthesis of 1,2,4-Benzotriazine 1,4-Dioxide Mustards. Our synthetic approaches to the desired tirapazamine-mustard derivatives were informed by previous reports describing the synthesis of tirapazamine and its analogues.74,75 The compound 6-fluoro-1,2,4-benzotriazine 1-oxide 13 was prepared via condensation of 5-fluoro-2-nitroaniline with cyanamide.74,75 Oxidation of 13 with trifluoroacetic acid/80% H2O2 gave the di-N-oxide 14 in 43% yield (Scheme 5). Treatment with diethanolamine in acetonitrile afforded a 73% yield of 15. Attempts to convert 15 to the tosyl mustard 17b via treatment with tosyl chloride gave a complex mixture of products, from which we isolated 19 as a major component. The structure of 19 was confirmed by single-crystal X-ray crystallographic analysis (Supplementary Figure S1). This product may arise via the initial attack of the 4-oxide unit of 15 on tosyl chloride, followed by a nucleophilic deoxygenative rearrangement.76,77 As an alternate route to the target mustards, 13 was treated with diethanolamine in N-methylpyrrolidone to give the mono-N-oxide 16 (94%). This product was converted in good yields to the ditosylate 17b by treatment with tosyl chloride or to the dimesylate 17a by treatment with mesyl chloride. Oxidation to the di-N-oxides 18 was effected by oxone or m-CPBA in modest yields (15−37%). The chlorinated mustard 17c was synthesized in 94% yield via treatment of 17b with lithium chloride in DMF, and 18c was prepared in 30% yield by oxidation with oxone.

To investigate how the 3-amino substituent and the location of the mustard unit on the benzotriazine ring system affects reactivity of an appended nitrogen mustard unit, we sought to prepare the 3-desamino tirapazamine analogues 21, 22, 25, and 26. Toward this end, 13 was deaminated by treatment with tert-butylnitrite in dimethylformamide to give 20 (50% yield, Scheme 6). Nucleophilic aromatic substitution with diethanolamine, followed by treatment with tosyl chloride, gave the mono-N-oxide mustard 21 in 67% yield for the two steps. Oxidation of 21 with oxone gave the di-N-oxide mustard 22 (10%). Synthesis of compounds with the mustard located on the 7-position of the benzotriazine ring system started with preparation of 7-fluoro-1,2,4-benzotriazine 1-oxide 23 via condensation of 6-fluoro-2-nitroaniline with cyanamide (Scheme 7).74,75 Treatment with tert-butylnitrite in dimethylformamide gave the deaminated analogue 24 in 50% yield. Subsequent reaction of this compound with diethanolamine, followed by tosyl chloride, gave a 57% yield of 25. Finally, oxidation with either trifluoroacetic anhydride/70% H2O2 in CH2Cl2 or oxone gave the target di-N-oxide 26 in low (1%) yield. Formation of the desired compound 26, in this case, was...
accompanying extensive degradation during the reaction and workup.

**Reactivity of the 1,2,4-Benzotriazine Oxide Mustards.**
This work explores the idea that mustard units in the parent di-N-oxides will be relatively unreactive, while the mustard units in the mono-N-oxide deoxygenation products will be active alkylating agents. To learn whether the mustard units in the mono-N-oxides 17, 21, and 25 are in fact more reactive than those in the corresponding di-N-oxides (18, 22, 26) as illustrated in Scheme 4, we first examined the hydrolytic stability of these agents in an organic/aqueous solvent mixture. Hydrolysis of aromatic nitrogen mustards typically proceeds via a rate-determining formation of the aziridinium ion intermediate. We used 1H NMR to monitor the hydrolysis of these mustard derivatives (~1 mM) in 50:50 CD3CN/D2O at 50 °C. This mixed organic/aqueous solvent system ensured solubility of the compounds at the concentrations required for the NMR experiments and also served to slow aziridinium ion formation and hydrolysis to easily measurable rates. The disappearance of the compounds followed first-order kinetics (Figure 1). The mesylate mustard analogue of tirapazamine 18a disappeared with a half-life of 15.4 ± 0.6 d (Table 1), while the corresponding mono-N-oxide mustard 17a disappeared approximately 5-fold faster, with a half-life of 2.7 ± 0.4 d. The tosylates displayed a similar trend, in which the di-N-oxide 18b was more stable (t1/2 = 15.4 ± 0.6 d) than the mono-N-oxide 17b (t1/2 = 5.5 ± 0.6 d). The similar reactivities of the tosyl (17b/18b) and mesyl (17a/18a) systems is consistent with previous analyses showing that tosylate and mesylate are comparable leaving groups in aqueous/organic solvent mixtures.

The chlorinated mustard analogues 17c and 18c were quite stable, with 17c displaying an estimated half-life of more than 51 d under the conditions of our NMR experiment (85% of the starting material remained after 10 d). Compound 18c showed no detectable reaction over the course of 10 d. Due to the sluggishness of these reactions we did not examine the hydrolysis of 17c and 18c further.

The products resulting from decomposition of the nitrogen mustards in these NMR experiments were identified as the expected diol (16) and the morpholino compound (27), presumably derived from intramolecular attack of the hydroxyethyl arm of the half-mustard intermediates (28) on the adjacent mustard group. Analogous metabolites have been observed for the clinically used mustard 2.

![Figure 1. Rate of decay for compounds 17a, 17b, 18a, and 18b in 1:1 acetonitrile-d6/D2O measured by 1H NMR at 50 °C.](image)

"Reagents and conditions: (a) t-BuNO2, DMF, 60 °C, 50%; (b) HN(CH2CH2OH)2, CH3CN; (c) TsCl, NaOH, THF 0 °C, 57% from 2 steps; (d) TFA, 70% H2O2, CH2Cl2, 0 °C, 1%.

The desamino analogues 21 and 22, bearing a tosyl mustard at the 6-position, disappeared with half-lives that were the same within experimental error (Table 1). The half-lives of these compounds (t1/2 ≈ 13 d) were very similar to that of the deactivated dioxide tosyl mustard 18b in the 3-amino series. The desamino analogues 25 and 26, bearing the tosyl mustard unit on the 7-position, also disappeared at the same rate within experimental error (Table 1). The decomposition rates of the 7-mustards 25 and 26 (t1/2 ≈ 6.5 d) was approximately two times faster than the decomposition of the desamino 6-mustards 21 and 22 (t1/2 ≈ 13 d). In the Conclusion, we consider possible reasons why there is no significant difference between the reactivities of the desamino mono-N-oxide and di-N-oxide mustards.

We also measured decomposition of the mesyls mustards 17a and 18a (250 mM) at 50 °C in a predominantly aqueous solvent mixture composed of sodium phosphate buffer (25 mM, pH 7) containing DMF (2.5% v/v). We used HPLC analysis to monitor the disappearance of the starting mustards (17a and 18a). As expected, the hydrolysis rates of 17a and 18a were substantially faster in this solvent as compared to the 50:50 CD3CN/D2O mixture employed in the NMR experiments. Compounds 17a and 18a disappeared with half-lives of 12 ± 0.5 h and 96 ± 21 h, respectively, in phosphate-buffered water. Under these conditions, the “activated” mono-N-oxide metabolite 17a reacted approximately seven times faster than the parent dioxide 18a. Comparison with authentic synthetic standards revealed that the major products generated in the decomposition of 17a were the diol 16 and the morpholino 27, both presumably formed via the half-mustard 28 (Supplementary Figure S2).

**Measurement of the Electron-Withdrawing Power of the Triazine Ring Systems in 17 and 18.** We felt it would be useful to quantitatively measure the electron-withdrawing power of the triazine ring systems in 17 and 18. Electronic effects exerted by various substituents on an aromatic ring typically are assessed using Hammett sigma (σ) constants. Hammett σ values are determined by measuring the affect that a substituent exerts on the acidity of benzoic acid, where
σ_{substituent} = \log \left( \frac{K_a(\text{substituted benzoic acid})}{K_a(\text{benzoic acid})} \right). Therefore, we prepared the carboxylic acid derivatives 29 and 30 via reaction of guanidine with 3-fluoro-4-nitrobenzoic acid under basic conditions, as shown in Scheme 8.83 We measured the acidity of 29 by monitoring the changes in its UV−vis spectrum as a function of pH (Supplementary Figure S3). From the measured pK_a of 2.9, we were able to calculate a σ value of 1.3 for the mono-N-oxide 29. The establishes the 1,2,4-triazine-1-oxide ring as a strongly electron-withdrawing substituent comparable to a p-sulfonyl cyanide group (−SO_2CN).82 Unfortunately, we were not able to measure the pK_a for the di-N-oxide 30 because the UV−vis spectral changes associated with protonation of the carboxylic acid group were obscured by another process, perhaps involving protonation of the oxygen in the 4-oxide group.

Electronic properties of substituents can also be measured using a σ^- parameter that is obtained by determining the effect of substituents on the acidity of phenol.82,84 While the σ^- parameter is probably less relevant than σ to the aziridinium ion-forming reactions that are the subject of this work,86 we felt this measurement would nonetheless provide a useful evaluation of the electronic properties of the triazine mono-oxide and dioxide ring systems. Therefore, we synthesized the phenol derivatives 31 and 32 by reaction of the corresponding fluoro compounds with basic hydrogen peroxide, followed by workup with sodium thiosulfate (Scheme 9).85 We then measured the acidity of these compounds by monitoring the

### Table 1. Decay Rates of 1,2,4-Benzotriazine Mustards in Acetonitrile-d_6/D_2O (1:1) at 50 °C Measured by ^1H NMR

| compd | R_1 | R_2 | R_3 | oxidation | k (d^-1) |
|-------|-----|-----|-----|-----------|----------|
| 17a   | H   | N(CH_2CH_2OMs)_2 | H   | 1-oxide   | 0.259 ± 0.060 |
| 18a   | H   | N(CH_2CH_2OMs)_2 | H   | 1,4-dioxide | 0.045 ± 0.002 |
| 17b   | NH_2 | N(CH_2CH_2OMs)_2 | H   | 1-oxide   | 0.127 ± 0.014 |
| 18b   | NH_2 | N(CH_2CH_2OMs)_2 | H   | 1,4-dioxide | 0.045 ± 0.002 |
| 21    | H   | N(CH_2CH_2OTs)_2 | H   | 1-oxide   | 0.050 ± 0.004 |
| 22    | H   | N(CH_2CH_2OTs)_2 | H   | 1,4-dioxide | 0.055 ± 0.006 |
| 25    | H   | H   | N(CH_2CH_2OTs)_2 | 1-oxide | 0.107 ± 0.013 |
| 26    | H   | H   | N(CH_2CH_2OTs)_2 | 1,4-dioxide | 0.106 ± 0.001 |

### Scheme 8. Synthesis of 6-Carboxy-1,2,4-benzotriazines

We measured the acidity of 29 by monitoring the changes in its UV−vis spectrum as a function of pH (Supplementary Figure S3). From the measured pK_a of 2.9, we were able to calculate a σ value of 1.3 for the mono-N-oxide 29. The establishes the 1,2,4-triazine-1-oxide ring as a strongly electron-withdrawing substituent comparable to a p-sulfonyl cyanide group (−SO_2CN).82 Unfortunately, we were not able to measure the pK_a for the di-N-oxide 30 because the UV−vis spectral changes associated with protonation of the carboxylic acid group were obscured by another process, perhaps involving protonation of the oxygen in the 4-oxide group.

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### Scheme 9. Synthesis of 6-Hydroxy-1,2,4-benzotriazines

**Reagents and conditions:** (a) guanidine, THF, EtOH 90 °C; (b) KOtBu, THF, 90 °C, 95% after 2 steps; (c) TFA, 70% H_2O_2, 50 °C, 36%.

We measured the acidity of 29 by monitoring the changes in its UV−vis spectrum as a function of pH (Supplementary Figure S3). From the measured pK_a of 2.9, we were able to calculate a σ value of 1.3 for the mono-N-oxide 29. The establishes the 1,2,4-triazine-1-oxide ring as a strongly electron-withdrawing substituent comparable to a p-sulfonyl cyanide group (−SO_2CN).82 Unfortunately, we were not able to measure the pK_a for the di-N-oxide 30 because the UV−vis spectral changes associated with protonation of the carboxylic acid group were obscured by another process, perhaps involving protonation of the oxygen in the 4-oxide group.

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**Figure 2.** UV−vis spectra of compounds 31 (top) and 32 (bottom) from pH 3.4 to 9.0 (31) and pH 2.4 to 9.0 (32).
changes in their UV–vis spectra as a function of pH (Figure 2). The pKₐ of 31 was found to be 6.3, and that of 32 was 5.3 (Supplementary Figure S4). The pKₐ measurements allowed us to calculate σ values of 1.81 for the 1,2,4-triazine 1-oxide "substituent" in 31 and 2.31 for the 1,2,4-triazine 1,4-dioxide "substituent" in 32. These values indicate that both the triazine mono-N-oxide and the di-N-oxide rings are strongly electron-withdrawing. By way of comparison, the sulfonyl perfluoropropane group (SO₂(CF₂)2CF₃) has a σ value of 1.75, and the diazonium group (–N₂⁺) has a σ value of 3.43. Importantly, there is a substantial difference (0.5) between the σ values of the 1,2,4-triazine 1-oxide and 1,2,4-triazine 1,4-dioxide substituents in 31 and 32. This is similar to the difference between σ values of the nitro group (1.27) and the acetyl group (0.84). Overall, the results validate the idea that transformation of the 1,4-di-N-oxide (9) to the 1-N-oxide (12) is accompanied by a substantial change in electron density at the 6-position of the benzo ring.57

**DNA Alkylation by 17a.** We next examined the abilities of 17a and 18a to alkylate DNA. Nitrogen mustards alkylate DNA primarily at the N7-position of guanine residues, with smaller amounts of reaction also occurring at the N3-position of adenine residues.6,8,9,88 The resulting lesions can be converted to strand breaks by treatment of the DNA with warm piperidine (Maxam–Gilbert workup).87 We incubated mustards 17a and 18a with the 5',32P-labeled DNA duplex 33 in HEPES buffer (50 mM, pH 7) containing NaCl (100 mM) and DMF (10% v/v), followed by piperidine workup (Figure 3). The resulting labeled DNA fragments were resolved on a 20% denaturing polyacrylamide gel and visualized by phosphorimager analysis.86 We incubated mustard 17a (0.25 mM) in sodium phosphate buffer (25 mM, pH 7) with 17a at 0.25, 0.5, and 1 mM concentrations, Lane 5: G-sequencing of duplex 33, (C) Lane 1: duplex 33 (no mustard), Lanes 2: duplex 33 with 17a, Lane 5: duplex 33 with 2, Lane 6: G-sequencing of duplex 33, (C) Lane 1: duplex 33 (no mustard), Lanes 2–4: duplex 34 with 17a at 0.25, 0.5, and 1 mM concentrations, Lane 5: duplex 34 with 1 (0.25 mM), Lane 6: duplex 34 with 2 (0.25 mM), and Lane 7: G-sequencing of duplex 34.

Compounds 17a and 18a showed a marked selectivity for alkylation at G2 than 18a (lanes 3 and 4, Figure 3B). The control diol 16 did not induce strand cleavage that was significantly above background (lane 2, Figure 3B). We compared DNA alkylation by 17a to that by the clinically used nitrogen mustard chlorambucil (2, lanes 4 and 5, Figure 3B). Chlorambucil generated piperidine-labile lesions predominantly at the guanine residues in duplex 31, alongside weaker cleavage that may arise from alkylation at adenine residues in the DNA.88

**Hypoxia-Selective, In Vitro Metabolic Conversion of 15 to 16.** Successful deployment of the activated mustard 17a requires hypoxia-selective enzymatic reduction of the dioxide 18a. Thus, it was important to examine whether the dialkylamine substituent at the 6-position of the tirapazamine analogue perturbs bioreductive, deoxygenative metabolism of the 1,2,4-benzotriazine 1,4-dioxide "core". To simplify product analysis in these experiments, we examined the in vitro hypoxic metabolism of an analogue, 15, bearing the chemically stable bis(2-hydroxyethyl)amino substituent. We employed recombinant human NADPH:cytochrome P450 reductase as the bioreductive enzyme because this, or a closely related enzyme, is thought to be responsible for bioactivation of 9 and related compounds in mammalian cells.68,89–91

**Figure 3. DNA Alkylation by 17a.** Conditions: HEPES buffer (50 mM, pH 7), NaCl (100 mM), mustard (1 mM unless otherwise specified), 10% DMF, 37 °C for 3 d followed by piperidine workup (1 M, 95 °C 30 min). Following incubation labeled DNA fragments were resolved on a 20% denaturing polyacrylamide gel. Labeled DNA was visualized by phosphorimager analysis. (A)32P-labeled duplexes 33 and 34 used to examine DNA-alkylating properties of mustards. (B) Lane 1: duplex 33 (no mustard), Lane 2: duplex 33 with 16, Lane 3: duplex 33 with 18a, Lane 4: duplex 33 with 17a, Lane 5: duplex 33 with 2, Lane 6: G-sequencing of duplex 33, (C) Lane 1: duplex 33 (no mustard), Lanes 2–4: duplex 34 with 17a at 0.25, 0.5, and 1 mM concentrations, Lane 5: duplex 34 with 1 (0.25 mM), Lane 6: duplex 34 with 2 (0.25 mM), and Lane 7: G-sequencing of duplex 34.
the results provide evidence that 15 can undergo hypoxia-selective metabolism in a manner similar to that of the parent compound tirapazamine (Figure S7).

**CONCLUSIONS**

In the work described here, we explored the idea that the hypoxia-selective enzymatic reduction of 1,2,4-benzotriazine 1,4-dioxides to the corresponding 1,2,4-benzotriazine 1-oxide metabolites can be exploited for the selective generation of DNA-alkylating species in the oxygen-poor cells found in tumor tissue. In the case of the tirapazamine analogue 18a bearing a nitrogen mustard unit at the 6-position, it was found that removal of the 4-oxide from the parent di-N-oxide to generate the mono-N-oxide analogue 17a does indeed cause a substantial increase in reactivity of the mustard unit, as measured by the rates of mustard hydrolysis. Hammett $\sigma$ and $\sigma^*$ values measured for the 3-amino-1,2,4-triazine 1-oxide and 3-amino-1,2,4-triazine 1,4-dioxide “substituents” in 17a and 18a, respectively, confirmed that removal of the 4-oxide causes a significant decrease in the electron-withdrawing effects exerted by the triazine ring system on substituents at the 6-position of the benzo ring.

In contrast to the tirapazamine analogues 17 and 18, the reactivities of the mustard units in the desamino series 21, 22, 25, and 26 were unaffected by the presence (or absence) of the 4-oxide group. It is interesting to consider why the reactivities of the mustard groups in the 3-desamino analogues (21, 22, 25, and 26) are not “switched” by deoxygenation. First, it may be noteworthy that the N=\(\equiv\)N(O) group in 17 and 18 is cross-conjugated with the mustard nitrogen at the 6-position and the 3-amino group. Thus, in these tirapazamine analogues, the 3-amino group may serve to mitigate the electron-withdrawing properties of the N=\(\equiv\)N(O) group. On the other hand, in analogues 21 and 22 lacking the 3-amino group, the N=\(\equiv\)N(O) group may exert a strong electron-withdrawing (deactivating) effect on the mustard unit in the 6-position that altogether prevents participation of the nitrogen lone pair in aziridinium ion formation. The hydrolysis rates of 25 and 26 are higher than those of 21 and 22 but still are unaffected by the presence or absence of the 4-oxide unit. Evidently, the N=\(\equiv\)N(O) unit in the meta position relative to the mustard unit is less electron-withdrawing than when it resides in the para position. In this regard, the N=\(\equiv\)N(O) group behaves similarly to the nitro group. The observation that the reactivity of the mustard unit in the 7-position is not affected by the presence or absence of the 4-oxide unit may be rationalized by the fact that the nitrogen lone pair of the mustard substituent in this location is not “through conjugated” with the 4-oxide unit.

The DNA-alkylating properties of the nitrogen mustard unit in the mono-N-oxide 17a are “switched on” compared to those of the parent di-N-oxide 18a. Under our assay conditions, the activated analogue 17a generates approximately 30-fold greater yields of guanine alkylation than does 18a. The mono-N-oxide 17a displays a striking and unexpected preference for reaction at guanine residues located in 5'-GT/5'-AC sequences. In contrast, typical mustards such as mechlorethamine (1) alkylate guanine residues with modest sequence selectivity (lane 5 of Figure 3C).\(^{38}\) We speculate that the sequence specificity of 17a arises via formation of Hoogsteen-type hydrogen bonds between the 3-amino-1,2,4-benzotriazine ring system and the adenine residue in the target 5'-GT/5'-AC sequence. Such an interaction could deliver the mustard unit of 17a to the N7-atom of the guanine residue in the major groove of the duplex (Figure 5). A similar scenario has been proposed to explain the unusual 5'-GC sequence specificity for uramustine.\(^{38}\) Finally, we provided evidence that the dialkylamino substituent on the 6-position of the 3-amino-1,2,4-benzotriazine 1,4-dioxide ring in analogues 18 is compatible with the hypoxia-selective enzymatic deoxygenative metabolism required to deploy the activated mustards such as 17a.

The compounds described here result from the combination of two well-studied anticancer drugs. The drug tirapazamine has oxygen sensing properties and generates oxidative DNA damage selectively under hypoxic conditions,\(^{34,35,46,52,57}\) while the nitrogen mustards are clinically used DNA-alkylating agents.\(^{3}\) Our work provides evidence that a judicious union of these two anticancer drug motifs can yield new agents with the potential to deliver both DNA-alkylating and DNA-oxidizing power selectively to oxygen-poor tumor tissue. A potential disadvantage of tirapazamine is that the agent kills only the small subset of tumor cells in which bioreductive metabolism occurs. Addition of a DNA-alkylating unit to the tirapazamine scaffold may yield agents with an ability to diffuse into—and kill—neighboring cells that exist under conditions of both more modest and more severe hypoxia. More generally, our results provide evidence that the 1,2,4-benzotriazine 1,4-dioxide unit can serve as an oxygen-sensing prodrug platform for the selective release or activation of various bioactive agents in hypoxic tissue.

**Figure 4.** HPLC chromatogram showing the in vitro metabolic conversion of 15 to 16 by NADPH:cytochrome P450 reductase under anaerobic conditions.

**Figure 5.** Molecular model depicting Hoogsteen base pairing by 17a with the adenine residue in a 5'-GT/5'-AC sequence.
Materials and Methods. All chemicals were used as purchased. The compound 3-fluoro-2-nitroaniline was purchased from Akr Scientific. The compound 3-fluoro-4-nitrobenzoic acid was purchased from Oakwood Chemical. Human cytochrome P450 P450 reductase was purchased from Sigma-Aldrich. For the kinetic measurements in the NMR, SS00 select series NMR tubes from Norell were used. NMR spectra were recorded at 500 MHz for 1H NMR and 125 MHz for 13C NMR. Oligonucleotides were obtained from Integrated DNA Technologies. T4 polynucleotide kinase was purchased from New England Biolabs. [γ-32P]-ATP (6000 Ci/mmol) was purchased from PerkinElmer. Compounds 9, 12, 13, 14, and 23 were prepared according to literature methods.65,74,75,92 Mass spectra were recorded using ESI-QTOF MS.

Synthesis of 3-Amino-6-(bis(2-hydroxyethyl)amino)-1,2,4-benzotriazine 1,4-Oxide (15). Compound 14 (240 mg, 1.3 mmol) was suspended in a mixture of acetone (35 mL), water (1 mL), and diethanolamine (1.4 g, 10 equiv) for 5 h. The resulting orange precipitate was collected by vacuum filtration and washed with cold ethanol followed by diethyl ether and dried in an oven at 110 °C for 2 h under a N2 atmosphere before being cooled on ice and mixed with ice-cold water (20 mL). The resulting yellow precipitate was collected by vacuum filtration, washed with water and diethyl ether, and then dried in a desiccator to give 17c (204 mg, 94% yield): 1H NMR (500 MHz, DMSO-d6) δ 9.9 (d, J = 9.6 Hz, 1H), 7.04 (d, J = 9.7, 1.8 Hz, 2H), 6.98 (bs, 2H), 6.56 (s, 1H), 4.38 (t, J = 5.3 Hz, 4H), 3.90 (t, J = 5.2 Hz, 4H), 3.17 (s, 6H); 13C NMR (125 MHz, DMSO-d6) δ 160.6, 153.3, 150.8, 122.0, 120.9, 114.4, 110.6, 67.4, 49.7, 37.1; HRMS (ESI) m/z calculated for C11H14Cl2N5O (M + H+) 302.0570, found 302.0576.

Preparation of 3-Amino-6-(bis((methylsulfonyl)oxy)ethyl)amino)-1,2,4-benzotriazine 1,4-Dioxide (18a). Compound 17a (70 mg, 0.17 mmol) and NaHCO3 (86 mg, 6 equiv) were suspended in methanol (25 mL). Oxone (245 mg, 1.2 equiv) was added, followed by water (10 mL). The mixture was stirred at 50 °C under a N2 atmosphere for 24 h. Thin layer chromatographic analysis indicated that most of the reaction progress occurred within the first 5 h. The reaction was cooled to room temperature, and the white solid was removed by vacuum filtration and washed with CH2Cl2 (10 mL). The filtrate was extracted with CH2Cl2 (2 × 5 mL), dried with anhydrous sodium sulfate, and evaporated under reduced pressure, and the resulting residue was subjected to column chromatography on silica gel eluted with a gradient of 1–5% methanol in CH2Cl2 to give 18a as an orange solid (11 mg, 15%): 1H NMR (500 MHz, DMSO-d6) δ 8.01 (d, J = 9.8 Hz, 1H), 7.82 (bs, 2H), 7.28 (dd, J = 9.9, 2.7 Hz, 2H), 7.09 (d, J = 2.7, 1H), 4.43 (t, J = 5.3 Hz, 4H), 3.97 (t, J = 5.3 Hz, 4H), 3.19 (s, 6H); 13C NMR (125 MHz, DMSO-d6) δ 150.3, 151.9, 140.1, 123.7, 123.2, 116.2, 92.8, 58.0, 53.2; HRMS (ESI) m/z calculated for C11H13Cl2N5O4S2 (M + H+) 574.0736, found 574.0737.

Preparation of 3-Amino-6-(bis(2-chloroethyl)amino)-1,2,4-benzotriazine 1,4-Dioxide (18b). Compound 17b (200 mg, 0.35 mmol) and LiCl (148 mg, 10 equiv) were dissolved in dimethylformamide (1.5 mL) and heated with 110 °C for 2 h under a N2 atmosphere before being cooled on ice and mixed with ice-cold water (20 mL). The resulting yellow precipitate was collected by vacuum filtration, washed with water and diethyl ether, and then dried in a desiccator to give 17b (104 mg, 94% yield): 1H NMR (500 MHz, DMSO-d6) δ 9.75 (d, J = 9.6 Hz, 1H), 7.04 (dd, J = 9.7, 2.5 Hz, 1H), 6.98 (bs, 2H), 6.48 (d, J = 2.5 Hz, 1H), 3.89 (t, J = 6.6 Hz, 4H), 3.80 (t, J = 6.6 Hz, 4H); 13C NMR (125 MHz, DMSO-d6) δ 160.7 (d, J = 253.9 Hz), 152.1, 150.8, 122.8, 114.3, 109.1, 51.8, 49.0; HRMS (ESI) m/z calculated for C11H12Cl2N5O4S2 (M + H+) 302.0570, found 302.0576.
Synthesis of 3-Amino-5-tosyl-6-(bis(2-hydroxyethyl)amino)-1,2,4-benzotriazine 1-Oxide (19). Compound 15 (52 mg, 0.18 mmol) was suspended in a stirred solution of pyridine in an ice bath, and tosyl chloride (172 mg, 5 equiv) was added. The mixture was stirred in an ice bath for 12 h, poured into ice-cold water (20 mL), and extracted with methylene chloride (5 × 25 mL). The combined organic fractions were washed with brine and dried over Na₂SO₄, and solvent was removed by rotary evaporation. Column chromatography on silica gel eluted with 1% methanol in CHCl₃ gave a pale white solid (438 mg, 50%): mp 127 °C. The resulting precipitate was collected by filtration. The precipitate was washed with water and diethyl ether and dried in an oven overnight at 70 °C.

Preparation of 6-(Bis(2-tosloyloxy)ethylamino)-1,2,4-benzotriazine 1,4-Dioxide (21). Compound 22 (40 mg, 0.04 mmol) and NaHCO₃ (30 mg, 1 equiv) were suspended in methanol (6.25 mL). Oxone (110 mg, 5 equiv) was added, followed by water (2.5 mL), and the mixture was stirred at 50 °C under an atmosphere of nitrogen gas for 16 h. The reaction was cooled to room temperature and extracted with methylene chloride (5 × 10 mL). The organic layers were combined, washed with brine, and dried over anhydrous sodium sulfate. Column chromatography on silica gel eluted with 1% MeOH in CHCl₃ gave compound 22 as a red-orange solid (2 mg, 10%) with 50% recovery of starting materials: 1H NMR (500 MHz, DMSO-δ) δ 8.89 (s, 1H), 7.73 (d, δ = 9.4 Hz, 1H), 7.56 (d, δ = 8.2 Hz, 4H), 7.44 (d, δ = 9.5, 2.8, 1H), 7.16 (d, δ = 8.1 Hz, 4H), 6.86 (d, δ = 2.8 Hz, 1H), 4.19 (t, δ = 5.0 Hz, 4H), 3.69 (t, δ = 5.0 Hz, 4H), 2.17 (s, 6H). 13C NMR (125 MHz, DMSO-δ) δ 150.2, 145.2, 140.7, 135.8, 132.0, 129.5, 127.7, 125.0, 94.8, 67.5, 49.1, 21.2. HRMS (ESI) m/z calculated for C₉H₈N₂O₃S (M + H⁺) = 559.1316, found 559.1317.

Synthesis of 7-(Bis(2-tosloyloxy)ethylamino)-1,2,4-benzotriazine 1,4-Dioxide (26). Using a procedure adapted from Pchakel and Hay, trifluoroacetic anhydride (300 μL) and methylene chloride (1.5 mL) were mixed with stirring in an ice bath, and 70% H₂O₂ (105 μL) was added dropwise. The mixture was stirred for 10 min and then allowed to warm to room temperature. This solution was cooled in an ice bath and slowly added to an ice-cold solution of 25 (110 mg, 0.2 mmol) in methylene chloride (10 mL). The reaction was stirred in an ice bath for 30 min before being diluted with methylene chloride (100 mL) and washed with cold water, cold NaHCO₃ (saturated), and then brine. The organic layer was dried over anhydrous sodium sulfate, and column chromatography on silica gel eluted with 0.5% MeOH in CHCl₃ gave 26 as a red solid (1 mg, 1%): 1H NMR (500 MHz, DMSO-δ) δ 9.15 (s, 1H), 7.96 (d, δ = 9.8 Hz, 1H), 7.57 (d, δ = 8.2 Hz, 4H), 7.25–7.18 (m, 5H), 6.84 (d, δ = 2.8 Hz, 1H), 4.21 (t, δ = 5.0 Hz, 4H), 3.70 (t, δ = 4.9 Hz, 4H), 2.25 (s, 6H). 13C NMR (125 MHz, DMSO-δ) δ 152.0, 145.3, 140.7, 135.8, 132.0, 129.5, 127.7, 125.0, 94.8, 67.4, 49.1, 21.2. HRMS (ESI) m/z calculated for C₉H₈N₂O₃S (M + H⁺) = 575.1265, found 575.1263.

Synthesis of 3-Amino-6-(tosloyloxy)morpholino-1,2,4-benzotriazine 1-Oxide (27). Compound 13 (503 mg, 2.8 mmol) and morpholine (0.72 mL, 3 equiv) were suspended in 1-methyl-2-pyrrolidinone (4 mL) and heated to 100 °C overnight. The resulting orange suspension was cooled to room temperature, diluted with water (40 mL), and filtered. The precipitate was washed with water and diethyl ether and then dried in an oven overnight at 70 °C. The orange-yellow powder was collected by vacuum filtration to give 27 (650 mg, 94%): mp 245–250 °C dec. 1H NMR (500 MHz, DMSO-δ) δ 7.94 (d, δ = 9.6 Hz, 6.
1H), 7.16 (dd, J = 9.7, 2.5 Hz, 2H), 7.00 (bs, 2H), 6.60 (d, J = 2.5, 1H), 3.73 (t, J = 4.7 Hz, 4H), 3.38 (t, J = 4.8 Hz, 4H); 1^1^C NMR (125 MHz, DMSO-\textit{d}_6) δ 160.7, 155.3, 150.8, 123.3, 120.9, 115.2, 103.0, 65.8, 46.8; HRMS (ESI) m/z calculated for C\textsubscript{7}H\textsubscript{10}N\textsubscript{4}O\textsubscript{2} (M + H\textsuperscript{+}) 179.0516, found 179.0516.

**Measurement of Mustard Hydrolysis Rates Using \textsuperscript{1}H NMR.** Solutions containing the 1,2,4-benzotriazine nitrogen mustards (1 mM final concentration) were prepared in CD\textsubscript{3}CN/D\textsubscript{2}O (1:1) containing disodium maleate (5 mM) as an internal standard. Solutions were placed in a capped S500 Norell NMR tube and warmed in a 50 °C water bath. At various time points the NMR tubes were removed from the heat bath and cooled in a room temperature water bath, and \textsuperscript{1}H NMR spectra were obtained using a 500 MHz NMR equipped with a 5 mm HCN cryo-probe. The probe temperature was 298 K during the experiment. Total number of scans was acquired with 32 repetitions delay of 4.1719923 s to ensure that the integration of the CH\textsubscript{2} resonances of the starting material in comparison to the CH\textsubscript{2} peak of the internal standard at each time point. The pseudo-first-order rates for the hydrolysis reactions were obtained by a least-squares fit to the equation ln A/A\textsubscript{0} = −kt.

**Measurement of Mustard Hydrolysis Rates and Decomposition Products Using HPLC.** Solutions of the mono- or di-N-oxide mustards (250 \textmu M) were prepared in sodium phosphate buffer (25 mM, pH 7) containing 2.5% DMS (v/v). The samples were incubated at 50 °C for 24 h. At various time-points aliquots were removed and frozen at −20 °C for later analysis. The samples were then analyzed by HPLC using a Varian Microsorb-MV C\textsubscript{18} column (100 A sphere size, 5 μm pore size, 250 mm length, and 2.6 mm i.d. eluted with a gradient mobile phase composed of solvent A (0.5% AcOH in H\textsubscript{2}O) and solvent B (methanol). For compound 17a a gradient of 25−50% B over 5 min, followed by 50−100% B over 5 min, followed by 100% B for 5 min was used. The mobile phase was returned to 25% B over 5 min and held at 25% B for 5 min postrun. For compound 18a the mobile phase was held at 25% B for 5 min following injection, before increasing to 50% B over 5 min and holding at 50% B for 4 min. The mobile phase was then returned to 25% B over 1 min and held at 25% B for 5 min postrun. The products were detected by monitoring absorbance at 280 nm. The identity of the major hydrolysis products was confirmed by comparison to authentic synthetic standards.

**Determination of pK\textsubscript{A}s for 29, 31, and 32.** UV−vis spectra were taken for compounds 29, 30, 31, and 32 (50 \textmu M) in solutions with pH values from 0 to 9 using HCl solutions and universal buffers prepared as in Britton and Robinson except containing 0.5% DMS (v/v). Buffer pH was determined with a pH meter. Absorbance values were measured at 256 and 282 nm for 29, 260 and 270 nm for 30, 345 and 400 nm for 31, and 360 and 400 nm for compound 32. Changes in absorbance were plotted against pH and the pK\textsubscript{A} values were calculated by fitting the data to the equation: pK\textsubscript{A} = pH + log[(d\textsubscript{A} − d)/ (d\textsubscript{d} − d\textsubscript{A})], where d\textsubscript{A} is the absorbance for the un-ionized species (starting absorbance), d\textsubscript{d} is the absorbance of the ionized species (final absorbance), and d\textsubscript{A} is the absorbance at any point in the pH titration. Taking the midpoint of the titration data gave a very similar result. To confirm that pK\textsubscript{A} values measured reflected protonation of the 6-COOH or 6-OH substituents in compounds 29, 31, and 32, pH-ab sorption measurements were performed with 9 and 12 to show that these control compounds displayed no significant changes in absorbance in the pH regions of interest.

**Examination of the DNA-Alkylation Properties of 17a and 18a.** The 2′-deoxyoligonucleotides (33 and 34, Figure 3) were labeled on the 5′-end with \textsuperscript{32}P and annealed to their complements using standard methods. \textsuperscript{32}P DNA duplexes were mixed with the indicated compounds (1 mM final concentrations) and incubated at 37 °C for 3 h in HEPES buffer (50 mM, pH 7) containing NaCl (100 mM) and DMF (10% v/v). The DNA was then ethanol precipitated, washed with 80% ethanol twice, and briefly dried in a SpeedVac concentrator (5 min at room temperature). The DNA was resuspended in piperidine (1 M in water) and heated to 95 °C for 30 min. The piperidine solution was removed under vacuum in a SpeedVac concentrator, and the resulting residue was resuspended in formamide.
loading buffer, warmed briefly, and loaded onto a 20% denaturing polyacrylamide gel. The gel was electrophoresed for 3 h at 1500 V to resolve the labeled DNA fragments. The labeled DNA fragments in the gel were visualized using phosphorimaging.

Hypoxia-Selective Enzymatic Reduction of N-Mustard Containing 1,2,4-Benzotriazine 1,4-Dioxides with Cytochrome P450. For experiments involving in vitro hypoxic metabolism, sodium phosphate buffer and HPLC-grade water were degassed by bubbling argon through the solutions for at least 30 min inside a glovebag. Stock solutions of compounds with argon gas. Stock solutions of compounds were detected by their absorbance at 420 nm, and the identity of products was confirmed by comparison to authentic synthetic standards.

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