Photochemical DNA Cleavage by the Antitumor Agent 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (Tirapazamine, WIN 59075, SR4233)

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Introduction

The compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, WIN 59075, SR4233, **1**), currently undergoing phase II and III clinical trials as an antitumor agent, is thought to derive its therapeutic activity by selectively damaging DNA in oxygen-poor (hypoxic) tumor cells.[1,2] DNA damage by **1** is triggered by enzymatic reduction of the heterocycle.[2] It is commonly suggested[1,2] that the one-electron reduced form of the drug (**2**) reacts directly with DNA, although recent studies have provided evidence that **2** undergoes fragmentation to yield the potent DNA-damaging agent hydroxyl radical (Scheme 1).[3] The selective cytotoxicity of **1** toward hypoxic cells probably is due to the fact that, in the presence of molecular oxygen, **2** is readily oxidized by molecular oxygen, thereby regenerating **1** and producing superoxide radical ($O_2^{\bullet-}$),[2,4] whose cytotoxicity is mitigated by cellular enzymes such as superoxide dismutase, glutathione peroxidase, and catalase.[5]

During the course of previous investigations, we observed light-dependent DNA cleavage by **1**. We further investigated this phenomenon because light-dependent formation of reactive species can be a medicinally important process.[6−8] In addition, we recognized that heterocyclic *N*-oxides are known to be rich in photochemistry[9−13] but that the photochemistry of benzotriazine *N*-oxides was virtually unexplored.

Results

DNA cleavage in our studies was monitored by measuring the conversion of form I (supercoiled) plasmid DNA to form II (open circular) that occurs upon single strand cleavage. In these experiments, buffered aqueous solutions containing DNA and the various compounds of interest were irradiated with 366 nm (λ_{max}) light in sealed Pyrex tubes (cutoff <280 nm) at room temperature. Under conditions of ambient oxygenation, photolysis of **1** in the presence of DNA does not lead to significant DNA cleavage (Table 1). Interestingly, light-dependent DNA cleavage by **1** under *anaerobic* conditions is significant (Table 1). Anaerobic cleavage reactions were degassed by three freeze−pump−thaw cycles, and the photolysis was performed in Pyrex tubes sealed under vacuum. The oxygen sensitivity of this photoreaction suggests that, in aerobic solution, light-dependent DNA cleavage by **1** may be inhibited by an energy transfer from photoexcited **1** to molecular oxygen which yields singlet oxygen ($^1\Delta_g$). Such photosensitized excitation of ground-state molecular oxygen to singlet oxygen is a well-known process.[14,15] Thus, although singlet oxygen is known to be capable of causing DNA cleavage,[16,17] production of singlet oxygen by photoexcited **1** in aerobic solution, in the context of DNA cleavage, appears to represent a deactivation pathway (Scheme 2). That is, the reactive species derived from photoexcitation of **1** under anaerobic conditions is a more efficient DNA-cleaving agent than singlet oxygen produced under aerobic conditions.[18]

The notion that singlet oxygen is produced in the irradiation of **1** under aerobic conditions (Scheme 2) is supported by two observations. First, while aerobic photolysis of **1** normally does not produce detectable DNA cleavage under the conditions employed here, when aerobic photolysis reactions containing **1** are performed in a buffered D_2O/CD_3CN solvent mixture, some DNA cleavage becomes evident (Table 1). The lifetime of singlet oxygen is markedly increased in deuterated solvents;[19] thus, it is reasonable to suspect that the DNA cleavage observed during aerobic photolysis in deuterated solvents is due to singlet oxygen. Further support for the presence of singlet oxygen in the aerobic photolysis of **1** is provided

Scheme 1

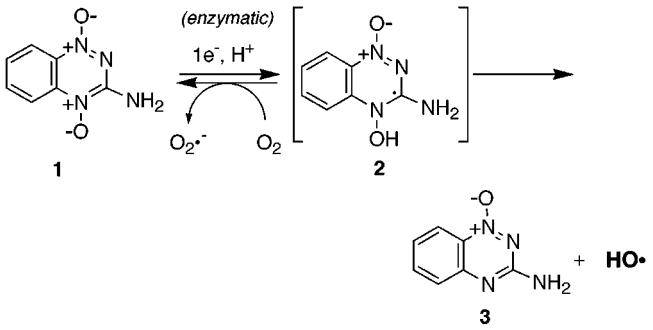

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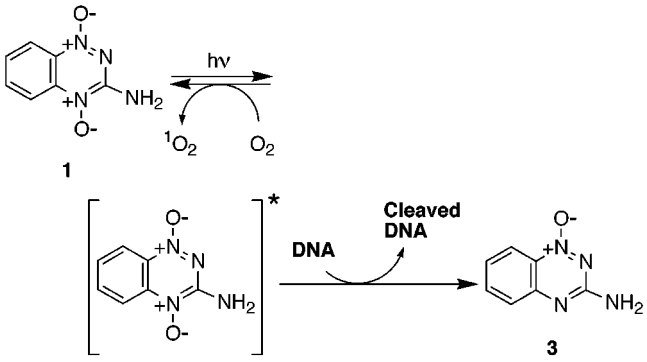
Table 1. Photocleavage of Plasmid DNA by 1

reaction[a]	% form I	% form II	*S* value[b]
aerobic; DNA + *hν*	88	12	0.13
anaerobic; DNA + *hν*	75	25	0.29
aerobic; 250 μM **1** + *hν*	87	13	0.14
anaerobic; 250 μM **1** + *hν*	30	70	1.20
aerobic; D_2O/CD_3CN; 250 μM **1** + *hν*	70	30	0.36
aerobic; 250 μM **1** + thiol[c] (1 mM) + *hν*	69	31	0.37
anaerobic; 250 μM **3** + *hν*	75	25	0.29

[a] Cleavage reactions contained supercoiled plasmid DNA (pBR322, 37 μM bp) in a solution of sodium phosphate buffer (50 mM, pH 7.0) and 10% acetonitrile by volume. Anaerobic reactions were freeze−pump−thaw degassed three times and photolyzed with >350 nm light under vacuum in sealed Pyrex tubes for 5.5 h at 25 °C. Aerobic reactions were performed in Pyrex glass tubes under ambient atmosphere. Reactions and densitometry were performed as described in the Experimental Section. [b] *S* is the mean number of strand breaks per plasmid molecule and is calculated using the following equation: $S = -\ln(\%\text{ form I DNA})$.[52] Reported values reflect the average of multiple experiments. The standard error of the reported values is approximately 5%. [c] 2-Mercaptoethanol.

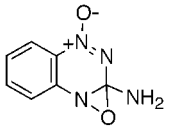
Scheme 2



by the observation that addition of thiols to aerobic photolysis reactions slightly increases the DNA-cleavage efficiency (Table 1), consistent with a previous report by Sies and co-workers that thiols increase the efficiency of spontaneous DNA strand scission by singlet oxygen.[20]

To better understand the chemical mechanism of photolytic DNA cleavage by **1**, we investigated the products resulting from the photolysis of this *N*-oxide under aerobic and anaerobic conditions. Photolysis of **1** under anaerobic conditions in aqueous buffer affords the deoxygenated compound **3** (Scheme 2) as a major product. Control experiments show that compound **3** does not mediate efficient DNA photocleavage under the anaerobic conditions employed in this study (Table 1). Significantly, no detectable amounts of **3** are produced in the aerobic photolysis of **1** under otherwise identical conditions. Thus, both light-dependent DNA cleavage and deoxygenation occur selectively under anaerobic conditions, thereby suggesting that a reactive intermediate formed during the deoxygenation of **1** may be responsible for the light-dependent DNA cleavage by this compound under anaerobic conditions (Scheme 2). Photolytic deoxygenation is a well-known reaction of heterocyclic *N*-oxides,[9−13,21] and importantly in the context of our work, formation of strong oxidizing species has previously been observed to accompany photolytic deoxygenation of these compounds.[11,22] It is well-known that oxidation of DNA can lead to spontaneous strand scission.[23]

Several mechanisms involving the formation of possible DNA-cleaving intermediates in the light-dependent deoxygenation of **1** may be considered. In some cases, photochemical deoxygenation of *N*-oxides is thought to occur through loss of atomic oxygen in its triplet ground state (oxene, $O(^3P)$).[11,22,24] Atomic oxygen is a highly reactive species that is capable of oxidizing organic substrates such as cyclohexane and benzene[22] and almost certainly would be capable of mediating oxidative DNA cleavage. Alternatively, it has been proposed that photolytic deoxygenation of heterocyclic *N*-oxides may involve oxaziridine intermediates (e.g. **4**).[9,11,25] Oxaziridines are known to be



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potent oxidizing agents[26] and might be capable of reacting with DNA. Finally, it is common for photoexcited states of organic molecules to have increased oxidizing potential relative to their ground states.[27−30] Thus, photoexcited *N*-oxides such as **1** may be capable of directly oxidizing DNA[30−33] either by electron transfer or hydrogen atom abstraction.[29,34−36] Importantly, a possible byproduct of such a reaction would be the one-electron reduced intermediate **2**, which, under anaerobic conditions, would produce further DNA damage (as shown in Scheme 1) while undergoing deoxygenation to **3**.[3]

At this time, any of the mechanisms described above are viable explanations for the observed light-dependent DNA cleavage by **1**; however, some additional insight regarding the detailed nature of reactive intermediates generated in the photolysis of 1,2,4-benzotriazine 1,4-dioxides may be provided by the isolation of 3-(4-methoxyanilino)-1,2,4-benzotriazine 1,4-dioxide (**6**) from the photolysis of 3-acetamido-1,2,4-benzotriazine 1,4-dioxide

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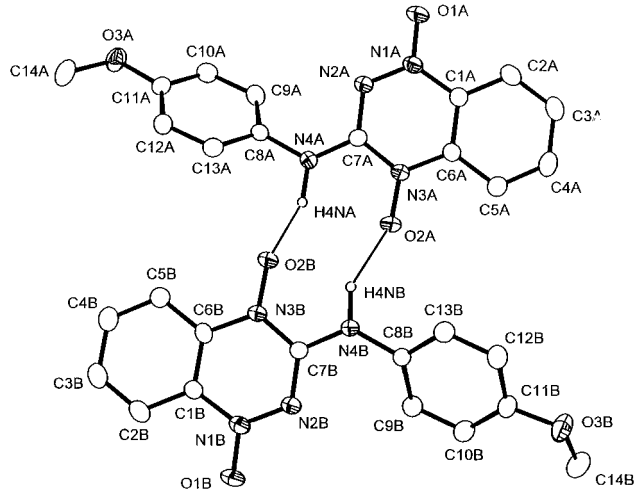
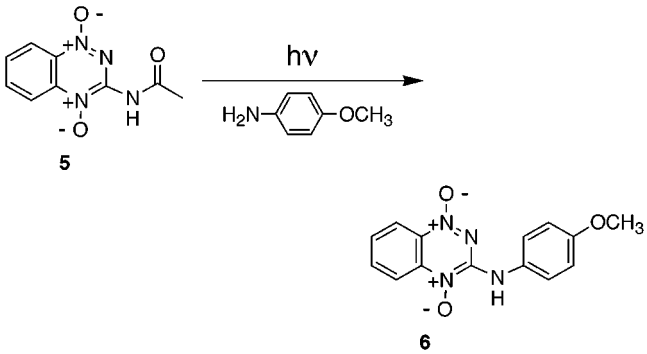


Figure 1. X-ray crystal structure of 3-(4-methoxyanilino)-1,2,4-benzotriazine 1,4-dioxide.

Scheme 3



(**5**) in the presence of *p*-anisidine (Scheme 3). Compound **6** was fully characterized by NMR, mass spectroscopy, and X-ray crystallography (Figure 1) and might reasonably be expected to arise through trapping of an oxaziridine intermediate derived from **5** by *p*-anisidine. Alternatively, **6** could arise through one-electron oxidation of *p*-anisidine by photoexcited **5**, followed by combination of the radical species and elimination of acetamide. No detectable quantities of compound **6** are formed when *p*-anisidine and **5** are stirred in the absence of light.

Discussion

In summary, we find that compound **1** effects efficient photocleavage of DNA specifically under anaerobic conditions. In aerobic solution, efficient DNA photocleavage by **1** is inhibited by energy transfer from photoexcited **1** to molecular oxygen, thus generating singlet oxygen. These results serve as a reminder[21,37−41] that, unless precautions are taken to exclude light, photochemical reactions can potentially affect the outcome of biochemical experiments involving heterocyclic *N*-oxides and that photodegradation of clinical preparations is a possibility. In addition, light-dependent formation of reactive species is medicinally relevant because such processes can cause undesired drug phototoxicity.[6−8]

Compounds that generate reactive intermediates upon photoexcitation have potential as phototherapeutic agents that can be employed, along with tissue-permeable visible light,[42] to deliver damaging, reactive species directly to tumor cells[43] and to tumor vasculature.[44−46] Most photodynamic therapy agents derive activity through the production of the toxic species singlet oxygen via so-called type II photosensitization reactions;[14,43,47] thus, these agents depend on the presence of molecular oxygen for activity.[47] Our findings indicate that compound **1**, in conjunction with light, produces singlet oxygen under aerobic conditions and, interestingly, also yields a reactive intermediate that can damage biomolecules under *anaerobic* conditions. In fact, in the context of DNA cleavage, energy transfer from photoexcited **1** to molecular oxygen represents a deactivation pathway; the reactive species generated by this *N*-oxide under anaerobic conditions is a more efficient DNA-cleaving agent than singlet oxygen.

Therapeutically useful agents that are highly photoactive in vivo typically absorb long wavelength light efficiently;[43] however, compound **1** has a relatively modest extinction coefficient. Therefore, compound **1** does not mediate efficient photocleavage of DNA with extremely high efficiency in vitro and perhaps should not be expected to be highly phototoxic in vivo. Nonetheless, our results indicate that light-dependent DNA cleavage by **1** is a chemically interesting process and that this compound might provide a foundation for the design of new antitumor agents that, in combination with direct irradiation by visible light, can specifically kill tumor cells by three distinct pathways: (a) hypoxia-selective, bioreductively activated DNA cleavage, (b) hypoxia-selective, light-dependent DNA cleavage; (c) photodynamic generation of singlet oxygen in oxygenated tumor vasculature.[48]

Experimental Section

Materials and Methods. Chemicals were purchased from the following suppliers and were of the highest purity available: EDTA, sodium phosphate, Aldrich Chemical Co.; sodium acetate, glycerol, Tris, and boric acid, Sigma Chemical Co.; HPLC grade solvents, Fisher; ethidium bromide, Boehringer Mannheim; Seakem ME agarose, FMC; ethanol, McCormick Distilling Co, Inc.; bromophenol blue and sodium dodecyl sulfate (SDS), United States Biochemical; pBR322 supercoiled plasmid DNA was isolated from *Escherichia coli* and purified by cesium chloride gradient centrifugation or purchased from Boehringer Mannheim. TLC was performed on silica gel plates, 0.25 mm, with F_{254} fluorophore (Merck) and compounds visualized with UV_{254} light. Column chromatography was performed using 230−400 mesh silica gel (Merck) with technical grade solvents that were distilled prior to use. Pyrex brand standard borosilicate

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glass tubing (o.d. 12 mm; wall thickness 1 mm) was purchased from either ChemGlass or Ace Glass. Reactions were irradiated with a hand-held long-wave UV lamp (UVP model UVGL-25, $\lambda_{max} = 366$ nm). In these reactions the Pyrex reaction tubes serve as a filter for < 280 nm light. Densitometry of ethidium-stained agarose gels was performed using an Alpha Innotech IS-1000 digital imaging system. Compounds **1**, **3**, **5**, and 1,2,4-benzotriazine (fully deoxygenated **1**) were prepared as previously described.[49]

Photocleavage of Plasmid DNA by 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (1). For anaerobic reactions, a Pyrex tube (sealed with a conical closure at one end) containing plasmid DNA (pBR322, 37 μM bp) and **1** (250 μM) in a solution of sodium phosphate (50 mM, pH 7.0) and acetonitrile (HPLC grade, 10 vol %) was subjected to three freeze−pump−thaw cycles followed by flame sealing under vacuum. The reaction was then photolyzed with >280 nm light at room temperature (fan cooled) for 5.5 h. For aerobic reactions, a Pyrex tube containing plasmid DNA (pBR322, 37 μM bp) and **1** (250 μM) in a mixture of sodium phosphate buffer (50 mM, pH 7.0) and acetonitrile (10 vol %) was sealed with a rubber septum under ambient atmosphere and photolyzed as described above. Following photolysis, the reaction vessels were opened, 5 μL of 50% glycerol loading buffer (containing 0.1% bromophenol blue, 150 mM EDTA, 1% SDS in 2 M Tris, 1 M acetate, pH 8) was added to the reactions, and the resulting mixture was loaded onto a 0.9% agarose gel. The gel was electrophoresed for approximately 4 h at 80 V in TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8) and then stained in an aqueous ethidium bromide solution (0.3 μg/mL) for 1−2 h. DNA in the gel was visualized by UV-transillumination and the gel image recorded using an Alpha Innotech IS-1000 digital imaging system. The values reported are uncorrected for the differential staining of form I and II plasmid DNA.[50]

Investigation of Products Resulting from Aerobic and Anaerobic Photolysis of 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (1). A solution of **1** (250 μM) in a mixture of sodium phosphate (50 mM, pH 7.0) and acetonitrile (HPLC grade, 10 vol %) was freeze−pump−thaw degassed (3×) and sealed under vacuum in a Pyrex tube. The solution was photolyzed with >280 nm light for 5.5 h at room temperature. The reaction was analyzed by HPLC (C18 reverse-phase Microsorb-MV column, 100 Å sphere size, 5 μM pore size, 25 cm length × 4.6 mm i.d. eluted with a 74:25:1 mixture of water/methanol/acetic acid) with monitoring at 254 nm. The formation of **3** in the photolysis of **1** was indicated by the presence of a peak eluting at ∼17.5 min (**1** elutes at ∼5.5 min). Under the conditions employed here neither **1** or **3** undergoes detectable conversion to the fully deoxygenated 3-amino-1,2,4-benzotriazine (retention time ∼16.5 min). The identity of the mono-*N*-oxide (**3**) was confirmed by comparison of retention times to that of an authentic sample and by co-injection with an authentic sample of **3**. Yields of **3** in the photolysis of **1** were determined by comparison of HPLC peak areas to a calibration curve prepared by measuring the HPLC peak areas resulting from injection of known concentrations of authentic **3**. Under the anaerobic conditions described above, photolysis of **1** produces approximately a 5% yield of the mono-*N*-oxide **3**. The major component of the reaction mixture is unchanged starting material (**1**). Under aerobic conditions, no detectable amounts (<0.5% yield) of **3** are obtained in the photolysis of **1**. Again, the major product of this reaction is unchanged starting material (**1**).

Photolysis of 3-Acetamido-1,2,4-benzotriazine 1,4-Dioxide (5) in the Presence of *p*-Anisidine: Isolation of 3-(4-Methoxyanilino)-1,2,4-benzotriazine 1,4-Dioxide (6). To a stirred solution of **5** (1.12 g, 5.2 mmol) in a mixture of acetonitrile (30 mL, HPLC grade) and aqueous sodium phosphate buffer (12 mL, 50 mM, pH 8) at 24 °C, a solution of *p*-anisidine (2.58 g, 21 mmol) in acetonitrile (3 mL) was added. The resulting light brown solution was photolyzed (>280 nm) with stirring at 25 °C for 90 h. After 90 h, TLC analysis of the reaction mixture revealed that unreacted starting materials and *N*-acetylated *p*-anisidine were major components of the reaction mixture. More interestingly, TLC analysis indicated that several other products had been formed. Control experiments revealed that the formation of these additional products is light-dependent. The reaction was filtered, the filtrate was extracted with diethyl ether (5 × 15 mL), and the combined extracts was dried over sodium sulfate. The dried diethyl ether extract was filtered and evaporated under reduced pressure to yield an oil. The oil was placed on a silica gel column and eluted with a hexane/ethyl acetate gradient (0→100% ethyl acetate) followed by elution with 95:5 ethyl acetate/methanol. From this column, 20 mg (1.5% yield) of the pure *p*-anisidine adduct **6** was isolated as a light red solid: $R_f = 0.28$ (100% ethyl acetate); mp 210−213 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.98 (bs, 1H), 8.35 (d, 2H), 7.90 (t, 1H), 7.52 (m, 3H), 6.94 (d, 2H), 3.81 (s, 3H); ^{13}C NMR (125.9 MHz, $CDCl_3$) δ 157.34, 147.79, 138.04, 136.01, 131.17, 128.46, 127.64, 123.01, 121.81, 117.72, 114.63, 55.55; HRMS (EI) *m/z* calcd for $C_{14}H_{12}N_4O_3$ 284.0909, found 284.0915. Crystals of **6** suitable for X-ray diffraction were obtained by slow evaporation of a chloroform solution. Crystal data for **6**: monoclinic, space group $P2_1/n$, $a = 9.976(1)$ Å, $b = 11.353(1)$ Å, $c = 23.319(2)$ Å, $\beta = 100.065(1)°$, $V = 2600.4(3)$ Å^3, $\rho_{calcd} = 1.45$ mg/cm^{-3}, $2\theta_{max} = 45°$, Mo Kα radiation ($\lambda = 0.710\ 69$ Å) for $Z = 8$. Intensity data were collected with use of the Siemans SMART system at 298 K. Least-squares refinement based on 2483 reflections with $I_{net} > 2.0\sigma(I_{net})$ (out of 3371 unique reflections) and 379 parameters on convergence gave a final value of $R = 0.050$. All crystallographic calculations were conducted using SHELXL-93[51] locally implemented on an IBM-compatible PC.

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Supporting Information Available: Tables of complete X-ray data and methods for **6** (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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