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Brief Article

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Hypoxia Activated Prodrugs of a 9-Aza-anthrapyrazole Derivative That Has Promising Anticancer Activity

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Supporting Information

ABSTRACT: Mono- and bis-*N*-oxides of a 9-aza-anthrapyrazole derivative having two 2-(dimethylamino)ethyl appendages were prepared by using a mild oxaziridine reagent. Biochemical and cell culture assays indicate that the bis-oxide is an inactive prodrug that readily converts to the active parent molecule under hypoxic conditions that are analogous to those present within certain tumors.

INTRODUCTION

Human solid tumors grow within a unique microenvironment that, among other features, is characterized by significantly lower oxygen levels compared to those found in normal tissues.[1] The median oxygen tension for all human tumors ranges from 1.3% to 3.9%, with most of the measurements in solid tumors being less than 0.3%. Alternatively, values for normal cells are in the range 3.1−8.7%.[2] The hypoxia in solid tumors can be attributed to its vasculature which is diminished and abnormal in architecture, leading to insufficient supply of oxygen and nutrients. Low oxygen tension, in turn, is associated with low intracellular pH, low glucose concentrations, and high lactate concentrations because glycolysis, the oxygen-independent metabolic pathway, becomes the primary mechanism to generate ATP.[3,4] There is also convincing evidence that hypoxia promotes metastasis[5] and angiogenesis[6] and may led to the selection of cells with a more malignant phenotype.[7]

Treatment of hypoxic tumors has been limited by systemic toxicity, inefficient delivery of drugs to cells that are distant from blood vessels, and reduced uptake of drugs by nondividing cancer cells. These features have rendered hypoxic tumor cells intrinsically more resistant to conventional treatments by ionizing radiation[8] and chemotherapeutic drugs.[9] Alternatively, from a drug design standpoint, the same morphological and physiological differences between hypoxic tumor cells and normal cells may be able to be exploited to selectively target tumor cells.[10,11]

The anthracycline antitumor and antibiotic agents doxorubicin and daunorubicin (Figure 1) have been used for the treatment of several types of cancer such as breast and ovarian cancers, acute nonlymphocytic leukemia in adults, and acute lymphocytic leukemia in adults and children.[12] Anthracyclines intercalate between DNA bases, thus blocking DNA synthesis and transcription. They also inhibit the activity of topoisomerase II (Topo II), leading to breaks in genomic DNA.[13] However, these anticancer agents possess serious side effect toxicity, especially cardiotoxicity. Although mitoxantrone

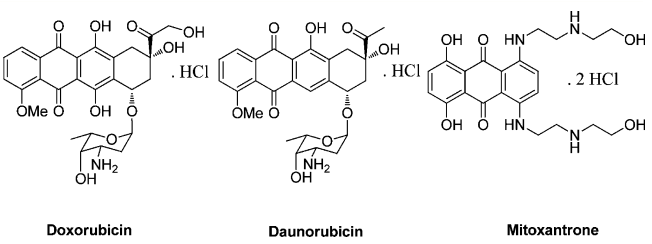

Figure 1. Structures of clinically useful anthracyclines.

(Figure 1), a structurally similar anticancer agent, was found to have an improved tolerance profile, it is still not devoid of serious side effects that are associated with myelosuppression and cardiotoxicity. Moreover, cell histotypes that developed resistance to doxorubicin due to the overexpression of P-glycoprotein (Pgp) also showed resistance to mitoxantrone.[14] The biochemical basis for cardiotoxicity is not fully understood, but it is thought that the in vivo reduction of the quinone moiety plays an important role. The addition of an electron to the quinone leads to the formation of a semiquinone free radical that, in turn, transfers an electron to molecular oxygen to generate superoxide radical anions. The so-formed radical anions lead to hydroxyl radicals that can damage cardiac tissue.

Several published studies have discussed structural modification of mitoxantrone in attempts to reduce its adverse side effects.[15−17] These studies led to the discovery of 9-aza-anthrapyrazole analogues whose general structure is shown in Figure 2. Because they were expected to be less prone to undergo bioreduction, the anthrapyrazole analogues were designed to have increased antitumor activity and reduced cardiotoxicity. In addition, the presence of two amine-containing side chains (e.g., R_1 and R_2 being 2-(dimethylamino)ethyl in Figure 2) can enhance their DNA

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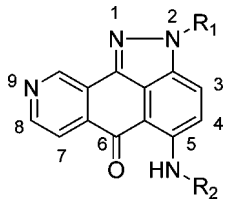


Figure 2. General structure of 9-aza-anthrapyrazole.

binding affinity and cytotoxicity, which is believed to be achieved through topoisomerase inhibition. However, selective targeting of such drugs to cancer cells remains an important issue because these analogues still have the capability to intercalate with the DNA of normal cells and that of cancer cells.[18]

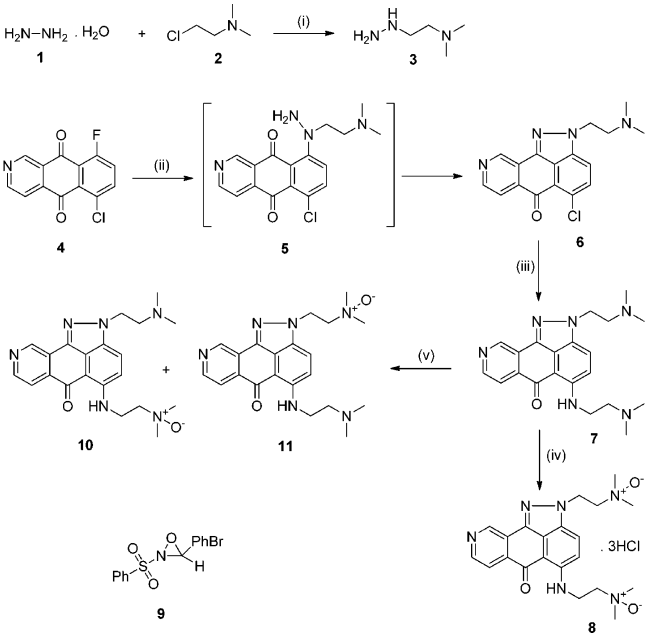
One approach to achieve selective targeting is to mask the chemotherapeutic agent as a prodrug that becomes unmasked at the cancer site.[19] This strategy has been described previously by Cerecetto et al., Lee et al., and Nishida et al.[11,20,21] relative to the analogous anthracene system. Herein, we describe the synthesis of 9-aza-anthrapyrazole *N*-oxide analogues as "bioreductive-dependent agents and as potential hypoxia-selective cytotoxins for cancer therapy".[20] The lower pH and oxygen-deprived (reductive) nature of hypoxic cancer cells may be able to reduce these inactive prodrugs to their active amine forms. Cellular reduction of the *N*-oxide functionalities will then result in "bis-armed" anthrapyrazole amines that regain DNA binding affinity and cytotoxicity. Such anthrapyrazole moieties are unique DNA-complexing agents that may then exhibit reduced or no cardiotoxicity because of their diminished tendency to form semiquinone free radicals at sites other than hypoxic tumor cells.[18]

CHEMISTRY

Our syntheses of the anthrapyrazole *N*-oxide analogues were adapted from the route previously reported by Krapcho et al.[15] who used it within the analogous anthrapyrazole system. We began by preparing the substituted hydrazine **3** that is essential for constructing the pyrazole ring via the reaction of hydrazine monohydrate **1** and dimethylaminoethyl chloride **2** under basic conditions (Scheme 1).[22] Regioselective construction of the pyrazole ring with an N-substituent can be achieved by treating anthraquinone **4** with hydrazine **3**.[15] Two aspects merit highlight in this respect. First, on the basis of previous SAR studies where regioisomers bearing nitrogen atoms at position 7, 8, 9, or 10 of the anthrapyrazole chromophore have been prepared;[23] the most active 9-aza-regioisomer has been selected for this synthesis. Second, the regioselective construction of the pyrazole ring was induced by choosing the fluorine and chlorine atoms as substituents at C-6 and C-9 of anthraquinone **4**, respectively, because of the difference in their electrophilicity during S_NAr displacements.[23]

Thus, the most nucleophilic nitrogen of the hydrazine adduct selectively displaced the C-9 fluorine atom, yielding intermediate **5** which under the reaction conditions also underwent spontaneous cyclization to yield anthrapyrazole **6**. Refluxing **6** with *N*,*N*-dimethylaminoethylamine in anhydrous pyridine under a nitrogen atmosphere displaced the C-5 chlorine atom and resulted in the bis-substituted anthrapyrazole diamine **7**.[15] Oxidation of anthrapyrazole **7** using 2.5 equiv of the mild oxidizing reagent oxaziridine **9** resulted in anthrapyrazole bis-*N*-oxide **8**.[20] Oxidizing **7** with 1 equiv of **9** afforded the

Scheme 1. Synthesis of 9-Aza-anthrapyrazole *N*-Oxide Analogues[a]



[a]Reagents and conditions: (i) K_2CO_3, reflux, then NaOH, 62%; (ii) DIPEA, THF, room temp, 75%; (iii) [2-(dimethylamino)ethyl]amine, anhydrous pyridine, 90 °C, 60%; (iv) 2.5 equiv of **9**, CH_2Cl_2/MeOH (3:1), 0−5 °C, then HCl (g), 87%; (v) 1 equiv of **9**, CH_2Cl_2/MeOH (3:1), 0−5 °C, 49%.

anthrapyrazole mono-*N*-oxide adducts in a 1:1 mixture of **10** and **11** as determined by NMR. These spectra displayed eight distinct peaks corresponding to the eight different methylene groups in **10** and **11**, while there were only four peaks in anthrapyrazole bis-*N*-oxide analogue **8**. The oxaziridine-containing, mild oxidizing reagent **9** was synthesized according to a literature procedure.[24]

BIOLOGY AND DISCUSSION

Preliminary biological experiments using the regioisomeric mixture of "partial prodrug" mono-*N*-oxides **10** and **11** compared to using the "full prodrug" bis-*N*-oxide **8** indicated that more consistent behavior was observed for the latter. Thus, a focus was placed on characterizing the profile of **8**. Toward that end, parent compounds **7** and **8** were first studied in a biochemical assay for inhibition of Topo II. A gel electrophoresis for the control runs in these experiments shows a single band for DNA, whereas multiple bands result when Topo II is added. Compound **7** was able to inhibit Topo II's fragmentation of DNA at 10 μM, while its prodrug **8** remained inactive up to 50 μM. Interactions with DNA were likewise assessed in a biochemical assay wherein displacement of the standard ligand, ethidium bromide, can be readily measured by the change in fluorescence. In this case, mitoxantrone was also tested as a standard. Mitoxantrone's effective concentration to inhibit ethidium bromide binding by 50% (IC_{50}) was 4.5 μM. The IC_{50} for **7** was reasonably similar (6.8 μM), while prodrug **8** was unable to displace ethidium bromide up to 50 μM.

Cell culture studies were used to assess cytotoxicity.[25] These investigations were conducted under oxygen rich ("oxic") and hypoxic ("anoxic") conditions using three cell lines: LoVo from colon cancer; MCF-7/S from breast cancer sensitive to

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doxorubicin; MCF-7/Dox from breast cancer that has become resistant to doxorubicin. Mitoxantrone was again utilized as a standard agent. Cells were cultured in 96-well plates for 74 h with varying concentrations of test agent. Cytotoxicity was determined by sulforhodamine B assay where viable cells convert the colorless solution to blue. Gradations of color versus test agent concentration allowed for calculation of IC_{50}, in this case the latter reflecting 50% inhibition of cultured cell growth. The hypoxic medium was created by incubating cells in an anerobic chamber flushed with 95% N_2 and 5% CO_2. As shown in Table 1, in the LoVo and MCF-7/S cell lines, mitoxantrone and **7** showed higher activity under oxic conditions compared to the hypoxic conditions. Alternatively, **8** proved to be significantly more active under hypoxic conditions wherein it was able to achieve levels comparable to those of mitoxantrone and **7**. Interestingly, while mitoxantrone's activity fell off in the hypoxic MCF-7/Dox panel, thus reflecting the multidrug resistance that this cell line can display toward chemotherapeutics, **7** and **8** were able to maintain their potency. Since one of the principle components of multidrug resistance is overexpression of the P-glycoprotein (Pgp) efflux transporter, it would appear that **7** and **8** are poor substrates for Pgp.

Table 1. IC_{50} (μM) Derived from the Cytotoxicity Assay under Oxic and Anoxic Conditions[a]

cell line	drug	oxic	anoxic
LoVo	mitoxantrone	0.1	0.9
	7	1.3	3.3
	8	>25	1.5
MCF-7/S	mitoxantrone	0.1	1.0
	7	0.9	4.0
	8	>25	5.0
MCF-7/Dox	mitoxantrone	4.7	>10
	7	1.0	1.2
	8	>25	1.3

[a]Data represent the average of two determinations across at least three different concentrations wherein the entire range of values for each point was always within 10% of the average.

To further evaluate **8** as a potential prodrug, we examined the ability for the cytochrome P450 system (CYP450) to perform the requisite reduction when under hypoxic conditions. As part of these tests, **7** and **8** were incubated with the S-9 fraction from rat liver in normal air or in 1% O_2 for 2 h at 37 °C. After precipitation of proteins by ethanol, samples were centrifuged and the supernatants used in the same tests for Topo II activity and cell culture cytotoxicity under oxic conditions. While **7** displayed comparable activity after either pretreatment, **8** demonstrated activity only from the metabolic incubations that were performed under hypoxic conditions. Finally, **8** and hypoxic CYP450-treated **8** were studied side by side in an alternative assay for DNA binding.[26] These results are depicted in Figure 3. While the scans for untreated **8** (lower panel) did not change upon sequentially increasing the concentrations of DNA, those for treated **8** (upper panel) displayed increases in hypochromicity accompanied by a minor bathochromic shift. These results further demonstrate that **8** does not interact significantly with DNA until it becomes reduced, in this case by CYP450.

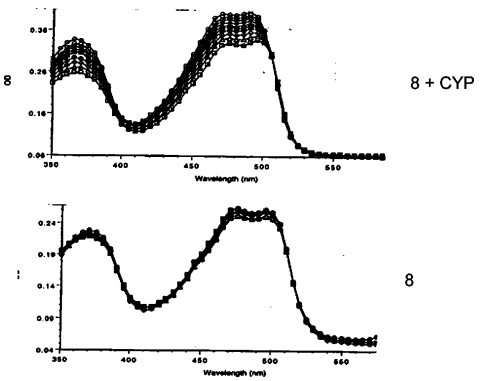

Figure 3. Binding curves for prodrug **8** and sequentially increasing concentrations of DNA without prior incubation with CYP450 (lower panel) and after incubation with CYP450 (upper panel). Axes *y* and *x* are absorbance and wavelength. See text for details.

SUMMARY

The composite of our results suggests that **8** serves as an effective prodrug for **7** by undergoing reduction under hypoxic conditions. Thus, there may be an added degree of selectivity that **8** will be able to display toward solid tumors compared to healthy cells. Further studies are underway in advanced pharmacological models to substantiate the additional promise demonstrated by this prodrug.

ASSOCIATED CONTENT

Supporting Information

Experimental methods and details for synthesis of the oxidizing reagent; biology testing methods and raw data for the biochemical studies; and NMR spectra for all intermediates and target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

CYP450, cytochrome P450; Pgp, P-glycoprotein; Topo II, topoisomerase II

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