important to the understanding of the disease process. Furthermore, this study suggests that clinical analysis of intact erythrocytes by spin-echo NMR may offer a valuable method of monitoring the progress of therapy in rheumatoid arthritis which is less subjective than the clinical assessments currently in use.

Experimental Section

Whole blood was collected in heparinized anticoagulant tubes from 10 healthy normal volunteers and 20 patients with classical or definite rheumatoid arthritis. Patients were not on any prescribed second-line or cytotoxic therapy. Separation of the blood was commenced on the day of collection. It was centrifuged at 3000 rpm (1000*g*) for 10 min at 4 °C, and the plasma was drawn off.

The isolated red-cell pellet obtained as described above was washed once in ^2H_2O/NaCl (0.154 M)/Na_2HPO_4 (0.125 M) to facilitate oxygen uptake and twice in ^2H_2O/saline (0.154 M NaCl). A volume of 0.4 mL of packed erythrocytes was then placed in a 5-mm NMR tube with 0.1 mL of ^2H_2O/saline to maintain a degree of fluidity within the cell suspension. NMR spectra were recorded using a Hahn spin-echo pulse sequence (90°-t-180°-t) with a delay time (t) of 60 ms. A Bruker 250 MHz spectrometer was used to record all spectra. Samples were maintained at 20 °C during data collection, and the data from 2000 complete pulse sequences were accumulated for each spectrum. The free induction decay (FID) was collected in the minimum memory size, either 4K, 2K, or 1K depending on the strength of the signal obtained from the sample. The FID was zero filled to 16 K, and a 0.5-Hz line-broadening function was applied, prior to Fourier transformation. The 90° pulse was generated with a 7.5-μs pulse width. The acquisition times varied with the size of memory employed to store the FID, being 0.64, 0.34, and 0.17 s, respectively. A small presaturation pulse was applied to the water resonance during relaxation delay (D1 = 0.5 s).

Patient Profiles. Twenty patients (5 males, 15 females; mean age 57.7 ± 2.5 years) with rheumatoid arthritis were recruited into the study. In all cases the clinical diagnosis of rheumatoid arthritis followed the criteria for classical or definite rheumatoid arthritis as defined by the American Rheumatism Association.[9] The patients were not on any second-line or cytotoxic therapy. This study was approved by the local ethical committee.

Statistics. The data were analyzed using the Mann-Whitney U test.

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Registry No. Penicillamine, 52-67-5; glutathione, 70-18-8; oxidized glutathione, 27025-41-8.

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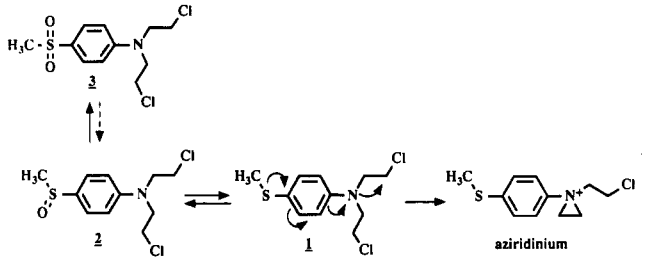
Communications to the Editor

p-(Methylsulfinyl)phenyl Nitrogen Mustard as a Novel Bioreductive Prodrug Selective against Hypoxic Tumors

Many conventional anticancer drugs display relatively poor selectivity for neoplastic cells. Solid tumor cells are particularly resistant to radiation and chemotherapy. While there may be few useful kinetic and/or biochemical differences between solid tumor cells and normal cells that can be exploited, there are important microenvironmental properties unique to solid tumors, e.g., localized hypoxia, nutrient deprivation, and low pH.[1] There has been considerable interest in designing drugs selective for hypoxic environments.[2-9]

Scheme I



Sulfoxides are known to be susceptible to bioreduction, and the reduction of sulfoxides by mammalian tissues is a complex process which may involve both soluble and membrane-bound enzyme systems.[10] One of the earliest reports on the hepatic reduction of sulfoxides was that of 4,4′-diaminodiphenyl sulfoxide which was readily reduced by 10000*g* supernatant fractions of rat liver in the presence

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Table I. Relative Alkylating Activity and Cytotoxicity Profiles of 1-3

compd	k[a]	IC_{50} (μM)[b] 3T3	B16-F10
1	1.03×10^{-1}	1.2	11.7
2	2.50×10^{-3}	18.7	125
3	3.29×10^{-4}	100	110

[a] The value k represents a pseudo-first-order rate constant representing relative rates of alkylation as determined by the standard NBP assay except the incubation temperature was 50 °C. [b] Survival was measured by clonogenic assay. Results from one experiment carried out at five drug concentrations. Drug exposure times were 13 h for Balb/c 3T3 mouse embryo fibroblasts and 3 h for B16-F10 murine melanoma cells.

of NADPH but not NADH.[11] The amino acid methionine in its oxidized form cannot be utilized as such for protein synthesis; this suggested that the methionine sulfoxide (Met SO) must be reduced to methionine in vivo.[12] In vitro reduction of Met SO by rat liver and kidney showed activity mainly in the cytosol of liver and was enhanced by NADH.[13] Sulindac, a well known non-steroidal anti-inflammatory drug, undergoes two major biotransformations: reversible reduction of the sulfoxide compound (parent drug) to the sulfide metabolite and irreversible oxidation to the sulfone metabolite.[14] The oxidation to the sulfone metabolite is the dominant process under normal physiological condition; however the reduction process becomes significant under anaerobic conditions.[15]

These literature data suggested that properly designed sulfoxide compounds, which can be converted to and/or generate alkylating species upon bioreduction, might serve as useful hypoxia-selective anticancer agents. *p*-(Methylsulfinyl)phenyl nitrogen mustard (**2**) was synthesized and examined as a prototype bioreductive prodrug of *p*-(methylthio)phenyl nitrogen mustard (**1**). The sulfoxide **2** should be chemically stable because of the electron-withdrawing effect by the sulfoxide moiety; hence the nitrogen lone pair of the nitrogen moiety is not available to readily form the reactive aziridinium species. However, upon reduction in the hypoxic environments of tumor cells, **2** is expected to generate the reactive sulfide **1** (Scheme I). Under the normal oxidative conditions found in normal cells, **2** is expected to be metabolized to the more chemically stable sulfone metabolite (**3**).

Chemistry. *p*-(Methylthio)-*N*,*N*-bis(2-chloroethyl)-aniline (**1**) was prepared essentially as described in the literatures[6,16,17] by (a) alkylating *p*-(methylthio)aniline with ethylene oxide or with 2-chloroethanol and (b) chlorinating the resulting diol, *p*-(methylthio)-*N*,*N*-bis(2-hydroxyethyl)aniline with phosphoryl chloride. *p*-(Methylsulfinyl)-*N*,*N*-bis(2-chloroethyl)aniline (**2**) and *p*-(methylsulfonyl)-*N*,*N*-bis(2-chloroethyl)aniline (**3**) were prepared by controlled oxidation of **1** using 3-chloroperoxybenzoic acid.

Table II. Formation of the Reactive Sulfide Metabolite 1 from the Incubation of 2 with Rat S-9 Fractions As Detected by HPLC[a]

conditions[b]	% of 2 converted to 1
O_2, control	0
O_2, -NADPH generating system	1.0
O_2, +NADPH generating system	2.6
N_2, control	0
N_2, -NADPH generating system	2.3
N_2, +NADPH generating system	4.3

[a] The HPLC system consists of: a Waters Model 440 detector; a reversed-phase Bio-sil ODS-10 C_{18} column (250 mm × 4 mm); isocratic eluent MeOH/H_2O (2:1), flow rate = 2 mL/min; detected at 254 nm (0.05 AUFS). t_R = 7.33 min for 1 and t_R = 2.30 min for 2. [b] NADPH generating system consists of 5 mM glucose 6-phosphate, 5 mM NADP, and 0.8 mM $MgCl_2$ in 0.025 M sodium phosphate buffer, pH 7.4. The anaerobic condition was induced by gently bubbling N_2 through the tubes sealed with rubber septa for 10 min. The S-9 fraction was boiled for 5 min before use in control incubation mixtures.

Biological Methods. The relative chemical reactivities of these compounds as determined by NBP [4-(4-nitrobenzyl)pyridine] assay[18,19] showed that **1** was the most reactive, followed by **2** and **3** (Table I). The relative cytotoxicity profile of these compounds against 3T3 cell line correlated well with their chemical reactivity data, albeit qualitative; the cytotoxicity decreased in the order of **1**, **2**, and **3** (75:5:1, respectively). In B16-F10 melanoma cell line, despite that **1** was more toxic (10-fold) than **2** or **3** as expected, there appeared to be no apparent difference in cytotoxicity between **2** and **3** (Table I).

To test if the sulfoxide **2** can be bioreduced to the more reactive sulfide **1**, **2** was incubated with the rat hepatic S-9 fraction under normal and anaerobic conditions. Significant alkylating activity as determined by NBP assay was observed (data not shown) under both aerobic and anaerobic conditions, suggesting that the sulfoxide compound produced and/or was converted to alkylating species by enzyme system(s) in the S-9 fraction. Since the NBP assay does not discriminate types of alkylating species, the alkylating activity observed from the aerobic incubation may have been due, at least in part, to chloroacetaldehyde, the possible oxidative N-dealkylation product of the chloroethylamine side chain.

In order to obtain a more accurate picture of the reaction processes, the reaction mixture was analyzed by HPLC (Table II). The data showed that **2** underwent reduction to the reactive **1** under both aerobic and anaerobic conditions. The relative percentage of the sulfide formation under anaerobic condition was approximately twice as much as that under aerobic condition. The actual difference in the formation of **1** between the aerobic and anaerobic conditions is possibly much greater, since **1** is

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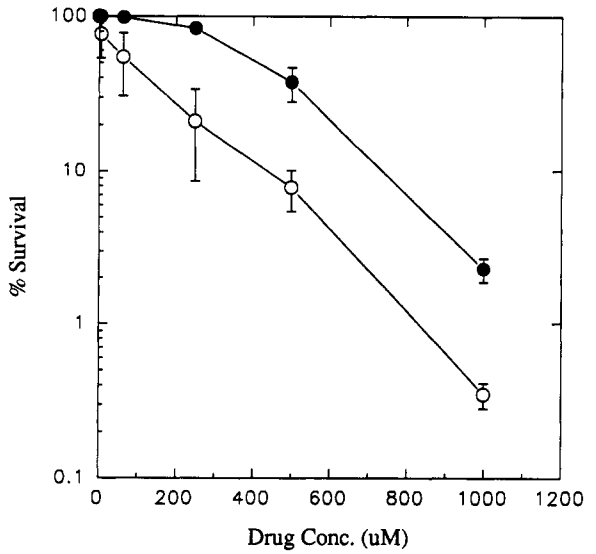

Figure 1. Survival of CHO cells following treatment with **2**. Cells were subjected to aerobic or hypoxic conditions for 2 h. Survival was measured by using a clonogenic assay: (●) aerobic, (○) hypoxic. The data points represent the mean ± SE of at least triplicate determinations for each of three separate experiments.

Table III. Cytotoxicities of **1** and **2** under Normal Aerobic and Hypoxic Conditions against CHO Cells

compd	IC_{50} (μM)[a] aerobic	hypoxic
1	3.50 ± 0.46	ND
2	362 ± 63	90 ± 38

[a] Mean ± SE of three experiments carried out at least five drug concentrations. Cells were subjected to aerobic or hypoxic conditions for 2 h before drug exposure. Drug exposure time was 3 h. Survival was measured by a clonogenic assay. ND = not determined. $p < 0.01$ for **2**.

chemically unstable and the other potential oxidative metabolites, e.g., monoalkylating metabolite(s) and **3**, could not be quantitated and therefore not included in the calculation. The observation that more of **1** was generated in the presence of NADPH generating system suggests a possible involvement of NADPH-dependent cytochrome P-450 reductase, which has been shown to catalyze the reduction of nitromin to nitrogen mustard,[20] in the reduction of **2** to **1**. These results provided the first evidence supporting the concept of enhanced bioreduction of **2** to **1** under hypoxic condition.

Subsequently, **2** was tested for its hypoxia-selective cytotoxicity against the Chinese hamster ovary (CHO) cell line under aerobic (95% air/5% CO_2) and hypoxic (95% N_2/5% CO_2) conditions (Figure 1). CHO cells have been shown to possess NADPH cytochrome P-450 reductase and DT-diaphorase which reductively metabolize mitomycin C, an alkylating agent that requires bioreductive activation.[4] The published procedure of Fracasso and Sartorelli[21] with some modifications was used to test for hypoxia selectivity. The results indicated that **1**, a compound which does not require bioactivation for its toxicity, was 100-fold more cytotoxic than **2** under aerobic conditions (Table III). When **2** was incubated with CHO cells under hypoxic conditions, there was a 4-fold enhancement of cytotoxicity as compared to aerobic conditions, on the basis of the IC_{50} values of the cell survivals. The hypoxia selectivity was however less substantial at higher drug concentrations with the enhancement of ca. 1.5–2-fold at the level of IC_{90} or greater.

In summary, these results support the exploration of properly designed sulfoxide compounds as potential anticancer agents against solid tumors, and to our knowledge, this is the first time that metabolism of sulfoxide has been applied in the design of hypoxia-selective alkylating agents. Research is in progress in the application of this methodology to provide more potent and selective cytotoxic drugs.

Chul-Hoon Kwon,* Daria R. Blanco, Nesrine Baturay
Department of Pharmaceutical Sciences
College of Pharmacy and Allied Health Professions
St. John's University
Jamaica, New York 11439
Received March 2, 1992

Cyclic Pentapeptide Endothelin Antagonists with High ET_A Selectivity. Potency- and Solubility-Enhancing Modifications

Endothelin (ET)-1, a potent vasoconstrictor, consisting of 21 amino acids, was first isolated from porcine aortic endothelial cell culture supernatant.[1] Subsequent studies including a human genomic analysis revealed the existence of two additional related peptides, ET-2 and ET-3.[2,3] The concept is now widely accepted that many mammalian species produce these three isopeptides and that the peptides elicit diverse biological effects on vascular[1,4,5] and nonvascular tissues[6-8] through at least two distinct ET receptor subtypes termed ET_A (selective for ET-1 and ET-2) and ET_B (equally sensitive to all three peptides).[9-11]

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