117.41, 123.86, 124.05, 127.41, 133.26, 149.07, 150.03, 151.83. Anal. ($C_{30}H_{36}Cl_2N_4 \cdot H_2O$) C, H, N.

Screening Methods. In vitro activity against *P. falciparum* was determined using a modification of the semiautomated microdilution technique of Desjardins et al.[20] and Milhous et al.[21] Two *P. falciparum* malaria parasite clones, designated as Sierra Leone (D-6) and Indochina (W-2), are used in susceptibility testing. The former is resistant to mefloquine, and the latter to CQ, pyrimethamine, sulfadoxine, and quinine. Test compounds are dissolved in dimethyl sulfoxide, and solutions are serially diluted with culture media. Erythrocytes with 0.25 to 0.5% parasitemia are added to each well of a 96-well microdilution plate to give a final hematocrit of 1.5%. Inhibition of uptake of tritiated hypoxanthine is used as an index of antimalarial activity. Results are reported as IC_{50} (ng/mL) values.

In vivo activity against *P. berghei* was obtained against a drug-sensitive strain of *P. berghei* (strain KBG 173).[22] Each test compound is administered sc to five male mice per dilution in a single subcutaneous dose 3 days after infection. Results are expressed in T-C values which indicate the mean survival time of the treated mice beyond that of the control animals; untreated mice survive on average 6.2 days. Compounds are classified as active (A) when the mean survival time of the treated mice is twice that of the controls (>6.2 days), and curative (C) when one or more test animals live 60 days postinfection. Deaths from 0–2 days post-treatment are attributed to toxicity (T).

Acknowledgment. This work was funded in part by DHHS/NIH/NIAID Grant No. 1 R15 AI28012-01. 2-Methylpentamethylenediamine and 1,12-dodecanediamine were graciously provided by Grace A. Pulci and Frank E. Herkes of the Du Pont Company, Petrochemicals Dept.

Registry No. 1, 140926-75-6; 2, 140926-76-7; 3, 140926-77-8; 4, 71595-17-0; 5, 140926-78-9; 6, 140926-79-0; 7, 140926-80-3; 8, 140926-81-4; 9, 140926-82-5; 10, 140926-83-6; 11, 140926-84-7; 12, 140926-85-8; 13, 140926-86-9; $H_2N(CH_2)_2NH_2$, 107-15-3; $H_2NCH_2CH(CH_3)NH_2$, 78-90-0; $H_2N(CH_2)_3NH_2$, 109-76-2; $H_2N(CH_2)_4NH_2$, 110-60-1; $H_2N(CH_2)_5NH_2$, 462-94-2; $H_2N(CH_2)_3CH(CH_3)CH_2NH_2$, 15520-10-2; $H_2N(CH_2)_6NH_2$, 124-09-4; $H_2N(CH_2)_7NH_2$, 646-19-5; $H_2N(CH_2)_8NH_2$, 373-44-4; $H_2N(CH_2)_9NH_2$, 646-24-2; $H_2N(CH_2)_{10}NH_2$, 646-25-3; $H_2N(CH_2)_{12}NH_2$, 2783-17-7; *trans*-1,2-cyclohexandiamine, 41013-43-8; 4,7-dichloroquinoline, 86-98-6.

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Clinical Analysis by ^1H Spin-Echo NMR. 2.† Oxidation of Intracellular Glutathione as a Consequence of Penicillamine Therapy in Rheumatoid Arthritis

J. Reglinski,*,‡ W. E. Smith,‡ M. Brzeski,§ M. Marabani,§ and R. D. Sturrock§

Department of Pure and Applied Chemistry, Strathclyde University, Glasgow G1 1X1, U.K., and The Centre for Rheumatic Diseases, University Department of Medicine, Glasgow University, Glasgow G4 0EA, U.K. *Received October 16, 1991*

Spin echo NMR analysis is used to monitor the effect of penicillamine on intact erythrocytes obtained from patients suffering from rheumatoid arthritis during a 12-week period of therapy. The results are compared to the previously reported in vitro effects of the compound (McKay, C. N. N.; et al. *Biochim. Biophys. Acta* **1986**, *888*, 30–35). At clinical assessment at week 12, the 20 patients were divided into responder and nonresponder groups. The intracellular glutathione in the responder group is more oxidized ($P < 0.01$) than in the nonresponder group. A retrospective analysis of the two patient groups at the initial assessment following the commencement of therapy indicated that in the nonresponder group intracellular glutathione was significantly more reduced ($P < 0.02$) than in the responder group. It is postulated that penicillamine stimulates cellular defense against the oxidation of the cell membrane at the expense of cytosolic glutathione. This initial study suggests that spin-echo NMR analysis of erythrocyte glutathione can act as an early indicator of a clinical response to penicillamine therapy.

Introduction

Penicillamine is one of a limited number of disease-modifying antirheumatic drugs used in the treatment of rheumatoid arthritis. The chemical processes underlying its action are still unknown. However, it may act by reacting with sulfhydryl sites on plasma proteins,[4] in the cytosol,[5] or at the cell membrane.[3,6] In a previous in vitro study of the action of penicillamine on intact, viable erythrocytes using ^1H spin-echo nuclear magnetic resonance spectroscopy (NMR),[5] we reported that intracellular glutathione became more oxidized. The effect was multiplicative with more cytosolic thiol being affected than penicillamine thiol applied. This clinical study was initiated in an attempt to discover whether the in vitro re-

† Presented as a communication at the British Rheumatology Society in London, 1988.[1] For part 1 of this series, see ref 2.
‡ Strathclyde University.
§ Glasgow University.

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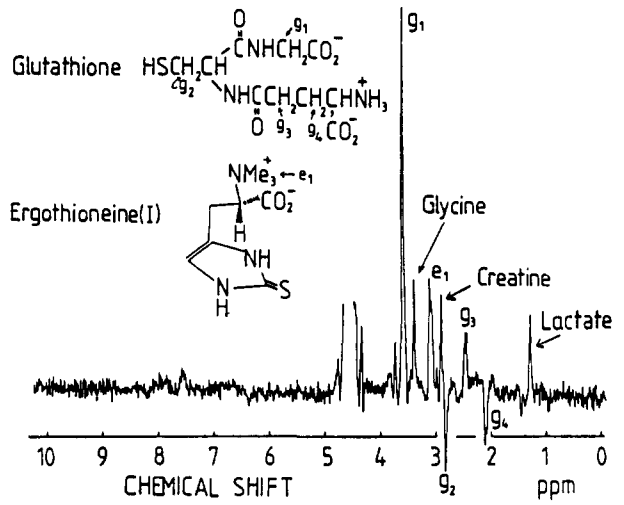

Figure 1. The 250-MHz spin-echo NMR spectrum of the human erythrocyte. Assignments of resonances are as previously reported.[2]

sponse applies in vivo and whether this mechanism is a part of the sequence of events leading to improvement in disease activity.

Clinical analysis using cells usually requires that the cell membrane is disrupted prior to analysis. Substantial oxidation of the analyte can occur during this step, making the results more difficult to interpret. By using modern NMR methods, it is now possible to assay intact cells such as erythrocytes[2,5-8] directly in a nondestructive manner. Sample preparation is reduced to simple washing of the cells with saline thus minimizing interference in the cellular chemistry. NMR analysis of the cellular components is achieved using a spin-echo pulse sequence (e.g. Hahn or Carr–Purcell–Meiboom–Gill (CPMG)) which takes advantage of the differences in relaxation times between large and small molecules in solution, tuning out the signal arising from the large molecules, producing a spectrum comprised solely of the small molecules in the cytosol.

Results and Discussion

In an earlier manuscript[2] we were able to comprehensively demonstrate the capabilities of ^1H spin-echo NMR spectroscopy as an approach to the clinical analysis of intact erythrocytes. A typical spin-echo NMR spectrum of the human erythrocyte is shown in Figure 1. Of great importance to our studies on changes in the redox balance of the cytosol is the g_2 signal (2.7 ppm) which arises from the β-cysteinyl residue of glutathione and the g_4 (2.0 ppm) signal which arises from the glutamyl residue of glutathione. If glutathione is in the reduced state (GSH), g_2 is negative and larger than g_4, but in the oxidized state as glutathione disulfide (GSSG), it is smaller. Using *tert*-butyl hydroperoxide to oxidize the cytosolic glutathione[7,8] in intact red cells, it was possible to construct a calibration chart (Figure 2)[2] which could then be used to obtain details of the intracellular redox balance, principally the GSH/GSSG ratio as a percentage contribution to the mixture. An important control for these measurements is the repeated analysis of untreated erythrocyte samples.[2] These are known to remain constant over many hours. Although the error involved in these measurements can be high (5%), it is preferable to wet-assay methods which rely on prior cell disruption and protein sedimentation, procedures which may alter the redox status of the complex mixture and release pools of bound glutathione.

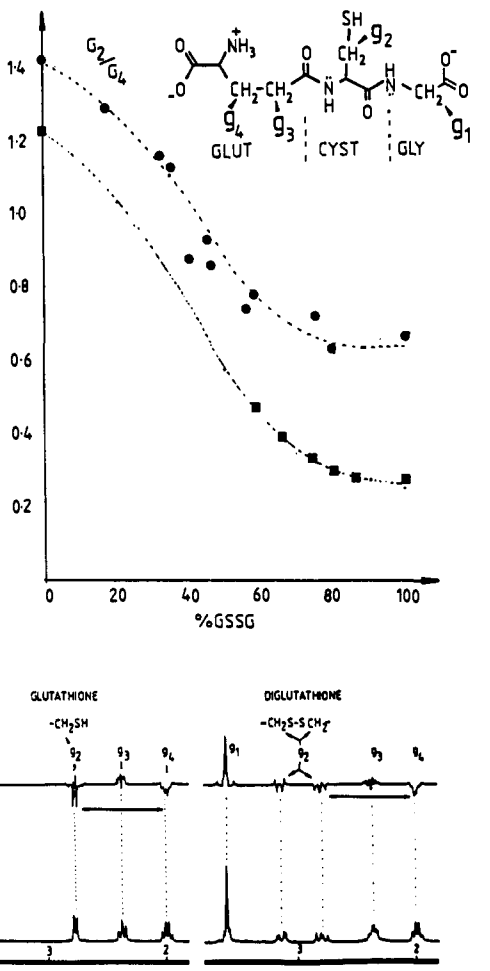

Figure 2. The change in the g_2/g_4 ratio as a consequence of the percent GSSG in the GSH/GSSG mixture. For full details see ref 2.

Erythrocytes from normal healthy volunteers and from patients with rheumatoid arthritis prior to the commencement of penicillamine therapy were analyzed by spin-echo NMR. Using the calibration chart (Figure 2), the GSH/GSSG ratios were calculated (Figure 3). As would be expected from our earlier study,[2] there is an equilibrium between the reduced and oxidized forms of glutathione. Furthermore, there is no significant difference between the normal volunteer and untreated diseased groups.

At 12 weeks the patient groups were separated into nonresponders and responders using standard clinical assessments (Figure 3). NMR analysis of the group identified as responders shows a significant change in the redox balance of the cytosol to the oxidized form ($P < 0.01$) against the week 0 group, against the week 12 nonresponder group ($P < 0.01$), and furthermore against the normal control group ($P < 0.01$, Figure 4). In contrast,

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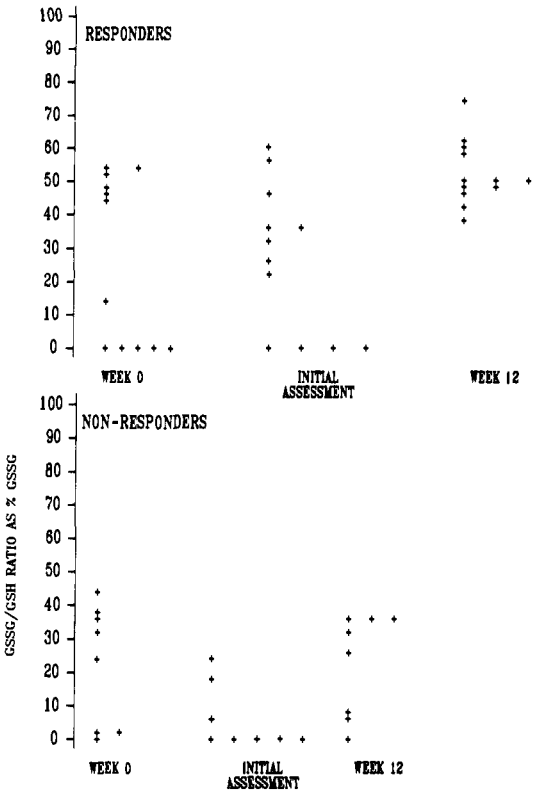

Figure 3. The measurement of the redox balance in patients suffering from rheumatoid arthritis, before (week 0) and after (at the initial assessment at weeks 1 or 2 and at week 12) penicillamine therapy. At week 12 a clinical assessment of the patients was made which is used to separate the groups retrospectively.

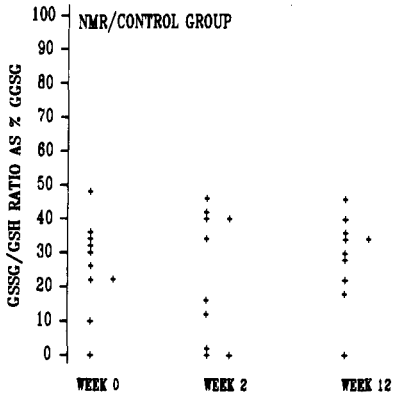

Figure 4. The measurement of the redox balance in normal healthy volunteers over a 12-week period (weeks 0, 1, 12) to show possible fluctuations over the study period.

the group identified as nonresponders shows no statistical difference between week 0 and week 12.

Perhaps the most significant point obtained from this study arises from the retrospective analysis of the two groups at assessment (1 or 2 weeks) after the commencement of therapy. There exists, even at this early stage in the therapeutic regime, a significant difference ($P < 0.02$) between the responding and nonresponding group. The cytosolic glutathione pool of the nonresponder group is observed to become more reduced after 1–2 weeks ($P < 0.01$) and subsequently to reoxidize to pretherapy levels. The cellular glutathione of the responder group is significantly more oxidized than the nonresponder group ($P < 0.02$) at the initial assessment although there is little change within the responder group itself between 0 and 2 weeks. It is possible to discriminate between the nonresponding and responding group using this technique. Should a patient show a substantial oxidation of the cellular glutathione (>30%) at the initial assessment, it could be taken as a sign that the therapy is providing some benefit. The opposite effect however, where the glutathione pool moves to a more reduced state, may not be an immediate indication of the lack of benefit with penicillamine.

A group of normal volunteers were also monitored over a similar 12-week time span to characterize normal fluctuations in erythrocyte glutathione redox status with time. Figure 4 indicates that the glutathione status of the cell would seem to remain relatively unchanged. Thus, the dip at 1–2 weeks in the nonresponder group and the pronounced oxidation of the responder group at 12 weeks is a function of penicillamine therapy.

In our previous clinical study we also reported that erythrocytes from patients with rheumatoid arthritis were low in ergothioneine. Data pertaining to this moiety were analyzed here as a function of penicillamine therapy, but no changes were identified.

In a previous in vitro study it was found that the effect of penicillamine on erythrocyte glutathione was that it becomes more oxidized.[5] This result has been repeated using Captopril,[6] another therapeutic agent containing a thiol moiety. In the latter case it was postulated that the thiol acts on the membrane sulfhydryl population which in turn shifts the redox status of the cytosol (eq 1). The

$$\text{RSH} + \text{membrane–SS–membrane} \rightleftharpoons \text{RSS–membrane} + \text{membrane–SH}$$

$$\text{RSS–membrane} + 2\text{GSH} \rightleftharpoons \text{RSH} + \text{membrane–SH} + \text{GSSH} \quad (1)$$

membrane disulfide is postulated to arise from disease-modulated membrane oxidation. This is a site of specific chemical damage, and the cell tends to regenerate the thiol groups to achieve its normal configuration.

Penicillamine is known to prefer mixed disulfides and will exchange with the oxidized protein-sulfhydryl sites on the outer membrane surface (eq 1). This stimulus is sufficient for intracellular glutathione to be activated, being oxidized at the inner membrane surface. This response releases electrons in an attempt to remove the foreign species at the membrane surface by reducing the penicillamine–membrane mixed disulfide. This process is multiplicative with many more electrons being produced than the original stimulus introduces. Subsequently, the outer surface of the cell may be protected by this additional reducing power. A mechanism such as this makes it possible for small amounts of therapeutically active materials to influence the redox gates in the cell membrane, stimulating the cell into quenching extracellular oxidative stress (eq 2) using the cytosolic thiol system. If

$$2(\text{membrane–SH}) \rightleftharpoons \text{membrane–SS–membrane} \quad (2)$$

the disease is active, the membrane will be reoxidized with time.

The time scale of in vitro studies[5] is short (hours) compared to the in vivo studies. However, in vitro the concentration of the active compounds and the small amount of biomaterial will contribute significantly to the increased kinetics of the changes. In vivo, it might be expected that the changes will be slower to manifest themselves and will require additional doses of penicillamine to reinforce the chemical change. It is significant that those patients who have responded to penicillamine therapy and a significant subgroup of the patients after 2 weeks of therapy show changes in their erythrocyte glutathione which are consistent with our previous in vitro study. This suggests that the chemical model identified by the in vitro studies is

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 (1000g) 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.

Acknowledgment. We thank the Scottish Home and Health Department and the Nuffield Foundation for financial support to J.R.

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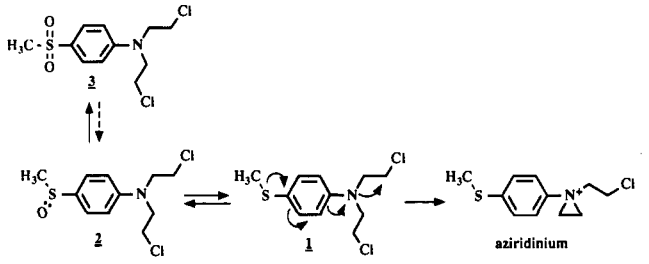
Communications to the Editor

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 10000g supernatant fractions of rat liver in the presence

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