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Measurement of Tissue Oxidation-Reduction State with Carbon-13 Nuclear Magnetic Resonance Spectroscopy[1]

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ABSTRACT

The oxidation state of tissues influences their response to cancer therapy. We have devised a novel approach to the measurement of thiol redox which is based on the relative nuclear magnetic resonance signal intensity from carbon-13 adjacent to sulfur in metabolites of the redox-sensitive phosphorothioate drug, *S*-2-(3-methylaminopropylamino)ethylphosphorothioic acid (WR3689). Incubation of WR3689 metabolites under oxidizing conditions results in quantifiable changes in the ^{13}C nuclear magnetic resonance spectrum stoichiometrically related to the degree of oxidation in mouse liver homogenate *in vitro*. Drug oxidation is competitive with the oxidation of tissue-derived thiol groups under these conditions. Noninvasive measurement of redox state may assist in designing more effective strategies for altering normal and malignant tissue response to cancer therapy.

INTRODUCTION

The reduction-oxidation status of cells affects their response to therapy for cancer. In particular, a reducing environment will often decrease and an oxidizing environment will amplify the effects of many cytotoxic agents, such as ionizing radiation and chemotherapeutic drugs. For example, the presence of oxygen increases the toxicity of photon radiation by nearly a factor of three (1). The ability to assess the oxidation-reduction balance (redox[4] state) in cells should aid in understanding differing responses of normal and cancer cells to various treatments. Also, knowledge of the cellular response to treatment under varying redox conditions may enable the use of drugs to favorably alter the relative responses of healthy *versus* tumor tissue.

This research is based upon extensive work done on aminoalkylphosphorothioates which have demonstrated radioprotective activity (2-4). These phosphorothioates undergo a sequence of reactions *in vivo* wherein the form of the drug is altered (5-7). The drug is administered as the phosphorothioate and the phosphorous-sulfur bond is cleaved by phosphatase enzymes. The released thiol presumably equilibrates with all other thiols and disulfides in its environment (Fig. 1).

Previous attempts to quantify the cellular thiol status by measuring the major cellular thiols and disulfides have required invasive sampling of tissue and extensive chemical work-up, potentially perturbing the measured equilibrium (8, 9). Chromatographic techniques using pre- and postcolumn derivatization as well as electrochemical detection have been employed. Stabilizing the thiols in acidic media or derivatization and inactivation of electron transport enzymes is helpful but not adequate.

Our technique is based on the NMR chemical shift of the carbon adjacent to sulfur in the phosphorothioate: WR3689. As shown below, the phosphorothioate, thiol, and disulfide forms of this drug are spectroscopically distinct by NMR; equilibration of the drug with tissue thiols and disulfides and regional quantification of these drug forms should provide a measure of the thiol-to-disulfide ratio in tissues and therefore an estimate of the tissue thiol redox state. We report here preliminary data showing the feasibility of the method and *in vitro* validation measurements and discuss extensions to *in vivo* spectroscopy of thiol redox state.

MATERIALS AND METHODS

Drug Synthesis. [^{13}C]WR3689 was synthesized by the following route: (*a*) nucleophilic displacement of bromine in ^{13}C-labeled ethyl bromoacetate by the sodium salt of bis-toluenesulfonamide, (*b*) reduction of the carbonyl to an alcohol, (*c*) concurrent deprotection and formation of bromide *via* treatment with HBr, and (*d*) displacement of bromine with sodium phosphorothioate. The first of these steps is described briefly here; the remainder of this synthesis was a modification of a previously described synthesis of [^{3}H]WR3689 (10). *N*-Methyl-*N,N'*-trimethylenebis-*p*-toluenesulphonamide (11) (12.0 mmol) was dissolved under nitrogen in anhydrous dimethylformamide. Sodium hydride (12.4 mmol) was added and the solution stirred. Ethyl 2-bromo-1-^{13}C-acetate (12.0 mmol; MSD Isotopes, Montreal, Quebec) was added dropwise and stirred under N_2 for 2 h. The solution was poured into ice water and stirred while the crude product solidified. The gummy solid was dissolved in $CHCl_3$, washed with water, dried ($MgSO_4$), and concentrated to give the product as a gum (5.79 g). This material was used in subsequent steps, ultimately producing *S*-2-(3-methylaminopropylamino)-[^{13}C]ethylphosphorothioate (0.8 g, 60%), mp 147°C, lit. 148°C. WR3689-thiol was prepared from the phosphorothioate by acid hydrolysis (12).

Sample Preparation. Samples of synthetic material (unlabeled and labeled WR3689 and metabolites) were dissolved in deoxygenated D_2O at 20 mM in an appropriate NMR sample tube. Mixtures of phosphorothioate, thiol, and disulfide at approximately equal concentrations in deoxygenated D_2O were used for the determination of NMR relaxation times.

Mouse liver homogenate was prepared from C3H mice (Stanford University, Stanford, CA). Mice were killed by cervical dislocation and the livers were quickly removed, weighed, and homogenized in an equal volume (50%) or three times volume (33%) of 0.1 M potassium phosphate buffer, pH 7.4. Liver cytosol was prepared from this homogenate by centrifugation in a Beckman U5 ultracentrifuge at 105,000 × *g* for 60 min.

Biological samples for NMR spectroscopy were prepared by combining 0.1 M potassium phosphate buffer (pH 7.4), liver cytosol or homog-

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[4] The abbreviations and trivial names used are: redox, oxidation-reduction; NMR, nuclear magnetic resonance; WR3689, S-2-(3-methylaminopropylamino)ethylphosphorothioic acid; GSH, glutathione; diamide, diazene dicarboxylate-*N,N'*-dimethylamide; T_1, longitudinal relaxation time; T_2, spin-spin relaxation time.

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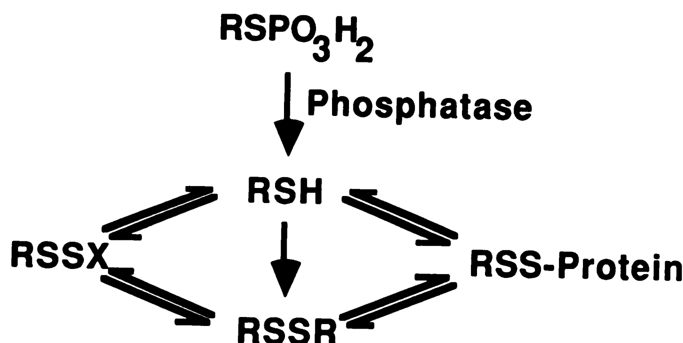

Fig. 1. Equilibration of WR3689 and its metabolites with tissue thiols and disulfides. $R = CH_3NHCH_2CH_2CH_2NHCH_2CH_2$; X = glutathione or other biological thiol.

enate, 0.1 ml D_2O (to provide a frequency lock), WR3689 or WR3689-thiol, and diamide (Sigma, St. Louis, MO) in an NMR tube. When present, WR3689 or WR3689-thiol was dissolved in phosphate buffer at 20 mM just prior to the experiment and an appropriate volume was added to each sample tube to achieve the final concentration stated in the figure legend. When present, one equivalent of diamide was added for every two equivalents of thiol, to take into account the known 1:2 stoichiometry for this reaction (13). When WR3689 was incubated, alkaline phosphatase (1.0 IU/ml; Sigma) was added to the incubation to hydrolyze the phosphate moiety. When WR3689-thiol was used in an experiment, care was taken to prevent artifactual drug oxidation by performing all sample manipulations under argon in a glove box. Samples were incubated at room temperature as specified in the figure legends and were chilled on ice during transport and storage, and the analysis was performed in the spectrometer at 22°C.

NMR Measurements. NMR spectra of synthetic products were acquired with a Varian VXR-300 spectrometer with a dual tuned (^1H, ^{13}C) 5-mm probe. For standard ^{13}C acquisition, a pulse time of 8.7 μs (65-degree pulse), 5.4-s recycle delay, and a sweep width of 218.77 ppm (16501.7 Hz) were used. Heteronuclear-broadband decoupling was accomplished *via* a Waltz-16 sequence centered at 4 ppm in the proton spectrum. For standard ^1H acquisition, a pulse time of 20 μs, recycle delay of 3 s, and sweep width of 13.3 ppm (4000 Hz) were used.

For T_1 determinations, the standard 180°-τ-90° pulse sequence was chosen with phase rotation of the composite 180° pulse. The τ values for a typical experiment were 0.125, 0.25, 0.5, 1, 2, and 4 s with a recycle time of 20 s (5 × T_1). Typically each acquisition was signal averaged 64 to 128 times. Broad band heteronuclear decoupling was used throughout the experiment. Fast fourier transform and exponential fit of data were done with standard Varian programs.

Certain measurements were taken with a Bruker CXP-200 spectrometer operating at 50.31 MHz for carbon-13. Acquisition settings included a sweep width of 5000 Hz, a 4.6-s recycle delay, and 8192 points per FID from a 10-mm probe. Proton decoupling was accomplished by broad band decoupling during acquisition. For T_1 measurements, 64 points were acquired per delay.

In certain biological experiments, a General Electric CSI-II, 2-Tesla, 30-cm bore imager/spectrometer was used. The samples were contained in 20-mm scintillation vials and the vials placed in a solenoid coil tuned for ^{13}C (21.5 MHz). The ^{13}C coil and sample were centered inside the GE 6-inch imaging coil which was used for heteronuclear broad band ^1H decoupling with the Waltz-16 sequence. ^{13}C acquisition parameters included a sweep width of 5000 Hz, free induction decay size of 2048 points, and a recycle delay as noted in Fig. 4, *legend.*

RESULTS AND DISCUSSION

We measured the ^{13}C spectra (Fig. 2) of the phosphorothioate, thiol, and disulfide forms of WR3689 and found that the chemical shifts of the carbon closest to sulfur (α) and its neighboring carbon (β) are sensitive to the sulfur oxidation state. Chemical shifts of the α- and β-carbons and the α-carbon T_1 values are shown in Table 1. Since our experiments utilized NMR spectrometers operating at different frequencies, measurement of the T_1 values of each compound was necessary to either confirm that each resonance is fully relaxed between pulses or to allow correction for partial relaxation effects. Accurate estimates of T_1 of the different chemical forms of the drug are necessary to quantify partially saturated spectra. The T_1 values of these compounds are affected by the chemical environment in which the spectra are acquired; T_1 values in biological material such as liver homogenate are somewhat shorter due to the relaxation effects caused by paramagnetic components of macromolecules (possibly the ferrous iron in metalloproteins) (14). The T_1 values measured in buffer have been taken as maximal for the compounds in question.

Carbon-13 spectra acquired from living tissue show a broad peak centered around 30 ppm due to endogenous lipid methylenes (15). In order to detect levels of drug achievable *in vivo*, ^{13}C-enriched WR3689 was synthesized. This material had 90% enrichment of ^{13}C in the α-carbon in 50% of the molecules and 90% enrichment in the β-carbon in the other 50% of the molecules. We initially thought that this scrambling of ^{13}C between the α- and β-carbons was the result of aziridine formation during the pentultimate (bromination) step. Upon examination,[5] the cause of scrambling was found to occur under conditions required for the displacement of bromide by sodium phosphorothioate.

Incubation of the drug under oxidizing conditions resulted in quantifiable changes in the NMR spectrum. Fig. 3*A* shows the results of an experiment in which drug was mixed with phosphatase, buffer, and the thiol oxidant drug diamide. In this chemically defined system, a 1:1 relationship between disappearance of thiol and appearance of disulfide was observed. When glutathione (GSH) was included in the incubation, additional equivalents of diamide were required to affect complete oxidation of the drug, indicating competition between WR3689-SH and GSH for the oxidant. Incubation of drug thiol (WR3689-SH) with diamide in buffer, mouse liver cytosol, or mouse liver homogenate produced the results shown in Fig. 3*B*. In this experiment, additional oxidizing equivalents were needed in the presence of cellular constitutients to fully oxidize the WR3689-SH and eliminate its resonance (~20 ppm) with concomittant conversion to disulfide, producing maximal resonance at ~33 ppm. We interpret these results as showing that WR3689-SH competes for oxidizing equivalents with cellular thiols and disulfides. If one thiol or the other were preferentially oxidized, the lines of Fig. 3 would have a step-like character. If WR3689-thiol was preferentially oxidized, the curve (■) of Fig. 3*A* would overlay the curve (●); if GSH were preferentially oxidized, the curve (■) would stay at 100% until one equivalent of diamide had been consumed and then would fall to zero with a slope equal to that of the curve (●). The fact that the slopes of these curves are altered indicates that the drug redox equilibrium is a measure of the total thiol redox equilibrium of its environment.

The unique value of this method is in its potential for noninvasive redox measurements *in vivo*. For this value to be realized, a large-bore NMR spectrometer must be used. Therefore, we tested the method with an *in vitro* model system of WR3689 incubated with alkaline phosphatase and oxygen in mouse liver homogenate observed with the 2T CSI spectrometer. Fig. 4 shows serial spectra acquired over a span of several

[5] This point was investigated by synthesizing WR3689 labeled with deuterium in the alpha position (by reducing the precursor ester with lithium aluminum deuteride) and following the retention of deuterium in the alpha position throughout the synthesis.

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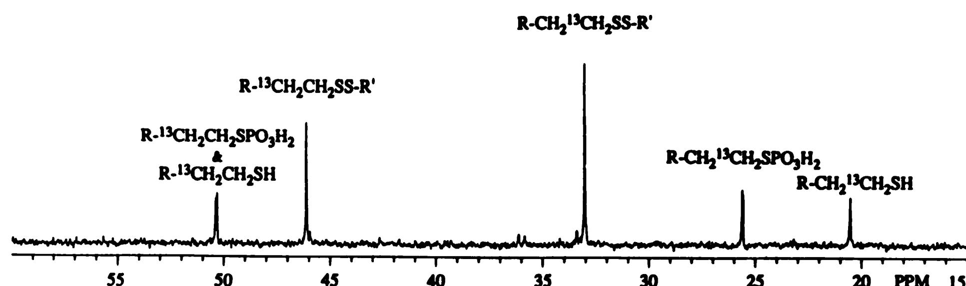

Fig. 2. [^{13}C]NMR spectrum of a mixture of WR3689, WR3689-SH, and WR-3689-SS acquired on the Varian VXR-300 spectrometer. The assignments of the observed resonances are shown and correspond to the chemical shifts reported in Table 1.

Table 1 *Chemical shifts and relaxation times*

Chemical Form	Chemical shift (ppm)		α Carbon T_1 (s)[a]		
			In buffer		In liver
	α-carbon	β-carbon	200 MHz	300 MHz	(300 MHz)
Phosphorothioate	25.6	50.4	0.33 (0.03)[b]	0.27 (0.02), 0.34 (0.06)[c]	0.5[c]
Thiol	20.5	50.3	1.11 (0.07), 1.5 (0.1)[b]	1.7 (0.3)[c]	1.1[c]
Disulfide	33.1	46.1	0.34 (0.02)[b]	0.43 (0.04)[c]	NM[d]

[a] Numbers in parentheses reflect standard deviation to exponential fit, duplicate values reflect reproducibility of values.
[b] Measured on the Bruker CXP-200 spectrometer.
[c] Measured on the Varian VXR-300 spectrometer.
[d] NM, not measured.

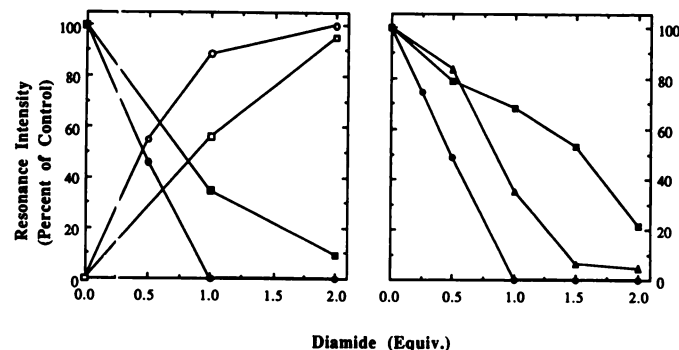

Fig. 3. *A*, changes in [^{13}C]NMR signal intensity as a result of changes in the oxidation state of sulfur in [^{13}C]WR3689. Each 5-mm NMR tube contained equal amounts of [^{13}C]WR3689 (0.05 mM in 0.02 ml of 0.1 M phosphate buffer at pH = 7.4), D_2O (0.1 ml) for lock signal, and alkaline phosphatase (1.0 IU/ml). The samples were incubated at 25°C for 30 min, and the *in situ* generated thiol was treated as follows: Control sample received buffer only. Samples received 0.5, 1.0, or 2.0 equivalents of diamide (○, ●). Samples received 0.05 mmol of GSH and 1.0 or 2.0 equivalents of diamide (□, ■). The volume of each sample was adjusted with buffer to the final volume of 0.7 ml. The NMR spectra were acquired and processed using identical parameters. *Solid symbols*, values for the thiol at 20.5 ppm; *open symbols*, resultant disulfide at 33.1 ppm. *B*, endogenous thiols in mouse liver cytosol and mouse liver homogenate compete with [^{13}C]-WR3689 derived thiol for oxidizing equivalents of diamide. WR3689-SH (0.0034-mmol in 0.020-ml buffer) was placed into a 5-mm NMR tube, D_2O (0.1 ml) was added for the lock, and buffer (●), liver cytosol (▲), or liver homogenate 25% in buffer (■) was added (0.525 ml). In each category, one sample received buffer only and served as control; the other samples received one (0.0017 mmol) or two (0.0034 mmol) equivalents of diamide. The acquisition and processing parameters were identical for each tube.

hours. The metabolism of the phosphorothioate is accompanied by appearance of thiol without apparent disulfide formation, indicating a highly reduced state for the tissue homogenate (16, 17). When oxidation of the sample was promoted by bubbling the sample with oxygen, disulfide resonance was observed. In the 72-h spectrum, the disulfide resonance is the major peak observed, while some thiol is still present, and no parent drug remains. These results suggest that the detection of drug in biological media is possible with a large-bore system and that further effort to define parameters necessary for *in vivo* spectroscopy should be pursued.

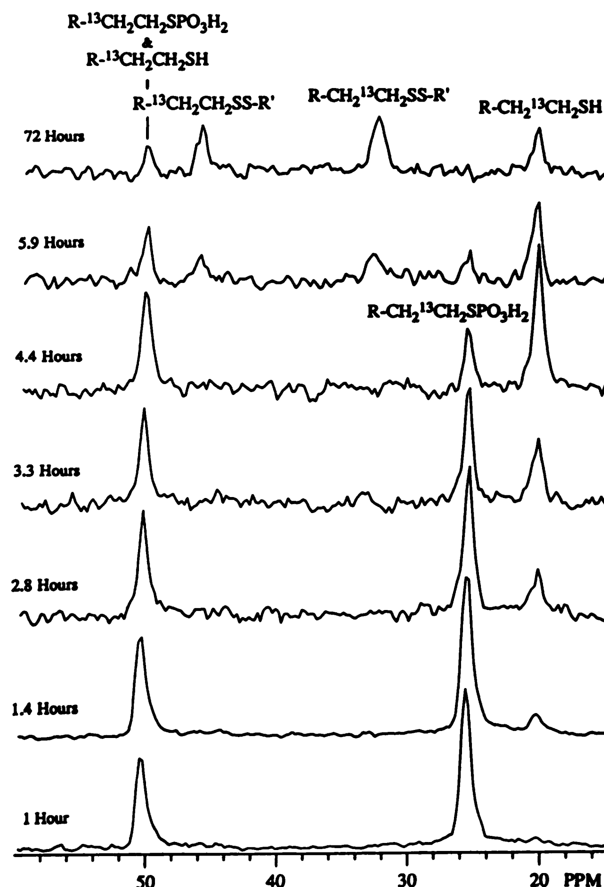

Fig. 4. Spectral changes accompanying liver oxidation. 50 mg of [^{13}C]WR-3689 per 10 ml of liver homogenate were used as a sample for which the initial recycle time and number of acquisitions were 1 s and 300 scans, respectively. Magnetic field strength in this experiment was 2T. Alkaline phosphatase was added to the sample at 165 and 195 min after scanning was initiated with 100 scans per spectrum at 5 s recycle time. O_2 was bubbled through the emulsion after 265 min of acquisition. Additional spectra were accumulated at 72 h. Reference was made to the parent/thiol peak at 50 ppm.

Several technological difficulties must be overcome in order to move to *in vivo* spectroscopic measurement of redox. Drug concentrations used in these *in vitro* experiments are somewhat higher than what can tolerably be achieved after an *in vivo* injection. However, we have used these concentrations to decrease the time required to acquire signal from multiple sam-

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ples. If a relatively stable redox equilibrium exists, spectra could be measured over many minutes. Concentrations greater than 1.0 mM can be achieved in liver in mice (10, 18) and at this concentration, 15–20 min are required for adequate signal acquisition to achieve an acceptable signal to noise ratio. An additional difficulty is the strong endogenous methylene peak at 30 ppm which may mask the signal from the α-carbon of the disulfide. Possible solutions include selective decoupling of only the drug protons, or using the β-carbon peak at 46 ppm as a measure of the disulfide concentration. It may also be possible to design a sulfur-containing drug whose thiol and disulfide peaks are distinct from endogenous C-13. Finally, certain NMR physics parameters are potential difficulties in the transition to *in vivo* spectroscopy of redox status. The signal-to-noise ratio of our tracer may decrease as we attempt to localize our spectra to specific tissues of interest, due to both decreased RF coil filling factor and line broadening from magnetic field inhomogeneity *in vivo*. Adequate signal-to-noise at 2 Tesla may be maintained by using RF coils in combination with either Faraday shielding or a magnetic gradient slice-selection pulse sequence to eliminate noise from extraneous tissues. Line broadening is dependent on how well localized the spectra is; for a small region in the mouse, the field homogeneity may be very good.

These studies show that our tracer molecule equilibrates with various thiols and disulfides, including protein thiols. Combination of substantial portions of the drug with large, slowly tumbling macromolecules could result in NMR-invisible drug, as the short T_2 relaxation times would preclude NMR observation of these bound molecules under the conditions employed. Preliminary experiments confirmed that drug binds to rat plasma proteins to an extent dependent on the drug form and experimental conditions.[6] After 1 h of incubation at 37°C, phosphorothioate, thiol, and disulfide forms of WR3689 attach to rat plasma proteins to 15%, 45%, and 40%, respectively. Since drug binding was nearly identical for the thiol and disulfide drug forms, the ratio of resonance intensities of these forms would not be artifactually skewed by selective macromolecular binding of any single drug form.

These measurements are designed to assess the thiol redox ratio of biological materials. The pyridine nucleotide cofactor systems are also components of cellular redox and are intimately linked with the thiol redox status through the GSH/glutathione disulfide redox couple, with NADPH as a source of reducing equivalents. Experiments are in progress to assess the ability of a thiol/disulfide probe such as WR3689 to reflect the redox status of the pyridine nucleotide system.

The cellular environment is a compartmentalized volume containing mitochondrial, endoplasmic reticulum, and cytosolic spaces. It is not known to which of these spaces WR3689 and its metabolites will penetrate. Autoradiographic evidence (10) is inadequate to determine whether drug metabolites penetrate to the mitochondrial space, but suggests a nearly homogeneous distribution in most tissues. Available evidence suggests that labeled material is not preferentially concentrated in the nucleus, which contains the major target(s) for radiation and chemotherapy (19).

The utility of these observations lies in the importance of thiol redox in cellular defense against toxicants. Derangement of thiol redox accompanies or is a cause of many types of cellular toxicities (20). Furthermore, thiol redox is a determinant of cellular response to radiation and chemotherapy; manipulation of this parameter in normal tissues may offer an adjuvant advantage in cancer therapy where normal tissue tolerance is frequently dose-limiting. Techniques to noninvasively measure thiol redox status should help to elucidate the role of this parameter in cellular defense mechanisms.

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