Metabolism of SR 4233 by Chinese Hamster Ovary Cells: Basis of Selective Hypoxic Cytotoxicity

Margaret A. Baker, Elaine M. Zeman, V. Kate Hirst, and J. Martin Brown
Department of Therapeutic Radiology, Division of Therapeutic Radiology, Stanford University, Stanford, California 94305

ABSTRACT
The metabolism of SR 4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide), recently reported as highly toxic to hypoxic cells in vitro, was studied by using suspensions of Chinese hamster ovary cells. The rates of formation of two known reduction products, the 1-oxide and the unoxgenated 3-amino-3-benzotriazine, were measured in aerobic and hypoxic cell suspensions for drug treatments producing both hypoxic and aerobic cytotoxicity. Formation of the 1-oxide and a small amount of the 3-amino-3-benzotriazine occurred preferentially in hypoxic suspensions. These metabolites were relatively nontoxic to either aerobic or hypoxic cells, implying another mechanism of toxicity. The activation of SR 4233 by single electron transfer, hypothetically forming a toxic drug radical, was explored. Aerobic stimulation of oxygen consumption in respiration-inhibited cells and malondialdehyde release from aerobic cells in the presence of SR 4233 indicated the formation of active oxygen species during drug activation. Increased malondialdehyde release in hypoxic cells and its attenuation by the hydrogen donor, dimethylthiourea, implied the presence of an oxidizing radical. Unlike the nitroimidazoles, misonidazole, hypoxic metabolism of SR 4233 did not deplete intracellular glutathione or result in increased binding of drug metabolites to cellular macromolecules. These results are consistent with macromolecular damage caused by an oxygen sensitive, nonbinding, drug-free radical intermediate with oxidizing properties as the mechanism of selective hypoxic toxicity of SR 4233.

INTRODUCTION
SR 4233 is the lead compound in a series of benzotriazine-N-oxides we are evaluating as potential chemotherapeutic and radiosensitizing agents. This class of compounds has not yet been tested for cancer chemotherapeutic use, although some have been used as antimalarials (1, 2). The basis for the proposed activity of SR 4233 is its high selective toxicity for hypoxic cells (3) and its ability, when combined with the vasodilating agent hydralazine, to produce extensive killing of tumor cells in vivo (4). The differential toxicity of SR 4233, 50- to 200-fold more for hypoxic than for aerobic cells, depending on the cell line, is higher than for most other bioreductively activated drugs, such as the nitroimidazoles and quinone antitumor antibiotics (5–7). The one bioreductively activated compound for which a differential cytotoxicity similar to that for SR 4233 has been reported (8) is a bifunctional compound, RSU 1069, which has both a nitro group and an aziridine ring. The selective hypoxic toxicity of these compounds makes them potentially useful as adjuncts to radiation therapy for the treatment of solid tumors with a significant hypoxic fraction, or in combination with vasodilating agents which selectively increase tumor hypoxia (4).

Reduction products of SR 4233 and the sequence of their formation can be predicted from the chemical reduction steps for the parent compound (Fig. 1). First is the loss of the 4-position oxide requiring two electron transfer to form either a hydroxide ion or, depending on the extent of protonation, a water molecule as the leaving group, thus producing the mono-N-oxide, SR 4317. The second site for reduction is the 1-position oxide, also requiring two electrons to produce a hydroxyl radical or water and the triazine, SR 4330 (Fig. 1). Sequential production of SR 4317 and SR 4330 by radiolytic generation of reducing equivalents in an anaerobic system containing formate, or in an anaerobic solution of xanthine oxidase plus xanthine has been reported (9). In the presence of air, reduction of SR 4233 was not measurable.

The differential toxicity of SR 4233 is greatest for CHO3 cells and for murine tumor cell lines when tested in vitro (3). We therefore used a CHO cell line to examine the differential metabolism of SR 4233 in aerobic and hypoxic cells. We also measured the toxicity of the metabolites themselves to hypoxic and aerobic cells. Possible mechanisms of hypoxic and aerobic toxicity such as oxidative damage, glutathione depletion, and adduct formation were also tested. In addition, the formation of active oxygen species in aerobic cells was examined.

MATERIALS AND METHODS
Cells and Drugs. SR 4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide) and its reduction products, SR 4317 (3-amino-1,2,4-benzotriazine-1-N-oxide) and SR 4330 (3-amino-1,2,4-benzotriazine), were synthesized at SRI International, Menlo Park, CA, by Dr. William W. Lee under contract from the National Cancer Institute. Chinese hamster ovary (CHO-To) cells (from Dr. A. M. Rauth, Ontario Cancer Institute, Toronto, Ontario, Canada) were grown in suspension in α-MEM medium (Grand Island Biologicals Laboratories, Santa Clara, CA) containing 10% fetal bovine serum (Flow Laboratories, Inc., Inglewood, CA), 126 mg/ml penicillin, and 146 mg/ml streptomycin. MISO and [2,14C]MISO were obtained from the National Cancer Institute. The TRI-CARTRAC was synthesized and generously supplied by Dr. Keith R. Laderoute (Ontario Cancer Institute). Silicon oil (Dow Corning 550 fluid) was purchased from K. R. Anderson Co., Inc., Santa Clara, CA, and mineral oil was purchased from E. R. Squibb and Sons, Princeton, NJ. DMTU and 2-vinylpyridine were purchased from Aldrich Chemical Co., Milwaukee, WI. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Benzotriazine Metabolism by CHO Cells. Cells were harvested in exponential phase of growth by centrifugation for 5 min at 300 x g and resuspended at 5 x 106 cells/ml in BBS. BBS contained per liter: 7.53 g NaCl, 2.20 g NaHCO3, 0.40 g KCl, 0.46 g KH2PO4, 0.14 g CaCl2, 0.10 g MgCl2·6H2O, 0.10 g MgSO4·7H2O, 0.9 g glucose (5 mM), and 0.01 g phenol red. Use of BBS decreased the HPLC background caused by medium and serum components and allowed the cell metabolism experiments to be done with the same gas mixtures as were used in the cytotoxicity assays (containing 5% CO2), while maintaining a constant pH of 7.4.

The cell suspension was added in equal amounts (15 ml) to each of several 25-ml double side-armied flasks (Wheaton, Millville, NJ) and...
incubated at 37°C while stirring under a continuous flow of humidified gas into one of the side arms. After 1 h, stock solutions of SR 4233 (6.0 mM), SR 4317 (0.45 mM), or SR 4330 (3.0 mM) in 25 mM sodium phosphate-buffered saline, pH 7.5, were added to the cell suspension. Immediately and at 30-min intervals, aliquots of the suspensions were removed through the open side arm and added to an equal volume of 50% methanol/2% acetic acid on ice. The samples were centrifuged at 13,000 × g for 10 min and the supernatant was removed and stored at 4°C until analysis by HPLC.

HPLC: SR 4233, SR 4317, and SR 4330 were all assayed with the same HPLC system. The system was isocratic reverse phase chromatography by using a Waters WISP (Model 710B), Waters pumps (Model 600A), a steel 30-cm C18-Bondapak column, and a Waters variable wavelength detector (Model 450) at 240 nm. Elution time and peak area were integrated by Waters data module 730. The mobile phase was 25% methanol/1% acetic acid/74% distilled water at a flow rate of 1.3 ml/min. SR 4233 eluted at 4.60 min. The limit of detection, using peak area, was ≤5 μM in standards made up in a background of cell extract (treated as for samples). SR 4317 eluted at 12.81 min, with a limit of detection ≤5 μM. SR 4330 eluted at 12.02 min, with a limit of detection ≤2 μM. Loss of SR 4233 and formation of SR 4317 were calculated from the peak areas by using area per concentration ratios derived from standards for each of the species run on the same day as the samples.

Cytotoxicity Assay. For drug toxicity experiments, SR 4233, SR 4317, or SR 4330 was dissolved in serum-free medium, diluted in complete medium, and gassed with air or nitrogen containing 5% CO2 in the same Wheaton flasks and stirring apparatus used in the metabolism studies. Exponentially growing CHO-To cells were harvested from suspension culture by centrifugation, resuspended at 5 × 10^7 cells/ml in complete medium, and loaded in 1-ml plastic syringes with an 18-gauge needle. Syringes were incubated at 37°C for 15-20 min to allow cells to become hypoxic through respiration of any residual oxygen remaining in the dense suspension. After a 1-h interval for gassing of the drug solution (including the 15- to 20-min incubation of cells in syringes), an aliquot of the cell suspension was introduced into each Wheaton flask containing drug. The final concentration of cells in each vessel was 10^6 cells/ml. Samples of cell suspension were then removed at 30-min intervals for up to 3 h after drug addition, diluted, and plated in appropriate numbers to assay for colony formation 8 days later. Cell suspensions were maintained at 37°C throughout the experiment.

Cellular O2 Consumption. Oxygen utilization was monitored by using a Clark type electrode and Yellow Springs Instrument Model 53 oxygen monitor (Yellow Springs, OH). For measurement of oxygen consumption by CHO cells, 2 × 10^6 cells/ml were suspended in Dulbecco's phosphate-buffered saline containing 5 mM glucose and 3 mM NaCN. A 3-ml aliquot of cells was brought to 37°C while stirring, and the oxygen probe was inserted into the chamber. After a background rate was established, a concentrated solution of either SR 4233, SR 4317, or SR 4330 in dimethyl sulfoxide was added to the chamber by using a Hamilton syringe fitted with a 6-cm blunt-ended needle.

GSH Assays. Cells were suspended in serum-free α-MEM at 3.6 or 1.8 × 10^6 cells/ml. Cells at 3.6 × 10^6 cells/ml were gassed with 95% air/5% CO2 for 1 h before adding 1 volume of serum-free α-MEM containing 4 mM SR 4233, to give a final drug concentration of 2 mM. For the hypoxic groups, cells at 1.8 × 10^6/ml were gassed with 95% N2/5% CO2 for 1 h before 0.01 volume 4 mM SR 4233 in serum-free α-MEM was added (to give a final drug concentration of 40 μM SR 4233). Immediately after drug addition and at 1-h intervals, 1-ml aliquots were removed from each flask and carefully layered into a previously prepared microfuge tube containing 0.2 ml 5% sulfosalicylic acid plus 20% glycerol (bottom layer) and 0.35 ml silicon oil:mineral oil, 84:16 (top layer). The tubes were centrifuged at 12,000 × g for 5 min, causing the cells to separate from the extracellular medium (the fraction overlaying the oil) and move through the oil to the bottom layer where metabolism stopped and GSH was extracted. A 0.8-ml aliquot of the extracellular layer (top) was added to 0.2 ml 5% sulfosalicylic acid, the remaining top layer and most of the oil was removed, and 0.150 ml was removed from the bottom layer with a P-200 pipettor. The samples were frozen overnight at -20°C before assay for total GSH and GSSG, using a modification of the method of Tietze (10) and for GSSG after GSH derivitization, using 2-vinylpyridine (11).

Drug Binding to Cellular Macromolecules. For [1^4C]MISO binding during CHO cell incubations, 5.0 mM [1^4C]MISO (specific activity, 0.02 μCi/ml) was added to cells (4 × 10^6 cells/ml) suspended in α-MEM after 1 h of pregassing with either 95% air or 95% N2 plus 5% CO2 at 37°C in 25-ml Wheaton spinner flasks. For [3^4C]SR 4233 binding, 0.2 mM [3-^4C]SR 4233 (specific activity, 0.150 μCi/ml) was added to aerobic or hypoxic cell suspensions. Immediately after drug addition and at various times thereafter, a 4-ml aliquot of suspension was removed, centrifuged at 300 × g, washed once in 5 ml cold saline, and resuspended in 2 ml cold saline. The cells were then sonicated to break the cell membranes and release trapped but unmetabolized drug. Tri-chloroacetic acid was added (to a final concentration of 7%) to precipitate DNA and protein, and after 15 min on ice, precipitated material was pelleted by centrifugation at 3000 × g for 20 min. The supernatant fraction was removed, the pellets were triturated in 0.5 ml 50 mM NaOH to which 4.5 ml Aquasol (New England Nuclear) was added, and radioactivity was monitored in a LKB Model 1212 Minibeta counter. Drug bound in pmol was calculated from the specific activity of each drug used.

Thiobarbituric Reactive Substance. TBARS were detected in the extracellular medium of a CHO cell suspension after incubation with 0.20 mM SR 4233. The cells were harvested by centrifugation at 300 × g for 10 min and resuspended in serum-free α-MEM at 3–7 × 10^6 cells/ml. The cell suspension was added to six double side-armed flasks (Wheaton; 25 ml) and stirred under a continuous flow of either 95% air/5% CO2 or 95% N2/5% CO2, DMTU (5 mM), a potent hydroxyl radical scavenger (12), was added at the beginning of gassing to one aerobic and one hypoxic flask. SR 4233 was added after 1-h of gassing. Three h later, 4 ml of cell suspension was centrifuged at 300 × g and the extracellular medium was removed from the cell pellet. TBARS were assayed by adding 0.5 ml of a 1% TBA solution in 50 mM NaOH and 0.5 ml of a 2.8% trichloroacetic acid solution to 1 ml of extracellular medium from each incubation and heating to 95°C for 15 min. TBARS in the cellular material were assayed by resuspending the cell pellets in 2.8% trichloroacetic acid, centrifuging the resulting precipitate, and heating a volume of the supernatant fraction with an equal volume of 1% TBA for 15 min. The absorbance at 532 nm was monitored and the samples were scanned at different wavelengths to verify that the peak absorbance was 532 nm and not a shoulder of other absorbing material.

For each of three experiments the absorbance at 532 nm for samples SR 4233 was subtracted from the values for the incubation with drug, and with and without DMTU, and then normalized for the cell concentration for that experiment. TBARS values are expressed as MDA equivalents, using E = 156,000 μM -1 cm^-1 for the chromogen (13). Statistics reported are P values for a two-tailed unpaired Student’s t test.

RESULTS

Drug Metabolism. High pressure liquid chromatograms of the 3-aminobenzotrazines are shown in Fig. 2, A–D. The chromatogram of 0.20 mM SR 4233 in the supernatant fraction of CHO cells in HPLC eluent (Fig. 2A) is indistinguishable from the chromatogram of the supernatant of extract (diluted 2-fold) from a hypoxic cell suspension to which 0.20 mM SR
SR 4233 METABOLISM AND HYPOXIC CYTOTOXICITY

Fig. 2. HPLC chromatograms of 0.1 mM SR 4233 in CHO cell extract (A); hypoxic CHO cell suspension plus 0.2 mM SR 4233 immediately after drug addition and 2 × dilution (B); a mixture of 0.05 mM SR 4233 (Peak I), 0.01 mM SR 4330 (Peak III), and 0.015 mM SR 4317 (Peak II) in cell extract (C); and extract from hypoxic CHO cells plus 0.2 mM SR 4233 after 3 h (D).

Fig. 3. Time course of loss of SR 4233 and formation of SR 4317 during hypoxic incubation of CHO cells with 0.20 mM SR 4233. ○, SR 4233; △, SR 4317; ●, total concentration of both drugs.

4233, was added and assayed immediately (Fig. 2B). A mixture of SR 4233 (Peak I), SR 4317 (Peak II), and SR 4330 (Peak III) is shown in Fig. 2C. The pattern produced by hypoxic incubation of CHO cells with 0.20 mM SR 4233 for 3 h is shown in Fig. 2D. SR 4317, but no SR 4330, was detected in the sample. Several other peaks eluting after SR 4233 and before SR 4330 were seen. The nature of this material has not been determined; however, there is a slow loss of methanol extractable benzotriazine from the mixture which may be accounted for by these peaks and material at the solvent front. Fig. 3 shows the concentrations of SR 4233, SR 4317, and total triazine in nmol per ml of cell suspension plotted for each time point. The rate of loss of SR 4233 and loss of total triazine were fitted to exponential equations giving a half-life (with SE) of 17.1 ± 0.9 h. The correlation coefficients were 0.996 and 0.993, respectively. Regression of the amount of SR 4317 on the residual concentration of SR 4233 at each time gave a ratio of 0.67 SR 4317 formed per SR 4233 lost (r = 0.998). When SR 4317 and SR 4330 were tested separately with hypoxic cells under identical conditions, no detectable metabolism occurred.

Aerobic metabolism of SR 4233 was tested at the same concentration as for hypoxic cells, 0.20 mM, as well as at a concentration which caused toxicity to aerobic cells, 2.0 mM. Neither loss of parent, SR 4233, nor the formation of SR 4317 or SR 4330 was detected in cell suspension incubated with 0.2 mM SR 4233. In the aerobic cell suspension incubated with 2.0 mM SR 4233, although the small rate of loss of parent could not be reliably determined, SR 4317 was formed at a rate of 4.14 nmol/h over the 3-h incubation period.

Cell Killing. Survival curves for the three compounds are shown in Fig. 4. In the metabolism experiments, a supralethal SR 4233 treatment (0.2 mM for 3 h under hypoxia) was used, so the two experiments cannot be strictly compared. Aerobic toxicity and toxicity of SR 4317 and SR 4330 are shown at the same concentrations as used for metabolic studies. The colony survival values for 0.1 and 0.2 mM SR 4317 on hypoxic cells were very close (Fig. 4B) as were 0.2 and 0.5 mM SR 4330 (Fig. 4C). The lack of cytotoxicity of SR 4317 and SR 4330 could not be accounted for by an inability of these drugs to cross the cell membrane. Using a modification of the "spin-through-oil" technique described for the determination of intracellular GSH levels, these drugs were found to actually concentrate intracellularly by severalfold. The hypoxic cytotoxicity ratio for SR 4233, calculated as the ratio of drug concentration required in air to yield approximately the same survival in hypoxia was 20–50 for this cell line.

Oxygen Utilization. Oxygen uptake by respiration-inhibition CHO cells was stimulated by SR 4233 at an average rate of 0.34 ± 0.05% (SE) of dissolved oxygen consumed per mM drug per min per 10⁶ cells (data not shown). MISO tested in the same cell preparations gave 0.029 ± 0.005% (SE) consumed per mM per min per 10⁶ cells or 12-fold less than the SR 4233 rate. Neither SR 4317 nor SR 4330 stimulated oxygen consumption.

Effect on Cellular Glutathione Levels. Neither GSH oxidation nor depletion was detected in cells treated with approximately equitoxic concentrations of SR 4233 in air (2.0 mM) and hypoxia (0.04 mM). However, it is possible that GSH oxidation to GSSG could have been a consequence of the metabolism of SR 4233 without observing a net increase in GSSG since levels of oxidized glutathione can increase only when the cellular capacity for its reduction by GSH reductase and NADPH are exceeded. SR 4233 or its activation product were present at sufficient levels under hypoxic conditions to alter intracellular GSH. The GSH concentration in the CHO cells as cultured averaged 3.8 nmol/10⁶ cells [or an intracellular concentration of roughly 3.0 mM assuming a total cell volume of 13 μl/10⁷ cells (14)]. The GSH concentration of the cell suspension containing 1.8 × 10⁶ cells/ml was therefore 6.8 nmol/ml.

5 E. M. Zeman, unpublished observations.

6 It is of interest that the hypoxic cytotoxicity ratio for this suspension culture-adapted CHO-To line was found to be less than that for the CHO HA1 subline, whose in vitro cytotoxic response to SR 4233 was described elsewhere (3). The lower hypoxic cytotoxicity ratio resulted from an increase in the aerobic cytotoxicity of SR 4233, rather than a change in the hypoxic response, and may have resulted from difference in methodology between the two sets of experiments. In the present experiments, both a higher cell density, a smaller total cell suspension volume, and glass vessels with lower surface to volume ratio were used; these might all have contributed to lowered oxygen tensions, even in the "aerobic" suspensions, with, possibly, a resultant increase in SR 4233 toxicity. In addition, the dilution and plating scheme in these experiments was such that residual SR 4233 was present in the Petri dishes during the colony-forming period, especially for the highest drug concentrations tested. Since the hypoxic toxicity of SR 4233 has not been characterized, it was possible that the 8- to 10-day exposure to low levels of drug could have also contributed to the aerobic toxicity.
Hence, SR 4233 (40 nmol/ml) was well in excess of intracellular GSH and, according to our calculated rate of hypoxic reduction, would have been 35% metabolized at 3 h. Since no loss of intracellular GSH occurred, conjugation of GSH to SR 4233 or its metabolites either enzymatically or chemically either did not occur or the GSH synthetic rate exactly matched rate of loss.

Binding of SR 4233 to Macromolecules. Misonidazole is differentially toxic to hypoxic cells, and this increased toxicity has been attributed to formation and binding of reduction products formed uniquely in hypoxic cells (7, 15). The possibility of formation of binding intermediates from SR 4233 was examined by using a protocol which demonstrates hypoxia-specific binding of [14C]MISO to acid-precipitable cellular material (Fig. 5). MISO binding in hypoxia was 2.9 nmol/10^6 cells after 3 h, which was 21-fold higher than MISO binding in air, and 30- and 50-fold more than SR 4233 associated with the cell pellet in hypoxia or air, respectively. The lack of hypoxia-specific binding of [14C]SR 4233 labeled in the triazine ring demonstrates that neither parent drug, the known hypoxically formed metabolites, nor other structures containing the 3-position carbon bind selectively to cellular material. Nonspecific interactions of low levels of drug with cellular macromolecules such as DNA intercalation, cannot be eliminated by these experiments, however.

Although MISO is metabolized to different products than is SR 4233 and the concentration of MISO used was 25-fold higher than that of SR 4233 (5 mM versus 0.20 mM), the comparison of drug binding characteristics is justifiable on the basis of the rates of activation being similar for the respective drug concentrations used (see Oxygen Utilization). On the basis of toxicity, the comparison is weighted heavily in favor of SR 4233. The hypoxic toxicity of 0.20 mM SR 4233 is immeasurably larger (refer to Fig. 4) than that of 5 mM MISO under the same conditions (data not shown). Therefore, because binding of hypoxically produced metabolites of SR 4233 to cellular material was lower than MISO binding in hypoxia on an absolute basis, lower on the basis of ratio bound per total drug, and not higher than for aerobic cells, this appears not to be the cause of selective toxicity.

TBARS Formation. The release of TBARS from cells incubated with 0.20 mM SR 4233 for 3 h under aerobic or hypoxic conditions, and the effect of adding the hydroxyl radical scavenger DMTU, are shown in Fig. 6. TBARS released from hypoxic cells (0.24 nmol/10^6 cells) were more than 5-fold higher (P < 0.01, paired t test) than from aerobic cells. Also, the TBARS released from aerobic cells incubated with SR 4233 were not significantly decreased by the addition of 5.0 mM DMTU, whereas a decrease in TBARS was observed in the hypoxic cells by adding DMTU (P = 0.06). Absorbance at 532 nm in cell pellets was equally low in all groups, indicating that all TBARS were soluble and that macromolecular precursors of TBARS which may react upon heating with TBA were not formed (13).

DISCUSSION

The metabolism of SR 4233 to SR 4317 demonstrates that reduction of the compound takes place in CHO cells. The lack of toxicity and hypoxic selectivity of the two electron reduction
product SR 4317 lead to our earlier hypothesis that SR 4233 toxicity to hypoxic cells might be the result of a one electron reduction product, necessarily a free radical (3). When stoichiometric calculations were applied to radiation reduced solutions of SR 4233 containing formate, fewer than 2 electrons were required to produce SR 4317 (9), which also suggested a chain reaction catalyzed by a radical intermediate. The results presented here support the formation of an oxidizing, nonbinding radical which is unstable in the presence of oxygen. Also, as noted below, our data show a quantitative relationship between reductive metabolism and cytotoxicity, which suggests that the radical formed by drug reduction is responsible for cellular toxicity. Drug metabolism and cell survival curves were measured under identical conditions of cell density, gas flow, and stir rate. The initial rate of formation of the stable reduction product, SR 4317, with 2.0 mM SR 4233 in air, was 4-fold less than the initial rate of SR 4317 formation in a cell suspension under nitrogen with 0.20 mM SR 4233 (16 nmol/h). Assuming a linear relationship between initial drug concentration and metabolic rate (suggested by the exponential loss of drug during hypoxic metabolism), this is effectively a 40-fold difference in rate of formation of SR 4317 under aerobic and hypoxic conditions. The fact that the hypoxic cytotoxicity ratio is 20:50 for these conditions suggests that the differential cytotoxicity can be accounted for quantitatively by the differential metabolism. It suggests further that the SR 4233 metabolism leading to SR 4317 formation is responsible for cellular toxicity. A possible scheme is shown in Fig. 7.

Further evidence of free radical formation in cells comes from the oxygen utilization data. Because the Clark electrode responds only to molecular oxygen and not to superoxide anion or to hydrogen peroxide, the measurement of oxygen consumption in cyanide-treated cells implies that oxidation-reduction cycling of SR 4233 occurs [in a manner analogous to that occurring with nitroaromatic compounds (16)]. Capture of the electron by molecular oxygen from the putative SR 4233 radical anion (thereby causing back oxidation to the parent drug and forming superoxide radical) results in a net consumption of oxygen. Superoxide dismutates spontaneously or via cellular superoxide dismutases to form equal amounts of hydrogen peroxide and molecular oxygen. Hydrogen peroxide breakdown by catalase (also releasing molecular oxygen) was prevented in our system by the addition of cyanide to cells. Therefore, assuming that each drug radical reacts with oxygen to form superoxide, the net reaction is that one molecule of oxygen is consumed for every two SR 4233 radicals formed. We have also demonstrated the formation of hydrogen peroxide in solutions of SR 4233 activated by ferridoxin reductase (ferrodoxin:NADP⁺ oxidoreductase, EC 1.18.1.2) and NADPH by the release of oxygen when catalase was added (data not shown).

The rate of drug activation in respiration-inhibited aerobic cells can be calculated from the oxygen consumption rate measured (assuming that dissolved oxygen concentration in the suspension is initially 240 nmol/ml) as 0.16 nmol of O₂ consumed/min/10⁶ cells with 0.20 mM SR 4233 or 0.32 nmol SR 4233 radical formed/min. However, the measured rate of hypoxic formation of SR 4317 was 0.05 nmol/min/10⁶ cells with 0.20 mM SR 4233. The discrepancy may result from the fact that metabolism of cyanide-treated cells are not metabolically equivalent to untreated cells, or may be due to the rate of back oxidation of the SR 4233 radical by residual O₂ which is present in the stirred dense cell solutions under N₂ (17). We hope to measure these low oxygen concentrations in future work in order to clarify this, but the effect of cyanide on electron transport and other enzymes besides catalase is not known.

Although the formation of oxygen radicals in cells can lead to oxidative stress and toxicity, it is significant that the release of MDA equivalents was higher in hypoxic cells than in aerobic cells treated with an equal concentration of SR 4233. Even for aerobic cells, the levels produced by SR 4233 were approximately 3-fold higher than for acetaminophen-treated hepatocytes (18), and MISO produced little MDA in aerobic A549 cells unless GSH was depleted (19). MDA can be formed during lipid peroxidation by a mechanism requiring hydrogen abstraction from unsaturated fats, peroxide formation by hydrogen addition, and subsequent rearrangements (13). MDA can also be formed by hydrogen abstraction reactions with deoxyribose [even within DNA (20)], some other sugars, and a few amino acids (21). The protection afforded by DMTU, a hydrogen donor (12, 22), lead us to conclude that this is the mechanism of the putative SR 4233 radical reaction with cellular material. The lack of binding of reduced SR 4233 to cellular macromolecules is also consistent with the hydrogen abstraction hypothesis. Disproportionation reactions of the radicals may also occur by producing one SR 4233 and one SR 4317 from two radicals (23) without causing cellular damage. The extent to which disproportionation as opposed to the reactions described...
above accounts for SR 4317 formation in the cellular environment is an interesting direction for future research.

Formation of SR 4317 accounts for 67% of the loss of SR 4233 in hypoxic CHO cell suspensions. The remaining lost material cannot be attributed to either GSH adduct formation, since no intracellular GSH depletion occurred, or to metabolite binding to cellular material. The chromatogram shown after 3 h of hypoxic incubation of SR 4233 with CHO cells (Fig. 4D) contains newly formed, unidentified peaks. Unidentified peaks in high pressure liquid chromatograms and loss of material was also reported for radiation-reduced SR 4233 (23). It is therefore possible that a nonenzymatically formed drug product may be represented by these peaks.

The results presented here are consistent with the scheme shown in Fig. 7. Both aerobic and hypoxic activation of SR 4233 are presumed to occur at the same intracellular sites via single electron transfer reactions. Under aerobic conditions, the resulting radical will react preferentially with oxygen to give up an electron, regenerate parent drug, and form superoxide radical. As the local oxygen tension decreases, the radical may abstract hydrogen from labile sites, producing lethal cellular lesions. We found both DNA single and double strand breaks produced in hypoxic cells incubated with SR 4233 (24). As is the case for H₂O₂ (which also leads to hydrogen abstraction), more strand breaks were produced for the level of toxicity when compared to radiation. Further characterization of reactions of the SR 4233 radical, the lesions produced in cells, and the stringency of hypoxia required for cell killing will be important in exploring the potential of this drug or an analogue as an anticancer agent in the clinic.

ACKNOWLEDGMENTS

The authors thank Cecelia Pearson for technical assistance and Chiyoye Adachi for secretarial help.

REFERENCES

Metabolism of SR 4233 by Chinese Hamster Ovary Cells: Basis of Selective Hypoxic Cytotoxicity

Margaret A. Baker, Elaine M. Zeman, V. Kate Hirst, et al.