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Role of NADPH:Cytochrome P450 Reductase in the Hypoxic Accumulation and Metabolism of BRU59-21, a Technetium-99m-Nitroimidazole for Imaging Tumor Hypoxia

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ABSTRACT. Nitroimidazoles labeled with technetium-99m are being investigated as non-invasive markers of tumor hypoxia. They are bioreductive compounds that require enzymatic reduction for retention in hypoxic cells, but little is known about the cellular factors affecting their accumulation in hypoxic cells. If the absolute accumulation of hypoxia markers is affected by enzyme levels, an inaccurate assessment of the hypoxic cell fraction in tumors may occur. BRU59-21, ^{99m}Tc-oxo[[3,3,9,9-tetramethyl-6-[(2-nitro-1H-imidazol-1-yl)methyl] 5-oxa-4,8-diazadioximato]-(3-)-*N*,*N*′,*N*″,*N*‴] technetium (V), a technetium-99m-nitroimidazole that is being studied as a potential marker of tumor hypoxia, was used in the present study to evaluate the effect of NADPH:cytochrome P450 reductase (EC 1.6.2.4) levels on BRU59-21 accumulation and metabolism. Metabolism of BRU59-21 in hypoxic cellular lysates derived from Chinese hamster ovary cells overexpressing NADPH:cytochrome P450 reductase was 8-fold greater than in control cells. This effect required the presence of exogenous NADPH. The increased metabolism of BRU59-21 in lysates overexpressing NADPH:cytochrome P450 reductase was inhibited at 4° and by the addition of NADPH:cytochrome P450 reductase inhibitors. The addition of inhibitors of other nitroreductase enzymes had no effect on BRU59-21 metabolism in these lysates. When the accumulation and metabolism of BRU59-21 were studied in stirred suspension cultures, it was found that cells overexpressing NADPH:cytochrome P450 reductase exhibited about a 3-fold increase in both the hypoxic metabolism and the accumulation of BRU59-21. These findings suggest that NADPH:cytochrome P450 reductase is an important enzyme in BRU59-21 metabolism in model systems of tumor hypoxia. BIOCHEM PHARMACOL **60**;5:625–634, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. BRU59-21; 2-nitroimidazole; hypoxia; P450 reductase; enzyme

Clinical investigations have revealed that tumor hypoxia is a limitation for the local control of some solid tumors by radiation [1]. Recent evidence also suggests that hypoxic tumors may predict a more aggressive and metastatic phenotype [2–4]. In light of these findings, there has been considerable interest in developing techniques to assess the oxygenation status of individual tumors. A routine measurement of tumor hypoxia would be a useful diagnostic tool, as it would allow the selection of patients who could benefit from adjuvant therapies to treat these resistant tumors. Among the techniques available to assess tumor hypoxia is the use of nitroimidazole compounds, which are selectively metabolized and retained in hypoxic cells. When labeled with gamma- or positron-emitting radioisotopes, these compounds may allow the non-invasive assessment of tumor hypoxia using nuclear medicine imaging techniques [5, 6].

BRU59-21 (Fig. 1) is a nitroimidazole labeled with ^{99m}Tc,§ which was synthesized as a marker for imaging hypoxic regions in tumors and in heart disease [7]. This compound is retained selectively in hypoxic cells, and has been investigated in murine tumor models, where it was found to have properties that support its usefulness for the assessment of tumor hypoxia [8].

Quantitation of the extent of hypoxia requires that nitroimidazole binding be primarily dependent on the oxygen concentration in the tumor cells. Since tumor cells may vary in their nitroreductase levels [9], it has been suggested that enzyme levels within hypoxic cells may also

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§ *Abbreviations*: 2′-AMP, adenosine 2′-monophosphate; b_5 reductase, NADH:cytochrome b_5 reductase; CHO, Chinese hamster ovary; DCPIP, 2,6-dichlorophenolindophenol; DPIC, diphenyleneiodonium chloride; DTPA, diethylenetriaminepentaacetic acid; DTD, DT-diaphorase; DTT, dithiothreitol; α-MEM, alpha minimal essential medium; *p*HMB, *p*-hydroxymercuribenzoic acid; P450 reductase, NADPH:cytochrome P450 reductase; PTU, 6-*n*-propyl-2-thiouracil; and ^{99m}Tc, technetium-99m.

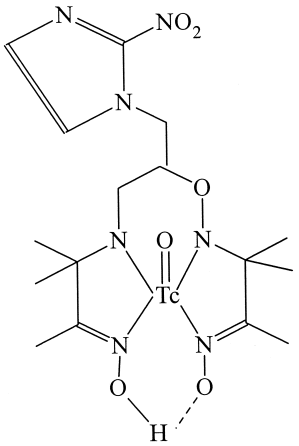

FIG. 1. Structure of BRU59-21.

affect the accumulation of the hypoxia marker [10]. Thus, increased nitroimidazole binding within tumors might be dependent not only on the oxygen concentration in the tumor, but also on the nitroreductase levels in the hypoxic tumor cell population.

Several enzymes, including P450 reductase (EC 1.6.2.4), b_5 reductase (EC 1.6.2.2), xanthine oxidase (EC 1.1.3.22), and DTD (EC 1.6.99.2), may be involved in the metabolism of bioreductive drugs [11]. P450 reductase has been shown to be involved in the reduction of the nitroimidazole misonidazole [11], and DTD is believed to play a role in misonidazole reduction and binding [12]. Transfection of P450 reductase or DTD into COS-1 cells resulted in an increased binding rate of 2-nitroimidazoles to cellular macromolecules, but there was a much greater effect observed with P450 reductase [13].

To date, there have been limited reports on the effect of nitroreductase levels on the accumulation and metabolism of nitroimidazole hypoxic cell markers. Purified xanthine oxidase was found to reduce both [^{18}F]FMISO [14] and the ^{99m}Tc-nitroimidazole BMS181321 (Ballinger JR, unpublished data), but it has also been reported that P450 reductase is a major enzyme involved in the reduction of a fluorinated 2-nitroimidazole hypoxia probe [15].

In the present investigation, the role of P450 reductase in the hypoxic accumulation and metabolism of BRU59-21 was studied in clones transfected with human P450 reductase. This is believed to be a more direct means of assessing the role of this enzyme in drug metabolism, since it will minimize the possibility that other factors, in addition to P450 reductase levels, affect drug metabolism. As well, previous investigations were performed at much higher drug concentrations (μM) than those used with ^{99m}Tc-labeled compounds. The high specific activity of ^{99m}Tc enables the use of compounds labeled with this isotope at very low (pM) drug concentrations [16]. The effect of nitroreductase levels on the accumulation of these compounds when present at low drug concentrations is not known. In the present study, the role of P450 reductase on the hypoxic accumulation and metabolism of BRU59-21 was determined in transfected CHO cells that overexpress human P450 reductase.

MATERIALS AND METHODS

Chemicals and Reagents

The ligand BRU59 (Bracco Research) was a gift from Drs. K. Linder and A. Nunn of Bracco Research. The reaction of the ligand with ^{99m}Tc-pertechnetate to form BRU59-21 has been reported previously [8]. In brief, 0.4 mg of the ligand was dissolved in 0.8 mL of isotonic saline, and 0.1 mL of ^{99m}Tc-pertechnetate (185 MBq) was added. A 0.1 mL aliquot of stannous DTPA (Techneplex, Squibb Diagnostics), which was reconstituted with 4 mL of saline, was added to the ligand and pertechnetate mixture. The formation of BRU59-21 was complete within 10 min at room temperature, with greater than 90% of the radioactivity associated with the ligand as measured by thin-layer chromatography [8]. For cellular lysate studies, the sample was purified by ethanol elution through a C_{18} solid phase extraction cartridge (Waters Corp.) to separate the labeled drug from any impurities and free ligand. Ethyl acetate, α-MEM, fetal bovine serum, NADPH, NADH, 2′-AMP, DPIC, *p*HMB, PTU, DCPIP, and dicumarol were obtained from the Sigma Chemical Co.

Cells

The CHO-VC (vector control) and CHO-FpT-27 (P450 reductase overexpressing transfectant) cell lines used in this study were provided by Dr. A. C. Sartorelli of Yale University. The CHO-FpT-27 cells were established by transfection of human P450 reductase cDNA into a CHO cell line deficient in dihydrofolate reductase (CHO-k1/dhfr$^-$) as described previously [17]. The cells were grown in monolayer cultures at 37° in α-MEM containing 10% fetal bovine serum (growth medium). CHO-AA8-4 cells were obtained originally from Dr. L. H. Thompson of Lawrence Livermore Laboratories and grown in suspension cultures in growth medium at 37°.

Preparation of Cellular Lysates

The preparation of cellular lysates has been described previously [18]. Briefly, exponentially growing cells were trypsinized from monolayer cultures. Following centrifugation, cell pellets were washed in ice-cold hypotonic buffer (10 mM HEPES/potassium hydroxide, pH 7.4, 1.5 mM magnesium chloride, 10 mM potassium chloride, 0.05 mM DTT). Cells were suspended in 1.5 mL of buffer and were lysed by exposure to three 10-sec ultrasound pulses at 10-sec intervals using a Vibra Cell Sonicator (Sonics and Materials). The suspensions were allowed to stand on ice for a further 10 min and then were centrifuged for 15 min at 7800 *g*. The lysate was removed and stored at −70°. The protein concentration of the cellular lysates was determined using the Bradford method [19] with BSA as the standard.

Measurement of Enzyme Activities

Methods for the determination of enzyme activities in cellular lysates have been described previously. P450 reductase activity was measured as the NADPH-dependent reduction of cytochrome *c* in the presence of KCN [18], b_5 reductase activity was measured as the *p*HMB-inhibitable NADH-dependent reduction of cytochrome *c* [20], and DTD activity was measured as the dicumarol-inhibitable NADPH-dependent reduction of DCPIP [21]. All assays were performed at room temperature. The relative concentration of NAD(P)H in the CHO-VC and CHO-FpT-27 cells was measured in cell suspensions using flow cytometric analysis of the autofluorescence of NAD(P)H as described previously [22].

Lysate Incubations

Incubations were performed in 4-mL glass vials (Bayer Corp.) at 37°. The incubation mixture consisted of 0.1 mL of cellular lysate (~1 mg/mL protein concentration), 0.2 mL of buffer (0.2 M phosphate buffer, pH 7.4), 0.1 mL of either NADPH (1 mM) or NADH (1 mM), and an inhibitor: either 2′-AMP (25 mM), DPIC (1.2 mM), *p*HMB (0.1 mM), PTU (60 mM), or dicumarol (10 μM). Argon was passed over the liquid surface for 13 min and bubbled in the liquid for an additional 2 min. The reaction was initiated by the addition of 0.1 mL of BRU59-21 (final activity ~ 4 MBq/mL) to the reaction vials, and the mixture was bubbled with argon for another minute. Samples were removed as a function of time without disturbing the oxygenation status of the vial. The samples were added to a mixture consisting of 2 mL ethyl acetate and 2 mL PBS, vortexed, and centrifuged at 200 *g* for 30 sec to separate the phases. The percent of total radioactivity extractable into ethyl acetate was determined by counting both phases in an automatic γ-well counter (Picker-Pace 1, Picker Corp.).

Initial experiments using an HPLC apparatus (System Gold model 125, Beckman Instruments Inc.) with a reverse-phase C_{18} column (Ultrasphere ODS, 4.6 × 250 mm, Beckman) and radioactivity detector set for ^{99m}Tc (model 171, Beckman) were performed to determine whether ethyl acetate extraction values were able to quantify the fraction of radioactivity attributed to BRU59-21. BRU59-21 was incubated in hypoxic suspension cultures of CHO-AA8-4 cells for 4 hr. The extracellular medium was isolated, passed through a centrifugal filter (10,000 NMWL, Ultrafree-MC, Millipore, >90% radioactivity recovery) to remove serum protein, and then analyzed by HPLC using a 40/60 (v/v) solvent mixture of acetonitrile and ammonium acetate buffer (0.1 M, pH 4.6) at a flow rate of 1 mL/min.

Drug Accumulation in Suspension Cultures

Exponentially growing cells were trypsinized from monolayer cultures, centrifuged, and resuspended in fresh growth medium at a concentration of 1×10^6 cells/mL. Glass vials containing 10 mL of the stirred cell suspension were placed in a water bath at 37° as described previously [23]. The cells were equilibrated for 30 min with a continual flow of a pre-humidified gas mixture of 95% air plus 5% CO_2 (aerobic exposure) or 95% N_2 plus 5% CO_2 (<10 ppm O_2, hypoxic exposure). BRU59-21 was added to each vial at a final activity of approximately 0.25 MBq/mL and at a total drug concentration (BRU59 + BRU59-21) of approximately 0.2 μM. The concentration of labeled drug (BRU59-21) was approximately 50 pM.

Duplicate 0.3-mL aliquots were removed from the vials as a function of time after incubation with BRU59-21. The aliquots were added to 1 mL of isotonic saline at 4° and centrifuged at 10,000 *g* for 3 min in a microcentrifuge tube. The aqueous growth medium and the saline were aspirated, 0.5 mL of saline was passed over the pellet, the residual saline was removed, and the tube tip containing the cell pellet was clipped and counted in the γ-well counter. From these counts, the ratio of radioactivity that would be found in 0.1 mL of packed cells (C_{in}), which was found to contain approximately 6×10^7 cells using a Constable tube as described previously [24], was determined. The amount of radioactivity in an equal volume of growth medium (C_{out}) was calculated, and the cellular accumulation was expressed in the form of the ratio C_{in}/C_{out}.

Metabolism in Suspension Cultures

The chemical form of the radioactivity in the medium of the stirred cell suspension cultures was investigated. A 0.3 mL sample was removed from the vial and centrifuged at 10,000 *g* for 3 min at room temperature. A 0.1 mL aliquot of the supernatant was vortexed in a mixture containing 2 mL ethyl acetate and 2 mL PBS at room temperature and centrifuged at 200 *g* for 30 sec to separate the phases. The phases were transferred into individual tubes and counted in the γ-well counter, and the percent of total counts extractable into the organic phase was determined.

HPLC analysis of the extracellular medium at the conclusion of the experiment was performed as described previously [8]. Briefly, 0.9 mL supernatant aliquots were passed through a centrifugal filter and analyzed by HPLC with a radioisotope detector set for ^{99m}Tc. A solvent system consisting of a 40/60 (v/v) mixture of acetonitrile and ammonium acetate buffer (0.1 M, pH 4.6) at a flow rate of 1 mL/min was used. The form of the radioactivity inside the cells was not determined in the present study.

Statistics

Data are presented as the means ± SEM of three or more independent experiments. Student's *t*-test was used to determine the significance of differences, with a value of $P \leq 0.05$ considered significant.

TABLE 1. Nitroreductase activities of vector-transfected control (CHO-VC) and human P450 reductase cDNA-transfected (CHO-FpT-27) cell clones

	Enzyme activity (nmol/min/mg protein)		
Cell line	P450 reductase	b_5 Reductase	DTD
CHO-VC	2.6 ± 0.4	58.2 ± 8.3	16.9 ± 2.8
CHO-FpT-27	12.0 ± 0.8	63.9 ± 4.8	15.3 ± 2.2

Values are the means ± SEM of three or more independent experiments.

RESULTS

Measurement of Enzyme Activity in Transfected Cells

The activities of nitroreductase enzymes implicated in the bioreductive metabolism of nitroimidazoles were determined in the CHO-VC and CHO-FpT-27 cell lines (Table 1). The CHO-FpT-27 cell line exhibited 5-fold higher P450 reductase activity than the CHO-VC cell line, whereas the activities of b_5 reductase and DTD were not significantly different between the two cell lines, consistent with previous investigations [17]. Neither of these cell lines contain detectable levels of xanthine oxidase or xanthine dehydrogenase [17]. As well, these cell lines did not differ in NAD(P)H levels as determined by flow cytometry analysis (data not shown).

Measurement of BRU59-21 Metabolism Rate

To develop a simple and rapid measurement of BRU59-21 metabolism, the validity of using ethyl acetate extraction measurements to quantify the metabolism of BRU59-21 was investigated. BRU59-21 was incubated in suspension cultures of CHO-AA8-4 cells under hypoxic conditions for 4 hr. The extracellular medium was isolated, passed through a centrifugal filter to remove serum protein, and analyzed by HPLC. Two distinct peaks were observed (data not shown) and isolated. The more lipophilic peak (retention time = 8 min) corresponded to BRU59-21, and the more hydrophilic peak (retention time = 4 min) was believed to correspond to free pertechnetate.

Mixtures of the isolated peaks, containing known ratios of the drug and hydrophilic product, were extracted into ethyl acetate, and the percent of total radioactivity extractable into ethyl acetate was plotted versus the percent of total drug in the mixtures (Fig. 2). When the mixture contained only BRU59-21, 92% of the radioactivity was extractable into ethyl acetate. This value is consistent with the lipophilicity of BRU59-21 and with previous investigations [8]. Decreasing the amount of BRU59-21 and increasing the amount of hydrophilic product in the mixture resulted in a linear decrease in the percent of total radioactivity that was extractable into ethyl acetate. When the mixture consisted only of the hydrophilic product, 20% of the radioactivity was extractable into ethyl acetate.

The ability of ethyl acetate extraction to predict the fraction of parent drug in the radioactive mixture was confirmed by comparing the predicted fraction of BRU59-21 as determined by ethyl acetate extraction values with the values obtained by HPLC analysis. The radioactivity was isolated from cellular lysates, and samples were analyzed by ethyl acetate extraction and HPLC. It was found that the values for the fraction of BRU59-21 in the lysates predicted through ethyl acetate extraction were consistent with the values obtained through HPLC analysis (data not shown). These results indicated that using the percent of total radioactivity that is ethyl acetate-extractable as a measure of BRU59-21 metabolism in cellular lysates is valid.

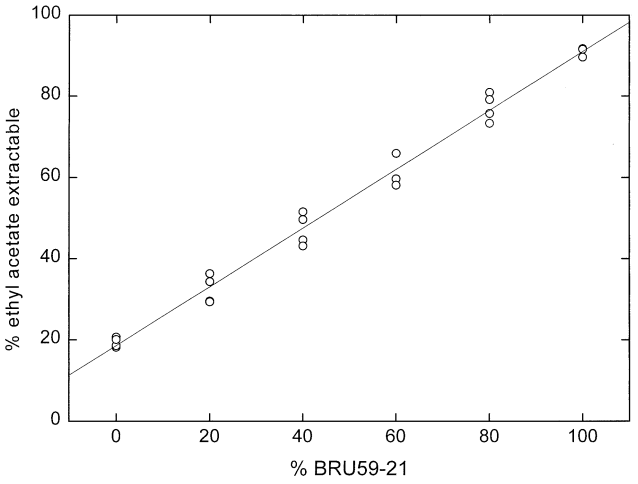

FIG. 2. Standard curve of percent ethyl acetate-extractable radioactivity versus mixtures containing various fractions of BRU59-21 and the hydrophilic product. BRU59-21 was incubated under hypoxic conditions for 4 hr in suspension cultures of CHO-AA8-4 cells. The extracellular medium was isolated and analyzed by HPLC. Two peaks were isolated, one corresponding to BRU59-21 and the other to a hydrophilic product. Mixtures containing various amounts of the parent compound and hydrophilic product were made and extracted into a mixture containing equal volumes of ethyl acetate and PBS. The percent of total radioactivity extractable into the organic phase was determined. Points represent independent determinations. The equation of the line: $y = 0.72x + 18.6$, $r = 0.994$.

Metabolism of BRU59-21 in Lysates Derived from Transfected Cells

To determine whether P450 reductase is important in the hypoxic metabolism of BRU59-21, the rate of metabolism of BRU59-21 in hypoxic lysates derived from vector-control cells and cells overexpressing P450 reductase was examined. The incubation mixture was supplemented with NADPH (1 mM), and the reaction was initiated by the addition of purified BRU59-21. In lysates derived from vector-control cells (CHO-VC), BRU59-21 was metabolized at a rate of 2 fmol/min/mg of lysate protein (Fig. 3). In contrast, lysates derived from CHO-FpT-27 cells, which overexpress P450 reductase, showed an 8-fold increase in BRU59-21 metabolism, suggesting that this enzyme is involved in the hypoxic metabolism of BRU59-21.

The enhanced metabolism of BRU59-21 in CHO-

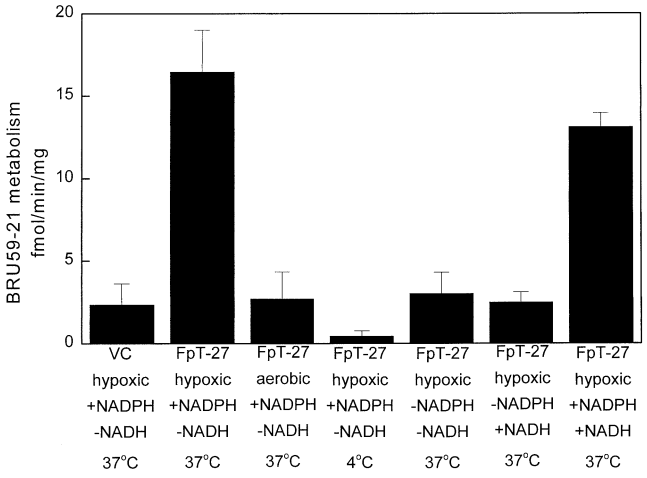

FIG. 3. Metabolism of BRU59-21 in CHO-VC and CHO-FpT-27 cellular lysates. The conditions of the incubation mixture are outlined on the figure. Samples were removed from the incubation mixture over 4 hr, and the amount of radioactivity extractable into ethyl acetate was determined. Using the standard curve shown in Fig. 2 and knowing the amount of radioactivity added to the incubation mixture, the specific activity of ^{99m}Tc, and the protein concentration present in the lysates, the metabolism of BRU59-21 was expressed as femtomoles per minute per milligram of lysate protein. Bars are the means ± SEM of three independent experiments.

FpT-27 lysates did not occur under aerobic conditions (Fig. 3). As well, the hypoxic metabolism of BRU59-21 by CHO-FpT-27 lysates was inhibited at 4°, suggesting that an enzymatic process is required for the metabolism of the drug. This effect was not statistically different from the effect observed in aerobic lysates. The hypoxic metabolism of BRU59-21 in CHO-FpT-27 lysates was dependent upon the presence of NADPH and did not occur with the addition of NADH (Fig. 3). The addition of both cofactors did not show a statistically significant change in the metabolism of the drug compared with incubations with NADPH alone, showing that the presence of both cofactors did not produce an additive effect on BRU59-21 metabolism.

To further demonstrate that P450 reductase is involved in the hypoxic metabolism of BRU59-21, the ability of P450 reductase inhibitors to affect the metabolism of BRU59-21 in cellular lysates was determined. 2′-AMP is a reversible inhibitor of P450 reductase that competes for the NADPH binding site of the enzyme [25], and DPIC is an irreversible inhibitor of P450 reductase that covalently binds to the flavin mononucleotide binding site of the enzyme [26–28]. Experiments were initially performed to determine the concentrations of 2′-AMP and DPIC needed to inhibit the NADPH-dependent reduction of cytochrome *c* (Fig. 4). Spectrophotometric analysis revealed that 2′-AMP was able to inhibit the NADPH-dependent reduction of cytochrome *c* in a concentration-dependent manner, and activity was inhibited by 85% at a 2′-AMP concentration of 25 mM (Fig. 4A). DPIC inhibition of P450 reductase activity was also concentration-dependent, and at a concentration of 1.2 mM DPIC, P450 reductase activity was inhibited by 80% (Fig. 4B).

The hypoxic metabolism of BRU59-21 was inhibited by ~80% by 2′-AMP (25 mM) and by ~90% by DPIC (1.2 mM) when supported by NADPH (Fig. 5). Neither inhibitor had any effect on the NADH-dependent reduction of cytochrome *c* (data not shown) or on the NADH reduction of BRU59-21 in cellular lysates (Fig. 5).

To assess the possible role of other enzymes, the effects of *p*HMB and PTU, inhibitors of b_5 reductase [29, 30], and of dicumarol, an inhibitor of DTD [21], on P450 reductase activity and the hypoxic metabolism of BRU59-21 were determined. The concentration of *p*HMB and PTU required to inhibit the activity of b_5 reductase was determined spectrophotometrically using lysates overexpressing b_5 reductase [31] (data not shown). It was found that none of the inhibitors had any effect on P450 reductase activity in CHO-FpT-27 lysates (data not shown). Consistent with this finding, it was found that the addition of either *p*HMB (0.12 mM), PTU (60 mM), or dicumarol (10 μM) had no statistically significant effect on the hypoxic metabolism of BRU59-21 in CHO-FpT-27 lysates supported by either NADPH or NADH (Fig. 5). These results suggested that the metabolism of BRU59-21 in CHO-FpT-27 hypoxic lysates is due to P450 reductase and is not caused by b_5 reductase or DTD.

Accumulation of BRU59-21 in Stirred Cell Suspensions

The effect of hypoxic or aerobic conditions on the accumulation of BRU59-21 in CHO-VC and CHO-FpT-27 cells as a function of time from 0 to 4 hr was determined (Fig. 6A). Aerobic cells of both cell types showed little accumulation of radioactivity at 5 min with a C_{in}/C_{out} value of ~3, and thereafter there was a small increase in

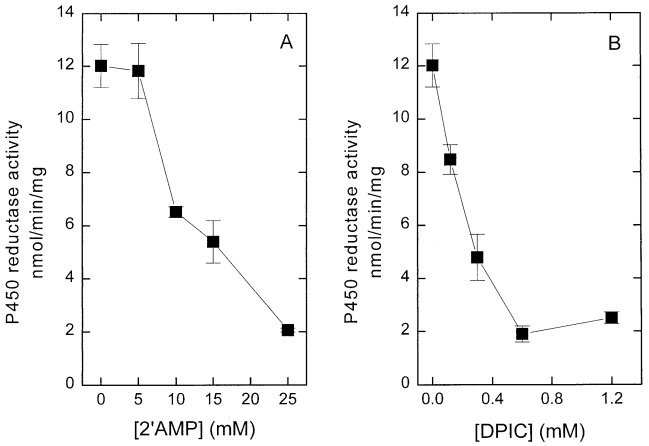

FIG. 4. Effect of 2′-AMP and DPIC on P450 reductase activity. P450 reductase activity was measured as the NADPH-dependent reduction of cytochrome *c* in the presence of KCN. (A) Effect of 2′-AMP, a reversible inhibitor of P450 reductase, on enzyme activity. (B) Effect of DPIC, an irreversible inhibitor of P450 reductase, on enzyme activity. Points are the means ± SEM of three independent experiments.

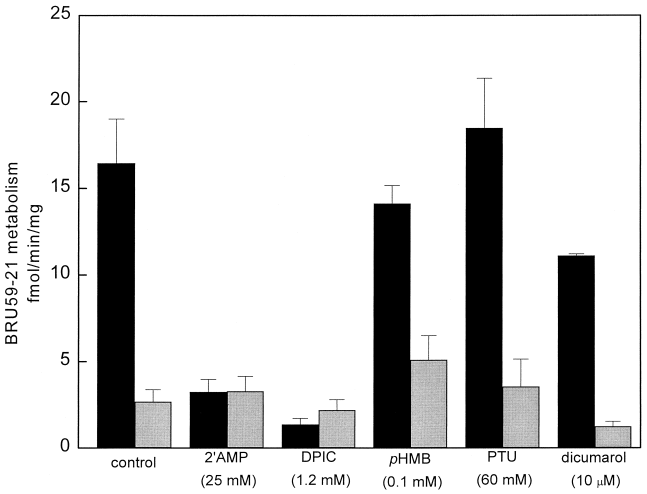

FIG. 5. Hypoxic metabolism of BRU59-21 in cellular lysates derived from CHO-FpT-27 cells in the presence of enzyme inhibitors. All determinations were performed under hypoxic conditions at 37°. Control conditions represent the metabolism of BRU59-21 in the absence of inhibitors. Incubations were performed with the addition of NADPH (1 mM, black bars) or NADH (1 mM, grey bars). Inhibitors were added before the addition of BRU59-21. 2′-AMP and DPIC are P450 reductase inhibitors, *p*HMB and PTU are b_5 reductase inhibitors, and dicumarol is a DTD inhibitor. Inhibitor concentrations are shown on the graph. After the addition of BRU59-21, samples were removed from the incubation mixture over 4 hr, and the amount of radioactivity extractable into ethyl acetate was determined. Using the standard curve shown in Fig. 2 and knowing the amount of radioactivity added to the incubation mixture, the specific activity of ^{99m}Tc, and the protein concentration present in the lysates, the metabolism of BRU59-21 was expressed as femtomoles per minute per milligram of lysate protein. Bars are the means ± SEM of three independent experiments.

accumulated activity up to 4 hr due to evaporation of the extracellular medium. There was no difference in the aerobic accumulation of radioactivity between CHO-VC and CHO-FpT-27 cells. Under hypoxic conditions, there was a constant increase in accumulated activity in CHO-VC cells, so that at 4 hr these cells had a C_{in}/C_{out} value of 60, representing a 20-fold increase in accumulated activity in hypoxic CHO-VC cells compared with aerobic cells. CHO-FpT-27 cells also exhibited an increase in accumulated radioactivity under hypoxic conditions. However, the increase was more rapid, and at 4 hr these cells had a C_{in}/C_{out} value of 175, which represents a 60-fold increase in accumulated activity compared with aerobic cells, and a 3-fold increased hypoxic accumulation of radioactivity compared with CHO-VC cells at 4 hr.

Metabolism of BRU59-21 in Stirred Suspension Cultures

The extractability into ethyl acetate of the radioactive material remaining in the supernatant of cellular aliquots under the same conditions as described above was also investigated (Fig. 6B). For aerobic CHO-VC and CHO-FpT-27 cells, over 90% of the radioactivity was initially extractable into ethyl acetate, and this value is consistent with the high lipophilicity of the drug. The fraction of radioactivity that was extractable into ethyl acetate when exposure occurred under aerobic conditions did not change appreciably over the time course of the experiment. In contrast, the percent of activity extractable into ethyl acetate decreased over time for hypoxic CHO-VC cells, and at 4 hr only ~70% of the activity was extractable into ethyl acetate. Supernatants from CHO-FpT-27 hypoxic cells also showed a greater decrease in the amount of radioactivity extractable into ethyl acetate, since only ~50% of the radioactivity was extractable into ethyl acetate at 4 hr. This finding is consistent with increased hypoxic metabolism of BRU59-21 in cells overexpressing P450 reductase.

The extracellular medium of BRU59-21 incubations was further investigated by HPLC (Fig. 7). After 4 hr, 90% of the radioactivity in the supernatant of aerobic CHO-VC and CHO-FpT-27 cells was the parent compound. In contrast, incubation for 4 hr under hypoxic conditions resulted in a substantial loss of the parent compound, such that it constituted only ~65% and ~50% of the total peak area in CHO-VC and CHO-FpT-27 cells, respectively. The remainder of the radioactivity was attributed to the hydrophilic metabolite. The differences in the amount of BRU59-21 remaining in the supernatant at the conclusion of the experiment between CHO-VC and CHO-FpT-27 cells are consistent with increased hypoxic metabolism of BRU59-21 in cells overexpressing P450 reductase. As well, it was found that using either ethyl acetate extraction

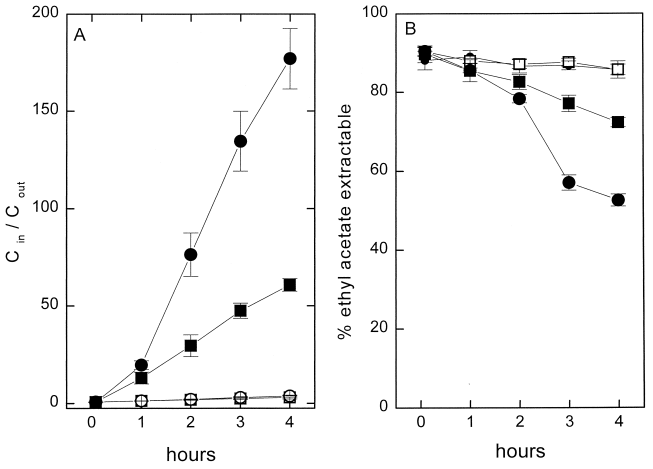

FIG. 6. Accumulation and metabolism of BRU59-21 in CHO-VC and CHO-FpT-27 cells. Cells were equilibrated for 30 min under aerobic or hypoxic conditions before the addition of BRU59-21. (A) The accumulation of BRU59-21 is expressed by the ratio C_{in}/C_{out} after the addition of BRU59-21. Symbols: aerobic CHO-VC (□), hypoxic CHO-VC (■), aerobic CHO-FpT-27 (○), and hypoxic CHO-FpT-27 (●) cells. Points are the means ± SEM of three independent experiments. (B) Metabolism of BRU59-21 as measured by the percent of total counts in the external medium that partitioned into ethyl acetate versus PBS as a function of time after the addition of BRU59-21. Symbols are the same as in panel A. Points are the means ± SEM of three independent experiments.

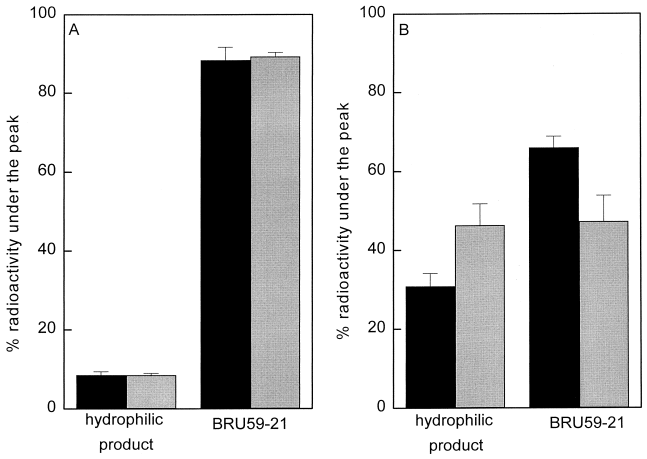

FIG. 7. HPLC analysis of the extracellular medium of cells after a 4-hr incubation with BRU59-21 under aerobic and hypoxic conditions. CHO-VC cells (black bars) and CHO-FpT-27 cells (grey bars) were incubated with BRU59-21 in suspension cultures under (A) aerobic and (B) hypoxic conditions. The extracellular medium was isolated, filtered, and analyzed by HPLC with a radioactivity detector set for ^{99m}Tc. Two distinct peaks were observed, and the data were plotted as the percent radioactivity under each of these peaks. Bars are the means ± SEM of four independent experiments.

values or HPLC analysis gave consistent values for the fraction of BRU59-21 in the external medium (data not shown).

DISCUSSION

The use of nitroimidazoles, which are selectively metabolized and retained in hypoxic cells, has been advocated as a means of measuring tumor hypoxia [32]. Assessment of tumor hypoxia requires that the cellular accumulation of these compounds be dependent on the oxygen concentration of the cell. Studies using ^{99m}Tc-labeled nitroimidazoles indicate that binding of the drug to cells is oxygen-dependent [8, 33]. However, quantitative measurements of tumor hypoxia require that the degree of drug accumulation be dependent only on oxygen concentration, and not on cellular factors needed for nitroimidazole accumulation.

Although considerable evidence suggests that the reduction and binding of 2-nitroimidazoles is dependent on bioreductive enzyme levels in the cell [11, 13, 15], little is known about the effect of enzyme levels on the accumulation and metabolism of ^{99m}Tc-nitroimidazoles. The high specific activity of ^{99m}Tc-nitroimidazoles enables the use of these compounds at very low (pM) drug concentrations [16], and it is possible that at such low drug concentrations, enzyme levels are not limiting, and the extent of drug accumulation is not affected.

The present study was conducted in cells that differed only in their P450 reductase levels [17]. This is considered a more direct approach for assessing the role of the enzyme in the accumulation of the drug, since the cells are of a common origin, presumably differing only in their P450 reductase levels. This reduces the possibility that any observed effect is due to genetic heterogeneity among the cells. The CHO-FpT-27 cell line, which overexpresses P450 reductase, appeared to differ from the vector-control cell line (CHO-VC) only in the activity of this enzyme. There was no difference in the levels of DTD or b_5 reductase between the two cell lines. However, the cells differed in the absolute values for enzyme activity obtained for P450 reductase as compared with previously published values [17]. It has been shown that temperature and phosphate buffer concentration can substantially increase P450 reductase activity [34]. However, altering both of these parameters had little effect on the activity of P450 reductase measured in the present study (data not shown). These quantitative differences in enzyme activity do not affect interpretation of the results in our study, since CHO-FpT-27 cells still show a highly significant increase in P450 reductase activity compared with control cells.

To assess the role of P450 reductase in the hypoxic metabolism of BRU59-21, cellular lysates of the P450 reductase-overexpressing and vector-control cells were prepared. Then the drug was incubated in the lysate mixture, which was supplemented with exogenous cofactor (NADPH or NADH). It was found that in hypoxic cellular lysates, overexpression of P450 reductase resulted in an 8-fold increase in BRU59-21 metabolism, and little metabolism of BRU59-21 was observed under aerobic conditions. These findings imply that the metabolism of BRU59-21 is hypoxia-specific and is an enzymatic process in which P450 reductase is an important enzyme.

Although the metabolism of BRU59-21 was reduced over 8-fold, there was not complete inhibition of BRU59-21 metabolism under air or at 4°. Control experiments performed without lysate, but under hypoxic conditions and with the addition of cofactor, indicated that there was some decrease in the amount of radioactivity extractable into ethyl acetate (data not shown). This indicates that the metabolism of BRU59-21 observed under aerobic conditions and at 4° is a result of background activity inherent in the assay and not indicative of true metabolism of the compound by cellular lysates.

The hypoxic metabolism of BRU59-21 in lysates overexpressing P450 reductase was dependent on the presence of NADPH. No increase in metabolism was observed with the addition of NADH, and the addition of both cofactors did not result in increased metabolism of BRU59-21 over NADPH alone. Since P450 reductase activity is dependent on the presence of NADPH, this finding further implicates P450 reductase in the hypoxic metabolism of BRU59-21 and suggests that metabolism of BRU59-21 may be limited by NADPH concentration.

To examine the role of P450 reductase in the hypoxic metabolism of BRU59-21 further, the effect of enzyme inhibitors on BRU59-21 metabolism was investigated. 2′-AMP and DPIC, inhibitors of P450 reductase [25–28], decreased the hypoxic metabolism of BRU59-21 in CHO-FpT-27 lysates in the presence of NADPH, using inhibitor

concentrations similar to those described previously [35]. To assess the possible role of other enzymes, the effects of inhibitors of b_5 reductase and DTD on the metabolism of BRU59-21 were determined. The hypoxic metabolism of BRU59-21 was not inhibited by *p*HMB or PTU, inhibitors of b_5 reductase [29, 30], or by the addition of dicumarol, an inhibitor of DTD [21]. These findings suggest that b_5 reductase and DTD are not involved in the hypoxic metabolism of BRU59-21 in lysates overexpressing P450 reductase.

The accumulation of BRU59-21 in suspensions of CHO-FpT-27 and CHO-VC cells was studied. The accumulation of BRU59-21 in both cell lines was hypoxia-specific, with cells overexpressing P450 reductase exhibiting 3-fold higher accumulation at 4 hr than vector-control cells. This finding shows that P450 reductase is important in the hypoxic accumulation of BRU59-21 in stirred suspension cultures and is consistent with the lysate results.

Previous studies investigating the accumulation of BRU59-21 in CHO-AA8-4 cells found a lower overall accumulation as compared with the present results from the P450 reductase-overexpressing cells [8]. The P450 reductase activity in CHO-AA8-4 cells (8 nmol/min/mg) is lower than the activity in CHO-FpT-27 cells (data not shown). Therefore, differences in the P450 reductase levels between the CHO-AA8-4 cells and the transfected cells used in the current study may account for these differences in the overall accumulation of BRU59-21. However, since the CHO-AA8-4 and CHO transfected cells are not of a common origin, the possibility that other genetic factors contributed to these differences in accumulation cannot be discounted.

The metabolism of BRU59-21 in the stirred suspension cultures of CHO-FpT-27 and CHO-VC cells was also investigated. Metabolism of BRU59-21 was hypoxia-specific, with cells overexpressing P450 reductase exhibiting increased metabolism compared with vector-control cells.

An area of active research in our laboratory is the identification of the products of BRU59-21 metabolism. Radiochemical reduction of BRU59-21 followed by HPLC analysis suggests that the hydrophilic product detected in our experimental system is free pertechnetate.* This suggests that BRU59-21 is unstable following reduction, leading to the release of pertechnetate from the compound. This may require that time be given for the washout of free radioactivity from the tumor before images are obtained. It should be noted, however, that the products of BRU59-21 metabolism that are retained in hypoxic cells have not been characterized. It is known that ~50% of the radioactivity accumulated in cells is retained after removing hypoxic cells from BRU59-21 exposure (Ballinger JR, unpublished data), indicating that there is significant trapping of metabolites. Additional investigation into these findings will allow further assessment of the use of BRU59-21 as a marker of tumor hypoxia.

The production of $^{99m}TcO_4$ as a metabolite of BRU59-21 bioreduction is thought to arise via reduction of the nitro group to form nitroso and/or hydroxylamine compounds known to be highly reactive with cellular macromolecules [6]. It is possible that the reduced nitroimidazole can react intramolecularly with the chelating moiety, contributing to the release of technetium. It has been shown previously that the hypoxic selective accumulation does not occur for a BRU59-21 analogue lacking the nitroimidazole [8]. This suggests that the redox center is the nitroimidazole. This is of some concern, since ^{99m}Tc-HL91, which also binds under bioreductive conditions selectively to hypoxic cells, lacks a nitroimidazole as a reduction site. It has been postulated that ^{99m}Tc in HL91 may be in a dioxo form rather than the mono-oxo form assumed for BRU59-21 [36], and reduction might be at the metal center itself. Further work will be required to confirm both the role of nitroreduction in the selective accumulation of BRU59-21 and the possible role of the metal center itself in metabolism of the drug.

Although it appears that P450 reductase is involved in the hypoxic metabolism and accumulation of BRU59-21, the possibility that other enzymes may contribute to the metabolism of this drug cannot be discounted. The cells used in the present study have also been transfected with rat DTD [37] and human b_5 reductase [31]. Overexpression of these enzymes resulted in increased hypoxic sensitivity to mitomycin C. However, the hypoxic accumulation of BRU59-21 in these cells was not increased compared with that in the vector-control cells (data not shown). This finding is consistent with the results using lysates overexpressing P450 reductase, which found that inhibitors of b_5 reductase and DTD had no effect on the hypoxic metabolism of BRU59-21.

Other cellular factors may also affect the accumulation of hypoxic markers. For instance, drug accumulation can be affected by known mechanisms of drug resistance, such as P-glycoprotein. However, it has been shown that the accumulation of the ^{99m}Tc-nitroimidazole BMS181321 is not dependent on P-glycoprotein [38], suggesting that this method of drug resistance may not affect the accumulation of this class of hypoxic markers. Nitroimidazole binding may also be affected by concentration gradients between blood vessels and necrotic regions that exist for nutrients other than oxygen. Specifically, low glucose concentrations have been shown to inhibit binding of misonidazole to tumor cells *in vitro* [39]. However, the effects of these cellular factors on ^{99m}Tc-nitroimidazole binding, at the very low drug concentrations used, have not been determined.

To further understand the role of P450 reductase in affecting the use of BRU59-21 as a hypoxic cell marker, isogenic tumors in mice might be developed that differ only in their P450 reductase levels. Through this approach, one would be able to discern whether increased P450 reductase

* Dr. Xiuguo Zhang, Ontario Cancer Institute, personal communication. Cited with permission.

levels result in increased measures of hypoxic cell fraction as determined by nuclear medicine imaging. If hypoxic marker binding in these isogenic tumors is strongly affected by P450 reductase, then BRU59-21 may not be able to provide quantitative measurements of tumor hypoxia. In this case, BRU59-21 may be most useful as a method of monitoring oxygenation within the same tumor over the course of therapy. Using such an approach, the tumor would act as the point of reference for drug accumulation and subsequently tumor oxygenation. By monitoring the changes in marker binding throughout the course of therapy in comparison with the initial accumulation, marker binding would reflect changes in oxygenation within the particular tumor irrespective of nitroreductase levels in the tumor.

In conclusion, the present study indicates that P450 reductase is involved in the hypoxic accumulation and metabolism of BRU59-21 in some models of tumor hypoxia, and further studies on the effect of nitroreductase levels on the use of BRU59-21 and other compounds as markers of tumor hypoxia are warranted.

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