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Modifying the *In Vitro* Accumulation of BMS181321, a Technetium-99m-Nitroimidazole, with Unlabelled Nitroaromatics

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ABSTRACT. BMS181321, [^{99m}Tc]oxo[[3,3,9,9-tetramethyl-1-(2-nitro-1*H*-imidazol-1-yl)-4,8-diazaundecane-2,10-dione dioximato]-(3)-*N*,*N*′,*N*″,*N*‴]technetium, is a ^{99m}Tc-nitroimidazole that is being investigated as a hypoxic marker in tumors. Due to the high specific activity of ^{99m}Tc, the concentration of BMS181321 used in its applications is very low. Metabolic depletion and non-specific binding of the drug may limit its ability to fully map out hypoxic regions. An attempt has been made to modify the *in vitro* accumulation of BMS181321 in hypoxic Chinese hamster ovary (CHO) cells with unlabelled nitroaromatics. The 2-nitroimidazole etanidazole (0.08 to 8 mM) caused a concentration-dependent decrease in BMS181321 accumulation to 70–28% and metabolism to 70–40% of the control level in hypoxic cells at 4 hr. In contrast, the 5-nitroimidazole tinidazole (0.09 to 9 mM) caused a concentration-dependent increase in BMS181321 accumulation to 110–170% and metabolism to 100–150% of the control level in hypoxic cells at 4 hr. Nitroaromatics with an electron affinity similar to or greater than that of BMS181321 inhibited its accumulation and metabolism, and 5-nitroimidazoles, which have an electron affinity lower than that of BMS181321, enhanced its accumulation and metabolism. The enhanced accumulation with the addition of metronidazole was not observed in the presence of low oxygen levels or of a nitrofuran of higher electron affinity than BMS181321. These results suggest that a competition for reducing equivalents and/or for the BMS181321 radical anion itself can occur in cells, leading to the inhibition of BMS181321 reduction in the presence of nitroaromatics of similar or greater electron affinity. A transfer of electrons from the radical anion form of the reduced 5-nitroimidazole to the more electron affinic BMS181321 compound may occur, causing increased hypoxic accumulation of BMS181321. BIOCHEM PHARMACOL **54**;6:685–693, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. BMS181321; 2-nitroimidazole; hypoxia; bioreductive; diagnosis; CHO cells

Hypoxic cells are thought to be a limitation for the local control of some types of solid tumors by radiation [1], but studies suggest that even for a given tumor type, not all tumors contain a significant fraction of hypoxic cells [2]. Unfortunately, there is no widely accepted method of assessing the extent of hypoxia in an individual tumor, although a number of approaches are being evaluated [3]. The general availability of a marker for tumor hypoxia would be a beneficial diagnostic tool, since it would allow the selection of patients who could benefit from therapies designed to target these treatment-resistant cells.

In 1979, it was first suggested that radiolabelled nitroimidazoles, which are selectively metabolized and retained in hypoxic cells, might be used to measure tumor hypoxia [4]. Misonidazole labelled with ^{3}H has shown localized accumulation in patients [5], and nitroimidazoles labelled with ^{18}F and ^{123}I have been investigated as possible non-invasive markers of hypoxia [6, 7]. However, these non-invasive compounds are not widely applicable because of the need for expensive isotopes, special chemical synthesis, and/or costly imaging equipment. The synthesis of BMS181321§ (Fig. 1), a 2-nitroimidazole labelled with ^{99m}Tc, offered the possibility of more routine and inexpensive imaging of hypoxia in damaged normal tissue or in tumors [8].

BMS181321 has been studied as a hypoxic marker in myocardial and cerebral ischemia. In isolated rat hearts, BMS181321 was found to accumulate under ischemia [9], and there was selective retention of BMS181321 in acutely ischemic rat brain following artery occlusion [10]. The use of BMS181321 to detect hypoxia in tumors has been

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§ *Abbreviations:* BMS181321, [^{99m}Tc]oxo[[3,3,9,9-tetramethyl-1-(2-nitro-1*H*-imidazol-1-yl)-4,8-diazaundecane-2,10-dione dioximato]-(3)-*N*,*N*′,*N*″,*N*‴]technetium; BMS181032, 4,8-diaza-3,3,9,9-tetramethyl-1-(2-nitro-1*H*-imidazol-1-yl)undecane-2,10-dione dioxime; etanidazole, *N*-(2-hydroxyethyl)-2-nitro-1*H*-imidazole-1-acetamide; misonidazole, 1-(2-hydroxy-3-methoxypropyl nitroimidazole); CHO, Chinese hamster ovary; α-MEM, minimum essential medium; NF167, 5-nitro-2-furaldehyde-5,3-diethyl-aminopyrole semioxamazone hydrochloride; RP8979, (hydroxy-2-ethyl)-1-methyl-2-nitro-4-imidazole; tinidazole, ethyl-2-(2′-methyl-5′-nitro-1′-imidazolyl)ethyl sulfonate; metronidazole, 2-methyl-5-nitroimidazole-1-ethanol; and ^{99m}Tc, technetium-99m.

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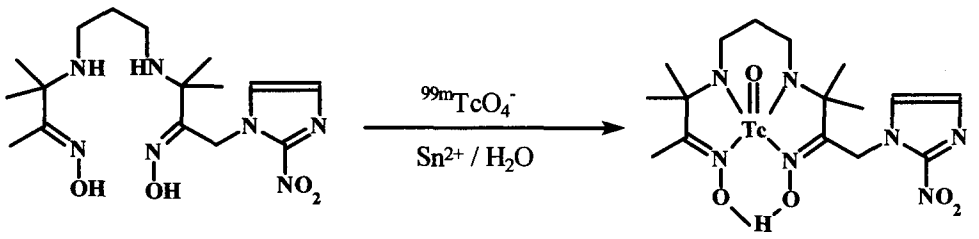

BMS181032 BMS181321

FIG. 1. Structure of BMS181321. BMS181321 is formed from the precursor compound BMS181032 by stannous chloride reduction of pertechnetate.

reported recently. It was found that BMS181321 is localized in rodent tumors *in vivo* and is accumulated preferentially in hypoxic cells *in vitro* [11]. Results from these *in vitro* studies showed that under hypoxia there was metabolic depletion of BMS181321, particularly at higher cell concentrations. Due to the high specific activity of ^{99m}Tc, BMS181321 is used at picomolar concentrations for imaging, which has led to a concern about the degree that this compound would be able to diffuse throughout the hypoxic regions present in the tumor without being metabolically consumed. Since BMS181321 is, in fact, a different compound than its ligand (BMS181032), one cannot vary its specific activity in a simple fashion. Increasing the concentration of BMS181321 is limited by its radiation hazard to the patient and surrounding personnel.

The need for nitroimidazoles to have complete access to the hypoxic cells present in the tumor has been addressed by others [12, 13]. If metabolic depletion of the nitroimidazole occurred in its clinical application, it could cause an underestimate of the hypoxic fraction present in the tumor. In the present work, the feasibility of using unlabelled nitroaromatics in conjunction with BMS181321 to modulate its rate of hypoxic accumulation was investigated in an *in vitro* model. These studies were designed to assess the feasibility of using these unlabelled nitroaromatics in conjunction with BMS181321 to alter its biodistribution *in vivo*. In the course of these studies, it was observed that the presence of added nitroaromatics could be stimulatory as well as inhibitory to BMS181321 accumulation and metabolism, depending on the electron affinity of the unlabelled nitroaromatic that was present.

MATERIALS AND METHODS

Chemicals and Reagents

Synthesis of the ligand BMS181032 (mol. wt = 383; Bracco Research, Princeton, NJ) and the reaction of the ligand with ^{99m}Tc-pertechnetate to form BMS181321 (mol. wt = 495) have been reported previously (Fig. 1) [8]. In brief, 0.4 mg of the ligand was dissolved in 0.8 mL of saline, and 0.1 mL of ^{99m}Tc-pertechnetate (250 MBq) was added. A 0.1-mL aliquot of stannous pentetic acid (DTPA) (Techneplex, Squibb Diagnostics, Princeton, NJ), which was reconstituted with 4 mL of saline, was added to the ligand and pertechnetate mixture. The formation of BMS181321 was complete within 10 min at room temperature. The radiochemical purity of BMS181321 was determined by paper chromatography and was greater than 90%. The majority of the impurity was free pertechnetate. The decomposition half-time of BMS181321 has been reported to be only 16–24 hr [11]. Dibutyl phthalate, ethyl acetate, α-MEM, and fetal bovine serum were from the Sigma Chemical Co. (St. Louis, MO). Vegetable oil was from Best Foods Inc. (Etobicoke, Canada). Etanidazole and misonidazole were gifts from the National Cancer Institute (Bethesda, MD). NF167 was from the Norwich Pharmacal Co. (Norwich, NY). RP8979 was from May & Baker Ltd. (London, England). Tinidazole was from Ortho Pharmaceutical (Don Mills, Canada) and metronidazole was from Poulenc Ltd. (Montreal, Canada).

Cells

CHO cells (subline AA8-4), obtained originally from Dr. L. H. Thompson of Lawrence Livermore Laboratories, Livermore, CA, were grown in suspension culture at 37° in α-MEM containing 10% fetal bovine serum (growth medium). Their doubling time was 12–14 hr when maintained in exponential growth.

Accumulation and Metabolism Studies

Exponentially growing cells were removed from suspension culture (2–4 × 10^5 cells/mL), centrifuged, and resuspended in fresh growth medium at a cell 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 [14]. Incubations were carried out in the absence or in the presence of either unlabelled etanidazole, misonidazole, NF167, tinidazole, metronidazole, or RP8979. Unlabelled drugs were added at 1-, 10- or 100-fold dilutions of maximum 5–9 mM final concentration in the growth medium. 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). The radioactive compound was then added to each vial at a final activity of approximately 0.25 MBq/mL and at a total drug concentration (BMS181321 + BMS181032) of approximately 0.2 μM. The concentration of labelled drug (BMS181321) was approximately 50 pM. These drug levels and the amount of radioactivity used were similar to those used for previous *in vitro* and *in vivo* studies [11].

Duplicate 0.1-mL aliquots were removed from the vials after 5, 60, 120, 180, and 240 min of incubation with BMS181321. The aliquots were layered over 1 mL of oil (dibutyl phthalate:vegetable oil, 4:1) at 4° and centrifuged at 10,000 *g* for 2 min in a microcentrifuge tube. The aqueous growth medium and the oil were aspirated, and the tube tip containing the cell pellet and residual oil was clipped and counted in an automatic γ-well counter (Picker-Pace 1, Picker Corp., Northford, CT). The counts were corrected for carry-through of radioactivity from the growth medium. When medium containing radioactivity but no cells was layered over oil and centrifuged, this carry-through of radioactivity was found to be approximately 0.7% of the counts in 0.1 mL of radioactive medium [11]. The ratio of radioactivity in 0.1 mL of packed cells (C_{in}), which contains approximately 5×10^7 cells [15], to the amount of radioactivity in an equal volume of growth medium (C_{out}) was calculated, and the data are presented in the form of the ratio C_{in}/C_{out}.

The amount and status of the radioactivity in the medium at each of the time points were investigated. A 0.3-mL sample was removed from the vial and centrifuged at 10,000 *g* for 2 min. A 0.1-mL aliquot of the supernatant was removed and its activity determined. A second 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 0.5 min to separate the phases. BMS181321 is neutral and lipophilic and extracts primarily (90–95%) into the organic phase, whereas the decomposition products and metabolites of BMS181321 partition into the aqueous phase. The phases were transferred to individual tubes and counted in the γ-well counter, and the percent of total counts extractable into the organic phase was calculated.

Cell Viability

Cell viability was determined by a colony formation assay. At the conclusion of the experiment, cells were plated in growth medium in 60-mm tissue culture dishes (Nunc, Roskilde, Denmark) and incubated for 8 days at 37° in a humidified incubator. The plates were then stained, and colonies of 50 or more cells were counted.

Statistics and Calculations

Points are the means ± SEM for three or more independent experiments. Linear fits were obtained by the method of least squares. Some accumulation and metabolism data for the unlabelled drug competition with BMS181321 are presented as a percentage of the control at 4 hr. The percent of control was calculated for each individual experiment and is equal to:

$$100\% \cdot [(\text{hypoxic}_B - \text{aerobic}_B)/(\text{hypoxic}_A - \text{aerobic}_A)]$$

where A is the 4-hr time point on the regression line for samples with only BMS181321 added (control samples) and B is the 4-hr time point on the regression line for samples with both BMS181321 and unlabelled nitroaromatic added. Student's *t*-test was used to determine the significance of difference between the means with a value of $P \leq 0.05$ considered significant.

RESULTS

The effect of hypoxic or aerobic conditions on the accumulation of BMS181321 in CHO cells as a function of time from 0–4 hr is illustrated in Fig. 2A. Aerobic cells showed extensive accumulation of activity at 5 min, and thereafter there was little further increase in accumulated activity up to 4 hr with a C_{in}/C_{out} value of approximately 20. This value indicated a 20-fold increase in the total cell-associated radioactivity over that in the same volume of medium. Under hypoxic conditions, there was a similar initial accumulation at 5 min, followed by a constant increase in accumulated activity, so that at 4 hr the amount of radioactivity within the cell was approximately 5 times greater than under aerobic conditions. These same results were obtained whether or not BMS181321 was separated from its unlabelled precursor BMS181032 before adding it to cells. The increase that was observed at 4 hr for aerobic incubations compared with the initial value was due to evaporation of the medium. However, the extent of evaporation (as measured at the conclusion of the experiment) was identical between hypoxic and aerobic samples, and did not influence the ratio of drug accumulation of hypoxic compared with aerobic incubations.

The extractability of the radioactive material remaining in the supernatant of cellular aliquots into ethyl acetate under the same conditions was also investigated (Fig. 2B). For aerobic cells, 90% of the activity was extractable into ethyl acetate, and this value is consistent with the amount of radiochemical impurity that is present in the drug preparation as measured by a paper chromatography technique. The amount of radioactivity that was extractable into ethyl acetate under aerobic conditions did not change appreciably over the time–course of the experiment. In contrast, the percentage of activity extractable into ethyl acetate decreased over the time–course of the experiment for hypoxic cells, and at 4 hr only 75% of the activity was extractable into ethyl acetate. Control experiments in which hypoxic incubations of the drug were carried out without cells showed results similar to those obtained for aerobic cells [11]. This decrease in the amount of activity extractable into ethyl acetate may be due to hypoxia-

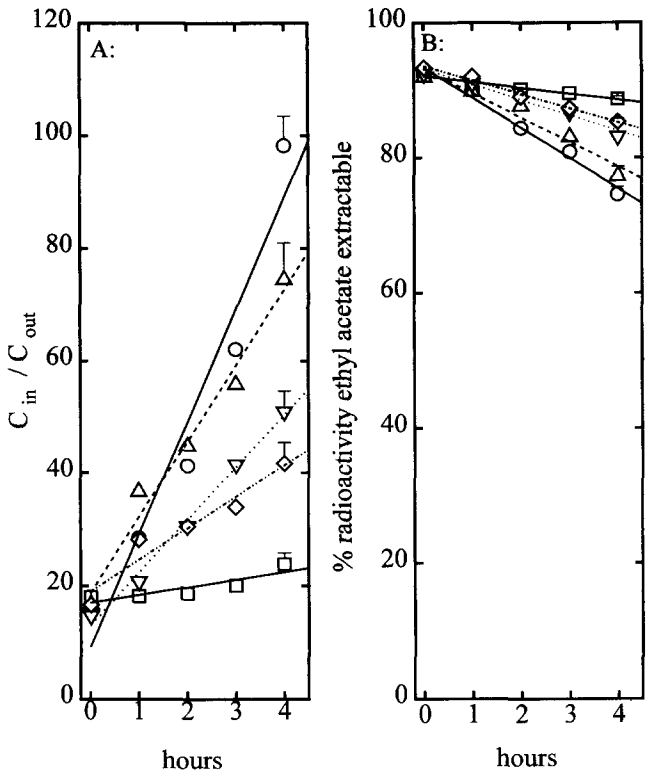

FIG. 2. Accumulation and metabolism of BMS181321 in the presence of unlabelled etanidazole in the growth medium. CHO cells were incubated with unlabelled etanidazole for 30 min while the system was equilibrating under aerobic and hypoxic conditions. Values are the means ± SEM for three or more independent experiments. (A) The accumulation of BMS181321 as expressed by the ratio C_{in}/C_{out} in the presence of unlabelled etanidazole plotted versus time after the addition of BMS181321. Symbols: aerobic control (□, –), hypoxic control (○, –), hypoxic incubation with 0.08 mM etanidazole (△, ----), hypoxic incubation with 0.8 mM etanidazole (▽, . . .), and hypoxic incubation with 8 mM etanidazole (◇, –..). (B) The percentage of total counts (~0.025 MBq) in the external medium that partitions into ethyl acetate versus PBS. Coding of the symbols is the same as in panel A.

dependent cellular metabolism of the drug followed by the release of more hydrophilic metabolites back into the external medium.

The amount of radioactivity remaining in the supernatant of aerobic cells did not change appreciably over 4 hr (data not shown). However, hypoxic cells showed a constant decrease in the amount of radioactivity in the supernatant, and at 4 hr there was a decrease in the total amount of radioactivity in the supernatant compared with the initial value. The activity depleted from the supernatant at 4 hr is approximately equal to the activity accumulated in the hypoxic cells. This indicates that a sequestration of radioactivity in hypoxic cells is occurring, and illustrates the potential problem of metabolic depletion of BMS181321 under hypoxia.

The 2-nitroimidazole etanidazole was selected for initial investigation of the effect of exogenous unlabelled drug on the accumulation of BMS181321 under aerobic and hypoxic conditions. The influence of three concentrations (0.08, 0.8, and 8 mM) of etanidazole on the accumulation of BMS181321 is also illustrated in Fig. 2A. Etanidazole did not affect BMS181321 accumulation in aerobic cells at the concentrations of etanidazole tested (data not shown). The addition of 0.08 mM etanidazole diminished BMS181321 accumulation in hypoxic cells to about 70% of the control level. The addition of higher concentrations of etanidazole decreased the amount of BMS181321 accumulation under hypoxic conditions and this effect was dose dependent; 0.8 mM etanidazole decreased the accumulation of BMS181321 to 40% of the control level and 8 mM etanidazole diminished the accumulation to 25% of the control level.

TABLE 1. Electron affinity of nitroaromatics used in the competition studies

Compound	Electron Affinity*	Reference
NF167	−0.28 V†	16, 17
Etanidazole	−0.41 V	17
Misonidazole	−0.41 V	16, 17
Tinidazole	−0.49 V	16, 17
Metronidazole	−0.51V	17
RP8979	−0.57 V‡	16, 17

* The electron affinities listed in this table take into account revisions to reference potentials [17] and data for closely related compounds [16]. Details of the reference compounds used have been described previously [16, 17].

† Assumed to be the same electron affinity as the mean electron affinity of nifuroxime, nitrofurazone, and nitrofurantoin.

‡ Assumed to be the same electron affinity as (2-hydroxy-3-methoxypropyl)-1-nitro-4-imidazole.

For hypoxic cells that were also incubated with unlabelled etanidazole, the total amount of radioactivity that was ethyl acetate extractable increased in proportion to the etanidazole concentration when compared with hypoxic cells with no unlabelled etanidazole added (Fig. 2B). These results indicate that etanidazole is able to decrease the net metabolism of BMS181321 in hypoxic cells.

The effect of other unlabelled nitroaromatics on BMS181321 accumulation and metabolism was investigated next. The unlabelled competitors used in these studies varied in their electron affinity (Table 1) to allow a determination of the generality and possible dependence on electron affinity for the accumulation and metabolism of BMS181321 in hypoxic cells. Full curves like those in Fig. 2 were determined for each drug. The results for etanidazole, misonidazole, and NF167 are summarized and expressed as a percent of the control results at 4 hr and are illustrated in Fig. 3. Misonidazole also inhibited the hypoxic accumulation and metabolism of BMS181321 and this inhibition was concentration dependent, with the greatest effect observed at a misonidazole concentration of 8 mM, where the accumulation of radioactivity was inhibited to approximately 20% (Fig. 3A) and metabolism to 30% (Fig. 3B) of the control level. Comparing etanidazole and misonidazole, there was no statistical difference in their ability to inhibit the accumulation and metabolism of

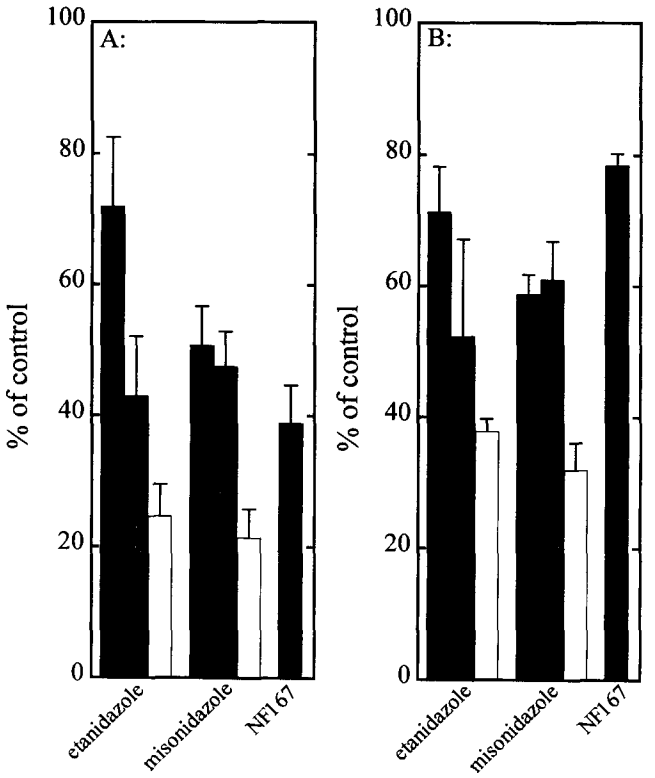

FIG. 3. Accumulation and metabolism of BMS181321 in hypoxic CHO cells incubated for 4 hr in the presence of different concentrations of unlabelled etanidazole, misonidazole, and NF167 in the growth medium. Values are the means ± SEM for three or more independent experiments. (A) Accumulation of BMS181321 expressed as a percent of the control cells (~0.005 MBq) with no added unlabelled drug. Different concentrations of the indicated unlabelled drug used were: black bars 0.08, 0.08, and 0.09 mM for etanidazole, misonidazole, and NF167, respectively; gray bars represent 0.8 mM etanidazole and misonidazole; and white bars represent 8 mM etanidazole and misonidazole. (B) Metabolism expressed as a percent of the BMS181321 that was ethyl acetate extractable in the supernatant of cells (~0.025 MBq) incubated with no added unlabeled drug. Coding of the bars is the same as in panel A.

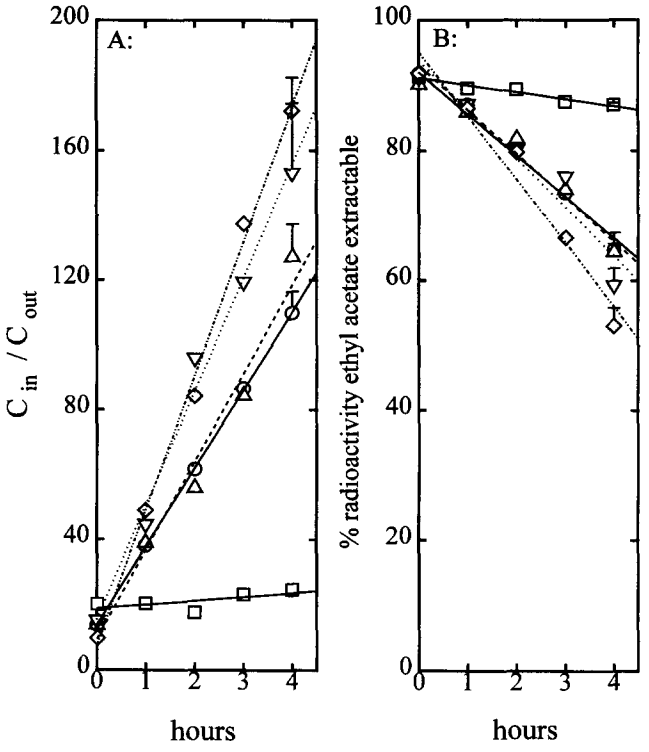

FIG. 4. Accumulation and metabolism of BMS181321 in the presence of unlabelled tinidazole in the growth medium. CHO cells were incubated with unlabelled tinidazole for 30 min while the system was equilibrating under aerobic and hypoxic conditions. Values are the means ± SEM for three or more independent experiments. (A) The accumulation of BMS181321 as expressed by the ratio C_{in}/C_{out} in the presence of unlabelled tinidazole plotted versus time after the addition of BMS181321. Symbols: aerobic control (□, –), hypoxic control (○, –), hypoxic incubation with 0.09 mM tinidazole (△, ----), hypoxic incubation with 0.9 mM tinidazole (▽, . . .), and hypoxic incubation with 9 mM tinidazole (◇, –..). (B) The percentage of total counts in the external medium (~0.025 MBq) that partitions into ethyl acetate versus PBS. Coding of the symbols is the same as in panel A.

BMS181321 under hypoxic conditions at equal concentrations (Student's *t*-test, $P > 0.05$). NF167 (0.09 mM) inhibited the accumulation of BMS181321 to 40% of the control level. Due to the high molar toxicity of NF167 compared with that of the nitroimidazoles [18], its effects at higher concentrations were not investigated. The results for the one concentration of NF167 tested were not significantly different from misonidazole and etanidazole at approximately equal concentrations in the accumulation studies ($P > 0.05$). These effects suggest that the results for etanidazole could be generalized to nitroaromatics of equal or greater electron affinity than BMS181321.

Compounds of lower electron affinity than the 2-nitroimidazoles were also investigated as possible modulators of BMS181321 accumulation and metabolism. Unexpectedly, the 5-nitroimidazole tinidazole caused a concentration-dependent stimulation of BMS181321 accumulation in hypoxic cells (Fig. 4A). The greatest stimulation was observed in the presence of 9 mM tinidazole, where the C_{in}/C_{out} value was 170 at 4 hr, which is 180% of the control level. Similarly, 9 mM tinidazole appeared to increase the metabolism of BMS181321 in hypoxic cells to 150% of the control level at 4 hr as only 50% of the total counts at 4 hr were extractable into ethyl acetate (Fig. 4B).

This stimulation in BMS181321 accumulation and metabolism was not unique to tinidazole. The 5-nitroimidazole metronidazole also caused a dose-dependent increase in BMS181321 accumulation and metabolism. The greatest stimulation was observed at the highest metronidazole concentration studied (5 mM), where the C_{in}/C_{out} value was 230% of the control level at 4 hr (Fig. 5A), and the percent of total activity extracted into ethyl acetate was 170% of the control level (Fig. 5B). There was no statistical difference between the stimulation observed with tinidazole and metronidazole (Student's *t*-test, $P > 0.05$). The 4-nitroimidazole RP8979, with an electron affinity even

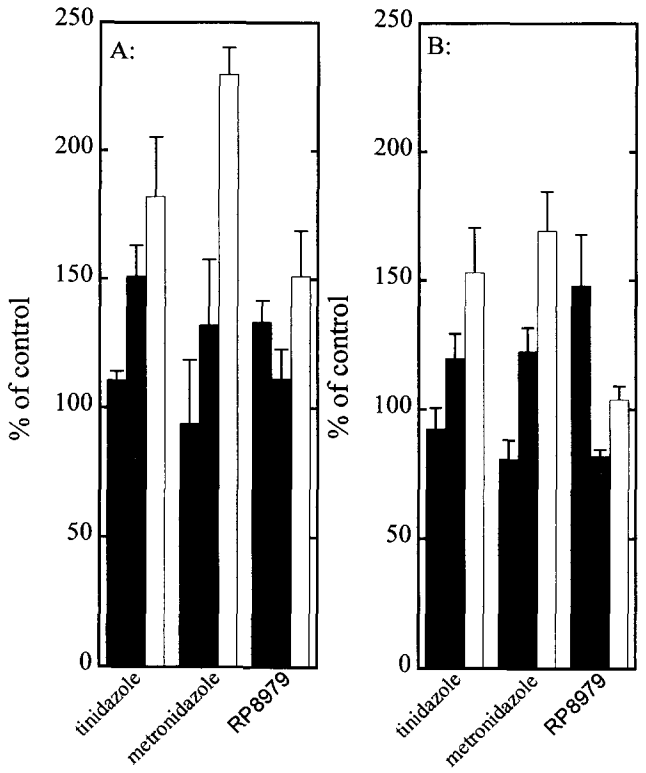

FIG. 5. Accumulation and metabolism of BMS181321 in hypoxic CHO cells incubated for 4 hr in the presence of different concentrations of tinidazole, metronidazole, and RP8979 in the growth medium. Values are the means ± SEM for three or more independent experiments. (A) Accumulation of BMS181321 expressed as a percent of the control cells (~0.005 MBq) with no added unlabelled drug. Different concentrations of the indicated unlabelled drug used were black bars 0.09, 0.05, and 0.08 mM for tinidazole, metronidazole, and RP8979, respectively; gray bars represent 0.9, 0.5, and 0.8 mM respectively, and white bars represent 9, 5, and 8 mM, respectively. (B) Metabolism expressed as a percent of the BMS181321 that is ethyl acetate extractable in the supernatant of cells (~0.025 MBq) incubated with no added unlabelled drug. Coding of the bars is the same as in panel A.

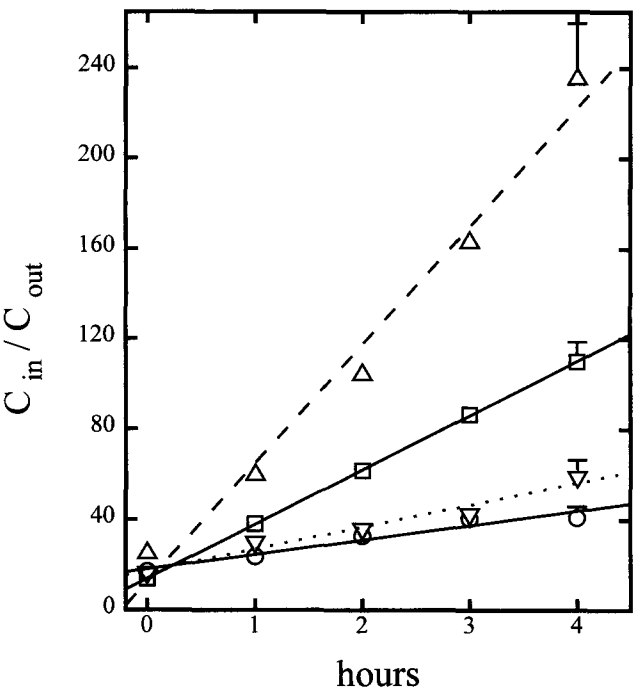

FIG. 6. Accumulation of BMS181321 in the presence of unlabelled metronidazole (5 mM) in the growth medium under hypoxic (95% N_2, 5% CO_2, <10 ppm O_2) and low oxygen (0.35% O_2, 5% CO_2, balance N_2, <100 ppm O_2) conditions. Symbols: hypoxic control (□, –), low oxygen control (○, —), hypoxic with metronidazole (△, – – –), and low oxygen with metronidazole (▽, – – –). Values are the means ± SEM for three or more independent experiments.

lower than that of the 5-nitroimidazole class of compounds, appeared to have little effect on BMS181321 metabolism and accumulation in hypoxic cells up to an RP8979 concentration of 8 mM (Fig. 5).

Previous studies have shown that BMS181321 accumulation is inhibited by low levels of oxygen, where a 50% inhibition of BMS181321 accumulation was seen at an oxygen concentration of approximately 40 ppm [11]. Therefore, the effect of low oxygen concentrations on the stimulation of BMS181321 accumulation and metabolism in the presence of unlabelled metronidazole was investigated. A gas mixture that contained 0.35% oxygen, which is equivalent to a dissolved oxygen concentration of less than 100 ppm [19], decreased the accumulation of BMS181321 in hypoxic cells to a C_{in}/C_{out} value of 50 at 4 hr (Fig. 6). The addition of unlabelled metronidazole (5 mM) had no effect on the accumulation of BMS181321 at this low oxygen concentration.

The effect of the simultaneous addition of both metronidazole (5 mM) and NF167 (0.09 mM) on the accumulation and metabolism of BMS181321 was also investigated. There was no enhancement of BMS181321 accumulation (Fig. 7A) and metabolism (Fig. 7B) under hypoxic conditions despite the addition of the same concentration of metronidazole that caused stimulation when added alone. In fact, the co-incubation of these two nitroaromatics caused an inhibition of BMS181321 accumulation and metabolism that was no different than that observed when NF167 was added alone (Student's t-test, $P > 0.05$).

DISCUSSION

BMS181321, a ^{99m}Tc-labelled 2-nitroimidazole, has been studied as a potential marker for tumor hypoxia. BMS181321 was found to preferentially accumulate in hypoxic cells, where at 4 hr there was approximately a 5-fold increase in the amount of radioactivity in hypoxic cells compared with aerobic cells. This is consistent with results that have been reported recently for CHO cells in the same system, though the reported accumulation was higher in that study [11]. Hypoxic cells showed a constant rate of accumulation in radioactivity over the time–course of the experiment. Such accumulation does not occur when the experiment is performed at 4° [11], which is consistent

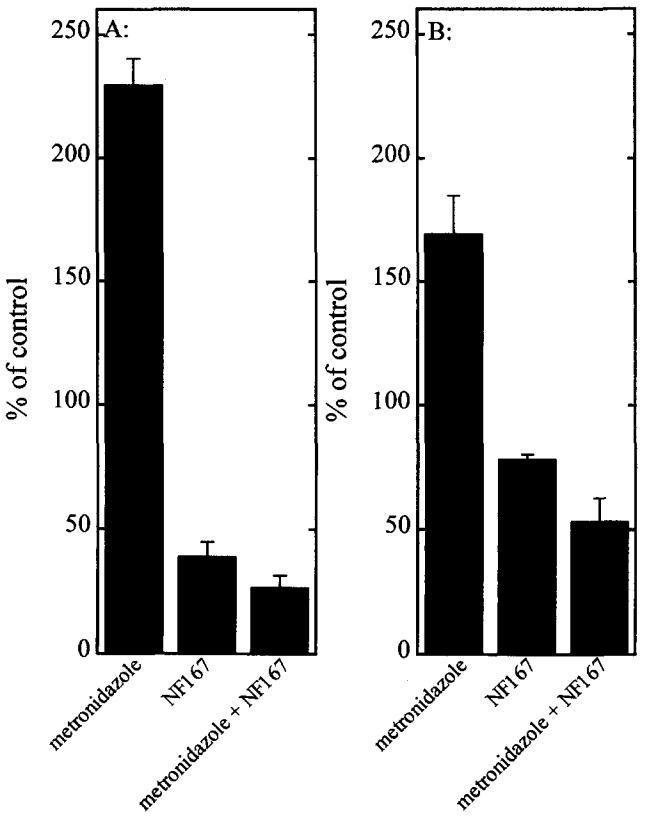

FIG. 7. Accumulation and metabolism of BMS181321 in hypoxic CHO cells incubated for 4 hr in the presence of metronidazole (5 mM) or NF167 (0.09 mM) in the growth medium, or a coincubation with metronidazole (5 mM) and NF167 (0.09 mM). Values are the means ± SEM for three or more independent experiments. (A) Accumulation of BMS181321 expressed as a percent of the control cells (~0.005 MBq) with no added unlabelled drug. (B) Metabolism expressed as a percent of the radioactivity that was ethyl acetate extractable in the supernatant of cells (~0.025 MBq) incubated with no metronidazole or NF167.

with an enzymatic process causing the sequestration of radioactivity in hypoxic cells.

Aerobic cells show an initial (5 min) accumulation of radioactivity, with a C_{in}/C_{out} value of approximately 20. There was little further accumulation of radioactivity in aerobic cells after this initial uptake. This high C_{in}/C_{out} value has been suggested to be due to the high lipophilicity of BMS181321, which has an octanol:water partition coefficient of 40 [11].

The form of the radioactivity present in the external medium during hypoxic incubations changes with time. In hypoxic cells, there is a decrease in the amount of radioactivity that is extractable into ethyl acetate. This change in the form of the radioactivity into more water-soluble products is thought to be due to hypoxia-specific intracellular metabolism of the drug, and the subsequent release of a portion of this altered form of radioactivity into the external medium [11]. The fact that there is no change in the form of the radioactivity when hypoxic exposure occurs with no cells present substantiates the proposal that the change in the lipophilicity of the radioactivity requires cellular processes. As well, when BMS181321 was reduced in the presence of xanthine oxidase, there was a substantial decrease in the amount of radioactivity extractable into ethyl acetate.* This suggests that reduction of the nitro group may be responsible at least partially for the formation of products that are less lipophilic than BMS181321 [8]. No further characterization of these putative reduction products is available at the present time. The metabolism of BMS181321 yielding the formation of more hydrophilic species is consistent with studies of hypoxic myocardium, in which it was found that BMS181321 underwent a metabolic change to more hydrophilic species [20].

Although some of the radioactivity appears to diffuse from the cell, there is sufficient accumulation of activity in hypoxic cells to indicate that BMS181321 possesses properties that allow it to act as a marker for hypoxic cells. However, in order for nitroimidazoles to act as quantitative markers for imaging tumor hypoxia, it is essential that the nitroimidazole have complete access to the target population of hypoxic cells present in the tumor [12]. Nitroimidazoles that are labelled with short half-life gamma emitters, such as ^{99m}Tc, are used at extremely low drug concentrations due to the high specific activity of the labelled drug. Therefore, if metabolic depletion of these drugs occurs, it is possible that the drug would not be able to diffuse to the entire hypoxic population, and an underestimate of the hypoxic fraction present in the tumor would occur.

When the amount of radioactivity in the external medium is measured over time, the total amount of radioactivity decreases substantially only for hypoxic cell incubations after exposure to BMS181321. This depletion can be explained by accumulation in hypoxic cells. Metabolic depletion of BMS181321 has been shown in CHO and HeLa cells at cell concentrations greater than 1×10^6 cells/mL using the same system reported in this study, where it was found that depletion of BMS181321 from the external medium led to a non-linear accumulation of radioactivity within the cells [11].

This paper addresses the feasibility of using unlabelled nitroaromatics to modulate the accumulation of BMS181321 under hypoxic conditions. In essence, the binding of BMS181321 in individual tumor cells would be decreased by adding nitroaromatics that might compete for the uptake, metabolism, and/or binding of BMS181321 in hypoxic cells. In theory, this should help to circumvent the problem of metabolic depletion of the hypoxic marker, because less of the drug would be metabolized in each cell, leaving more unmetabolized drug free to diffuse and label other hypoxic cells within the tumor. Decreasing the number of molecules of the hypoxic marker bound per cell would inevitably decrease the sensitivity of detection of small fractions of hypoxic cells, but this loss of sensitivity

* Unpublished data from this laboratory.

may be offset by more complete labelling of the hypoxic cell fraction in the tumor.

Etanidazole and misonidazole were investigated initially because of previous clinical experience and interest in using these 2-nitroimidazoles as hypoxic cell radiosensitizers [1]. Both drugs inhibited the accumulation and metabolism of BMS181321 only under hypoxic conditions. The inhibition was concentration dependent and was not caused by an increased cellular toxicity due to the co-incubation of these unlabelled nitroimidazoles with BMS181321, since very little toxicity was seen using colony formation assays (data not shown). NF167 also caused hypoxia-specific inhibition of BMS181321. The inhibition observed could be due to competition between BMS181321 and the unlabelled nitroaromatic for cellular reducing equivalents that are involved in the hypoxia-specific reduction of nitroimidazoles. Alternatively, a transfer of electrons from the radical anion species of BMS181321 to the unlabelled nitroaromatic present at an excess concentration may occur.

The concentrations of etanidazole and misonidazole needed to inhibit BMS181321 accumulation and metabolism under hypoxic conditions were over a million-fold higher than the concentration of BMS181321. It took an 8 mM concentration of either etanidazole or misonidazole to inhibit the hypoxic accumulation and metabolism of BMS181321 by approximately 80%, but significant inhibition was also observed at etanidazole and misonidazole levels that were over 100-fold lower than these initial concentrations. The high extracellular levels that are required are not due to low intracellular concentrations of unlabelled drug since misonidazole has been shown to partition equally inside and outside of the cell [15].

The maximum drug levels of etanidazole and misonidazole studied are approximately 10-fold higher than plasma levels observed in patients treated with nitroimidazoles as hypoxic cell radiosensitizers [21, 22]. However, such applications require multiple doses of drug to be administered over the course of fractionated radiotherapy. Single doses of nitroimidazoles have been administered that yield blood levels close to the maximum drug levels studied in these experiments. Although these results are not particularly encouraging from a clinical standpoint, they do offer some important insights into the mechanisms modulating the retention of nitroimidazoles in hypoxic cells.

Unexpectedly, the 5-nitroimidazoles metronidazole and tinidazole stimulated both the accumulation and metabolism of BMS181321 under hypoxic conditions. In previous studies, the nitrofuran AF-2 was found to stimulate the accumulation of misonidazole in 9L rodent cells [23]. This effect was surprising because AF-2 is more electron affinic than misonidazole. The most probable explanation for this observed increase in BMS181321 accumulation is that there is a transfer of electrons from the reduced 5-nitroimidazoles to BMS181321. The radical anion form of the 5-nitroimidazoles is less electron affinic than 2-nitroimidazoles [24], and would be predicted to be able to donate electrons to the 2-nitroimidazole. This would increase the bioreductive metabolism of BMS181321, leading to the observed increased hypoxic accumulation of BMS181321. The nitro radical anion of the 5-nitroimidazoles could also donate electrons to other metabolites of BMS181321. Further investigation into the validity of this model is needed.

RP8979, a 4-nitroimidazole that has an electron affinity even lower than that of the 5-nitroimidazole class of compounds, had little effect on either BMS181321 accumulation or metabolism under hypoxic conditions. If an electron transfer process between the less electron affinic radical anion species to BMS181321 is occurring, it is postulated that the electron affinity of RP8979 may be too low to be effectively reduced and to be able to transfer electrons to BMS181321. It has also been suggested that the lifetime of radical intermediates could play a role in the interaction of nitroimidazoles in mammalian cells [23]. If RP8979 radicals are much shorter lived than metronidazole or tinidazole radicals, then RP8979 could be bioreductively reduced to non-radical metabolites before its radical species can efficiently transfer electrons to BMS181321.

The stimulation in BMS181321 accumulation with the addition of 5-nitroimidazoles occurred only under conditions of extreme hypoxia, and higher but still very low oxygen levels (<100 ppm oxygen) inhibited the stimulation of BMS181321 accumulation. The most probable explanation for this observed lack of stimulation in BMS181321 accumulation at low oxygen concentrations is the oxygen sensitivity of metronidazole metabolism. It has been shown that less than 100 ppm of oxygen in the gas phase inhibits nitroreduction of metronidazole by 90% [25]. Thus, even an oxygen concentration as low as 100 ppm oxygen would be predicted to decrease the formation of the radical anion species of metronidazole substantially. Without adequate concentrations of the metronidazole radical anion, efficient transfer to BMS181321 would not be possible, and no enhanced accumulation of BMS181321 would be observed.

There was an observed inhibition of BMS181321 accumulation and metabolism when metronidazole (5 mM) and NF167 (0.09 mM) were added at levels that exhibited maximal stimulation and inhibition, respectively, when added separately. If the donation of electrons from less electron affinic compounds to more electron affinic counterparts is occurring, one would expect the least electron affinic compound to transfer electrons to the most electron affinic compound in the system. In this case, the radical anion form of metronidazole would be predicted to transfer electrons to NF167, at the expense of electron donation to BMS181321.

In conclusion, the initial purpose of this work was to evaluate the use of unlabelled nitroaromatics as an adjuvant drug to decrease the potential problem of metabolic depletion. It was found that nitroaromatics of a similar or higher electron affinity than BMS181321 could inhibit the accumulation of this hypoxic marker *in vitro*, while nitroaromat-

ics of lower electron affinity could stimulate the accumulation of the marker. This increased accumulation of BMS181321 in hypoxic cells with the addition of 5-nitroimidazoles offers the possibility of increasing the contrast between hypoxic and normal tissue during imaging. However, in both situations the effect on BMS181321 accumulation may not be practical clinically, since maximally effective drug concentrations would not be tolerated by normal tissues. This situation could change with the development of less toxic analogues of these compounds.

For metabolic depletion to limit the distribution of BMS181321, the rate of metabolism of the drug must be greater than its diffusion through the tumor mass. Recent work using an *in vitro* multilayer cell system [26] indicates that the rate of metabolism of BMS181321 may be slow relative to the diffusion rate.* Further work will need to be done to confirm this observation and the effects that unlabelled nitroaromatics have on the diffusion of BMS181321 through a tumor-like environment.

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* Unpublished data from this laboratory.