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REDUCTION OF FLUOROMISONIDAZOLE, A NEW IMAGING AGENT FOR HYPOXIA

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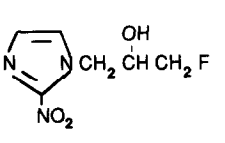
Abstract—[^{18}F]Fluoromisonidazole (1-(3-[^{18}F]fluoro-2-hydroxypropyl)-2-nitroimidazole, [^{18}F]FMISO) is a nitroimidazole compound that is being used as a new imaging agent for hypoxia. Because its uptake in hypoxic tissue is dependent on reduction of the nitro group on the imidazole ring, it is necessary to verify the availability of nitroreductase enzymes in a variety of tissues. FMISO reduction was studied using chemical and enzymatic reducing systems and mammalian cells. FMISO reduction by iron/HCl eliminated the absorbance peak at 325 nm caused by the nitro group. FMISO reduction by xanthine oxidase, as measured by a decrease in absorbance at 325 nm, occurred at a rate of 2.4 ± 0.3 nmol/min/unit enzyme (mean ± SEM, N = 15). This reaction was inhibited by allopurinol. Separation of the parent drug from its reduction product following chemical and enzymatic reductions indicated that iron/HCl reduced the majority of the FMISO molecules present, while xanthine oxidase did not. Reduction of FMISO by NADH dehydrogenase could not be demonstrated spectrophotometrically. Measurement of the reduction of FMISO in V79 cells based on the binding of [^{3}H]FMISO to cellular macromolecules was performed using a cell suspension in a three-neck flask. Hypoxic V79 cells bound [^{3}H]FMISO at the rate of 0.26 ± 0.07 pmol/10^{6} cells/min (N = 8). When specific inhibitors of two nitroreductase enzymes and a general inhibitor of electron transport were added to the cell suspension, no consistent, statistically significant inhibition of FMISO binding could be shown. We conclude that while inhibition of FMISO reduction by a purified nitroreductase can be shown, nitroreductase activity in cells is not inhibited so easily. This supports the hypothesis that nitroreductases are plentiful and will not limit the rate of FMISO reduction and uptake in hypoxic tumors or nonmalignant tissues.

Drugs such as misonidazole (MISO), which sensitize oxygen-depleted cells to the cytotoxic effects of inonizing radiation, have been promoted as markers for hypoxic cells in tumors and normal tissues. Radiolabeled MISO binds covalently to cellular macromolecules in an inverse relationship to oxygen concentration [1]. The potential of [^{18}F]-fluoromisonidazole (1-(3-[^{18}F]fluoro-2-hydroxypropyl)-2-nitroimidazole, [^{18}F]FMISO) as a radiopharmaceutical for positron emission tomography has been shown recently, both in tumors [2] and in ischemic myocardial tissue [3]. While the chemistry and biochemistry of MISO are well understood, less is known about FMISO. The work presented here confirms the similarity of FMISO and MISO and provides information about the enzymatic reduction of FMISO in mammalian cells.

FMISO (Fig. 1) has an octanol:water partition coefficient of 0.41 [4], allowing easy passage across cell membranes and rapid distribution to all tissues. Once in the cell, metabolism of FMISO is believed to be similar to that of MISO and other nitroaromatics (Fig. 1; [5]). Initial reduction of the nitro group to the nitro radical anion is accomplished by enzymes having nitroreductase activity. A number of redox-type enzymes are believed to cause reduction of nitroimidazoles [5]. In the presence of oxygen, the electron of the nitro radical anion is abstracted by oxygen, due to its higher electron affinity, resulting in regeneration of the unreduced drug and no net change in the cellular concentration of the drug (the "futile cycle" of nitroimidazole metabolism [6]). In

STRUCTURE OF FLUOROMISONIDAZOLE



REDUCTION OF NITROIMIDAZOLES

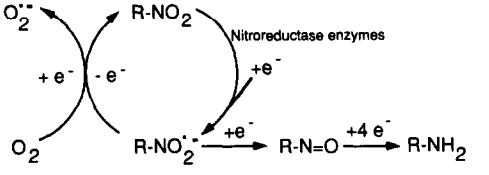


Fig. 1. Structure of FMISO and reduction of nitroimidazoles. FMISO is an analog of the nitroimidazole MISO, where the methoxy group on the side chain of MISO has been replaced by a fluorine atom. In the reduction scheme, nitroimidazoles are represented as an organic molecule (R-) with a nitro group (-NO_2) attached. The scheme shows the addition of the first electron and reoxidation by oxygen (futile metabolism) and the competing reaction, the complete reduction of the nitro group to the amine in the absence of oxygen.

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the absence of oxygen, the drug undergoes further reduction to a series of intermediates, which bind to cellular macromolecules, restricting diffusion out of the cell. The end result is a net accumulation of the drug in cells that are viable but oxygen-depleted. If the reduction of FMISO follows this scheme, then the administration of [^{18}F]FMISO should enable one to image hypoxic cells using positron emission tomography.

An understanding of the mechanism of radiopharmaceutical uptake is important to effective imaging and image interpretation. To this end, this paper addresses some questions about FMISO biochemistry. Two hypotheses are tested: one, that reduction of FMISO is similar to that of MISO; and two, that nitroreductases which will reduce FMISO are plentiful and numerous in a given cell type. The first hypothesis, if true, would make a great deal of MISO research relevant to predicting the behavior of FMISO. The second hypothesis is crucial to the use of FMISO as an imaging agent in a variety of tumors derived from different tissues in humans.

The similarity of FMISO reduction to MISO reduction was tested using a chemical reducing system (iron/hydrochloric acid) and two enzymes, xanthine oxidase (EC 1.2.3.2) and NADH dehydroxygenase (EC 1.6.99.3). The iron/HCl reaction is known to reduce aromatic nitro compounds to their amine derivatives and was used to characterize the final product of FMISO reduction. The enzymes were chosen because of their commercial availability, their ubiquitous distributions, and their known ability to reduce aromatic nitro compounds. Xanthine oxidase has been shown to cause nitroreduction of MISO and other nitroimidazoles [7, 8]. It is found in many tissues in dehydrogenase form and is converted to xanthine oxidase under hypoxic conditions. NADH dehydrogenase, a mitochondrial enzyme, is also widely distributed and has been shown to cause reduction of nitrobenzenes [9]. It is thus possible that these enzymes, among others, would be involved in nitroreduction of FMISO *in vivo* and it was deemed appropriate to test their *in vitro* ability to accomplish the same reaction.

If FMISO is to be used as an imaging agent, the degree of binding should ideally be determined only by the oxygen level and should not be limited by the availability of nitroreductase enzymes or other chemical factors in cells. Therefore, the second hypothesis addressed in this paper is that nitroreductases are plentiful and ubiquitous in a given cell type. This was tested using V79 cells in suspension, where reduction of FMISO was correlated with binding to cellular macromolecules [10]. Allopurinol (a specific inhibitor of xanthine oxidase), 2′-AMP (a specific inhibitor of NADH dehydrogenase), and KCN (a general inhibitor of electron transport) were added to cell suspensions and their effects on the binding rate were measured.

MATERIALS AND METHODS

FMISO and MISO were gifts of the Division of Cancer Treatment of the National Cancer Institute. FMISO was tritiated in our laboratory [4]. Xanthine oxidase, xanthine, allopurinol, NADH dehydrogenase, NADH, cytochrome *c*, and 2′-AMP were obtained from the Sigma Chemical Co. (St. Louis, MO) and were used without further analysis or purification. Other chemicals used were analytic or reagent grade. Materials used in tissue culture were also obtained from the Sigma Chemical Co. with the exception of fetal bovine serum, which was obtained from Flow Laboratories, Inc., Rockville, MD. Eagle's Minimum Essential Medium was supplemented with HEPES buffer (25 mM), penicillin (10 I.U./mL), streptomycin (10 mg/mL) and fetal bovine serum (10%, v/v). Hank's balanced salt solution (HBSS, calcium- and magnesium-free) was used as a diluent and washing agent.

Chemical reduction of FMISO utilizing iron and hydrochloric acid was modified from the procedure described by West [11]. FMISO (0.5 g, 2.66 mmol) spiked with [^{3}H]FMISO (800 kBq/mg, 153 MBq/mmol, 1.15 mmol) was dissolved in 20 mL methanol (final concentration 133 mM, 8.8 kBq/mL) and 1 mL concentrated HCl was added. After the solution was heated to boiling, 0.5 g iron (100 mesh) was added to the solution in four aliquots. The solution was refluxed for 2.5 hr, and timed samples were taken for spectrophotometry and thin-layer chromatography (TLC). Silica gel plates (EM Science $60F_{254}$, 5 × 20 cm) and a solvent system of $CHCl_3$:methanol (93:7) were used for TLC. Unreduced [^{3}H]FMISO was used as a standard on the TLC plates, each lane of which was cut into 1-cm pieces following development. For liquid scintillation counting, 5 mL of scintillation fluid (Instagel, Packard Instrument Co., Inc., Downers Grove, IL) was added to each 1-cm piece of the TLC plate in a liquid scintillation vial. The vials were counted in a tritium window with a Packard Tricarb 2660 liquid scintillation counter. Quench correction was performed by the external standard method. Optical spectroscopy was performed on a Varian DMS 100 UV/visible spectrophotometer.

Enzymatic reduction of FMISO by xanthine oxidase was measured spectrophotometrically using the method of Josephy *et al.* [7]. Briefly, xanthine (0.33, 0.67 or 1.33 mM) and FMISO (0.17 or 0.10 mM) were used as the reducing and oxidizing substrates, respectively. The solutions were made anoxic by bubbling for 15 min with argon gas. At 37°, one unit of xanthine oxidase in 0.5 mL was added to 2.5 mL of reaction mixture and the absorbance at 325 nm was monitored for 10–45 min. The reaction rate was determined from the initial slope of the plot of absorbance versus time, and converted to nanomoles per minute using the FMISO extinction coefficient 7500 $M^{-1}\,cm^{-1}$ at 325 nm. Allopurinol and 2′-AMP were added to test inhibition. To compare the chemical and enzymatic reductions of FMISO, the above procedure was followed with [^{3}H]FMISO (153 MBq/mmol, 1.15 mmol) added to the cuvette prior to addition of the enzyme. In addition to spectrophotometric measurements, 0.05-mL aliquots were removed at timed intervals and applied to TLC plates as described above.

Enzymatic reduction using NADH dehydrogenase [12] employed NADH (6 mM) as the reducing substrate and FMISO (300 mM) as the oxidizing

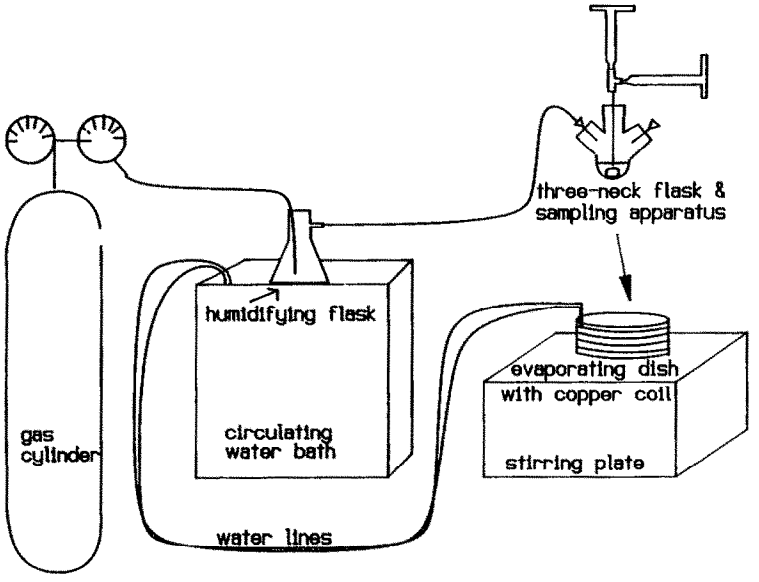

Fig. 2. Experimental apparatus for measurement of FMISO reduction and binding in V79 cells. The water bath circulates warm (40°) water through Tygon tubing to a copper coil, which sits in a 50 × 100 mm crystallizing dish filled with water. Some cooling occurs in transit, so that the evaporating dish temperature is 37°. Gas from the tank passes through sterile deionized water in a humidifying flask at 40° before entering the three-neck flask via a 20-gauge needle plugged with sterile cotton. An identical vent needle allows the gas to escape, and a 3-in, 22-gauge needle in the center neck, connected via a three-way stopcock to two 1-cc. syringes, is used for sampling the cell suspension.

substrate. Again, the reaction was followed spectrophotometrically following addition of 0.05 to 1.5 units of NADH dehydrogenase (final enzyme concentration 0.02 to 0.5 units/mL). The effect of 2′-AMP and allopurinol on the natural reaction (NADH plus cytochrome *c*) was measured.

V79 Chinese hamster fibroblasts (from Dr. Eric Hall, Columbia University) were used for *in vitro* FMISO binding studies. The cell line was maintained by monolayer growth and twice weekly subculture in Eagle's Minimum Essential Medium with 10% fetal bovine serum. The technique for measuring FMISO binding in V79 cells was derived from the method of Chapman *et al.* [10]. Rather than using a custom-made glass vessel, a three-neck, round-bottom flask (15 mL capacity) was used. A suspension of V79 cells ($1–2 \times 10^7$ cells in 10 mL) was placed in the flask along with a magnetic stirring bar. The three ports of the flask were covered with serum stoppers, two of which were pierced with sterile, cotton-plugged needles for gas inlet and gas outlet. The third port was pierced with a 22-gauge, 3-in. needle connected to a three-way stopcock and 1-cc syringes for sampling. The flask was placed in a water bath at 37° and stirred continuously at 200 rpm. The cell suspension was gassed for 1 hr with 95% N_2, 5% CO_2 for hypoxia experiments or 95% air, 5% CO_2 for aerobic experiments, at a rate of at least 180 mL/min [13]. The gas mixture was humidified by bubbling it through deionized water at 37° before it entered the three-neck flask. This apparatus is diagrammed in Fig. 2.

Binding of [^{3}H]FMISO was measured as follows: after 60 min of gas flow, [^{3}H]FMISO (6.7 MBq/mg, 1.27 GBq/mmol, 170 nmol) was added to the cell suspension ([^{3}H]FMISO concentration 17 mM, 22 kBq/mL), which was maintained for 4 hr with stirring and continued gas flow at 37°. At half-hour intervals, 0.5 mL of the cell suspension was removed. From each sample, 0.2 mL was used to determine cell concentration and 0.2 mL was added to 2 mL of cold trichloroacetic acid (TCA) solution (5%, w/v) to precipitate the macromolecular or acid-insoluble fraction. The TCA solution was filtered through a 0.45 μm filter (Millipore Corp.), washed with two aliquots of cold TCA (3 mL each), and placed in a scintillation vial. The filters were dried for 15 min in a 65° oven. Activity of tritium was determined by liquid scintillation counting and expressed as dpm/10^6 cells. The results were plotted versus time and the slope was determined by linear regression.

FMISO binding *in vitro* had been shown to occur over a range of oxygen levels bracketing that for radiobiological hypoxia [14]. To verify the creation of a radiobiologically hypoxic environment, the entire apparatus shown in Fig. 2 was placed in front of a 6000 Ci ^{137}Cs beam irradiator (J. L. Shepherd & Associates, model 81–14) and aerobic and hypoxic cell survival measured. The cell flask was placed in the water bath at 37° and maintained for 1 hr under air/CO_2 or N_2/CO_2 at a flow rate of at least 180 mL/min, with stirring. The cell concentration was 1.2×10^6 cells/mL for the hypoxic experiment and 3.8×10^5/mL for the aerobic experiment. The suspension was then irradiated in 2-Gy increments at a rate of 1.3 Gy/min to a total of 14 Gy. Cell

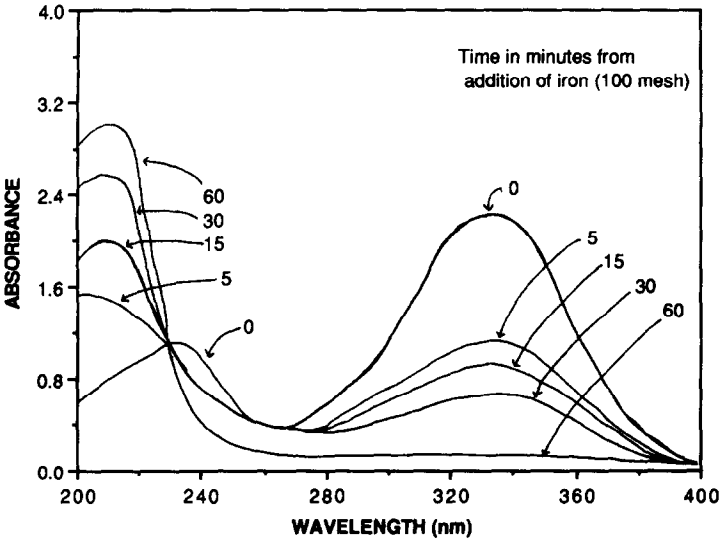

Fig. 3. Serial spectra of FMISO during reduction by iron (100 MESH) and HCl. Timed samples were taken from the FMISO/iron/HCl reaction, diluted 1:500, and analyzed spectroscopically. Spectra from samples at 0 (addition of first iron aliquot), 5, 15, 30, and 60 min are shown on the same graph. Total loss of absorbance at 325 nm occurred by 60 min. Details of the reduction conditions are given in Materials and Methods.

survival was determined by a standard colony formation assay. The surviving fraction was plotted versus radiation dose on a semilogarithmic plot and the points were fitted to the equation:

$$S = 1 - (1 - e^{-D/D_0})^n$$

where S = surviving fraction, D = dose, D_0 = the dose that reduces the survival by 63% in the linear portion of the curve, and n = extrapolation number. The oxygen enhancement ratio (OER) was calculated as the ratio of the hypoxic and aerobic D_0 values [15].

To study the effects of general and specific enzyme inhibitors on [^{3}H]FMISO binding by V79 cells, two cell suspensions of the same volume and concentration were prepared from the same subculture. These were put into identical three-neck flasks and each was placed into an apparatus like that of Fig. 2. One flask then received a solution of inhibitor dissolved in medium, while the other flask received an equal volume of unaltered medium. The inhibitors used and their concentrations were: allopurinol (300 mM) [10], 2'-AMP (500 mM) [16], and KCN (5 mM) [17]. The flasks were gassed for 1 hr with 95% N_2, 5% CO_2, after which [^{3}H]FMISO (17 mM, 22 kBq/mL) was added to each flask. Samples were taken and treated as above. At the end of each experiment, a sample of the cell suspension was removed to a Petri dish for a colony formation assay, to ensure that the inhibitors were not toxic to the cells. Uptake for each pair of flasks was plotted versus time and the slopes were obtained by linear regression. Thus, for each experiment, the binding rates of the control and inhibited cell suspensions could be directly compared. Student's t-test was used to test the hypothesis that the ratio of the slopes (inhibited:control) was equal to one. A paired t-test was used to test the hypothesis that the difference of the slopes (control-inhibited) was equal to zero.

RESULTS

Chemical reduction was performed as a non-enzymatic comparison to the enzyme experiments. Reduction by iron caused complete loss of the absorbance peak at 325 nm (Fig. 3). TLC showed loss of the original compound and an increase in the reduced derivative (Fig. 4). Reduction of FMISO based on TLC and liquid scintillation counting indicates that iron/HCl reduced 80% and xanthine oxidase only about 35% of the starting amount of FMISO. The R_f values for FMISO and its reduced derivative matched those obtained previously in this laboratory following reduction of FMISO with a Pd/charcoal catalyst and H_2 gas, a reaction known to produce the amine derivative of MISO [18].

The normal reactivity of xanthine oxidase was shown by demonstrating production of uric acid (measured by the increase in absorbance at 295 nm) in the presence of oxygen and the lack of uric acid production in anaerobic solution. The lack of FMISO metabolism in the presence of oxygen was demonstrated with a reaction mixture of xanthine and FMISO made fully aerobic by bubbling it with pure O_2. No loss of absorbance at 325 nm was seen in this instance. The rate of reaction of FMISO and xanthine with xanthine oxidase under anoxic conditions is given in Table 1. Also shown is the reaction rate of xanthine and xanthine oxidase with MISO, with literature values for comparison. FMISO was less reactive by a factor of 2.4 when compared to its parent drug, MISO.

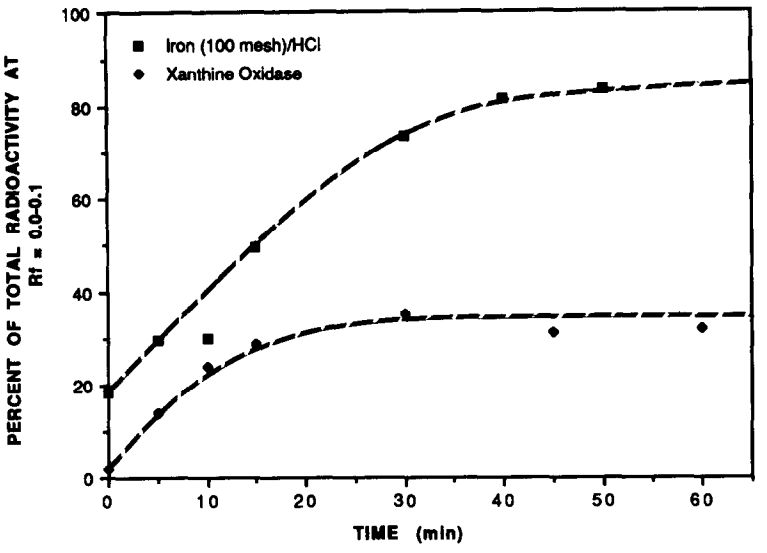

Fig. 4. Chemical versus enzymatic reduction. Details of both reduction methods are in Materials and Methods.

Table 1. Reaction rates of xanthine oxidase with various substrates

Oxidizing substrate	Oxygen status	Reaction rate (nmol/min/unit enzyme)	Number of experiments	Literature value and reference
Oxygen	Aerobic	370 ± 4	5	1750 [5]
FMISO	Anaerobic	2.4 ± 0.27	15	
MISO	Anaerobic	5.7 ± 0.7	2	6 [5]
Oxygen*	Aerobic	310	1	8 [6]
FMISO*	Anaerobic	2.2 ± 1.2	2	

The reducing substrate was xanthine. Reaction rates are given as means ± SEM, with the number of replicate experiments given, or mean ± range where N = 2.
* In these experiments, 2'-AMP was added to the reaction cuvette.

Allopurinol was shown to inhibit both oxidation of xanthine to uric acid and aerobic solution and FMISO reduction in anaerobic solution. In both cases, as the allopurinol concentration was increased, the reaction rate decreased dramatically. This is illustrated for FMISO in Fig. 5. A specific inhibitor of NADH dehydrogenase, 2'-AMP, did not cross-react with xanthine oxidase (Table 1).

To compare enzymatic with chemical reduction, [^{3}H]FMISO was added to the xanthine oxidase reaction mixture, and TLC was performed on timed samples. Unreduced FMISO migrated with an R_f of 0.30. The reduction product of the iron/HCl experiment migrated to an R_f of 0.07 in this TLC system, whereas the reduction product of the xanthine oxidase experiment did not migrate from the origin. Figure 4 shows the proportion of radioactivity in the reduction product(s) for each experiment.

NADH dehydrogenase reduces cytochrome *c* with a reaction rate of 6.5 ± 0.8 nmol/min/unit (N = 4) [12]. Inhibition of this reaction by 2'-AMP was also shown. The reaction rate of cytochrome *c* reduction by NADH dehydrogenase in the presence of 333 mM allopurinol was essentially the same as the reaction rate in the absence of this inhibitor of xanthine oxidase. However, FMISO did not react with NADH and NADH dehydrogenase under anaerobic conditions, as measured spectrophotometrically. This was still true after adding up to 1.5 units of enzyme to the cuvette.

Binding of [^{3}H]FMISO to acid-precipitated macromolecules in V79 cells incubated under hypoxic conditions was measured at a rate of 0.26 ± 0.07 pmol/10^6 cells/min (N = 8). Figure 6 shows the increase in dpm/10^6 cells over time in hypoxic cells in a sample experiment. The binding rate was 0.15 pmol/10^6 cells/min. Also shown in Fig. 6 is a binding curve for cells equilibrated in air/CO_2. With aerobic conditions, the binding did not increase over time as indicated by the slope of 0.003 pmol/10^6 cells/min. Some trapped radioactivity is seen, but the amount of binding did not increase over time. This negative result under aerobic conditions verified that adequate gas exchange was occurring.

A truly hypoxic environment is the most crucial

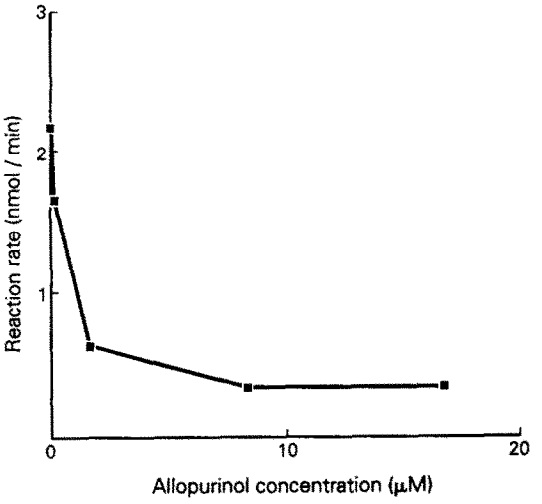

Fig. 5. Inhibition of FMISO reduction by allopurinol. Increasing amounts of allopurinol were added to a reaction mixture of xanthine, FMISO, and xanthine oxidase, and the reaction rate was measured.

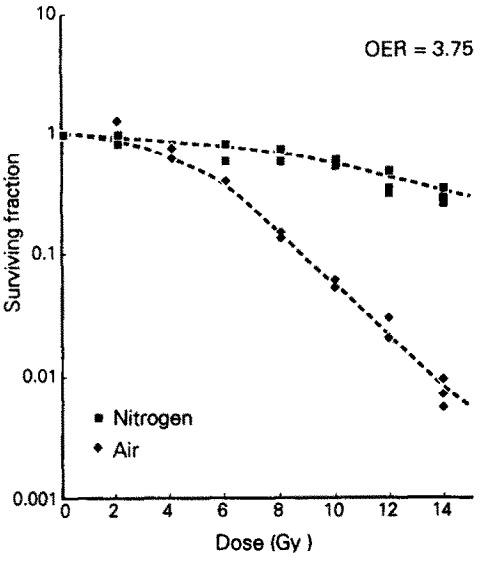

Fig. 7. Cell survival of V79 cells following irradiation in air or nitrogen. Each point represents the average surviving fraction of three plates plated with the same cell density at the given dose. The points are fitted to the equation $S = 1 - (1 - e^{-DD_0})^n$, where S = surviving fraction, D = dose, D_0 = the dose that reduces the survival by 63% in the linear portion of the curve, and n = the number of the targets. The oxygen enhancement ratio (OER) was calculated by dividing the hypoxic D_0 by the aerobic D_0.

factor for FMISO reduction and binding, so the existence of hypoxia was tested independently based on cell survival following irradiation [15]. Survival of V79 cells irradiated in this system are shown in Fig. 7. The D_0 values were measured to be 2.0 and 7.7 Gy for the aerobic and hypoxic cell suspensions, respectively. The oxygen enhancement ratio, calculated by dividing the hypoxic D_0 by the aerobic D_0, was 3.75.

Two FMISO binding experiments were done with each of the specific inhibitors. One experiment with KCN only and two experiments with both 2′-AMP and KCN were also done. The results of the seven experiments are summarized in Table 2. The slopes of the binding curves for the paired cell suspensions are given, as well as their ratio (inhibited/control) and the difference (control − inhibited).

Statistical analysis used either the ratio or the

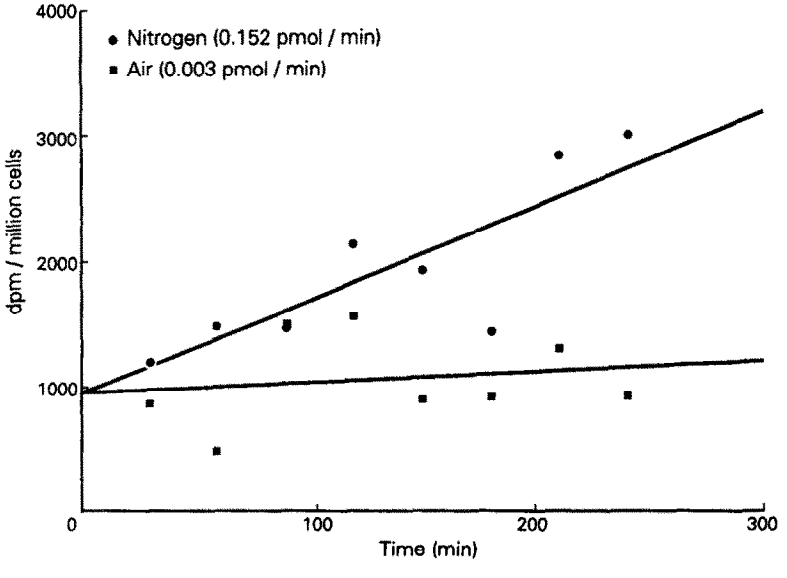

Fig. 6. Binding of [^{3}H]FMISO to the acid-insoluble fraction of V79 cells under hypoxic or aerobic conditions. The graph shows the result of a single experiment.

Table 2. Reaction rates of [^{3}H]FMISO in V79 cells with various inhibitors

Inhibitor	Slope of binding curve (dpm/million cells/min) Control	Inhibited	Ratio	Difference
Air	7.6	0.2	0.03	
KCN	4.0	3.0	0.8	1.0
2′-AMP	6.3	10.0	1.6	−3.7
2′-AMP	9.1	10.6	1.2	−1.5
Allopurinol	13.7	9.4	0.7	4.3
Allopurinol	7.0	8.7	1.2	−1.7
2′-AMP + KCN	34.4	20.8	0.6	13.6
2′-AMP + KCN	22.3	18.4	0.8	3.9
Mean			1.0	2.2
SEM			0.1	2.2

difference of the control and inhibited slopes [19]. In the former case, the null hypothesis is that the two slopes are equal, or that the ratio of the slopes is one. If the null hypothesis is true, the ratio of the slopes should obey a normal distribution, and statistical analysis using Student's *t*-test is appropriate. In the latter case, the difference of the two slopes is taken, and the null hypothesis is that the average of the differences is zero. Again, if the null hypothesis is true, the difference should obey a normal distribution; this is the basis of the paired *t*-test. For both tests, the *P* value for the test statistic was greater than 0.10, indicating that the null hypothesis may not be rejected, or that there was no significant difference between the two slopes.

DISCUSSION

An understanding of the biochemical basis of tracer localization of a radiopharmaceutical is necessary to determine its appropriate use for imaging. While it is possible to interpret nuclear medicine images just from the standpoint of pattern recognition and correlation, more information is available if the mechanisms of localization are understood. For example, gallium-67 citrate was developed in the 1970s to image infections and malignancies, but fell out of favor in part because its uptake mechanism in tissues was not adequately understood [20, 21]. Some of the problems were: inconsistent uptake among tumors (even within the same histologic class); inconsistent uptake from site to site within the same patient; and unacceptably high false positive and false negative rates. These problems emphasize the value of delineation of the mechanism(s) of uptake of FMISO in normal and abnormal tissues.

Chemical and enzymatic reduction experiments verify the similarity of FMISO to MISO. The reduction of FMISO by iron/HCl showed the loss of the 325-nm absorbance peak, as has been shown for MISO [22]. Xanthine oxidase reduced FMISO, as has been observed for MISO [7, 8], and also demonstrated the absence of FMISO metabolism in the presence of oxygen. TLC measurement of the reaction product of these two reducing systems indicated that FMISO reduction by xanthine oxidase was not complete (Fig. 5). In the xanthine oxidase system, reduced FMISO may be bound to the enzyme (as it binds to macromolecules in cells), rather than existing in solution in reduced form. This FMISO-enzyme product is probably being measured, rather than the reduced derivative alone, accounting for the differences in R_f values in the two TLC experiments.

As shown in Table 1, FMISO reacted more slowly with xanthine oxidase than did MISO. In the MISO experiment, the absorbance-vs-time curve leveled off at a lower value than did FMISO. However, because radiolabeled MISO was not available for these studies, it was not possible to quantitate what proportion of that nitroimidazole was reduced. The evidence in these experiments supports the hypothesis that FMISO reduction is similar to, albeit slower than, MISO reduction.

Raleigh *et al.* [9] demonstrated that NADH dehydrogenase reduces nitrobenzenes. If any nitroreductase can reduce any nitroaromatic, then NADH dehydrogenase should also reduce FMISO. Our results indicate that it does not. Differences between Raleigh's methods and our work may reconcile the disparate conclusions. Raleigh *et al.* worked in an aerobic environment and used the reporter molecule AF-2, which fluoresces when reversibly reduced, to show reversible reduction of nitrobenzenes by NADH dehydrogenase (i.e. the "futile cycle" of nitroaromatic metabolism). The sensitivity of the AF-2 method may be much greater than the spectrophotometric method. Also, AF-2 may modify the activity of the enzyme or may even react with the enzyme itself, since it too is an electron-rich aromatic compound. Alternatively, nitrobenzenes may be more reactive with NADH dehydrogenase than the nitroimidazoles. These differences between the two experimental approaches are enough to make their comparison less significant and do not challenge the hypothesis of the similarity of FMISO to MISO.

The second hypothesis, that the nitroreductases are plentiful in mammalian tissues, was tested in V79 cells incubated with FMISO under hypoxic conditions. The choice of a three-neck round-bottom flask rather than a custom-made glass vessel was a variation from published methods. Its selection was based on evidence that the rate of gas exchange is proportional to the ratio of surface area to volume [13], which is increased in a round-bottom flask as compared to other glass vessels. The ability of this apparatus to maintain either an aerobic or a radiobiologically hypoxic environment (<1000 ppm O_2; 0.76 mm Hg) was verified operationally. The creation of an aerobic environment by air/CO_2 was shown by a lack of binding of FMISO to cellular macromolecules (Fig. 6), consistent with the futile metabolism of nitroimidazoles (Fig. 1). The creation of a hypoxic environment was shown by an OER of 3.75. An OER of 2.5 to 3.0 is taken as evidence of full radiobiological hypoxia [15]. The D_0 values for

the hypoxic and aerobic V79 cell suspensions may be compared to the results of Hall and Roizin–Towle [23] obtained with the same cell line. They measured D_0 values of 2.17 and 7.36 Gy for aerobic and hypoxic cells, respectively, for an OER of 3.39. This is higher than the commonly reported OER of 2.5 to 3.0 but similar to our value of 3.75.

Binding of FMISO to cellular macromolecules under hypoxic conditions was measured to be 0.26 ± 0.07 pmol/10^6 cells/min. The large standard error (28% of the mean) may be due to other secondary factors besides oxygen tension that affect FMISO uptake, including cell age, glucose and nonprotein thiol levels [14]. To eliminate this variability, the inhibition studies were done with paired control experiments, so that the cells with and without inhibitor had the same life history. This was important in allowing a more meaningful interpretation of the results.

Two inhibitors were chosen to test the nitroreductase activity of those enzymes most closely associated with MISO reduction. Allopurinol specifically inhibits xanthine oxidase [24]. 2′-AMP inhibits both NADH dehydrogenase and NADPH cytochrome reductase (EC 1.6.2.4) [25, 26], the latter having demonstrated MISO reduction capability [27] but is not commercially available. The third inhibitor, KCN, is a potent inhibitor of electron transport in general and inhibits xanthine oxidase specifically. Because of a report the NADH dehydrogenase is stimulated by KCN [12], a combination of KCN and 2′-AMP was used in two experiments. A comparison of FMISO binding rates of control and inhibited suspensions in Table 2 shows that no consistent decrease in reaction rate was seen on addition of one or more inhibitors.

Several reasons may be postulated for this result. First, the inhibitor may not enter the cell. Allopurinol and KCN are small enough to cross cell membranes through the aqueous pores [28]; 2′-AMP, due to its larger size, may not [29]. Second, the inhibitors may be metabolized in other ways in the cells rather than inhibiting a specific enzyme. Third, if the inhibitors do enter the cell and inhibit the specific enzyme, other nitroreductases present may still be adequate to reduce FMISO. The third explanation is consistent with the hypothesis of this paper.

A number of researchers have tried to inhibit reduction and/or binding of nitroaromatics with specific enzyme inhibitors, and those who have been successful in showing inhibition have either used subcellular particles such as microsomes or have purified specific enzymes to show inhibition [30–32] Chapman *et al.* [10] could not inhibit MISO binding when they added allopurinol to EMT-6 cells. The same authors also showed cysteamine inhibition of MISO binding in EMT-6-cells. Rasey *et al.* [14] specifically looked at the effect of cysteamine on FMISO binding in V79 cells and showed an increase in binding levels with increasing cysteamine concentration.

From the work reported here, several conclusions may be drawn. FMISO reduction is similar to that of MISO, for both chemical and enzymatic reduction processes. In V79 cells, addition of specific inhibitors of nitroreductases does not inhibit FMISO reduction and binding. These data, although limited, support the hypothesis that the availability of intracellular nitroreductases will not limit FMISO binding *in vivo*. Additional experiments suggested by this research include studying the *in vitro* reduction of FMISO by purified NAD(P)H cytochrome reductase (EC 1.6.99.2); sonicating or otherwise disrupting the cell membrane before adding inhibitor and measuring the rate of FMISO binding to the cellular extract; using inhibitors of other potential nitroreductases, such as NAD(P)H cytochrome reductase, aldehyde oxidase (EC 1.2.3.1), and dihydrolipoamide reductase (EC 1.6.4.3) in V79 cells; and performing similar experiments on normal, cancerous, and hypoxic mammalian tissues, including brain and heart. Experiments such as these will help to explain the biochemical basis for cellular uptake and subsequent imaging with FMISO.

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