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BIOREDUCTIVE METABOLISM OF AF-2 [2-(2-FURYL)-3-(5-NITRO-2-FURYL)ACRYLAMIDE] COMBINED WITH 2-NITROIMIDAZOLES

IMPLICATIONS FOR USE AS HYPOXIC CELL MARKERS

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Abstract—Metabolism of misonidazole under hypoxic conditions depletes the parent drug and causes about 4% of the reduced-drug-products to form adducts with cellular macromolecules (binding), and this process has been used to detect hypoxia in cells and tissues. The nitrofuran, AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide] has been shown to increase both the metabolic depletion of misonidazole and its binding. In the present study, factors which might affect this process have been examined, in an *in vitro* system, to test the hypothesis that metabolic depletion of misonidazole could limit its ability to diffuse freely to the hypoxic cell population. Drastic reductions in glucose concentrations from their normal value of 5–10 mM to less than 0.5 mM had no significant effect on the metabolism of either misonidazole or AF-2. Similarly, glucose concentration did not influence the binding of misonidazole, even when concentrations of both oxygen (extreme hypoxia) and glucose were near zero—a very toxic biochemical environment. Similarly, the metabolism of the nitroheterocyclics had no effect on glucose consumption. The bioreductive depletion of misonidazole in extreme hypoxia appeared to be independent of drug concentration between 25 and 100 μM: this nearly zero-order rate of drug metabolism prevented the possibility of working at constant drug concentration. AF-2 exacerbated this effect by greatly enhancing the metabolic depletion of misonidazole. AF-2 was found to increase both the metabolic depletion and binding of misonidazole by the same factor. An unexpected finding was that metabolism of etanidazole, a 2-nitroimidazole closely related to misonidazole, was not enhanced by AF-2. Micromolar amounts of oxygen inhibited the reductive activation of AF-2, and also the interaction between AF-2 and misonidazole. Our results suggest that metabolic depletion of nitroheterocyclics could influence their ability to diffuse adequately to hypoxic tissues, particularly at the low drug concentrations that have been used to measure tissue hypoxia *in vivo*.

An important goal in oncology research is the identification and elimination of treatment-resistant cells. Hypoxic cells are often used as a model for treatment resistance since lowered levels of oxygen (and also cytotoxic drugs) are caused by the relative isolation of these cells from the blood supply [1, 2].

Evaluation of the importance of hypoxic cells in determining treatment response requires accurate measurements of the extent and degree of hypoxia in individual tumors. Hypoxic cells in human tumors have been measured directly using oxygen microelectrodes [3–5], and have been inferred in numerous other ways [6, 7]. A relatively new approach to oxygen measurement, with the potential for subcellular resolution, is based upon observations made more than a decade ago regarding the metabolism of nitroimidazoles [8–12]. These compounds are metabolized at rates that increase dramatically as the oxygen concentration decreases, leading to adduct formation between the metabolized nitroimidazole and cellular macromolecules (binding). The binding can be monitored by several invasive or noninvasive detection techniques, with the resolution depending on the detection technique used [13–17].

Most binding studies have used the 2-nitroimidazole misonidazole, or one of its derivatives, as the oxygen-detecting drug, but quantitative measurements have shown a great divergence in absolute binding rates in different cells or tissues that were maintained at the same oxygen concentration, and in the relative change in binding as a function of oxygen concentration [18–21]. Thus, misonidazole may be useful as a relative indicator of varying oxygen concentration, but perhaps requires extensive, cell/tissue-dependent calibration to be used as an absolute monitor of oxygen concentration [21].

Several studies have shown that binding of misonidazole to hypoxic cells can be modified substantially [19, 22]. In principle enhanced binding could result in an increased ability to detect hypoxic cells with the following restrictions: (1) the increased rate of binding should not increase the variability of the relationship between binding and oxygen concentration; and (2) drug levels should not be lowered significantly by the increase in binding rate.

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The latter restriction is considered a fundamentally important property for compounds like the nitroimidazoles which must have essentially complete access to the target population of hypoxic cells [1], but based on modelling of drug reduction and binding in tissue culture and radiochemical model systems, we have been concerned that drug depletion in hypoxic tissue could indeed lead to reduced drug delivery to the target cells [19, 23]. The measurement of nitroaromatic drug depletion at very low drug concentrations is especially important for their use as oxygen detectors, since the drug concentrations used for this purpose have been extremely low—in the micromolar to nanomolar range depending on the detection method [13, 24, 25].

Both the addition of the nitrofuran AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide] and the depletion of cellular GSH have been shown to increase dramatically the binding of misonidazole to hypoxic cells [19, 22]. The effect of AF-2 is surprising, since it is much more electron affinic than misonidazole itself. Thus, one might expect that AF-2 would compete for and hence diminish cellular reducing equivalents available for misonidazole reduction [22, 26]. A possible complicating factor from some previous experiments with AF-2 was the use of highly cytotoxic drug concentrations (>200 μM) and very high cell densities (5×10^7 cells/mL), sometimes in relatively simple salt buffers [22, 27, 28]. Based on published measurements of cellular glucose consumption [29, 30] and our observations of substantial decreases in pH and glucose concentration even at much lower cell densities ($2–5 \times 10^6$ cells/mL—see below), it became apparent that reducing equivalents available from the metabolism of exogenous glucose could have been limiting in the former studies. Any significant decrease in glucose could be important, because it has been shown that the metabolic effects of misonidazole were greatly diminished under such circumstances [31].

Although the rate of drug metabolism for agents such as misonidazole is known for some tissue models (e.g. hypoxic perfused rat liver [32, 33]), similar data are not available for tumors or tumor cells. A recent attempt to measure the rate of misonidazole metabolism by cells in tissue culture was unable to detect measurable drug loss [22].

In view of the above-mentioned concerns over drug depletion, we felt that the addition of drugs like AF-2, which greatly enhance metabolism of misonidazole, might inadvertently lead to lack of drug availability. The present experiments evaluated the interactions of AF-2 at relatively less toxic levels, with both misonidazole and etanidazole using moderate cell densities. A preliminary assessment of the importance of oxygen in the drug interactions has also been investigated. The present results suggest, in contrast to the two restrictions listed above, that AF-2 considerably modifies the oxygen dependence of binding, and that drug consumption can be a substantial problem even at the relatively low cell densities used in tissue culture.

METHODS

Chromatography

An Alltech (Alltech Associates, Deerfield, IL) reverse phase column (C18, 250 mm length, 4.6 mm i.d., 5 μm, Adsorbosphere HS) was used in the separation of AF-2 and the nitroimidazoles. Separations were obtained at a flow rate of 0.9 mL/min, 50°, using a mobile phase containing 100 mM monochloroacetic acid, 1% dimethylformamide and 3.3 mM heptane sulfonic acid, pH 2.60 [34]. Molecules of varying hydrophilicity were separated by modifying the methanol content (0% for etanidazole, 10% for misonidazole, 30% for *cis*- and *trans*-AF-2). Pumps (models 510) and detectors (4 channel spectrometer, model 490) were from Waters Associates (Milford, MA). In some experiments, radioactivity of selected nitroimidazoles was monitored by a radioactivity detector (β-RAM, IN/US, Fairfield, NJ), which was in series with the spectrometer. Signals were monitored by chart recorders from Houston Instruments and the data collection computer of the β-RAM. In all cases, peak heights were found to be proportional to concentrations of drug. Medium samples or cells were treated with a final concentration of 0.3 M trichloroacetic acid (TCA), and then centrifuged at ~10,000 *g* for 10 min to remove macromolecules, using a Fisher 59A centrifuge. Automatic injection of samples (20–200 μL) was made using a Waters 712 WISP. At the relatively low cell densities used in the present experiments ($<3 \times 10^6$/mL), the fraction of drug consumed was often very small. Thus, corrections were made for slight (2–4%) decreases in medium volume caused by evaporation, and the sample preparations (e.g. acid addition) were made by weight. Multiple samples were often analyzed to further reduce sampling errors when necessary. With the above corrections, random (dilution based) and consistent (evaporative losses) errors of 10–15% were reduced to the order of 2–3%.

In vitro *studies*

Drug consumption, binding and toxicity. Two cell types were used. The first was a subline (WNRE) of V79 Chinese hamster fibroblasts, selected for its ability to grow both in suspension and in monolayer culture (obtained from Dr. J. D. Chapman, Fox Chase Cancer Center, Philadelphia). The second was the rat-derived 9L glioblastoma (obtained from Dr K. T. Wheeler, Bowman Gray University). The cells were thawed from frozen stock on a roughly semi-annual basis, and tests were made routinely to ensure that the cultures were free from Mycoplasma and other contaminations. The cells were cultured (37°, 95% air + 5% carbon dioxide, 100% relative humidity) in the exponential phase of growth by twice weekly transfers using Eagle's Minimal Essential Medium containing 12.5% (v/v) serum (fetal bovine for V79 and calf for 9L) and antibiotics (culture solutions from GIBCO or Sigma). On the day before an experiment, cells were trypsinized and plated onto glass Petri dishes—approximately $2–5 \times 10^5$ cells confined to the central area of the dish as described previously [35]. For drug consumption experiments, up to 2×10^6 cells were plated uniformly over the surface of the dish. Cell plating was followed by overnight incubation at 37°. The dishes were then removed from the incubator and

cooled, and their medium was replaced with drug-containing medium, twice as a rinse (1 mL) which was simply aspirated and then as the actual medium used for the experiment (1.3 mL). A 0.3-mL sample was then removed from the dish to assess initial concentration, and the dish bottoms were weighed immediately. (We have found that a film of water forms on the dish lid's inner surface which can lead to erroneous estimations of evaporative loss, so lids were not included in the weight determinations, i.e. since samples were added to and taken from only the dish bottom.) Dishes were placed in leak-proof aluminum chambers that were connected to a manifold allowing them to be deoxygenated with a series of gas exchanges taking approximately 30 min [36]. The confinement of cells to the central area of the dish (at the lower cell numbers), and the use of a small volume of medium allows very rapid equilibration of the gas and liquid phase to improve the control of oxygen concentration (the time constant for gas–liquid equilibration is about 6 sec [35]). After gas phase equilibration, the chambers were incubated at 37°. To prevent minor gradients of oxygen or potentially larger gradients of nutrients/metabolites, the chambers were also shaken gently (1 Hz, 2.5-cm stroke). Oxygen content of the gas during equilibration was monitored by a sensitive polarographic sensor [37]. The gas phase of individual chambers could also be sampled at the end of the experiment by gas transfer to a previously evacuated monitoring chamber containing the same sensor. At the end of the incubation period, the chambers were opened, dish bases reweighed and medium samples immediately saved in polypropylene microfuge tubes on ice.

To determine binding of radioactive nitroheterocyclics under defined experimental conditions, acid-soluble and acid-insoluble radioactive products retained by the cells (after extensive rinsing) were assayed as described previously [18, 19]. Since the cell number, drug concentration and specific activity, and efficiency of the liquid scintillation counter (1940 TR; Packard, Meriden, CT) were all known, the absolute incorporation of adducts could be calculated as picomoles per cell. V-79 WNRE cells have a volume of about 0.75 pL [38], whereas 9L cells have a 2-fold larger volume. Therefore, the unit of "picomole per cell" is close to picomole per picoliter, or "molar".

Cytotoxicity of the nitroheterocyclics was assessed using similar techniques but with non-radioactive drug. Similar metabolic effects of AF-2 were observed whether the drug was dissolved directly in medium, with extensive stirring, or using prior solubilization at 50–100 mM in tissue culture grade dimethyl sulfoxide (Sigma; final concentration less than 0.5%). After incubation with or without drug-containing medium, cells were trypsinized and plated for colony formation as described previously [19, 39]. In previous experiments, drug-containing medium typically consisted of Earle's based MEM, with buffering by 20 mM HEPES and 5 mM sodium bicarbonate, usually supplemented by the addition of 10–15% fetal bovine serum (the predominantly HEPES based buffer allows the use of gases without carbon dioxide). However, it was found that neither the buffering capacity nor the normal physiological glucose concentration (5.5 mM) was adequate for the higher cell numbers under extreme hypoxia. In addition, the pH indicator typically present—phenol red—was found to co-chromatograph with *cis*-AF-2 under our standard HPLC conditions. Thus, a special formulation of medium, containing no phenol red, twice the normal glucose concentration (11 mM) and 75 mM HEPES (initial pH 7.5, with osmolarity held constant by the addition of water) was used. This medium formulation was not detectably different from the standard formulation at low cell densities with respect to growth and plating efficiency of the cells.

Glucose concentration in the medium was measured with standard assay kits from Sigma (Kit No. 510, based on glucose oxidase followed by peroxide measurement, and Kit No. 16, based on the production of NADH using ATP, hexokinase and glucose-6-phosphate-dehydrogenase). Although the nitroaromatic compounds, at the concentrations used, did not interfere with either assay, the latter procedure based on hexokinase appeared to be substantially more reproducible.

Cellular respiration was measured in custom-designed vials with standard taper ground glass upper joints, fitted with a machined ceramic stopper with matching taper. The stopper included an additional tapered hole into which the oxygen sensor fitted exactly. Stirring was effected by a custom-designed ceramic-enclosed magnet, with shaft and bearing (also ceramic) allowing stirring without grinding of cells on the base of the vial. The leak-proof design and elimination of virtually all plastics allow the measurement of oxygen consumption over a very broad range of oxygen levels including the very low concentrations over which changes in radiation sensitivity occur [40].

Drugs. The drugs used, their abbreviations, and their sources were AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide], Dr J. Raleigh, University of North Carolina; misonidazole [3-methoxy-1-(2-nitroimidazol-1-yl)-2-propanol], and etanidazole [*N*-(2 - hydroxyethyl) - 2(2-nitroimidazol - 1-yl)acetamide], both unlabeled and 2-^{14}C-labeled, from Dr R. Haugwitz, NCI, Drug Synthesis and Chemistry Branch. Specific activity of the radioactive 2-nitroimidazoles was approximately 70 μCi/mg.

RESULTS

In preliminary experiments, substantial toxicities were noted for 9L cells at relatively high cell density with no other treatment than incubation in extreme hypoxia, and this toxicity was traced to metabolic depletion of glucose. The 9L cells showed a substantial Pasteur effect, as reported by Li [29], and surprisingly, intermediate levels of oxygen did not greatly inhibit glucose consumption (Fig. 1, Table 1) so that testing and correcting for glucose depletion was essential in experiments at cell densities greater than $\sim 2 \times 10^6$/mL. The Chinese hamster fibroblasts had a negligible Pasteur effect and somewhat lower rate of glucose consumption, but their rate of glucose consumption was still high enough to require glucose supplementation, from

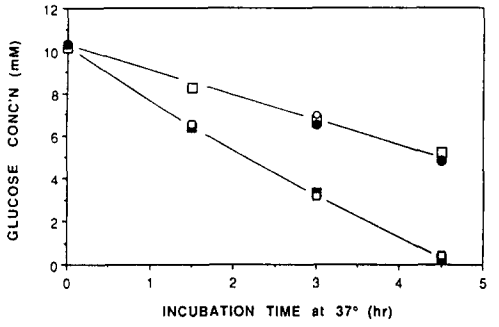

Fig. 1. Glucose consumption by Chinese hamster fibroblasts (●, ○) or 9L rat glioma (■, □, □) as a function of time under various conditions of oxygenation. Cell numbers were chosen to give similar values for consumption in air (○, □). Other oxygen levels of incubation were 0.4% oxygen (□) and nitrogen (●, ■). The data for Figs. 1 and 6, in contrast to the other figures, were generated using relatively high densities (~3×10^6 9L cells/mL or 5×10^6 WNRE cells/mL) to allow drug consumption to be measured.

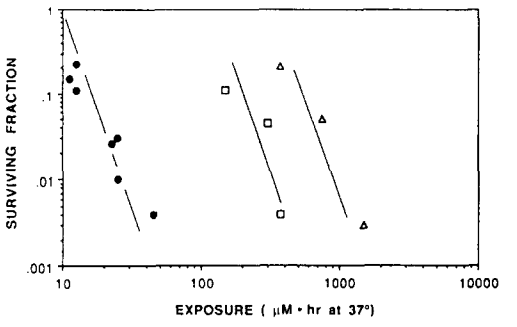

Fig. 2. Survival as a function of the logarithm of drug exposure for WNRE Chinese hamster fibroblasts incubated with *cis*-AF2 under conditions of near zero oxygen (●), 0.1% oxygen (□), and 0.4% oxygen (△).

the normal level of 5.5 mM to 11 mM (see Methods) at relatively high cell densities (Fig. 1).

Under conditions of extreme hypoxia, AF-2 was toxic at low drug concentrations (Fig. 2). Very low concentrations of oxygen led to a substantial reduction in this toxicity, so that at even 0.1% oxygen in the gas phase, nearly 10-fold larger concentrations of drug were required for the same level of toxicity. Increasing the oxygen partial pressure to 0.4% caused an additional 3-fold reduction in toxicity, but between 0.4% oxygen and air (20.9% oxygen) little additional change occurred. The extremely high dependence of toxicity on oxygen concentration was also found for 9L cells (data not shown). With reference to zero oxygen, cells would be 2-fold more sensitive to radiation exposure at 0.4% oxygen, but are more than 20-fold less sensitive to drug toxicity.

Under conditions of extreme hypoxia, binding of misonidazole (20 μM) was enhanced 5-fold by low concentrations of AF-2 (10 μM), but this effect was not observed for binding of etanidazole (Fig. 3). The rate of binding of etanidazole was substantially lower than that of misonidazole but the differences are complex, being both cell-line and drug concentration dependent [12, 19]. Additionally, binding of misonidazole was not enhanced by AF-2 when low concentrations of oxygen were present (Fig. 4).

Several experiments were performed to derive approximate drug consumption rates and determine optimal timing and drug levels (data not shown). Then, measurements of binding and metabolic consumption, at approximately 3×10^6 9L cells/mL, of misonidazole and AF-2, alone and in combination,

Table 1. Drug metabolism rates by 9L cells

Condition	Cell	Drug metabolism rates (pmol/cell/sec) Air	0.4% Oxygen	Nitrogen
Glucose (±drugs)	9L	8.5×10^{-5}	1.6×10^{-4}	1.6×10^{-4}
	WNRE	1.2×10^{-4}	1.2×10^{-4}	1.2×10^{-4}
Oxygen (±RNO_2)	9L	3.5×10^{-5}	3.5×10^{-5}	
	WNRE	2.7×10^{-5}	2.7×10^{-5}	
AF-2 [25 μM]	9L			3.4×10^{-6}
AF-2 [25 μM] (+ 100 μM misonidazole)	9L			1.4×10^{-6}
Misonidazole [100 μM]	9L			7.4×10^{-7}
Misonidazole [100 μM] (+ 25 μM AF-2)	9L			3.4×10^{-6}
Misonidazole [100 μM] (binding)	9L			1.4×10^{-7}
Misonidazole [100 μM] (+ 25 μM AF-2) (binding)	9L			1.1×10^{-6}

The rates should be considered as initial rates at the concentrations indicated, although misonidazole appears to be consumed in a nearly zero order process. The standard errors of the measurements are approximately ±10%, and between-experiment variability is somewhat larger, approximately ±15–30%.

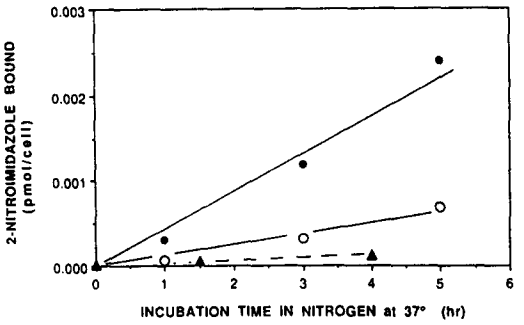

Fig. 3. Effect of AF-2 on binding of misonidazole and etanidazole in WNRE cells. Measurement of binding of 2-nitroimidazoles (20 μM) as a function of time in near zero oxygen for hamster fibroblasts in the presence (●, ▲) or absence (○, △) of AF2 (10 μM). Monitored binding agents were misonidazole (●, ○) or etanidazole (▲, △). In all figures, combined acid-soluble plus -insoluble counts are plotted with the latter representing about 20–30% of the total.

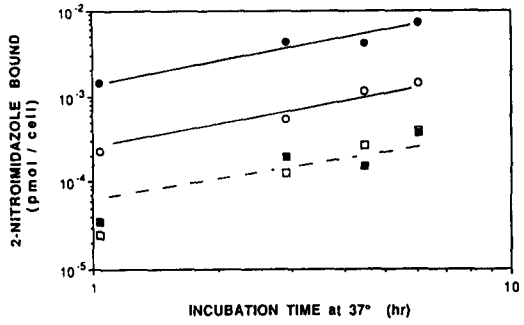

Fig. 4. Effect of low oxygen concentrations on the interaction of AF-2 and misonidazole in WNRE cells. Zero oxygen (○, ●) vs 0.2% oxygen (□, ■). The monitored binding agent was 100 μM misonidazole, either alone (○, □) or with 100 μM AF-2 (●, ■). To accommodate the large changes in absolute binding rate, both axes employ a logarithmic scale (i.e. binding is linear with time as in Fig. 3).

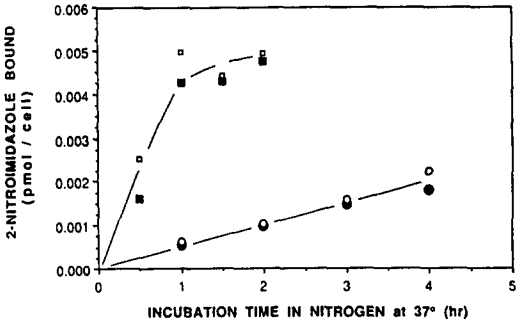

Fig. 5. Influence of glucose on binding of 100 μM misonidazole, either alone (○, ●) or with 25 μM AF-2 (□, ■). Initial glucose concentration was ~11 mM (●, ■) vs ~0.5 mM (○, □).

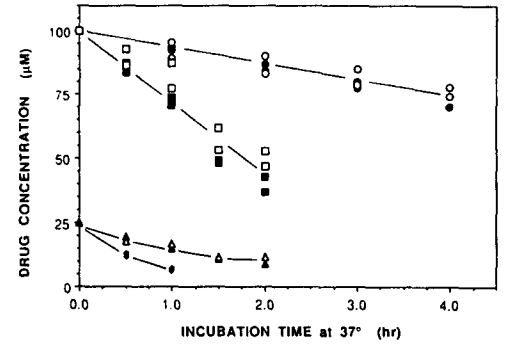

Fig. 6. Drug metabolism in the presence of high (closed symbols) vs low (open symbols) glucose levels (initial concentrations 11 mM vs 0.5 mM, respectively). Metabolism of misonidazole (initial concentration of 100 μM) was measured either alone (○, ●) or in combination with 25 μM AF-2 (□, ■). Similarly AF-2 (initial concentration of 25 μM) was measured either alone (◇, ◆) or in combination with 100 μM misonidazole (△, ▲).

with and without glucose were compared in a single large experiment (Figs. 5–7). No effect of severe glucose depletion was seen on binding (Fig. 5) (at time zero, the medium was not completely glucose free, since ~0.5 mM was contributed by the 10% serum component). The binding rate for misonidazole in the presence of AF-2 decreased at the longest times, perhaps because of drug consumption (see Fig. 6) or toxicity (see Fig. 1). Metabolic depletion of 100 μM misonidazole was enhanced 5-fold by 25 μM AF-2, whereas depletion of AF-2 was inhibited by the presence of misonidazole (Fig. 6). The combination of 100 μM misonidazole and 25 μM AF-2 was much less toxic than 25 μM AF-2 alone (data not shown). The rate of nitroheterocyclic depletion was not detectably different when near-glucose-free

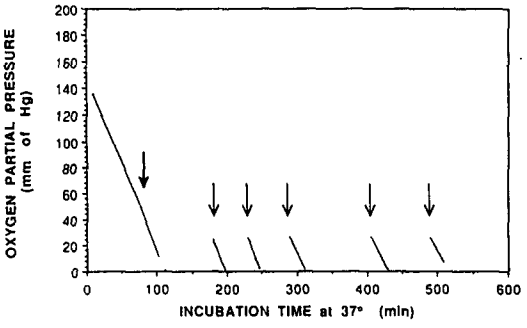

Fig. 7. Effect of AF-2 on cellular respiration rate. AF-2 was added (200 μM) at a relatively high oxygen concentration (heavy arrow). After the oxygen concentration had decreased to zero, catalase and small amounts of hydrogen peroxide were added to allow bursts of increased oxygen concentration (light arrows).

medium was used (Fig. 6). Glucose consumption was not affected by the presence of either drug or their combination (data not shown). The rates of depletion per cell of both nitroheterocyclic drugs were similar for both the 9L rat glioma and Chinese hamster fibroblasts, when corrections were made for total cell mass (data not shown). Very low concentrations of oxygen (0.4%) caused drug consumption to decrease to such a small value as to make accurate measurements very difficult at these cell densities (data not shown). Metabolism of misonidazole appeared to be constant with time over the concentration range studied, suggesting a less than first-order reaction, whereas metabolism of AF-2 was clearly concentration dependent.

Since all of the interesting interactions of AF-2 appeared to be inhibited by low concentrations of oxygen, we wondered if metabolism of AF-2 could also modify metabolism of oxygen. Relatively large concentrations of AF-2 (200 μM) enhanced oxygen consumption (by approximately 30%) of WNRE cells at high oxygen concentrations, whereas the opposite effect was observed at low oxygen concentrations [approximately 50% inhibition after several hours (Fig. 7)]. Lower levels of AF-2 were found to have no discernible effect on cellular respiration. When misonidazole was also present, only a slight stimulation of respiration at high oxygen levels was observed (data not shown). Although 200 μM AF-2 would not have been very toxic at the high oxygen level of first addition, it would have very quickly caused the clonogenic killing of all the cells (see Fig. 2) at the very low oxygen levels imposed by the metabolic depletion of oxygen. Thus, the 50% decrease in oxygen consumption rate after several hours is difficult to interpret.

DISCUSSION

Our original goal was to provide additional evaluation of the use of AF-2 as an adjuvant drug to enhance the binding of misonidazole and hence perhaps to increase its sensitivity of hypoxic cell detection. Of the two restrictions established for the utility of such an adjuvant drug (see introduction), neither was satisfied by AF-2: first, it stimulated binding only under conditions of extreme hypoxia, with no effect seen with even trace concentrations of oxygen present; second, it caused an excessive metabolic depletion of misonidazole which *in vivo* could lead to the inability of misonidazole to fully diffuse throughout substantial regions of hypoxia. Furthermore, AF-2 failed to enhance the binding of etanidazole, a 2-nitroimidazole differing from misonidazole only in its side chain. The extreme inverse oxygen dependence of AF-2's enhancement of misonidazole binding was also seen in other aspects of metabolism of AF-2. Oxygen concentrations of only 0.1% in the gas phase of the chambers caused a dramatic reduction in toxicity, with almost ten times more drug required to produce the same toxicity as that required under extreme hypoxia. The gas phase oxygen level represented an upper limit to the equilibrium value expected in the medium surrounding the cells (~1.0 μM for 0.1% oxygen in gas phase) because of oxygen depletion by respiration, even with the thin film system and shaking of the cell-containing dishes.

Our results have demonstrated substantial levels of cellular metabolism of misonidazole by cells in culture at moderate cell densities under conditions of extreme hypoxia. Since we know that drug reduction as well as many of the other effects of misonidazole are very susceptible to inhibition by trace levels of oxygen [19], it is possible that previous reports of much lower levels of drug metabolism [22] were caused by trace contamination by oxygen. With our present knowledge, it is difficult to predict the importance of tissue-induced drug depletion *in vivo*. On the one hand, the fractional volume of viable tumor tissue existing at near zero oxygen levels might be expected to be low. On the other hand, we have seen complete consumption of 50 μM misonidazole by 9L cells in about 240 min at 2–3 million cells per mL (or per dish, since each dish contained only 1 mL), and since cell densities in tissue are several hundred-fold larger, the lifetime of similar misonidazole concentrations in hypoxic tissue may be only a fraction of a minute. Additionally, consumption of misonidazole appears to be nearly independent of drug concentration over the range studied (~25–100 μM), which means that the lifetime of the drug would decrease with concentration. Most drug pharmacokinetic studies have assumed a first-order depletion of drug, based upon blood and (predominantly aerobic) tissue lifetimes at relatively high drug concentrations. Since the major present use for these nitroheterocyclics is as oxygen detectors at very low drug concentrations [13, 41], our results suggest that metabolic drug depletion must be evaluated at the concentrations that are actually being used *in vivo*. Although it has not been demonstrated conclusively that the rate of binding accurately reflects the rate of drug metabolism, binding has been shown to increase as the square root of misonidazole concentration [12, 18, 19]. Although this kinetic behavior was interpreted previously in terms of a free radical reaction [18], it is also possible that it results from some combination of zero-order and first-order metabolic consumption reactions.

Our present estimates for the rates of metabolite and drug consumption are listed in Table 1. The ordering of metabolic rates found in this study was: glucose > oxygen > AF-2 > 2-nitroimidazole > binding, the factors being roughly 5, 10, 5 and 3, respectively. This ordering offers a possible explanation for the apparent lack of effect of glucose concentration on drug metabolism found in the present studies. "Normal" metabolic requirements for glucose are in vast excess of specific drug-reduction requirements, even for very toxic drugs like AF-2. It is possible that at higher drug concentrations, and still lower glucose concentrations, an inhibition of bioreductive metabolism will occur. However, this would represent an extremely toxic environment.

We are in the process of continuing these studies with other nitroheterocyclics which we believe are better suited to oxygen detection than is misonidazole, and at higher and possibly more relevant oxygen concentrations than "near zero".

Nevertheless, it appears that our previous estimate of drug consumption versus binding may be relevant. To restate the background for this estimate, we calculated the mole fraction of drug bound per drug consumed (metabolized or otherwise chemically reduced) using data from various model systems. This fraction varied from much less than 0.01 to ~0.2, with the latter, highest value found for radiochemical reduction of 2-nitroimidazoles under extreme hypoxia in the presence of thiol-containing proteins (protein thiol levels in the submillimolar range) [19, 23]. Knowing the rate of binding as a function of drug and oxygen consumption, and assuming that the relationship between drug consumption and binding remained the same at increased oxygen levels, we calculated that drug consumption could be an important limiting factor if the binding fraction were at the low end of the above-stated range.

For cells in culture under conditions of extreme hypoxia, and at misonidazole concentrations of about 50 μM, the fraction of misonidazole metabolites that are retained by cells appears to be about 0.18 to 0.3, with roughly 25% of this associated with the acid precipitable and presumed macromolecular cell components (this study). Although this fraction is therefore at the high end of estimates made previously [23], it remains to be shown what the actual result may be in tumor tissue at possibly much lower drug concentration. This can be stated in another way: suppose a typical tumor blood flow rate were 0.1 mL/min/g, and the blood contained 10 μM misonidazole; then the blood would be providing $\sim 1.7 \times 10^{-11}$ mol of drug/sec/g of tumor. Misonidazole consumption rates in this study have been found to be of the order of 7×10^{-19} mol/cell/sec. At 5×10^8 cells/g, a completely hypoxic tumor would consume 3×10^{-10} mol/sec of drug. Thus, the consumption rate is 20-fold more than the supply rate. Thus, even a 5% hypoxic fraction could consume all available drug. In actual practice, large diffusion limited gradients would be likely to occur. From the data presented, addition of a drug like AF-2, which enhances both binding and metabolism, would compound the drug consumption problem.

If an overall goal is to maximize binding to hypoxic cells, then a number of alternate approaches are possible: (1) the simple use of higher concentrations of misonidazole or other nitroaromatic drug; this would appear to be far less toxic than would an AF-2/misonidazole combination, and would entail a much reduced risk of drug depletion problems; (2) drugs or conditions that would enhance the mole-fraction of bound to metabolized drug should be investigated; this requires a more detailed understanding of the molecular/biochemical mechanisms of drug activation and binding; and (3) the proportion of cellular adducts/metabolites that are associated with cellular macromolecules (precipitable in 5% TCA) should be maximized. We have shown previously that this proportion increases with drug hydrophobicity and accessibility to the 3,4 carbons of the imidazole ring [19, 42].

Finally, it should be emphasized that although drug pharmacology becomes increasingly difficult at low drug concentrations, it will be important to test for the possibility of less than first-order drug consumption by any agents that are being considered for use at low concentrations *in vivo*.

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REFERENCES

1. Adams GE, Hypoxia-mediated drugs for radiation and chemotherapy. *Cancer* **48**: 696–707, 1981.
2. Kennedy KA, Teicher BA, Rockwell S and Sartorelli AC, The hypoxic tumor cell: A target for selective cancer chemotherapy. *Biochem Pharmacol* **29**: 1–8, 1980.
3. Cater DB and Silver IA, Quantitative measurements of oxygen tensions in normal tissues and in the tumors of patients before and after radiotherapy. *Acta Radiol* **23**: 233–256, 1960.
4. Wendling P, Manz R, Thews G and Vaupel P, Heterogeneous oxygenation of rectal carcinomas in humans. A critical parameter for pre-operative irradiation. *Adv Exp Med Biol* **180**: 293–300, 1984.
5. Gatenby RA, Kessler HB, Rosenblum JS, Coia LR, Moldofsky PJ, Hartz WH and Broder GJ, Oxygen tension in human tumors: *In vitro* mapping using CT-guided probes. *Radiology* **156**: 211–214, 1985.
6. Thomlinson RH and Gray LH, The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br J Cancer* **9**: 539–579, 1955.
7. Bush RS, Jenkins RDT, Allt WEC, Beale FA, Bean H, Dembo AJ and Pringle JF, Definitive evidence for hypoxic cells influencing cure in cancer therapy. *Br J Cancer* **37** (Suppl III): 302–306, 1978.
8. Taylor YC and Rauth AM, Differences in the toxicity and metabolism of 2-nitroimidazole misonidazole (Ro-07-0582) in HeLa and Chinese hamster ovary cells. *Cancer Res* **38**: 2745–2752, 1978.
9. Taylor YC and Rauth AM, Sulphydryls, ascorbate and oxygen as modifiers of the toxicity and metabolism of misonidazole *in vitro*. *Br J Cancer* **41**: 892–900, 1980.
10. Varghese AJ and Whitmore GF, Binding to cellular macromolecules as a possible mechanism for the cytotoxicity of misonidazole. *Cancer Res* **40**: 2165–2169, 1980.
11. Chapman JD, Hypoxic sensitizers—Implications for radiation therapy. *N Engl J Med* **301**: 1429–1432, 1979.
12. Chapman JD, Baer K and Lee J, Characteristics of the metabolism-induced binding of misonidazole to hypoxic mammalian cells. *Cancer Res* **43**: 1523–1528, 1983.
13. Urtasun RC, Koh CJ, Franko AJ, Raleigh JA and Chapman JD, A novel technique for measuring human tissue hypoxia at the cellular level. *Br J Cancer* **54**: 453–457, 1985.
14. Chapman JD, Lee J and Meeker BE, Keynote address: Cellular reduction of nitroimidazole drugs: Potential for selective chemotherapy and diagnosis of hypoxic cells. *Int J Radiat Oncol Biol Phys* **16**: 911–917, 1989.
15. Raleigh JA, Franko AJ, Treiber EO, Lunt JA and Allen PS, Covalent binding of a fluorinated 2-nitroimidazole to EMT-6 tumors in Balb/C mice: Detection by F-19 nuclear magnetic resonance at 2.35 T *Int J Radiat Oncol Biol Phys* **12**: 1243–1245, 1986.
16. Olive PL and Chaplin DJ, Oxygen and nitroreductase-dependent binding of AF-2 in spheroids and murine tumors. *Int J Radiat Biol Oncol Phys* **12**: 1247–1250, 1986.
17. Rasey JS, Grunbaum Z, Magee S, Nelson NJ, Olive PL, Durand RE and Krohn KA, Characterization of radiolabeled fluoromisonidazole as a probe for hypoxic cells. *Radiat Res* **111**: 292–304, 1987.

18. Koch CJ, Stobbe CC and Baer KA, Metabolism induced binding of ^{14}C-misonidazole to hypoxic cells: Kinetic dependence on oxygen concentration and misonidazole concentration. *Int J Radiat Oncol Biol Phys* **10**: 1327–1331, 1984.
19. Koch CJ, "The reductive activation of nitroimidazoles; modification by oxygen and other redox-active molecules in cellular systems". NATO International Scientific Programme Advanced Research Workshop, Fermo, Italy, 1990. In: *Selective Activation of Drugs by Redox Processes* (Eds. Adams GE, Breccia A, Fielden EM and Wardman P), pp. 237–247. Plenum Press, New York, 1991.
20. Franko AJ and Koch CJ, Binding of misonidazole to V79 spheroids and fragments of Dunning rat prostate and human colon carcinoma *in vitro*: Diffusion of oxygen and reactive metabolites. *Int J Radiat Oncol Biol Phys* **10**: 1333–1337, 1984.
21. Franko AJ, Koch CJ, Garrecht BM, Sharplin J and Howorko J, Oxygen dependence of binding of misonidazole to rodent and human tumors *in vitro*. *Cancer Res* **47**: 5367–5376, 1987.
22. Olive PL, Enhanced misonidazole binding in the presence of AF-2. *Radiat Res* **109**: 490–499, 1987.
23. Raleigh JA and Koch CJ, The importance of thiols in the reductive binding of 2-nitroimidazoles to macromolecules. *Biochem Pharmacol* **40**: 2457–2464, 1990.
24. Koh W-J, Rasey JS, Evans ML, Grierson JR, Lewellan TK, Graham MM, Krohn KA and Griffin TW, Imaging of hypoxia in human tumors with [F-18]-fluoromisonidazole. *Int J Radiat Biol Oncol Phys* **22**: 199–212, 1991.
25. Parliament MB, Chapman JD, Urtasun RC, McEwan AJ, Goldberg L, Mercer JR, Mannan RH and Wiebe LI, Non-invasive assessment of human tumour hypoxia with ^{123}I-iodoazomycin arabinoside: Preliminary report of a clinical study. *Br J Cancer* **65**: 90–95, 1992.
26. Olive PL, Rasey JS and Durand RE, Comparison between the binding of [^{3}H]misonidazole and AF-2 in Chinese hamster V79 spheroids. *Radiat Res* **105**: 105–114, 1986.
27. Olive PL, Evidence suggesting that the mechanism for aerobic and hypoxic cytotoxicity of nitroheterocycles is the same. *Int J Radiat Oncol Biol Phys* **8**: 687–691, 1982.
28. Olive PL and Durand RE, Fluorescent nitroheterocycles for identifying hypoxic cells. *Cancer Res* **43**: 3276–3280, 1983.
29. Li CKN, The glucose distribution in 9L rat brain multicell tumor spheroids and its effect on cell necrosis. *Cancer* **50**: 2066–2073, 1982.
30. Freyer JP and Sutherland RM, A reduction in the *in situ* rates of oxygen and glucose consumption of cells in EMT6/Ro spheroids during growth. *J Cell Physiol* **124**: 516–524, 1985.
31. Ling LL, Streffer C and Sutherland RM, Decreased hypoxic toxicity and binding of misonidazole by low glucose concentrations. *Int J Radiat Oncol Biol Phys* **12**: 1231–1234, 1986.
32. Smith BR, Born JL and Garcia DJ, Influence of hypoxia on the metabolism and excretion of misonidazole by the isolated perfused rat liver—a model system. *Biochem Pharmacol* **32**: 1609–1612, 1983.
33. Workman P and Brown JM, Reduction of nitro-heterocyclic compounds by mammalian tissues *in vivo* *Cancer Chemother Pharmacol* **6**: 39–49, 1981.
34. Shaw LM, Bonner HS, Turrisi AT, Norfleet AL and Kligerman M, Measurement of *S*-2(3-amino-propylamino) ethanethiol (WR-1065) in blood and tissue. *J Liquid Chromatogr* **9**: 845–859, 1986.
35. Koch CJ, A thin-film culturing technique allowing rapid gas–liquid equilibration (6 sec) with no toxicity to mammalian cells. *Radiat Res* **97**: 434–442, 1984.
36. Koch CJ and Howell RL, Combined radiation-protective and radiation-sensitizing agents. II. Radiosensitivity of hypoxic or aerobic Chinese hamster fibroblasts in the presence of cysteamine and misonidazole: Implications for the "oxygen effect" (with appendix on calculation of dose-modifying factors). *Radiat Res* **87**: 265–283, 1981.
37. Koch CJ, *Polarographic Oxygen Sensor*, United States Patent, 5030036, July 9, 1991.
38. Koch CJ, Stobbe CC and Baer KA, Combined radiation-protective and radiation-sensitizing agents. III: Radiosensitization by misonidazole as a function of concentrations of endogenous glutathione or exogenous thiols. *Int J Radiat Oncol Biol Phys* **12**: 1151–1155, 1986.
39. Koch CJ and Howell RL, Misonidazole: Inter-related factors affecting cytotoxicity. *Int J Radiat Oncol Biol Phys* **8**: 693–696, 1982.
40. Koch CJ, Stobbe CC and Bump EA, The effect on the K_m for radiosensitization at 0°C of thiol depletion by diethylmaleate pretreatment: Quantitative differences found using the radiation sensitizing agent misonidazole or oxygen. *Radiat Res* **98**: 141–153, 1984.
41. Rasey JS, Hoffman JM, Spence AM and Krohn KA, Hypoxia mediated binding of misonidazole in non-malignant tissue. *Int J Radiat Oncol Biol Phys* **12**: 1255–1358, 1986.
42. Born JL, Smith BR, Harper N and Koch CJ, Metabolism and radiosensitization of 4,5-dimethylmisonidazole, a ring-substituted analog of misonidazole. *Biochem Pharmacol* **43**: 1337–1344, 1992.