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METABOLIC BINDING OF MISONIDAZOLE TO MOUSE TISSUES

COMPARISON BETWEEN LABELS ON THE RING AND SIDE CHAIN, AND THE PRODUCTION OF TRITIATED WATER

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Abstract—The 2-nitroimidazole, misonidazole, is of current interest as an imaging agent for hypoxic regions in tumors and in vascular disease such as stroke. The basis of this technique is the reductive activation and binding of nitroheterocycles which is much more efficient in the absence of oxygen. The appropriate molecular location for an active isotope on the nitroheterocyclic probe depends on the nature of the metabolites retained in tissues after the parent drug has been cleared. Previous studies with tumor cells *in vitro* indicated that a ring label (2-^{14}C) and a side-chain label (^{3}H) were retained equally efficiently in the acid-insoluble fraction, whereas 1.5 to 3 times more side-chain label was retained in the total pool (acid soluble plus acid insoluble) of metabolites in several normal murine tissues. We show here that the excess side-chain label in six normal tissues, plasma and EMT6 tumors was found entirely in the acid-soluble fraction as a volatile component. This volatile component was tentatively identified as tritiated water. It appeared that, in general, molecular products of misonidazole metabolism were retained in mouse tissues, with the exceptions that a small excess of ring label was found in liver and heart and that tritiated water appeared in the acid-soluble fraction of all tissues. Tritiated water would not be important in imaging studies but could be a factor in studies in which scintillation counting of tritiated nitroheterocyles is used.

The 2-nitroimidazole, misonidazole, has been investigated extensively as a radiosensitizer of hypoxic cells (reviewed in Ref. 1). More recent interest in this compound has focused on its selective toxicity to hypoxic cells (reviewed in Refs 2 and 3), its potentiation of chemotherapeutic agents (reviewed in Refs 3 and 4) and its use as a marker of hypoxic tissue in both tumors ([5–7], reviewed in Ref. 8) and normal tissues [9, 10]. The last application is based on the observation that nitroheterocyclic compounds (nitroimidazoles, nitrofurans and nitrothiazoles) can be reductively activated to intermediates which bind to macromolecules in hypoxic cells or subcellular preparations [11–16].

Oxygen at 200 μM reduces the rate of activation by factors between 5 and 50 in a variety of tumor lines and normal tissues, and the concentration required to inhibit activation by a factor of 2 is near 3 μM for most tumor and normal tissues studied to date [10, 17, 18]. Although the use of misonidazole and its analogues is still in the developmental phase, it appears that the distribution of 2-nitroimidazole adducts as visualized in tissue sections by autoradiography [5, 19] or fluorescence immunohistochemistry [20] is the best technique currently available for estimating tissue pO_2 at the cellular level. The concentration of 2-nitroimidazole adducts formed in tumor tissue has been shown to correlate with the fraction of tumor cells which is hypoxic and thus radioresistant [19, 21, 22]. This raises the possibility that estimates of hypoxia-mediated tumor radioresistance may be made non-invasively if suitably-labelled analogues of misonidazole or other nitroheterocyclic compounds were used. Similarly, regions of ischemic tissue arising from infarct or stroke might be detected. Preliminary work with fluorinated 2-nitroimidazoles indicates that *in vivo* ^{19}F magnetic resonance spectroscopy [23] or ^{18}F positron emission tomography [24] may be useful in this regard.

The 2-nitroimidazole metabolite that is metabolically bound to macromolecules in hypoxic cells is not known with certainty. It is known, however, that side-chain and ring labels in misonidazole are bound to the same extent to hypoxic V79 WNRE Chinese hamster cells and EMT6 tumor cells *in vitro* [25]. This clearly indicates that the reductively-activated intermediate incorporates the whole carbon skeleton of the original 2-nitroimidazole. It was somewhat surprising, therefore, to discover in a variety of normal mouse tissues that 1.5 to 3 times more side-chain label (^{3}H) than ring label (^{14}C) was retained in most of the tissues 24 hr after injection of labelled misonidazole [26]. One difference between the *in vitro* cell studies and *in vivo* tissue studies is that the cells *in vitro* were anoxic during labelling, whereas the normal tissue *in vivo* should have been well-oxygenated. A second difference is that, under the conditions used for the studies with V79 and EMT6 cells, metabolites which were not

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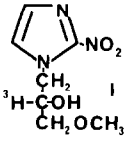
bound to macromolecules should have been removed, whereas in the study of normal tissues both bound and unbound metabolites were measured.

We report here further characterization of the metabolites of misonidazole retained in EMT6 tumors and normal mouse tissues 24 hr after injection of the drug. The quantities of acid-soluble metabolites and those covalently bound to macromolecules (acid insoluble) were measured separately, and a preliminary identification of the excess side-chain label is presented. The results are relevant not only to the design of markers of hypoxia, but also to an understanding of the metabolism of misonidazole by hypoxic and aerobic tissues.

METHODS

Animals and tumors. Balb/c female mice were purchased from the Jackson Laboratory, Bar Harbor, ME, and used at ages of 12–16 weeks. EMT6/Ed tumors were implanted by subcutaneous injection of 2×10^5 cells in each flank and excised 10 days later at sizes of 100–200 mg. The cells were maintained in tissue culture, with passage in animals every 3 months.

Drugs. Misonidazole labelled on the side chain with tritium (2-[^{3}H]-1-(2-hydroxy-3-methoxypropyl)-2-nitroimidazole)(I) was prepared according to a published procedure [27]. The specific activity was 470 μCi/mg. Misonidazole labelled with carbon-14 at the 2-position of the ring was purchased from SRI International, Menlo Park, CA. The specific activity was 90 μCi/mg. Equal quantities of each isotope were mixed in sterile saline and injected intraperitoneally into mice at 20 mg/kg. Additional injections of 10 mg/kg were given 1 and 2 hr after the first injection, to maintain the plasma concentration at 50–100 μM for 3 hr.



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Processing of tissue samples. Mice were anesthetized with tribromoethanol (Aldrich, Milwaukee, WI) 24 hr after the last injection. A blood sample was collected from the femoral artery and centrifuged in heparinized tubes. The plasma was stored frozen until further processing. The following tissues were excised: EMT6 tumors, liver, spleen, kidney, heart, brain and a sample of skeletal muscle from the hind legs. They were stored frozen in sealed test tubes for up to 2 weeks before further processing. Subsequently, it was established that small amounts of tritiated water evaporated from the tissues under these conditions. Following intraperitoneal injection of tritiated water (New England Nuclear, Boston, MA) and excision of tissues 7 hr later, it was found that 20% of the tritiated water was lost from spleen during a 2-week period of frozen storage, whereas a negligible proportion was lost from liver. This difference is likely the result of the much larger surface-to-volume ratio of the spleen, as well as the 10-fold greater weight of liver tissue stored.

Each tissue was minced with scissors, mixed thoroughly, and a sample of 0.1 to 0.8 g was selected and weighed. The sample was homogenized (Ten Broeck, Fisher, West Haven, CT), placed on ice, and suspended in cold 5% trichloroacetic acid (TCA). Cold TCA was added directly to the plasma samples. Centrifugation at 1200 *g* yielded a pellet of acid-insoluble material and a clear supernatant fraction of acid-soluble material. Duplicate samples of the supernatant fraction were removed, added to scintillation fluid (Scintiverse I, Fisher), and counted on a double channel scintillation counter (Beckman Instruments Inc., model LS 7000, Fullerton, CA) with energy channels chosen to facilitate determinations of ^{3}H and ^{14}C. Additional samples were dried at room temperature, redissolved in 5% TCA, and counted. The remaining supernatant fraction was removed completely, and the pellet was dissolved in KOH (1 N) and neutralized with HCl. This solution was added to scintillation fluid and counted. The counts in each channel were converted to disintegrations per minute from ^{3}H and ^{14}C by reference to standard curves generated from known quantities of [^{3}H]H_2O and [^{14}C]toluene (New England Nuclear) added separately to non-radioactive tissue samples chosen to provide a full range of quenching. The quenching level in each sample was determined from the counts recorded after a brief irradiation by a ^{133}Ba source within the scintillation counter. The concentrations of ^{3}H and ^{14}C in each tissue sample were calculated from the specific activity of the two isotopes and expressed in terms of moles per liter of tissue for both the acid-soluble and acid-insoluble portions of the total radioactivity.

Statistical analysis. Differences in concentrations were evaluated for statistical significance using Student's *t*-test [28]. Ten mice were used for the analysis reported here.

HPLC analysis of the acid-soluble material. Misonidazole and its metabolites were detected in the acid-soluble fraction of plasma from blood collected at 2 and 24 hr after a single i.p. injection of 20 mg/kg tritiated misonidazole into the mice. An aliquot (100 μl) of each acid-soluble sample was analyzed directly by HPLC. The remainder of the acid-soluble sample was then evaporated to dryness under a stream of N_2 gas, and the residue was redissolved in 5% trichloroacetic acid. Aliquots (100 μl) of these samples were chromatographed on a Partisil 10 ODS reversed phase column (4.6 mm × 25 cm; Whatman Chemical Separation, Inc., Clifton, NJ). Misonidazole and its metabolites were eluted with 10% aqueous acetonitrile flowing at 2.0 ml/min. A Varian (Sunnyvale, CA) 5000 series HPLC pump was used for this. Fractions of the column effluent (0.5 ml) were collected in scintillation vials, 10 ml of Scintiverse I (Fisher) was added, and the tritium content of the fractions was measured by scintillation counting.

RESULTS

The quantities of side-chain label (^{3}H) and ring label (^{14}C) retained in the acid-insoluble fraction of six normal mouse tissues, EMT6 tumors and plasma are shown in Table 1. For each isotope the quantities

Table 1. Concentration of metabolites of misonidazole retained in mouse tissues and plasma which were isolated in the acid-insoluble fraction

	Acid-insoluble fraction	
	$^3H^*$ (μM)	$^{14}C\dagger$ (μM)
EMT6 Tumor	3.38 ± 0.50‡	3.10 ± 0.45
Liver	3.30 ± 0.35	3.65 ± 0.29
Spleen	0.27 ± 0.09	0.37 ± 0.12
Kidney	0.57 ± 0.10	0.59 ± 0.12
Heart	0.72 ± 0.19	0.79 ± 0.21
Muscle	0.32 ± 0.07	0.31 ± 0.09
Brain	0.34 ± 0.03	0.28 ± 0.03§
Plasma	0.19 ± 0.02	0.15 ± 0.01

* Label on the side chain.
† Label on the ring (2-C).
‡ Values are means ± 95% confidence limit.
§ Difference between ^3H and ^{14}C was statistically significant ($P < 0.05$).

retained varied substantially among the tissues, but for each tissue the quantities of ^3H and ^{14}C were nearly equal. Statistically significant excesses of ^3H were seen in brain and plasma, but these differences were relatively small compared to the differences among tissues.

The acid-soluble fractions of the tissues, shown in Table 2, clearly contained appreciably more ^3H than ^{14}C. The excess ^3H was found to be volatile. Removal of the volatile component by evaporation resulted in equal amounts of ^3H and ^{14}C in the acid-soluble fraction or, in the case of heart and liver, an excess of ^{14}C (Table 2). In contrast, evaporation had no effect on the quantity of ^{14}C in the acid-soluble fraction. The quantity of ^3H removed by evaporation appeared to be relatively constant in all tissues (column 6, Table 2). Only the difference between spleen and plasma was statistically significant ($P < 0.05$). It was shown that some evaporation of the volatile component occurred during frozen storage in test tubes in which case the loss of volatile material would be expected to be the greatest for spleen which has the largest ratio of surface area to volume and the smallest mass for the storage conditions employed.

At 2 hr after i.p. injection of tritiated misonidazole (6 plasma half-lives) [29], three major peaks of radioactivity appear in the HPLC chromatogram (Fig. 1A). Two of the peaks had retention times corresponding to those of misonidazole and its demethylated metabolite, Ro-05-9963. The third, most polar component, which had the lowest retention time, proved to be volatile and could be removed completely by evaporation under a stream of N_2 gas at room temperature (Fig. 1B). An authentic sample of tritiated water had the same retention time as the volatile component—not only in this solvent system but in a second system (C-18 μBondapak, Waters, Milford, MA, 60% aqueous acetonitrile 0.2 ml/min) in which the components eluted more slowly albeit with poorer resolution (results not shown). At 24 hr after i.p. injection of tritiated misonidazole, very little of either misonidazole or Ro-05-9963 was detectable in the acid-soluble fraction of the mouse plasma. Almost all of the tritium now appeared in the most polar, volatile component (Fig. 2A).

DISCUSSION

The quantities of ring and side-chain label of misonidazole retained in the acid-insoluble fractions of most tissues studied were identical within experimental error of approximately 10%, whereas differences of 25% were seen for brain and plasma (Table 1). This is in agreement with a previous observation that equal quantities of both labels are bound to macromolecules in cells labelled in the absence of oxygen *in vitro* [25]. The data shown in Table 2 are also in agreement with a previous report [26] which demonstrated that an excess of side-chain label (^3H) from misonidazole is retained by several mouse tissues, compared to the quantity of ring label (^{14}C) retained at times after injection that are long relative to the rate of clearance of non-metabolized drug. All of the excess side-chain label was shown to be present in the acid-soluble fraction in a volatile form (Table 2). After removal of the volatile material, essentially equal quantities of both labels

Table 2. Concentrations of metabolites of misonidazole retained in mouse tissues and plasma which were isolated in the acid-soluble fraction

	Acid-soluble fraction		Acid-soluble fraction after evaporation		Quantity of ^3H removed by evaporation of acid-soluble fraction (μM)
	$^3H^*$ (μM)	$^{14}C\dagger$ (μM)	$^3H^*$ (μM)	$^{14}C\dagger$ (μM)	
EMT6 Tumor	3.07 ± 0.27‡	2.04 ± 0.22§	2.07 ± 0.26	2.02 ± 0.26	1.00 ± 0.09
Liver	1.95 ± 0.31	1.46 ± 0.19§	0.98 ± 0.16	1.49 ± 0.21§	0.97 ± 0.18
Spleen	1.29 ± 0.21	0.46 ± 0.15§	0.42 ± 0.14	0.46 ± 0.18	0.87 ± 0.14
Kidney	1.45 ± 0.24	0.58 ± 0.16§	0.46 ± 0.14	0.57 ± 0.15	0.99 ± 0.17
Heart	1.40 ± 0.26	0.71 ± 0.19§	0.44 ± 0.13	0.67 ± 0.15§	0.96 ± 0.20
Muscle	1.24 ± 0.18	0.42 ± 0.15§	0.32 ± 0.15	0.43 ± 0.15	0.92 ± 0.17
Brain	1.42 ± 0.21	0.37 ± 0.05§	0.36 ± 0.05	0.37 ± 0.06	1.06 ± 0.18
Plasma	1.27 ± 0.24	0.08 ± 0.04§	0.06 ± 0.04	0.07 ± 0.04	1.21 ± 0.21

* Label on the side chain.
† Label on the ring (2-C).
‡ Values are means ± 95% confidence limit.
§ Difference between ^3H and ^{14}C was statistically significant ($P < 0.05$).

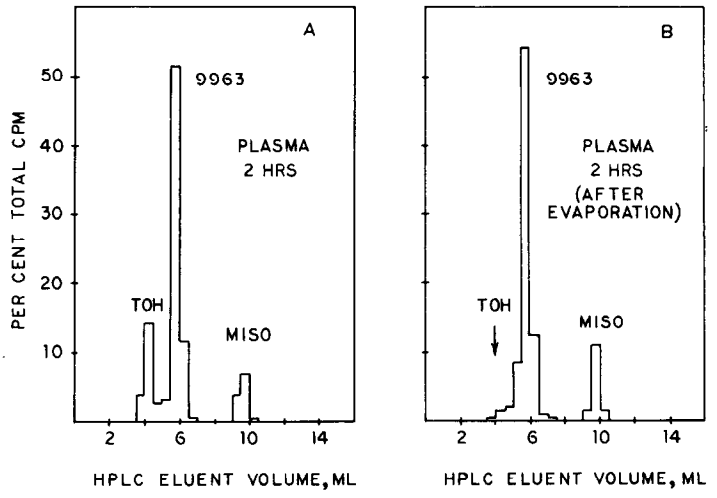

Fig. 1. HPLC of the acid-soluble fraction of mouse plasma 2 hr after [^{3}H]misonidazole injection. (A) HPLC profile before evaporation, and (B) HPLC profile after evaporation and reconstitution of the acid-soluble fraction. TOH is where authentic tritiated water appears in the chromatogram.

were also found in the acid-soluble material from most tissues, with the exceptions of liver and heart, where 50% excesses of ring label were found. Thus, the predominant species among both the non-volatile adducts to macromolecules and the metabolites retained as small molecules by EMT_6 tumors and various normal mouse tissues at long times after injection appear to contain both the 2-carbon and the side chain.

It has been argued previously in respect to binding to the acid-insoluble material of hypoxic cells *in vitro* that the simplest interpretation of this result is that a molecular product of misonidazole reduction is responsible for binding of both the side chain and the 2-carbon to macromolecules [25]. The essence of this argument is that, while a wide variety of fragmentation products of misonidazole metabolism has been identified and several different paths of fragmentation have been proposed (reviewed in Ref. 8), none leads to fragments which have appreciable binding potential that contain both the side chain and the 2-carbon. The possibility that the binding of a variety of fragments might coincidentally lead to equal amounts of macromolecular binding of the two labels was considered to be less plausible. Using the same arguments, it may be postulated on the basis of the data presented here that molecular products of misonidazole reduction (e.g. hydroxylamine or nitroso derivatives) or free radical intermediates which comprise the complete carbon skeleton of misonidazole are responsible for most of the macromolecular binding—not only in hypoxic tumor cells but in aerobic normal mouse tissues as well. Furthermore, the metabolites found in the acid-soluble fraction, whether bound to small molecules or in the form of poorly diffusible species, also appear to be

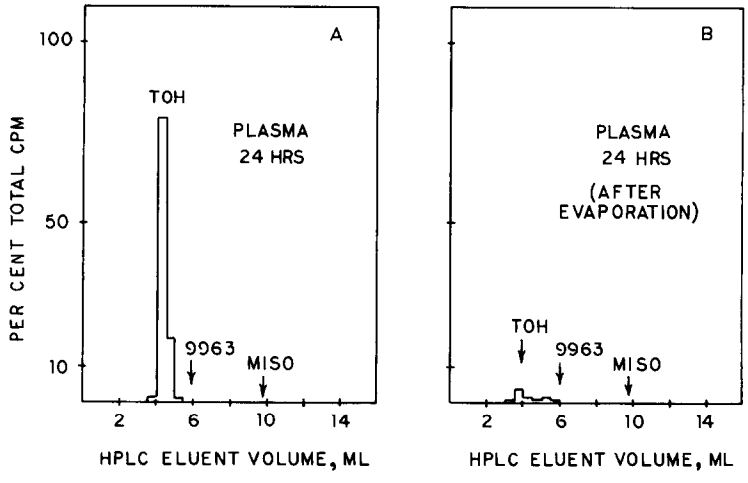

Fig. 2. HPLC of the acid-soluble fraction of mouse plasma 24 hr after [^{3}H]misonidazole injection. (A) HPLC profile before evaporation, and (B) HPLC profile after evaporation and reconstitution of the acid-soluble fraction. TOH is where authentic tritiated water appears in the chromatogram.

primarily molecular products of misonidazole metabolism, with the exception of contributions to the acid-soluble fractions of liver and heart from a fragmentation product containing the 2-carbon of the ring and not the side chain. The conclusion derived from previous work [25] that a molecular species is involved was considerably strengthened by the data in Tables 1 and 2, because it seems even less likely that two different fragmentation products would be found in nearly equal quantities in both acid-soluble and acid-insoluble fractions of seven different tissues and plasma.

The results of the analysis of the acid-soluble material of the plasma by HPLC (Figs 1 and 2) indicate that the volatile material was tritiated water. Tritiated water would be expected to be distributed uniformly among all tissues, and the results in Table 2 are consistent with this requirement—particularly when the possibility of some differential evaporation of water during storage of the excised tissues is considered, as noted above. The turnover time of tritiated water in mice (biological half-life = 1.1 days [30]) is consistent with the retention of an appreciable quantity of volatile tritiated material 21 hr after the last injection of [^{3}H]misonidazole. Although the evidence cannot be considered as conclusive, we believe that a plausible mechanism for the production of tritiated water from [^{3}H]misonidazole involves metabolic oxidation at the C-2 position of the side chain of misonidazole.

Assuming that the volatile metabolite was indeed tritiated water, it may be concluded that metabolic oxidation at the 2-position of the side chain in misonidazole is a relatively minor route for its degradation. Only a 1 μM concentration of the volatile metabolite was observed in the acid-soluble fraction 21 hr after a concentration of 100 μM misonidazole had been maintained for 3 hr in the mouse tissues (Table 2). It is possible that the metabolite resulting from the loss of tritium has a greater binding potential than misonidazole itself and thus could contribute to the differential retention of the ring label. In particular, if it were formed primarily in a single organ, then some excess retention of ring label over side-chain label might be expected in that organ. The data in Table 1 indicate that this may be the case for the acid-insoluble fraction of liver and heart. The acid-soluble material from these organs (Table 2) also exhibited a small (statistically non-significant) excess of ring label. Thus, the liver and heart may be the primary sites of production of tritiated water.

These results are relevant to the design of labelled compounds as markers for tissue hypoxia using non-invasive assays such as nuclear magnetic resonance, positron emission tomography, and single photon emission tomography. The volatile product of misonidazole reduction was almost certainly tritiated water and, therefore, contained none of the side-chain carbon atoms. Thus, the generation of tritiated water from the side chain should have no effect on the ratio of the quantities of other isotopes such as ^{19}F or ^{14}C retained in tumors and normal tissues, regardless of the location of the isotopes on the misonidazole structure. This conclusion, in conjunction with the suggestion that molecular species are responsible for retention of essentially all non-volatile metabolites of misonidazole at long times after injection, indicates that side-chain labels and ring labels will be equally efficient for non-invasive assays of tissue hypoxia.

A major qualification to the foregoing conclusion is that in the present work only the 2-carbon position of the ring has been compared with the label on the side chain. This is currently the most important comparison because much of the work which has established misonidazole as a useful marker for hypoxia was performed with a 2-^{14}C label [5–8], while several studies with side-chain labels are currently in progress. These include human trials with [^{3}H]misonidazole [31] and developmental studies with fluorinated analogues [23, 24].

Labels at other positions in the ring are potentially of interest. It may be expected that these would be equally useful as markers for hypoxia because molecular products of misonidazole appear to be responsible for most of the retention of labels reported here. However, fragmentation of reductively-activated 2-nitroimidazoles is a significant process and may lead to species with binding potential. For example, glyoxal in either free or sequestered form arises from the 4- and 5-carbons of the ring during fragmentation [32–34]. In this connection, a product of misonidazole metabolism has been recovered in hypoxic CHO cells and from human urine by Varghese and Whitmore [35, 36] which reacts with guanosine to yield a glyoxal-guanosine adduct. They proposed that the reactive intermediate is not glyoxal, but an unstable product of hydrolysis of the hydroxylamine stage of misonidazole reduction. However, recent data favor the interpretation that glyoxal itself is the reactive species [34]. Regardless of the nature of this reactive species, the fact that it appears in urine implies that it may be either insufficiently reactive or too freely diffusible to provide good discrimination between hypoxic and aerobic regions of tissues separated by distances of tens of micrometers. In contrast, there is considerable evidence indicating that the reactive species produced by hypoxic cells which contain the 2-carbon and the side chain are largely restricted to the cell in which they are produced [7, 37, 38]. Thus, further investigation will be required to determine whether misonidazole labelled at other positions in the ring will be a useful marker for hypoxia.

A small proportion of reactive species binds to macromolecules in the plasma (Table 1). It is known that very little reactive material escapes from hypoxic cells *in vitro* [7, 37], and it may be that this binding is the result of production by aerobic tissues of small quantities of molecular intermediates which can bind to plasma components. Misonidazole amine apparently can be activated to bind to aerobic tissue *in vivo* [13]. If the amine were to be formed by the reduction of misonidazole by intestinal flora, for example [13, 39], it would represent a diffusable substrate that could be activated to a binding molecular intermediate in aerobic tissue. In any case, it raises the possibility that a proportion of the material retained in a given tissue may have been produced elsewhere in the animal and thus should not be related to the local pO_2. This would be a very minor problem for tissues which metabolize relatively large

quantities of misonidazole such as liver and tumors, but it may be significant in using misonidazole retention to estimate local pO_2 in many other tissues.

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