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Characteristics of the Metabolism-induced Binding of Misonidazole to Hypoxic Mammalian Cells[1]

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ABSTRACT

[^{14}C]Misonidazole (MISO) becomes bound to macromolecules of mammalian cells upon hypoxic incubation. Intracellular enzyme processes are implicated since the temperature dependence for this process showed an activation energy of 33.5 kcal/mol. The sensitizer bound to both hypoxic and aerobic cells was associated with the macromolecular fraction and the soluble fraction in the proportion, 23 and 77%, respectively. The initial rate of binding of [^{14}C]MISO to the macromolecular (acid-insoluble) fraction of hypoxic EMT-6 mouse tumor and V-79 hamster cells increased proportionally with the square root of extracellular concentration of MISO up to at least 5 mM. High concentrations of dimethyl sulfoxide (an effective $OH^{\cdot}$ radical scavenger), allopurinol (an effective inhibitor of xanthine oxidase), and diamide (a chemical which can deplete cellular levels of glutathione) had little or no effect on this metabolism-induced binding process. The addition of high concentrations of exogenous cysteamine to hypoxic cell cultures resulted in almost complete inhibition of binding. Extracellular bovine albumin at high concentration in hypoxic cell cultures had little effect on the production of adducts to cell macromolecules and only small amounts of [^{14}C]MISO were found to bind to the extra-cellular bovine albumin. This result suggests that MISO preferentially binds to molecules within the cell in which it is metabolically activated.

In experiments where cells labeled under hypoxic conditions with [^{14}C]MISO were subsequently permitted to proliferate in aerobic monolayers, a half-life of the acid-insoluble addition products of ~55 hr was measured. A large number of [^{14}C]-MISO adducts (~10^9/cell) can be generated in hypoxic cells without any evidence of cytotoxicity, and they are slowly cleared from cells. These are favorable characteristics as regards the development of this technique as a marker for hypoxic cells in solid tumors.

INTRODUCTION

Solid tumors in both animals and man are known to consist of cells which are heterogeneous in proliferation status as well as oxygenation status. Thomlinson and Gray (26) showed that the histological structure of some human cancers suggested the presence of hypoxic cells and potentially an inherent resistance to treatment with ionizing radiations. Tannock (23–25) studied the effects of pO_2 on cell proliferation kinetics in 2 transplantable mouse tumor systems and demonstrated that the microarchitecture of the animal tumors was similar to that of some human tumors. This work also showed that the proliferating zone of tumor cells occurred within a radius of 100 ± 30 (S.D.) μm around blood vessels, a distance which closely corresponded to computed maximum oxygen diffusion distances when consumption of oxygen by respiration was taken into account. The cellular labeling index, radius of proliferating zone, and tumor growth rate were all lowered if animals were made to breathe air which contained only 10% oxygen. These classical studies provided conclusive evidence that oxygen concentration was an important factor in tumor growth kinetics and that tumor histology was a useful tool in studying such effects. The presence of hypoxic viable cells in tumors has always been histologically inferred since no direct assay or marker for such cells has been available.

Several clinical evidences exist which indicate that hypoxic cells in solid tumors can control the ultimate radiocurability of the disease (5). Hypoxic cells are known to be about 2.5 to 3.0 times more resistant to ionizing radiation than are oxygenated cells (12). Radioresistant hypoxic tumor cells which survive a course of radiotherapy can become reoxygenated, proliferate, and result in a local recurrence of the disease. Several of the experimental modalities in modern radiation therapy, including neutron therapy, the use of high-pressure oxygen chambers, and hypoxic cell radiosensitizers, are directed at the treatment resistance of hypoxic cells in solid tumors. It would seem that the presence of hypoxic cells in tumors and their role in tumor responsiveness will always have clinical relevance and techniques for diagnosing hypoxia within individual tumors could play an important role in understanding the problem.

MISO[3] becomes bound to molecules selectively in hypoxic mammalian cells (29). The selective uptake of [^{14}C]MISO has been used to determine the location of hypoxic cells in histological sections of multicellular spheroids and a murine tumor (6). The presence of bound sensitizer was determined by autoradiography of residual label after the unbound drug was washed from the sections. In this manuscript, several characteristics of the sensitizer-binding process in hypoxic mammalian cells are defined which might help to define the mechanisms of hypoxic cell cytotoxicity and might be useful in the development of a radioactively labeled marker of hypoxic cells. Previous studies had shown that the total activity of [^{14}C]MISO bound to EMT-6 tumor cells *in vitro* was distributed between the acid-soluble and acid-insoluble fractions in proportions of 77 and 23%, respectively (20). The characteristics of sensitizer binding reported herein relate to the 23% of total counts bound to the acid-insoluble fraction of cells.

MATERIALS AND METHODS

MISO was generously provided by Hoffmann-La Roche, Ltd., Vau-

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[3] The abbreviations used are: MISO, misonidazole, 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol; BA, bovine albumin; MEM, Eagle's minimal essential medium.

dreuil, Quebec, Canada. [^{14}C]MISO (labeled at C-2 of the imidazole ring) was generously supplied by Dr. W. E. Scott, Hoffman-La Roche, Inc., Nutley, N. J., at specific activities of 144.5 and 293 μCi/mg. [^{14}C]-Demethylmisonidazole (NSC-261036) and [^{14}C]SR2508 (NSC-301467) were supplied by Dr. Robert Engle, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., at specific activities of 66.5 and 61.0 μCi/mg, respectively. [^{14}C]Nitrofurazone, [^{14}C]nitrofurantoin, and [^{14}C]furaltadone were supplied by Dr. F. Ebetino, Chemistry Research Division, Norwich-Eaton Pharmaceuticals, Norwich, N. Y., at specific activities of 17.7, 11.4, and 16.1 μCi/mg, respectively. [^{14}C]-Metronidazole was supplied by Dr. Robert Sarrazin, Rhône-Poulenc Pharma, Inc., Montreal, Quebec, Canada, at a specific activity of 57.6 μCi/mg. Dimethyl sulfoxide (spectrograde) was purchased from Fisher Scientific Ltd., Edmonton, Alberta, Canada, and allopurinol, BA, diamide, and cysteamine were purchased from Sigma Chemical Co., St. Louis, Mo.

EMT-6 fibrosarcoma mouse tumor cells and V-79 Chinese hamster fibroblasts were cultured as monolayers and transferred twice weekly in Waymouth's media and standard MEM, respectively. Cells grown to near confluence on 150-sq cm flasks were trypsinized, centrifuged, and resuspended in spinner MEM (Mg^{2+} and Ca^{2+} free) which contained 7% fetal calf serum. Cells at a concentration of 5×10^6 cells/ml were transferred to glass chambers designed to permit control of both temperature of the liquid culture and oxygen levels in the gas phase. These chambers were originally designed for radiobiological studies with suspensions of mammalian cells and have been described previously (4). The cell suspensions were gassed with humidified gas mixtures, 5% CO_2 plus air for aerobic conditions and 5% CO_2 plus 95% ultrapure nitrogen (oxygen content <5 ppm) for hypoxic conditions. Radiobiological hypoxia was achieved in cell suspensions stirred slowly in these chambers after 40 min of degassing with nitrogen mixtures at 1 liter/min (4), and consequently a nitrogen gas flow rate of 1 liter/min was used in these experiments to achieve hypoxia. An air mixture flow rate of one-fourth to one-half liter/min was used to maintain aerobic conditions in other cell suspensions.

Solutions of [^{14}C]MISO and various drugs were prepared at 20 to 50 times the final desired concentration and were added in small volumes to the cell suspensions in the chambers. At various times, samples of the incubated cell suspensions were removed through the sampling port in the glass chambers. These were added to 10 volumes of cold trichloroacetic acid at 5% in aqueous solution to effect the precipitation of the macromolecular fraction. Samples were maintained on ice until they were filtered through cellulose acetate filters (Sartorius; pore size, 0.45 μm) held in a Millipore sampling manifold to collect the macromolecular fractions. Filters were washed twice with cold 5% trichloroacetic acid, dried in an oven at 65°, and placed in scintillation vials to which was added 10 ml of toluene Omnifluor scintillation fluid, and ^{14}C activity was determined with a liquid scintillation counter (LS7000, Beckman Instruments, Inc.). The counting efficiency of ^{14}C by this technique was routinely between 70 to 80%.

V-79 and EMT-6 cells were prepared at a concentration of 5×10^6 cells/ml in spinner MEM which contained BA at a final concentration of 0.1% and [^{14}C]MISO at 10 μM. At various times after incubation under hypoxia, samples were removed and centrifuged at 2000 rpm for 10 min to separate the cells into a pellet from the BA suspension. Trichloroacetic acid (5%) was added to both the pellet and the supernatant, and acid-precipitable material was collected and counted for ^{14}C activity as described above.

Suspensions of either V-79 or EMT-6 cells were incubated in standard spinner MEM with 7% fetal calf serum under hypoxic conditions in the presence of 5 μM [^{14}C]MISO for 3 hr. Cells were removed from the radioactive media by centrifugation, resuspended in unlabeled culture media, plated at a concentration of 10^6 (V-79) or 0.5×10^6 (EMT-6) cells/dish, and incubated under aerobic conditions for several days. Both cell number and [^{14}C]MISO bound to total cellular material were assayed at various times.

RESULTS

Characteristics of the metabolism-induced binding of MISO to hypoxic mammalian cells might be useful in defining cytotoxic and mutagenic properties of this drug. Nevertheless, the major emphasis of this current work was to determine if the adducts formed between MISO and cellular molecules had properties which might be exploited in the development of a marker for hypoxic cells in human tumors.

Experiments reported previously (20) indicated that the metabolism-induced binding of MISO to EMT-6 tumor cells was about 50 times more efficient under hypoxic conditions than under aerobic conditions. It was reported in that same study that the bound [^{14}C]MISO was associated with the macromolecular fraction and the soluble fraction in the proportion, 23 and 77%, respectively of both hypoxic and aerobic EMT-6 tumor cells. The 23% of [^{14}C]MISO bound to the macromolecular fraction was distributed among RNA (17%), lipid (4%), DNA (1%), and protein (1%).

Preliminary studies with different concentrations of [^{14}C]MISO had shown that the initial rate of adduct formation in hypoxic Chinese Hamster V-79 cells was dependent upon sensitizer concentration (8). Chart 1 indicates that the binding of ^{14}C from labeled MISO to the macromolecular fraction of Chinese hamster cells is linear with time for at least 3 hr. Best-fitted straight lines through data points up to 150 min on rectilinear graph paper defined the initial rates of binding. Additional studies have been performed with both EMT-6 tumor and Chinese hamster V-79 cells utilizing a broad concentration range of [^{14}C]MISO. Chart 2 shows the results of these studies. The initial rate of ^{14}C adduct formation in both cell lines is given in units of pmol/10^6 cells/hr. It can be seen that the initial rate of binding of ^{14}C from labeled MISO to the macromolecular fraction of hypoxic cells increases with increasing MISO concentration. The absolute rates of binding of ^{14}C-MISO to EMT-6 tumor cells are approximately double those measured for Chinese hamster V-79 cells over the entire concentration range. It is of interest to note that a straight line

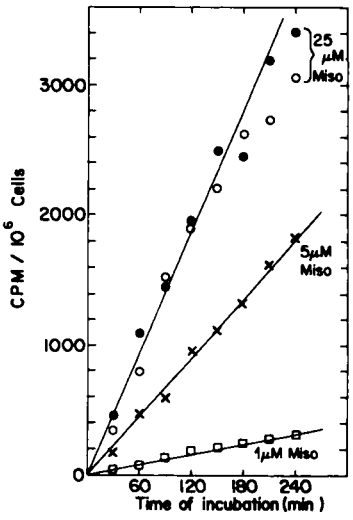

Chart 1. Amount of [^{14}C]MISO bound to the acid-insoluble fraction of Chinese hamster V-79 cells at various times of hypoxic incubation at 37°. One experiment at 1 μM [^{14}C]MISO (□) and 5 μM [^{14}C]MISO (×) and duplicate experiments at 25 μM [^{14}C]MISO (●, ○) are shown. Best-fitted straight lines through data points up to 150 min define the initial rates of binding.

fitted through these measured rate constants for both cell lines has a slope of approximately 0.5.

It has been suggested that a metabolism-induced and/or an enzyme-activated product of MISO is the chemical species which can react with cellular biomolecules to form addition products (29). The initial rate of binding of [^{14}C]MISO at 10 μM to EMT-6 tumor cells in hypoxia was studied over the temperature range 37 to 20°. Chart 3 shows these initial rates plotted *versus* the reciprocal of absolute temperature. An activation energy of 33.5 kcal/mol can be computed from this plot. This number strongly suggests that cellular enzyme processes are involved in the formation of MISO adducts with cellular biomolecules under hypoxic conditions.

If a metabolism-activated product of MISO can bind to cellular macromolecules, it is of interest to know whether such activated species can leave the cell in which they become activated, diffuse to another cell, and form adducts at that more distant site. If this could happen, it was postulated that a high concentration of extracellular biomolecules might serve as a trap for such activated species and result in a decreased rate of binding to the biomolecules within the cells. Chart 4 shows the results from one experiment where EMT-6 cells were incubated in culture medium containing 0.1% BA and 10 μM [^{14}C]MISO under hypoxic conditions. After incubation, the cells were separated from the BA by centrifugation, and the amount of radioactivity bound to the macromolecular fraction of the cells and to the BA was determined. These data indicate that, at times when large numbers of [^{14}C]MISO adducts were bound to the cellular macromolecules, a much smaller amount of [^{14}C]MISO became bound to the BA. This result suggests that the activated MISO species which can bind to cellular biomolecules are probably highly reactive and that few actually diffuse away from the cells in which they were activated. Similar experiments performed with Chinese hamster V-79 cells showed even less or no ^{14}C activity bound to extracellular BA.

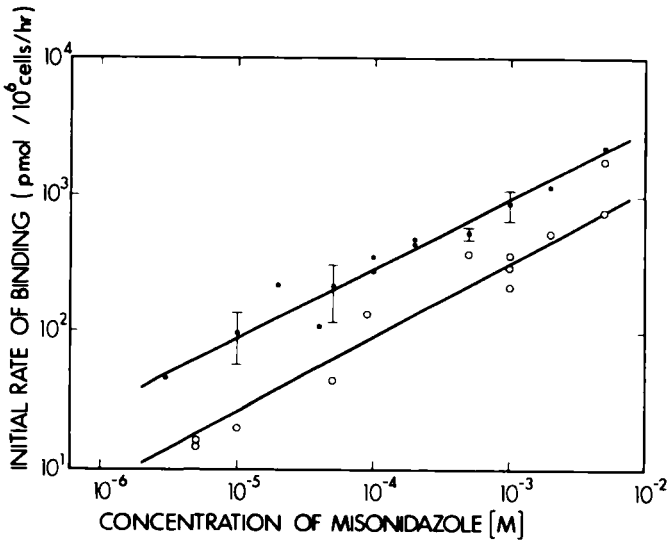

Chart 2. Initial rate of binding of [^{14}C]MISO to the acid-insoluble fraction of hypoxic EMT-6 mouse tumor (●) and hypoxic Chinese hamster V-79 (○) cells at 37° as a function of MISO concentration. Points, derived from independent experiments; *bars*, S.D., shown for concentrations with 3 or more determinations.

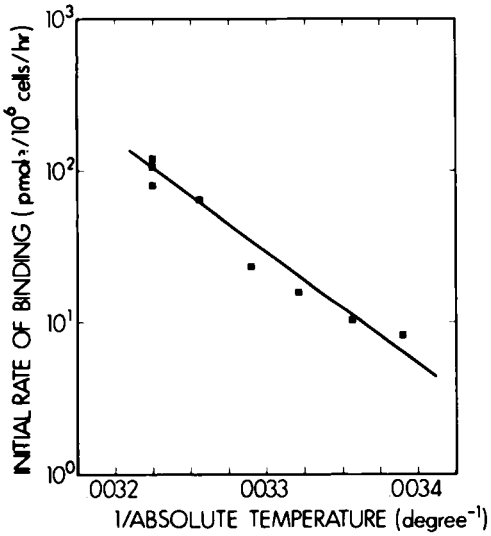

Chart 3. Initial rate of binding of [^{14}C]MISO at 10 μM to hypoxic EMT-6 tumor cells *versus* the reciprocal of absolute temperatures for each determination. The slope of the straight line shown yields an activation energy of 33.5 kcal/mol.

The nitrosubstituent of aromatic compounds can be reduced by a 6-electron process to the amino product (9). Intermediate species in such a reduction scheme would be the nitroradical anion, nitroso, and hydroxyl amino aromatic derivatives. It has not been determined which cellular electron-donating systems are responsible for the reduction of nitroaromatics in hypoxic mammalian cells. One well-characterized electron-donating system in cells is xanthine oxidase, and allopurinol is an efficient inhibitor of this enzyme (21). The data in Table 1 indicate allopurinol at concentrations known to inhibit xanthine oxidase activity *in vitro* (30) did not diminish the initial rate of binding of ^{14}C from labeled MISO to the macromolecules of hypoxic EMT-6 tumor cells. Intracellular nonprotein sulfhydryl (at least 90% of which is glutathione) is known to be an efficient radical-scavenging agent (1) and an important antioxidant in the cellular aqueous compartments. Diamide has been shown to decrease cellular levels of nonprotein sulfhydryl (16) and can influence cellular radiation response (17). Diamide over a concentration range of 20 to 500 μM had little effect on the initial rate of binding of [^{14}C]MISO to hypoxic EMT-6 tumor cells (Table 1). A slight reduction in binding rate was observed at high concentrations when in fact enhanced binding might have been expected if intracellular glutathione had been efficiently oxidized. Glutathione levels in these cells were not determined. Cysteamine added exogenously to hypoxic cultures of EMT-6 tumor cells reduced the initial rate of binding of [^{14}C]MISO to the macromolecular fraction of cells with initial binding rates inhibited by ~80% with concentrations of 5 to 10 mM. Dimethyl sulfoxide is an efficient scavenger of OH· in cells and a common solvent for several drugs. Its presence in hypoxic cell suspensions at 1% did not alter the initial rate of binding of ^{14}C from labeled MISO to the macromolecular fractions.

Adducts of radiosensitizing drugs to hypoxic cells might be exploited as a noninvasive probe for tumor hypoxia (and consequently tumor radioresistance) if their lifetime is significantly longer than the clearance time of unbound sensitizer from the surrounding aerobic tissues (10, 31). The stability and lifetime of the [^{14}C]MISO adducts to the macromolecular fraction of both EMT-6 and Chinese hamster V-79 cells were measured. Chart 5 shows both the proliferative capacity (consequently viability) and

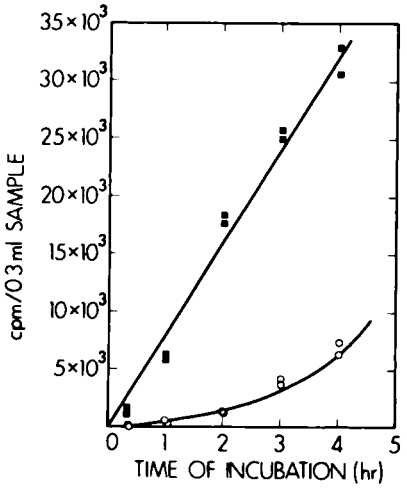

Chart 4. Amount of [^{14}C]MISO at 10 μM bound at 37° to the acid-insoluble fraction of 1.5 × 10^6 EMT-6 cells in 0.3 ml of sample (■) and to the extracellular BA of the same sample (○) at various times of hypoxic incubation.

Table 1

Effect of exogenous chemicals on initial rate of binding of [^{14}C]MISO to hypoxic EMT-6 cells

[^{14}C]MISO (μM)	Drug	Concentration	Rate of binding[a] (pmol/10^6 cells/hr)
10	Control		84 ± 17[b]
	Dimethyl sulfoxide	At 1%	97 (115)[c]
	Diamide	20 μM	96 (114)
	Diamide	50 μM	75 (89)
	Diamide	200 μM	56 (67)
	Diamide	500 μM	50 (60)
	Allopurinol	50 μM	67 (80)
	Allopurinol	100 μM	111 (132)
	Allopurinol	300 μM	76 (90)
20	Control		116 ± 10[b]
	Cysteamine	0.2 mM	146 (126)
	Cysteamine	0.5 mM	107 (92)
	Cysteamine	1.0 mM	56 (48)
	Cysteamine	2.0 mM	47 (41)
	Cysteamine	5.0 mM	33 (28)
	Cysteamine	10.0 mM	24 (21)

[a] Unless otherwise noted, All other rates are single experiment determinations where the error might be expected to be ±20%.

[b] Mean ± S.D. rate of binding for 10 and 20 μM, respectively, [^{14}C]MISO with no additional chemicals.

[c] Numbers in parentheses, binding rate as a percentage of the control.

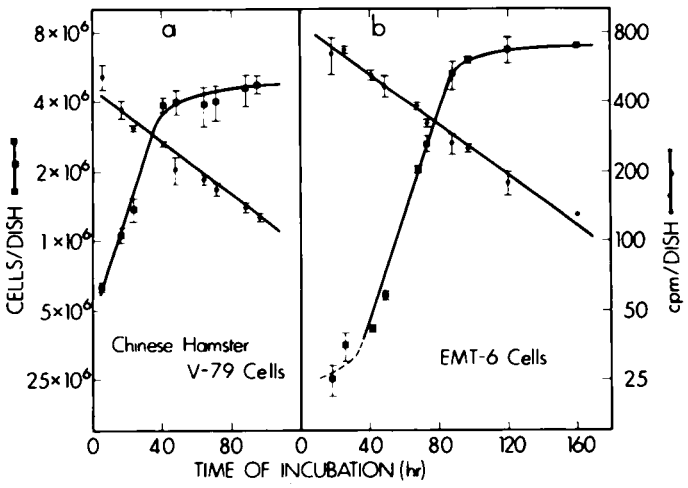

Chart 5. Chinese hamster V-79 (*a*) and EMT-6 mouse tumor (*b*) cells were labeled with 10 μM [^{14}C]MISO for 3 hr, reoxygenated, distributed into Petri dishes, and incubated at 37°. The number of cells per dish (■) and the total acid-insoluble ^{14}C cpm per dish (●) are plotted *versus* time of subsequent incubation in hr.

the amount of bound [^{14}C]MISO remaining in the expanded cell populations. Cells were initially labeled for 3 hr in hypoxia in the presence of 5 μM [^{14}C]MISO and subsequently reoxygenated, distributed into Petri dishes, and cultured at 37°. The exponential growth rate of both EMT-6 and Chinese hamster V-79 labeled cells is similar to that of unlabeled controls. The half-life of the [^{14}C]MISO adducts to macromolecules in both cell lines is approximately 50 to 55 hr. The half-life of MISO in both the serum and various tissues of mice is 25 to 45 min (10) and in those of humans is approximately 10 hr (11, 27).

MISO was selected for this study from several nitroaromatic drugs which had hypoxic cell radiosensitizing activity in both tissue cultures and animal tumor models. It has been administered at high dosages to a large number of cancer patients undergoing experimental radiation therapies (22) and consequently has the advantage of a recent literature. On the other hand, other nitroaromatic drugs might be more efficient in forming metabolism-induced adducts with cellular biomolecules in hypoxic cells and be optimum for this diagnostic application. Additional 2-nitroimidazole, 5-nitroimidazole, and 5-nitrofuran compounds labeled with ^{14}C were incubated at 10 μM with hypoxic EMT-6 tumor cells. These drugs have shown good radiosensitizing activity when tested at much higher concentrations in this hypoxic cell culture system. The initial rates of binding measured in these studies are shown in Table 2. [^{14}C]Demethylmisonidazole, [^{14}C]nitrofurazone, and [^{14}C]furaltadone have rates of binding similar to that measured for MISO. The rates of binding of [^{14}C]nitrofurantoin, [^{14}C]SR2508 and [^{14}C]metronidazole are 50, 10, and 8% of the MISO rate, respectively. It would seem that MISO is as efficient a metabolism-induced macromolecule binding agent in these hypoxic cells as are any of the other sensitizing drugs.

DISCUSSION

MISO is selectively cytotoxic to hypoxic mammalian cells (15). It has been proposed that the toxicity of nitroaromatic drugs results from metabolism-produced "activated" products of such

Table 2

Rates of binding of ^{14}C-labeled radiosensitizers (at 10 μM) to hypoxic EMT-6 cells

Drug	Binding rate (pmol/10^6 cells/hr)
Misonidazole	109 ± 36[a]
Demethylmisonidazole	113 (2)[b]
SR-2508	13 (2)
Metronidazole	10 (2)
Nitrofurazone	81 (3)
Nitrofurantoin	51 (3)
Furaltadone	109 (1)

[a] Mean ± S.D.

[b] Numbers in parentheses, number of independent determinations which were averaged to obtain the listed value.

Table 3

MISO adducts and cytotoxicity in Chinese hamster V-79 cells

Concentration (mM)	Time to kill[a] 50% (hr)	Rate of binding (pmol/10^6 cells/hr)	MISO adducts/cell: Insoluble fraction (× 10^6)	MISO adducts/cell: Whole cell (× 10^9)
1	4.3	320	8.3	3.6
2	3.2	480	9.3	4.0
5	1.85	800	8.9	3.9
Average			8.8	3.8

[a] Data from Chapman, et al. (7).

drugs (19, 28) and that the addition of such activated species to specific cellular biomolecules might constitute the toxic mechanism (29). The data reported in this manuscript are consistent with this postulate. The binding of [^{14}C]MISO to the macromolecular fraction of hypoxic EMT-6 cells is shown to have a temperature dependence consistent with cellular enzyme functions. An intracellular activation energy of 33.5 kcal/mol defines the thermodynamics of the rate-limiting step of the cellular activation process and could be useful in identifying the specific enzyme function(s). The data presented also indicate that both V-79 and EMT-6 tumor cells can accumulate many [^{14}C]MISO adducts without any deleterious effect on cellular proliferative capacity and/or cloning ability. Previous studies (20) had indicated that EMT-6 tumor cells could tolerate nearly 1×10^9 MISO adducts/cell without evidence of cellular toxicity. In Table 3, similar calculations are shown for hypoxic V-79 cells utilizing previously published cytotoxicity data (7) and the rates of binding of [^{14}C]MISO to these cells. These calculations indicate that 50% of the cells lose their ability to form colonies when approximately 9×10^8 MISO adducts are formed with the macromolecular fraction. Little or no toxicity is observed in cells which have bound one-third to one-half the amounts of bound [^{14}C]MISO required to produce 50% kill. Such nontoxic cellular burdens of radiosensitizer adduct might be detectable by several analytical procedures.

The rate of binding of [^{14}C]MISO to cellular macromolecules in EMT-6 tumor cells is approximately double that measured for Chinese hamster V-79 cells. The cellular volume of EMT-6 tumor cells is approximately twice that of V-79 cells, and consequently a doubling of sensitizer adduct to an increased macromolecular weight might be expected. It would appear that both cell lines can tolerate almost 10^9 adducts/cell without significant toxicity. From other data (20), it is evident that sensitizer adducts are widely distributed over both the soluble and insoluble cell biomolecules. If hypoxic cell cytotoxicity by radiosensitizing drugs is the direct result of addition reactions, the task of identifying the specific critical adduct(s) against a background of at least 10^9 noncritical adducts should prove a formidable task. The mechanism of cellular cytotoxicity by radiosensitizing drugs has yet to be defined and might not be related to addition reactions.

The data presented in this paper are encouraging as regards the utility of sensitizer adducts to hypoxic cells as an important tool in tumor biology and as a possible diagnostic probe for hypoxia in human cancers. The rate of binding of MISO to the biomolecules of mammalian cells was at least 50 times higher under the hypoxic conditions of these experiments as compared to aerobic conditions. Solid animal tumors are known to have deficient oxygenation status, and the fraction of hypoxic clonogenic cells can be as high as 30 to 40% as determined by radiobiological techniques (18). Studies with multicellular spheroids (13, 14) and EMT-6 mouse tumors growing in BALB/c mice (5) suggest that [^{14}C]MISO bound to molecules of hypoxic cells can be detected by autoradiographic techniques and indicate the oxygenation status of cells in tissues. Such a marker for hypoxic cells could prove useful in studies relating to tumor radioresistance, tumor chemoresistance, tumor reoxygenation after therapy, and tumor cell kinetics. There is clinical evidence which indicates that hypoxic cells determine the curability of solid tumors by radiation (2) and possibly by some chemotherapeutic drugs and any technique which might contribute to a better definition and understanding of this treatment-resistant subpopulation within tumors could have clinical use.

MISO adducts to the biomolecules of hypoxic mammalian cells might be detected by noninvasive techniques and consequently find application in the diagnosis and staging of human cancers. After a 2- to 10-hr exposure to MISO, the adducts in hypoxic cells will constitute a small percentage (1 to 10%) of the total drug distributed to most tissues. Consequently, to observe a significant signal from such adducts, most of the unbound drug would have to be cleared from various tissues by normal excretion and detoxification mechanisms.

The data of Chart 5 show an exponential loss with time of ^{14}C bound to the macromolecular fraction of both V-79 and EMT-6 cells. No difference in the rate of loss of ^{14}C adducts from cells in rapid growth or in stationary phase was observed. Whether or not this loss of ^{14}C is strongly temperature dependent or indicative of an active repair process in these cells has yet to be studied. These data do suggest that a similar loss of ^{14}C adduct from labeled MISO might be expected from tissues at 37° in animals.

The data presented indicate that the quantity and stability of sensitizer adducts to the macromolecules of both V-79 and EMT-6 cells are adequate for such an application. Furthermore, the evidence that the vast majority of adducts are formed within hypoxic cells in which the sensitizer is activated suggests that the probability of finding radiolabeled adducts in surrounding oxygenated tissue is relatively low. A similar conclusion was reached from experiments with EMT-6 and V-79 spheroids labeled with [^{14}C]MISO under controlled oxygen environments (14). MISO and demethylmisonidazole showed equal rates of binding to the macromolecules of hypoxic EMT-6 cells and the highest rates of the 7 radiosensitizers tested. These drugs might serve as the initial well-characterized sensitizers to which radionuclides might be attached. It is interesting to note that the more hydrophilic 2-nitroimidazole radiosensitizer SR2508 had a rate of binding approximately 10 times lower than that observed for MISO or demethylmisonidazole. This could indicate that the intracellular site and/or the molecular site at which sensitizer activation occurs are lipid associated and drugs with higher lipid solubilities will be preferentially activated. An activation energy of 33.5 kcal/mol is at the high end of the range for cellular enzyme processes when determined *in vitro* and might be indicative of membrane or other cellular structure-associated function.

The conventional techniques of radiopharmacy and nuclear medicine might be one system amenable to detect sensitizer adducts in hypoxic cells. The attachment of an appropriate γ-emitting radionuclide to an appropriate hypoxic cell radiosensitizer has been suggested (3, 9). The data presented in this manuscript suggest that radiochemical and radiopharmaceutical studies with γ-labeled radiosensitizers are warranted, and attempts to label radiosensitizers with bromine, indium, and iodine are in progress.

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