Increased Cell Killing by Metronidazole and Nitrofurazone of Hypoxic Compared to Aerobic Mammalian Cells

Jagjit K. Mohindra and Andrew M. Rauth

Ontario Cancer Institute [A. M. R.] and Department of Medical Biophysics University of Toronto [J. K. M., A. M. R.], Toronto, Ontario, M4X 1K9 Canada

SUMMARY

Nitroimidazole and nitrofuran derivatives comprise a large family of compounds, some of which have been shown to be hypoxic cell specific radiosensitizers in vivo and in vitro. The effects of metronidazole (2-methyl-5-nitroimidazole-1-ethanol) and nitrofurazone (5-nitro-2-furaldehyde semicarbazone) were studied on cell viability in vitro in the presence of air or nitrogen in the absence of radiation. Exponential-phase Chinese hamster ovary cells were placed in suspension culture in complete medium in the presence of air, made hypoxic by flowing nitrogen (<0.001% oxygen), and exposed to various concentrations of these drugs. As a function of time, aliquots were removed and plated to determine cell viability. After 8 hr of incubation of Chinese hamster ovary cells in 29 mM metronidazole or 500 μM nitrofurazone, the absolute plating efficiency remains relatively constant (80 to 40%) in the presence of air. In contrast, under hypoxic conditions the plating efficiency of the cells dropped to 1% after 6 hr of incubation in 29 mM metronidazole or 500 μM nitrofurazone. This phenomenon of hypoxic cell specific toxicity was found to be dependent upon cell type, concentration of drug, temperature of incubation, and oxygen concentration. The results of these experiments indicate an increased toxicity of these drugs under hypoxic conditions and suggest that further investigation into the mechanism and specificity of these effects is warranted.

INTRODUCTION

In the last few years a number of nitro group-containing compounds have been shown to sensitize hypoxic cells preferentially in experimental systems in vivo and/or in vitro to ionizing radiation (2, 4, 6, 7, 21, 25). Two of these compounds, nitrofurazone and metronidazole, are of particular interest since they are already used in humans as antibacterial and antiprotozoal agents (16, 19). Unfortunately, subsequent attempts to demonstrate hypoxic cell radiosensitization by nitrofurazone in vivo have met with little success (21), and recently it has been shown that at least some nitrofuran derivatives lose their ability to radiosensitize at high mammalian cell densities such as one might encounter in solid tumors in vivo (1, 27). In contrast, metronidazole, which radiosensitizes cells in vitro at both low and high cell densities (27), has been shown to radiosensitize hypoxic tumor cells in vivo in a number of test systems (4, 21, 25). Another difference between these 2 drugs is that metronidazole sensitizes hypoxic cells in the 1 to 10-mM concentration range, whereas nitrofurazone is effective at low cell densities in vitro at concentrations of the order of 10 to 100 μM.

These experiments were undertaken to study the toxicity of these 2 drugs for mammalian cells in the absence of radiation in order to understand better the differences in their mode of action in vivo and in vitro. Initially, studies were carried out on mammalian cells growing in air in the absence and presence of different drug concentrations. During the course of these studies Sutherland (26) reported the preferential killing by metronidazole of noncycling, possibly hypoxic cells, in his in vitro spheroid model tumor system in the absence of radiation. At approximately the same time, B. Palcic (personal communication) reported the increased toxicity of the 2-nitroimidazole, Ro07-0582, toward suspension cultures of Chinese hamster cells in vitro under hypoxic compared to aerobic conditions. In general, these effects required longer incubation times than those used for the standard radiosensitization experiments and the 2 effects could be separated experimentally. As a result of these observations the present studies were extended to measure the effects of nitrofurazone and metronidazole on cell growth and viability in the absence of oxygen to see whether metronidazole was indeed toxic to hypoxic cells and whether this effect could also be seen with a nitrofuran.

For most of these studies use was made of a Chinese hamster cell line, but since the ultimate application of the drug is directed toward humans, some use was made of human cells as well. The results to be reported indicate that both metronidazole and nitrofurazone exert an increased toxicity toward cells in the absence of oxygen. This differential toxicity toward hypoxic relative to aerobic cells was found to be a function of drug type, drug concentration, time of drug exposure, cell type, temperature, and oxygen concentration. These results suggest that this class of compounds may be useful as chemotherapeutic agents, as well as radiosensitizers specific for hypoxic cells.

MATERIALS AND METHODS

Cells. Studies were carried out with CHO2 cells (obtained...
from Dr. W. Dewey, Colorado State University, Fort Collins, Colo.), human HeLa cells (obtained from Dr. T. P. Brent, St. Jude Children’s Hospital, Memphis, Tenn.), and human bone marrow cells obtained directly from aspiration samples. CHO and HeLa cells were routinely maintained in suspension culture in complete α medium supplemented with antibiotics (24) and 10% FCS (v/v) (Flow Laboratories, Rockville, Md.). Normal cell-doubling times were 16 to 20 hr for both cell lines. Cell number was determined with an electronic particle counter (Coulter Electronics, Hialeah, Fla.). For most experiments, asynchronous populations of cells in exponential growth were used.

**Chemicals.** Metronidazole (2-methyl-5-nitroimidazole-1-ethanol) was obtained from Poulenc Ltd., Montreal, Canada, and nitrofurazone (5-nitro-2-furaldehyde semicarbazone) from Norwich Pharmacal Co., Norwich, N. Y. Both drugs were usually dissolved in complete α medium containing 10% FCS at concentrations of 58 or 1 mM, respectively. Medium containing drugs was filtered, using a 0.45-μm Nalgene (Nalge Sybron Corporation, Rochester, N. Y.) filter unit, and desired dilutions were made from this stock.

**Toxicity Tests.** Chronic toxicity of metronidazole and nitrofurazone toward HeLa and CHO cells was determined by plating the cells in 60-mm Falcon Petri dishes (Falcon Plastics, Oxnard, Calif.) containing 5 ml of complete α medium plus 10% FCS (v/v), with various concentrations of the drugs. The plates were left undisturbed for 10 to 12 days at 37° in a humidified 5% carbon dioxide plus 95% air incubator. Methylene blue in water:ethanol (1:1) was used for staining the colonies. Colonies of 50 or more cells were scored to determine the plating efficiency.

The method of Iscove et al. (12), as modified by Messner (14), was used to determine the capacity of human marrow suspensions to form colonies in culture in the presence of various concentrations of these drugs. Briefly,uffy coats prepared from suspensions obtained by aspiration were plated in medium consisting at final concentration of 0.8% methyl cellulose, 16% FCS, 10% human leukocyte-conditioned medium, and α medium. Incubation was done at 37° in a 7.5% carbon dioxide balance air incubator. After 14 days the colonies containing more than 20 cells were counted with the aid of an inverted microscope.

For long-term acute toxicity experiments in air, various drug concentrations were prepared in α medium plus 10% FCS. The CHO cells were suspended in drug-containing medium in stoppered 50-ml roller tubes that were placed in a roller wheel (New Brunswick Scientific Co., Inc., New Brunswick, N. J.). The samples that showed cell growth were diluted when the cell number reached 4 to 6 × 10⁶ cells/ml, to keep them in exponential growth. Aliquots were removed as a function of time, counted and plated in fresh medium after dilutions, and plating efficiency was determined.

Short-term acute toxicity experiments in reduced levels of oxygen were done as follows. Exponentially growing cells were resuspended at cell concentrations of 5 to 7 × 10⁶ cells/ml in α medium minus bicarbonate plus 10% FCS containing various concentrations of the drugs. Nitrogen-oxygen gas mixtures were flowed over a 7-ml cell suspension in 26-ml glass Polyshell vials (Richards Glass Co., Toronto, Ontario, Canada). The gas used was supplied by Gas Dynamics Ltd., Toronto, Ontario, Canada, and oxygen levels were determined by them, using combustion techniques. Absolute measurements with a Hersch cell (10) were in agreement, within experimental error, with oxygen levels in nitrogen quoted by the supplier. The vials were placed on a magnetic stirrer in a warm room at 34° and stirred continuously during the time of the experiment. The gases were humidified by bubbling through water and passed through a manifold so that a number of vials could be gassed at once. Gas was flowed at a rate of 1 liter/min through an 18-gauge needle in the stopper of each vial. A glass tube inserted through the stopper allowed the gas to escape. As a function of time, 10- or 100-μl samples were removed through the glass tube, cell number was determined, and cells were plated in fresh growth medium after appropriate dilutions.

In control samples exposed to air, cells were suspended in complete α medium containing 10% FCS at the same concentration of drugs under the same experimental conditions, except 5% carbon dioxide and 95% air were flowed instead of nitrogen-oxygen mixtures.

**Oxygen Concentration Measurements.** The problem of removing oxygen from aqueous solutions by passing a stream of nitrogen over them is not trivial (3, 15). A Hersch cell (10) was used to determine the oxygen concentration in the aqueous medium being gassed. Vials containing 7 ml of air-equilibrated distilled water were gassed with various nitrogen-oxygen mixtures exactly as described above for the short-term acute toxicity testing of cells. Samples of water were removed as a function of gassing time and analyzed for their relative oxygen content, using the Hersch cell as discussed by Dewey and Gray (8). The results are shown in Chart 1. As can be seen initially, the relative oxygen concentration in the stirred samples falls exponentially and then plateaus at the expected levels for 0.1% and 0.01% oxygen in nitrogen within 1.5 to 2 hr of gassing. Due to the sensitivity limit of the present Hersch cell, oxygen levels below 0.0002% of that in air-equilibrated water were not detectable. Thus, the plateau of the oxygen level in the vials gassed with 0.001% oxygen in nitrogen could not be detected. In the following results it has been assumed that it equilibrates at the expected level.

**RESULTS**

The toxicity of metronidazole and nitrofurazone toward mammalian cells was measured as a function of concentration of the drug and the time and conditions of exposure.

**Chronic Toxicity In Air.** In these experiments, the toxicity of different concentrations of metronidazole and nitrofurazone was measured by incubating the cells continuously in the drug-containing medium during their assay for colony formation. The plating of CHO and HeLa cells was done by the monolayer technique, while the plating of the human marrow cell suspension was done in methyl cellulose-containing medium (see “Materials and Methods”). Chart 2 shows that CHO, HeLa, and human marrow cells respond similarly to increasing concentrations of metronidazole and nitrofurazone in the plating medium. The concentrations of
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were resuspended in suspension culture in growth medium containing different concentrations of metronidazole and nitrofurazone at 34°C. The cells were diluted into fresh medium of the same composition whenever required, before they reached stationary phase. As a function of time, aliquots of the suspensions were removed, counted, and plated to determine their colony-forming ability. Chart 3, a to d, shows the results of these experiments. At the highest concentration of metronidazole, 58 mM, which is the solubility limit of the drug, the relative cell number decreased gradually with time, and the plating efficiency of CHO cells was reduced to about 5% in 50 hr of incubation. Almost no growth of cells was observed at 29 mM; nevertheless, their plating efficiency was reduced to only 30% after 100 hr incubation in the drug. At a metronidazole concentration of 14.5 mM, the growth rate of the cells continually slowed down and was essentially 0 after 100 hr; however, there was only a gradual drop in plating efficiency over the duration of the experiment. At lower metronidazole concentrations there were small effects of the drug on cell growth but no effect on cell-plating efficiency. Thus, in air, metronidazole concentrations that stop cell growth do not result in rapid loss of cell colony-forming ability. Somewhat similar results were obtained for nitrofurazone at drug concentrations 500-fold less than for metronidazole. For example, as seen in Chart 3b, a nitrofurazone concentration of 55 μM inhibited cell growth but did not result in rapid cell death.

Acute Toxicity in the Absence of Oxygen. Exponentially growing CHO cells were suspended under hypoxic conditions in media containing various concentrations of metronidazole or nitrofurazone, and as a function of time, samples were removed, cell counts were made, and aliquots were plated to determine the cell colony-forming ability. Additional samples were also run at the same time, in which air was flowed instead of nitrogen. As can be seen from Chart 4, a and b, these drugs cause cell death, as measured by a decrease in cell-plating efficiency, under hypoxic conditions. The results for air, which are not shown, at all concentrations of drug tested were identical with the no nitrogen curve. The toxicity seen, over the 10-hr exposure time, for CHO cells under hypoxia but in the absence of the drug (0 mM) is apparently not due to the hypoxia itself. In these earliest experiments it was noted that rapid stirring reduced cell viability with time; therefore, in later experiments the cells were stirred more slowly and full viability was maintained (see Chart 4, c and d, and charts 5 and 7; 0 mM, N2 curves).

Thus, metronidazole and nitrofurazone are much more toxic to cells in the absence of oxygen than in its presence. Even the lower concentrations of these drugs which have little effect on the growth of the cells or their colony-forming ability in air over a 2-day period, e.g., 7 mM metronidazole and 55 μM nitrofurazone in Chart 3, shows some signs of toxicity under hypoxic conditions after only 10 hr.

Acute Toxicity in Air. Exponentially growing CHO cells

Chart 1. Relative oxygen concentration in the aqueous phase of vials flushed with 0.1 (% , 0), 0.01 (■, O), or 0.001% (▲, Δ) O2 in N2 under standard conditions; 25-ml glass vials containing 7 ml stirred distilled water, gas flow rate 1 liter/min/vial, in a 34°C warm room. Theoretical equilibrium levels for the above gas mixtures are indicated. Arrow, sensitivity limit of the Hersch cell used for the measurements.

Chart 2. Relative plating efficiency of CHO cells (% , O), human HeLa cells (▲, Δ), and human bone marrow aspirants (■, O) plated in different concentrations of drug. Cells were left for the duration of the assay (10 to 14 days) without media change.

the drugs that inhibit colony-forming ability by 50% in air are approximately 10 mM for metronidazole and 20 μM for nitrofurazone, a difference of 500 in concentration. These results indicated that to assay cell survival, after exposure to high drug concentrations, the drug concentration must be reduced below 5 μM for nitrofurazone and 1 mM for metronidazole to avoid residual toxicity.

Acute Toxicity in Air. Exponentially growing CHO cells were resuspended in suspension culture in growth medium containing different concentrations of metronidazole and nitrofurazone at 34°C. The cells were diluted into fresh medium of the same composition whenever required, before they reached stationary phase. As a function of time, aliquots of the suspensions were removed, counted, and plated to determine their colony-forming ability. Chart 3, a to d, shows the results of these experiments. At the highest concentration of metronidazole, 58 mM, which is the solubility limit of the drug, the relative cell number decreased gradually with time, and the plating efficiency of CHO cells was reduced to about 5% in 50 hr of incubation. Almost no growth of cells was observed at 29 mM; nevertheless, their plating efficiency was reduced to only 30% after 100 hr incubation in the drug. At a metronidazole concentration of 14.5 mM, the growth rate of the cells continually slowed down and was essentially 0 after 100 hr; however, there was only a gradual drop in plating efficiency over the duration of the experiment. At lower metronidazole concentrations there were small effects of the drug on cell growth but no effect on cell-plating efficiency. Thus, in air, metronidazole concentrations that stop cell growth do not result in rapid loss of cell colony-forming ability. Somewhat similar results were obtained for nitrofurazone at drug concentrations 500-fold less than for metronidazole. For example, as seen in Chart 3b, a nitrofurazone concentration of 55 μM inhibited cell growth but did not result in rapid cell death.

Acute Toxicity in the Absence of Oxygen. Exponentially growing CHO cells were suspended under hypoxic conditions in media containing various concentrations of metronidazole or nitrofurazone, and as a function of time, samples were removed, cell counts were made, and aliquots were plated to determine the cell colony-forming ability. Additional samples were also run at the same time, in which air was flowed instead of nitrogen. As can be seen from Chart 4, a and b, these drugs cause cell death, as measured by a decrease in cell-plating efficiency, under hypoxic conditions. The results for air, which are not shown, at all concentrations of drug tested were identical with the no nitrogen curve. The toxicity seen, over the 10-hr exposure time, for CHO cells under hypoxia but in the absence of the drug (0 mM) is apparently not due to the hypoxia itself. In these earliest experiments it was noted that rapid stirring reduced cell viability with time; therefore, in later experiments the cells were stirred more slowly and full viability was maintained (see Chart 4, c and d, and charts 5 and 7; 0 mM, N2 curves).

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The same experiment was repeated with HeLa cells. Under normal conditions of cell culture in air there is no difference in the toxicity of metronidazole and nitrofurazone toward HeLa and CHO cells (Chart 2). Nevertheless, HeLa cells appear to be approximately twice as sensitive to
Toxicity of Metronidazole and Nitrofurazone

Effects of Temperature. All the above experiments were done at 34°. The specific toxicity of metronidazole and nitrofurazone was studied at room temperature to see whether the hypoxic cell-specific toxicity of these drugs was temperature dependent. In this experiment, cells were suspended in the absence of drugs, in 29 mM metronidazole, or in 500 μM nitrofurazone. Hypoxia was maintained as described above and cells were exposed under identical conditions at 20° or 34°, and colony-forming ability as a function of time was determined. Metronidazole and nitrofurazone are much less toxic to the cells under hypoxic conditions at room temperature than at 34° (Chart 5). When this experiment was done at 4°, even the small degree of cell killing seen at 20° was lost (data not shown).

Effect of Oxygen Concentrations. In all of the above experiments use was made of air (21% oxygen) or nitrogen (<0.001% oxygen). To determine whether intermediate levels of oxygen could lead to differential toxicity, the following experiment was done. CHO cells were suspended in media containing no drug, 29 mM metronidazole, or 500 μM nitrofurazone and put in vials. Nitrogen containing less than 0.001% oxygen (<10 ppm), 0.01% (100 ppm), 0.113% (1130 ppm), 1.02% (10,200 ppm), or 5% CO₂ balance air was flowed through separate vials. As can be seen from Chart 6, metronidazole shows the greatest differential killing of cells for less than 0.001% oxygen. At 0.01% oxygen the killing is much reduced. Although nitrofurazone shows greatest toxicity at the lowest oxygen concentration, appreciable cell killing is still seen at 0.1% oxygen, and only at 1% or greater is the toxicity lost. Thus, some difference in cell killing is seen for the 2 drugs as a function of oxygen concentration.

Stationary Phase Cell Toxicity. All the above experiments were done using cells growing exponentially at the time they were put in the vials for the differential toxicity tests. To see whether the toxicity of these drugs was dependent on the growth state of the cells before exposure to the drug, early stationary-phase cells were used rather than exponential-phase cells. Cells were grown in suspension culture in complete media. Cell counts were taken every 24 hr and when no increase was seen for 24 hr, the cells were considered to be in early stationary phase. The cells were centrifuged and resuspended in 50% of the original medium plus 50% fresh medium containing no drug, 58 mM metronidazole, or 1 mM nitrofurazone, so as to give the same cell number as the original culture from which they were taken. Differential toxicity was studied under air and nitrogen as described above. As seen in Chart 7, 29 mM metronidazole

Chart 3. Growth of CHO cells in suspension culture in air in the presence of various concentrations of drug and percentage of plating efficiency of aliquots of the same cells removed after the indicated growth times and plated in the absence of the drug; growth curves in (a) metronidazole and (b) nitrofurazone; plating efficiency of cells grown in (c) metronidazole and (d) nitrofurazone.
Chart 4. Plating efficiency of cells exposed to various drug concentrations for various times under hypoxic (<0.001% O₂ in N₂) conditions at 34°C. Cells were suspended in a medium minus HCO₃ and continually gassed with N₂. CHO cells exposed to (a) metronidazole, and (b) nitrofurazone and HeLa cells exposed to (c) metronidazole, and (d) nitrofurazone.

Chart 5. Plating efficiency of CHO cells exposed to metronidazole (29 mM) or nitrofurazone (500 μM) under hypoxic (<0.001% O₂ in N₂) or air conditions for various times at 20°C or 34°C. ---, same curve shown in Chart 4b.

and 500 μM nitrofurazone showed similar toxicity as was observed previously, using exponential-phase cells in Chart 4. The metronidazole data of Chart 4 agree quite well with the data in Chart 7. However, the shoulder seen in Chart 7 for stationary-phase cells with nitrofurazone is absent in the data for exponential-phase cells in Chart 4. In general, however, the growth state of the cell prior to drug exposure does not seem to have a large effect on the degree of differential toxicity observed.

DISCUSSION

From these results it can be seen that hypoxic cell-specific radiosensitizers metronidazole and nitrofurazone act...
Toxicity of Metronidazole and Nitrofurazone

The toxicity of these drugs is strongly time and temperature dependent. In addition, the radiosensitizing effects of the drugs in general are independent of the oxygen concentration below 0.1% (5), while the differential toxicity is strongly dependent on the oxygen concentration in the range 0.1 to 0.001% as seen in Chart 6. Since most in vitro radiosensitization experiments referred to above involve exposure of hypoxic cells to drug for less than 2 hr usually at room temperature, the radiosensitizing effects of these drugs would not have been complicated by the phenomenon of differential toxicity.

The explanation for the effect of temperature and the difference in the hypoxia necessary to demonstrate a differential toxicity for metronidazole and nitrofurazone may be directly due to the biochemical state of the cells and possible mechanisms for these effects are briefly discussed below. Demonstration of the differential toxicity of metronidazole requires much lower oxygen levels (<0.01%) than for nitrofurazone. Some care must be exercised to assure that the nitrogen used does have low oxygen levels and that equilibrium is reached by the gassing system (see Chart 1).

In this system times of the order of 1.5 hr were required for equilibrium to occur. It is possible that the shoulder seen for a decrease in cell survival for CHO cells in metronidazole (Charts 4 to 6) could be due to this experimental artifact. However, experiments in which cells and drug were prepared separately for 2 hr before mixing under hypoxic conditions, yielded essentially the same results as above.

Therefore, the shoulder of the metronidazole curve is probably not a result of this equilibrium time and may be a property of the cells themselves.

Sutherland (26) reported that exposing spheroids of V-79 hamster cells to 15 mM metronidazole for 24 hr at 37° resulted in the preferential killing of the central noncycling, and probably hypoxic cells of the spheroid. When CHO cells are exposed to 14.5 mM metronidazole under hypoxia (<0.001% oxygen) for 24 hr using the techniques described in this paper, their survival was reduced to 0.1% of the control (data not shown). These results are consistent with the observations of Sutherland and indicate that the differential toxicity was most probably due to the hypoxic state of the interior cells of the spheroid. Olive and McCalla (18) have shown that nitrofurans produce single-strand DNA breaks in mammalian cells; the number of breaks increases under reduced oxygen tensions, consistent with the increased toxicity at low oxygen concentrations in these experiments.

The increased sensitivity of hypoxic compared to aerobic cells to the 2-nitroimidazole, Ro07-0582 (22), has been observed in this system also. Preliminary data indicate that various degrees of increased sensitivity under hypoxic conditions are seen for the 5-nitroimidazole, tinidazole, the 4-nitroimidazoles, R.P. 8532 and R.P. 8979, as well as the nitrobenzene, paranimroacetophenone. Thus, hypoxic cell toxicity in a broad sense seems to be a characteristic of many nitro groups containing hypoxic cell radiosensitizers.

How unique this toxicity is for nitrofurans and nitroimidazoles remains to be seen. Other chemotherapeutic agents have not been tested specifically in this way, although such tests are currently under way in the present in vitro system. Some tests of cancer chemotherapeutic agents on chromosome damage in plant and animal cells in vitro have revealed that their effects can be inhibited, independent of, or enhanced by the lack of oxygen. The most common result was no change or less effect under anoxia (23).

Nitrofurans and nitroimidazoles are in general more effective as antibiotics toward anaerobic bacterial and protozoal infections than aerobic infections (11, 19, 20). At least 3 possible mechanisms for the differential toxicity of metronidazole and nitrofurazone have been suggested: (a) an increased permeability of the drug into cells under hypoxic compared to aerobic conditions (17); (b) a hypoxia-dependent enzymatic activation of the drug to a toxic form (11, 13, 18, 20); and (c) inhibition of a biochemical pathway that is unique or required for cell viability under hypoxic culture conditions (19). Further work will be required to determine whether any of these mechanisms can explain the differential toxicity observed in these experiments.

To what degree these agents will be useful in vivo will of course depend on the extent to which hypoxic cells are a problem in human tumors. In radiotherapy, hypoxic cells are considered to be a potential problem in obtaining cures with some tumors (9). This is of course the reason why hypoxic cell radiosensitizers were sought in the first place. The fact that such agents may also exert a specific toxicity toward hypoxic cells in the absence of radiation makes them even more promising in this application.
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