Nitrofurans as Radiosensitizers of Hypoxic Mammalian Cells

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SUMMARY

Our studies showed that various nitrofuran derivatives have excellent radiosensitizing properties in hypoxic Chinese hamster cells at concentrations at which there is no effect on plating efficiency. The radiosensitizing effect is primarily a dose-modifying effect, with nitrofurantoin, nitrofurazone, and nifuroxime (each at 500 μM) in complete medium giving enhancement ratios of 1.65, 2.0, and 2.2, respectively. In parallel studies, the radiosensitizing effect of molecular oxygen (air-saturated conditions) was found to be dose modifying, with an enhancement ratio of ~2.9. Selective radiosensitization in hypoxia has been demonstrated in proliferating populations of cells in every phase of the cell cycle, as well as in nonproliferating populations which were contact inhibited at the time of irradiation. In all cell populations tested, these compounds did not alter radiosensitivity in air-saturated conditions.

The permeability of phospholipid membranes to nitrofurazone in 0.1 M NaCl has been measured, and a permeability constant (K) of 1.5 (± 0.7) x 10^-5 cm/sec obtained. No diffusion time lag was observed. In the presence of 0.1 M NaCl and 0.5 mM MgCl₂, the permeability constant was increased to 2.5 (± 0.4) x 10^-5 cm/sec, and a diffusion lag time of 12 ± 3 min was obtained. Comparison of this constant with permeability constants for other molecules suggests that the phospholipid membrane presents no great barrier to the penetration of this compound.

Hypoxia is shown to enhance the rate of radiation-induced binding of the label from nitrofurazone-14C to bovine serum albumin, DNA, polynucleotides, and Chinese hamster cells. Mechanisms of radiosensitization are discussed in relation to the observed binding and the antibacterial mode of action of the nitrofurans. The potential application of this class of compounds in the radiotherapy of tumors in which hypoxia is suspected is discussed in relation to their known pharmacological properties and current clinical usage.

INTRODUCTION

It has been suggested that the concentration of oxygen dissolved in tissues at the time of irradiation may be a factor in determining the success of radiotherapy for some tumors (17, 18). Subsequent experiments indicated that cells in solid tumors irradiated in vivo are inactivated by ionizing radiation according to a multicomponent survival curve that may be related to a variation in oxygen concentration across the tumor mass (22, 36). If the radiosensitivity of hypoxic cells within solid tumors could be made to coincide with that of the oxygenated cells of the same tumor, the success of current radiotherapy regimes would be greatly enhanced. Attempts to modify the radiosensitivity of suspected hypoxic centers in tumors by oxygen have been frustrated by difficulties and in general have not been successful. Kaplan (23) has stated, regarding clinical trials of hyperbaric oxygen radiotherapy, that “Unfortunately these randomized clinical trials have to date failed to reveal any significant improvement in survival or, in most instances, even in the frequency of local eradication of several types of cancer treated under hyperbaric oxygen . . . .” Another approach to the problem of hypoxia in radiotherapy would be the use of a different chemical radiosensitizer. Advantages could possibly be gained if the sensitizer were not rapidly metabolized by tissue so that concentrations displaying radiosensitizing potential could be established in the vicinity of the hypoxic cells during the radiation treatment.

Bridges (9) has reviewed the topic of chemical radiosensitization, and one of the most promising classes of radiosensitizers for application in tumor therapy appears to be the electron-affinic type described by Adams et al. (1-4). PNAP² was shown to be strongly electron affinic (1) and, subsequently, was shown to be an effective radiosensitizer of hypoxic mammalian cells (2, 13). A study with analogs of PNAP (10) has demonstrated that the NO₂ substituent of the benzene ring is essential for demonstration of radiosensitization in mammalian cells. Furthermore, the extent of radiosensitization could, to some degree, be correlated with the electronegativity of the nitrobenzene analogs tested. Studies have been extended to some nitrofuran derivatives which are known to be more electronegative than their corresponding nitrobenzene derivatives (39) and which have the attraction of established clinical acceptability (32). Preliminary experiments indicated that nitrofuran derivatives are excellent radiosensitizers of hypoxic mammalian cells (37). In this paper, we report additional radiobiological data demonstrating the radiosensitizing effectiveness of 3 nitrofuran derivatives, along with data pertaining to the toxicity, permeability, and mechanism of radiosensitization of these compounds.

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² The abbreviations used are: PNAP, p-nitroacetophenone; poly(A), poly(adenylate); poly(C), poly(cytidylate); poly(I), poly(inosinate); poly(U), poly(uridylate); HBSS, Hanks' balanced salt solution; TCA, trichloroacetic acid; ER, enhancement ratio.
MATERIALS AND METHODS

Chinese hamster cell line V79-379-A was routinely grown in suspension culture consisting of minimal essential medium with spinner salts (Grand Island Biological Co., Grand Island, N. Y.) supplemented with antibiotics and fetal calf serum (7% by volume: Becton and Dickinson Ltd., Clarkson, Ontario, Canada). Cells grown in this manner at 37° had a doubling time of ~10 hr and could be transferred from spinner culture to monolayer culture (attached to glass) and back again with ease.

5-Nitro-2-furaldehyde-semicarbazone (nitrofurazone), \(N\)-(5-nitro-2-furfurylidine-1-aminohydantoin (nitrofurantoin), and nitrofurazone-formyl-\(^{14}\)C (specific activity, 31.1 μCi/mg) were generously provided by Norwich Pharmacal Co., Norwich, N. Y. \(N\)-(5-nitro-2-furaldoxime (nifuroxime) was obtained from Aldrich Chemical Co., Milwaukee, Wis. Bovine serum albumin (Fraction V), salmon sperm DNA (highly polymerized), poly(A), and poly(C) were obtained from Sigma Chemical Co., St. Louis, Mo. Poly(I) and poly(U) were purchased from Schwarz/Mann, Orangeburg, N. Y. In all of our cellular experiments, the drugs were dissolved in complete growth medium unless specified to the contrary.

The radiation source, radiation quality, and dose rate in a cellular monolayer have been described elsewhere (13).

Toxicity studies with cells growing \textit{in vitro} were performed to determine both the cytostatic and cytotoxic action of these drugs. The cytostatic effect was measured by determining the effect of various concentrations of each nitrofurans on net cell proliferation over a 24-hr growth period. These experiments were performed with cells growing attached to Petri dishes, and the data were reduced to a percentage of normal proliferation. We determined the cytotoxic effect of each nitrofuran derivative by exposing small numbers of hamster cells attached to Petri dishes to various concentrations of each drug for various lengths of time. The ability of cells treated so that they would subsequently proliferate in drug-free medium and form normal colonies in 5 to 7 days was assayed. The cellular proliferative capacity (or colony-forming ability) was the end point used in all radiobiological experiments, as well.

We prepared cells for irradiation by diluting growing cultures and plating an appropriate number of cells in glass Petri dishes (60 × 15 mm) in 3 ml of medium. Cells were permitted to attach and grow at 37° for 2 to 3 hr. The cell number per dish was adjusted so that, depending upon irradiation conditions and dose, approximately 100 colonies per dish would be evident after 6 days of incubation at 37° for hamster cells. Prior to irradiation, the medium in which the cells had attached was removed from the dishes and replaced with 0.75 ml of complete medium with or without drug. The air-tight chamber used to hold the Petri dishes in the X-ray field and degassing procedures used to establish hypoxia have been described elsewhere (13).

Synchronously growing populations of Chinese hamster cells were established by a mitotic selection procedure (12). Cells growing in spinner culture were plated in 16-oz bottles (3 to 4 × 10^6 cells/bottle) and were incubated overnight at 37°. The monolayers were then shaken at hourly intervals, and the second or third population selected was used. Radiation doses for synchronously growing cells were adjusted so that the resulting level of survival was approximately the same for cells irradiated under the various conditions. Whole survival curves were also constructed for cells in the most sensitive and most resistant parts of the cell cycle.

We prepared confluent cultures by plating ~10^6 cells into glass Petri dishes in an adequate volume of medium and incubating them until the cells were observed to have reached confluence. Contact-inhibited cells (at confluence for ~48 hr) were irradiated in a manner identical with that described for asynchronously growing cell monolayers. The cells were covered with 0.75 ml of medium (with and without sensitizer) in air and in nitrogen environments. After irradiation, the cells were trypsinized, counted, and diluted, and appropriate numbers were plated into plastic Petri dishes. The plating efficiency of cells handled in this manner was always greater than 75%.

Reconstituted phospholipid membranes, prepared by procedures previously described (33, 34), were used as models for an examination of the permeability of membranes to the nitrofurazone-\(^{14}\)C radiosensitizer. Through the use of a perfusion technique (34), all of the nitrofurazone-\(^{14}\)C that diffused through the phospholipid membrane was collected in the perfusate as a function of time. The nitrofurazone-\(^{14}\)C activity in the volume of perfusate collected each hr was measured by liquid scintillation counting, as previously described (34).

Solutions containing 3.2 mg bovine serum albumin and about 10^6 cpn (20 μg) of nitrofurazone-\(^{14}\)C in 1.25 ml of 0.053 M phosphate buffer, pH 7.2, were irradiated in 25-ml suction flasks with γ-rays from a \(^{137}\)Cs source. The dose rate as determined by a Victoreen dosimeter was 330 R/min. Hypoxic conditions were achieved by blowing H₂O-saturated N₂ over the solution for 10 min prior to radiation, as well as during irradiation.

After irradiation, the solution was applied to a Sephadex G-25 column and eluted with water. Protein-containing fractions were located by their UV absorbance. One ml from the peak tube was added to 15 ml of dioxane fluor and counted in a Nuclear-Chicago Unilux 1 counter.

Polynucleotides (0.6 mg) were dissolved in 0.015 M sodium citrate:0.15 M NaCl buffer (pH 7.0), irradiated, and chromatographed as described previously. The concentration of polynucleotide in the peak tube was determined from A₂₆₀ measurements after acid hydrolysis.

DNA solutions (0.15 M NaCl solution: citrate buffer) were irradiated in the presence of nitrofurazone and then were deproteinized by shaking with chloroform:isoamyl alcohol (9:1) after the addition of sodium dodecyl sulfate (1%). The deproteinized step was repeated (usually 3 times) until the aqueous phase reached constant activity. The solution was then chromatographed as described above. The amount of protein present in the polynucleotide preparations was determined by the folin procedure of Lowry et al. (28), with bovine serum albumin as a reference.

We demonstrated radiation-induced binding to hamster cells by preparing a standard suspension of cells and nitrofurazone-\(^{14}\)C in HBSS and irradiating them in a γ-cell (Atomic Energy of Canada Ltd.; dose rate, ~18,000 rad/min). Cells growing asynchronously in spinner culture were...
harvested by centrifugation (700 X g for 10 min), washed once in HBSS, and resuspended at a final concentration of 10^6 cells/ml in HBSS that contained 100 μM nitrofurazone-formyl-14C (specific activity, 31.1 μCi/mg). Aliquots (0.05 ml) were dispensed into the bottom of test tubes and were allowed to equilibrate with environments of air or pure nitrogen which were circulated over the droplets prior to and during irradiation. After irradiation, 1 ml of ice-cold 5% TCA was added to each test tube, and the samples were held in an ice bath for 30 min. The acid precipitates were collected on membrane filters, washed with ice-cold 5% TCA, dried, and counted in toluene base liquid scintillator in a Nuclear-Chicago Mark II liquid scintillation spectrometer.

RESULTS

In Vitro Toxicity of Nitrofuran Derivatives. We measured the cytostatic effect of each nitrofuran derivative by exposing hamster cells growing at 37° in complete growth medium to various concentrations of each drug for 24 hr and assaying the increase in cell number. Chart 1 shows growth inhibition responses for nitrofurazone, nitrofurantoin, and nifuroxime, and the concentrations of drug resulting in 50% inhibition of proliferation are 44, 72, and 44 μM, respectively. The cytotoxic effect of various concentrations of the nitrofurans was measured, and the data are shown in Chart 2. It is apparent that nifuroxime is at least 2 to 3 times more toxic to hamster cells in culture than are nitrofurazone and nitrofurantoin. In all of the subsequent experiments reported in this paper, the time of exposure of the the nitrofurans to cells was always less than 30 min so that, even at very high concentrations of the drugs, negligible reduction in plating efficiency was measured in parallel toxicity runs.

Characterization of Nitrofuran Radiosensitization of Cells. To estimate the radiosensitizing effectiveness of potential radiosensitizers of hypoxic cells, we routinely use a rapid screening technique based on a single dose of irradiation. Chart 3 shows the ratio of surviving fractions for hamster cells irradiated with 1650 rads in hypoxia in the presence and absence of various concentrations of the drugs tested. All concentrations of the nitrofurans showed radiosensitizing ability which increased with concentration. The concentrations of nifuroxime, nitrofurazone, and nitrofurantoin that give a “ratio of surviving fractions” of 0.10 (equivalent to an enhancement ratio of ~1.7 for purely dose-modifying effects) were 100, 200, and 600 μM, respectively. The radiosensitizing effectiveness of nitrofurazone, nitrofurantoin, and nifuroxime was measured, and the data are shown in Chart 2. It is apparent that nifuroxime is at least 2 to 3 times more toxic to hamster cells in culture than are nitrofurazone and nitrofurantoin.
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Furfuraldehyde semicarbazone has been measured over a similar concentration range, and the data are also shown in Chart 3. It is evident that the introduction of a NO₂ group on the furan ring greatly enhances the radiosensitizing effectiveness of that structure. The radiosensitizing effectiveness of oxygen in air-saturated conditions is shown in the same plot, and the extent of radiosensitization by nitrofurazone and nifuroxime at 500 μM (and greater) approaches that of oxygen.

Complete survival curves were constructed to demonstrate the radiosensitizing effectiveness of each of the drugs at 500 μM in complete medium, and the data are shown in Chart 4. The radiosensitizing effect of the nitrofurans as well as molecular oxygen is primarily dose modifying. The data in Chart 4 are from an experiment performed on the same cell population; ER's as calculated from the D₀ values of the curves for radiosensitization by nitrofurantoin, nitrofurazone, nifuroxime, and molecular oxygen (air-saturated conditions), are 1.66, 1.95, 2.06, and 2.85, respectively. Small variations in ER are observed from day to day for any 1 concentration of drug, and the average ER's determined from 3 independent experiments for each of the sensitizing conditions mentioned above are 1.65, 2.0, 2.2, and 2.9. At 500 μM, these drugs had no effect on the radiosensitivity of hamster cells in air-saturated environments. What was previously reported (37) as a slight radioprotective effect of the nitrofurans in air has since been shown to result from a small amount (0.3%) of dimethyl sulfoxide that had been used initially to dissolve the drugs prior to dilution in complete medium.

Whole survival curves for hypoxic hamster cells irradiated in the presence of various concentrations of nifuroxime showed the radiosensitizing effect to be dose modifying at each concentration, much like the radiosensitizing effectiveness of oxygen as measured by Elkind et al. (15). Significant radiosensitization (ER, 1.22) was measured with nifuroxime at 10 μM, a concentration of drug which is nontoxic by both criteria used to estimate cellular toxicity (see Charts 1 and 2).

The radiosensitization of mammalian cells by oxygen has been shown to be independent of position within the cell cycle (13, 24, 25), and this is also true for the electron-affinic radiosensitizer PNAP (13). Experiments were performed to determine the cell cycle dependency of the radiosensitizing effectiveness of nitrofurazone and nitrofurantoin. Cells selected at mitosis and plated were irradiated at hourly intervals during the 1st cell cycle. Chart 5 shows the surviving fractions from an experiment in which cells were irradiated with 1055 rads in air, 2970 rads in N₂, and 1615 rads in N₂ in the presence of either 500 μM nitrofurazone or 1 mM nitrofurantoin. The cell cycle response for all of these cases shows the same general shape; cells in G₁ were most sensitive and cells in late S were resistant. Nitrofurazone appears to be slightly more effective in radiosensitizing G₁ than in radiosensitizing S cells. This increased effect on G₁ cells is demonstrated in Chart 6, which displays whole survival curves for G₁ cells (2 hr after selection and plating) and S cells (8 hr after selection and plating). The ER's for 500 μM nitrofurazone in this run were 2.18 for G₁ cells and 1.86 for S cells.
Hypoxic cells in tumors are likely to be part of the nonproliferating or slowly proliferating population. Stationary phase cultures at confluence have been studied as an in vitro model system more closely approximating nonproliferating cells in vivo (7, 19, 26, 27). Our hamster cell line, when brought to a nonproliferating state at contact inhibition, is blocked in a postmitotic, pre-DNA-synthetic part of the cell cycle (11), and it has a radiation sensitivity similar to that of G1 cells in synchronously growing cultures if the cells are irradiated as confluent cultures and immediately dispersed by trypsin for an assay of colony-forming ability (J. D. Chapman, unpublished results). Confluent cultures were irradiated in air and in hypoxia, with and without 500 μM nitrofurazone; the survival data are shown in Chart 7. An ER of 1.82 for nitrofurazone in hypoxia was obtained. This value is not significantly different from those measured in proliferating cell populations.

The charts that show cellular radiosensitivity (Chart 3 to 7) were constructed with the use of data from single-cell populations, and the number of colonies counted for each survival determination was such that the error for each was less than 7% (near the size of the symbols on the charts).

Permeability of Model Membranes to Nitrofurazone. Chart 8a illustrates the results obtained in a 7-hr experiment for a 0.06-sq cm membrane in 0.1 M NaCl. Q(t) is the time integral of the nitrofurazone-14C counts. It is seen that Q(t) increases linearly with time. From the slope of the line, the amount of radiosensitizer permeating in a unidirectional manner through the membrane per unit time, dQ/dt, is determined. The permeability constant K (cm/sec) is obtained as previously described (34) from the equation

\[ \frac{dQ}{dt} = KA \Delta c \]

where A is the area of membrane in sq cm and Δc is the concentration difference of nitrofurazone-14C across the membrane (≈10^6 cpm/cu cm). In 0.1 M NaCl, the permeability constant was determined to be 1.5 (± 0.7) × 10^-5 cm/sec, and there was no diffusion time lag, indicating that the drug partitions readily into the lipid membrane phase. In 0.1 M NaCl and 0.5 mM MgCl2, the Q(t) versus t curves (Chart 8b) were basically similar, except that the value of K was somewhat higher [≈ 2.5 (± 0.4) × 10^-5 cm/sec], and a
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Chart 9. Radiation-induced binding of $^{14}$C from labeled nitrofurazone to protein. $\circ$ and $\circ$, Experiment 1; $\bullet$ and $\bullet$, Experiment 2; K rads, kilorads.

diffusion time lag of 12 ± 3 min was demonstrated. The diffusion time lag obtained by extrapolating the steady state portion of the curve to the time axis is a measure of the time required for the nitrofurazone to reach an equilibrium distribution within the different regions of the membrane, particularly the interfaces. The values of $K$ are about 1 order of magnitude less than the permeability constant for water (34), and they suggest that the radiosensitizer should readily penetrate the membranes of individual cells.

Radiation-induced Binding of Nitrofurazone-$^{14}$C. Chart 9 shows the binding of $^{14}$C to protein when solutions of labeled nitrofurazone and serum albumin were irradiated with $\gamma$-rays. The amount bound increases with dose and is greater under hypoxia than in air by a factor of 4 to 5 at 20,000 rads. Irradiation of a nitrofurazone solution, followed by the addition of protein, did not result in binding that was significantly greater than that found in unirradiated controls.

The radiation-induced binding experiments were performed with radiation doses approximately 10 times greater than the dose range used to characterize the cellular radiosensitizing effects. This dose range was chosen primarily because of the relatively low specific activity of the labeled sensitizer. Nevertheless, the linear increase in sensitizer bound with increasing radiation dose (Charts 9 and 10) indicates that the observed binding characteristics can be expected to hold for the biological dose range as well.

Table 1 shows that there is extensive radiation-induced binding of $^{14}$C from labeled nitrofurazone to DNA and to polynucleotides. Under the conditions of our hypoxic experiments, 1.6 to 6.4 million counts were bound per mmole of nucleotide, representing the order of 1 molecule of nitrofurazone per 5,000 nucleotide units. With the exception of poly(A), about 15 times as much label was bound under hypoxic conditions as were bound under aerobic conditions at 20,000 rads.

The protein content of poly(1), poly(U), and poly(A) was below 0.25%, so binding to contaminating protein would not have amounted to more than about 4,500 cpm/m mole nucleotide. The poly(C) contained 2.5% protein which might have bound up to 45,000 cpm/m mole nucleotide. Even this value is so far below the total amount bound to the nucleotides that protein binding can be considered to be negligible.

Similarly, little radioactivity was bound to polymers in unirradiated controls or when the polymer was added to previously irradiated nitrofurazone solutions.

Hamster cells were suspended in HBSS containing 100 µM nitrofurazone-$^{14}$C and irradiated in air and hypoxia. Chart 10 shows the amount of $^{14}$C label from the sensitizer bound to the TCA-insoluble fraction of whole cells as a function of radiation dose. Radiation-induced binding in hypoxia is

Table 1

| Compound | Nitrofurazone input (cpm x 10^{-5} / 1.25 ml) | Amount bound (cpm x 10^{-5}/mmole nucleotide) in
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Air</th>
<th>Hypoxia</th>
<th>N_{2}:air</th>
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<tr>
<td>DNA</td>
<td>3.25</td>
<td>2.6*</td>
<td>37*</td>
<td>14</td>
</tr>
<tr>
<td>Poly(1)</td>
<td>2.3</td>
<td>3.2</td>
<td>64</td>
<td>17</td>
</tr>
<tr>
<td>Poly(4)</td>
<td>2.2</td>
<td>2.6</td>
<td>16</td>
<td>6.2</td>
</tr>
<tr>
<td>Poly(C)</td>
<td>2.3</td>
<td>1.8</td>
<td>27</td>
<td>15</td>
</tr>
<tr>
<td>Poly(U)</td>
<td>2.3</td>
<td>3.2</td>
<td>48</td>
<td>15</td>
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*Assuming an average molecular weight of 310 for the nucleotides of DNA.
enhanced by a factor of 7.0 to 7.5, as compared with oxygenated conditions. This enhancement ratio for binding to whole cells is greater than that measured for binding to protein and is less than that for DNA, suggesting that a variety of cellular molecules probably are reacting with the nitrofurazone-\textsuperscript{14}C.

**DISCUSSION**

Most of the data presented in this paper pertain to the toxic and radiosensitizing properties of some nitrofuran derivatives in an *in vitro* mammalian cell system. This cell system is convenient for rapidly estimating the merits of compounds selected as potential radiosensitizers. It is hoped that these data will provide a useful basis upon which to construct experimental designs for the radiotherapy of solid tumors in animals.

All radiosensitizers of the "electron-affinic type" (1) will probably be toxic to mammalian cells at high concentrations. One aim of our research has been to define drug concentration limits within which good selective radiosensitization of hypoxic cells can be demonstrated and which show no toxicity. Regarding the cytostatic effect of the nitrofurans, those tested inhibit cell proliferation at concentrations 6 to 9 times lower than that of PNAP (13). This result agrees with a previous observation that cellular toxicity as estimated by growth inhibition increases with increasing electronegativities of drugs (10). Cytocidal studies (Chart 2) with these nitrofuran derivatives show that cells can tolerate exposures to these drugs for a few hr at concentrations that completely inhibit growth without any subsequent effect on the ability of the cells to proliferate and form normal colonies. These studies predict that either 500 \(\mu\)M concentrations of nitrofurazone and nitrofurantoin or 125 \(\mu\)M concentrations of nifuroxime can be tolerated by cells for exposures of up to 5 hr. The order of toxicity of these 3 nitrofuran derivatives, as determined by growth inhibition (cytostatic) and plating efficiency (cytocidal), is identical with the order established for chronic and acute toxicity of these drugs in mice (32).

The radiosensitizing effectiveness of these drugs in mammalian cells is greater than that of most radiosensitizers characterized to date (2, 5, 9, 13, 31). At concentrations that do not interfere with cell proliferation (< 20 \(\mu\)M), both nitrofurazone and nifuroxime radiosensitize cells with ER's of between 1.2 and 1.4. This extent of radiosensitization is significant and would be expected to enhance the effectiveness of present radiotherapy regimes if all hypoxic cells within a tumor could be sensitized to this extent (see Ref. 22). The observation that nitrofurazone can sensitize both nonproliferating and proliferating cells in every phase of the cell cycle demonstrates that the radiosensitizing mechanism is not influenced to any great extent by the metabolic state of the cell or specific metabolism in distinctive parts of the cell cycle. On the basis of the characterizations presented in this paper, the nitrofurans act in a manner similar to those of other electron-affinic radiosensitizers (2, 13) and are intermediate between PNAP and molecular oxygen in effectiveness.

Studies with nitrofuran derivatives have shown that plasma concentrations of up to 50 \(\mu\)M can be detected between 30 and 60 min after a dose that is well below the acutely toxic level (32) has been administered, p.o., to rats. The results presented in this paper show that significant levels of radiosensitization (ER's of 1.3 to 1.5) could be realized with nitrofuran concentrations of this magnitude in the vicinity of hypoxic cells. In clinical studies in man, urinary concentrations of nitrofurantoin up to and exceeding 1 mM have been detected after its administration p.o. (8, 38). The establishment of high concentrations of nitrofurantoin in the urine prior to and during irradiation may very well result in a more successful radiotherapy of bladder tumors. Radiation studies with hypoxic cells with doses of 500 rads and less (doses more in line with normal radiotherapy procedures) show the radiosensitizing effect of the nitrofurans to be dose modifying, even on the shoulder portion of the survival curves.

The demonstration that nitrofurazone can readily penetrate the bimolecular lipid membrane structure correlates with the observation that radiation-induced damage in the DNA of hamster cells (as estimated by single-strand-break analysis) is enhanced for cells irradiated in hypoxia in the presence of these nitrofuran derivatives (14). If we assume that the mechanism of radiosensitization involves short-lived, free radical species, then the drug would have to be in the vicinity of the nucleus and DNA at the time or irradiation in order to have an enhancing effect at that level. Studies with hamster cells at 37\(^\circ\) indicate that the maximum radiosensitizing effect is achieved with the drug (500 \(\mu\)M nitrofurazone) in contact with the cells only 5 min prior to irradiation. These data demonstrate that the nitrofurans probably penetrate the cell membrane rapidly and enhance radiation-induced damage within the cell. The possibilities that only a few nitrofuran molecules at some site in the cell are required for the effect or that the radiation target for cell lethality is at the cell membrane cannot be excluded on the basis of this work.

The radiation-induced binding of the label of nitrofurazone-\textsuperscript{14}C to macromolecules and to whole cells and the enhancement of this binding by the absence of oxygen suggest that sensitizer binding may be involved in radiosensitization. While this enhanced binding is nonspecific, in that it has been demonstrated for protein, DNA, and polynucleotides, considerably more enhancement (14- to 17-fold) is observed with DNA, poly(I), poly(U), and poly(C) than with protein. With poly(A) and protein, enhancements of 4- to 5-fold are seen when oxygen is excluded. Since radiation-induced strand breakage of hamster cell DNA is enhanced in hypoxia by the nitrofurans to an extent similar to that for cell killing (14), it is possible that enhanced binding to cellular DNA may result in enhanced radiation-induced damage in the DNA and, ultimately, in enhanced cell killing.

Recent studies on the antibacterial mode of action of nitrofurazone have shown that activation of this drug by reductive metabolism in bacteria leads to the binding of nitrofurazone-\textsuperscript{14}C to the acid-insoluble fraction (29) and to the formation of single-strand breaks in DNA (30). The enzymatic activation of the nitrofurans by bacteria and the subsequent binding, DNA strand breakage, and lethality seem to be parallel to the radiation-enhanced effects obtained with the same compounds.

The nitrofurans have been used with good success as
antibacterial agents in the treatment of infections in humans and animals (6, 32). Friedgood and Green (16) reported marked atrophy of the testes following administration of p.o. doses of nitrofurazone to mice, as well as a retardation of the growth of a transplantable fibrosarcoma (16). This observation led to the experimental treatment with nitrofurazone of primary and metastatic seminomas in humans (20, 21, 35, 40). Nitrofurazone chemotherapy was usually initiated after both surgery and radiotherapy had failed to arrest the metastatic invasion of testicular cancer to the lungs and its subsequent growth. In only 1 case was nitrofurazone chemotherapy used in parallel with an X-ray therapy regime which involved 4200 R over a 34-day period (20), and regression of this pulmonary metastases from embryonal carcinoma of the testis was reported. This isolated case history leaves many questions unanswered regarding whether the drug, or the X-rays, or a combination of both led to the observed regression. It is safe to say that the use of nitrofuran derivatives in conjunction with radiotherapy of tumors when hypoxia is suspected has not as yet been clinically evaluated. The demonstration in this paper of the radiosensitizing potential of this class of drugs warrants a careful evaluation of their potential use in the radiotherapy of human cancers.

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