RADIOPHARMACOLOGY AND TOXIC EFFECTS OF
RO-07-0582 IN HYPOXIC MAMMALIAN CELLS

by

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B.Sc., University of British Columbia, 1973

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Churchill College of Medical Genetics)

We accept this thesis as conforming

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ABSTRACT:

Cells experiencing a low oxygen tension show relative resistance to the lethal effects of radiation. It is believed that the effectiveness of the treatment of certain human tumors is hindered by the existence of such radioresistant cells within the tumor. The purpose of this work was to study the drug Ro-07-0582 both for its toxic effects and its ability to preferentially sensitize hypoxic cells to the lethal effects of radiation (radiosensitize). These properties were examined in vitro in two Chinese hamster cell lines, CHO and CH2B2, and also in the mouse tumor cell line EMT6.

Ro-07-0582 is shown to have a chemotherapeutic potential in that it demonstrates a very selective toxicity for hypoxic
cells after a few hours exposure. It is much less toxic to aerobic cells. These toxic properties were studied extensively, both in hypoxic and aerobic cell suspensions. The measured endpoint was the ability of a cell to multiply and form a colony of 50 or more cells within an allotted incubation time. Hypoxic toxicity was greater at 37°C than at 22°C and was affected by small changes (≈30ppm) in $O_2$ concentration in the cell suspension. The toxic effects were similar in the three cell lines.

The radiosensitizing capability of Ro-07-0582 was determined by measuring the Dose Modifying Factors (DMF's) for various drug concentrations with each cell line. DMF's were calculated by comparison of survival curves for cell suspensions irradiated under hypoxia in the presence of drug with the survival curve for cell suspensions irradiated under hypoxia in the absence of drug. The DMF for the irradiation of aerobic cells in the absence of drug is called the Oxygen Enhancement Ratio (OER) and was approximately 3.0 in all three cell lines.

Ro-07-0582 was found to selectively radiosensitize hypoxic cells in suspension with high efficiency. For each cell line, sensitization was observed with drug concentrations as low as 0.1mM, while concentrations of 10mM or greater yielded DMF's within the measured range of OER values. The presence of 1mM
Ro-07-0582 during irradiation of hypoxic cells yields a DMF of 1.8. Introduction of the drug before or after irradiation, instead of during irradiation, had little if any effect.

Radiosensitization measurements were also carried out at high cell concentrations (cell pellets), where many sensitizers are ineffective. Results showed that the 0582 radiosensitization attained in cell pellets is quite comparable with that attained in dilute suspension.

The attributes of Ro-07-0582 as a potential radiosensitizer were considered. The sensitization achieved by Ro-07-0582 is very good, and surpasses that of metronidazole, a chemical under study for clinical use. For drug doses necessary to achieve high levels of sensitization the toxicity of Ro-07-0582 to aerobic cells is quite acceptable. The toxicity to hypoxic cells, however, is much increased over the toxicity to aerobic cells, and this may prove to be a useful adjunct to the drug's sensitizing properties in destroying hypoxic tumour cells.
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ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. L.D. Skarsgard, under whose supervision this project was carried out.

I would like to thank Dr. B. Palcic for his helpful suggestions in the course of this work.

I am grateful to Dr. C. Gregory for supplying the EMT6 cells.

I am thankful to my colleague, Bill Hohman, for our many informative and refreshing discussions.

I am indebted to Mrs. I. Harrison and Mrs. R. Dudley for their valuable technical assistance.

And finally, special thanks to Patty Filan for her assistance, encouragement and great patience during the project and for her thoughtfulness in typing this thesis.
1. INTRODUCTION

1.1 CANCER AND RADIATION THERAPY

The effectiveness of the treatment of certain human tumors by radiation is believed to be hindered by the existence of relatively radioresistant cells within the tumors. This radioresistance is due to the low oxygen tension which arises in the tumor interior when it outgrows the surrounding vascular supply. The relative radioresistance of cells with a low oxygen tension as compared to well oxygenated cells, a phenomenon referred to as the "oxygen effect", represents a considerable problem to radiotherapy since the maximum tumor dose is limited by the tolerance of the surrounding healthy, oxygenated tissue. It is therefore desirable to obtain some means of reducing the oxygen effect.

A clinical approach to this problem has been the use of hyperbaric oxygen tanks. In this technique, radiotherapy is performed while the patient is exposed to oxygen at 3 atmospheres pressure. The aim is to increase the distance that oxygen diffuses from the capillaries and thus improve the oxygenation of hypoxic regions of the tumor. However, results of clinical trials so far have shown the cure rate to be improved only slightly, so attention has now shifted from this approach.
The use of neutron beams in cancer therapy has also grown in recent years. The major advantage of neutrons is a reduced oxygen effect relative to that of γ-rays (Broerse and Barendsen, 1966). It has been suggested by Thomlinson (1971) that while neutrons will probably not, in general, provide a large improvement over x-rays or γ-rays, there are likely to be special situations where neutrons will greatly increase the probability of cure. As well, he suggests that neutrons would generally be of greatest value at the start of therapy when both the number and percentage of hypoxic cells is maximum. The increased killing of hypoxic cells by neutrons as compared to x-rays or γ-rays would result in an increased availability of oxygen to the surviving tumor cells, thus starting the process of reoxygenation. The extent of reoxygenation would determine the advantage of further neutron doses over x-ray or γ-ray doses. Aside from the possible advantages, the expense of neutron generators is presently a limiting factor in the application of fast neutrons to cancer radiotherapy.

A third approach to reducing the oxygen effect is to find chemical radiosensitizers which increase the sensitivity of hypoxic cells to a level near or equal to that of oxygenated cells. The criteria employed by those searching for chemical radiosensitizers are:

1) the ability to preferentially sensitize hypoxic cells to the lethal effects of ionizing radiation,
2) acceptable toxicity,
3) metabolic stability, so that the sensitizer is not inactivated prior to irradiation,

4) suitable cell cycle dependence, so that hypoxic cells which may have stopped at some stage of the cell cycle are effectively sensitized.
1.2 RADIATION DAMAGE

When living cells are irradiated with ionizing radiation such as x-rays or γ-rays, interactions with molecules within the cells result in ejection of energetic electrons. These electrons quickly transfer their energy to surrounding molecules through further ionizations and excitations. "Direct effect" is the term given to any ionization or excitation which results in the direct chemical alteration (damage) of some cellular molecule.

The large water content of the mammalian cell accounts for 70-80% of the cell's total energy absorption. This energy absorption results in the breakage of water molecules into very reactive free radicals, the most important of which are \(\text{OH}^\cdot\), \(\text{H}^\cdot\) and \(\text{e}_{\text{aq}}^\cdot\). Some of these free radicals react with target molecules, resulting in target free radical formation. This type of damage is said to result from indirect effects. Once the target molecule is damaged, whether by direct or indirect effect, further reactions may make the alteration irreversible, a process called damage fixation. Such fixed damage may result in cell death. Alternatively, the target molecule may revert to its original form by a process referred to as damage repair.

There has been some controversy as to the relative importance of direct and indirect effect in cell killing. Most results indicate that indirect effects predominate. Sanner and
Pihl (1969) concluded that about 50% of lethal damage in their *E. coli* B bacterial system was the result of indirect effect, while Ebert, Dodd and Nias (1970) found indirect effects accounted for 70% of the lethal damage in mammalian (HeLa) cells. More recently, Chapman and co-workers (1974) suggested, on the basis of results from the use of OH· radical scavengers as radioprotective agents (Chapman et al., 1973b), that 60-70% of lethal damage to aerobic Chinese hamster cells is the result of fixation of indirect damage caused by OH·, $e_{aq}^-$ or H·.

It is generally accepted that the OH· radical, a strong oxidizing agent, is the most important agent of indirect damage in aerobic cells. Supporting evidence has been provided by Blok et al. (1967) who looked at inactivation and mutation in an aerobic suspension of ÐX174 DNA in the presence of various OH· scavengers; by Roots and Okada (1972) who used the method of alkaline sucrose gradients (McGrath and Williams, 1966) to study the production of single strand breaks in the DNA of aerobic mammalian cells irradiated during exposure to a variety of OH· scavengers; and by Chapman et al. (1973b), whose work using OH· scavengers as radioprotectors of mammalian cells led to the conclusion that approximately 60% of the lethal effects of radiation upon aerobic hamster cells is due to the indirect action of OH·. Chapman and his co-workers also suggested that ≤10% of radiation lethality to aerobic mammalian cells results from the indirect action of the reducing radicals $e_{aq}^-$ and H·. They suggested that target radical anions, formed
by the interaction of $e_{aq}^-$ with the target molecules, would be quickly repaired by electron transfer to endogenous molecules having a greater electron affinity than the target molecule. Studies by Adams et al (1972) of electron transfer in irradiated nucleotide solutions provide support for this suggestion. It is interesting to note that $O_2^-$, which is considerably electron affinic, may thus protect target molecules from potentially lethal target radical anion damage.

In summary, Chapman et al (1973b) have suggested that in aerobic cells, about 80% of the inactivation is due to fixation of target radicals, with about 60% resulting from $OH^-$ and about 20% from direct effect. As well, it was proposed that about 65% of inactivation results from fixation by oxygen, the remainder resulting from fixation by other endogenous substances. Of the remaining 20% of aerobic inactivation, these workers suggested that at least a portion could result from outright lethal damage in the target molecule by direct effect.

Chapman and co-workers (1974) reported that under hypoxia approximately 30% of lethal damage to hamster cells is caused by the indirect effect of $OH^-$, compared to about 60% in aerobic cells. It is therefore possible that of the total biological lethality, the portions resulting from direct effects and from the indirect effects of the reducing species $e_{aq}^-$ and $H^-$ may be greater under hypoxic conditions than under aerobic conditions.
1.3 MECHANISMS OF RADIOSENSITIZATION

Oxygen is the best known and most extensively studied radiosensitizing agent. To be effective at all, oxygen must be present either during or immediately following irradiation. Shenoy et al (1975) have shown that the addition of oxygen to hypoxic Chinese hamster cells only 5 milliseconds after irradiation yielded only a slight increase in sensitivity. Thus very short-lived processes are involved in oxygen sensitization. The oxygen-fixation hypothesis (Alexander, 1962) has suggested that target free radicals, whether from direct or indirect effects, react quickly with oxygen to form peroxy radicals:

\[ T^- + O_2 \rightarrow TO_2^- \]

This reaction was considered irreversible, thus resulting in the fixation of potentially lethal damage. The fixation step was proposed to compete with repair of the damage via hydrogen donation from intracellular radical-reducing species.

It has been suggested that another possible mechanism of chemical radiosensitization is the binding of intracellular sulphydryl compounds. Thus the sensitizing ability of several sulphydryl-binding compounds, notably N-Ethylmaleimide (NEM), has been studied. Intracellular sulphydryl compounds have been described as radioprotective substances, repairing damage by hydrogen donation to target free radicals (Alexander and Charlesby, 1954) or to organic free radicals (Loman, Voogd...
and Blok, 1970). Binding of these sulphydryl compounds would prevent such radioprotection.

The importance of intracellular sulphydryl compounds in cell survival has been disputed. Harris, Painter and Hahn (1969) measured the radiosensitivity of Chinese hamster cells for various concentrations of intracellular non-protein sulphydryl compounds. They concluded that most endogenous non-protein sulphydryl compounds did not produce a radioprotective effect. In 1969, Mullenger and Omerod proposed a second mechanism by which NEM might sensitize; a reaction with a radiolytic species producing an activated molecule which reacts with and damages the target molecule.

It was suggested by Adams and Dewey (1963) that the property of radiosensitization is related to the electron affinity of a compound, as most radiosensitizers, including NEM, were known or thought to be electron affinic. The mechanism initially proposed was that the sensitizers enhanced the indirect effect by capturing $e^{aq\text{-}}$ species and releasing them to a supposedly more electron affinic target molecule. This would have the effect of prolonging the life of the $e^{aq\text{-}}$ radical. However, this model does not explain the oxygen effect because the high electron affinity of $O_2^-$ makes electron transfer from $O_2^-$ to a target molecule unlikely.

A second model of Adams and Cooke (1969) suggested that electron affinic sensitizers might act by enhancing the effects of direct damage. Following ionization of a target molecule,
the electron was proposed to localize at some electron affinic site on the molecule. Electron transfer to a sensitizer molecule of greater electron affinity would reduce the chance of recombination and increase the chance of decay of the positive target ion to a free radical and subsequent damage fixation. Applying this mechanism to oxygen sensitization, we have:

\[
\begin{align*}
\text{ionization} & \xrightarrow{\text{transfer}} \text{recombination} \\
\text{+} \quad \text{+} \\
\text{electron} & \xrightarrow{\text{transfer}} \text{+0}_2^- \\
\text{+H}^+ \\
\text{free radical}
\end{align*}
\]

In 1973 Chapman and co-workers formulated a model to explain both radiosensitization and radioprotection. The model is a more general form of the oxygen-fixation hypothesis and suggests that the redox state of the cellular target environment is the critical property determining the lethal effects of radiation. This redox state determines whether neutral target radicals, once formed, will be repaired by reduction or fixed by oxidation (fig. 1). Only neutral target radicals are considered of importance since, as previously mentioned, these workers have suggested that target radical anions caused by e\text{aq}^- are repaired by electron transfer to endogenous molecules of greater electron affinity. It was suggested that a radiosensitizer can act either:

1) by adding to the cellular pool of radical oxidizing species,

2) by oxidation of the endogenous pool of radical reducing species,
DIRECT EFFECT
\[ \gamma_{\text{hv}} \xrightarrow{} \text{WWW} \]

\[ \xrightarrow{} + \text{WWW} \]

\[ \xrightarrow{} \text{OH}^* \]

\[ \text{H}_2\text{O} \quad \text{or} \quad -\text{H}^+ \]

Indirect EFFECT
\[ \gamma_{\text{hv}} \xrightarrow{} \text{H}_2\text{O} \]

\[ \xrightarrow{} \text{OH}^* \quad \text{H}^+ \quad e_{\text{aq}}^- \]

\[ \text{Reduction (inactivation)} \]

\[ \text{Repair by Oxidation (ie by electron transfer)} \]

\[ \text{Repair by Reduction} \]

\[ \text{e.g. SH, NADH} \]

\[ \text{Inter-radical reactions} \]

\[ 
\begin{align*}
\text{Fixation by Oxidation} \\
\text{ie by adduct formation and/or electron transfer} \\
\text{Oxidation of P} \\
\text{P} - \text{radical reducing species} \\
\text{S} - \text{radical oxidizing species}
\end{align*}
\]

FIGURE 1. Schematic Representation of Proposed Mechanisms of Radiosensitization and Radioprotection

Direct or indirect effects on a target molecule (WWW) result in target radical formation. The mechanisms of sensitization (1, 2) and protection (3, 4) are discussed in section 1.3.
or by a combination of these mechanisms. It was suggested that oxidation might occur either by electron transfer to the more electron affinic compound or by the binding of compounds. Radioprotectors would act either:

3) by adding to the endogenous pool of radical reducing species, thus increasing the probability of target radical reduction, or

4) by competing with target molecules for OH·, H· and other radicals, thus inhibiting indirect damage by these species.

These mechanisms are represented schematically in fig. 1.

The above model is capable of explaining both the oxygen effect and the radioprotection and radiosensitization observed with many compounds. It receives further support from the work of Redpath and Willson (1973), who used pulse radiolysis to show that ascorbic acid, a reducing agent, reacts with and reduces organic radicals. In particular, the presence of ascorbic acid eliminated the sensitizing effects on 

*Serratia marcescens*

by the stable free radical triacetoneamine-N-oxyl (TAN). This loss of sensitization was attributed to the reduction of TAN by ascorbic acid, which agrees with protective mechanism (2) of the above model.
1.4 RADIOSENSITIZERS

The first reports of selective chemical radiosensitization of hypoxic mammalian cells were from Parker, Skarsgard and Emmerson (1966, 1969) using the organic nitroxide free radical triacetoneamine-N-oxyl (TAN). Many compounds have since been shown to possess radiosensitizing ability, some being much more effective than others.

The search by Adams and co-workers for electron affinic compounds as sensitizers led to the testing of several substituted phenones. One of these, para-nitroacetophenone (PNAP) was found to sensitize hypoxic mammalian cells in vitro at concentrations in the sub-millimolar range (Adams et al, 1971; Chapman, Webb and Borsa, 1971). However, this drug failed to be useful in vivo because its low water solubility presented difficulties in administration. A more soluble derivative of PNAP, N-dimethylparanitro-2-propiophenone (NDPP), has yielded some sensitization in vivo (Denekamp and Michael, 1972), but only for drug doses of near lethal values. As well, despite its sensitizing ability in vitro, TAN has also been found to have little effect in vivo (Hornsey, 1972).

A stimulus to the search for radiosensitizers came with the discovery (Reuvers, Chapman and Borsa, 1972; Chapman et al, 1972) that various nitrofuran derivatives have excellent radiosensitizing properties in hypoxic Chinese hamster cells at drug concentrations which are non-toxic. Metronidazole, a
nitroimidazole, was found to sensitize hypoxic mammalian cells both in vitro (Foster and Willson, 1973; Chapman, Reuvers and Borsa, 1973; Palcic, Agnew and Skarsgard, 1974; Asquith et al, 1974; Thomson and Rauth, 1974) and in vivo (Begg, Sheldon and Foster, 1974; Denekamp, Michael and Harris, 1974; Rauth and Kaufman, 1975; Denekamp and Harris, 1975). Even though rather high concentrations of metronidazole were required to achieve sensitization, the drug's very low toxicity and relatively slow rate of metabolism, both in animals and in man, made the results quite encouraging.

The success with metronidazole stimulated the search for other nitroheteroaromatic sensitizers which might sensitize at lower concentrations while retaining the low toxicity characteristic. Initial tests with another nitroimidazole, Ro-07-0582, have shown this compound to surpass metronidazole as a sensitizer of hypoxic mammalian cells in vitro (Asquith et al, 1974(b); Moore, Palcic and Skarsgard, 1975) and in vivo (Denekamp, Michael and Harris, 1974; Denekamp and Harris, 1975).

The purpose of the work described in this thesis was to study several aspects of radiosensitization by Ro-07-0582 and, as well, the toxicity of the drug.
2. MATERIALS AND METHODS

2.1 TISSUE CULTURE TECHNIQUES

2.1.1 Cell Lines

Three mammalian cell lines have been used in these experiments. Two lines, CH2B₂ and CHO are Chinese hamster cell lines. The CH2B₂ line (Agnew and Skarsgard, 1974) was derived from a line CHEF, originally described by Prescott and Bender (1963). The CHO line (Hahn and Bagshaw, 1966) is a subline of Chinese hamster ovary cells. The third cell line, EMT6, is a tumour cell line selected in tissue culture from the KHJJ tumour line which, in turn, was derived from a primary mammary tumour arising in a BALB/c mouse (Rockwell, Kallman and Fajardo, 1972).

2.1.2 CH2B₂ Cell Culture

The CH2B₂ cells were grown attached to plastic in screw cap flasks of growth area 25 or 75 cm². The growth medium consisted of MEM F-16 (Grand Island Biological Company (GIBCO), Grand Island, New York) supplemented with 10% fetal calf serum (GIBCO) and 0.8% penicillin-streptomycin solution (10,000 units penicillin and 10,000 mcg streptomycin per ml, GIBCO). The cells were incubated under standard conditions (37°C in an atmosphere of 5% CO₂, 95% air and 100% humidity), and had a doubling time of 12-14 hours.
To maintain the cell line in log phase growth (cells actively growing and dividing), the cells in monolayers were subcultured by trypsinization every 2-4 days. This involved a 5 second wash with 5 ml of 0.1% trypsin (Bacto-trypsin, Difco Laboratories, Detroit, Michigan) preheated to 37°C, followed by an 8 minute treatment with 5 ml trypsin. During the last minute of treatment cells were shaken free of the surface. The trypsin action was stopped by immediately adding the trypsin-cell suspension to 10 ml growth medium. The cells were centrifuged 6 minutes at 50 g, resuspended in growth medium and counted.

For the cell line, grown in 25 cm² flasks, aliquots of between 10⁵ and 4 x 10⁵ cells were plated into fresh flasks containing 10 ml growth medium. The growth medium was usually replaced daily and occasionally every 2 days. Cells for experiments were plated 2 days prior to the experiment at 1.2 x 10⁶ cells per 75 cm² flask containing 15 ml growth medium. Fresh growth medium was added the following day and the cells trypsinized the day of the experiment, using the technique described above.

2.1.3 CHO Cell Culture

The CHO cells, which have a doubling time of 12-13 hours, were grown at 37°C in suspension in spinner culture in α-medium lacking nucleic acids (Flow Laboratories, Rockville, Md., Cat. #1F-094C) supplemented with 10% fetal calf serum and
0.8% penicillin-streptomycin solution. The cells were maintained in log phase growth (cell concentrations between $5 \times 10^4$ and $5 \times 10^5$ cells/ml) by regular dilution with fresh medium.

2.1.4 EMT6 Cell Culture

EMT6 cells, which have a doubling time of approximately 13 hours, were grown attached to plastic in 25 or 75 cm$^2$ screw cap flasks. The growth medium consisted of α-medium (lacking nucleic acids) supplemented with 15% fetal calf serum and 0.8% penicillin-streptomycin solution. The cells were incubated under standard conditions and trypsinized every 2-4 days by the technique described in section 2.1.2. The growth medium was replaced every day or every second day. Cells for experiments were plated 2 days prior to the experiment at $10^6$ cells per 75 cm$^2$ flask containing 15 ml growth medium. The growth medium was replaced the day prior to the experiment.
2.2 RADIATION SOURCE

Irradiations were performed with two $^{60}$Co therapy units (Theratron Model F and Eldorado Super G, Atomic Energy of Canada Limited), providing 1.17 and 1.33 MeV gamma rays. Dose rates were between 330 and 680 rads/minute.
2.3 CELL SUSPENSION EXPERIMENTS

2.3.1 Preparation of the Cell Suspension

Cells that were to be used for toxicity tests or irradiated in suspension were obtained from log phase monolayers in the case of CH2B2 and EMT6 cells, or from suspensions of log phase CHO cells. Following CH2B2 or EMT6 trypsinization (see 2.1.2) or removal of CHO cells from spinner culture, the cells were centrifuged and resuspended in drug treated or drug free growth medium.

For cell suspension survival experiments (involving irradiation) the cells were resuspended at 2.5 X 10^5 cells/ml in growth medium lacking sodium bicarbonate. The absence of sodium bicarbonate held the pH at about 7.2 while gassing in the absence of CO2 (see 2.3.2). A 15-20 ml volume of cell suspension was placed in special glass irradiation vessels (fig. 2(a)) and stirred with a magnetic stirrer. All survival experiments were performed at 22°C. In one survival experiment it was necessary to use 10^6 cells/ml. This experiment is described in section 3.2.1.

For toxicity tests, cells were resuspended at 3.2 X 10^4 cells/ml in growth medium containing sodium bicarbonate. The presence of both sodium bicarbonate and 5% CO2 during gassing (see 2.3.2) maintained the pH at 7.2. A 25-30 ml volume of cell suspension was placed in a 125 ml Erlenmeyer flask (fig. 2(b)) and stirred with a magnetic stirrer. Experiments were
Nitrogen was flowed at 0.7 litres/minute over a stirred 15-20 ml suspension of 2.5 X 10^5 cells/ml for 45 minutes (or longer) prior to and during irradiation. Samples were obtained by removing the stopper briefly and lowering a pipet down the neck of the vessel into the suspension. All experiments were performed at 22°C.

(b) The Glass Vessel for Cell Suspension Toxicity Tests

Nitrogen (5% CO2) was flowed over a stirred 25-30 ml suspension of 3.2 X 10^3 cells/ml contained in a 125 ml Erlenmeyer flask. Samples were obtained by removing the small stopper briefly and lowering a pipet down the glass tubing into the suspension. Experiments were performed at 22°C and 37°C.
performed at 22° and 37°C, the flasks being situated in a
temperature-regulated water bath.

2.3.2 **Hypoxic and Aerobic Conditions**

Since cells experience an increased resistance to the
lethal effects of radiation when they are irradiated in the
absence of oxygen, it is most convenient to describe hypoxia
as the state in which radiation resistance is maximum. In
previous work in this laboratory, Agnew (1972) showed that by
gassing a stirred 15 ml suspension in an irradiation vessel
with prepurified N\textsubscript{2} (<10 ppm O\textsubscript{2}) at 0.7 litres/minute, maximum
radiation resistance is attained within 15 minutes. In the
cell suspension survival experiments reported in this thesis,
hypoxic conditions were achieved by flowing N\textsubscript{2} (Liquid Carbonic,
Vancouver) at 0.7 litres/minute over the suspension for 45
minutes (or longer) prior to and during irradiation.

Hypoxic conditions were achieved and maintained during
toxicity tests by continually flowing N\textsubscript{2} + 5% CO\textsubscript{2} (Liquid
Carbonic, Vancouver) over the suspensions, with time \(t=0\)
corresponding to the start of gassing. The flow rate was
maintained at 0.7 litres/minute for 1 hour, then reduced to
about 0.4 litres/minute for the remainder of the experiment.
As well, the gas was passed through a water-filled gas washing
bottle before reaching the cell suspension. The resulting
humidified gas minimized evaporation from the cell suspension.
One toxicity test was performed using prepurified nitrogen
(no CO\textsubscript{2}). The experiment is described in section 3.1.1.3.
Aerobic conditions for suspension experiments were obtained by flowing O₂ (Canadian Liquid Air, Vancouver) instead of N₂.

2.3.3 Plating and Colony Formation

Aliquots removed from a suspension during an experiment were immediately added to normal growth medium, thus diluting the drug. The samples were then centrifuged and the cells resuspended in fresh growth medium. The cells were diluted and plated into 60 X 15 mm plastic tissue culture petris (Falcon Plastics, Oxnard, California, and Lux Scientific, Thousand Oaks, California) containing 6 ml of normal growth medium plus, for CHO and CH₂B₂ cells only, 10⁵ heavily irradiated (6000 rads under aerobic conditions) feeder cells per petri. No EMT6 feeder cells were used because these cells absorb stain, making colony counting difficult.

The cells were incubated under standard conditions, allowing surviving cells to proliferate and form colonies, a colony being defined as a tight group of 50 or more cells. The incubation time amounted to 7 days for CHO cells, 8 days for CH₂B₂ cells, and 9 days for EMT6 cells. Following incubation, the medium was poured from the petri and the colonies stained by a 6 minute exposure at room temperature to a methylene blue solution (2 g/litre).
2.3.4 Plating Efficiency and Surviving Fraction

Since the experiments reported in this thesis use colony forming ability as the measured end point, a survivor is defined as a cell which will form a colony of 50 or more cells within the allotted incubation time (see 2.3.3). Even for unirradiated populations, not all the cells will form colonies, as some will undergo limited or no proliferation. Thus the term "plating efficiency" will be used in toxicity tests to represent the percentage of the cells plated (counted by Coulter Counter) which achieve colony status. For experiments involving irradiation, the ratio of the plating efficiency of irradiated cells to untreated, or control cells, will be referred to as the "surviving fraction". The surviving fraction is simply a normalized plating efficiency, where all points are normalized to the plating efficiency of control cells.

2.3.5 Survival Curves and Dose Modifying Factors

The survival of mammalian cells exposed to radiation is generally shown by means of a survival curve, in which the log of the surviving fraction is plotted against the dose absorbed. The curves usually have a rounded, or "shoulder" region in the range of high surviving fraction, followed by an approximately linear region representing exponential survival. All survival curves presented in this thesis are of this form. The reciprocal of the slope of the straight line region of a survival curve is labelled $D_0$. If the linear portion is
extrapolated to zero dose, the intercept with the ordinate is called the extrapolation number.

If a set of survival curves have a similar shape, any two curves can be approximately superimposed by the application of a scaling factor to the dose values. This factor is called the dose modifying factor (DMF), and is a single numerical representation of the comparison of two entire survival curves. Of course, if linear segments are to shift and overlap completely, a common extrapolation number is required for all survival curves. This condition is described by saying the cause of the shift between survival curves is simply "dose modifying". For each of the 3 cell lines, the survival curves presented in this thesis can be adequately described by dose modification.

In this thesis the DMF represents a comparison of the cell survival curve obtained for a drug-treated suspension with the survival curve obtained concurrently for a drug-free suspension. The DMF is calculated either from the ratio of the doses required to reduce cell survival to 0.01 or from the ratio of the two D_0 values.

The oxygen enhancement ratio (OER) is defined as the DMF obtained when comparing the survival curves of aerobic and hypoxic suspensions (both drug-free).
2.4 CELL PELLET SURVIVAL EXPERIMENTS

2.4.1 Formation of the Cell Pellet

Cells were harvested from log phase growth (2.1.2, 2.1.3) and resuspended at about $10^7$ cells/ml in normal growth medium lacking sodium bicarbonate and with or without dissolved drug. Eight ml of suspension was placed into each of one or more 50 ml conical glass centrifuge tubes. Hypoxia was achieved by flowing humidified $N_2$ at 0.7 litres/minute over each suspension (see fig. 3(a)), the samples being shaken regularly during this period. After a minimum of ten minutes gassing, the tubes were sealed to maintain hypoxia, centrifuged for 8 minutes at 120 g, reconnected to the $N_2$ flow, and the supernatant removed by aspirator (see fig. 3(b)). The $N_2$ was humidified by passage through a water-filled gas washing bottle, the humidified gas being required to prevent dehydration of the cell pellet.

Each cell pellet was irrigated with various doses at times between 15 and 110 minutes after the end of centrifugation, the total time from initial $N_2$ flow to irradiation being not less than 45 minutes. All cell pellet experiments were performed at $22^\circ$C.

2.4.2 Irradiation and Cell Survival of Pellet Samples

Following the required accumulated doses of radiation, samples of the pellets ($5 \times 10^5$-$10^7$ cells) were drawn into the
FIGURE 3. The Vessel Used for Cell Pellet Experiments

(a) Nitrogen was flowed at 0.7 litres/minute over an 8 ml suspension of 10^7 cells/ml for a minimum of 10 minutes. 
(b) The tubes were then sealed, the vessel centrifuged for 8 minutes at 120 g, the N_2 flow re-established, and the supernatant removed by an aspirator. Irradiations commenced 45 minutes or more after the start of gassing. Pellet samples were removed by pipet (c and d). All steps were performed in such a manner as to maintain hypoxia. Every cell pellet experiment was performed at 22°C.
ends of 1 ml plastic disposable pipets (Falcon Plastics), hypoxia being maintained within the tube during the procedure (figs. 3(c) and (d)). The samples were resuspended in 7 ml of normal growth medium at 22°C, counted, diluted, and plated into 15 X 60 mm plastic petris containing 6 ml of normal growth medium plus $10^5$ heavily irradiated feeder cells per petri. Incubation, staining and determination of cell survival was as described in sections 2.3.3 and 2.3.4.
The sensitizer used in this work was Ro-07-0582, 1-(2-nitro-1-imidazonyl)-3-methoxy-2-propanol, $C_7H_{11}O_4N_3$ (M.W. 201.18), synthesized by Roche Products Ltd.

To check the stability of Ro-07-0582 in solution, a sample of 7 mM drug was dissolved in normal growth medium lacking sodium bicarbonate and stored at $2^{\circ}C$ in the absence of light. After 41 days the drug was diluted and used in a CH2B2 suspension experiment. Figure 4 shows that the sensitization observed with this preparation of the drug was equivalent to that obtained with fresh preparations.
Drug was dissolved in normal growth medium lacking sodium bicarbonate and stored 41 days at 2°C in the absence of light. The drug was then diluted and used in a CH2B₂ suspension experiment. The "fresh" samples were prepared immediately prior to the experiment.
3. RESULTS

3.1 TOXICITY TESTS WITH Ro-07-0582

In examining the toxicity of potentially useful compounds one is normally concerned primarily with the toxic effects of the compound on normal, aerobic tissue since these are the tissues which one wishes to spare in the treatment. Thus, prior to the start of this work, it was common to measure toxic effects in aerobic cell cultures. The discovery that 0582 exhibits increased toxicity to hypoxic cells was of interest both for a further control of malignant disease and for possible detrimental effects upon normally hypoxic body tissues. Therefore the toxicity of this drug was studied using several cell lines under both aerobic and hypoxic conditions.

3.1.1 CH2B2 Cell Line

3.1.1.1 Toxicity of 0582 to aerobic and hypoxic CH2B2 cells in suspension at 37°C

Figure 5 shows the results of experiments in which aerobic CH2B2 cells in suspension at 37°C were incubated in 0582 for various times. Single aliquots were processed and the plating efficiency determined by colony forming ability. The plating efficiencies were normalized against plating efficiencies of samples taken at the same time from drug-free aerobic solutions. A reduced plating efficiency is observed at
FIGURE 5. Percent Plating Efficiency of Aerobic CH2B₂ Cells as a Function of Incubation Time at 37°C in 5, 10, 25 or 50 mM Ro-07-0582
FIGURE 6. Percent Plating Efficiency of Hypoxic CH2B2 Cells as a Function of Incubation Time at 37°C in 0, 1.0, 5, 15, or 50 mM Ro-07-0582

Error bars represent the standard deviation between independent measurements, and in many cases are smaller than the plotted symbols.
4 hours incubation in 25 or 50 mM 0582, whereas no toxic effects are seen in the 5 and 10 mM samples.

Figure 6 shows the plating efficiency of CH2B2 cells plotted as a function of incubation time at 37°C in Ro-07-0582 under hypoxic conditions. For each point, two aliquots of cell suspension were processed and plated independently. The points and error bars on fig. 6 represent the average of the two resulting plating efficiencies (from a single experiment) and the standard deviation, respectively. Points without error bars have a standard deviation too small to plot. The drug is considerably toxic to cells under hypoxic conditions. A concentration as low as 1 mM reduces the plating efficiency tenfold within 7 hours. The highest concentration tested, 50 mM, showed very large toxicity within 2 hours. Note that there has been no normalization of plating efficiencies against the drug-free controls (open squares).

Comparison of figs. 5 and 6 shows very clearly a dramatic difference between aerobic and hypoxic toxicity of 0582. For example, a 3 hour incubation in 10 mM 0582 produced no toxic effects for aerobic cells (fig. 5), while fig. 6 shows that the plating efficiency of hypoxic cells would be less than 10% after such an incubation.

3.1.1.2 Temperature dependence of acute toxicity under hypoxic conditions

To test for temperature dependence of acute toxicity under hypoxic conditions, an experiment was performed with CH2B2
cells incubated in Ro-07-0582 at 22.5°C. The results are shown in fig. 7, where each point is again the average of two independent measurements. Comparison of fig. 7 and fig. 6 shows that the hypoxic toxicity of 0582 is substantially dependent on temperature (note the expanded ordinate scale of fig. 7).

3.1.1.3 Hypoxic toxicity using prepurified nitrogen

To check the possible effect of trace amounts of O₂ present in hypoxic toxicity tests, an experiment was performed on hypoxic CH2B₂ cells in suspension. Hypoxia was obtained by flowing N₂ containing about 10 ppm O₂ (measured with a Hersch Oxygen Meter) over the suspension at a rate of 0.7 litres/minute. The O₂ content was likely less than that for experiments using N₂ + 5% CO₂, for which measurements of approximately 40 ppm O₂ have been obtained from similar mixtures. The results are plotted on fig. 8. For each point, two aliquots of cell suspension were processed and plated independently. Points without error bars have too small a standard deviation to plot. The results of fig. 8 show the 1 and 5 mM exposures to be more toxic than their counterparts in fig. 6. There is considerable difference in the 5 mM curves, indicating that the hypoxic toxicity response is effected by small amounts of remaining O₂. However, the fact that the cell killing for the 50 mM exposure in fig. 8 does not exceed that for fig. 6 suggests that perhaps these two curves represent the upper limit for toxic effects on this cell line.
FIGURE 7. Percent Plating Efficiency of Hypoxic CH2B2 Cells as a Function of Incubation Time at 22.5°C in 0, 1.0, 5, or 50 mM Ro-07-0582

Note the expanded ordinate scale compared to fig. 6.
FIGURE 8. Percent Plating Efficiency of CH2B7 Cells Gassed with Prepurified Nitrogen, Shown as a Function of Incubation Time at 37°C in 0, 1, 5, or 50 mM Ro-07-0582.

The nitrogen contained about 10 ppm O₂.
3.1.2 Toxicity of 0582 In Aerobic and Hypoxic CHO Cells In Suspension at 37°C

Figure 9 shows the plating efficiency of aerobic CHO cells as a function of time of incubation at 37°C in the presence of Ro-07-0582. For each point, three aliquots of cell suspension were processed and plated independently. The points and error bars on fig. 9 represent the average of the three plating efficiencies and the standard deviation. For comparison, fig. 10 shows the plating efficiency of CHO cells plotted as a function of incubation time at 37°C in Ro-07-0582 under hypoxic conditions. For each point, two aliquots of cell suspension were processed and plated independently.

Clearly the hypoxic toxicity of fig. 10 is much greater than the aerobic toxicity of fig. 9. For example, 2 hours incubation of CHO cells at 37°C in 50 mM Ro-07-0582 yields a plating efficiency of 1.2% under hypoxic conditions (fig. 10), while the same drug concentration under aerobic conditions produces no detectable toxicity (fig. 9). As well, comparison of figs. 6 and 10 shows that 0582 has a similar hypoxic killing effect on the CHO and CH2B₂ cell lines.

3.1.3 Toxicity of 0582 In Aerobic and Hypoxic EMT6 Cell Suspensions

The EMT6 cell line was also used for tests of aerobic and hypoxic toxicity of 0582. As EMT6 is a mammalian tumor line, the information was a valuable addition to the results for the CH2B₂ and CHO lines.
FIGURE 9. Percent Plating Efficiency of Aerobic CHO Cells as a Function of Incubation Time at 37°C in 0, 1.0, 15, or 50 mM Ro-07-0582

Note the expanded ordinate scale compared to that of figs. 6 and 10.
FIGURE 10. Percent Plating Efficiency of Hypoxic CHO Cells as a Function of Incubation Time at 37°C in 0, 1.0, 5, 15, or 50 mM Ro-07-0582
Figure 11 shows the plating efficiency of aerobic EMT6 cells as a function of incubation time in 0582 at 37°C. Each point is once more the average of 2 independent measurements. For exposures to 13 hours with drug concentrations of 15 mM or less, there was little if any toxicity relative to the drug-free control (solid triangles and solid curve). Slight relative toxicity was observed for a 22 hour incubation in 15 mM 0582. Although incubation in 50 mM 0582 appeared to give large toxicity, this single experiment provided insufficient data to be conclusive.

Figure 12 shows the results for EMT6 cells incubated under hypoxic conditions at 37°C in the presence or absence of Ro-07-0582. Each point represents the average of two independent plating efficiencies. Comparison of figs. 6, 10 and 12 shows that the hypoxic toxicity is greater for the EMT6 line than either the CH2B2 or CHO line for incubation in 15 or 50 mM 0582. However, the 5 mM curves are all similar, and it appears from the 1 mM curves that the toxicity at low concentrations of 0582 is less for EMT6 than for CH2B2 or CHO cells, especially if one considers the low plating efficiency (at long incubation times) of the drug-free EMT6 suspension.
FIGURE 11. Percent Plating Efficiency of Aerobic EMT6 Cells as a Function of Incubation Time at 37°C in 0, 1.0, 5, 15 or 50 mM Ro-07-0582

Note the expanded ordinate scale.
FIGURE 12.  Percent Plating Efficiency of Hypoxic EMT6 Cells as a Function of Incubation Time at 37°C in 0, 1.0, 5, 15, or 50 mM Ro-07-0582
3.2 TREATMENT WITH 0582

3.2.1 Treatment of CH2B2 Cells in Suspension With 0582

In these experiments 0582 was added to the cell suspension (maintained at 22°C) just prior to the start of gassing and diluted immediately following irradiation by the addition of drug-free medium. In no instance was there toxicity from exposure to the drug. This was demonstrated by the plating efficiencies of zero dose control samples taken immediately prior to irradiation. Toxicity would not be expected for reasonable drug concentrations, as fig. 7 shows that at 22°C there is negligible toxicity even at 50 mM during the first 2 hours exposure to the drug.

Figure 13 shows the survival curves of an experiment in which CH2B2 cells in a drug-free suspension at 22°C were irradiated in the presence (squares) or absence (circles) of oxygen, the objective being to accurately determine the OER (see 2.3.5). To enable precise measurement of low survival values, it was necessary to use suspensions of 10^6 CH2B2 cells/ml in this experiment. The solid circles are survival values from a suspension which received an initial dose of 4100 rads (dose rate 420 rads/min.), thus minimizing the time required to deliver the high doses. This also reduces the enzymatic repair of sublethal damage which might occur over the course of irradiation. The difference between the hypoxic survival curves presumably represents the difference in the amount of enzymatic repair.
FIGURE 13.  The OER for CH2B2 Cells

CH2B2 cells were irradiated under aerobic (open squares) or hypoxic (circles) conditions. The suspension represented by solid circles received an initial dose of 4100 rads, thus minimizing the time required to deliver the high doses. The OER was approximately 3 (see 3.2.1).
The straight line segments of the survival curves of fig. 13 were fitted to the data points by the method of least squares. In order to omit data points lying on the "shoulder" of the curve, only points with a survival less than or equal to 0.25 were accepted for the least squares fit. OER values were calculated from the ratio of the slopes of the linear segments. When the hypoxic response was represented by the open circles, the calculated OER was 3.07±0.04. When open circle data was used for doses below 4000 rads and closed circle data for doses above 4000 rads, the OER was 2.84±0.06. These values lie in the range of OER measurements (2.7 to 3.1) reported by most experimenters using mammalian cells.

Figure 14 shows the survival curves for three drug concentrations present during irradiation of hypoxic CH2B2 cells at 22°C. Also included are data obtained the same day with only N2 or O2 present during irradiation of drug-free suspensions. Clearly the presence of 0582 sensitizes hypoxic cells, the DMF (see 2.3.5) increasing rapidly with drug concentration. The straight line segments of the survival curves of fig. 14 were fitted to the data points by the method of least squares, again using only survival values less than or equal to 0.25. The slopes of the linear fits were used to calculate DMF's of 1.37, 1.85 and 3.37, corresponding to 0.1, 1.0 and 15 mM 0582 respectively. The OER was 3.16.

In fig. 15 are summarized the results of a large number of survival response measurements similar to those shown in
FIGURE 14. Survival Curves for CH2B2 Cells Irradiated at 22°C in the Presence of Nitrogen, Nitrogen Plus 0.1, 1.0, or 15 mM Ro-07-0582, or Oxygen
FIGURE 15.  Sensitization and Toxicity of Ro-07-0582 with CH2B₂ Cells in Suspension as a Function of Drug Concentration

- DMF obtained under hypoxic conditions at 22°C;    limits of OER values;    Δ normalized plating efficiency after a 3 hour incubation at 37°C under hypoxic conditions (note the inverted ordinate scale);    ▲ normalized plating efficiency after a 6 hour incubation at 37°C under aerobic conditions.  Note:  The rather high maximum DMF and OER values shown in this figure probably result from the manner in which these parameters were calculated (see section 3.2.1).
fig. 14. DMF's and standard errors are plotted as a function of the concentration of 0582 present during irradiation under hypoxic conditions. It can be seen that sensitization occurs for drug concentrations as low as 0.1 mM. A DMF of 2.1 is attained with 1 mM 0582, while concentrations over 20 mM appear to give a DMF equal to the OER. As well, one experiment (fig. 16) showed that addition of 100 mM drug to oxygenated cells did not increase sensitization above that for O₂ alone.

The somewhat high OER range shown in fig. 15 probably results from comparing aerobic responses to hypoxic responses which had been carried to insufficiently high doses to accurately establish the final slope (minimum survival values were commonly 0.002). When the DMF's are calculated, not from slopes of the survival curves, but rather from the ratio of the doses required to reduce survival to 0.01, the 0582 DMF approaches a maximum value of approximately 2.8 at 100 mM, whereas the OER calculated in the same manner is 2.9 in these experiments.

In order to compare the sensitization and toxicity of Ro-07-0582, two time-cut toxicity experiments were carried out, one under hypoxic and one under aerobic conditions, using the method described in section 2. Multiple drug concentrations were tested in both cases. The toxicity was measured at 3 hours in the hypoxic and at 6 hours in the aerobic suspensions.

The results, shown in fig. 15, indicate for aerobic cells, that a 6 hour incubation in concentrations of 0582 up to
FIGURE 16. Survival Curves for CH2B, Cells Irradiated at 22°C in the Presence of Oxygen or Oxygen Plus 100 mM Ro-07-0582
10 mM produces negligible loss in plating efficiency although this concentration produces nearly maximum sensitization of hypoxic cells. The toxic effect of 0582 on hypoxic cells increases sharply for concentrations of 0582 greater than 1.0 mM such that a 3 hour incubation at 10 mM leaves less than 5% of the cells viable. Comparable time-cut toxicity responses for other incubation times under hypoxia can be constructed from fig. 6.

3.2.2 Treatment of CHO Cells In Suspension With 0582

Figure 17 shows survival curves for CHO cells irradiated at 22°C under hypoxic or aerobic conditions. As was done for the CH2B2 line, a least squares fit was obtained for the linear portion of each curve. The OER and the DMF values for each drug concentration were determined by comparing the doses which reduced survival to 0.01. The DMF’s calculated from a number of CHO survival curves are shown in fig. 18 (closed circles), and again above 10 mM 0582 the DMF approaches the OER value. Also shown for comparison in fig. 18 is the DMF response of CH2B2 cells (see fig. 15) calculated not from the slopes of the survival curves but by comparing the doses which reduced survival to 0.01. It can be seen that when DMF values are calculated in this way, the maximum DMF and OER approach a common value of approximately 3 for both cell lines.
FIGURE 17. Survival Curves for CHO Cells Irradiated at 22°C in the Presence of Nitrogen, Nitrogen Plus 0.08, 0.6, 3, or 25 mM Ro-07-0582, or Oxygen.
FIGURE 18. Ro-07-0582 Sensitization of CHO Cells in Suspension, Shown as a Function of Drug Concentration

The 0582 sensitization of both CHO cells (closed circles and solid curve) and CH2B2 cells (dashed curve; experimental points not shown) in suspension at 22°C is shown. DMF values were calculated by comparing the doses required to reduce the surviving fraction to 0.01.
3.2.3 Treatment of EMT6 Cells In Suspension With 0582

In fig. 19 are presented several survival curves for EMT6 cells irradiated at 22°C under aerobic or hypoxic conditions. Least squares fits and DMF's were obtained as described in section 3.2.2. The DMF's calculated from a number of EMT6 survival curves are shown in fig. 20 (closed circles), again with the CH2B2 response shown for comparison. The sensitization by 0582 of EMT6 cells in suspension is very similar to the sensitization of CH2B2 cells (and CHO, fig. 18), with drug concentrations of approximately 10 mM and greater producing a DMF approaching the OER.

3.2.4 Treatment of Cell Pellets With 0582

Cell pellet experiments were desirable as a possible means of checking for cell concentration dependent sensitization. An example of the above is provided by triacetoneamine-N-oxyl (TAN) which produces a much reduced sensitization in vivo as compared to sensitization achieved in in vitro tests. Agnew and Skarsgard (1972) observed a reduced sensitization by TAN for CH2B2 cells in a cell pellet as compared to cells in suspension. A similar effect was observed for several other drugs including PNAP (Agnew and Skarsgard, 1974), and it was suggested that the cell pellet experiment may prove to be a useful indicator of the effectiveness of radiosensitizers in vivo.
FIGURE 19. Survival Curves for EMT6 Cells Irradiated at 22°C in the Presence of Nitrogen, Nitrogen Plus 0.09, 0.45, or 2.2 mM Ro-07-0582, or Oxygen
FIGURE 20. **Ro-07-0582 Sensitization of EMT6 Cells in Suspension, Shown as a Function of Drug Concentration**

The 0582 sensitization of both EMT6 cells (closed circles and solid curve) and CH2B2 cells (dashed curve; experimental points not shown) in suspension at 22°C is shown. DMF values were calculated by comparing the doses required to reduce the surviving fraction to 0.01.
Figure 21 presents a comparison of the hypoxic response of drug-free CH2B2 cells irradiated at 22°C in dilute suspension or in cell pellet form. In order to prevent drying of the cell pellet the N2 was first humidified by passing through a water-filled gas washing bottle, a procedure followed in all cell pellet experiments. The results of fig. 21 show the cell pellet survival values to be slightly but consistently greater than those for cells in suspension. However, the 3 slopes, calculated from least squares fits of survival values of 0.25 or less, are equal to within the standard errors. Similar observations were obtained using CHO cells in pellet and suspension (fig. 22). It is fair to conclude from figs. 21 and 22 that high cell density does not greatly affect the radiation response of either cell line.

To check for possible time dependent loss of sensitization of cells in pellet form, as Agnew and Skarsgard (1974) observed for several drugs, cell pellets were prepared with equal drug concentrations and were irradiated at various times after formation of the pellets. The drug concentrations and incubation times were again such that no toxic effects were observed upon the zero dose control samples. The results are presented in fig. 23. In this experiment, cell pellets were formed with drug concentrations of 0 (1 pellet), 0.48 (3 pellets) and 4.8 mM (3 pellets). Pellets containing drug were then incubated at 22°C for 18, 50 or 90 minutes before they were irradiated under hypoxic conditions. The resulting survival
FIGURE 22. Survival Curves for CHO Cells Irradiated at 22°C While in a Suspension Gassed with Humidified Nitrogen or as a Cell Pellet Gassed with Humidified Nitrogen.
To test for time-dependent loss of sensitization the cell pellets were incubated at 22°C for 18, 50, or 90 minutes before irradiation.
responses were fitted to a straight line by the method of least squares, again using only survival values of 0.25 or less. These responses show no significant dependence on pellet incubation time. This observation with 0582 is in sharp contrast with earlier studies with TAN (Agnew and Skarsgard, 1972), all of which demonstrated a rapid loss of sensitizing capacity with pellet incubation time.

Survival curves were also obtained for CH2B2 cell pellets exposed to other drug concentrations and these data were pooled with those of fig. 23. DMF's were calculated from the ratio of slopes of the linear least squares fits, and in fig. 24 these are plotted with the corresponding standard error. Also shown in fig. 24 is the only result for a CHO cell pellet, also irradiated at 22°C. The curve, fitted by eye, shows CH2B2 pellet sensitization to be slightly less than the sensitization achieved in dilute suspension (fig. 15). However, 0582 concentrations of the order of 20 mM gave a pellet DMF of 2.6, approaching the full OER.
FIGURE 24.  Ro-07-0582 Sensitization of Hypoxic Cells in Pellet Form as a Function of Concentration of Ro-07-0582

Both CH2B2 cell pellets (solid circles) and the single CHO cell pellet (solid square) were irradiated at 22°C.
3.3 PRETREATMENT AND POST-TREATMENT WITH 0582

Because of the pretreatment and post-treatment effects observed with TAN by Agnew and Skarsgard (1972), it was of interest to determine whether such effects existed with 0582.

3.3.1 Pretreatment With 0582

CH2B2 cells suspended at a concentration of 2.5 $\times 10^5$ cells/ml in normal growth medium lacking sodium bicarbonate were treated for 1 hour at 22°C with 10 mM 0582 under aerobic conditions. The drug was then removed by twice centrifuging the suspension and resuspending the cells in drug-free medium lacking sodium bicarbonate. The suspension was placed in an irradiation vessel and, 1 hour after removal of the drug, irradiated under hypoxic conditions. Figure 25 shows the results of this experiment. Also shown are survival data obtained concurrently for hypoxic CH2B2 cells irradiated in drug-free medium lacking sodium bicarbonate. Within experimental accuracy the results show no pretreatment effect for 0582.

3.3.2 Post-Treatment With 0582

CH2B2 cells suspended at 2.5 $\times 10^5$ cells/ml were irradiated under hypoxic conditions. Immediately following irradiation three aliquots were removed from the hypoxic suspension and added to an aerobic solution of 0582 such that the final drug
FIGURE 25. Survival Responses for CH2B2 Cells (a) Irradiated at 22°C in the Presence of Nitrogen or (b) Irradiated Following Pretreatment for 1 Hour at 22°C with 10 mM Ro-07-0582.

The 0582 was removed by washing prior to irradiation.
concentration was 0 (control), 10 or 30 mM. After one hour the cells were removed from the drug by centrifuging and resuspending in drug-free medium. The cells were then plated to determine colony forming ability. The suspensions were maintained at 22°C prior to, during and after irradiation. The results, presented in fig. 26, show no post-treatment effect with 0582.
FIGURE 26. Survival Responses for CH2B2 Cells Subjected to a 1 Hour Post-treatment with 0, 10, or 30 mM Ro-07-0582 at 22°C, Following Irradiation Under Hypoxic Conditions in the Absence of 0582
4. DISCUSSION

4.1 TOXICITY OF Ro-07-0582

Each of the three cell lines tested showed that the toxic effects of 0582 in the absence of irradiation are quite selective for hypoxic cells. This can be seen by comparison of fig. 5 with fig. 6, fig. 9 with fig. 10, and fig. 11 with fig. 12. This suggests that the drug may have, in addition to an impressive radiosensitizing capacity, significant chemotherapeutic properties. In both cases the effects are selective for hypoxic cells. Thus, hypoxic tumour cells, irradiated in the presence of 0582, would be placed in a double jeopardy situation provided that the biological half-life of the compound in the patient was of the order of a few hours.

An example of the above may be constructed from our data with the CHO cell line. Let us consider the effect upon aerobic and hypoxic CHO cells subjected to a 4 hour incubation in 5 mM 0582, with 1000 rads delivered somewhere in this time interval. Let us also assume that the two effects are additive. Figure 18 indicates that hypoxic cells irradiated in the presence of 5 mM 0582 will experience a DMF of ~2.6. From the definition of the DMF (see 2.3.5), this implies the survival rate will be about the same as for drug-free hypoxic cells exposed to 2600 rads. Figure 17 shows the corresponding surviving fraction to be 0.015. Figure 17 also shows that
1000 rads reduces the surviving fraction of aerobic CHO cells to 0.005. Further, figs. 9 and 10 show that aerobic CHO cells will experience no toxicity while ~99% of hypoxic cells will be killed. The net surviving fractions of original viable cells are $0.15 \times 10^{-3}$ for hypoxic cells versus $5 \times 10^{-3}$ for aerobic cells.

The net survival of hypoxic cells treated as above is much lower than for hypoxic cells irradiated in the absence of drug, which yields a DMF of 1.0 and no selective toxicity. Figure 17 shows the surviving fraction of drug-free hypoxic cells exposed to 1000 rads to be 0.4. Comparison of the net surviving fractions indicates that the combination of radiosensitization and toxicity may be of value as an approach to the hypoxic cell problem.

There are several points which should be discussed at this stage. First, the assumption that the effects of toxicity and radiosensitization are additive is not unjust, as one would expect any interaction of the two would enhance cell killing and result in reduced survival for hypoxic cells, thus improving the effectiveness of the treatment.

Secondly, we should consider the effectiveness of irradiation and toxicity on cells exposed to various degrees of oxygenation. This is necessary because of the different oxygen concentrations that exist in various regions of some tumors. For a sufficiently large tumor, one would expect cells to range from about totally hypoxic to aerobic. If we consider only irradiation effects, the presence of oxygen will result in a
lower survival rate than that for hypoxic conditions, since oxygen is a radiosensitizer. Thus radiation treatment would be more effective on a tumor having cells exposed to various degrees of oxygenation than on a totally hypoxic tumor.

If we now consider only toxic effects, we might expect that a reduced overall toxicity would be observed for cells having a range of $O_2$ levels (such as might be found in a tumor) as compared to a group of acutely hypoxic cells. This is seen by comparison of figs. 6 and 8, which indicates that the hypoxic toxicity of 0582 is affected by fairly small changes in $O_2$ concentration in the cell suspension. However, the advantage to the selective hypoxic toxicity exhibited by 0582 is that the more hypoxic the cell's environment, and therefore the more radioresistant the cell, the greater the probability of death due to toxic effects of the drug.

Our discovery of differential toxicity with 0582 led to similar observations with other potential radiosensitizers. Mohindra and Rauth (private communication), working with CHO cells, have reported that the toxicities of metronidazole and nitrofurazone exhibit a dependence upon the $O_2$ content of the gas flowing over the cell suspensions. Following a 6 hour incubation at $34^\circ$C in 29 mM metronidazole the plating efficiency was reported one log decreased for cells gassed with $N_2$ containing 10 ppm $O_2$ (plating efficiency = 5%) as compared to $N_2$ containing 100 ppm $O_2$ (plating efficiency = 45%). These observations are compatible with the observations (see above and
incubation in 1 mM 0582 for 6 hours with N₂ (=10 ppm O₂, fig. 8) is shown to reduce the plating efficiency to 3%. Thus it is evident that the hypoxic toxicity displayed by Ro-07-0582 is considerably greater than that of metronidazole. This conclusion is supported by studies of metronidazole performed in our laboratory (Skarsgard and Harrison, private communication).

The greatly increased toxicity of 0582 under hypoxic conditions could be caused by a metabolic product of reduction of the drug, a reduction proceeding only in the absence of O₂. Olive and McCalla (1975) have shown that single-strand breaks are produced in mammalian cell DNA both in vitro and in vivo as a result of nitrofurazone treatment under hypoxic conditions. They also measured the rate of reduction of the drug under various O₂ levels and demonstrated that reduction was eliminated under normal aerobic conditions. Olive and McCalla concluded that the metabolic reduction of nitrofurazone is required for DNA single-strand break production. Support for this proposal is provided by the observation that while single-strand breaks were produced in the DNA of E. coli B/r by nitrofuran derivatives (including nitrofurazone), no detectable breaks were produced in an E. coli mutant lacking nitrofurazone-reductase activity (McCalla, Reuvers and Kaiser, 1971).

Our observation of increased acute toxicity under hypoxic conditions might therefore be similarly explained by the hypoxic reduction of Ro-07-0582 to form a toxic product.
A comparison of figs. 6 and 7 shows that the hypoxic toxicity of 0582 is substantially greater at 37°C than at 22°C, as might be expected if enzymatic reduction of the drug is involved. This temperature effect, however, could also be due to some secondary metabolic event involved in developing the toxic effect.
4.2 RADIOSENSITIZATION WITH Ro-07-0582

4.2.1 Pre- and Post-treatment

The absence of pre- and post-irradiation sensitization (see 3.3.1 and 3.3.2) indicates that 0582 employs a different mechanism of sensitization than does TAN, for which both pre- and post-irradiation sensitization were observed (Agnew and Skarsgard, 1972). It is likely that 0582 sensitizes according to the electron affinity hypothesis (see 1.3), which involves reactions with short-lived species, thus suggesting the absence of pre- and post-treatment sensitization. Further support for this suggestion is provided by the work of Whillans, Adams, and Neta (1975) who showed, by a study of one-electron transfer reactions, that the electron affinity of the 2-nitroimidazole structure (e.g. Ro-07-0582) is greater than that of the 5-nitroimidazole structure (e.g. metronidazole). Electron spin resonance studies supported this observation. Thus the electron affinity correlates with the effectiveness of the compound as a sensitizer.

The pre- and post-treatment effects observed with TAN indicate that this sensitizer does not sensitize largely by interaction with short-lived species. It has been suggested that TAN acts by producing stable unrepaired DNA lesions (Emmerson, 1969; Nakken, Sikkeland and Brustad, 1970; Hohman, Palcic and Skarsgard, private communication). Thus, although most attention is presently focused upon electron affinic
compounds, this by no means excludes other mechanisms of sensitization.

4.2.2 Radiosensitization in Dilute Suspension

The results of fig. 16 indicate that 0582 demonstrates one of the essential properties of a potential radiosensitizer: selective sensitization of hypoxic cells. Presence of both 0582 and oxygen during irradiation produces the same survival response as for oxygen presence alone. This observation is explained by the fact that oxygen is extremely electron affinic, much more so than any of the electron affinic radiosensitizers including 0582. According to the radiosensitization-radioprotection model of Chapman et al (see 1.3), oxygen and 0582 would compete for the fixation of damage and the oxidation of radical reducing species. It is likely that under fully aerobic conditions the oxygen present saturates the fixation and oxidation processes. Therefore, addition of 0582 results in no additional sensitization. Alternatively, the relatively low electron affinity of 0582 compared to oxygen may result in a non-measurable component of lethality under aerobic conditions.

Very similar sensitization was achieved when 0582 was present during the irradiation of hypoxic CH2B2, CHO (fig. 18), or EMT6 cell lines (fig. 20). Detectable sensitization occurs for drug concentrations as low as 0.1 mM, while concentrations of about 10 mM gave sensitization similar to that achieved with
oxygen. These results are similar to those of Asquith et al (1974) who reported the maximum sensitization of 0582 upon Chinese hamster cells (line V79-GL1) to yield a DMF of roughly 2.5 at a drug concentration of 5 mM. They also reported DMF's of 2.2 at 1 mM and 1.6 at 0.4 mM. These values are only slightly higher than our DMF values of 1.8 and 1.5 at 1.0 mM and 0.4 mM 0582 respectively, in CH2B2 cells.

The sensitization provided by Ro-07-0582 is better than that of metronidazole. The maximum DMF (1.8 to 1.9) for metronidazole is reached at a concentration of roughly 10 mM (Asquith et al, 1974; Agnew, Palcic and Skarsgard, unpublished data), whereas a DMF of 1.9 is attained with 1-2 mM 0582 (figs. 18 and 20). Furthermore, 0582 was observed to give DMF's greater than 1.9, eventually reaching the full OER in dilute cell suspension.

It should be mentioned that the possibility of synergism between the toxic and radiosensitizing properties of Ro-07-0582 should be unimportant in most of the sensitization studies since the time of exposure to the drug was generally 1.5 hours or less. Drug concentrations less than 50 mM show very little toxicity at these exposure times, even under hypoxia. Of course, if the toxic and sensitizing effects are only additive, the control samples would have normalized out the toxic effects. In clinical use of the drug, however, when it is likely to be present in significant concentrations for many hours, one would
expect that both the radiosensitizing capacity and the hypoxic toxicity of 0582 may be important (see 4.1).

4.2.3 Radiosensitization of Cell Pellets

The slight relative radioresistance of hypoxic cells irradiated in cell pellet form as compared to hypoxic cells irradiated in dilute suspension (figs. 21 and 22) is in agreement with the observations of Thomson and Rauth (1974), who used cell suspensions prepared directly from the KHT fibrosarcoma of C3H mice. When various cell densities were irradiated under hypoxic conditions, Thomson and Rauth observed a slight increase in survival at high cell densities. This was attributed to an increase in the extrapolation number N rather than a change in the slope of the curve. Our data appear to support this suggestion, but more precise measurements will be necessary before a firm conclusion can be reached.

Figure 24 shows that the 0582 sensitization attained in cell pellets is quite comparable with, and perhaps slightly less than that attained in dilute suspension (figs. 15 and 18). Denekamp and Harris have observed with mice that injection of 1 mg 0582 per gram body weight (which corresponds to 5-7.5 mM 0582 in the body fluids) yields a DMF of approximately 2 in two different in vivo assays (Denekamp, Michael and Harris, 1974; Denekamp and Harris, 1975). In our cell pellets, 6 mM 0582 gives a DMF of 2.4 (fig. 24). This provides support for
the idea that the *in vitro* cell pellet system may be useful as a test system intermediate between conventional *in vitro* and *in vivo* systems (see 3.2.4). Further support for this suggestion is provided by the work of Thomson and Rauth (1974) who showed that NF-167 (a nitrofuran derivative) lost the ability to sensitize hypoxic KHT tumor cells when the cell concentration approached $10^8$ cells/ml, while metronidazole showed little loss of sensitizing ability. This correlates well with the observed sensitization of hypoxic KHT cells in mice by metronidazole and the lack of *in vivo* sensitization by NF-167 (Rauth and Kaufman, 1975).
4.3 Ro-07-0582 AS A POTENTIAL RADIOSENSITIZER

In section 1.1 there were several properties listed which are considered desirable for an effective radiosensitizer. The work in this thesis and the work of others permits us to now consider the attributes of Ro-07-0582 as a potential radiosensitizer.

Ro-07-0582 definitely shows the ability to preferentially sensitize hypoxic cells to the lethal effects of ionizing radiation (see 4.2.2). The sensitization achieved in vitro is better than that of metronidazole. Ro-07-0582 has also been shown to sensitize hypoxic cells in vivo.

For drug doses necessary to achieve high levels of sensitization the toxicity of Ro-07-0582 to aerobic cells is quite acceptable (see 3.2.1 and fig. 15). The toxicity to hypoxic cells, however, is much increased over the toxicity to aerobic cells, and this may prove to be a useful adjunct to the drug's sensitizing properties in destroying hypoxic tumor cells.

It has been shown by Asquith et al (1974b), using Chinese hamster cells, that the sensitization achieved with 0582 is independent of the position of the cells in the mitotic cycle. This experiment involved measurement of the DMF by single dose irradiations of hypoxic synchronized cells, both in the presence of 4 mM 0582 and in drug-free suspensions. Although the sensitivity of the cells changed with position in the cell
cycle, being most sensitive near mitosis and most resistant in late S-phase, the DMF was constant.

The limited results of preliminary serum concentration measurements in man indicate that 0582 is absorbed rapidly and is eliminated from the serum with a half-life of 9-18 hours (Annual Report 1974, Gray Laboratory). Thus it is hoped that Ro-07-0582 has sufficient metabolic stability to be effective. As well, single doses of up to 10 grams 0582 have been taken orally, apparently with few or no side effects. This is very encouraging when the observations of Denekamp and Harris (1975) are considered. They reported that a drug dose of 0.1 mg per gram body weight gave a DMF of 1.35 in mice. This corresponds (by weight) to a dose of 6-7 grams to a human, less than doses already successfully administered.

From the above observations it is evident that Ro-07-0582 shows considerable promise as a radiosensitizer.


