

**ACCUMULATION AND METABOLISM OF BMS181321, A TECHNETIUM-99m-
LABELLED NITROIMIDAZOLE, IN VITRO**

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science,
Graduate Department of Medical Biophysics,
University of Toronto

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Abstract

Hypoxic conditions are believed to be a limitation for the local control of some solid tumours by radiation therapy. However, even for a given tumour type there is great heterogeneity in the extent of hypoxia. A technique to assess the extent of hypoxia in an individual tumour would allow the selection of patients who would most benefit from therapies that target these hypoxic cells. Nitroimidazoles, which are selectively retained in hypoxic cells, have been investigated as hypoxic cell markers. BMS181321, a 2-nitroimidazole labelled with technetium-99m, has offered the possibility of assessing tumour hypoxia using nuclear medicine techniques. Due to the high specific activity of technetium-99m, the concentration of BMS181321 used in its applications is very low. Metabolic depletion of the drug may result in incomplete detection of hypoxic regions in solid tumours. The goal of this work was to investigate the feasibility of using unlabelled nitroaromatics to modify the *in vitro* accumulation of BMS181321 in hypoxic cells and to assess the potential of using this approach to alter the biodistribution of BMS181321 in its *in vivo* application. It was found that nitroaromatics of a similar or higher electron affinity than BMS181321 inhibited the hypoxic accumulation of the drug. In contrast, nitroimidazoles of a lower electron affinity than BMS181321 stimulated its accumulation. This thesis addresses the potential clinical relevance of such an approach to modify the hypoxic accumulation of BMS181321, as well as the mechanisms involved in the retention of BMS181321 in hypoxic cells.

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In memory of my grandfather

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Chapter 1: Introduction

1.1 Introduction

Hypoxic cells are resistant to the lethal effects of ionizing radiation. The radioresistance of some human tumours is thought to be due to the presence of hypoxic cells. Tumours that appear identical by several clinical criteria may have differing hypoxic fractions, thus methods to assess the extent of hypoxia in tumours are necessary. A number of methods are available, but none of the available techniques is widely accepted. Amongst the proposed techniques is the use of nitroimidazoles as hypoxic cell markers. Nitroimidazoles are compounds that are specifically retained in hypoxic cells and which have been investigated as hypoxic cell cytotoxins and radiosensitizers. The synthesis of BMS181321, a 2-nitroimidazole that is labelled with technetium-99m, has offered the possibility of routine imaging of tumours to assess the hypoxic fraction. BMS181321 has been investigated as a hypoxic marker in heart and brain and its potential use as a hypoxic marker in tumours is under investigation.

1.2 Tumour Hypoxia

The vascular system of a tumour can be highly disorganized in both its structure and function where the vascularization is characterized by malformed and leaky vessels irregularly spaced throughout the tumour. It consists of vessels that are recruited from the pre-existing host vasculature by the angiogenic response of host vessels to cancer cells (Jain, 1989). In order for the tumour to grow, new vascularization is needed. The rate of vascularization is often insufficient relative to tumour growth and the ability of the tumour vasculature to supply oxygen throughout the tumour mass may be compromised and a portion of the tumour rendered hypoxic (Vaupel *et al.*, 1989). This chronic or diffusion limited hypoxia occurs because oxygen is

consumed as it diffuses away from blood vessels, creating a gradient of decreasing oxygen concentration as the distance from the vasculature increases.

In 1955, Thomlinson and Gray provided one of the first descriptions of chronic hypoxia in tumours (Thomlinson and Gray, 1955). Histological sections of bronchial carcinoma specimens contained viable tumour regions surrounded by vascular stroma from which the nutrient and oxygen needs of the cells were met. The area of viable tissue was about 150 μm in thickness, which was similar to the calculated diffusion distance of oxygen in respiring tissue.

Periods of acute or perfusion-limited hypoxia may also occur in tumours due to intermittent fluctuations in tumour blood flow caused by vascular collapse. Vascular collapse is caused by the malformed structure and leakiness of the vasculature, which results in increased intratumour pressure causing the region around the collapsed blood vessel to become hypoxic. Increased intratumour pressure is also related to a lack of lymphatics in the tumour and to tumour vascular spasm. Acute hypoxia in tumours has been demonstrated in rodent tumours (Chaplin *et al.*, 1987) and in human tumour xenografts (Horsman, 1995). As well, recent findings in human malignancies found a change in erythrocyte flux which is consistent with transient, perfusion-driven changes in tumour oxygenation (Hill *et al.*, 1996).

Tumour cells in hypoxic regions are resistant to ionizing radiation. When cells are irradiated, unpaired electrons are produced on DNA. Due to the high electron affinity of oxygen, oxygen reacts with these radicals, forming lesions which the cell has difficulty repairing. Thus, in the absence of oxygen, the number and severity of irreparable or stable lesions produced by ionizing radiation decreases and cell survival increases (Rockwell, 1992).

Chronic hypoxia may also influence chemotherapeutic drug action (Wilson *et al.*, 1989). Drug accessibility to the hypoxic cells that are distant from the tumour vasculature may be hindered due to diffusion limitations. As well, hypoxic cells progress slowly, if at all, through the cell cycle and may be less susceptible to cell cycle-specific drugs. Thus, tumour hypoxia creates regions in solid tumours that may be resistant to radiation or chemotherapy which limits therapeutic efficacy, and if reoxygenation of the surviving hypoxic cells occurs, these cells may proliferate and cause tumour recurrence.

1.3 Evidence for Tumour Hypoxia

The resistance of hypoxic cells to the lethal effects of ionizing radiation has been demonstrated extensively in cell culture systems (Hill, 1992). Hypoxic cells are approximately three times more resistant to radiation than aerobic cells. Low oxygen concentrations are needed for oxygen to lose its effectiveness as a radiosensitizer. The oxygen concentration for half-maximum radiosensitization (K_m) is approximately 5 μ M.

Hypoxic fractions have been measured in a variety of mouse and rat tumours (Moulder and Rockwell, 1984, Rockwell and Moulder, 1990). Most transplanted rodent tumours contain hypoxic cells and in many instances 10-20% of the clonogenic tumour cells are hypoxic. Hypoxic cells have also been found in human tumours xenografted into mice, with hypoxic fractions similar to those of transplanted rodent tumours (Rockwell and Moulder, 1990).

Evidence for the presence of hypoxic cells in human tumours and their effect on tumour cure has been reported. Analysis of survival data from patients with cervical carcinomas treated with radiation revealed that patients with hemoglobin levels greater than 12 mg/dl at the time of

treatment had a higher local control (Bush *et al.*, 1978). The oxygen saturation of hemoglobin in individual erythrocytes within blood vessels from human tumour specimens has been studied using cryospectroscopy (Mueller-Klieser *et al.*, 1981). A wide range of hemoglobin saturations was found and some erythrocytes also exhibited a level of saturation that would be expected only under severe hypoxia. As well, the use of high oxygen-content gas breathing, particularly under hyperbaric conditions, resulted in a significant benefit in local tumour control and survival in patients with cervical and advanced head and neck tumours treated with radiation (Overgaard and Horsman, 1996).

Recent studies using oxygen electrodes, have also demonstrated that human tumours may contain hypoxic cells and evidence for a relationship between an increased hypoxic fraction and treatment failure has been provided. In metastases of squamous cell carcinomas of the head and neck, tumours with a median pO_2 less than 8 mm Hg had a significantly decreased response to therapy relative to those tumours with a higher median pO_2 when tumour size was investigated 90 days after therapy (Gatenby *et al.*, 1988). In 22 patients with soft tissue sarcomas that underwent irradiation and hyperthermia treatment, the 18-month disease-free survival was 70% for patients with a pretreatment median tumour pO_2 value of greater than 10 mm Hg but only 35% for those with median pO_2 values of less than 10 mm Hg (Brizel *et al.*, 1996). In cancers of the uterine cervix, patients with low pO_2 tumours (median pO_2 less than 10 mm Hg) showed a decreased recurrence-free survival and overall survival relative to patients with high pO_2 tumours after a 19 month follow-up (Höckel *et al.*, 1993). As well, it was found that tumours of the same stage and size could vary widely in their hypoxic fraction. These clinical studies suggest that a routine measure of the hypoxic fraction in tumours could be a beneficial prognostic

tool, and might allow for the rational selection of patients who would benefit from treatment strategies that target hypoxic cells.

1.4 Methods to Treat Tumour Hypoxia

The resistance of hypoxic cells to treatment has led to the development of strategies to treat hypoxic cells in tumours. Treatment strategies include the use of fractionated radiotherapy regimens, which should minimize the effects of acute hypoxia in treatment resistance, as well as the effects of chronically hypoxic cells if reoxygenation of the hypoxic fraction occurs before another round of radiation. The use of hyperbaric oxygen has seen limited success in clinical trials (Dische, 1991). This technique is based on the principle that the concentration of free oxygen in blood should increase in patients breathing oxygen at high pressure, resulting in an increase in the diffusion gradient of oxygen to an extent that is sufficient to reach chronically hypoxic cells. Another strategy to treat hypoxic cells is the use of heavy particle radiotherapy (high linear energy transfer). This technique is based on the principle that the oxygen enhancement ratio decreases with an increased ionization density of the radiation, thus decreasing the adverse protective effect of tumour hypoxia. Unfortunately, this technique can be expensive and it may not completely limit the oxygen effect. The use of nitroimidazoles to specifically radiosensitize or kill hypoxic cells has been advocated as a means to treat hypoxic cells because it is a simple and potentially cost effective technique for routine clinical use.

1.4.1 Nitroimidazoles as Radiation Sensitizers

Nitroimidazoles are electron affinic compounds, a number of which have been found to be hypoxic cell radiosensitizers, since nitroimidazoles can mimic oxygen in its ability to sensitize hypoxic cells to radiation. Nitroimidazoles mimic oxygen in the chemical reactions that lead to DNA damage, and should not sensitize aerobic cells, since aerobic cells already have sufficient oxygen concentrations to allow maximal radiosensitization.

The 5-nitroimidazole, metronidazole, was the first nitroimidazole studied as a hypoxic cell radiosensitizer and it was shown to be an effective radiosensitizer both *in vitro* and *in vivo* (Foster and Willson, 1973, and Rauth and Kaufman, 1975). When 5-nitroimidazoles and subsequently 2-nitroimidazoles were used in the clinic, they only exhibited marginal success as radiosensitizers when used as adjuvants to radiation therapy (Overgaard, 1994). However, these clinical results do not necessarily preclude the future success of nitroimidazole radiosensitizers in the clinic. Adverse drug effects, including gastrointestinal toxicity and peripheral neuropathy were observed following administration of the nitroimidazole. This toxicity was dose-limiting, which precluded administering adequate drug doses to achieve maximal radiosensitization. As well, these clinical studies did not select for patients that had tumours with high hypoxic fractions; i.e. those patients that would be most likely to benefit from therapies to treat hypoxic cells. It is now believed that clinical trials should be limited to such patients. As well, future clinical investigations with nitroimidazole radiosensitizers should use drugs that may be delivered at a tolerable yet effective dose to the patient. The success of future clinical studies would require developing techniques that could allow one to routinely measure the hypoxic fraction in human tumours.

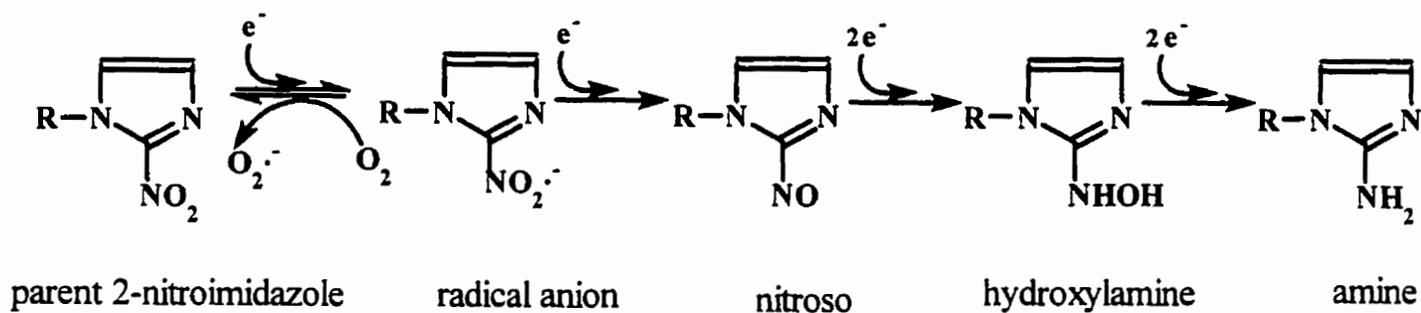


Figure 1.1: The bioreductive metabolism of nitroimidazoles. In this schematic, the parent 2-nitroimidazole is reduced to the radical anion form in both hypoxic and aerobic cells. In aerobic cells, the radical anion is back-oxidized to the parent compound by reaction with oxygen. In hypoxic cells, no back-oxidation to the parent compound occurs and the radical anion is further reduced to the nitroso, hydroxylamine and amine metabolites.

1.4.2 Nitroimidazoles as Hypoxic Cell Cytotoxins

In 1974, Sutherland found that metronidazole was preferentially toxic to hypoxic cells in spheroids (Sutherland, 1974). This effect was confirmed with a wide range of nitroimidazoles, where it was found that hypoxic cell cytotoxicity was related to the electron affinity of the nitroimidazole (Adams *et al.*, 1980). The mechanism of this cytotoxicity is believed to occur by enzymatic reduction of the nitroimidazole, yielding highly reactive metabolites.

Nitroimidazoles are reduced intracellularly in all cells to the radical anion form, but in the presence of adequate oxygen concentrations the radical anion is back-oxidized to the parent form (Edwards, 1993). In the absence of oxygen, the nitroimidazole undergoes further reduction reactions that form intermediates which bind to cellular macromolecules, resulting in the intracellular trapping of the compound. Figure 1.1 illustrates the bioreductive metabolism of nitroimidazoles. The formation of these metabolites is initiated by a nitroreductase-mediated single electron reduction of the nitro group to a free radical. Under hypoxic conditions, the reduction pathway can proceed in successive steps through nitroso and hydroxylamine derivatives to terminate at the amine derivative. The nitroso and hydroxylamine derivatives can give rise, through chemical rearrangement, to nitrenium ion intermediates which are thought to be reactive forms that bind to cellular macromolecules and ultimately cause cell death (McClelland *et al.*, 1985).

As is the case with the use of nitroimidazoles as hypoxic cell radiosensitizers, the success of therapeutic modalities using hypoxic cell cytotoxins as an adjuvant to traditional cancer therapies requires that a technique be developed that would allow the selection of patients that would most benefit from these therapies to treat hypoxic cells.

1.5 Methods of Measuring Hypoxia in Tumours

The National Cancer Institute in the United States held a workshop in 1992 to evaluate the proposed methods for assessing the oxygenation of human tumours (Stone *et al.*, 1993). Fourteen methods were described, which varied in stage of development, invasiveness, and tumour applicability. Table 1.1 provides a brief overview of some of the techniques discussed at the workshop.

1.5.1 Polarographic Oxygen Electrode

Included in the report was the polarographic oxygen electrode, which requires insertion of a needle (cathode) into the tumour and applying a small voltage between the cathode and a reference electrode applied to the skin surface. The current that flows is due to the reduction of oxygen at the cathode and is proportional to the oxygen concentration adjacent to the cathode. A histogram of pO_2 values is produced by step-wise advancement of the probe into the tumour. This technique provides a direct measure of oxygen concentration at different points in the tumour and a correlation between electrode measurements and treatment outcome has been reported (Gatenby *et al.*, 1988, Höckel *et al.*, 1993 and Brizel *et al.*, 1996). However, the oxygen electrode is an invasive technique, and as such it may cause local infection, hemorrhage, and dislodgement of tumour cell clusters along the measurement track. As well, the electrode cannot distinguish measurements made in necrotic tissue from those made in viable tissue and at low oxygen concentrations there is a low signal to noise ratio (Kavanagh *et al.*, 1996 and Raleigh *et al.*, 1996).

METHOD	INVASIVE	AVAILABILITY	WHAT IT MEASURES
polarographic oxygen electrode	yes	currently	extracellular pO ₂
tumour interstitial pressure	yes	currently	interstitial pressure
comet assay	yes	currently	radiosensitivity
alkaline elution assay	yes	future	radiosensitivity
ESR spectroscopy	no	future	pO ₂ at site of probe
oxyhemoglobin saturation	yes	currently	HbO ₂ saturation
³¹ P-MRS	no	currently	P metabolites
¹ H-MRS (lactate)	no	currently (brain)	lactate concentration
¹⁸ F-nitroimidazole with PET	no	currently	nitroimidazole binding
[¹²⁵ I]IAZA	no	currently	nitroimidazole binding

Table 1.1: Overview of some of the methods available to measure the hypoxic fraction in tumours that were profiled at the National Cancer Institute Workshop. (Adapted from Stone *et al.*, 1993).

1.5.2 Interstitial Fluid Pressure Measurements

Interstitial fluid pressure is elevated in tumours, and the interstitial fluid pressure decreases gradually from the centre of the tumour and at the tumour periphery there is a sharp drop in interstitial fluid pressure (Boucher *et al.*, 1990). A decrease in tumour pO_2 has been associated with an increased interstitial fluid pressure in the tumour, which may then cause vascular collapse and cessation of blood flow, leading to acute hypoxia. Tumour interstitial pressure has been measured in 7 patients with carcinomas of the uterine cervix before and after radiation treatment using the wick-and-needle technique (Roh *et al.*, 1991). In this technique, a needle with a side hole 3-4 mm from the needle tip, filled with saline and nylon filaments, is inserted into the tissue, and tubing connects the needle to a pressure transducer. The four patients that exhibited a decreased interstitial fluid pressure after radiation treatment had complete regression of the tumour 4 to 6 weeks after therapy. In contrast, the 3 patients that had an increased interstitial fluid pressure after therapy did not exhibit tumour regression. These results, though limited, appear to indicate that interstitial fluid pressure may decrease following radiation therapy of radioresponsive tumours. However, the ability of interstitial fluid pressure to be indicative of tumour oxygenation, and the ability for such a measure to have a predictive value requires further laboratory and clinical studies.

1.5.3 Measurements of DNA Strand Breaks

Two assays have been developed that exploit the relationship between the oxygen concentration and radiation sensitivity of DNA strand breaks. The comet assay measures single strand breaks in DNA in individual cells following damage (Olive *et al.*, 1990). Cells are

obtained from needle biopsy samples taken immediately after radiation therapy and electrophoresis of individual cell nuclei is used to assess damaged DNA by analysis of the amount of DNA released from individual cells during electrophoresis. This assay was carried out in 8 human tumours of the breast or the head and neck and an average hypoxic fraction of 5-10% was found in 7 of these tumours (Olive *et al.*, 1993).

The alkaline elution assay measures single strand breaks in DNA and DNA-protein crosslinks following damage to the tumour sample as a whole. In the alkaline elution assay, filters are used to discriminate DNA single strand sizes and variations of this technique can be used to measure single strand breaks and DNA-protein crosslinks. It is known that the type and quantity of DNA damage produced by ionizing radiation is dependent on the oxygen concentration around the DNA (Zhang and Wheeler, 1993). As the oxygen concentration decreases, the number of strand breaks decrease and the number of crosslinks increase during irradiation. The number of crosslinks and single strand breaks correlates to the hypoxic fraction of the tissue sample (Zhang and Wheeler, 1993). This technique is more laborious than the comet assay, it has the reputation of high variability between measurements, and studies in human tumours have not been reported.

Both the comet assay and the alkaline elution assay provide a direct measure of radiation damage. However, these are both invasive techniques which are technically challenging because processing must occur quickly in order to avoid the onset of DNA repair following radiation damage.

1.5.4 Electron Spin Resonance Spectroscopy

Electron spin resonance spectroscopy (ESR) measures the magnetic moment of unpaired electrons. The paramagnetic nature of molecular oxygen alters the ESR spectrum in proportion to the oxygen concentration in the vicinity of a probe. The probe may be a solid particle probe that is injected into the tumour and then imaged. The solid particle probe is not biodegradable, which allows the probe to remain in the tumour throughout tumour growth, allowing multiple measurements to be obtained. Soluble probes exist that can distribute to cellular water compartments, where the specificity of localization is determined by a side-group. In general, ESR spectroscopy techniques are advantageous because no background signal is produced, since there are few unpaired electrons from interfering species, so competition from signals other than molecular oxygen is avoided (Stone *et al.*, 1993). Currently, these ESR techniques are only experimental.

1.5.5 Measurement of Oxyhemoglobin Saturation

The measurement of oxyhemoglobin saturation, using either cryospectroscopy or near infrared spectroscopy, may provide a means to predict tumour radiosensitivity. The absorption spectrum of hemoglobin changes when oxygen is bound, thus monitoring the sample at appropriate wavelengths allows a ratio of oxygenated to deoxygenated hemoglobin to be obtained. A relationship between vascular density and oxyhemoglobin frequency distribution in 14 biopsies from 9 patients with squamous cell carcinomas of the oral cavity has been found using cryospectroscopy (Mueller-Klieser *et al.*, 1981). These techniques are labour intensive. As well,

measurements may not be related to radioresistance because of inter- and intra-tumour variability in oxygen consumption and vascular geometry.

1.5.6 Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) techniques detect differences in cellular metabolism that occur under hypoxia. ^{31}P -MRS:phosphorous is a non-invasive means of determining proportions of molecules containing ^{31}P :phosphocreatine, ATP, ADP, AMP, phosphomonoesters and inorganic phosphate. The ratio of these molecules differs in aerobic and hypoxic cells. In some patients with sarcomas of the extremities, a change in the ratio of ATP to inorganic phosphate was seen during radiation treatment, and this change was thought to reflect reoxygenation of the tumour (Stone *et al.*, 1993). Currently, this technique is only applicable to predictions of reoxygenation during therapy, since quantification of the hypoxic fraction is not possible because there is no clear relationship between the fraction of radiobiologically hypoxic cells and bioenergetic status.

^1H -MRS:lactate measures the cellular content of lactate, which is the endpoint of anaerobic metabolism and which is produced at higher concentrations by hypoxic cells. The pattern of lactate distribution and misonidazole binding has been found to be similar in RIF-1 murine tumours (Stone *et al.*, 1993). This technique is not widely applicable because the measurement and interpretation of ^1H -MRS data is difficult, and the lactate concentration does not always correlate with treatment outcome, since lactate may be trapped in necrotic tissue (Stone *et al.*, 1993). A measurement of lactate production would be a preferable approach, since this measure would be predicted to correlate better with the oxygenation status of the tumour.

1.5.7 Recommendations of the Workshop

Although the polarographic oxygen electrode was advocated as the best available method for the determination of the extent of hypoxia in tumours, it is limited to use on accessible tumours and is invasive, which limits the number of repeat measurements which may be taken. Of the non-invasive techniques described in the workshop, nitroimidazole binding in conjunction with nuclear medicine techniques seems to offer good resolution and sensitivity for assessing the extent of hypoxia in a tumour. As well, it is not limited to easily accessible tumours, and it has already shown promise in clinical trials (Parliament *et al.*, 1992, and Koh *et al.*, 1992). Developments in this area are discussed below.

1.6 Nitroimidazoles as Hypoxic Cell Markers

In 1979, it was first suggested that the nitroimidazole class of compounds could be possible radiopharmaceutical agents for determining the extent of hypoxia in tumours (Chapman, 1979). Nitroimidazole binding would be able to detect hypoxia at the cellular level. However, the use of nitroimidazoles as hypoxic cell markers may be affected by other cellular factors, such as the biochemical status and type of hypoxia (chronic or acute) present in the tumour. This may make it difficult to use the intensity of nitroimidazole binding as an absolute measure of tumour hypoxia. The report of the National Cancer Institute suggested that nitroimidazoles may be more applicable to measuring changes in the hypoxic fraction within the tumour throughout the course of treatment.

1.6.1 ^{14}C and ^3H -Labelled Nitroimidazoles

Nitroimidazoles which are ring-labelled with ^{14}C or side-chain-labelled with ^3H , were initially investigated as potential markers of hypoxic cells in tumours. Autoradiographed sections of spheroids exposed to ^{14}C -misonidazole contained a ring of radioactivity lying just outside the central necrotic region (Chapman *et al.*, 1981). This area of radioactivity deposition was thought to represent the layer of cells that have intact enzymes, and which have an oxygen concentration which is unable to efficiently oxidize the initial radical anion reduction product. Similar areas of misonidazole deposition have been found in animal and human tumours (Olive and Durand, 1989 and Urtasun *et al.*, 1986) and it was found that tumour cell survival was dramatically increased in acutely anemic mice, and this was correlated with an increase in ^{14}C -misonidazole binding to tumours (Hirst *et al.*, 1985). Although the use of ^{14}C - and ^3H -labelled nitroimidazoles has proven valuable in implicating hypoxia in the radioresistance of some solid tumours, interest has emerged in developing more clinically exploitable nitroimidazoles that are either non-invasive markers of tumour hypoxia because they are labelled with gamma-emitting isotopes or are not radioactively labelled but which are detected using immunohistochemistry techniques. The latter would allow greater specificity in detection of hypoxic regions in tumours.

1.6.2 Nitroimidazole Adducts Detectable by Antibodies

EF5 (Figure 1.2), which is an analogue of etanidazole with a pentafluorinated side chain, and the hexafluorinated misonidazole analogue CCI-103F (Figure 1.2), have been investigated as hypoxic cell markers. The binding of these compounds can be detected through a tumour biopsy and the extent of hypoxia quantified using antibodies which recognize bound adducts of

the drug that are formed under hypoxic conditions. A fluorescently tagged antibody is then used to detect labelled cells through immunohistochemistry on the tumour sections or flow cytometry on cell suspensions.

EF5 binding in tumour sections obtained from tumour-bearing animals showed high contrast regions within EMT6 mouse and Morris 7777 hepatoma rat tumours (Lord *et al.*, 1993) and EF5 binding to 9L gliomas was found to be specific and oxygen dependent (Evans *et al.*, 1995). The ability of EF5 binding to predict radiation resistance was studied in Fischer rats with 9L tumours, where a good correlation between relative radiation resistance or hypoxic survival and EF5 binding of moderately hypoxic cells in air breathing animals was found (Evans *et al.*, 1996).

CCI-103F was used to identify the prevalence and distribution of hypoxic tumour cells in spontaneous canine tumours (Cline *et al.*, 1994). Labelled cells were found in 31 of the 32 tumours examined and adducts were distributed randomly in the tumour. Binding of CCI-103F was not found near blood vessels, consistent with CCI-103F binding being in areas of low oxygen tension. In the same canine tumour model, an estimate of the change in tumour hypoxia in an individual tumour over time during radiation treatment by assaying tumour biopsy samples for CCI-103F antigen concentration was obtained (Thrall *et al.*, 1994). This suggests that CCI-103F may be a useful compound to assess the reoxygenation of a tumour over the course of radiation therapy.

Detecting bound adducts of nitroimidazoles with fluorescently tagged antibodies provides a direct and quantitative measure of the labelled area fraction in the tumour. Unfortunately, this is an invasive approach to monitor hypoxia and as such it is limited to readily accessible tumours.

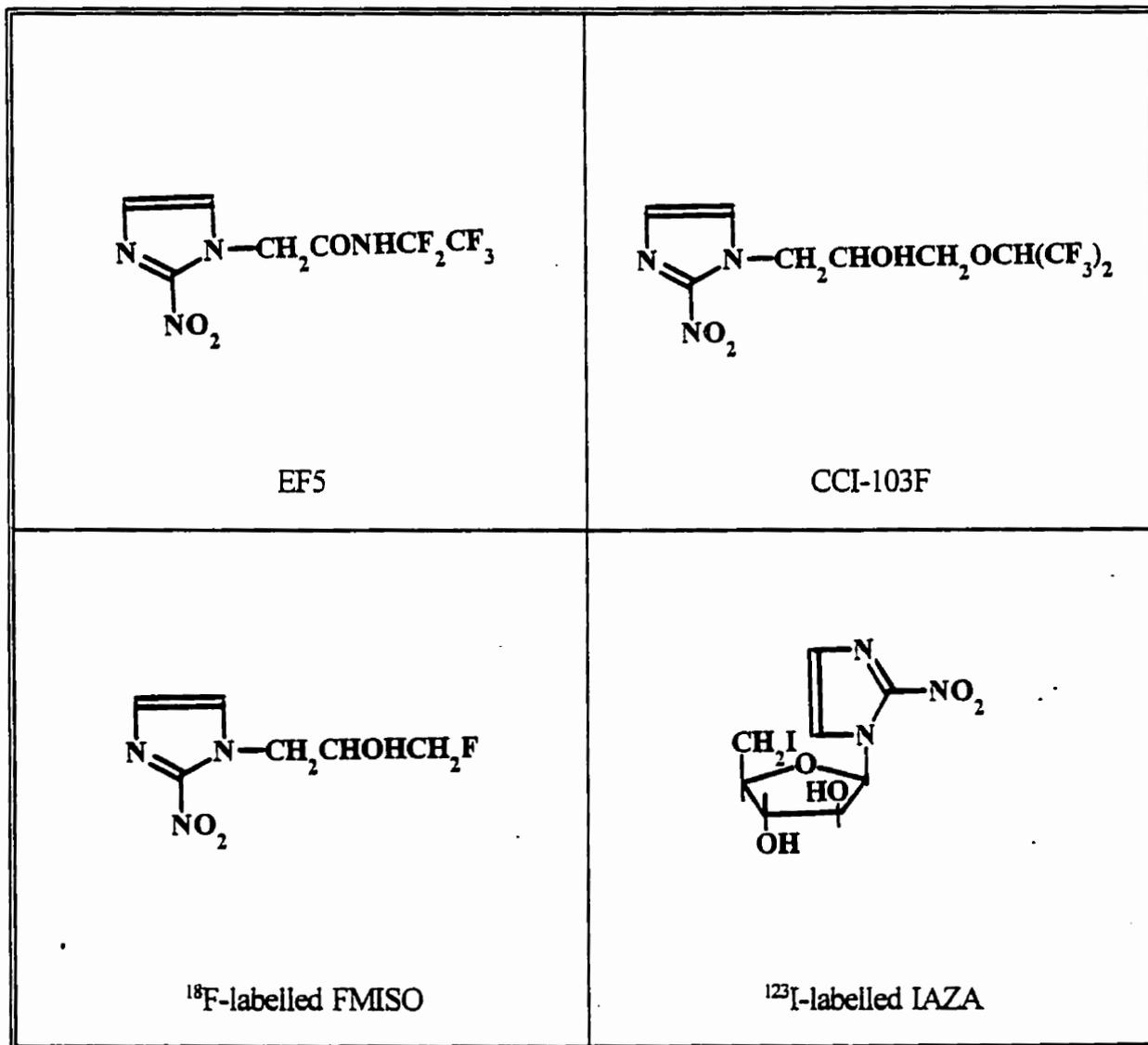


Figure 1.2: Structure of selected nitroimidazoles used as hypoxic cell markers.

As well, the usefulness of this technique will require that the biopsy be representative of the tumour population as a whole and in order for these compounds to assess reoxygenation of the tumour throughout the course of therapy, repeat doses of the nitroimidazole must be administered.

1.6.3 ¹⁸F-Labelled Nitroimidazoles

FMISO (Figure 1.2) is a misonidazole derivative that is labelled with the positron emitter ¹⁸F in place of the methoxy group at the end of the misonidazole side chain. The accumulation of this compound is measured non-invasively using positron emission tomography (PET), allowing images of several adjacent tumour planes to be obtained. For quantification, a fractional hypoxic volume (FHV) is defined as the number of image pixels within the tumour volume with a tumour to blood ratio of drug ≥ 1.4 at two or more hours after injection of the drug. In studies of 28 patients (Koh *et al.*, 1992, and Stone *et al.*, 1993), the FHV had a median value of 10-19%. Seven out of 8 tumours, in which images were obtained after therapy, showed a decrease in their FHV, and this decrease is believed to be due to reoxygenation of the tumour. Another clinical investigation obtained images before, during and after radiation treatment of non-small cell lung carcinomas (Koh *et al.*, 1995). FHV data on seven patients was obtained pretreatment and midtreatment, while six patients were investigated after therapy was completed. The initial FHV had a median value of 58%, the midtreatment FHV had a median value of 29%, while only one patient had essentially no detectable tumour hypoxia at the end of treatment. The results of this study suggest that midtreatment measurements with FMISO would allow an assessment of the reoxygenation of the tumour through treatment to be obtained and would help in the selection of patients where therapies directly targeted at hypoxic cells are to be included in the treatment.

Despite these clinical investigations, FMISO has several shortcomings. FMISO is a very

hydrophilic compound, and may accumulate at low concentrations within the lesion, thus providing limited contrast between normal and abnormal tissue. As well, the synthesis of the labelled compound requires specialized chemical expertise and equipment, and imaging is limited to PET centres. The spatial resolution that is obtained with PET imaging may no longer justify its use, since modern single photon emission computed tomography (SPECT) cameras also offer high spatial resolution, but at a much lower cost compared to PET imaging equipment.

1.6.4 ^{123}I -Labelled Nitroimidazoles

Iodinated sugars attached to nitroimidazoles have been investigated as non-invasive imaging agents to be used with SPECT. Of the ones synthesized, ^{123}I -iodoazomycin arabinoside (LAZA, Figure 1.2) has undergone extensive investigation (Mannan *et al.*, 1991). In clinical studies of head and neck squamous cell carcinoma metastases, tumours which accumulated LAZA before radiation showed no local control after three months (Raleigh *et al.*, 1996). Despite efficient transfer of LAZA across the blood/brain barrier and evidence of decreased perfusion, no uptake of LAZA was seen in patients with glioblastoma multiforma. This result is consistent with ^{18}F -misonidazole results (Valk *et al.*, 1992), but contradicts studies with oxygen microelectrodes (Rampling *et al.*, 1994). This suggests that the hypoxic fraction measured by nitroimidazole binding and polarographic electrodes may not be identical and this further illustrates the need for direct comparisons between the methods available to measure tumour hypoxia, to validate that the measurements obtained with the different techniques are consistent. The accumulation of LAZA in patients was also compared to the perfusion imaging agent $^{99\text{m}}\text{Tc}$ -HMPAO and it was found that in 8 of the 13 patients who showed decreased perfusion in their tumours, there was

an increase in the accumulation of LAZA, while only 5 of 14 tumours with increased or normal perfusion showed increased LAZA accumulation (Groshar *et al.*, 1993). The impact of these results is tentative, since there may not be a direct relationship between decreased blood flow and increased uptake of LAZA and any relationship that might exist may also be dependent on tumour type.

Iodinated nitroimidazoles offer the advantage of being readily imaged with widely available equipment that is applicable to most tumour sites. However, the expense of the isotope and the need for chemical expertise and specialized equipment to prepare the labelled drug limit the wide spread use of these iodinated nitroimidazoles as standard hypoxic cell markers.

1.6.5 Technetium-99m-Labelled Nitroimidazoles

In the early 1990s, interest began to emerge in synthesizing a nitroimidazole that could be labelled with technetium-99m (^{99m}Tc). The development of a ^{99m}Tc based hypoxia imaging agent, with the longer physical half-life of ^{99m}Tc (6 hours) compared to ^{18}F (112 minutes), is predicted to produce images of a higher quality than those obtained with FMISO. As well, the proposed labelling of compounds with ^{99m}Tc uses simple radiochemical techniques and the necessary SPECT imaging equipment is available in most nuclear medicine departments, resulting in decreased costs and wider applicability for routine assessment of tumour hypoxia. BMS181321, synthesized by Bristol Myers Squibb Pharmaceuticals, was the first reported nitroimidazole labelled with ^{99m}Tc that has undergone extensive investigation (Linder *et al.*, 1994).

1.7 BMS181321

BMS181321 was synthesized as a hypoxic marker to identify tissue at risk in myocardial and cerebral ischemia. BMS181321 is a 2-nitroimidazole that chelates ^{99m}Tc through a propylene amine oxime (PnAO) chelator that is connected to the imidazole ring through a methylene bridge. BMS181321 is formed by stannous chloride reduction of ^{99m}Tc -pertechnetate in the presence of the precursor compound, BMS181032 (Figure 1.3). These are chemically different compounds. The precursor has an octanol:water partition coefficient of 0.04, and the labelled compound has a partition coefficient of 40. The high lipophilicity of the labelled compound may cause BMS181321 to be retained in normal tissues, which may result in high background values during imaging.

Cyclic voltammetry has shown that there is a reversible reduction of BMS181321 at -1.48 V, which is only 0.01 V more positive than the value observed for misonidazole (Linder *et al.*, 1994). The value obtained for BMS181032 is -1.52 V, which indicates that complexing of the compound with ^{99m}Tc shifts the reduction potential slightly positive. It has also been shown that hypoxic incubations of BMS181321 with xanthine oxidase, a typical experimental model for one electron nitroreductases, causes a time-dependent loss of the nitro absorbance peak at 326 nm. This rate of reduction is approximately half of the value that has been reported for misonidazole, but similar to values obtained for FMISO.

The usefulness of BMS181321 as a hypoxic marker has been studied in a variety of model systems, and in limited clinical investigations. These results are summarized in the following sections.

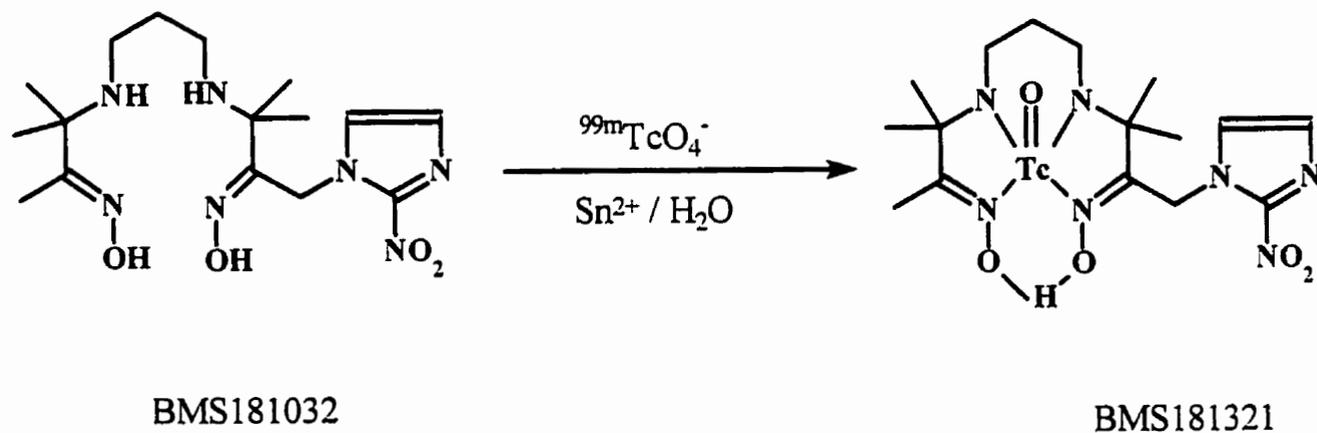


Figure 1.3: Formation of BMS181321. BMS181321 is formed by stannous chloride reduction of ^{99m}Tc -pertechnetate in the presence of the precursor compound BMS181032. (Adapted from Linder *et al.*, 1994).

1.7.1 Studies of BMS181321 in Heart

BMS181321 has undergone extensive investigation in myocardial model systems. The subcellular distribution of ^{99m}Tc following *in vitro* and *in vivo* experiments with BMS181321 showed that in myocardial cells, 51% of the activity was associated with membranes and 44% was in the cytosol; however, 42% of the activity in the membrane was intact BMS181321 compared to 5% in the cytosol, which contained hydrophilic and protein-bound metabolites (Jayatilak *et al.*, 1994). Metabolites are thought to include low molecular weight products that diffuse readily out of the cell, and higher molecular weight products that are retained in the cell because of binding to cellular macromolecules.

The effect of oxygen concentration on the kinetics and retention of BMS181321 in myocardial perfusion states has been addressed. In an isolated-rat-perfused-heart model, BMS181321 was found to be well trapped in ischemic myocardium after reperfusion when BMS181321 was injected before ischemia, whereas retention of BMS181321 was less pronounced in hypoxic myocardium (Kusuoka *et al.*, 1994). In the same model system, where a strong correlation between a decrease in pO_2 and increased retention of BMS181321 was reported (Rumsey *et al.*, 1995), the myocardial retention of BMS181321 was found to be coupled to the level of oxygen in hearts that were perfused retrogradely with buffer.

Contradictory reports on the target to background effectiveness of BMS181321 have been given. In an *in vivo* open-chest canine model of partial coronary occlusion, BMS181321 was found to preferentially accumulate in ischemic, but viable regions (Shi *et al.*, 1995). However, unfavourable target to background ratios were found in this model. The opposite conclusion was reached using a rat-perfused-heart model where favourable ischemic to normal tissue ratios of

12:1 were observed at maximum accumulation of the tracer in the heart. This value increased to 30:1 after BMS181321 was allowed to clear for 60 minutes (Okada *et al.*, 1996). However, this latter study did not report the target to liver ratios, which may not provide as optimistic values as those reported in the study, because BMS181321 has a high propensity to accumulate in the liver.

1.7.2 Studies of BMS181321 in Brain

In a middle-cerebral-artery-occlusion model in rats, BMS181321 was found to be selectively retained in acutely ischemic brain before disruption of the blood-brain barrier, but not in ischemic infarcts (DiRocco *et al.*, 1993). SPECT images obtained from rats after middle-cerebral-artery occlusion showed that the initial distribution of BMS181321 was consistent with regional cerebral blood flow, and selective retention of BMS181321 produced a positive image in the area of ischemia. The study suggested that BMS181321, which is the first ^{99m}Tc-labelled compound to indicate ischemic tissue at risk of infarction, may be able to contribute to the clinical management of acute stroke by allowing an early prediction of the severity of disease.

The use of BMS181321 to indicate occlusion of the cerebral artery has been studied clinically (Barron *et al.*, 1996). Patients who had a high probability of cerebral infarction were chosen. CT scans of the patients both before and after injection of 20-25 mCi of BMS181321 were obtained and SPECT images of the brain were obtained 15 min, 1 hour and 3 hours after BMS181321 injection. Two patients showed no abnormal uptake of BMS181321. A third patient had an abnormal focal region of increased radioactivity in the left striatal region. The CT scan performed 2 hours after onset was normal, but the scan performed at 36 hours showed a

region of hyperdensity involving the left frontal lobe. From this limited data, it appears that the biochemistry and imaging reported for animal models of cerebral ischemia using BMS181321 may be replicated in the clinic. However, further clinical investigations are needed to validate the usefulness of BMS181321 as an agent to image ischemic regions in brain.

1.7.3 Studies of BMS181321 in Tumours

BMS181321 was studied in KHT, SCC-VII and RIF-1 murine tumours in C3H mice using dynamic gamma-camera imaging (Ballinger *et al.*, 1996). It was found that after intravenous injection of BMS181321, the radioactivity rapidly distributed throughout the mouse and then cleared through the hepatobiliary system. The tracer cleared quickly from the blood and blood levels were found to stabilize at approximately 4% of the total injected dose. Tumour to blood ratios were less than one throughout the time course of the study, indicative of poor blood clearance of drug. Absolute uptake of BMS181321 in tumours was highest 10 minutes after injection but tumour to muscle ratios increased and then plateaued to values between 3.5 and 4, four to eight hours after injection of BMS181321. The absolute uptake of BMS181321 was found to be similar in the three tumour types investigated, suggesting that these three tumours might contain similar hypoxic fractions. This is consistent with results obtained in our Institute, where no difference in the hypoxic fraction of these three tumour types was found (Kavanagh *et al.*, 1996). Two drugs, hydralazine and nitro-L-arginine, which are known to increase the hypoxic fraction in tumours by decreasing blood flow (Wood *et al.*, 1993), caused tumour-selective increases in the retention of radioactivity when administered to mice in conjunction with

BMS181321 (Ballinger *et al.*, 1996). In contrast, hydralazine and nitro-L-arginine had little effect on the accumulation of radioactivity in normal tissues.

BMS181321 binding was studied *in vivo* using L2981 tumours in nude mice (Wen *et al.*, 1994). It was found that BMS181321 levels remained constant in the tumour at approximately 1% of the injected dose over four hours, whereas the contralateral hindflank had 0.2% of the injected dose accumulated over the same time period. The microvascular pO₂ levels in these tumours were found to be between 3-5 mm Hg. In contrast, the contralateral limb had an average pO₂ value of 14 mm Hg. This preferential binding of BMS181321 in the tumour compared to the contralateral limb is consistent with the preferential binding of BMS181321 in hypoxic tumours.

Compared to LAZA and FMISO, BMS181321 shows poorer pharmacokinetic properties for imaging in terms of tumour to blood ratios. LAZA and FMISO show higher localization in the tumour and lower blood levels than BMS181321, which may be partly attributed to the higher lipophilicity of BMS181321. However, the tumour to muscle ratios between these three compounds do not differ considerably (Ballinger *et al.*, 1996, Mannan *et al.*, 1991 and Gurnbaum *et al.*, 1987). It has yet to be assessed if this apparent pharmacokinetic deficiency of BMS181321 will be a hindrance to its applicability for imaging tumours clinically.

1.7.4 Evaluation of BMS181321 in Cell Culture

In an *in vitro* model system, Chinese hamster ovary (CHO) cells showed a selective accumulation of BMS181321 under hypoxic versus aerobic conditions using a spin-through-oil technique (Ballinger *et al.*, 1996). The K_m for this accumulation was 40 ppm oxygen tension,

which is 10 to 100 times lower than that reported for other 2-nitroimidazoles. Whether these differences in the K_m reported for 2-nitroimidazoles are relevant to *in vivo* accumulation or due to the experimental approach employed is not known. Washout experiments indicated that 30% of the radioactivity accumulated after a 2 hour hypoxic exposure with BMS181321 was retained at 24 hours. It was also found that the form of radioactivity present in the external media changed with time under hypoxic conditions. This is thought to be due to cellular metabolism of the drug and subsequent release of the drug from the cell. Only picomolar concentrations of BMS181321 were required for detection of uptake and accumulation in this system because of the high specific activity of the drug. This low concentration of labelled drug resulted in depletion of BMS181321 from the external medium at higher cell densities. This depletion may be a problem in the application of BMS181321 *in vivo* if the drug is fully consumed before it is able to diffuse to all the hypoxic cells present in the tumour. This thesis will address potential means of altering the biodistribution of ^{99m}Tc -nitroimidazoles using unlabelled nitroaromatics. The unlabelled nitroaromatic would compete for reduction of the ^{99m}Tc -nitroimidazole, allowing the labelled compound to diffuse throughout the tumour before metabolic depletion of the compound occurs. As well, the degree that metabolic depletion of these drugs is a problem limiting their usefulness will be addressed.

1.8 Thesis Outline

If nitroimidazoles are to act as absolute markers of tumour hypoxia, they must have complete access to the target population of hypoxic cells present in the tumour. It has been suggested that metabolic depletion of BMS181321 may cause an underestimate of the hypoxic

fraction. The purpose of the work reported in this thesis was to assess the feasibility of using unlabelled nitroaromatics to modulate the hypoxic accumulation of BMS181321.

Chapter 2 discusses the approach and experiments performed to address the feasibility of using unlabelled nitroaromatics in conjunction with BMS181321 in an *in vitro* cell culture model of tumour hypoxia. Not only did these experiments assess the feasibility of using this technique, but it provided some insights into the mechanisms involved in the retention and metabolism of BMS181321 in the cell.

Chapter 3 provides a summary and discussion of the experimental results. As well, possible future experimental directions are provided for further investigations into the use of BMS181321 and other ^{99m}Tc -nitroimidazoles as hypoxic cell markers.

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Chapter 2: Modifying the In Vitro Accumulation of BMS181321 with Unlabelled Nitroaromatics

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2.1 Abstract

BMS181321 is a technetium-99m-labelled nitroimidazole which has been shown to accumulate in hypoxic regions in heart and stroke disease models. Its potential use as a hypoxic marker in solid tumours is under investigation. Due to the high specific activity of technetium-99m, the concentration of BMS181321 used in its imaging applications is very low. Metabolic depletion of the drug may limit its ability to fully map out the hypoxic regions present in the tumour. An attempt has been made to modify the *in vitro* accumulation and metabolism of BMS181321 in hypoxic Chinese hamster ovary cells with unlabelled nitroaromatics, in order to assess the feasibility of using unlabelled nitroaromatics to alter the biodistribution of BMS181321 *in vivo*. The addition of the 2-nitroimidazole etanidazole (0.08-8 mM) caused a concentration dependent decrease in BMS181321 accumulation of from 70 to 28% and metabolism of from 70 to 40% of the control level in hypoxic cells at 4 hours. In contrast, the addition of the 5-nitroimidazole tinidazole (0.09-9 mM) caused a concentration dependent increase in BMS181321 accumulation of from 110 to 170% and metabolism of from 100 to 150% of the control level in hypoxic cells at 4 hours. In general, nitroaromatics with an electron affinity similar or greater than BMS181321 (etanidazole, misonidazole, NF167) inhibited its accumulation and metabolism whereas 5-nitroimidazoles (tinidazole, metronidazole), which have a lower electron affinity than BMS181321, enhanced its accumulation and metabolism. The enhanced accumulation with the addition of metronidazole was not observed in the presence of low oxygen levels (<100 ppm) or with the addition of NF167, a nitrofurantoin of higher electron affinity than BMS181321. RP8979, a 4-nitroimidazole of lower electron affinity than the 5-nitroimidazoles, had no effect on BMS181321 accumulation. These results suggest that a competition for reducing equivalents can

occur in the cells, leading to the inhibition of BMS181321 reduction in the presence of nitroaromatics of similar or greater electron affinity. It is hypothesized that a transfer of electrons from the radical anion form of the reduced 5-nitroimidazole to the more electron affinic BMS181321 compound occurs, resulting in an increased accumulation of BMS181321 under hypoxic conditions in the presence of these drugs.

2.2 Introduction

Hypoxic cells are thought to be a limitation for the local control of some types of solid tumours by radiation (Overgaard, 1994), but studies suggest that even for a given tumour type, not all tumours contain a significant fraction of hypoxic cells (Höckel *et al.*, 1993). Unfortunately, there is no widely accepted method of assessing the extent of hypoxia in an individual tumour, though a number of approaches are being evaluated (Stone *et al.*, 1993). The general availability of a marker for tumour hypoxia would be a beneficial diagnostic tool, since it would allow the selection of patients who could benefit from therapies designed to target treatment resistant cells.

In 1979, it was first suggested that radiolabelled nitroimidazoles, which are selectively metabolized and retained in hypoxic cells, might be used to measure tumour hypoxia (Chapman, 1979). Misonidazole labelled with ^3H has shown localized accumulation in patients (Urtasun *et al.*, 1986), and nitroimidazoles labelled with ^{18}F and ^{123}I have been investigated as possible non-invasive markers of hypoxia (Koh *et al.*, 1992 and Mannan *et al.*, 1991). However, these non-invasive compounds are not widely applicable because of the need for expensive isotopes, special chemical synthesis and/or costly imaging equipment. The synthesis of BMS181321, a

nitroimidazole labelled with technetium-99m (^{99m}Tc), offered the possibility of more routine and inexpensive imaging of hypoxia in damaged normal tissue or in tumours (Linder *et al.*, 1994).

BMS181321 has been studied as a hypoxic marker in myocardial and cerebral ischemia. In isolated rat hearts, BMS181321 was found to accumulate under ischemia (Kusuoka *et al.*, 1994), and there was selective retention of BMS181321 in acutely ischemic rat brain following artery occlusion (DiRocco *et al.*, 1993). The use of BMS181321 to detect hypoxia in tumours has recently been reported. It was found that BMS181321 is localized in rodent tumours *in vivo* and is preferentially accumulated in hypoxic cells *in vitro* (Ballinger *et al.*, 1996). Results from these *in vitro* studies showed that under hypoxia there was metabolic depletion of BMS181321, particularly at higher cell concentrations. Due to the high specific activity of ^{99m}Tc , BMS181321 is used at picomolar concentrations for imaging, which has led to a concern about the degree that this compound would be able to diffuse throughout the hypoxic regions present in the tumour without being metabolically consumed.

The need for nitroimidazoles to have complete access to the hypoxic cells present in the tumour has been pointed out by others (Koch *et al.*, 1993). If metabolic depletion of the nitroimidazole occurred in its clinical application, it could cause an underestimate of the hypoxic fraction present in the tumour. This concern about drug depletion in the application of nitroimidazoles as hypoxic cell markers has also been addressed in other tissue culture systems (Koch, 1991).

In the present work, the feasibility of using unlabelled nitroaromatics in conjunction with BMS181321 to modulate its rate of hypoxic metabolism was investigated in an *in vitro* model. These studies were designed to assess the feasibility of using these unlabelled nitroaromatics in

conjunction with BMS181321 to alter its biodistribution *in vivo*. In the course of these studies it was observed that the presence of added nitroaromatics could be stimulatory as well as inhibitory to BMS181321 accumulation and metabolism depending on the electron affinity of the unlabelled nitroaromatic that was present.

2.3 Methods

2.3.1 Chemicals and Reagents

Synthesis of the ligand BMS181032 (4,8-diaza-3,3,9,9-tetramethyl-1-[2-nitro-1*H*-imidazol-1-yl]undecane-2,10-dione dioxime; Bracco Research, USA) and the reaction of the ligand with ^{99m}Tc -pertechnetate to form BMS181321 (^{99m}Tc]oxo[[3,3,9,9-tetramethyl-1-(2-nitro-1*H*-imidazol-1-yl)-4,8-diazaundecane-2,10-dione dioximato]-(3-)-*N,N',N'',N'''*]technetium) has been reported previously (Linder *et al.*, 1994). In brief, 0.4 mg of the ligand was dissolved in 0.8 ml of saline and 0.1 ml of ^{99m}Tc -pertechnetate (250 MBq) was added. A 0.1 ml aliquot of stannous DTPA (Techneplex, Squibb), which was reconstituted with 4 ml of saline, was added to the ligand and pertechnetate mixture. The formation of BMS181321 was complete within 10 minutes at room temperature. The radiochemical purity of BMS181321 was determined by paper chromatography and was greater than 90%. The decomposition half-time of BMS181321 has been reported to be only 16-24 hours (Ballinger *et al.*, 1996). Dibutyl phthalate, α -medium and fetal calf serum were from Sigma Chemical Co. (St. Louis, MO). Vegetable oil was from Best Foods Inc. (Etobicoke, Canada). Trypan blue was from Gibco BRL (Gaithersburg, MD). Etanidazole (N-[2-hydroxyethyl]-2-nitro-1*H*-imidazole-1-acetamide) and misonidazole (1-[2-hydroxy-3-methoxypropyl nitroimidazole]) were gifts from the National Cancer Institute (USA). NF167 (5-

nitro-2-furaldehyde-5-3-diethyl-aminopyrole semioxamazone hydrochloride) was from Norwich Pharmacal Co. (Norwich, NY). RP8979 ([hydroxy-2-ethyl]-1-methyl-2-nitro-4-imidazole) was from May and Baker Ltd. (London, England). Tinidazole (ethyl-2-[2'-methyl-5'-nitro-1'-imidazolyl]ethyl sulfonate) was from Ortho Pharmaceutical (Don Mills, Canada) and metronidazole (2-methyl-5-nitroimidazole-1-ethanol) was from Poulenc Ltd. (Montreal, Canada).

2.3.2 Cells

Chinese hamster ovary cells (sub-line AA8-4), obtained originally from Dr. L.H. Thompson of Lawrence Livermore Laboratories, CA, were grown in suspension culture at 37°C in α -medium containing 10% fetal calf serum (growth medium). Their doubling time was 12-14 hours when maintained in exponential growth.

2.3.3 Accumulation and Metabolism Studies

Exponentially growing cells were removed from suspension culture ($2-4 \times 10^5$ cell/ml), centrifuged and resuspended in fresh growth medium at a cell concentration of 1×10^6 cell/ml. Glass vials containing 10 ml of the stirred cell suspension were placed in a water bath at 37°C as described previously (Whillans and Rauth, 1980). For some incubations unlabelled etanidazole, misonidazole, NF167, tinidazole, metronidazole or RP8979, whose chemical structures are shown in Figure 2.1, were added resulting in 1, 10 or 100 fold dilutions of maximum 5 - 9 mM concentrations. The cells were equilibrated for 30 minutes with a continual flow of a pre-humidified gas mixture of 95% air plus 5% CO₂ (aerobic exposure) or 95% N₂ plus 5% CO₂ (<10 ppm O₂, hypoxic exposure). The radioactive compound was then added to each

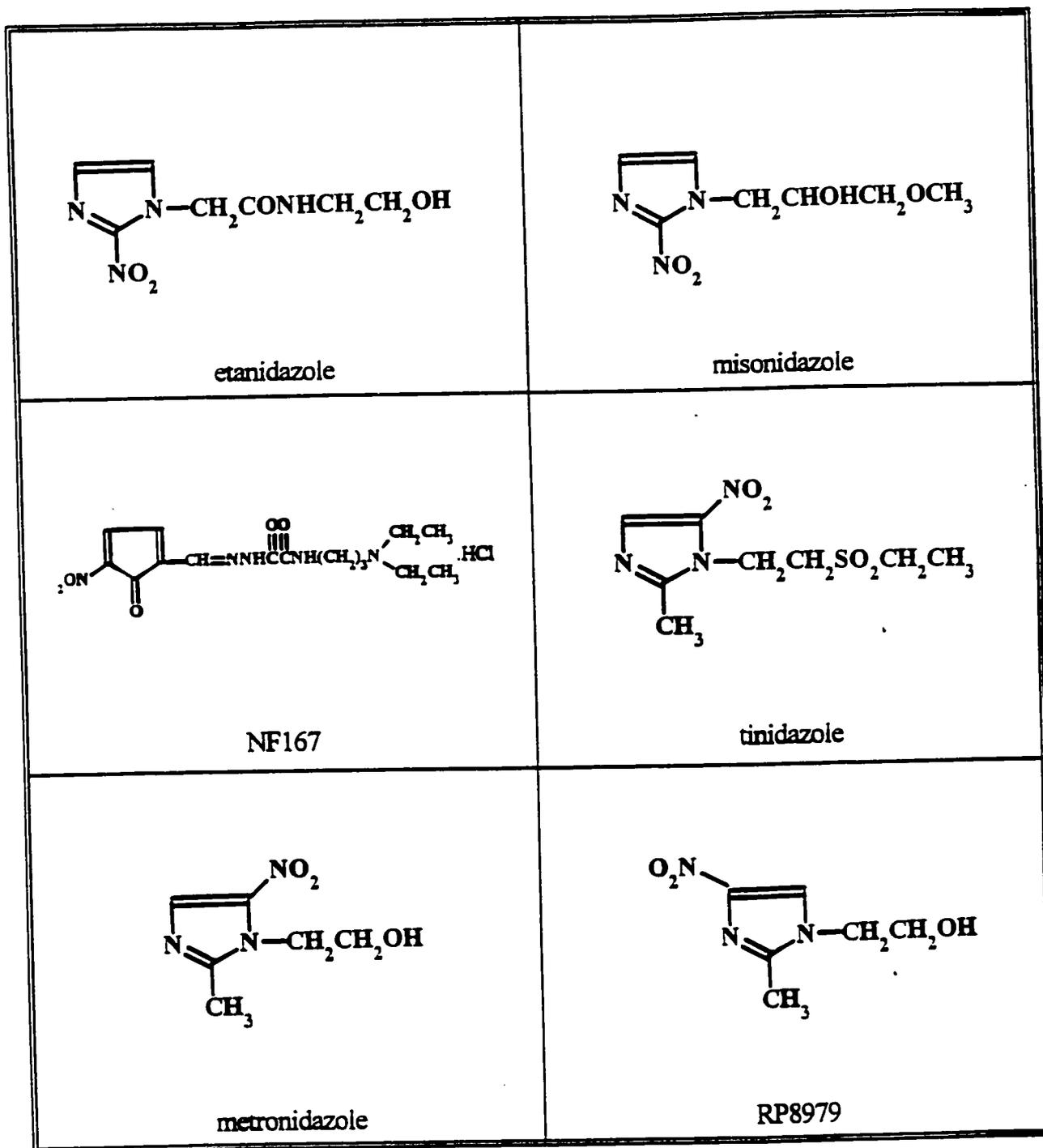


Figure 2.1: Structure of unlabelled nitroaromatics used in competition studies.

vial at a final activity of approximately 0.25 MBq/ml and at a total drug concentration (BMS181321 + BMS181032) of 0.7 $\mu\text{g/ml}$ ($\sim 0.2 \mu\text{M}$). The concentration of labelled drug (BMS181321) was approximately 55 pM.

Duplicate 0.1 ml aliquots were removed from the vials after 5, 60, 120, 180, and 240 minutes of incubation with BMS181321. The aliquots were layered over 1 ml of oil (dibutyl phthalate:vegetable oil, 4:1) at 4°C and centrifuged at 10000 g for 2 minutes in a microcentrifuge tube. The aqueous growth medium and the oil were aspirated and the tube tip containing the cell pellet and residual oil was clipped and counted in an automatic well counter (Picker-Pace 1, Picker Corp., Northford, CN). The counts were corrected for carry-through of radioactivity from the growth medium. This carry-through of radioactivity was found to be approximately 0.7% of the counts in 0.1 ml of radioactive medium (Ballinger *et al.*, 1996). The ratio of radioactivity in 0.1 ml of packed cells (C_{in}), which contains approximately 5×10^7 cells (Taylor and Rauth, 1978), to the amount of radioactivity in an equal volume of growth medium (C_{out}) was calculated and the data is presented in the form of the ratio $C_{\text{in}}/C_{\text{out}}$.

The amount and status of the radioactivity in the medium at each of the time points was investigated. A 0.3 ml sample was removed from the vial and centrifuged at 10000 g for 2 minutes. A 0.1 ml aliquot of the supernatant was removed and its activity determined. A second 0.1 ml aliquot of the supernatant was vortexed in a mixture containing 2 ml ethyl acetate and 2 ml PBS at room temperature and centrifuged at 200 g for 0.5 minutes to separate the phases. The phases were transferred to individual tubes and counted in the well counter and the percent of total counts extractable into the organic phase was calculated.

2.3.4 Cell Viability

Cell viability was determined by a colony formation assay. At the conclusion of the experiment, cells were plated in growth medium in 60 mm tissue culture dishes (Nunc, Roskilde, Denmark) and incubated for 8 days at 37°C in a humidified incubator. The plates were then stained and colonies of 50 or more cells were counted. As well, a 0.3 ml sample of the cell suspension was removed from the vial at the conclusion of the experiment and 0.02 ml of trypan blue was added, so that membrane integrity could be assessed.

2.3.5 Statistics and Calculations

The data is presented as the mean \pm standard error of the mean for three or more independent experiments. Lines were produced by the method of least squares. Some accumulation and metabolism data for the unlabelled drug competition with BMS181321 is presented as a percentage of the control at four hours. The percent of control was calculated for each individual experiment and is equal to

$$100\% \times [(\text{hypoxic}_B - \text{aerobic}_B) / (\text{hypoxic}_A - \text{aerobic}_A)]$$

where A is the 4 hour time point on the regression line for samples with only BMS181321 added (control samples) and B is the 4 hour time point on the regression line for samples with both BMS181321 and unlabelled nitroaromatic added. Student's t-test was used to determine the significance of difference between the means with a value of $p \leq 0.05$ considered significant.

2.4 Results

The effect of hypoxic or aerobic conditions on the accumulation of BMS181321 in CHO cells as a function of time from 0 - 4 hours is illustrated in Figure 2.2A. Aerobic cells showed extensive accumulation of activity at 5 minutes and thereafter there was little further increase in accumulated activity up to 4 hours with a C_{in}/C_{out} value of approximately 20. This value indicated a twenty fold increase in the total intracellular radioactivity over that in the same volume of medium. Under hypoxic conditions, there was a similar initial accumulation at 5 minutes, followed by a constant increase in accumulated activity, so that at 4 hours the amount of radioactivity within the cell was approximately 5 times greater than under aerobic conditions.

The extractability of the radioactive material remaining in the supernatant of cellular aliquots into ethyl acetate under the same conditions was also investigated (Figure 2.2B). For aerobic cells, 90% of the activity was extractable into ethyl acetate and this value is consistent with the amount of radiochemical impurity that is present in the drug preparation as measured by a paper chromatography technique. The amount of radioactivity that was extractable into ethyl acetate under aerobic conditions did not change appreciably over the time course of the experiment. In contrast, the percentage of activity extractable into ethyl acetate decreased over the time course of the experiment for hypoxic cells, and at 4 hours only 75% of the activity was extractable into ethyl acetate. Control experiments in which hypoxic incubations of the drug were carried out without cells showed results similar to that obtained for aerobic cells (Ballinger *et al.*, 1996). This decrease in the amount of activity extractable into ethyl acetate may be due

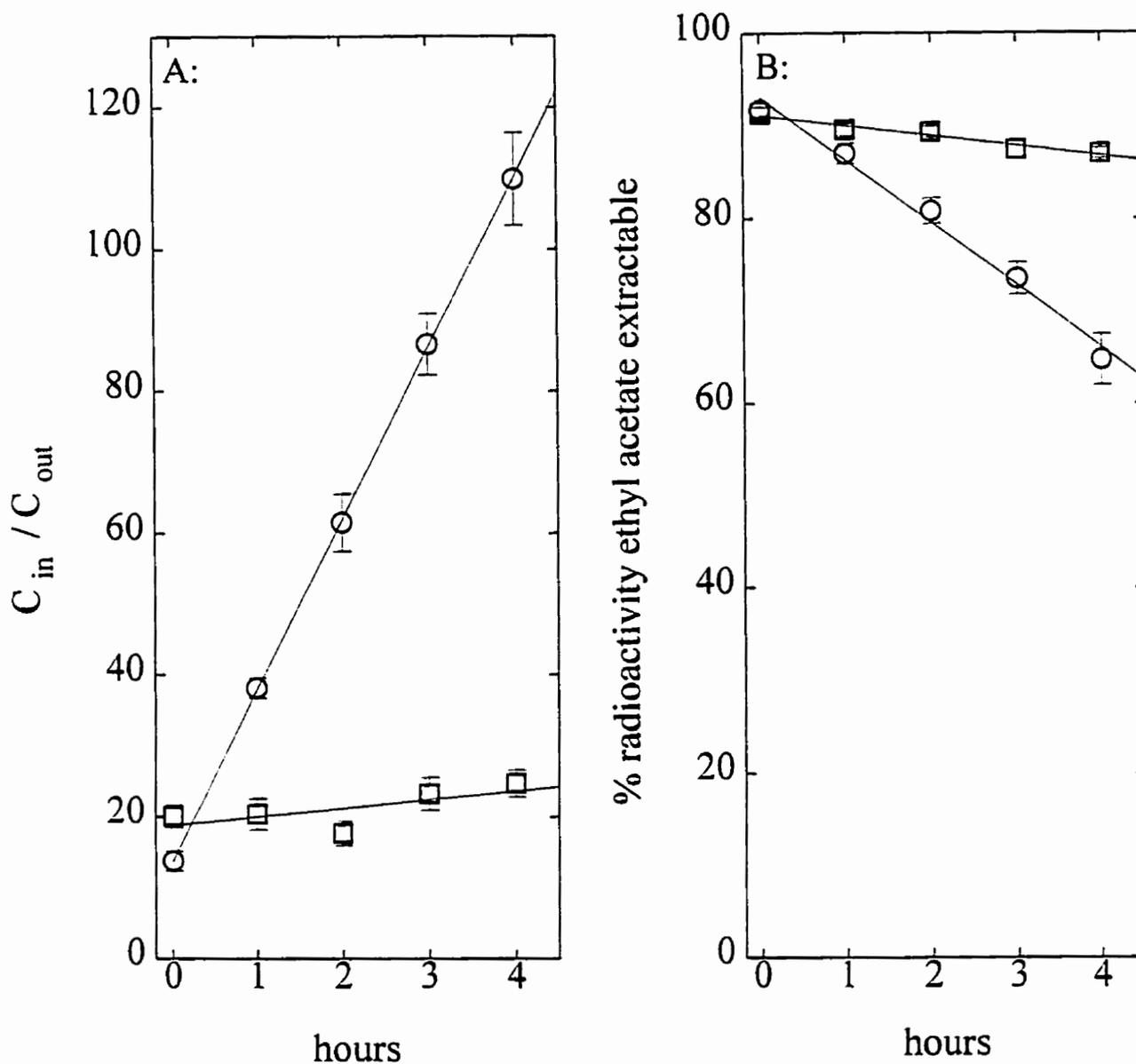


Figure 2.2: Selective accumulation of BMS181321 in hypoxic versus aerobic cells. CHO cells at a concentration of 1×10^6 cell/ml were incubated under aerobic (95% air, 5% CO_2 , \square) or hypoxic (95% N_2 , 5% CO_2 , <10 ppm O_2 , \circ) conditions in the presence of BMS181321. Points are the mean \pm standard error of the mean of three or more independent experiments. A: The accumulation of BMS181321. Data is calculated as the ratio of radioactivity in 0.1 ml of packed cells (C_{in}) to the radioactivity in 0.1 ml of external media (C_{out}) and plotted versus time after the addition of BMS181321. (B) The percentage of total counts in the external media that partitions into the ethyl acetate phase versus PBS for aerobic and hypoxic incubations as a function of time.

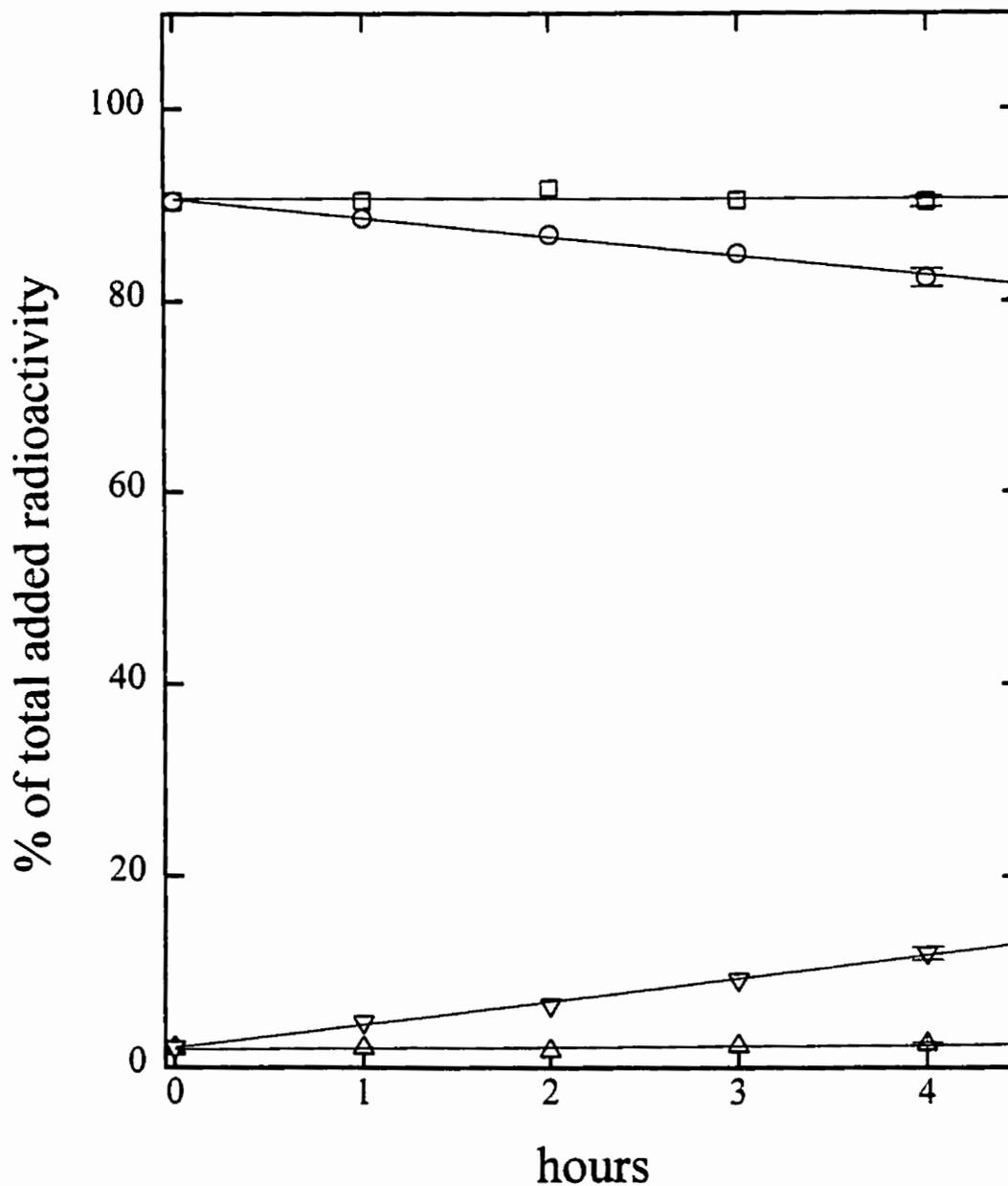


Figure 2.3: The percent of the total added radioactivity in the external medium and in the cells after incubation with BMS181321. The percent of total added radioactivity that is in the external media of aerobic (□) and hypoxic (○) incubations and the percent of total added radioactivity that is accumulated in aerobic (Δ) and hypoxic (▽) cells is plotted versus time. Points are the mean \pm standard error of the mean of nine individual experiments.

to hypoxia dependent cellular metabolism of the drug followed by the release of more hydrophilic metabolites back into the external medium.

The amount of radioactivity remaining in the supernatant of aerobic cells did not change appreciably over 4 hours (Figure 2.3). However, hypoxic incubations showed a constant decrease in the amount of radioactivity present in the supernatant over the 4 hour experiment. The activity depleted from the supernatant of hypoxic incubations at four hours is approximately equal to the activity accumulated in the hypoxic cells. This indicates a sequestration of radioactivity in hypoxic cells is occurring, and illustrates the potential problem of metabolic depletion of BMS181321 under hypoxia.

The 2-nitroimidazole etanidazole was selected for initial investigation of the effect of exogenous unlabelled drug on the accumulation of BMS181321 under aerobic and hypoxic conditions. The influence of three concentrations (0.08, 0.8 and 8 mM) of etanidazole on the accumulation of BMS181321 is illustrated in Figure 2.4A. Etanidazole did not affect BMS181321 accumulation in aerobic cells at the concentrations of etanidazole tested (data not shown). The addition of 0.08 mM of etanidazole diminished BMS181321 accumulation in hypoxic cells to about 70% of the control level. The addition of higher concentrations of etanidazole decreased the amount of BMS181321 accumulation under hypoxic conditions and this effect was dose dependent; 0.8 mM of etanidazole decreased the accumulation of BMS181321 to 40% of the control level and 8 mM of etanidazole diminished the accumulation to 25% of the control level.

For hypoxic cells that were also incubated with unlabelled etanidazole, the total amount of radioactivity that was ethyl acetate extractable increased proportional to the etanidazole

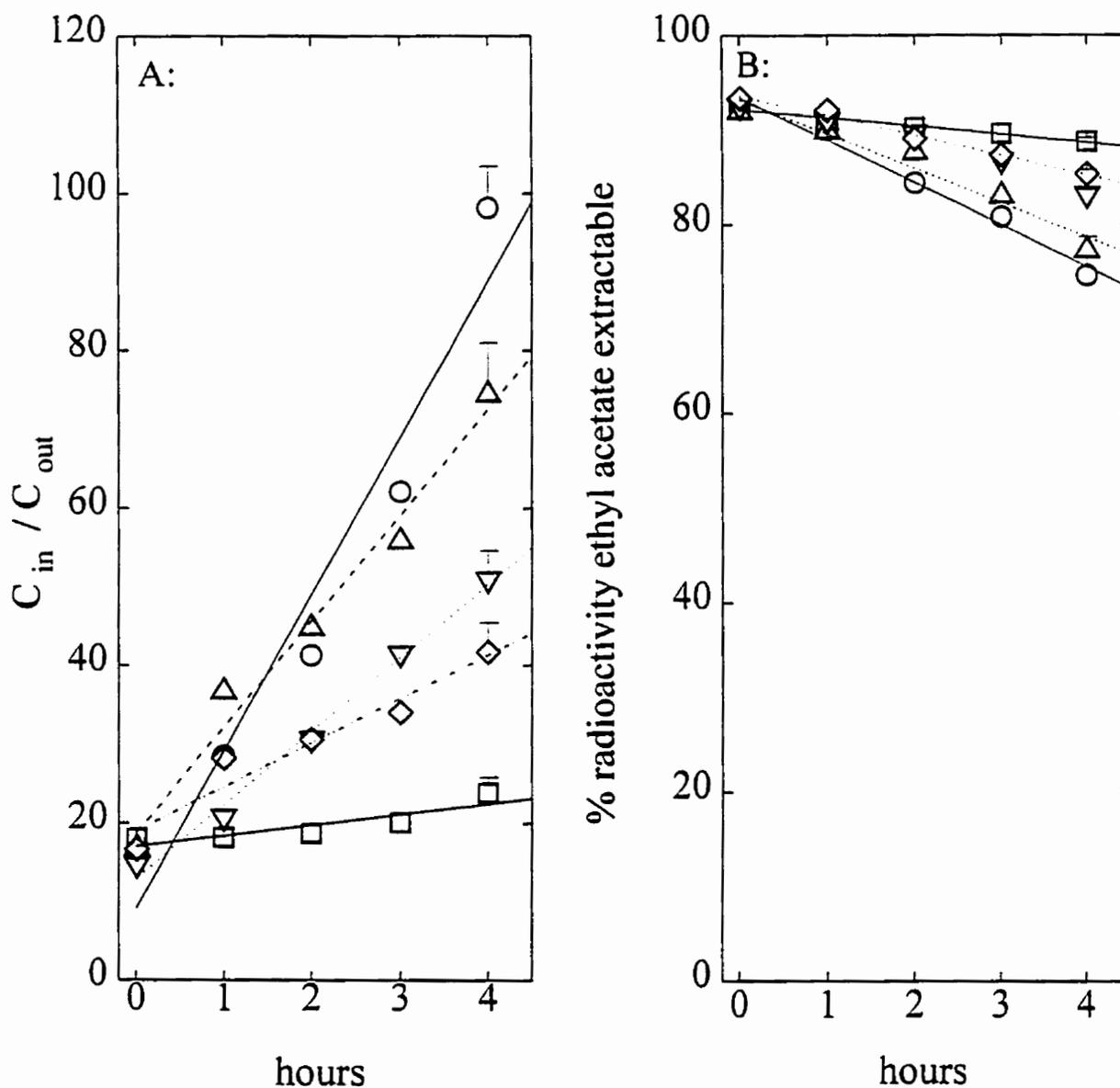


Figure 2.4: Accumulation and metabolism of BMS181321 in the presence of unlabelled etanidazole. CHO cells were incubated with etanidazole for 30 minutes while the system was equilibrating under aerobic and hypoxic conditions. Points are the mean \pm standard error of the mean of three or more independent experiments and lines were produced by the method of least squares. A: The accumulation of BMS181321, as expressed by the ratio C_{in}/C_{out} in the presence of unlabelled etanidazole, plotted versus time after the addition of BMS181321. Points are aerobic control (\square , -), hypoxic control (O, -), hypoxic incubation with 0.08 mM etanidazole (Δ , -), hypoxic incubation with 0.8 mM etanidazole (∇ , ...) and hypoxic incubation with 8 mM etanidazole (\diamond , -.). B: The percentage of total counts in the external medium that partitions into ethyl acetate versus PBS. Coding of the symbols is the same as in A.

Compound	Electron Affinity	Reference
NF167	-0.28 V [†]	Wardman, 1989 and Wardman, 1991
etanidazole	-0.41 V	Wardman, 1991
misonidazole	-0.41 V	Wardman, 1989 and Wardman, 1991
tinidazole	-0.49 V	Wardman, 1989 and Wardman, 1991
metronidazole	-0.51 V	Wardman, 1991
RP8979	-0.57 V [‡]	Wardman, 1989 and Wardman, 1991

Table 2.1: Electron affinity of nitroaromatics used in competition studies. The values listed in this table take account of revisions to reference potentials (Wardman, 1991) and data for closely related compounds (Wardman, 1989).

† Assumed to be the same as mean electron affinity of nifuroxime, nitrofurazone, and nitrofurantoin.

‡ Assumed to be the same electron affinity as (2-hydroxy-3-methoxypropyl)-1-nitro-4-imidazole.

concentration when compared to hypoxic cells with no unlabelled etanidazole added (Figure 2.4B). These results indicate that etanidazole is able to decrease the net metabolism of BMS181321 in hypoxic cells.

The effect of other unlabelled nitroaromatics on BMS181321 accumulation and metabolism was investigated next. The unlabelled competitors used in these studies varied in their electron affinity (Table 2.1) to allow a determination of the generality and possible dependence on electron affinity for the modification of the accumulation and metabolism of BMS181321 in hypoxic cells. Full curves like those in Figure 2.4 were determined for each drug. The results for etanidazole, misonidazole and NF167 are summarized and expressed as a percent of the control results at 4 hours and are illustrated in Figure 2.5. Misonidazole also inhibits the hypoxic accumulation and metabolism of BMS181321 and this inhibition is concentration dependent, with the greatest effect observed at a misonidazole concentration of 8 mM, where the accumulation of radioactivity was inhibited to approximately 20% and metabolism 30% of the control level. Comparing etanidazole and misonidazole, there is no statistical difference in their ability to inhibit the accumulation and metabolism of BMS181321 under hypoxic conditions at equal concentrations (Student's t-test, $p > 0.05$). NF167 (0.09 mM) inhibits the accumulation of BMS181321 to 40% of the control level. Due to the high molar toxicity of NF167 compared to the nitroimidazoles (Thomson and Rauth, 1974), its effects at higher concentrations were not investigated. The results for the one concentration of NF167 tested were not significantly different from misonidazole and etanidazole at approximately equal concentrations in the accumulation studies ($p > 0.05$). These effects suggest the results for etanidazole could be generalized to nitroaromatics of equal or greater electron affinity than

BMS181321.

Compounds of lower electron affinity than the 2-nitroimidazoles were also investigated as possible modulators of BMS181321 accumulation and metabolism. Unexpectedly, the 5-nitroimidazole tinidazole caused a concentration dependent stimulation of BMS181321 accumulation in hypoxic cells (Figure 2.6). The greatest stimulation was observed in the presence of 9 mM of tinidazole, where the C_{in}/C_{out} value was 170 at 4 hours, which is 180% of the control level. Similarly, 9 mM of tinidazole appeared to increase the metabolism of BMS181321 in hypoxic cells to 150% of the control level at 4 hours as only 50% of the total counts at 4 hours were extractable into ethyl acetate (Figure 2.6B).

This stimulation in BMS181321 accumulation and metabolism was not unique to tinidazole. The 5-nitroimidazole metronidazole also caused a dose dependent increase in BMS181321 accumulation and metabolism. The greatest stimulation was observed at the highest concentration of metronidazole studied (5 mM), where the C_{in}/C_{out} value was 230% of the control level at 4 hours (Figure 2.7A) and the percent of total activity extracted into ethyl acetate was 170% of the control level (Figure 2.7B). There was no statistical difference between the stimulation observed with tinidazole and metronidazole (Student's t-test, $p > 0.05$). The 4-nitroimidazole RP8979, with an electron affinity even lower than the 5-nitroimidazole class of compounds, did not appear to have a large effect on BMS181321 metabolism and accumulation in hypoxic cells up to an RP8979 concentration of 8 mM (Figure 2.7).

Previous studies have shown that BMS181321 accumulation is inhibited by low levels of oxygen, where a 50% inhibition of BMS181321 accumulation was seen at an oxygen concentration of approximately 40 ppm (Ballinger *et al.*, 1996). The effect of low oxygen

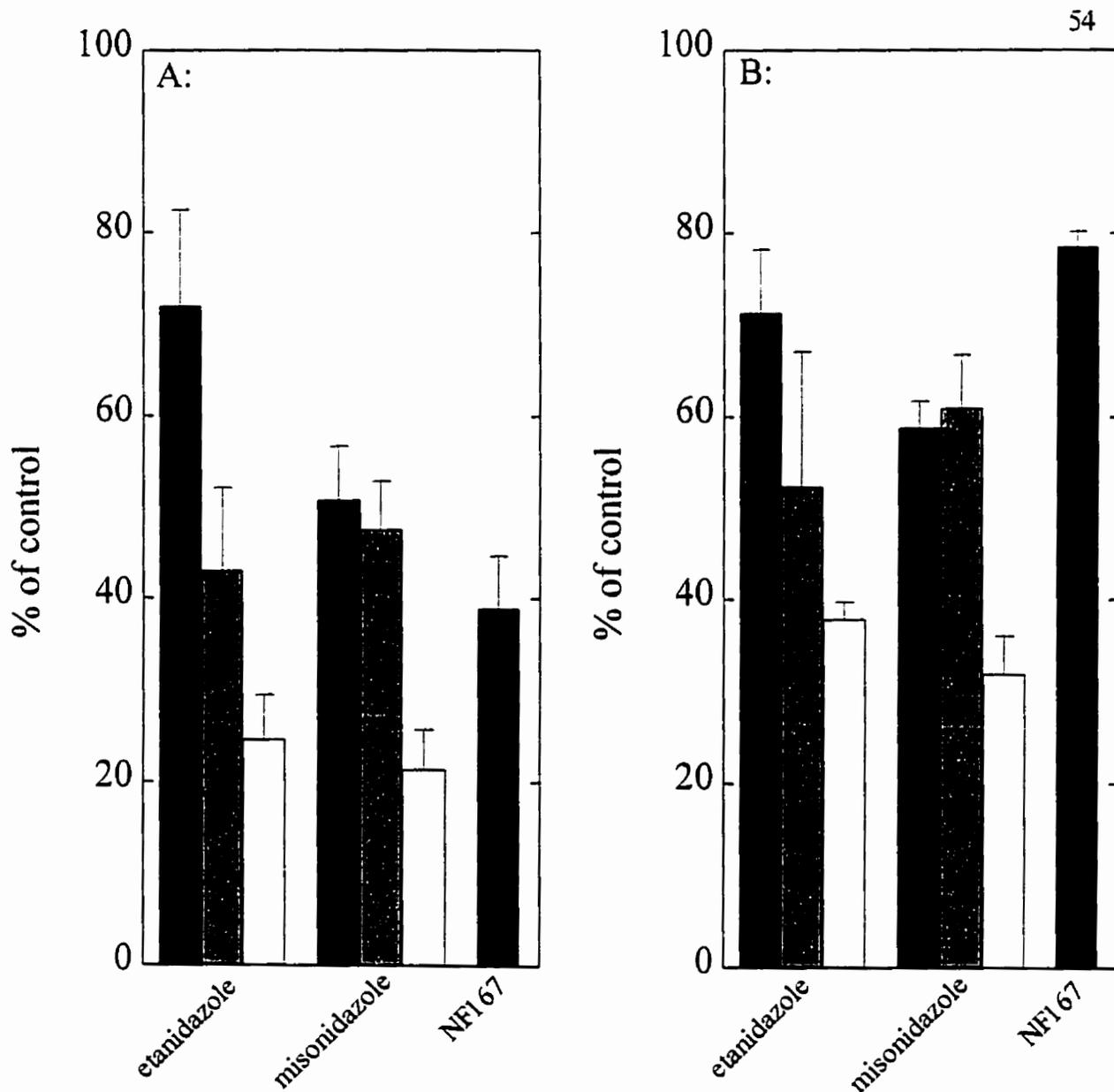


Figure 2.5: The accumulation and metabolism of BMS181321 in hypoxic CHO cells incubated for four hours in the presence of different concentrations of unlabelled etanidazole, misonidazole, and NF167. Points are the mean \pm standard error of the mean of three independent experiments. A: Accumulation of BMS181321 expressed as a percent of the control cells with no added unlabelled drug. Different concentrations of the indicated unlabelled drug used were black bars 0.08, 0.08 and 0.09 mM for etanidazole, misonidazole and NF167 respectively, grey bars represent 0.8 mM of etanidazole and misonidazole, and white bars represent 8 mM of etanidazole and misonidazole. B: Metabolism expressed as a percent of the radioactivity that was ethyl acetate extractable in the supernatant of cells incubated with no added drug. Coding of the bars is the same as in A.

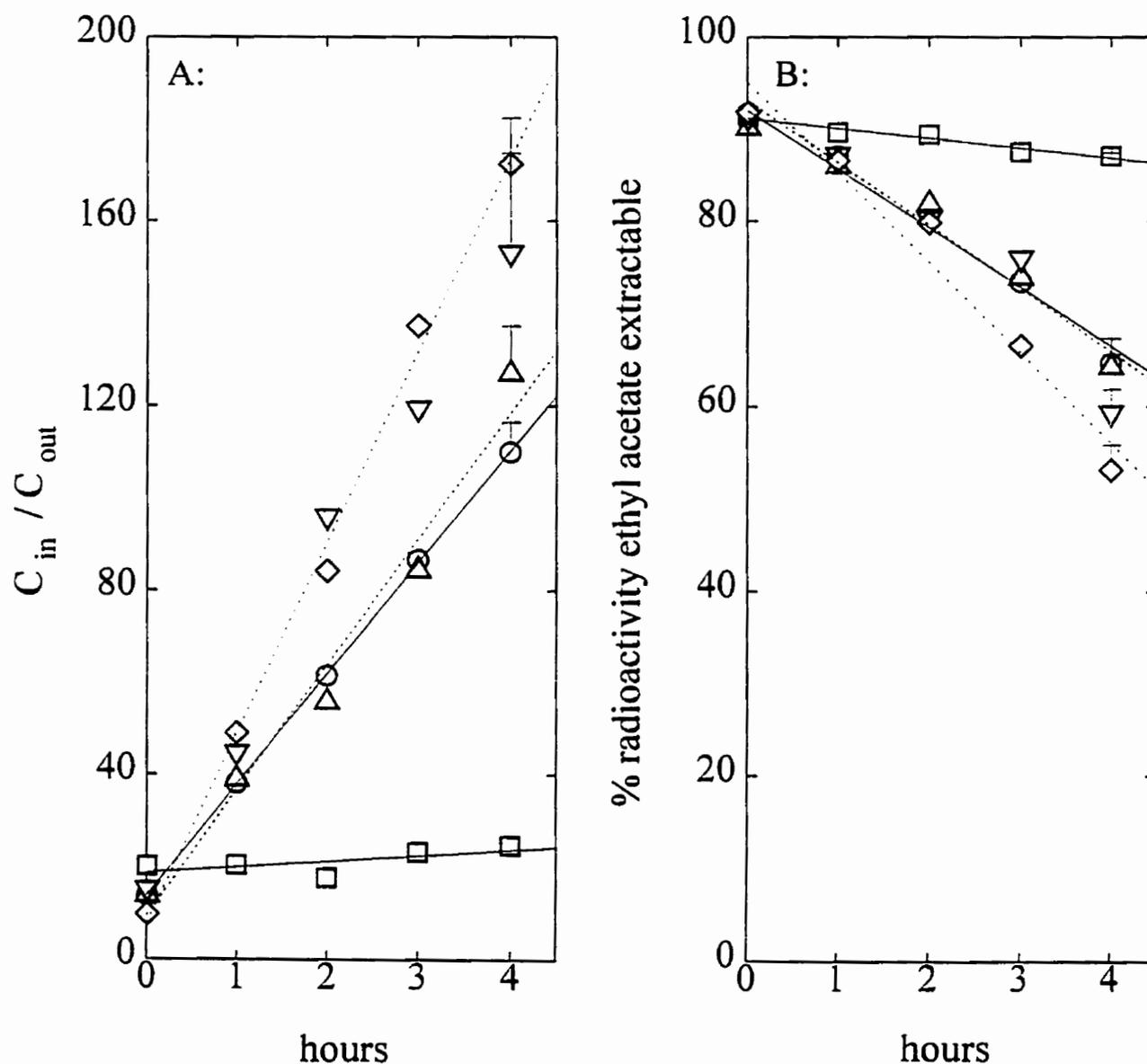


Figure 2.6: Accumulation and metabolism of BMS181321 in the presence of unlabelled tinidazole. CHO cells were incubated with unlabelled tinidazole for 30 minutes while the system was equilibrating under aerobic and hypoxic conditions. Points are the mean \pm standard error of the mean of at least three separate experiments and lines were produced by the method of least squares. A: The accumulation of BMS181321 as expressed by the ratio C_{in}/C_{out} in the presence of unlabelled tinidazole plotted versus time after the addition of BMS181321. Points are aerobic control (\square , -), hypoxic control (\circ , -), hypoxic incubation with 0.08 mM tinidazole (\triangle , -), hypoxic incubation with 0.8 mM tinidazole (∇ , ...), and hypoxic incubation with 8 mM tinidazole (\diamond , ...). B: The percentage of total counts in the external medium that partitions into ethyl acetate versus PBS. Coding of the symbols is the same as in A.

concentrations on the stimulation of BMS181321 accumulation and metabolism in the presence of unlabelled metronidazole was therefore investigated. A gas mixture that contained 0.35% oxygen, which is equivalent to a dissolved oxygen concentration of less than 100 ppm (Marshall *et al.*, 1986), decreased the accumulation of BMS181321 in hypoxic cells to a C_{in} / C_{out} value of 40 at 4 hours (Figure 2.8). The addition of unlabelled metronidazole (5 mM) had no effect on the accumulation of BMS181321 at this low oxygen concentration.

The effect of the simultaneous addition of both metronidazole (5 mM) and NF167 (0.09 mM) on the accumulation and metabolism of BMS181321 was also investigated. There was no enhancement of BMS181321 accumulation and metabolism under hypoxic conditions despite the addition of the same concentration of metronidazole that caused stimulation when added alone. In fact, the co-incubation of these two nitroaromatics caused an inhibition of BMS181321 accumulation and metabolism that was no different than that observed when NF167 was added alone (Student's t-test, $p > 0.05$) (Figure 2.9).

2.5 Discussion

BMS181321, a ^{99m}Tc -labelled 2-nitroimidazole, has been studied as a potential marker for tumour hypoxia. BMS181321 was found to preferentially accumulate in hypoxic cells, where at 4 hours there is approximately a five fold increase in the amount of radioactivity in hypoxic cells compared to aerobic cells. This is consistent with results that have been reported recently for CHO cells in the same system, though the reported accumulation was higher in that study (Ballinger *et al.*, 1996). Hypoxic cells showed a constant rate of accumulation in radioactivity over the time course of the experiment. Such accumulation does not occur when the experiment

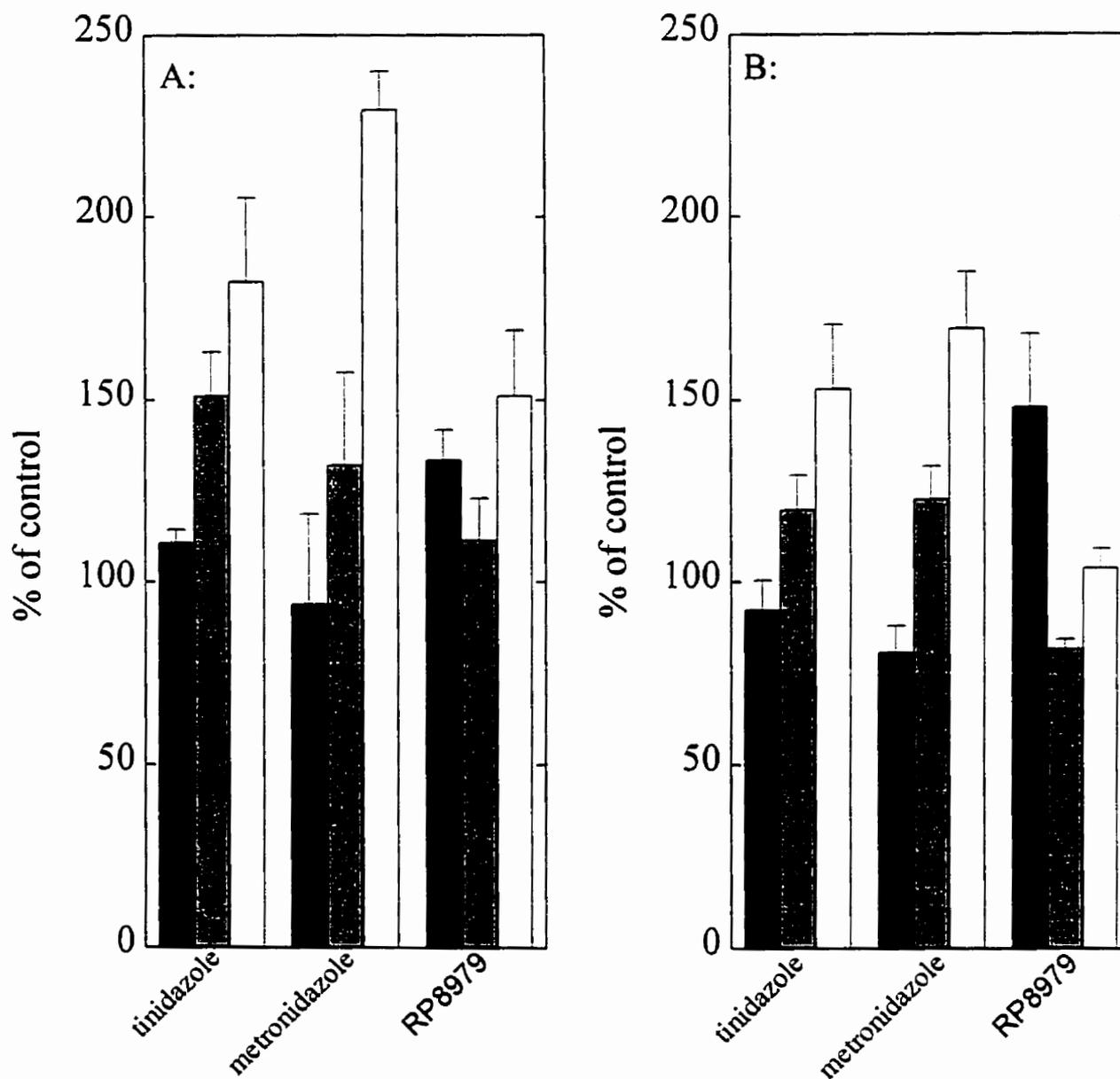


Figure 2.7: The accumulation and metabolism of BMS181321 in hypoxic CHO cells incubated for four hours in the presence of different concentrations of tinidazole, metronidazole and RP8979. Points are the mean \pm standard error of the mean of three independent experiments. A: Accumulation of BMS181321 expressed as a percent of the control cells with no added labelled drug. Different concentrations of the indicated unlabelled drug used were black bars 0.09, 0.05 and 0.08 mM for tinidazole, metronidazole and RP8979 respectively, grey bars represent 0.9, 0.5 and 0.8 mM respectively, and white bars represent 9, 5, 8 mM respectively. B: Metabolism expressed as a percent of the BMS181321 that is ethyl acetate extractable in the supernatant of cells incubated with no added drug. Coding of the bars is the same as in A.

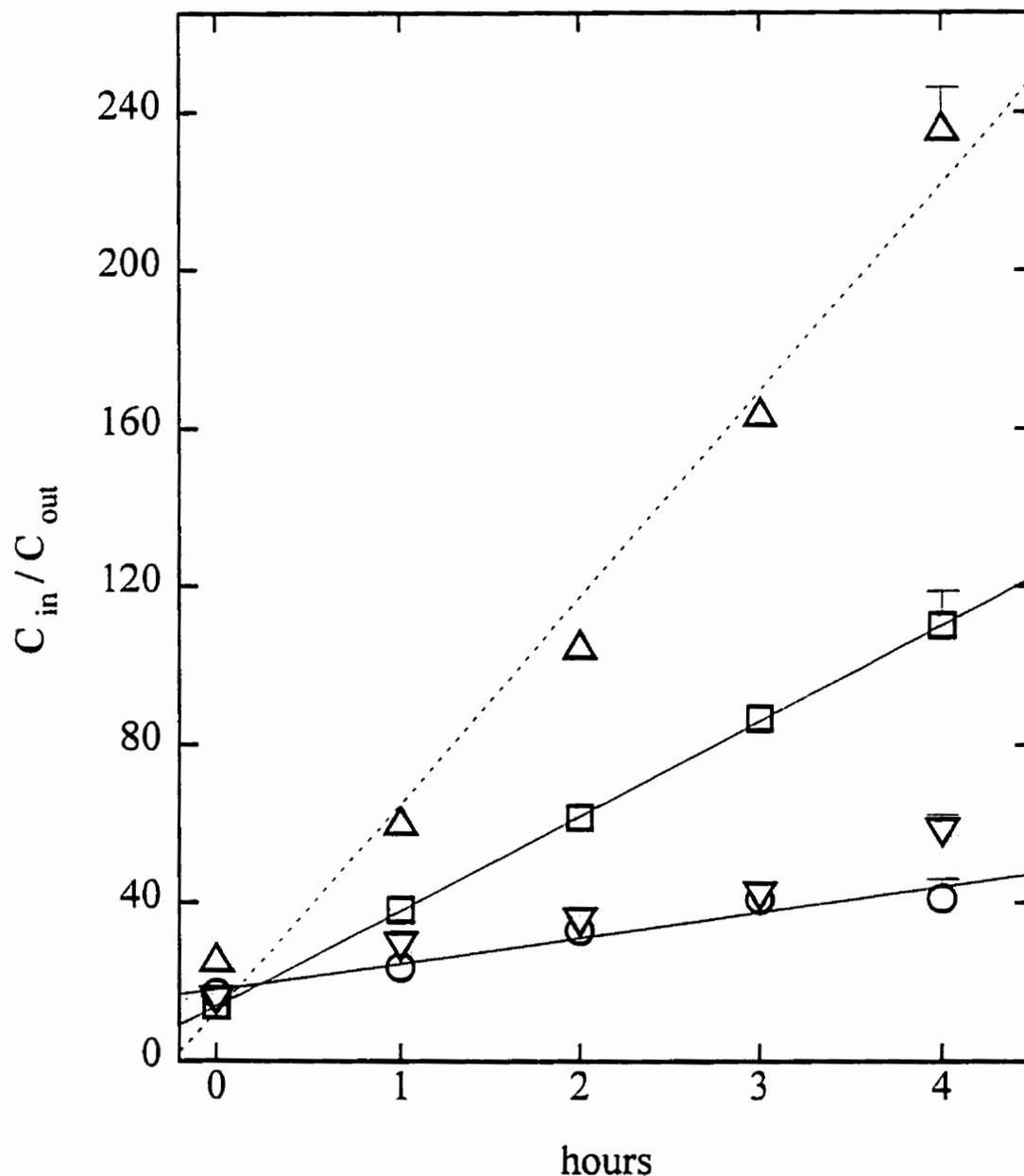


Figure 2.8: The accumulation of BMS181321 in the presence of unlabelled metronidazole (5 mM) under hypoxic (95% N₂, 5% CO₂, <10 ppm O₂) and low oxygen (0.35% O₂, 5% CO₂, balance N₂, <100 ppm O₂) conditions. Points are the mean \pm standard error of the mean of five independent experiments and lines were produced by the method of least squares. The accumulation of BMS181321 expressed by the ratio C_{in}/C_{out} in the presence of 5 mM unlabelled metronidazole plotted versus time after the addition of BMS181321. Points are hypoxic control (□, -), low oxygen control (○, -), hypoxic with metronidazole (Δ, —), and low oxygen with metronidazole (▽, —).

is performed at 4°C (Ballinger *et al.*, 1996), which is consistent with an enzymatic process causing the sequestration of radioactivity in hypoxic cells.

Aerobic cells show an initial (five minute) accumulation of radioactivity, with a C_{in}/C_{out} value of approximately 20. There was no further accumulation of radioactivity in aerobic cells after this initial uptake. This high C_{in}/C_{out} value has been suggested to be due to the high lipophilicity of BMS181321, which has an octanol:water partition coefficient of 40 (Ballinger *et al.*, 1996).

The form of the radioactivity present in the external medium during hypoxic incubations changes with time. In hypoxic cells, there is a decrease in the amount of radioactivity that is extractable into ethyl acetate. This change in the form of the radioactivity into more water soluble products is thought to be due to hypoxia specific metabolism of the drug, and the subsequent release of this altered form of radioactivity into the external media (Ballinger *et al.*, 1996). The fact that there is no change in the form of the radioactivity when hypoxic exposure occurs with no cells present substantiates the proposal that the change in the extractability of the radioactivity requires cellular processes. As well, when BMS181321 is reduced in the presence of xanthine oxidase, there is a substantial decrease in the amount of radioactivity extractable into ethyl acetate (Dr. Ballinger, personal communication). This suggests that reduction of the nitro group may be at least partially responsible for the formation of products that are less lipophilic than BMS181321 (Linder *et al.*, 1996). No further characterization of these putative reduction products is available at the present time.

The metabolism of BMS181321 yielding the formation of more hydrophilic species is consistent with studies of hypoxic myocardium, in which it was found that BMS181321

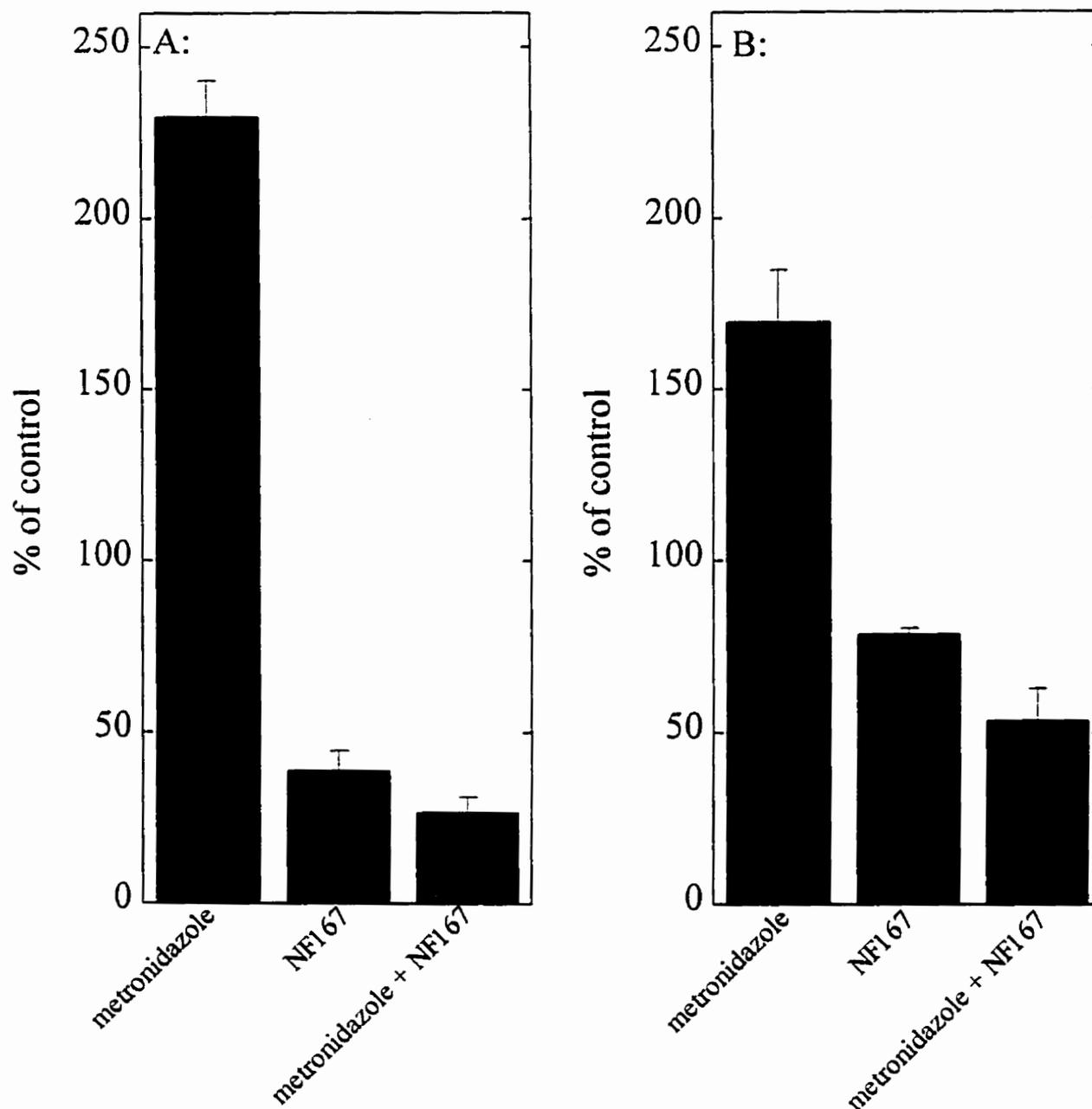


Figure 2.9: The accumulation and metabolism of BMS181321 in hypoxic CHO cells incubated for four hours in the presence of metronidazole (5 mM), NF167 (0.09 mM) or a coincubation with metronidazole (5 mM) and NF167 (0.09 mM). Points are the mean \pm standard error of the mean of at least three independent experiments. A: Accumulation of BMS181321 expressed as a percent of the control cells with no added unlabelled drug. B: Metabolism expressed as a percent of the radioactivity that was ethyl acetate extractable in the supernatant of cells incubated with no metronidazole or NF167.

underwent a metabolic change to more hydrophilic species (Jayatilak *et al.*, 1994). As well, this study reported that BMS181321 or its metabolites were bound to intracellular proteins, which provides an explanation for the sequestration of BMS181321 in hypoxic cells. Consistent with this finding, preliminary studies on the binding of radioactivity to cellular proteins in CHO cells found that the majority of radioactivity associated with the cellular fractions in hypoxic cells was associated with the trichloroacetic acid precipitable fraction (data not shown).

Although some of the metabolites of BMS181321 appear to diffuse from the cell, there is sufficient accumulation of radioactivity in hypoxic cells to indicate that BMS181321 possesses properties that allow it to act as a marker for hypoxic cells. However, in order for nitroimidazoles to act as quantitative markers for imaging tumour hypoxia, it is essential that the nitroimidazole have complete access to the target population of hypoxic cells present in the tumour (Koch *et al.*, 1993). Nitroimidazoles that are labelled with short half-life gamma emitters, such as ^{99m}Tc , are used at extremely low drug concentrations due to the high specific activity of the labelled drug. Therefore, if metabolic depletion of these drugs occurs, it is possible that the drug would not be able to diffuse to the entire hypoxic population, and an underestimate of the hypoxic fraction present in the tumour would occur.

When the amount of radioactivity in the external media is measured over time, the total amount of radioactivity decreases substantially only for hypoxic cell incubations after exposure to BMS181321. This depletion can be explained by accumulation in hypoxic cells. Metabolic depletion of BMS181321 has been shown in CHO and HeLa cells at cell concentrations greater than 1×10^6 cell/ml using the same system reported in this study, where it was found that depletion of BMS181321 from the external medium led to a non-linear accumulation of

radioactivity within the cells (Ballinger *et al.*, 1996).

The potential problem of metabolic depletion of other nitroimidazole hypoxic cell markers has been addressed by others (Koch *et al.*, 1993). In their model, a tumour was assumed to have a blood flow rate of 0.1 ml/min/g and a misonidazole concentration of 10 μM was contained in the blood. Based on a known rate of drug metabolism, it was calculated that a completely hypoxic tumour would consume the drug at a rate 20 times higher than the supply rate and even a 5% hypoxic fraction in the tumour would consume all of the available drug (Koch *et al.*, 1993). BMS181321 has been estimated to have a consumption rate in CHO cells which is about one-tenth the consumption rate of misonidazole (Ballinger *et al.*, 1996). However, BMS181321 is used at 55 pM concentrations, and when the same assumptions for blood flow rate and tumour size are used as above and the consumption rate for BMS181321 is corrected for this picomolar concentration, it can be seen that the consumption rate of BMS181321 will be higher than the supply rate. Thus, any concerns that exist about metabolic depletion for drugs used at 10 μM concentrations are even greater for drugs such as BMS181321, which are present at million fold lower levels. This concern, of course, is based on the assumption that metabolism of the hypoxic marker is fast with respect to the diffusion rate through the tumour.

This paper addresses the feasibility of using unlabelled nitroaromatics, that varied in their electron affinity, to modulate the accumulation of BMS181321 under hypoxic conditions. In essence, the binding of BMS181321 in individual tumour cells would be decreased by adding nitroaromatics that would be assumed to be metabolized and retained in the hypoxic cell by the same mechanism as BMS181321. In theory, this should help to circumvent the problem of metabolic depletion of the hypoxic marker, because less of the drug would be metabolized in

each cell, leaving more unmetabolized drug free to diffuse and label other hypoxic cells within the tumour. Decreasing the number of molecules of the hypoxic marker bound per cell would inevitably decrease the sensitivity of detection of hypoxic cells, but this loss of sensitivity may be offset by more complete labelling of the hypoxic cell fractions in the tumour.

Etanidazole and misonidazole were initially investigated because of the previous clinical experience and interest in using these 2-nitroimidazoles as hypoxic cell radiosensitizers (Overgaard, 1994). Both drugs inhibited the accumulation and metabolism of BMS181321 only under hypoxic conditions. The inhibition was concentration dependent and was not caused by an increased cellular toxicity due to the co-incubation of these unlabelled nitroimidazoles with BMS181321, since very little toxicity was seen using colony formation assays and membrane integrity was not compromised as observed by the cellular exclusion of trypan blue (data not shown). NF167 also caused hypoxia specific inhibition of BMS181321 under hypoxic conditions. The inhibition observed could be due either to competition for cellular reducing equivalents that are involved in the hypoxia specific reduction of nitroimidazoles (Figure 10A) or to competition for the reduced radical anion of BMS181321 (Figure 10B).

The concentrations of etanidazole and misonidazole needed to inhibit BMS181321 accumulation and metabolism under hypoxic conditions were over a million fold higher than the concentration of BMS181321. It took 8 mM of both etanidazole or misonidazole to inhibit the hypoxic accumulation and metabolism of BMS181321 by approximately 80%, but significant inhibition was also observed at etanidazole and misonidazole levels that were over 100 fold lower than these initial concentrations.

The drug levels of misonidazole and etanidazole needed to exhibit effective competition

are in the same concentration range as those which have been reported to cause toxicity to normal patient tissues when these compounds were studied in clinical trials as hypoxic cell radiosensitizers (Dische *et al.*, 1979 and Wasserman *et al.*, 1979). Patients exhibited a dose limiting peripheral neuropathy, which limited the success of these trials, since optimum doses to cause radiosensitization could not be administered. Thus, the use of etanidazole and misonidazole to decrease possible metabolic depletion of hypoxic cell markers is unlikely to be practical in the clinic, because maximally effective concentrations of the unlabelled nitroimidazole cannot be administered. Although these results are not encouraging from a clinical standpoint, they do offer some interesting insights into the mechanisms modulating the retention of nitroimidazoles in hypoxic cells.

Unexpectedly, the 5-nitroimidazoles, metronidazole and tinidazole, stimulated both the accumulation and metabolism of BMS181321 under hypoxic conditions. In previous studies, the nitrofurantoin AF-2 was found to stimulate the accumulation of misonidazole in 9L rodent cells (Olive, 1987). This effect was surprising because AF-2 is more electron affinic than misonidazole. It was suggested that this increase in misonidazole accumulation was due to glucose deprivation because of the high cell concentration used in the study (Koch *et al.*, 1993). Glucose deprivation would be predicted to cause a decrease in the number of reducing equivalents available from the metabolism of glucose. The increase observed in the accumulation and metabolism of BMS181321 under hypoxia with the addition of 5-nitroimidazoles was not due to glucose deprivation because the current studies were performed at a cell concentration of 1×10^6 cells/ml, which would be predicted to be too low to cause depletion of glucose from the media after the four hour incubation.

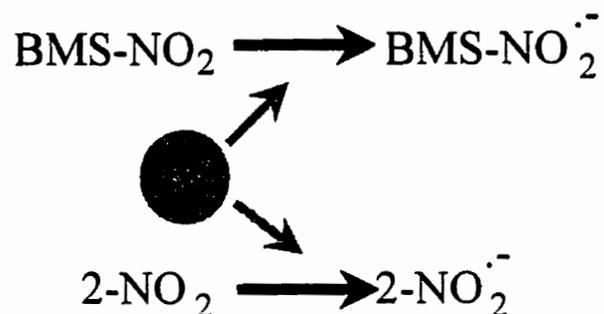
(A) Competition for reducing equivalents.**(B) Transfer of electrons from radical anion of:****(i) BMS181321 to compounds of higher electron affinity.****(ii) 5-nitroimidazoles to BMS181321.**

Figure 2.10: Models for the inhibition and stimulation of BMS181321 accumulation. BMS-NO₂, 2-NO₂ and 5-NO₂ represent BMS181321, 2-nitroimidazoles and 5-nitroimidazoles respectively. BMS-NO₂^{·-}, 2-NO₂^{·-} and 5-NO₂^{·-} represent the radical anion forms of BMS181321, 2-nitroimidazoles and 5-nitroimidazoles respectively.

The most probable explanation for this observed increase in BMS181321 accumulation is that there is a transfer of electrons from the reduced 5-nitroimidazoles to BMS181321 (Figure 10). The radical anion form of the 5-nitroimidazoles is less electron affinic than 2-nitroimidazoles (Wardman and Clarke, 1976), and would be predicted to be able to donate electrons to the 2-nitroimidazole. This would increase the bioreductive metabolism of BMS181321, leading to the observed increased hypoxic accumulation of BMS181321. The nitro radical anion of the 5-nitroimidazoles could also donate electrons to other metabolites of BMS181321. Further investigation into the validity of this model is needed.

RP8979, a 4-nitroimidazole that has an even lower electron affinity than the 5-nitroimidazole class of compounds, did not show any great effect on either BMS181321 accumulation or metabolism under hypoxic conditions. If an electron transfer process between the less electron affinic radical anion species to BMS181321 is occurring, it is postulated that the electron affinity of RP8979 may be too low to be effectively reduced and to be able to transfer electrons to BMS181321. It has also been suggested that the lifetime of radical intermediates could play a role in the interaction of nitroimidazoles in mammalian cells (Olive, 1987). If RP8979 radicals are much shorter lived than metronidazole or tinidazole radicals, then RP8979 could be bioreductively reduced to non-radical metabolites before its radical species can efficiently transfer electrons to BMS181321.

The stimulation in BMS181321 accumulation with the addition of 5-nitroimidazoles occurred only under conditions of extreme hypoxia, and even very low oxygen levels (< 100 ppm oxygen) inhibited the stimulation of BMS181321 accumulation. The most probable explanation for this observed lack of stimulation is the oxygen sensitivity of metronidazole metabolism. It

has been shown that less than 100 ppm of oxygen in the gas phase inhibits nitroreduction of metronidazole by 90% (Rauth *et al.*, 1984). Thus, even an oxygen concentration as low as 100 ppm oxygen would be predicted to decrease the formation of the radical anion species of metronidazole substantially. Without adequate concentrations of the metronidazole radical anion, efficient transfer to BMS181321 would not be possible, and no enhanced accumulation of BMS181321 would be observed.

There was an observed inhibition of BMS181321 accumulation and metabolism when metronidazole (5 mM) and NF167 (0.09 mM) were added at levels that exhibited maximal stimulation and inhibition respectively when added separately. If the donation of electrons from less electron affinic compounds to more electron affinic counterparts is occurring, one would expect the least electron affinic compound to transfer electrons to the most electron affinic compound in the system. In this case, the radical anion form of metronidazole would be predicted to transfer electrons to NF167, at the expense of electron donation to BMS181321.

In conclusion, the initial purpose of this work was to evaluate the use of unlabelled nitroaromatics as an adjuvant drug to decrease the potential problem of metabolic depletion. It was found that nitroaromatics of a similar or higher electron affinity than BMS181321 could inhibit the accumulation of this hypoxic marker *in vitro*, while nitroaromatics of lower electron affinity could stimulate the accumulation of the marker. This increased accumulation of BMS181321 in hypoxic cells with the addition of 5-nitroimidazoles offers the possibility of increasing the contrast between hypoxic and normal tissue during imaging. However, in both situations the effect on BMS181321 accumulation may not be practical clinically, since maximally effective drug concentrations would not be tolerated by normal tissues.

As stated earlier, in order for metabolic depletion to limit the distribution of BMS181321, the rate of metabolism of the drug must be greater than its diffusion through the tumour mass. Recent work using an *in vitro* multilayer cell system (Cowan *et al.*, 1996), indicates that the rate of metabolism of BMS181321 may be slow relative to the diffusion rate (Melo, unpublished data). Further work will need to be done to confirm this observation and the effects that unlabelled nitroaromatics have on the diffusion of BMS181321 through a tumour-like environment.

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Chapter 3: Discussion and Future Directions

Ideally, nitroimidazoles should have complete access to the target population of hypoxic cells in order to image all the hypoxic cells in tumours. There is a concern that metabolic depletion of ^{99m}Tc -nitroimidazole hypoxic cell markers, which are used at picomolar concentrations, could result in decreased drug delivery to the target cells. This would cause hypoxic regions in the tumour to be incompletely mapped out leading to an underestimate of the hypoxic cell fraction. The feasibility of using unlabelled nitroaromatics to modulate the accumulation of BMS181321, a ^{99m}Tc -nitroimidazole, under hypoxic conditions was the focus of this thesis. The work was intended as a prelude to assessing the feasibility of using unlabelled nitroimidazoles to alter the biodistribution of BMS181321 *in vivo* and to provide information about the mechanism of retention of BMS181321 in hypoxic cells.

3.1 BMS181321 as a Marker for Tumour Hypoxia

Nitroimidazoles used to image hypoxia have a wide variety of lipophilicity, protein binding, and plasma clearance characteristics. An optimal agent should localize in the tissue of interest quickly, and provide sufficient photon flux to permit images to be recorded in a short time (Nunn *et al.*, 1995). Contrast is determined by the relative retention in hypoxic tissue and clearance from normoxic tissue. This will be determined by the stability of the labelled drug, its partition coefficient, and rate of metabolism.

Our laboratory has been interested in evaluating BMS181321 and related compounds as possible agents to image tumour hypoxia. CHO cells in suspension culture show a selective accumulation of drug under hypoxic versus aerobic conditions, where there is approximately a five fold increase in the amount of accumulation in hypoxic cells compared to aerobic cells.

In the same model system, it was found that 30% of the radioactivity accumulated after a two-hour hypoxic exposure was retained at 24 hours (Ballinger *et al.*, 1996). In contrast, most of the radioactivity that accumulated in aerobic cells quickly washed out after removal of the drug from the medium, suggesting that retention in aerobic cells is not caused by binding to cellular macromolecules. However, when BMS181321 was studied in murine tumours, tumour to blood ratios were less than unity (Ballinger *et al.*, 1996), indicating that there was poor clearance of BMS181321 from the blood, resulting in high background values. The poor blood clearance of BMS181321 is not unexpected because of the high lipophilicity of the compound. Interest is emerging in synthesizing ^{99m}Tc -nitroimidazoles that are considerably less lipophilic and which would be predicted to exhibit improved pharmacological properties for imaging.

Ideally, a hypoxic cell marker should have a constant binding rate in different cells and tissues that are maintained at the same oxygen concentration in order to be used as an absolute monitor of oxygen concentration. The variability in misonidazole binding in different cell lines has been reported (Koch *et al.*, 1993). Included as an appendix in this thesis is the cell-line dependency of BMS181321 accumulation under hypoxic conditions. Three lines of CHO cells of differing P-glycoprotein status, as well as one rat and two human cell lines were investigated (Cowan *et al.*, 1996a). Human cells showed approximately a two-fold higher accumulation of BMS181321 than rodent cells, which is consistent with previous results for misonidazole (Taylor and Rauth, 1978), and which may reflect differences in nitroreductase activities between these cell lines. These differences in the accumulation of BMS181321 provide circumstantial evidence that BMS181321 may not be useful as an absolute monitor of oxygen concentration. However, this investigation should be extended to selected human cell lines of a common type, allowing

a more definitive statement about the cell type dependency of BMS181321 binding. If BMS181321 fails to be effective as an absolute marker of oxygen concentration, its usefulness to assess the reoxygenation of a tumour throughout the course of treatment is not precluded.

3.2 Metabolism of BMS181321

The form of the radioactivity in the external medium of cells incubated with BMS181321 under hypoxic conditions changes with time, since the amount of radioactivity extractable into ethyl acetate decreases. This is thought to be due to metabolism of BMS181321 to less lipophilic metabolites, and release of these metabolites into the external medium. This result is consistent with other investigations, that found that metabolism of BMS181321 resulted in the formation of more hydrophilic metabolites (Jayatilak *et al.*, 1994).

The exact nature of this changing form of radioactivity in the external medium of hypoxic incubations has not been determined. Preliminary investigations using xanthine oxidase catalyzed reduction of BMS181321 indicates that at least part of this changing form of radioactivity is due to reduction of the nitro group, since in the xanthine catalyzed system, there is also a decreasing amount of radioactivity that is extractable into ethyl acetate. However, the very unstable nature of BMS181321 (Ballinger *et al.*, 1996) does not preclude the possibility that these less lipophilic species may be degradation products. Further studies will need to be employed to deduce the nature of these products.

3.3 Altering the Accumulation of BMS181321 with Unlabelled Nitroaromatics

Several lines of evidence, both circumstantial and theoretical, suggest that metabolic depletion of nitroimidazoles may cause the drug to be unable to fully map out the hypoxic regions present in the tumour, resulting in an underestimate of the extent of hypoxia (Koch *et al.*, 1993, Koch *et al.*, 1991, and Ballinger *et al.*, 1996).

To circumvent this potential problem, the feasibility of using unlabelled nitroaromatics to alter the hypoxic accumulation and metabolism of BMS181321 was investigated. It was found that nitroaromatics that had an electron affinity that was similar or greater than BMS181321 were able to inhibit the hypoxic accumulation and metabolism of BMS181321 and this effect was concentration dependent. Surprisingly, 5-nitroimidazoles, which have a lower electron affinity than BMS181321, stimulated the hypoxic accumulation and metabolism of BMS181321. It is suggested that inhibition is due to competition for reducing equivalents or for the BMS181321 radical anion itself, while stimulation is due to the radical anion form of the 5-nitroimidazole donating its electrons to BMS181321. However, over a million fold excess concentration of the competitor or stimulator over that of BMS181321 was needed to exhibit any effect on BMS181321 accumulation and metabolism. These high concentrations suggest that such a technique may not be useful clinically, since they are known to be intolerable to the patient (Dische *et al.*, 1979 and Wasserman *et al.*, 1979).

The stimulation in BMS181321 accumulation with the addition of the 5-nitroimidazole metronidazole was inhibited by low oxygen levels (<100 ppm). Consistent with previous studies (Rauth *et al.*, 1986), the radical anion form of metronidazole would not be formed at this oxygen concentration and the transfer of electrons to BMS181321 would not be predicted.

Before dismissing the practicality of using 5-nitroimidazoles to increase BMS181321 accumulation, a thorough assessment of the validity of the concern of metabolic depletion of BMS181321 must be addressed. Preliminary results, which are discussed below, suggest that metabolic depletion of BMS181321 may not be a problem in the use of this compound. If this is the case, then the feasibility of using 5-nitroimidazoles to increase the contrast between normal and hypoxic tissue imaged with BMS181321 is worth investigating. Single doses of metronidazole have been given clinically (at plasma concentrations ~ 1 mM), and these metronidazole concentrations would be predicted to give approximately a 1.5-fold enhanced hypoxic accumulation of BMS181321. As mentioned earlier, the high lipophilicity of BMS181321 causes it to be slowly cleared from the blood, resulting in high background values when imaged (Ballinger *et al.*, 1996). Increasing the accumulation of BMS181321 exclusively in hypoxic tissue would provide a greater contrast between hypoxic and normal tissue. However, this prediction would merit further investigation only after a comparison of the diffusion and metabolism of BMS181321 in tumours is made.

3.4 Future Directions

3.4.1 Analysis of the Diffusion of ^{99m}Tc -nitroimidazoles

In order for metabolic depletion of the hypoxic marker to occur, metabolism of the drug must be faster than the diffusion of the drug through the tumour. It will be of interest to evaluate the magnitude of diffusion versus metabolic effects in the use of BMS181321 and other ^{99m}Tc -nitroimidazoles in tumours.

A new *in vitro* model for measuring the diffusion of drugs in a tumour-like environment

has been developed (Cowan *et al.*, 1996b). Chinese hamster V79 cells are grown for four days on microporous membranes at tissue-like densities with feeding by culture medium from both sides, resulting in symmetrical cellular multilayers that are up to 20 cell diameters thick with a central area of necrosis. The drug is added to one side of the multicellular membrane (compartment 1), the membrane floated in growth medium (compartment 2), and samples taken from compartment 2 as a function of time (Figure 3.1). The diffusion of the drug through the membrane may be monitored from these samples using HPLC techniques, or by measuring the amount of radioactivity. In studies already performed using these V79 cellular multilayers, it was found that the diffusion of misonidazole through multilayers was concentration independent over the range of 0.1-3 mM (Cowan *et al.*, 1996b). As well, it was found that the diffusion of misonidazole was not affected by the extent of hypoxia.

This cellular multilayer system has been reproduced in our laboratory and used to study the diffusion of BMS181321 through a tumour-like environment under both aerobic and fully hypoxic incubations. Our preliminary results indicate that less radioactivity appears in compartment 2 as a function of time under hypoxic conditions compared to that seen for aerobic conditions (Figure 3.2), and the form of this radioactivity changes more with time compared to aerobic conditions (Figure 3.3). Although there is decreased diffusion under hypoxic conditions, there is no evidence for extensive metabolic depletion of BMS181321, since radioactivity does continue to accumulate over the time course of the experiment.

These preliminary results suggest that the rate of diffusion of BMS181321 is greater than its rate of accumulation, and that metabolic depletion of BMS181321 may in fact not be a limitation for its *in vivo* application. These studies should be further enhanced using HPLC

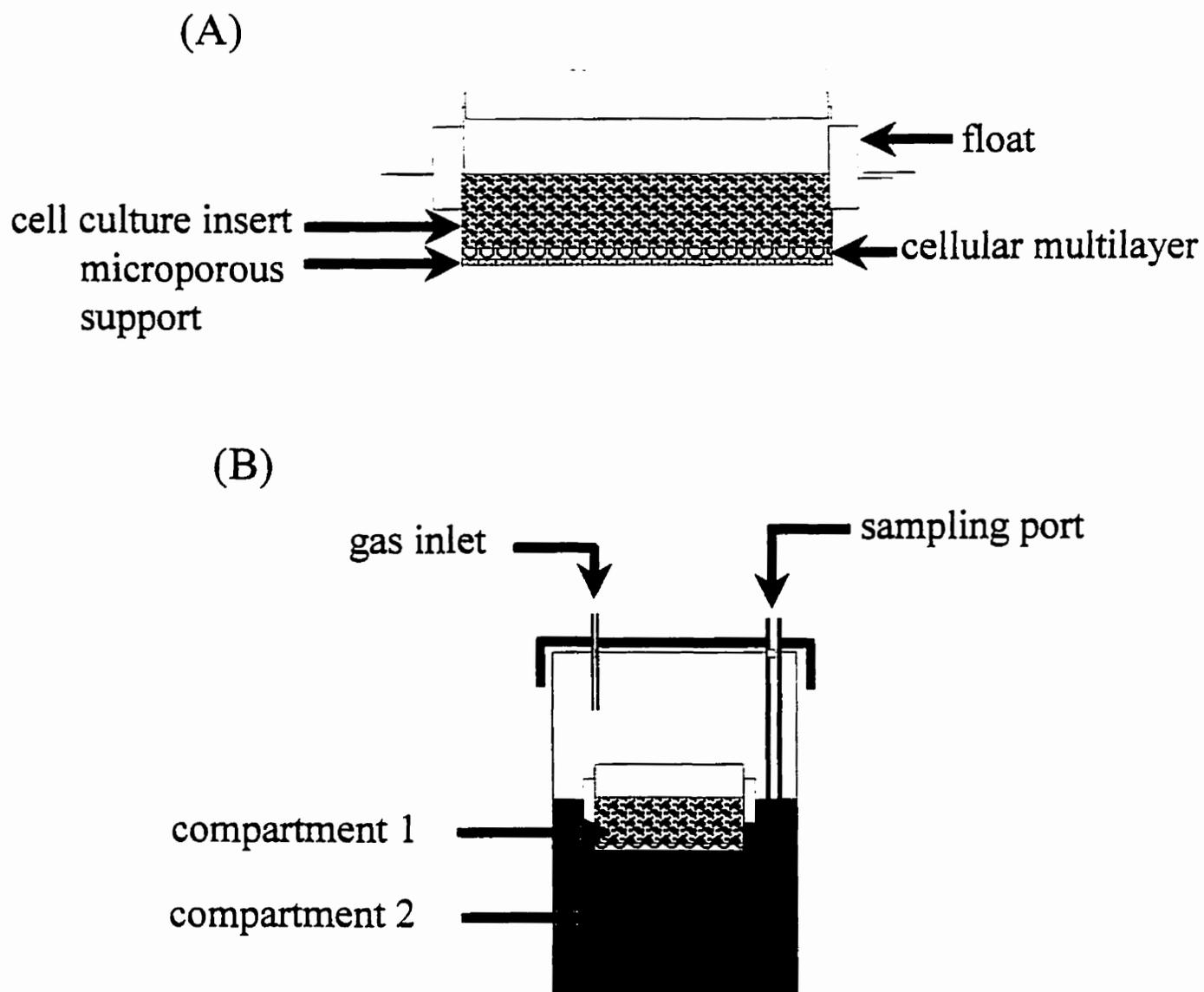


Figure 3.1: Multicellular membrane experimental setup. (A) Schematic of multicellular membrane which comprises compartment 1. (B) Apparatus for measurement of drug diffusion through the multicellular membrane. (Adapted from Cowan *et al.*, 1996b).

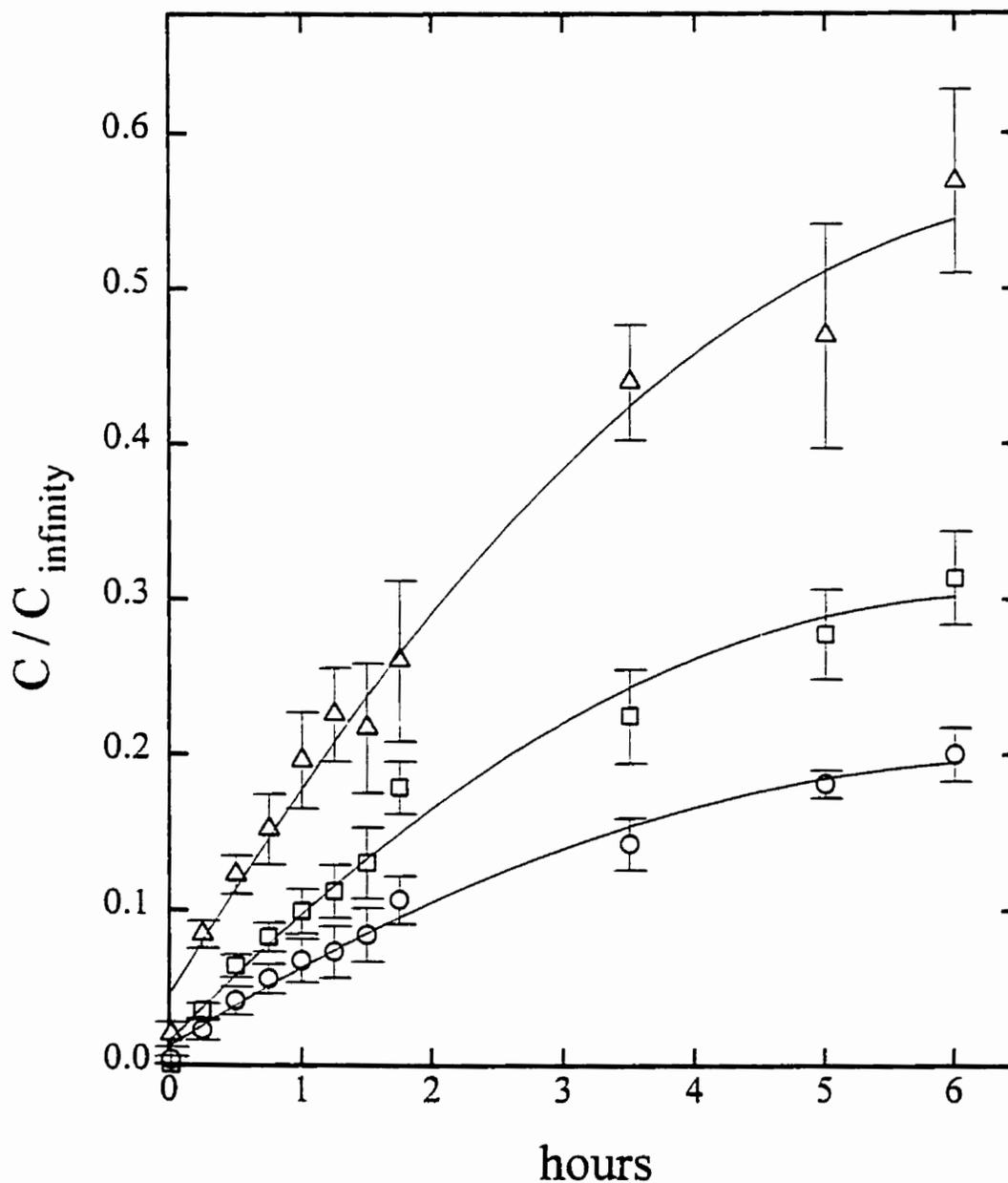


Figure 3.2: Diffusion of BMS181321 through V79 cellular multilayer. BMS181321 is added to the top of compartment one at time zero and the appearance of radioactivity in compartment two is measured as a function of time. The accumulation of radioactivity is expressed as the ratio C/C_{∞} which is the ratio of counts measured in compartment two over the total counts added to compartment one. Points are the mean \pm standard error of the mean of three separate experiments. Points are aerobic (95% air, 5% CO₂, □) and hypoxic (95% N₂, 5% CO₂, <10 ppm O₂, ○) gassing of the multilayer. The cell-free control (Δ) was gassed under hypoxic conditions.

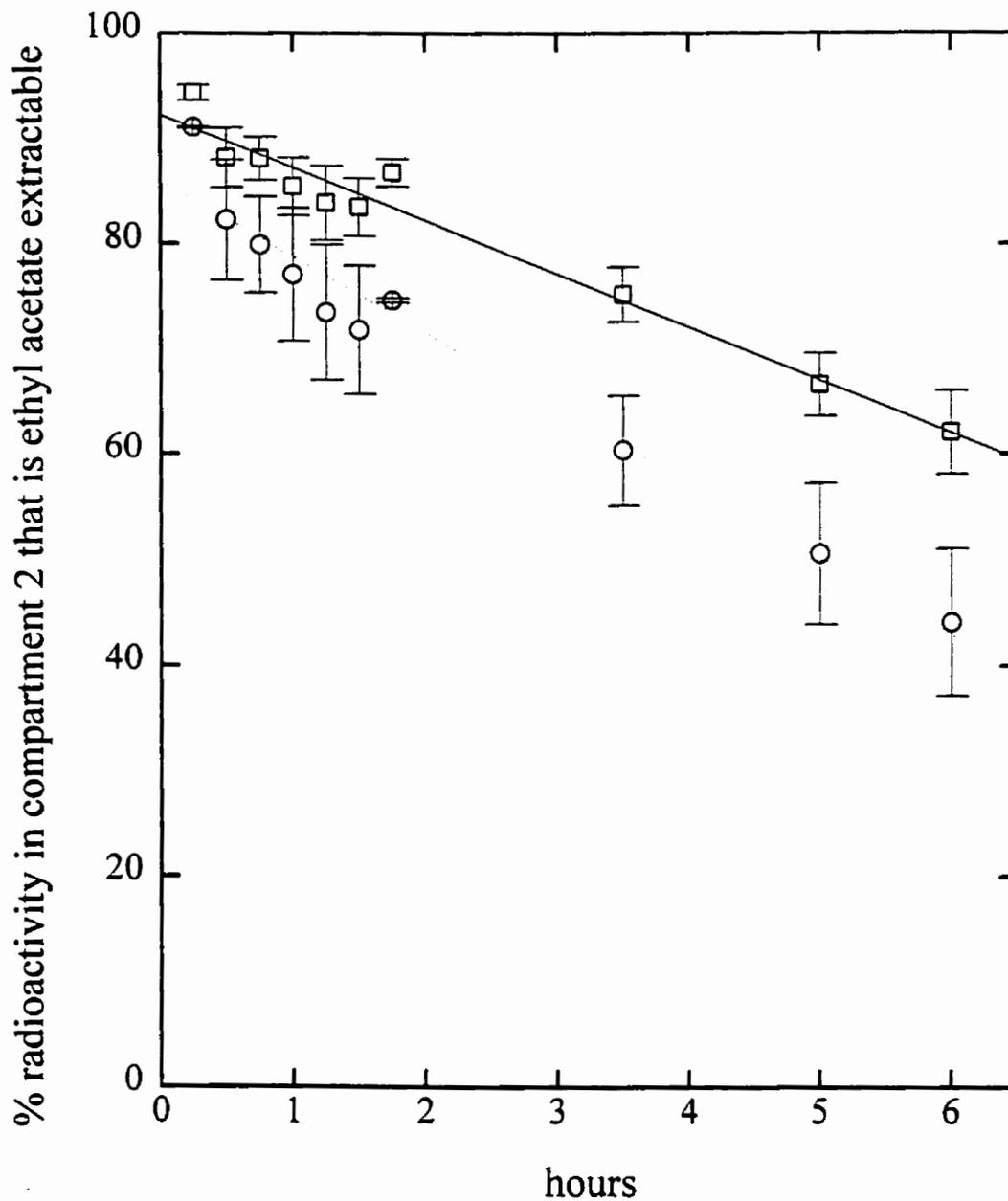


Figure 3.3: The ability of the radioactivity in compartment two of the V79 cellular multilayer system to be extracted into ethyl acetate. BMS181321 is added to the top of compartment one at time zero and the diffusion of BMS181321 through the V79 cellular multilayer is monitored. This graph shows the percentage of total counts in compartment two that partitions into the ethyl acetate phase versus PBS for aerobic (95% air, 5% CO₂, □) and hypoxic (95% N₂, 5% CO₂, <10 ppm O₂, ○) incubations as a function of time. Points are the mean \pm standard error of the mean of three separate experiments.

techniques to analyze the form of the radioactivity appearing in compartment 2. As well, other cell-lines should be examined, particularly those which have been shown to exhibit increased accumulation of BMS181321 (Cowan *et al.*, 1996a) and would be predicted to exhibit a slower diffusion of radioactivity under hypoxia because of increased metabolism and retention of the drug in the cell.

3.4.2 Analysis of New ^{99m}Tc-nitroimidazoles

Bracco Research USA has developed a second generation ^{99m}Tc-nitroimidazole, BMS194796, which is less lipophilic than BMS181321. As well, Resolution Pharmaceuticals is synthesizing a new generation of nitroimidazoles which are linked to peptidic chelators. These, and selected other ^{99m}Tc-nitroimidazoles that become available may be characterized in a manner similar to that described previously (Ballinger *et al.*, 1996). Such characterization should encompass chemical, *in vitro* and *in vivo* tests to identify useful agents for imaging hypoxia in solid tumours.

Our laboratory has already begun characterization of BMS194796 (Figure 3.4). BMS194796 is less lipophilic (octanol:water partition coefficient of 12) than BMS181321 (octanol:water partition coefficient of 40) and BMS194796 appears to be of higher stability than BMS181321 using a thin layer chromatography method. The differential accumulation of BMS194796 and BMS181321 has been compared in hypoxic versus aerobic cells (Figure 3.5). BMS194796 exhibited a lower accumulation under both conditions than was obtained with BMS181321, most likely because of the lower lipophilicity of BMS194796. However, there was no statistical difference between the ratio of activity accumulated in hypoxic versus aerobic cells

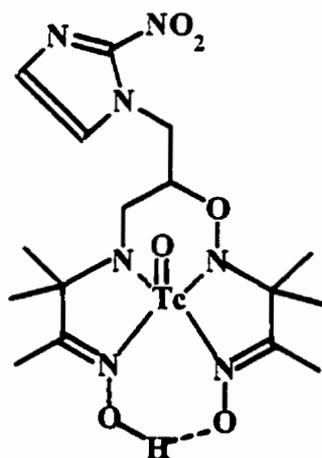


Figure 3.4: Structure of BMS194796.

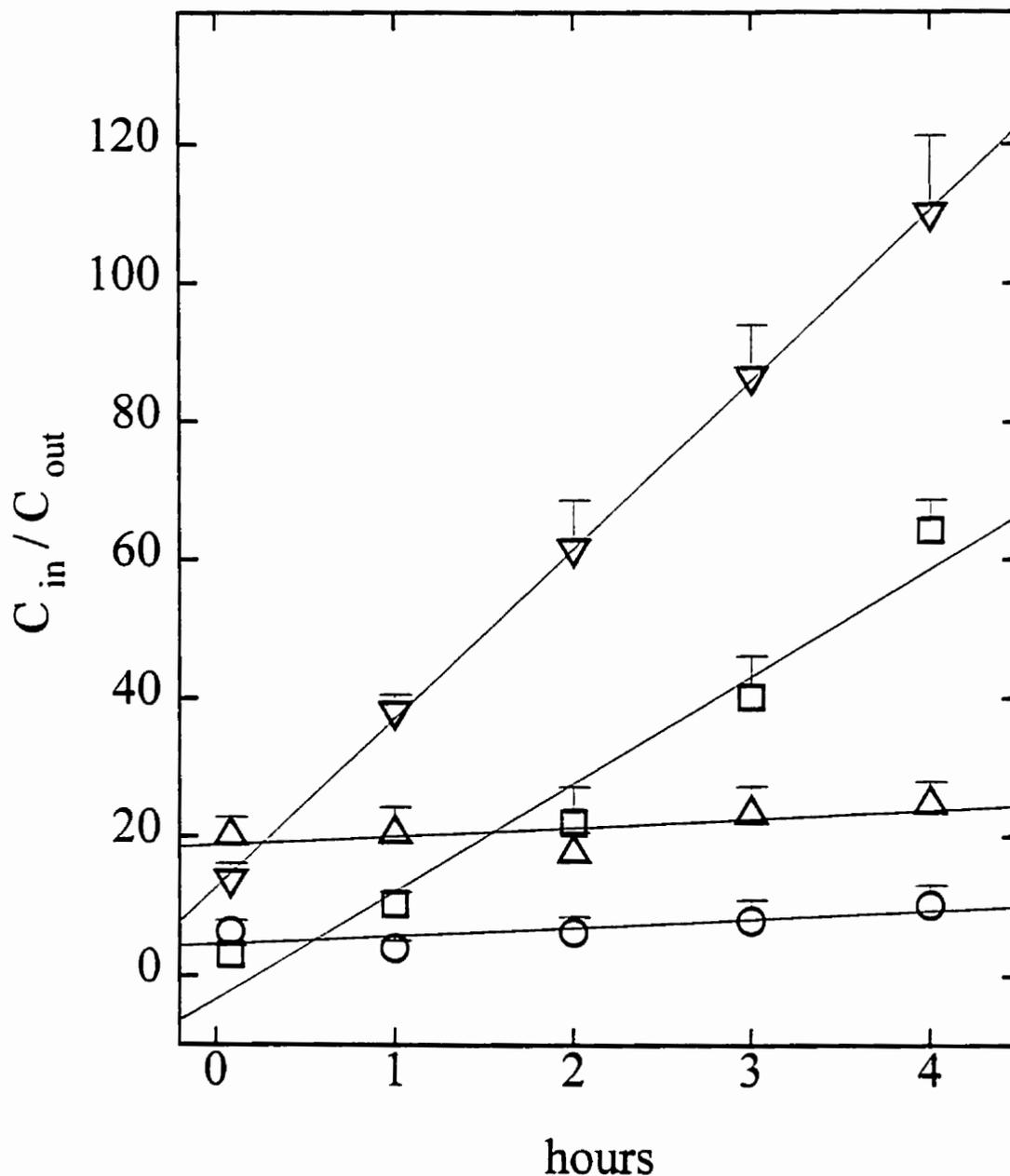


Figure 3.5: Comparison of the selective accumulation of BMS194796 versus BMS181321 in hypoxic versus aerobic cells. CHO cells at a concentration of 1×10^6 cell/ml were incubated under aerobic (95% air, 5% CO_2) or hypoxic (95% N_2 , 5% CO_2 , <10 ppm O_2) conditions in the presence of BMS194796 (\circ for aerobic, \square for hypoxic) or BMS181321 (Δ for aerobic, ∇ for hypoxic). Data is calculated as the ratio of radioactivity in 0.1 ml of packed cells (C_{in}) to the radioactivity in 0.1 ml of external medium (C_{out}) and plotted versus time after the addition of BMS194796. Points are the mean \pm standard error of the mean of at least three experiments and lines were produced by the method of least squares.

for BMS194796 compared to BMS181321 (Student's t-test, $p > 0.05$). Chronic toxicity tests done on the unlabelled compound and on the chelator showed that these compounds are not more toxic than misonidazole.

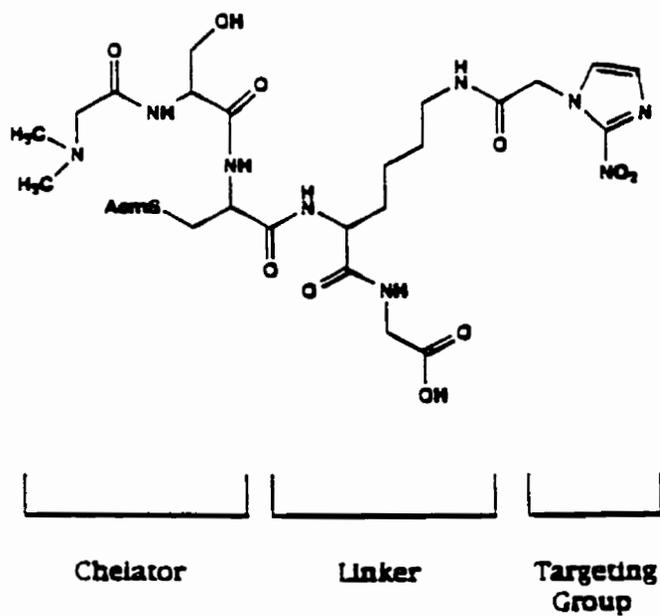
In preliminary *in vivo* studies in SCC VII tumours in mice, it was found that tumour/muscle ratios using BMS194796 were greater than those found with BMS181321. As well, tumour/blood ratios obtained with BMS194796 were greater than 1, which is an improvement from BMS181321, which had a ratio of 0.2 (Ballinger *et al.*, 1996). This indicates that BMS194796 has improved characteristics for imaging.

Resolution Pharmaceuticals has designed and synthesized a peptidic chelator for ^{99m}Tc . Using solid phase synthesis techniques, this peptidic chelator has been linked to a nitroimidazole. The structure comprises three components: a 2-nitroimidazole hypoxia-targeting moiety, a peptidic chelator for ^{99m}Tc , and a lysine linker (Figure 3.6). These structural components may be varied if desired. RP256, the first compound synthesized, had a very low partition coefficient (octanol:water partition coefficient of ~ 0.001) and did not demonstrate any hypoxic accumulation differential. Such a result is consistent with previous studies of the role of partition coefficient on cell uptake of simpler 2-nitroimidazoles (Brown and Workman, 1980). In collaboration with Resolution Pharmaceuticals, synthesis of derivatives of this compound has already begun, with the aim of attaining compounds of higher partition coefficient than this lead compound.

3.4.3 Studying the Metabolism and Cellular Localization of BMS181321

The metabolism-induced binding characteristics of ^{99m}Tc -nitroimidazoles should be determined in order to understand some of the cellular mechanisms of its binding. The

A:



B:

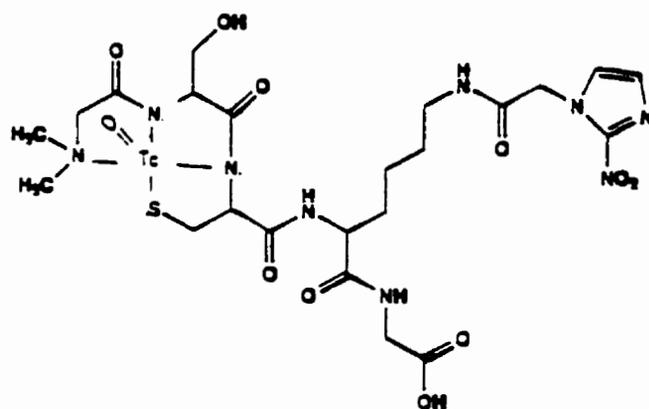


Figure 3.6: A: Chemical structure of the lead compound (RP256) synthesized by Resolution Pharmaceuticals. B: Structure of ^{99m}Tc complex of lead compound.

subcellular distribution of ^{99m}Tc following *in vitro* and *in vivo* experiments with BMS181321 showed that in myocardial cells, 51% of the activity was associated with membranes and 44% was in the cytosol. However, 42% of the activity in the membrane fraction was intact BMS181321 compared to 5% in the cytosol, which contained hydrophilic and protein-bound metabolites (Jayatilak *et al.*, 1994). Using cell-fractionation techniques, these studies may be extended to the *in vitro* system discussed in this thesis, to determine the cellular localization of BMS181321 and other ^{99m}Tc -nitroimidazoles under aerobic and hypoxic conditions using approaches described previously (Chapman *et al.*, 1983).

Incubation of BMS181321 with xanthine oxidase under hypoxic conditions shows a time-dependent loss of the nitro absorbance peak (Linder *et al.*, 1994). The nature of the products formed has not been established, but it is speculated that enzyme catalyzed reduction of the nitro group occurs. The chemical form of BMS181321 in the *in vitro* system employed in this thesis changes with time under hypoxic conditions. The nature of these products may be studied using reversed-phase HPLC methods. These products would be predicted to have earlier elution times than the parent drug because they appear to be more polar.

In order to identify whether the radioactivity released from the cell are metabolites, or at the very least to ascertain to what degree the nitroimidazole is involved in the altered state of the radioactivity, BMS1810321, the ligand of BMS181321, may be radiochemically reduced under conditions where it would be predicted to form the hydroxylamine product. The hydroxylamine or its rearrangement product may then be isolated, and reacted with ^{99m}Tc . This will require that reduction of the ligand does not change the ability of BMS181032 to chelate ^{99m}Tc . If successful, this product may then be used as a standard in the HPLC method used to analyze the metabolites

of BMS181321.

The BMS181321 ligand labelled with ^{14}C at the C-2 position of the imidazole ring has been synthesized by Bracco Research. This compound is labelled with two isotopes in different parts of the molecule. It will thus allow determination of whether the altered form of the radioactivity that we observe contains the imidazole group, or if degradation of the compound is occurring, causing the PnAO chelator to dissociate from the imidazole ring.

3.5 Summary

The presence of hypoxic cells is thought to be a limitation for the control of some solid tumours by radiation. Nitroimidazoles labelled with $^{99\text{m}}\text{Tc}$ have been investigated as possible non-invasive agents to assess the extent of hypoxia in an individual tumour. The work presented in this thesis provides some understanding of the processes involved in the metabolism and retention of these compounds in hypoxic cells. As well, the problem of metabolism limiting the diffusability of these compounds has been addressed. An understanding of the processes involved in the retention of these compounds will allow a rational selection of the best $^{99\text{m}}\text{Tc}$ -nitroimidazoles for detecting hypoxic cells in solid tumours and it will aid in the utilization of these agents clinically.

3.6 References

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Appendix: BMS181321 Accumulation in Rodent and Human Cells: The Role of P-Glycoprotein

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A1.1 Abstract

A 2-nitroimidazole with a side chain that contains technetium-99m as a chelate, BMS181321, is undergoing evaluation as an imaging agent for myocardial and cerebral ischemia, as well as a diagnostic probe for hypoxic cells in solid tumours. Its accumulation in hypoxic and aerobic populations of three lines of Chinese hamster ovary cells of differing P-glycoprotein status, as well as one rat and two human cell lines has been determined. There was selective accumulation of BMS181321 in hypoxic versus aerobic cells. P-glycoprotein level was not a factor in this accumulation and hypoxic human cells accumulated BMS181321 more rapidly than the rodent cells. These results indicate P-glycoprotein levels in tumour cells will not confound the use of BMS181321 as a hypoxic cell marker.

A1.2 Introduction

The potential therapeutic use of nitroimidazoles as hypoxic cell radiosensitizers and toxins is well-documented (Brown, 1989). Nitroimidazoles have also been studied as diagnostic markers for the presence of hypoxic cells in solid tumours (Nunn *et al*, 1995). Their selective accumulation in hypoxic versus aerobic cells is due to their enhanced reduction in the presence of low levels of oxygen to reactive nitroso and/or hydroxylamine derivatives which rapidly react and bind covalently to cellular macromolecules (McClelland *et al*, 1987). A variety of radioactive labels of the parent nitroimidazoles have been used to allow the invasive (Urtasun *et al*, 1986) and non-invasive (Koh *et al*, 1992) detection of hypoxic cells in solid tumours.

In the course of studies of 2-nitroimidazoles as hypoxic cell radiosensitizers, Cowan *et al* (1994) developed a compound that could be targeted to its site of action (DNA) by the

presence of an intercalating phenanthridine group in its side chain (5-[3-(2-nitro-1-imidazolyl)-propyl]-phenanthridinium bromide, 2-NLP-3, Figure A1.1). 2-NLP-3 had an increased molar efficacy over untargeted nitroimidazole, based both on extracellular concentration *in vitro* and administered dose *in vivo* (Cowan *et al*, 1994). In the course of the 2-NLP-3 studies a Chinese hamster ovary (CHO) cell line, C-1000, was isolated which was resistant to this drug. Through the study of drug analogues and comparison to the multi-drug resistant CHO cell line, CHR-C5 (C5, Ling and Thompson, 1974), a portion but not all of the resistance of C-1000 was shown to be due to the overexpression of P-glycoprotein, a putative drug pump in the membrane of C5 and C-1000 cells (Cowan *et al*, 1995).

Recently a 2-nitroimidazole with a side chain able to chelate technetium-99m [$\text{TcO}(\text{PnAO}-1-(2\text{-nitroimidazole}))$], BMS181321, Figure A1.1] has been developed and is being evaluated as an imaging agent for myocardial and cerebral ischemia (Linder *et al*, 1994). Both 2-NLP-3 and BMS181321 have bulky multi-ring side chain groups. 2-NLP-3 has a permanent positive charge and a partition coefficient in octanol/water of 0.5 (Cowan *et al*, 1994) while BMS181321 is uncharged but is much more hydrophobic with a partition coefficient of approximately 40 (Ballinger *et al*, 1996). The objective of the present work is to see if mechanisms that make C5 and C-1000 cells resistant to 2-NLP-3 and a variety of other chemotherapeutic agents known to be substrates for P-glycoprotein are able to affect the accumulation of BMS181321.

A1.3 Materials and Methods

The origin of the CHO wild type (wt), C-1000 and C5 cells and their routine culture as monolayers in α -medium plus 10% fetal calf serum (growth medium) have been described

previously (Cowan *et al*, 1995). The human HeLa cells (received from Dr. T. Mulcahy of Madison, Wisconsin), the rat mammary carcinoma cell line MatB (received from Dr. G. Batist, McGill University) and human breast carcinoma cell line MCF-7 (purchased from American Tissue Type Culture Collection, Camden, New Jersey) were all cultured under the same conditions as the CHO cell lines.

The BMS181321 was prepared, as described previously (Ballinger *et al*, 1996), from kits supplied by Bracco Research (Princeton, NJ) (Linder *et al*, 1994). Briefly, the ligand BMS181032 was dissolved in saline containing ^{99m}Tc -pertechnetate. A stannous DTPA kit was reconstituted with saline and an aliquot added to the ligand and pertechnetate. After 10 min. at room temperature the radiochemical purity (typically greater than 90%) of BMS181321 was determined by paper chromatography. BMS181321 was diluted appropriately before use.

For BMS181321 accumulation studies, exponentially growing cells were trypsinized, centrifuged and resuspended in fresh growth medium as stirred suspension cultures at 3×10^5 to 1×10^6 cells per ml, total volume of 10 ml in glass vials, flushed with 5% CO_2 balance air (aerobic conditions) or 5% CO_2 balance nitrogen (hypoxic conditions) as described previously (Whillans and Rauth, 1981). After a forty-minute pre-equilibration of the cell suspension at 37°C the drug plus ligand were added to yield a final activity of ~ 0.25 MBq/ml at a concentration of $0.7 \mu\text{g/ml}$ (Ballinger *et al*, 1996). As a function of time 0.1 ml aliquots were removed, spun through a 1 ml oil mixture to separate cells from medium and the cell pellets counted in a γ -well counter as previously described (Ballinger *et al*, 1993). Results were plotted as the cpm in the cell pellet, calculated as a 0.1 ml cell volume equivalent (C_{in}), divided by the cpm in 0.1 ml supernatant (C_{out}). This ratio of $C_{\text{in}}/C_{\text{out}}$ was a measure of the radioactivity accumulated in the

cells relative to the level in the medium (Ballinger *et al*, 1996).

A1.4 Results

Previous results have indicated that BMS181321 accumulation, measured by taking hourly points over a four-hour time period using the spin through oil technique, gave reproducible results using CHO wt cells (Ballinger *et al*, 1996). The accumulation of radioactivity in CHO wt and the 2-NLP-3 drug resistant C-1000 cells in the present experiment are shown in Figure A1.2, as the radioactivity associated with cells to that in the medium, C_{in}/C_{out} . Corrections have been made for non-cellular radioactivity carry-through in the spin through oil technique used (Ballinger *et al*, 1996). This correction was based on the carry-through occurring of BMS181321 in medium without cells layered over the oil phase and handled identically to the cell containing sample. Under aerobic incubation conditions there was a rapid increase in this ratio by five minutes followed by a very gradual increase from a value of fifteen to a value of twenty over the next four hours. The aerobic accumulation curve for C-1000 cells was the same as for CHO wt cells (data not shown). In contrast under acutely hypoxic conditions there was a continuous linear increase in the C_{in}/C_{out} ratio which at four hours was six- to seven-fold greater than the ratio under aerobic conditions for both CHO wt and C-1000 cells. Statistically there was no difference in accumulation under aerobic or hypoxic conditions for CHO wt versus C-1000 cells.

Similar accumulation curves under aerobic and hypoxic conditions were measured for C5, MatB, HeLa and MCF-7 cells from zero to four hours (data not shown). These curves were qualitatively similar to CHO wt and C-1000 cells in that the C_{in}/C_{out} reached a plateau level in aerobic cells within five minutes while with hypoxic cells there was a linear increase with time.

Quantitatively there were significant differences between cell lines in the ratio of C_{in}/C_{out} under hypoxic to C_{in}/C_{out} under aerobic conditions when measured after a two-hour incubation with BMS181321 (Figure A1.3). Both human cell lines (HeLa and MCF-7) showed a greater hypoxic:aerobic ratio than the four rodent cell lines which were not significantly different from one another.

A1.5 Discussion

A consideration in the rational design of chemotherapeutic and diagnostic agents is the degree to which their efficacy may be affected by known mechanisms of drug resistance in different tumour types. One such mechanism of drug resistance is the up-regulation of P-glycoprotein in cells resistant to a wide variety of drugs of varying structure but often containing hydrophobic, multi-ring structures and a positive charge at physiological pH (Endicott and Ling, 1989). An example of this is 2-NLP-3, in which the presence of the phenanthridine ring caused this 2-nitroimidazole to have decreased accumulation in cells with increased levels of P-glycoprotein (Cowan *et al*, 1995).

BMS181321 is the lead compound for a type of 2-nitroimidazole with a side chain that chelates the radionuclide ^{99m}Tc , widely used in nuclear medicine (Linder *et al*, 1994). Upon the chelation of ^{99m}Tc , BMS181321, a hydrophobic structure, is formed which has no net charge. The partition coefficient of BMS181321 in octanol/water is approximately 40, indicating this chelate is quite hydrophobic (Ballinger *et al*, 1996). Since this agent is being investigated as a diagnostic marker for hypoxic cells in solid tumours, it is important to determine if its ability to accumulate in hypoxic cells is P-glycoprotein dependent. The detailed results presented in Figure

A1.2 and survey results presented in Figure A1.3 indicate this is not a problem.

CHO wt and C-1000 cells have similar accumulation curves under aerobic and hypoxic conditions. A large portion, but not all, of the 2-NLP-3 resistance of C-1000 is caused by P-glycoprotein and is reversible by verapamil (Cowan *et al*, 1995). The remaining 2-NLP-3 resistance of C-1000 cells has yet to be explained but it obviously does not affect the accumulation of BMS181321. C5 cells, which appear to have higher levels of P-glycoprotein than C-1000 cells, also show similar accumulation of BMS181321 to CHO wt and C-1000 cells (Figure A1.3). Previous results have indicated that the increased P-glycoprotein levels in C-1000 and C5 cells have no effect on the aerobic or hypoxic toxicity of the 2-nitroimidazole misonidazole (Cowan *et al*, 1995), compared to CHO wt cells. This indicates that enzymes involved in the metabolism of 2-nitroimidazoles are not altered in C-1000 or C5 cells and is consistent with the present results for BMS181321 accumulation.

Limited data on P-glycoprotein overexpressing multi-drug resistant Adr^R MatB (Schechter *et al*, 1991) and Adr^R MCF-7 cells (Batist *et al*, 1986) showed similar BMS181321 accumulation curves under hypoxic and aerobic conditions (data not shown) compared to their wild type counterparts shown in Figure A1.3. Thus in both rodent and human cells P-glycoprotein, as well as other drug resistance mechanisms such as increased glutathione transferase levels present in the Adr^R cells, are not a factor in BMS181321 accumulation under either aerobic or hypoxic conditions. Whether a compound is a substrate for the transmembrane pump P-glycoprotein is not always easy to predict. The drug 2-NLP-3 has the characteristics of a classic substrate, containing a fused planar ring system and bearing a permanent positive charge (Endicott and Ling, 1989). However, the non-ring positively charged compound technetium-99m-sestamibi

(MIBI) used as a myocardial imaging agent, is also a substrate for P-glycoprotein (Piwnicka-Worms *et al*, 1993; Ballinger *et al*, 1995). In fact, this property has led to the suggestion that MIBI may be useful clinically for assessing P-glycoprotein status in solid tumours before and during drug treatment.

The increased accumulation of BMS181321 in the two human cell lines studied compared to the four rodent lines may indicate increased nitroreductase activity in the human versus rodent cell lines. Consistent with this interpretation is previous data that indicates hypoxic HeLa cells accumulate the 2-nitroimidazole misonidazole about twice as rapidly as CHO wt cells and this accumulation was shown to be due to enhanced drug metabolism in the human versus rodent cell lines (Taylor and Rauth, 1978).

Clearly any generalization as to the differences between hypoxic rodent and human cell lines in their ability to metabolize 2-nitroimidazole will require a wider survey of cell lines. Nevertheless the present results indicate the selective accumulation of BMS181321 in hypoxic regions of solid tumours will not be complicated by differential expression of P-glycoprotein and other related drug resistance mechanisms.

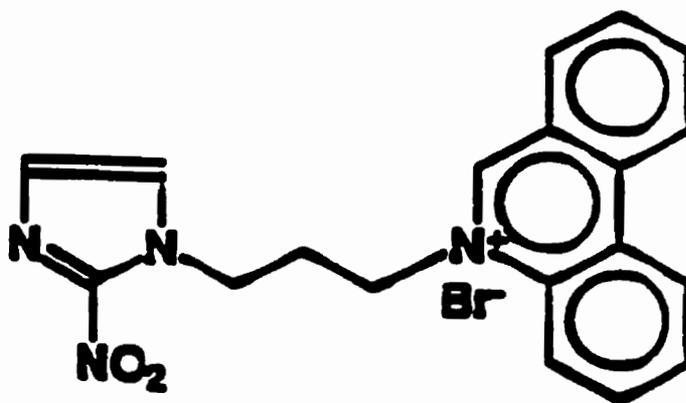


Figure A1.1: Structure of 2-NLP-3.

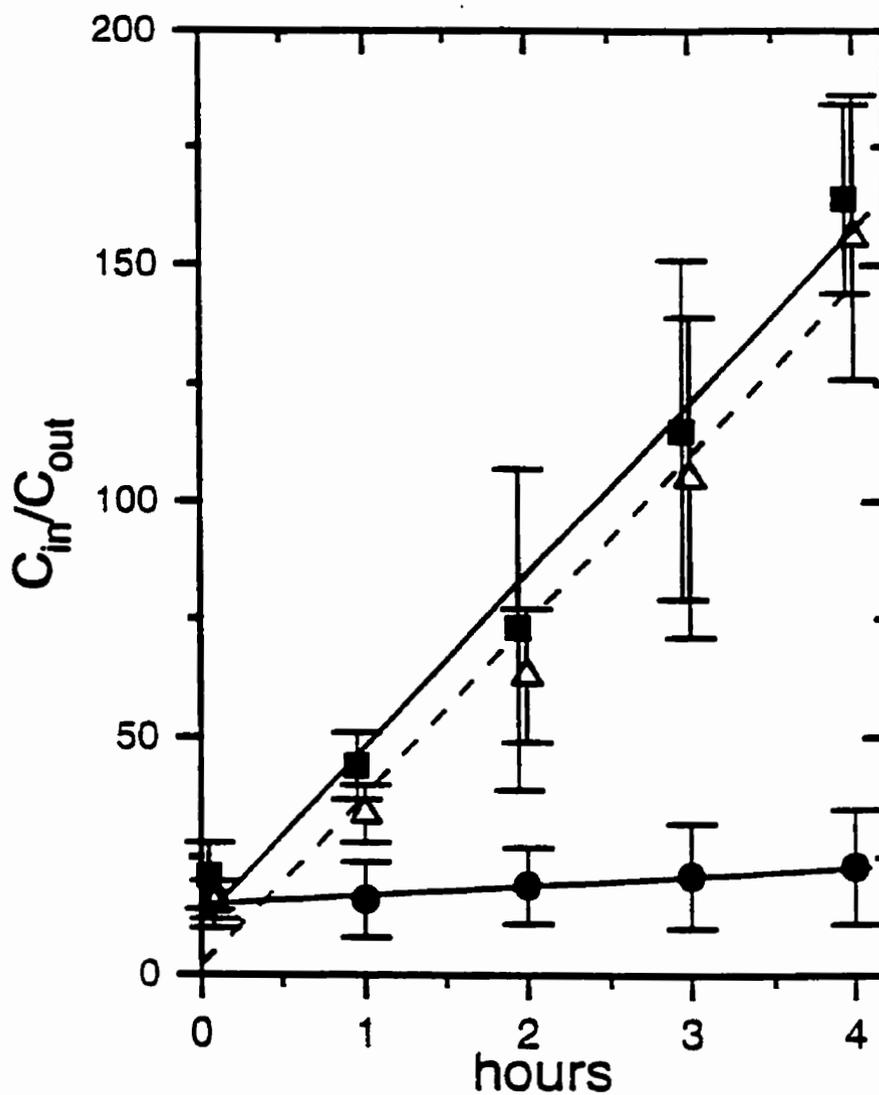


Figure A1.2: Accumulation of BMS181321 in CHO wt cells under aerobic (circles) or hypoxic (squares) conditions compared to C-1000 cells under hypoxic (triangles) conditions. The ratio for activity inside the cell to outside, C_{in}/C_{out} , was measured for cells as a function of time using a spin through oil technique. Points are the mean value of three or more determinations and error bars are standard deviations.

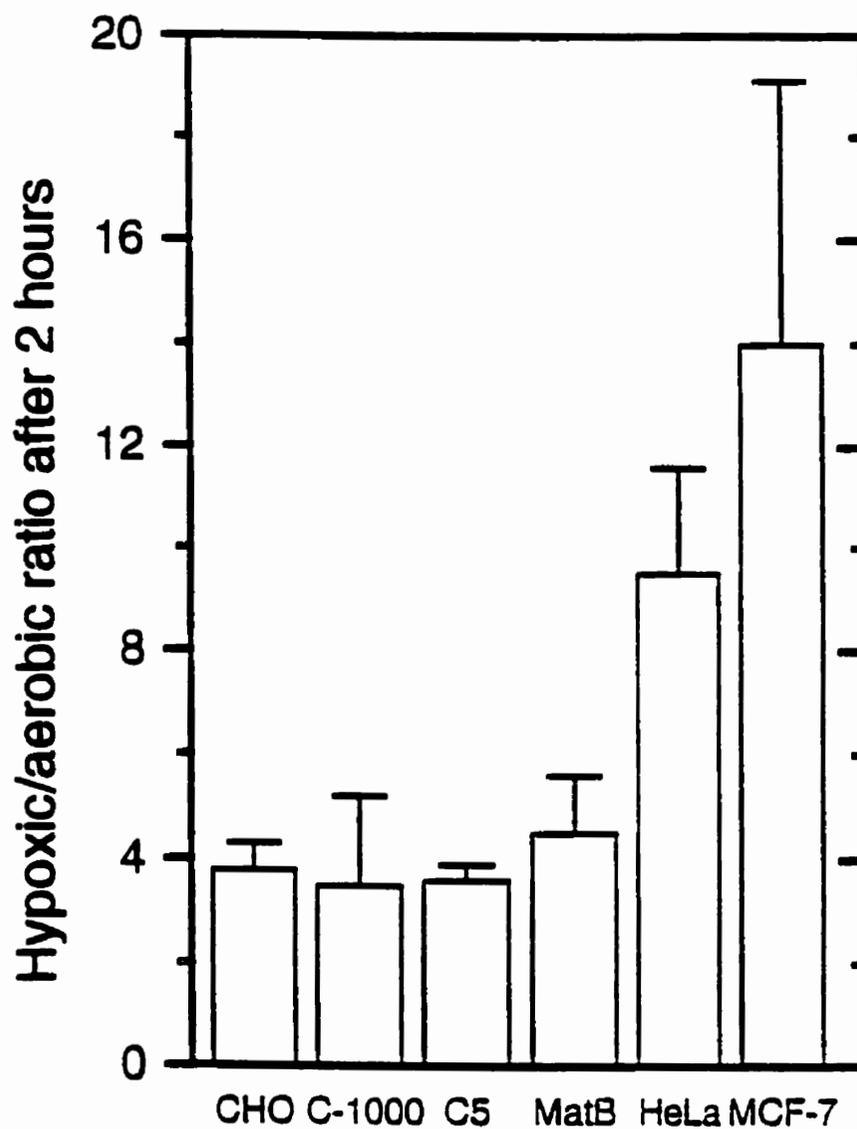


Figure A1.3: The ratio of C_{in}/C_{out} for a two-hour hypoxic incubation to C_{in}/C_{out} for a two-hour aerobic incubation for four rodent and two human cell lines. Bars are the mean value and error bars are standard deviations of the mean for three or more experiments.

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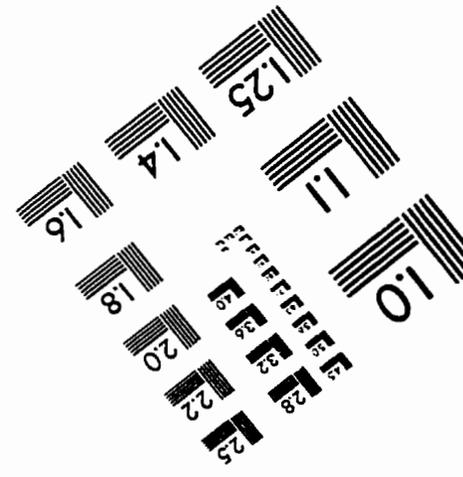
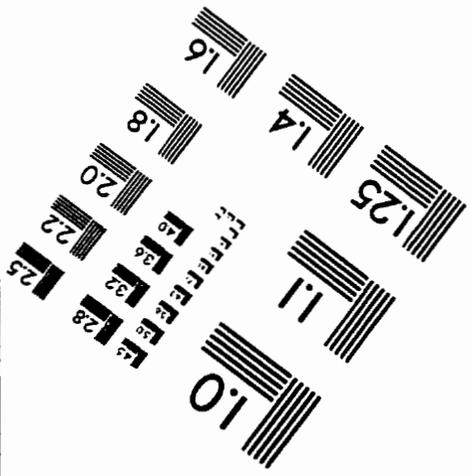
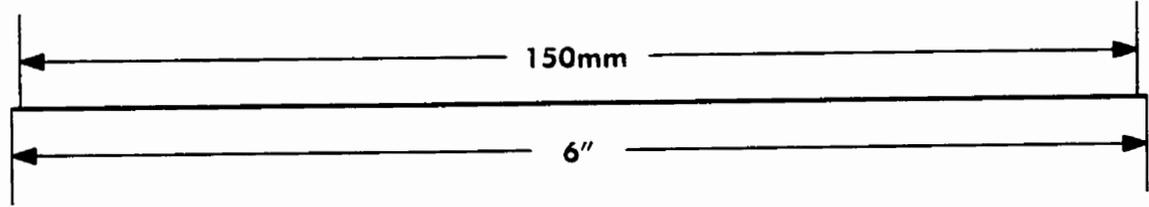
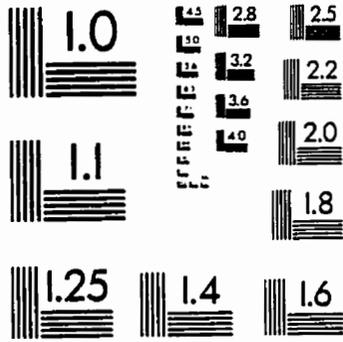
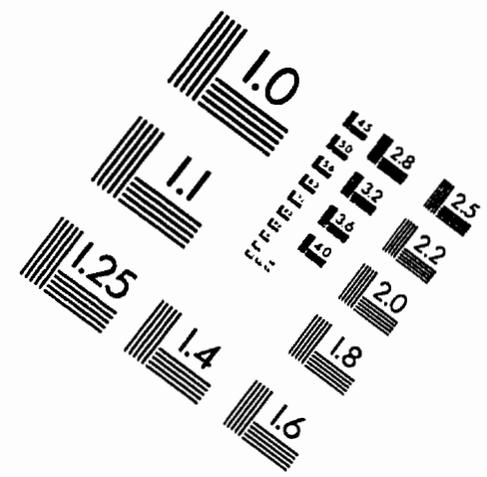
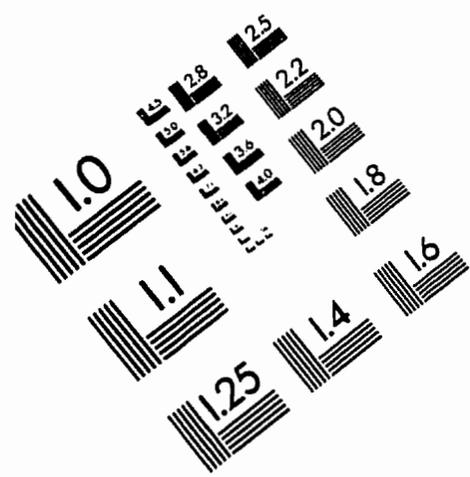
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IMAGE EVALUATION TEST TARGET (QA-3)



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