Anti-Cancer Drugs 1995, **6**, pp. 259–269

Fused pyrazine mono-*N*-oxides as bioreductive drugs III. Characterization of RB 90740 *in vitro* and *in vivo*

Matthew A Naylor,[1] Gerald E Adams,[1] Angela Haigh,[1] Shirley Cole,[1] Terence Jenner,[1] Naomi Robertson,[1] Dietmar Siemann,[2] Miriam A Stephens[1] and Ian J Stratford[1]

[1] Experimental Oncology Division, MRC Radiobiology Unit, Chilton, Didcot OX11 ORD, UK. Tel: (+44) 235 834393; Fax: (+44) 235 834776. [2] Tumor Biology Division, University of Rochester Cancer Center, Box 704, 601 Elmwood Avenue, Rochester, NY 14642, USA.

RB 90740 is the lead compound in a series of aromatic mono-*N*-oxide bioreductive drugs. The compound shows considerably greater toxicity towards hypoxic verses aerobic mammalian cells *in vitro*. The differential in concentration required to give the same level of cell killing under these conditions ranges from 3.5 in a human bronchio-alveolar tumor cell line up to 120 in a rodent cell line defective in the repair of DNA strand breaks. The ability of RB 90740 to cause DNA strand breaks under hypoxic conditions was confirmed by alkaline sucrose gradient and pulsed field gel electrophoresis techniques. Despite these properties demonstrated *in vitro*, RB 90740 was shown *not* to be cytotoxic to hypoxic cells in experimental murine tumors *in vivo*. This may be due, in part, to the level of hypoxia (<0.02% O_2) necessary to produce toxicity *in vitro*.

Key words: Bioreductive drugs, DNA strand breaks, hypoxia, *N*-oxides, reductive activation.

Introduction

Cellular hypoxia is believed to be one of the major factors influencing tumor response to radiotherapy. Reduced oxygenation relative to that in most normal tissues has been demonstrated in a variety of tumor types using micro-electrodes[1–4] and has been related to treatment outcome.[1,5] Further, the presence of hypoxia in some human tumors has been inferred from studies of intra-tumor binding of isotopically-labeled misonidazole and other related nitroimidazoles.[6]

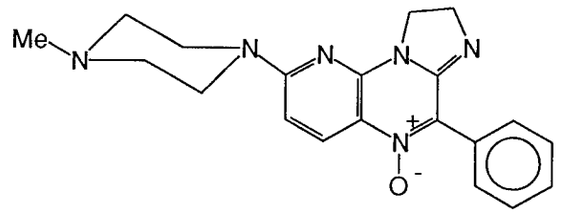
Hypoxic cells are relatively resistant to radiation and can be resistant also to various chemotherapeutic agents. Various strategies have been designed to overcome this resistance, including the use of bioreductive drugs. These agents are considerably more toxic to hypoxic compared to oxic cells and this is the basis for tumor selectivity. Various classes of bioreductive drug have been developed including (i) the 'dual function' alkylating nitroimidazoles, of which the lead compound is RB 6145, a pro-drug of RSU 1069;[7–10] (ii) benzotriazene-di-*N*-oxides represented by tirapazamine, SR 4233;[11–13] and (iii) some mitosenes such as the indoloquinone EO9.[14–17]

Bioreductive drugs are activated by metabolic reduction to form cytotoxic products. Some of the systems involved in the activation processes have been identified and these include: isozymes of the cytochrome P450 system, cytochrome P450 reductase, xanthine oxidase and aldehyde oxidase.[18–20] Under aerobic conditions, metabolic reduction can still occur but the initial one-electron step in this process can be reversed by oxygen. In contrast, reduction under anaerobic conditions can proceed to yield, ultimately, products that are toxic to the cell. The different pathways occurring under oxic and hypoxic conditions provide the basis for the differential effects of bioreductive drugs in hypoxic tumors.

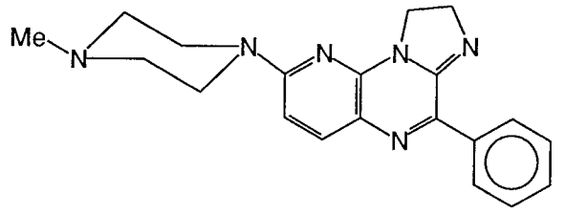
In regard to the potential of organic *N*-oxides as bioreductive drugs, many analogs of tirapazamine have been synthesized and evaluated.[12,21] Tirapazamine remains the lead compound in this series and studies with compounds of this type indicate that retention of the di-*N*-oxide structure is essential for bioreductive activity. The two-electron reduced form of tirapazamine, the mono-*N*-oxide SR 4317, shows no activity.[11] In contrast a novel series of fused pyrazine mono-*N*-oxides recently developed in our laboratory does show activity as bioreductive drugs.[22] The lead compound is RB 90740 (1,2-dihydro-8-(4-methylpiperazin-1-yl)-4-phenylimidazo[1,2-*a*]pyrido[3,2-*e*]pyrazine 5-oxide) (Figure 1).[23–25] This report describes a study, using a panel of rodent and human tumor cell lines, of the cytotoxic effects of RB 90740 under oxic and hy-

Supported by US NCI grant no. PO1-CA-55165 and the British Technology Group.

Correspondence to IJ Stratford



RB90740



RB92815

Figure 1. Structures of the fused pyrazine *N*–oxide RB 90740 and its two-electron reduced product RB92815.

poxic conditions as well as at a range of intermediate oxygen concentrations. The cell lines chosen for this work express different abilities for repair of DNA damage and also show different levels of some of the enzymes important for bioreductive activation. The ability of RB 90740 to cause single- and double-strand breaks in hypoxic cells was measured and assessment *in vivo* made of the activity of RB 90740 in experimental tumors.

Materials and methods

Cytotoxicity *in vitro*

Cells. Chinese hamster cells and mutants derived from them, together with a range of rodent and human tumor cells, were used to evaluate the cytotoxic properties of RB 90740. All cell lines, with the exception of KHT, were maintained in exponential growth in RPMI 1640 (ICRF Clare Hall Laboratories) supplemented with 2 mM glutamine and 10% fetal calf serum (FCS; Sera). KHT cells for *in vitro* experiments were maintained in α-MEM plus 10% FCS.

MTT assay. This proliferation assay is based on the ability of viable cells to convert a soluble tetrazolium salt, MTT (3-4,5-dimethylthiazole-2,5-diphenyl tetrazolium bromide), into purple formazan crystals.[26] The optical density of the dissolved crystals is proportional to the number of viable cells, although the sensitivity varies with cell line as the assay depends on the activity of mitochondrial dehydrogenases.[27] The conditions for carrying out the assay have been described for V79 cells[28] and for human cells.[27,29] The optimum conditions for the human tumor cells require plating 10^4 to 5×10^4 cells (depending on cell line) into each well of a 24-glass-well dish 3 h before exposure to the bioreductive agent for 3 h at 37°C either in air or hypoxia. The drug was then removed, 0.5 ml of fresh medium added and the cells allowed to grow for 4 days. In some experiments, aerobic exposure to the drug was allowed to continue for 4 days in 96-well plates.

Clonogenic assay. Assay of Chinese hamster cell survival was carried out either by plating 1.5×10^6 cells into 100 ml flat bottles and allowing the cells to grow for 48 h to reach confluence, or by seeding 2×10^2 or 2×10^3 cells for an exponentially growing culture into 6 cm glass plates and allowing them to attach for 2–3 h. Subsequently, cells were treated with various concentrations of RB 90740 for 3 h at 37°C under either hypoxic or aerobic conditions. Confluent cultures were then washed, trypsinized, diluted and cells plated for colony formation. For exponential cells attached to glass plates, the drug was removed, replaced with fresh medium and the surviving cells scored as colonies 7 days later. KHT, HT29 and A549 cells were inoculated (2×10^5 cells/ml) into medium containing drug that had been equilibrated with air or N_2 plus 5% CO_2 in glass vials.[30] Gas flow through the vials continued during the 4 h drug exposure at 37°C. Cells were removed at the end of treatment, rinsed, counted and various dilutions plated for colony formation.

Toxicity as a function of oxygen concentration

Chinese hamster V79 cells were used for these experiments and survival was measured by clonogenic assay. Exponentially growing cells (50 ml, 2×10^5 cells/ml) were set up in 250 ml spinner flasks in the presence or absence of 0.15 mM RB 90740.[31] Varying concentrations of O_2 in N_2 plus 5% CO_2 flowed over the surface of the stirred suspension for 4 h. After this time, samples of cells were removed, centrifuged to remove RB 90740, resuspended, counted, diluted and plated for colony for-

mation. In a parallel series of experiments V79 cells were irradiated at different oxygen tensions. This was done by allowing varying numbers of cells to attach to 6 cm glass Petri dishes for 2 h prior to adding drug to cells and placing dishes in Dural chambers.[32] These vessels could be gassed with the varying oxygen concentrations used in the above toxicity experiments. After gas equilibration for 1 h, the Dural containers were sealed and irradiated with 14 Gy γ-rays. Immediately after irradiation vessels were opened, drug solutions removed from plates and replaced with fresh medium. Dishes were then incubated for 7 days to allow for colony formation.

Induction of DNA strand breaks

V79-379A cells ($1.2–1.5 \times 10^6$) were seeded into 50 ml Falcon flasks with growth medium containing 0.5 μCi/mmol [^{3}H]thymidine (25 mCi/mmol; Amersham) in order to label the cells. Cultures were incubated for about 24 h at 37°C. The active medium was then replaced with fresh medium supplemented with cold thymidine (0.1 mg/ml final concentration) and cells incubated for 1 h. The cells were trypsinized, resuspended in label-free medium (MEM modified for suspension cultures) and treated with varying concentrations of RB 90740 under aerobic or hypoxic conditions for 3 h at 37°C. After the incubation, cell suspensions were placed on ice to minimize further damage and/or repair. RB 90740 was removed by centrifugation and the cell pellet resuspended in 0.2 ml of 10 mM Tris/1 mM EDTA/150 mM NaCl at pH 7.5.

DNA single- and double-strand breaks (ssbs and dsbs) were determined by sedimentation in isokinetic sucrose gradients following cell lysis and DNA release. The methods for cell lysis, gradient formation and subsequent density sedimentation under alkaline (ssbs) or neutral (dsbs) conditions have been described.[33,34] DNA dsbs were also measured using the technique of pulsed field gel electrophoresis (PFGE).[35,36] Briefly, after drug exposure, cells (4×10^6 cells/ml) were prepared as plugs by resuspending in low melting point agarose (LMPA) at 37°C and pipetting immediately into moulds at 4°C. Plugs were then placed in ice-cold lysis solution containing 1 mg/ml proteinase K in 2% lauroylsarkosine/0.5 M EDTA, pH 7.6, for 1 h followed by incubation at 37°C for 24 h. Subsequently, cell plugs were sectioned into samples of approximately 40 mm^3 and loaded into the wells of a 0.8% agarose gel (14 cm wide × 12 cm long × 1 cm thick). The wells were then sealed with 0.8% LMPA. The PFGE unit gives homogenous pulsed fields at 120° to each other (Bio-Rad CHEF-DRII). Electrophoresis conditions were: 0.5 × TBE buffer (Sigma), switching time 60 min, 45 V for 96 h. Buffer temperature was maintained at 16°C. Following electrophoresis, the gels were stained in 0.5 μg/ml ethidium bromide for 1 h then destained in distilled water for 2–4 h. Gels were photographed under UV illumination. Gels were then cut into 5 mm sections, melted slowly in 100 μl 1 M HCl, the liquefied samples mixed with scintillation fluid and the isotope activity determined as disintegrations per minute (c.p.m.). The number of dsb induced in DNA of irradiated cells is believed to be related to the fraction of DNA which is fragmented below the threshold size and so is able to migrate under PFGE. The fraction extracted (FE) is the ratio of isotope counts detected in the sample lane to the total counts, i.e.

$$\mathrm{FE} = \frac{\text{c.p.m. lane}}{\text{c.p.m. lane} + \text{c.p.m. well}}$$

All results are expressed as a percentage retained where:

$$\text{percentage retained} = [1 - (\mathrm{FE}_{\text{treated}} - \mathrm{FE}_{\text{control}})] \times 100$$

Tumor toxicity *in vivo*

Tumor models. The RIF-1 and KHT murine sarcoma lines were maintained as described previously.[37,38] Approximately 2×10^5 cells in 0.05 ml PBS were implanted intra-dermally into the mid-dorsal pelvic region of 8–12 week old C3H/He mice (category 4). Females were used for KHT and males for RIF-1. Tumors were used for treatment when they reached a volume of 100–200 mm^3, generally 11–15 days after implant. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, under project licence no. 30/0086.

Maximum tolerated dose (MTD). Escalating doses of RB 90740 were administered by the intraperitoneal route in a volume of 0.5 ml/25 g body weight to groups of two or three tumor-bearing mice. In these and all subsequent experiments RB 90740 was dissolved in phosphate buffered saline (pH 7.4), protected from light and injected within 10 min. The MTD was defined as the highest single dose which did not produce severe or persistant clinical signs or death of the animals within 24 h.

Assays of radiation response. The response of RIF-1 tumors to therapy was measured by growth delay. Treatment groups consisted of six to 10 mice. The volume of each tumor (calculated from three orthogonal diameters measured with graduated vernier callipers) was measured prior to treatment and subsequently three times weekly. The end-point was the time to reach four times the volume of tumor at treatment. The response of KHT tumors to treatment with radiation and RB 90740 was measured by clonogenic assay *in vitro*.[38] Twenty four hours after treatment, KHT tumors were excised, single cell suspensions prepared by trypsinization and filtration, and cells plated in soft agar with heavily irradiated KHT feeder cells and rat red blood cells. At 14 days later colonies of 50 or more cells were scored as being derived from a single surviving KHT tumor cell.

Irradiations. A Pantac X-ray was used to produce 250 kV X-rays (15 mA) at a dose rate of 3.8 Gy/min, with filtration giving an HVL equivalent to 1.3 mm Cu. Doses were monitored with an air chamber corrected for ambient temperature and pressure. Unanesthetized mice were restrained in polyvinyl jigs with lead shielding and a cut-away section to allow local irradiation of the tumor by the unilateral beam.[39] Up to four mice in jigs were mounted onto a collimator plate on the head of the X-ray set and jigs were turned through 180° halfway through the exposure time.

Experimental design. Tumor-bearing mice were given doses of irradiation that were chosen to kill most of the oxic cells in the tumors. Thus, the measured response will then reflect the survival of residual hypoxic clonogenic cells.[40] RB 90740 was given immediately after X-rays such that any therapeutic response greater than that achieved by radiation alone is likely to be a reflection of killing by the drug of the residual hypoxic cells. These conditions were achieved by giving 10 Gy to KHT tumors and 25 Gy to RIF-1 tumors. Each experiment also included mice exposed to radiation without drug and drug without radiation.

Results

Toxicity *in vitro*

The cytotoxicity of RB 90740 was assessed under both aerobic and hypoxic conditions in a range of tumor cell lines. Data from experiments with two rodent and two human tumor cell lines where clonogenic cell survival was used to assess the activity of RB 90740 are given in Figure 2. In each line, RB 90740 is considerably more toxic to hypoxic compared with aerobic cells. The differential toxicity is expressed as the ratio of the concentration required to reduce surviving fraction to 0.1 in aerobic cells to that in hypoxic cells. For V79 cells, for example, the concentrations are of 2.5 and 0.1 mM in air and hypoxia, respectively, giving a differential toxicity of 25. Values for all cell lines used are shown in Table 1. Data for V79 cells and three human tumor cell lines treated with RB 90740 under both aerobic and hypoxic conditions, but with toxicity measured using the MTT assay, are given in Figure 3. The value of differential toxicity is determined from the ratio of concentrations, under aerobic or hypoxic conditions, required to cause a 50% decrease in the optical density of treated cells relative to untreated controls. These values are given in Table 1 and are compared with those obtained using the clonogenic assay. Results for the nine cell lines shown in Table 1 illustrate the generality of the activity of RB 90740 as a bioreductive drug with values of differential toxicity ranging from 3.5 for H322 to 25 for V79 cells.

The greater toxicity of RB 90740 towards hypoxic cells is further demonstrated by results derived from exponential cultures of various Chinese hamster cell types and mutants derived from them. The characteristics of these cells and their responses to RB 90740 under aerobic and hypoxic conditions are shown in Table 2. All the cell lines have similar sensitivity to RB 90740 in air but responses under anaerobic conditions differ markedly and this is reflected in the different toxicity ratios. The CHO-MMC^R cell line has lower levels of cytochrome P450 reductase than wild-type CHO-K1 cells[45] and is almost twice as resistant to RB 90740, which could suggest that P450 reductase may be involved in activation of this drug. No cross-sensitivity in air or N_2 is seen in the bleomycin sensitive cell line, BLM-2.[44] In contrast, large hypoxic/aerobic differentials are found with the DNA repair-deficient mutants. The V79-IRS1 cell line is sensitive to ionizing radiation but shows marked cross-sensitivity to UV, alkylating agents and mitomycin C. This cell line is believed to be defective in the repair of DNA strand breaks.[42] The CHO-UV41 cells are also sensitive to UV, mitomycin C and cross-linking agents. The defect in this cell line is thought to be in the initial incisive step in DNA excision repair.[43] The increased sensitivity of these cell lines to RB 90740 under anaerobic conditions suggests that the DNA repair

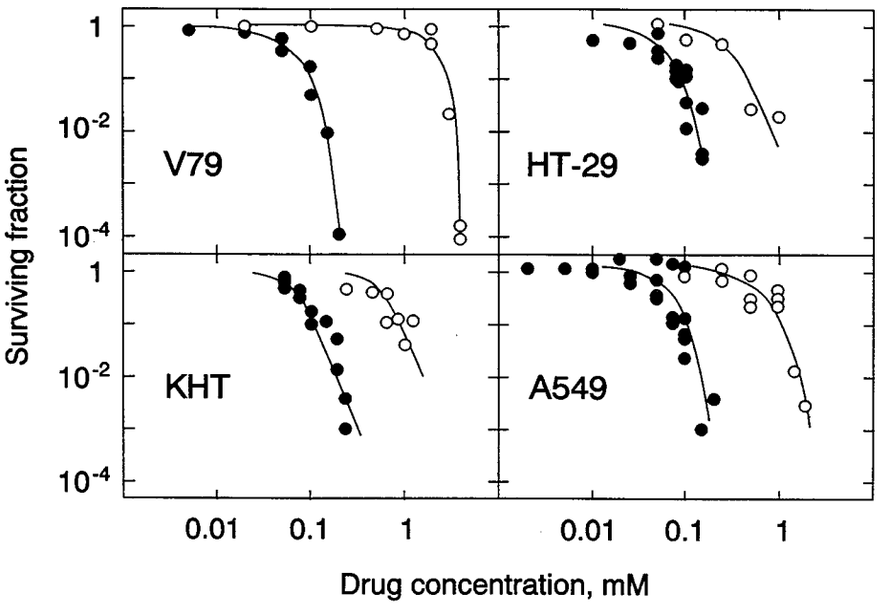


Figure 2. Toxicity of varying concentrations of RB 90740 towards mammalian cells *in vitro* measured by clonogenic assay. Confluent V79 cells were exposed to drug for 3 h.[41] The other cell types were derived from exponential cultures and exposed to RB 90740 for 4 h.[30] Each point represents an individual determination at each concentration. ●, Hypoxic exposure; ○, aerobic exposure.

Table 1. Toxicity of RB 90740 in mammalian cells *in vitro*

Cell line	Origin/histology	Assay[a]	Aerobic toxicity	Hypoxic toxicity	Ratio[b]
V79	hamster lung	MTT	0.8	0.05	16
	fibroblast	clonogenic	2.5	0.1	25
KHT	murine sarcoma	clonogenic	0.9	0.11	8.2
HT29	human	MTT	1.2	0.07	17
	colonic adenocarcinoma	clonogenic	0.46	0.09	12
A549	human lung	MTT	2.6	0.18	14
	adenocarcinoma	clonogenic	1.1	0.085	13
H647	human lung adeno-squamous carcinoma	MTT	1.8	0.15	12
H322	human, lung bronchio-alveola carcinoma	MTT	1.3	0.37	3.5
H460	human, lung large cell carcinoma	MTT	2.8	0.15	19
MDA468	human, breast adenocarcinoma	MTT	1.3	0.07	19
T47D	human, breast adenocarcinoma	MTT	1.5	0.11	14

[a] Values of toxicity quoted for the MTT assay are values of IC_{50}, the concentration of drug required to reduce optical density by 50% relative to untreated controls. For the clonogenic assay the values are IC_{90}, the concentration of drug required to reduce surviving fraction to 0.1. Concentrations are given in mmol/dm^3.
[b] Ratio = aerobic toxicity/hypoxic toxicity.

process defective in these cells may be important in influencing the response to RB 90740.

It is reasonable to suspect that the *N*-oxide function is a necessary requirement for the differential hypoxic cell toxicity of RB 90740. The two-electron reduction product of RB 90740 has been synthesized[22] (RB 92815) and the toxicity of its hydrochloride salt (RB 92816) determined in V79 cells under both hypoxic and aerobic conditions. The data in Figure 4 indicate that there is no clear difference

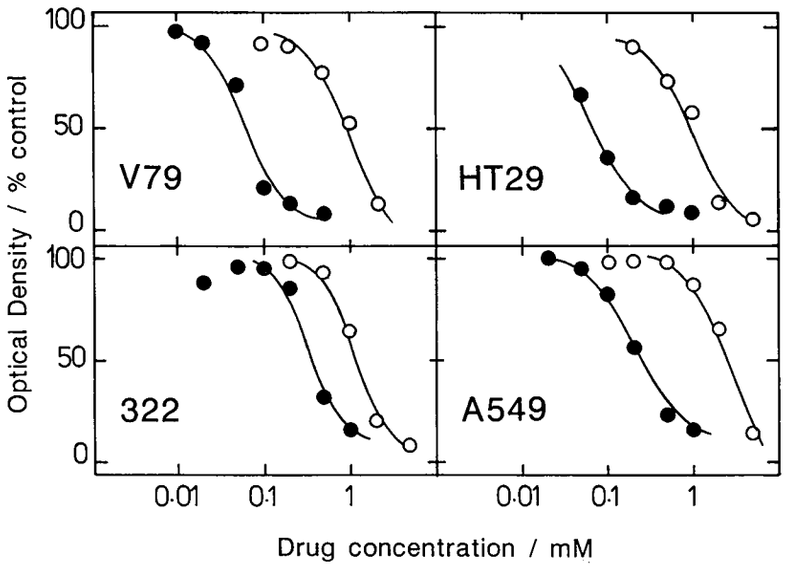


Figure 3. MTT assay of the toxicity of RB 90740 towards mammalian cells *in vitro*. Exposure was for 3 h at 37°C under hypoxic (●) or aerobic (○) conditions. Data points are mean values from up to eight independent experiments; curves were fitted by eye.

Table 2. Toxicity of RB 90740 in Chinese hamster cell lines and mutants derived from them

Cell line	Characteristic	IC_{90} (mmol/dm^3)[a]		Ratio
		Air	N_2	
V79	wild-type	2.1	0.07	30
V79-IRS1	radiation sensitive[42]	1.6	0.013	120
CHO-AA8	wild-type	3.0	0.08	37
CHO-UV41	DNA cross-line repair deficient[43]	2.0	0.022	85
CHO-K1	wild-type	2.0	0.16	13
CHO-BLM-2	bleomycin sensitive[44]	1.7	0.13	13
CHO-MMC^R	mitomycin C resistant[45]	2.0	0.26	7.7

[a] IC_{90} values derived from clonogenic assay of exponential cells exposed to RB 90740 for 3 h

between the two sets of data giving an IC_{50} value for both hypoxic and aerobic cells of 0.4 mM, which is 2-fold higher than the IC_{50} value for RB 90740 in air. These results provide strong support that the *N*-oxide function in RB 90740 is a necessary requirement for reductive activation to a cytotoxic metabolite, which cannot be the two-electron-reduced product.

Production of DNA damage

Damage to DNA is known to be involved in the hypoxic cytotoxicity of *N*-oxides such as tirapazamine.[46] Experiments were carried out, using assays of both ssbs and dsbs in DNA, to investigate the mechanisms of the cytotoxic effect of RB 90740. Hypoxic Chinese hamster V79 cells were exposed to different concentrations of RB 90740 for 3 h at 37°C. The yields of the ssbs and dsbs produced are plotted in Figure 5 as a function of drug concentration. The data show clearly that RB 90740 causes ssbs with an indication of some dsb induction also. Over the same concentration range there is no effect on aerobic cells (data not shown).

In order to assess the incidence of ssbs and dsbs measured by the sucrose sedimentation assay we have chosen the concentration of RB 90740 which correspond to the yield of ssbs induced by 110 Gy of X-irradiation under aerobic conditions. For ssbs the value of 2×10^{-8} representing the reciprocal molecular weight for 110 Gy corresponds to about 125 ssbs/10^{10} Da. The concentration of RB 90740 equivalent to 110 Gy of damage is about 0.2 mmol/dm^3 which is about 3 times greater than the IC_{50} value in these cells. This concentration of RB 90740

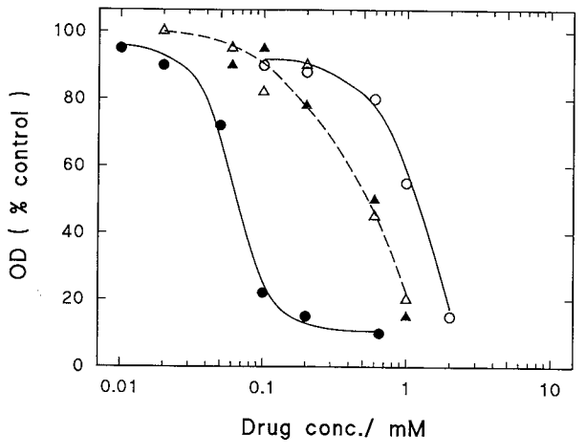


Figure 4. MTT assay of the toxicity of RB 92816 towards Chinese hamster V79 cells *in vitro* following exposure for 3 h at 37°C under hypoxic (▲) or aerobic (△) conditions. Each point represents an individual determination of survival at each concentration. Solid lines indicate the survival curves for RB 90740 in air (○) or hypoxia (●) in a parallel series of experiments.

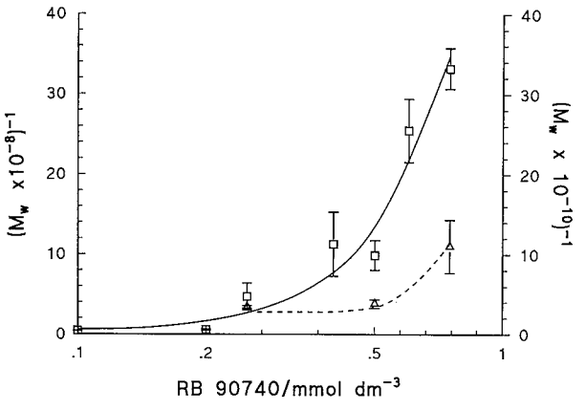


Figure 5. DNA strand breakage caused by RB 90740 under anaerobic conditions and measured by sucrose gradient techniques. Dependence of the yield of DNA breaks on concentration of RB 90740: ssbs (□); dsbs (△). Drug exposures were 3 h at 37°C. Error bars are mean values (±SE) from at least three separate experiments.

does not yield dsbs by this assay. However, the concentration of RE 90740 equivalent to 110 Gy of dsb damage (i.e. $1/MW = 5 \times 10^{-10})^{34}$ is 0.65 mmol/dm^3. This concentration produces at least one order of magnitude more ssbs than the concentration reported above. Therefore, it seems most likely that dsbs are produced by overlapping ssbs.

The production of dsbs in V79 cells following treatment with RB 90740 is confirmed by using the technique of PFGE (Figure 6). This is considered to be a more sensitive method of analysis[47] and results given in Figure 6 show that dsbs can be detected at lower concentrations of RB 90740 than is possible using sucrose gradients.

Tumor toxicity *in vivo*

The MTD of RB 90740 in tumor-bearing C3H mice was determined to be 100 mg/kg (0.28 mmol/kg) which is similar to the di-*N*-oxide SR 4233 (tirapazamine). The MTD or 50 or 20 mg/kg doses of RB 90740 were given to mice immediately after a dose of radiation to the RIF-1 or KHT tumors. Growth delay (RIF-1) and cell survival (KHT) data are summarized in Tables 3 and 4, respectively. The RIF-1 tumor takes approximately 6–7 days to increase in volume 4-fold from a starting volume of about 100 mm^3. Following 25 Gy the time required is 33 days. Giving RB 90740 immediately after irradiation appears to result in a small additional delay in the time for a 4-fold increase in volume, when compared with radiation alone. When RIF-1 tumors are given 30 Gy, the time taken for a 4-fold increase in tumor volume is 45.7 ± 6.2 days (n = 7) (Bremner and Stratford, unpublished). Hence, any increase in therapeutic efficiency by using RB 90740 in combination with radiation is equivalent to an enhancement ratio of less than 1.2. However, this effect is small when compared with that achievable with the bioreductive drugs RSU 1069 and tirapazamine

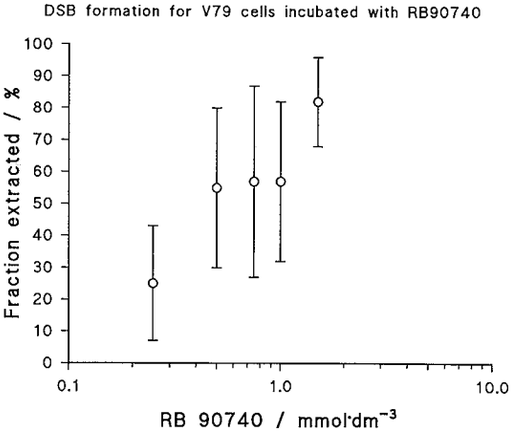


Figure 6. The dependence of the induction of double-strand DNA breaks on concentration of RB 90740 as determined by PFGE. Drug exposure was 3 h at 37°C. Error bars are mean values (± SE) derived from at least three separate experiments for each concentration.

Table 3. The effect of bioreductive drugs given intraperitoneal to C3H mice on the growth of the RIF-1 tumor (drugs were immediately given after a 25 Gy X-ray dose; assay was by growth delay)

Treatment	Drug dose (mg/kg)	Time to 4-fold volume (days)	n^a
Control	–	6.3 ± 0.8	29
RB90740 only	100	5.3 ± 0.4	14
25 Gy only (XR)		32.9 ± 1.4	41
XR + RB90740	100	35.0 ± 1.9	14
XR + RB90740	50	37.9 ± 2.3	28
XR + RB90740	20	40.7 ± 6.4	7
XR + RB90740	60 (i.v.)[b]	41.2 ± 4.1	6
XR + tirapazamine	50	59.6 ± 4.7	4 + 2 cures
XR + RSU 1069	80	45.2 ± 3.4	27

[a] These totals are made up from sub-groups of four to nine mice that were treated at different times over a period of 18 months.
[#b] MTD of RB 90740 by intravenous route.

Table 4. The effect of bioreductive drugs given immediately after 10 Gy X-rays on the survival of KHT tumor cells following treatment

Treatment	Drug dose (mg/kg)	Surviving fraction	No. of tumors
RB90740 alone	100	7.6×10^{-1}	6
10 Gy only (XR)	–	2×10^{-2}	15
XR + RB90740	100	9×10^{-3}	6
XR + RB90740	50	9×10^{-3}	8
XR + RB90740	20	1.8×10^{-2}	6
XR + tirapazamine	50	1.3×10^{-3}	6
XR + RSU 1069	80	$1.0 + 10^{-3}$	12

when they are administered to tumor-bearing mice immediately post-irradiation.

The relatively small effect of RB 90740 in the RIF-1 tumor is confirmed in the KHT tumor which has a 10-fold greater fraction of hypoxic cells.[48] Data in Table 4 show that 10 Gy alone reduces the survival of KHT cells to 2×10^{-2} and there is about a further 2-fold reduction when RB 90740 is given after irradiation. In contrast, when RSU 1069 or tirapazamine are given post-irradiation, an additional 10-fold increase in cell killing is obtained. This latter level of cell kill is equivalent to complete sterilization of the radiation resistant hypoxic cells in this tumor model.[40]

Discussion

In this paper we describe the evaluation of the fused pyrazine mono-*N*-oxide RB 90740 as a bioreductive drug *in vitro* and *in vivo*. This compound is the first heterocyclic mono-*N*-oxide to demonstrate greater toxicity towards hypoxic relative to aerobic cells. This has been demonstrated in 15 cell lines (eight rodent and seven derived from human tumors). The ratio of concentrations required to produce toxicity under aerobic versus hypoxic conditions in the human tumor cell lines varies from 19 to 3.5. It is likely that the variation in toxicity between cell lines will primarily have its basis in differing abilities to activate RB 90740. We have already profiled a panel of human breast and lung tumor cell lines for their activity of the reductase DT-diaphorase and measured a 5000-fold variation in enzyme levels.[29] Some previous data showed no trend for the aerobic toxicity of RB 90740 to be dependent on the expression of this particular reductive enzyme[23] and this is confirmed in the present work where cell lines with widely varying levels of DT-diaphorase show similar aerobic toxicities (e.g. MDA 468 and HT 29 have enzyme activities of 63 and 2750 nmol cytochrome *c*, reduced/min/mg protein and values of IC_{50} of 1.3

and 1.2 mmol/dm^3, respectively). In addition, little dependence of hypoxic toxicity on DT-diaphorase activity is apparent. In contrast, cytochrome P450 reductase may be implicated in the hypoxic activation of RB 90740. This is inferred from the slight increase in resistance of the CHO-MMC^R cells compared to wild-type CHO-K1 cells under anaerobic conditions.

Other studies with the mutant cells strongly suggest that DNA damage is important for expression of toxicity. The DNA repair deficient cell lines V79-IRS1 and CHO-UV41 show large values in differential toxicity (120 and 85, respectively) when compared with the wild-type lines. This is primarily due to an increase in toxicity under hypoxic conditions. Indeed, RB 90740 produces DNA ssbs and dsbs in V79 cells following treatment under anaerobic conditions. These breaks are measured under steady state conditions in that induction of damage and its repair will be going on during the exposure period. At this stage we have not examined the repair of the damage remaining after the 3 h drug exposure. Nevertheless, the data currently suggest that the dsbs may result from overlapping ssbs. Also, there may be some equivalence between damage caused by radiation and that caused by RB 90740 under anaerobic conditions (Jenner, unpublished data). However, the repair of such damage may be very different.

The two-electron reduction of RB 90740 yields the *N*-deoxy compound RB 92816. The effect of this agent has been determined in V79 cells and no differential hypoxic toxicity is observed. This implies that not only is the *N*-oxide moiety important but it is the process of reduction that leads to toxicity, i.e. one-electron reduction generating a radical species which may react with DNA to produce lesions.[24]

Despite the profound activity of RB 90740 *in vitro*, little if any activity is demonstrated *in vivo* using the KHT and RIF-1 tumor models. KHT cells *in vitro* show about a 10-fold hypoxic to aerobic differential cell killing. Hence, the lack of effect *in vivo* is unlikely to be due to deficiency of activating enzymes. Pharmacokinetic and drug distribution studies have been carried out with RB 90740 in C3H mice carrying KHT tumors. In these studies, extremely high and persistent levels of RB 90740 were achieved in tumors and tumor:blood ratios of about 10 were obtained. The pharmacokinetic AUC for RB 90740 in KHT tumors was 28.3 mg/ml/min or 78 mM/min.[25] Inspection of Table 1 shows that the IC_{90} for killing of hypoxic KHT cells is 0.11 mM, which was determined following 4 h exposure to drug. Thus, the product of concentration and time is 26.4 mM/min, which is considerably lower than the AUC. Therefore, it would be predicted that RB 90740 should show substantial toxicity towards hypoxic KHT tumor cells *in vivo*—this is not the case.

An explanation may come from the results of experiments on the oxygen concentration dependence for RB 90740 toxicity *in vitro*. Figure 7 shows a plot of surviving fraction of V79 cells exposed to 0.15 mM RB 90740 for 4 h under various conditions of oxygenation. The survival of cells under hypoxic conditions is about 10^{-3} and a similar level for survival is obtained at an equilibrium oxygen concentration of 0.02% in the gas phase. As the oxygen concentration is further increased, cells become progressively more resistant to RB 90740, such that at 0.1% oxygen the level of cell killing is only about 10^{-1}. Also shown in Figure 7 is the oxygen concentration dependence for killing of V79 cells by radiation—14 Gy γ-rays in air reduces survival to about 10^{-4}. The surviving fraction increases substantially as the oxygen concentration decreases from 2 to 0.1% and little additional cell killing occurs as oxygen levels are decreased further. It is apparent from this combined set of data that there is a window of oxygen concentration where neither radiation nor RB 90740 effectively kills cells. It is possible that this level of oxygenation is similar to that which limits the outcome of treatment of KHT and RIF-1 tumors with radiation *in vivo*. If so, it is not surprising that RB 90740 shows little enhancing effect when combined with radiation *in vivo*. Recently Koch[49] using V79 cells has demonstrated that *no* such 'resistance' window of oxygen concentration exists for tirapazamine when combined with

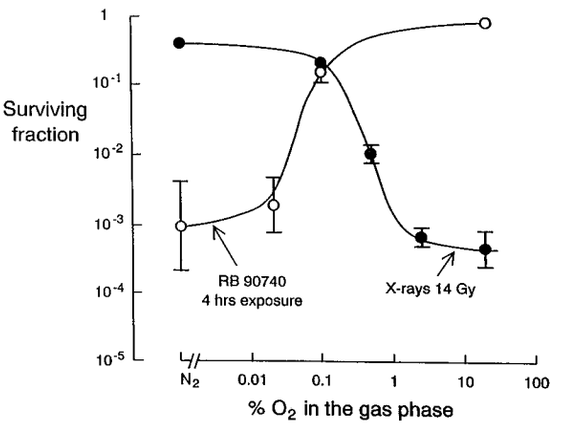


Figure 7. Surviving fraction of V79 cells as a function of oxygen concentration in the gas phase: ○, cells exposed to 0.15 mM RB 90740 for 4 h at 36°C; ●, cells given 14 Gy γ-rays.

radiation. This is consistent with the high activity of this agent when combined with radiotherapy to treat tumors *in vivo*.

In conclusion we have clearly demonstrated marked activity of the heterocyclic mono-*N*-oxide, RB 90740, as a bioreductive drug *in vitro*. This is not reflected by activity *in vivo*, which may be due to the requirement for extremely low levels of oxygen to be present before the toxicity of RB 90740 can be expressed. Further development of these aromatic mono-*N*-oxides is being carried out with a view to reducing this dependence on such extreme levels of hypoxia for toxicity.

Acknowledgments

We would thank Drs JCM Bremner, EM Fielden and P O'Neill for their helpful and constructive comments. Melanie King, Carol Williams and John Bowler are thanked for their skilled and dedicated help with the *in vivo* experiments.

References

1. Gatenby RA, Kessler HB, Rosenblum JS, *et al.* Oxygen distribution in squamous cell carcinoma metastases and its relationship to the outcome of radiation therapy. *Int J Radiat Oncol Biol Phys* 1988; **14**: 831–8.
2. Vaupel P, Schlenger K, Knoop C, *et al.* Oxygenation of human tumours: evaluation of tissue oxygen distribution in breast cancers by computerized O_2 tension measurements. *Cancer Res* 1991; **51**: 3316–22.
3. Hockel M, Schlenger K, Knoop C, *et al.* Oxygenation of carcinomas of the uterine cervix: evaluation by computerized O_2 tension measurements. *Cancer Res* 1991; **51**: 6098–102.
4. Lartigau E, Martin L, Lambin P, *et al.* Mesure de la pression partielle en oxygéne dans des tumeurs du col utérin. *Bull Cancer/Radiotherapie* 1992; **79**: 199–206.
5. Hockel M, Knoop C, Schlenger K, *et al.* Intratumoral pO_2 predicts survival in advanced cancer of the uterine cervix. *Radiother Oncol* 1993; **26**: 45–50.
6. Chapman JD. Measurement of tumour hypoxia by invasive and non-invasive procedures—a review of recent clinical studies. *Radiother Oncol* 1991; **20** (suppl 1): 13–20.
7. Jenkins TC, Naylor MA, O'Neill P, *et al.* Synthesis and evaluation of 1-(3-(2-haloethylamino)propyl-2-nitroimidazoles as pro-drugs of RSU 1069 and its analogues, which are radiosensitizers and bioreductively activated cytotoxins. *J Med Chem* 1990; **33**: 2603–10.
8. Cole S, Stratford IJ, Adams GE, *et al.* Dual function 2-nitroimidazoles and hypoxic cell radiosensitizers and bioreductive cytotoxins: *in vivo* evaluation in KHT murine sarcomas. *Radiat Res* 1990; **124**: S38–43.
9. Cole S, Stratford IJ, Bowler J, *et al.* Oral (po) dosing with RSU 1069 or RB 6145 maintains their potency as hypoxic cell radiosensitizers and cytotoxins but reduces systemic toxicity compared with parenteral (ip) administration of mice. *Int J Radiat Oncol Biol Phy* 1991; **21**: 387–95.
10. Cole S, Stratford IJ, Fielden EM, *et al.* Dual function nitroimidazoles less toxic than RSU 1069: selection of candidate drugs for clinical trial (RB 6145 and/or PD 130908). *Int J Radiat Oncol Biol Phys* 1992; **22**: 545–8.
11. Zeman EM, Brown JM, Lemmon MJ, *et al.* SR 4233: a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. *Int J Radiat Oncol Biol Phys* 1986; **12**: 1239–42.
12. Zeman EM, Baker MA, Lemmon MJ, *et al.* Structure–activity relationships for benzotriazene-di-*N*-oxides. *Int J Radiat Oncol Biol Phys* 1989 **16**: 977–82.
13. Brown JM, Lemmon MJ. Potentiation by the hypoxic cytotoxin SR 4233 of cell killing produced by fractionated irradiation of mouse tumours. *Cancer Res* 1990; **50**: 7745-49.
14. Speckamp WN, Oostveen EA. *International Patent WO 87/06227*; Indoloquinone compounds, 1987.
15. Walton MI, Smith PJ, Workman P. The role of NAD(P)H: quinone reductase (EC1.6.99.2,DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumour agent EO9. *Cancer Commun* 1991; **3**: 199–206.
16. Adams GE, Stratford IJ, Edwards HS, *et al.* Bioreductive drugs as post-irradiation sensitizers: comparison of dual function agents with SR 4233 and the mitomycin C analogue EO9. *Int J Rad Oncol Biol Phys* 1992; **22**: 717–20.
17. Adams GE, Bremner J, Edwards HS. Bioreductive drugs-dual function agents and related compounds. In: Dewey WC, *et al.*, eds. *Radiat research: a twentieth-century perspective.* New York: Academic Press 1992; **II**: 802–6.
18. Walton MI, Suggett N, Workman P. The role of human and rodent DT-diaphorase in the reductive metabolism of hypoxic cell cytotoxins. *Int J Radiat Oncol Biol Phys* 1991; **22**: 643–8.
19. Walton MI, Wolf CR, Workman P. Molecular enzymology of the reductive bioactivation of hypoxic cell cytotoxins. *Int J Radiat Oncol Biol Phys* 1989; **16**: 983–6.
20. Workman P, Walton MI, Enyme-directed bioreductive drug development. In: Adams GE, Breccia A, Wardman P, *et al.*, eds. *Selective activation of drugs by redox processes.* New York: Plenum 1990: 173–91.
21. Minchington AI, Lemmon MJ, Tracy M, *et al.* Second-generation 1,2,4-benzotriazine 1,4-di-*N*-oxide bioreductive anti-tumour agents: pharmacology and activity *in vitro* and *in vivo*. *Int J Radiat Oncol Biol Phys* 1992; **22**: 701–6.
22. Naylor MA, Stratford IJ, Stephens MA, *et al.* Heterocyclic mono-*N*-oxides with potential applications as bioreductive anti-tumor drugs: part 1. 8-Alkylamino substituted phenylimidazo[1,2-*a*]quinoxalines. *AntiCancer Drug Des* 1993; **8**: 439–61.
23. Adams GE, Stratford IJ, Bioreductive drugs for cancer therapy: the search for tumour specificity. *Int J Radiat Oncol Biol Phys* 1994; **29**: 231–8.
24. Naylor MA, Sutton BM, Nolan J, *et al.* Radiolytic and photochemical reduction of the hypoxic cytotoxin 1,2-dihydro-8-(4-methylpiperazinyl)-4-phenylimidazo [1,2-*a*] pyrido [3,2-*e*] pyrazine 5-oxide (RB90740) and a potential mechanism for hypoxia-selective toxicity. *Int J Radiat Oncol Biol Phys* 1994; **29**: 333–7.
25. Sutton BM, Reeves NJ, Naylor MA, *et al.* Pharmacoki-

netics, metabolism and distribution of RB90740 in C3H mice. *Int J Radiat Oncol Biol Phys* 1994; **29**: 339–44.
26. Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–61.
27. Carmichael J, De Graff WG, Gazdar AF, *et al.* Evaluation of a tetrazolium-based semi-automated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; **47**: 936–41.
28. Stratford IJ, Stephens MA. The differential hypoxic cytotoxicity of bioreductive agents determined *in vitro* by the MTT assay. *Int J Radiat Oncol Biol Phys* 1989; **16**: 973–6.
29. Robertson N, Haigh N, Adams GE, *et al.* Factors affecting sensitivity to EO9 in rodent and human tumour cells *in vitro*: DT-diaphorase activity and hypoxia. *Eur J Cancer* 1994; **30A**: 1013–9.
30. Siemann DW. Cytotoxicity of dual function nitrofurans in rodent and human tumor cells. *Int J Radiat Oncol Biol Phys* 1992; **22**: 697–700.
31. Stratford IJ. Split dose experiments with misonidazole (Ro-087-0582), a cytotoxic agent specific for hypoxic cells. *Br J Cancer* 1978; **38**: 130–6.
32. Cooke BC, Fielden EM, Johnson M, *et al.* Polyfunctional radiosensitizers, I. Effects of nitroxyl biradical on the survival of mammalian cells *in vitro*. *Radiat Res* 1976; **65**: 152–62.
33. Millar BC, Sapora O, Fielden EM, *et al.* The application of Rapid-lysis techniques in radiobiology. IV. The effect of glycerol and DMSO on Chinese hamster cell survival and DNA single strand break production. *Radiat Res* 1981; **86**: 506–14.
34. Jenner TJ, Sapora O, O'Neill P, *et al.* Enhancement of DNA damage in mammalian cells upon bioreduction of the nitroimidazole-aziridines RSU-1069 and RSU-1131. *Biochem Pharmacol* 1988; **37**: 3837–42.
35. Whitaker SJ, Macmillan TJ. Oxygen effect for DNA double strand break induction determined by pulsed field gel electrophoresis. *Int J Radiat Biol* 1992; **61**: 29–42.
36. Jenner TJ, De Lara CM, Stevens DL, *et al.* The induction of DNA double strand breaks in V79-4 cells by γ and α-radiations–complexity of damage. *Radiat Protect Dosim* 1994; **52**: 289–94.
37. Twentyman PR, Brown JM, Gray JW, *et al.* A new mouse tumor model system (RIF-1) for comparison of end-point studies. *J Natl Cancer Inst* 1980; **64**: 595–604.
38. Stratford IJ, Adams GE, Godden J, *et al.* Potentiation of the anti-tumour effect of melphalan by the vaso active agent hydralazine. *Br J Cancer* 1988; **58**: 122–7.
39. Sheldon PW, Hill SA. Further investigations of the effects of the hypoxic cell radiosensitizer, Ro-07-0582, on local control of a mouse tumour. *Br J Cancer* 1977; **36**: 198–205.
40. Stratford IJ, Adams GE, Godden J, *et al.* Induction of tumour hypoxia post-irradiation: a method for increasing the sensitizing efficiency of misonidazole and RSU1069 *in vivo*. *Int J Radiat Biol* 1989; **55**: 411–22.
41. Stratford IS, Walling JM, Silver ARJ. The differential cytotoxicity of RSU 1069: Cell survival studies indicating interaction with DNA as a possible mode of action. *Br J Cancer* 1986; **53**: 339–44.
42. Jones NJ, Cox R, Thacker J. Isolation and cross-sensitivity of X-ray-sensitive mutants of V79-4 hamster cells. *Mutat Res* 1987; **183**: 279.
43. Dulhanty AM, Li M, Whitemore GF. Isolation of Chinese hamster ovary cell mutants deficient in excision repair and mytomycin C bioactivation. *Cancer Res* 1989; **49**: 117–22.
44. Robson CN, Harris AL, Hickson ID. Isolation and characterization of Chinese hamster ovary cell lines sensitive to mitomycin C and bleomycin. *Cancer Res* 1985; **45**: 5304–9.
45. Hoban PR, Walton MI, Robson CN, *et al.* Decreased NADPH: cytochrome P-450 reductase activity and impaired drug activation in a mammalian cell line resistant to mytomycin C under aerobic but not hypoxic conditions. *Cancer Res* 1990; **50**: 4692–7.
46. Brown JM. SR 4233 (Tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. *Br J Cancer* 1993; **67**: 1163–70.
47. Blocher D, Einspenner M, Zajackowski J. CHEF electrophoresis, a sensitive technique for the determination of DNA double-strand breaks. *Int J Radiat Biol* 1989; **56**: 437–48.
48. Stratford IJ, Godden J, Howells N, *et al.* Manipulation of tumour oxygenation by hydralazine increases the potency of bioreductive radiosensitizers and enhances the effect of melphalan in experimental tumours. In: Fielden EM, Fowler JF, Hendry JH, *et al.*, eds. *Radiation Research*. London: Taylor & Francis 1987; **2**: 737–42.
49. Koch CJ. Unusual oxygen concentration dependence of toxicity of SR-4233, a hypoxic cell toxin. *Cancer Res* 1993; **53**: 3992–7.

(Received 24 October 1994; accepted 15 December 1994)