Anti-Cancer Drugs 1994, 5, pp. 467–472

Synthesis and bioreductive potential of a mono *N*-oxide derivative of the alkylating agent chlorambucil

D Lynn Kirkpatrick*, Heather L Schroeder and Katherine J Chandler
Department of Chemistry, University of Regina, Regina, SK S4S 0A2, Canada. Tel: (+1) 306 585 4239; Fax: (+1) 306 585 4894.

Chlorambucil *N*-oxide (CHLN-O) was synthesized and evaluated for *in vitro* bioreductive antitumor activity. A time-dependent hypoxic differential was observed when EMT6 cells were exposed to CHLN-O in the presence of rat liver microsomes and reducing equivalents. The cytotoxicity of the *N*-oxide was potentiated under hypoxia, and augmented further by a combination of low pH and hypoxia. Metabolic studies were also undertaken, which utilized previously described HPLC methodology for the analysis of CHLN-O loss from biological fluids. These demonstrated the requirement for microsomal enzymes and reducing equivalents, and also illustrated the time-dependent manner of CHLN-O loss from isolated microsomal preparations.

Key words: Bioreductive, chlorambucil, hypoxia, *N*-oxide, pH.

Introduction

It is well established that a significant portion of the cells in solid tumors of both rodent and humans are hypoxic.[1,2] It is also clear that these hypoxic cells limit the cure rate of standard radiotherapy[3,4] and possibly some anticancer drugs[5,6] in at least some types of human malignancy. Drugs which are being developed to deal with these hypoxic cells include radiosensitizers, chemosensitizers and agents selectively toxic toward hypoxic cells.[7] The focus of this work was to evaluate the antitumor activity of an agent which was designed to be reduced to a cytotoxic species by biochemical processes in the hypoxic environment of the solid tumor, commonly referred to as a bioreductive agent.

Conventional bioreductive agents have been found to be intrinsically toxic and/or require chronic hypoxia to maintain their cytotoxic activity.[8] A new approach which we have recently pursued is the development of *N*-oxide derivatives of conventional anticancer alkylating agents. These agents may act as prodrugs that can be bioreductively activated to persistent oxygen insensitive cytotoxic species. The approach is based on the fact that the *N*-oxide functional group will deactivate the extremely reactive alkylating agent, chlorambucil, thereby diminishing its toxicity. Yet, upon reduction in the hypoxic tumor milieu, the reactive chlorambucil should be released. The activated species might diffuse into surrounding tumor tissue which may not be hypoxic and the agent would still be active in contrast to other bioreductive agents presently undergoing clinical trials.[8]

This paper describes the synthesis of chlorambucil *N*-oxide (CHLN-O) and examination of the bioreductive potential of this agent. We examined the biological effect of CHLN-O *in vitro* under hypoxic and oxic conditions, using the EMT6 cell line grown as a monolayer. Due to the possibility of limited reducing enzymes in the cell line,[9] we compared the cytotoxicity of the agent with and without the addition of rat liver microsomes to the medium during the exposure period. The cytotoxicity of the *N*-oxide was compared with its metabolic activation in the presence of isolated enzymes and to the cytotoxicity and metabolism of chlorambucil under similar conditions. In addition, the effect of pH on the cytotoxicity of CHLN-O was also studied and compared with the results reported for chlorambucil.[10]

The studies reported here found that CHLN-O is markedly less cytotoxic than chlorambucil, and under hypoxic conditions its cytotoxicity and metabolism are potentiated by the presence of reducing enzymes. Additionally, in a similar fashion as was found with chlorambucil, the cytotoxicity of CHLN-O is potentiated at low pH and further by a combination of hypoxia, reducing enzymes and low pH.

Materials and methods

Synthesis

A modified method of Mann and Shervington[11] was used to synthesize CHLN-O (4-[*p*[bis(2-chloro-

This work has been supported by a Special Research Grant from the University of Regina. KJC was the recipient of a NSERC PGSA scholarship.

Correspondence to DL Kirkpatrick

ethyl)aminolphenyl]butanoic acid *N*-oxide). To a solution of chlorambucil (ICN, St Laurent, Quebec) 2 g, 6.6 mmol) in dichloromethane (10 ml) at 0°C, was added dropwise with stirring, 8 ml peroxyacetic acid (32% w/v in acetic acid). The mixture was stirred for 30 min. The resultant mixture was extracted three times with H_2O (50 ml), the organic layer dried over $CaCl_2$ and the solvent evaporated under reduced pressure at room temperature. The crude residue was chromatographed on a column of silica gel with petroleum ether (30–60°C) : ethyl acetate (1 : 1) gradienting to 3 : 4 followed by petroleum ether : ethyl acetate : ethanol (95%) (3 : 4 : 0.5). The fractions containing the product were dried and flash chromatographed with petrolem ether : ethyl acetate (3 : 4) as an eluent. The *N*- oxide was produced as a light colored low melting solid (m.p. 46.1°C). $C_{14}H_{19}Cl_2NO_3$l: calculated, C, 52.51%; H, 5.98%, N, 4.37%; found, C, 52.98%; H, 6.10%; N, 4.31%.

Cytotoxicity

EMT6 cells, obtained from Dr Sally Rockwell, Yale University, were maintained in monolayer in glass milk dilution bottles with Waymouth's MB 752/1 medium (pH 7.4) supplemented with 15% Clex (a semisynthetic serum supplement; Dextran Products, Scarborough, Ontario). The cells were passaged twice weekly and for experimentation 1×10^5 cells in 10 ml media were seeded into milk dilutions bottles 3 days prior to drug exposure. On the day of experimentation, the medium in the bottles was exchanged with 10 ml fresh, half of the bottles were fitted with rubber stoppers and gassed for 1.5 h with humidified 95% nitrogen/5% CO_2 to simulate the hypoxic environment of solid tumors. The other half of the bottles were kept in a CO_2 incubator under aerobic conditions (95% air/5% CO_2).

Both chlorambucil and CHLN-O were dissolved in ethanol just prior to cytotoxicity testing. Following the pregassing period, 100 µl of vehicle or drug was injected through the rubber septa of hypoxic bottles or directly into the medium of aerobic bottles to give final concentrations as reported in Figure 1. The cells were treated for 2–6 h, following which the medium was removed, the cells were washed with phosphate buffered saline and the cells were removed by trypsinization. An aliquot of cells were counted using a Coulter Counter (Coulter, Hialeah, FL) and cells were diluted into 60 mm tissue culture dishes. Following incubation for 10 days, the

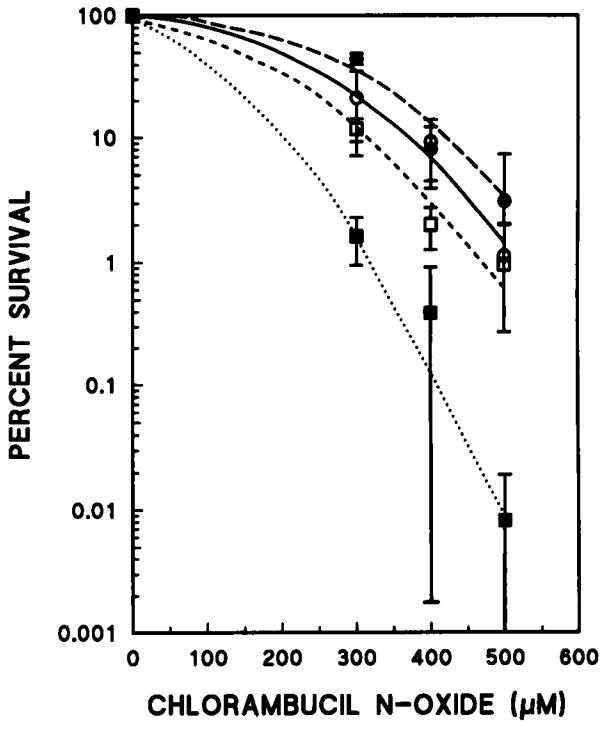

Figure 1. EMT6 cells treated *in vitro* with CHLN-O for 6 h under oxic or hypoxic conditions in the presence or absence of rat liver microsomes and NADPH: ○, oxic; □, with microsomes; ●, hypoxic; ■, with microsomes.

colonies were stained and those containing more than 50 cells were counted. The survival fractions were calculated by comparing the plating efficiencies of drug treated versus vehicle treated cells.

To evaluate the effect of externally added reducing enzymes and a nicotinamide cofactor, microsomes (0.3 µmol/min/ml) were added directly to the medium prior to the pre-gassing period. NADPH (1.3 mM) was dissolved in medium and added just prior to injection of the drug. The survival following exposure to chlorambucil or CHLN-O was measured as described above.

Metabolism

Isolation and preparation of rat liver microsomal fraction. Male Wistar rats (High Oaks Lab.) were killed by gassing with CO_2 and the livers were quickly removed, washed with ice cold sucrose (0.25 M), dried and weighed. The liver was then minced and homogenized on ice in a sucrose buffer [0.25 M sucrose/5 mM Tris/0.5 mM EDTA (pH 7.5)]. The homogenized fraction was centrifuged at 10 000 *g* for 15 min, the supernatant collected and centrifuged at 105 000 *g* for 80 min. The microsomal

pellet was resuspended in 20 ml sucrose buffer and frozen at 1°C per min to −80°C and stored until required.

Protein determination. The microsomal fraction was diluted and assayed for protein content using the Lowry method[12] with bovine serum albumin as a standard.

NADPH cytochrome P-450 reductase. NADPH cytochrome P-450 reductase activity was measured spectrophotometrically by monitoring the reduction of cytochrome *c* at 550 nm using the procedure of Masters *et al.*[13]. The activity was calculated and reported as the reduction of μmol cytochrome *c*/min/mg.

CHLN-O metabolism. Metabolism of CHLN-O by microsomal enzymes was measured under oxic and hypoxic conditions in the presence of NADPH (0.25 or 0.5 mM) or NADH (0.5 mM). All incubations were conducted at 37°C. Control incubations were conducted in the absence of cofactor, enzyme or in the presence of boiled microsome preparation. Milk dilution bottles containing microsomes (3 μmol/min in 12 ml) in 0.1 M Tris (pH 7.4) were fitted with rubber septa and gassed with N_2 95%/CO_2 5% for 30 min. Drug or vehicle, with or without cofactor, were injected through the rubber septum into the medium allowing for uninterrupted gassing. Parallel studies were carried out under oxic conditions where reaction mixtures were placed in an atmosphere of air 95%/CO_2 5%. Aliquots of the reaction mixture (1 ml) were removed at consecutive time points and added to two volumes of methyl ethyl ketone (MEK) containing internal standard. These mixtures were vortex mixed for 60 s then centrifuged at 1000 g for 5 min. The organic layer was removed to an evaporation tube and the aqueous layer extracted again with 1 ml MEK. The organic layers were combined and evaporated to dryness under vacuum at 20°C. The residues were either frozen at −20°C for future analysis or dissolved in 1 ml glass distilled methanol and filtered before analysis by HPLC as described previously.[14]

Results

Synthesis

Although a number of previously reported methods[15,16] were attempted for the synthesis of CHLN-O, only by modifying that of Mann and Shervington[17] was any product isolated with sufficient quantity to undertake cytotoxicity and metabolic studies. Once isolated and dried, CHLN-O was found to be stable when stored at 0°C or when in alcoholic solution.[14]

Cytotoxicity

Figure 1 illustrates the cytotoxic effects of treating EMT6 tumor cells with CHLN-O for 6 h under hypoxic or oxic conditions, with or without microsomes. In the absence of microsomes, hypoxia appears to produce similar survival as that seen under aerobic conditions. The addition of microsomes (0.3 μmol/min/ml) in the presence of 1.3 mM NADPH potentiates the cytotoxicity under both the aerobic and hypoxic conditions. The greatest potentiation of tumor cell kill was observed when the cells were exposed to both microsomes and reducing equivalents under hypoxia.

The corresponding data for survival following exposure to chlorambucil is illustrated in Figure 2. EMT6 cells were exposed to CHL for 2 h under

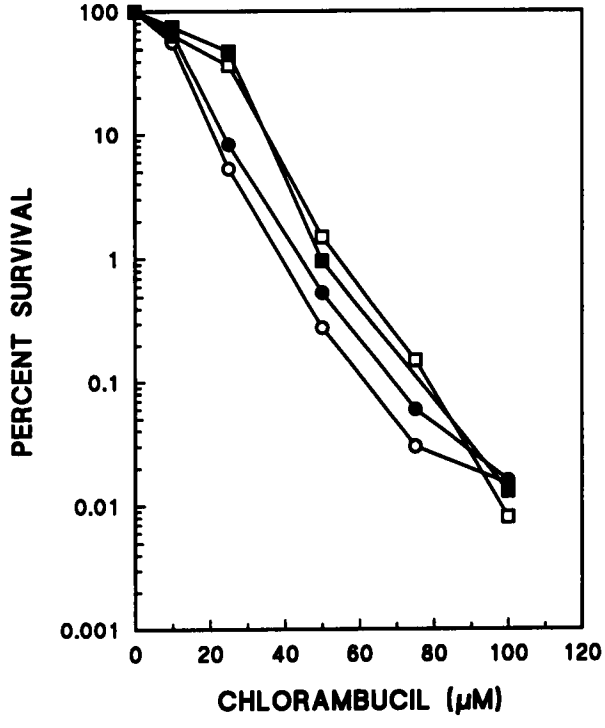

Figure 2. EMT6 cells treated *in vitro* with chlorambucil for 2 h under oxic or hypoxic conditions in the presence or absence of rat liver microsomes and NADPH: ○, oxic; □, with microsomes; ●, hypoxic; ■, with microsomes.

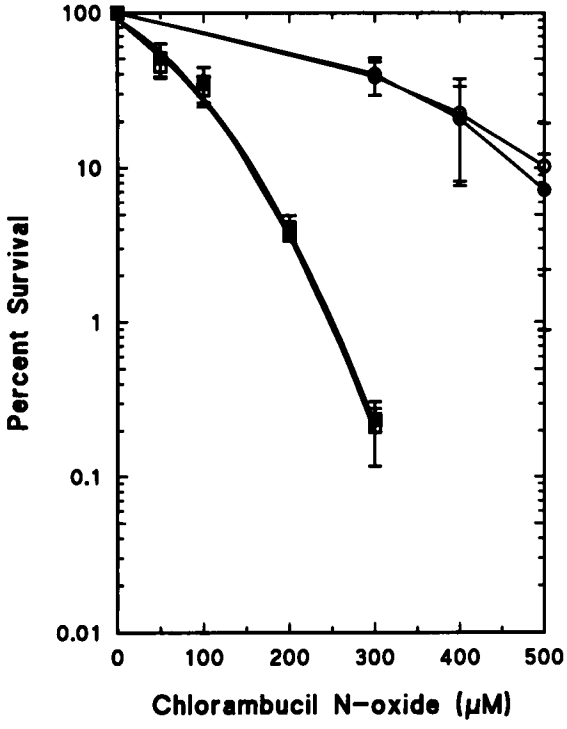

Figure 3. EMT6 cells treated with CHLN-O for 2 h under oxic or hypoxic conditions at pH 7.4 and 6.7: ○, pH 7.4 oxic; ●, pH 7.4 hypoxic; □, pH 6.7 oxic; ■, pH 6.7 hypoxic.

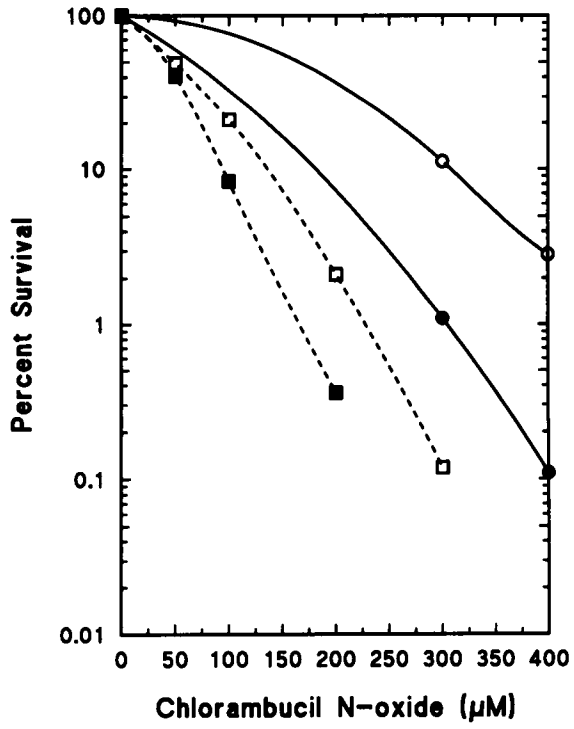

Figure 4. EMT6 cells treated with CHLN-O for 6 h under oxic or hypoxic conditions in the presence of microsomes and NADPH at pH 7.4 or 6.8: ○, pH 7.4 oxic; ●, pH 7.4 hypoxic; □, pH 6.8 oxic; ■, pH 6.8 hypoxic.

oxia and hypoxia, in the presence and absence of microsomes (0.3 μmol/min/ml). It can be noted that chlorambucil is far more toxic than CHLN-O and neither microsomes nor hypoxia caused any potentiation of cytotoxicity.

The effect of lowering the pH of the medium during drug exposure is illustrated in Figure 3. EMT6 cells were exposed for 2 h to CHLN-O, under hypoxic or oxic conditions at pH 7.4 or 6.75. The IC_{50} was reduced from 300 to 50 μM by decreasing the pH (IC_{90} reduced to 150 from 500 μM). There was little effect produced by exposure under hypoxia at lowered pH in the absence of microsomal enzymes and the 2 h time exposure was insufficient to demonstrate a preferential toxicity under hypoxia in the presence of microsomes (0.149 μmol/min/ml cytochrome P-450 reductase; 3.05 nmol/mL cytochrome P-450). However, increasing the exposure time to 6 h at pH 6.75, in the presence of microsomes (0.272 mmol/min/ml cytochrome P-450 reductase; 2.83 nmol/ml cytochrome P-450) and NADPH, produced a significant hypoxic cytotoxicity differential (Figure 4). The IC_{90} for CHLN-O under these conditions was 90 mM, compared with 240 mM which was observed under hypoxia with no microsomes at pH 7.4.

Metabolism

The loss of chlorambucil or CHLN-O from reaction mixtures containing microsomes was monitored using a previously published method for extraction and HPLC analyses.[14] Figure 5 illustrates the effect of exposing CHLN-O to microsomes (active or boiled) and NADPH under oxic and hypoxic conditions or NADH under hypoxia. Boiled microsomes produced little effect on the metabolism of CHLN-O over a 4 h period. Similar effects were seen when cofactor or enzyme was eliminated from the reaction mixture (data not shown). In air the presence of reducing equivalents (0.5 mM), 82% of *N*-oxide remained after a 1 h incubation, while after 4 h 78% of the initial CHLN-O remained in the microsomal mixture. Under hypoxic conditions with NADPH (0.25 or 0.5 mM) a 52 or 62% loss, respectively, of CHLN-O was observed after 1 h. Following a 4 h incubation, the reaction mixture with 0.25 mM NADPH contained approximately 10% of the original CHLN-O while that with 0.5 mM NADPH was virtually devoid of the *N*-oxide. Using NADH (0.5 mM) as a cofactor also increased the rate of metabolism of CHLN-O under hypoxia as compared with that seen in air. However, NADH did not appear to be as

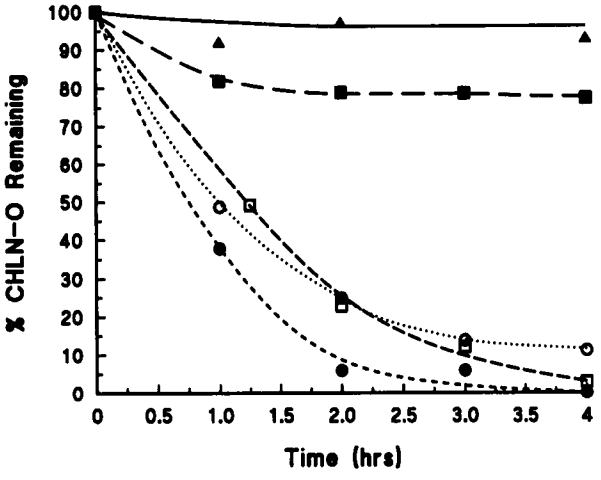

Figure 5. Metabolism of CHLN-O by rat liver microsomes *in vitro* under various exposure conditions. Hypoxia: ▲, boiled microsomes; □, 0.5 mM NADH; ●, 0.5 mM NADPH; ○, 0.25 mM NADPH. Oxia: ■, 0.5 mM NADPH.

efficient as NADPH, producing a 42% loss after 1 h and 97% after 4 h.

Discussion

These studies demonstrate that the *N*-oxide derivative of chlorambucil is far less toxic to tumor cells in culture than is the parent chlorambucil. It was found, however, that the toxicity of CHLN-O can be potentiated by co-incubation with microsomal enzymes and reducing equivalents especially under hypoxic conditions giving an air to hypoxia differential in IC_{50} of 2–4. Figure 1 illustrates the fact that the enzyme and reducing equivalents cause some potentiation even in air. These data were supported by the microsomal metabolism studies which also found some loss of *N*-oxide when incubated in air in the presence of microsome and reducing equivalents. Boiled microsomes were not able to cause this decrease, emphasizing the fact that the loss of CHLN-O was not due to a non-specific breakdown of the prodrug.

These data, however, are contrasted by those of Patterson,[17] who report that up to a 1000-fold differential in cytotoxicity in air versus hypoxia was produced by the incubation of 1,4-bis{[2-(dimethylamino - *N*-oxide)ethyl]amino}5,8 - dihydroxyanthracene-9,10-dione (AQ4N) in the presence of high levels of microsomes and reducing equivalents. The reasons as to why CHLN-O does not produce this large differential may be many, two of which are outlined. First, there may be a cell line specificity. The V79 cell line was used by Patterson[17] while the EMT6 line was used in the present studies and it is possible that different hypoxic/oxic differentials may be observed when using different cell lines. Secondly, the amount of microsomes and reducing equivalents used by Patterson[17] far exceeded what was used in these studies. Our metabolism data showed that by increasing the amount of reducing equivalents in the incubation mixture, the rate of loss of *N*-oxide also increased. In addition, when microsomes with lower activities were used for the cytotoxicity studies, a smaller potentiation of toxicity was observed under hypoxic conditions (data not shown). This suggested that by using the higher levels of microsomes and reducing equivalents in the cytotoxicity studies, we may have observed an even greater hypoxic/oxic differential. However, these conditions may not reflect those enzyme levels available in the tumor milieu.

In addition to the reductive activation of CHLN-O under hypoxia, the cytotoxicity of CHLN-O was also potentiated when cells were exposed in medium of lower pH (6.75 versus 7.4). Since there is evidence of both hypoxia and pH differences in solid tumors, these data suggest that CHLN-O may have greater bioreductive potential than what has been previously reported for this agent.[11]

Conclusions

These findings strongly suggest that CHLN-O might have selective solid tumor activity, especially against the hypoxic subcellular fraction and cells in environments of low pH. The hypoxic versus oxic cytotoxic differential measured for this agent is not as large as reported by other investigators and may be extremely dependent on the reductive enzyme profile of particular solid tumors. Further studies to identify the enzymes responsible for the activation and to examine the *in vivo* activity of this agent are ongoing.

Acknowledgments

The technical assistance of Wilson Lee is gratefully acknowledged.

References

1. Moulder JE, Rockwell S, Tumor hypoxia: its impact on cancer therapy. *Cancer Metastasis Rev.* 1987; **5**: 313–41.
2. Vaupel P, Kallinowski K, Okunieff, P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res* 1989; **49**: 6449–65.

3. Dische S, Anderson PJ, Sealy R, *et al.* Carcinoma of the cervix: anaemia, radiotherapy and hyperbaric oxygen. *Br J Radiol* 1983; **56**: 251–5.
4. Gatenby RA, Kessler HB, Rosenblum JS, *et al.* Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. *Int J Radiat Oncol Biol Phys* 1988; **14**: 831–8.
5. Tannock I, Guttman P. Response of Chinese hamster ovary cells to anticancer drugs under aerobic and hypoxic conditions. *Br J Cancer* 1981; **43**: 245–8.
6. Sartorelli AC, Therapeutic attack of hypoxic cells of solid tumors: Presidential address. *Cancer Res.* 1988; **48**: 775–8.
7. Workman P. New drugs against hypoxic cells (Part II). *Cancer Topics* 1983; **4**: 54–7.
8. Coleman CN, Doherty N, Hancock S, *et al. Proc. Bioreductive Agents: Activation, Detoxification and Clinical Development Workshop,* September 1992, Oxford, UK.
9. Workman P. *Proc. Bioreductive Agents: Activation, Detoxification and Clinical Development Workshop,* September 1992, Oxford, UK.
10. Skarsgard LD, Chaplin D, Wilson DJ, *et al.* The effect of Hypoxia and low pH on the cytotoxicity of chlorambucil. *Int J Radiat Oncol* 1992; **22**: 737–41.
11. Mann J, Shervington LA. Synthesis of novel *N*- and *S*-mustards as potential pro-drug activated by bioreductive processes. *J Chem Soc Perkin Trans* 1991; **1**: 2961–4.
12. Lowry OH, Rosenbrough NJ, Fair L, *et al.* Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; **193**: 265–75.
13. Masters B, Williams C, Kamin H. The preparation and properties of microsomal TPNH-cytochrome *c* reductase from pig liver. *Methods Enzymol* 1967; **10**: 563–73.
14. Chandler K, McCabe J, Kirkpatrick DL. *J Chrom* 1994; **652**: 195–202.
15. Degutis Y, Medekshene G. *N*-substituted *N*-2-chloroethylaniline *N*-oxides. *J Org Chem USSR* 1970; **6**: 1827–30.
16. Degutene A, Dzhyuvene D, Shukyalene D, *et al.* Synthesis and spontaneous decomposition of the *N*-oxides of di(2-chloroalkyl)aminophenylalknaoic acids. *J Org Chem USSR* 1986; **22**: 2191–7.
17. Patterson LH. Rationale for the use of aliphatic *N*-oxides of cytotoxic anthaquinones as prodrug DNA binding agents: a new class of bioreductive agent. *Cancer Metas Rev* 1993; **12**: 119–34.

(Received 14 April 1994; accepted 12 May 1994)