Pergamon

Biochemical Pharmacology, Vol. 50, No. 1, pp. 61–68, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00113-1

ISOLATION AND CHARACTERIZATION OF A CELL LINE RESISTANT TO 5-[3-(2-NITRO-1-IMIDAZOYL)-PROPYL]-PHENANTHRIDINIUM BROMIDE (2-NLP-3), A DNA-INTERCALATING HYPOXIC CELL RADIOSENSITIZER AND CYTOTOXIN

DAVID S. M. COWAN,* ROBERT A. McCLELLAND† and ANDREW M. RAUTH‡§

‡Experimental Therapeutics Division, The Ontario Cancer Institute, Toronto; and Departments of *Medical Biophysics, and †Chemistry, University of Toronto, Toronto, Ontario, Canada

(*Received* 9 *September* 1994; *accepted* 12 *January* 1995)

Abstract—A DNA-targeted hypoxic cell radiosensitizer and cytotoxin, 5-[3-(2-nitro-1-imidazoyl)-propyl]-phenanthridinium bromide (2-NLP-3), has been shown previously to have increased efficacy over untargeted analogues *in vitro*. To further study the mechanism of action of this compound, a cell line, CHO-1000, derived from Chinese hamster ovary (CHO) AA8-4 cells was isolated. This cell line is capable of continuously growing in a concentration of 2-NLP-3 approximately 10-fold greater than that tolerated by wild-type CHO cells. The resistance of CHO-1000 to 2-NLP-3 was compared with that of the P-glycoprotein overexpressing, multidrug resistant Chinese hamster cell line CH^R-C5 (C5). The resistance of CHO-1000 cells to the acute toxic effects of 2-NLP-3 under both hypoxic and aerobic exposure conditions was intermediate to that of the sensitive CHO wild-type cells and the resistant C5 cells. A similar pattern was seen for the hypoxic cell radiosensitizing ability of 2-NLP-3. 2-NLP-3 produced significant depletion of glutathione under both hypoxic and aerobic conditions in all three cell lines studied, and the degree of depletion was correlated with drug toxicity. CHO-1000 and C5 cells were significantly more resistant to colchicine and doxorubicin compared with wild-type cells. The toxicity pattern of 2-NLP-3 and its comparison phenanthridinium ion, P3, was not the same for CHO-1000 cells compared with C5 cells. Verapamil was an effective agent for reversing the hypoxic resistance to 2-NLP-3 in both CHO-1000 and C5 cells, but only a partial reversal of aerobic resistance was observed in CHO-1000 cells. These results indicate that the resistant phenotype of CHO-1000 is mediated to some degree by P-glycoprotein expression, but that other as yet unidentified factors are also involved.

Key words: drug resistance; P-glycoprotein; hypoxic cell radiosensitizer; 2-NLP-3; cytotoxicity

The presence of radioresistant hypoxic cells is one explanation for the failure of local control seen with some solid tumours treated with radiotherapy [1], and the production of oxygen-mimetic drugs has been one approach to overcome this problem. Compounds incorporating the electron affinic nitro group have been the most extensively studied class of chemical radiation sensitizers with the 2-nitroimidazole, Miso‖, being the classical agent of this type [2]. In an attempt to increase the molar efficiency of hypoxic cell radiosensitizers and cytotoxins, a 2-nitroimidazole targeted to DNA, its site of action, via the attachment of an intercalating phenanthridine moiety was developed [3]. This compound, 2-NLP-3, has been shown previously to be 10–100 times more effective than the untargeted 2-nitroimidazole, Miso, as an *in vitro* hypoxic cell radiosensitizer and cytotoxin [4]. In an effort to determine factors controlling the radiosensitizing ability and toxicity of 2-NLP-3, a series of cell lines that are resistant to the compound were developed by serial passage of CHO cells in increasing concentrations of the drug.

The experiments reported here concern the isolation and characterization of CHO-1000 cells that are capable of growing continuously in a concentration of 2-NLP-3 that is approximately 10 times higher (1000 μM) than that in which wild-type CHO cells can survive [4]. CHO-1000 cells were compared with wild-type CHO cells and the P-glycoprotein overexpressing cell line CH^R-C5 (C5) [5]. The cytotoxicity and radiosensitizing ability of 2-NLP-3 in these cell lines were examined, as well as the effect of 2-NLP-3 on intracellular thiol status. The possible role of P-glycoprotein in the observed

§ Corresponding author: Dr. Andrew M. Rauth, Experimental Therapeutics Division, The Ontario Cancer Institute, 500 Sherbourne St., Toronto, Ontario, Canada M4X 1K9. Tel. (416) 924-0671, Ext. 5057; FAX (416) 926-6529.

‖ Abbreviations: 2-NLP-3, 5-[3-(2-nitro-1-imidazoyl)-propyl]-phenanthridinium bromide; C5, colchicine-resistant Chinese hamster cell line-clone 5; CHO, Chinese hamster ovary; DTNB, dithiobis-2-nitrobenzoic acid; GSH, glutathione (reduced form); Miso, misonidazole; OER, oxygen enhancement ratio (the ratio of the doses of radiation required to produce a given endpoint under hypoxic versus aerobic conditions); P3, 1-(2-nitro-1′-imidazoyl)-3-bromopropane; SER, sensitizer enhancement ratio (ratio of the doses of radiation for a given endpoint to the doses of radiation plus drug to give the same endpoint); and TCA, trichloroacetic acid.

resistance of CHO-1000 cells was examined by testing for cross-resistance to other chemical compounds as well as the ability of a P-glycoprotein inhibitor, verapamil, to reverse the phenotype. The data obtained suggest that while the reduction of intracellular levels of the compound by P-glycoprotein plays a role in cellular resistance to 2-NLP-3, it is not sufficient to account for all of the decrease in sensitivity observed.

MATERIALS AND METHODS

Cells

The wild-type cell line used in the isolation of 2-NLP-3 resistant cell lines was the CHO cell line AA8-4. The P-glycoprotein overexpressing colchicine resistant cell line, C5, originally developed from CHO AuxB1 [5], was provided by Dr. V. Ling of The Ontario Cancer Institute. These cell lines grow well in both flasks and suspension culture, and routine maintenance was as described previously for wild-type CHO cells [4]. Fresh cultures of the lines for experimental use were obtained from frozen samples approximately once every 3 months.

Chemicals

The DNA-intercalating, hypoxic cell radiosensitizer and cytotoxin, 2-NLP-3, and the phenanthridinium comparison ion with a three-carbon side chain, P3, were prepared as described previously [3]. The untargeted radiosensitizer, Miso, was a gift of The National Cancer Institute (U.S.A.). Verapamil, colchicine, doxorubicin, and Hoechst 33342 were all purchased from the Sigma Chemical Co. (St. Louis, MO). All drugs were dissolved in PBS and filtered through a 0.45 μM syringe filter (Millipore, Mississauga, Canada) prior to use. For the thiol assays, GSH, NADPH, DTNB, and GSH reductase were all purchased from Sigma. TCA and ether were purchased from the Fisher Chemical Co. (Toronto, Canada).

Isolation of 2-NLP-3 resistant cell lines

The 2-NLP-3 resistant cell lines were derived through serial passage of wild-type CHO cells in monolayer culture in the continuous presence of increasing concentrations of the compound. The cell line chosen for routine experimentation was CHO-1000, which is capable of growing in at least a 1000 μM concentration of 2-NLP-3. These cells also grow in both tissue culture flasks and suspension culture and were maintained as described for wild-type CHO cells. Passage and storage of the line were done in the presence of drug. Cell size, doubling time, and plating efficiency of the resistant cell line were similar to those of the wild-type cells. There appeared to be little clonal variation in five different CHO-1000 clones tested in chronic toxicity experiments (data not shown).

Acute toxicity studies

The toxic effects of acute aerobic and hypoxic exposure of wild-type CHO, CHO-1000, and C5 cells to 2-NLP-3 were assayed as described previously [4].

Radiation survival studies

The radiosensitizing ability of 2-NLP-3 and the untargeted 2-nitroimidazole, Miso, toward the three cell lines was tested in a manner similar to the acute toxicity experiments except that cell samples were removed as a function of radiation dose rather than time [4]. Cells were irradiated with a ^{60}Co external beam therapy machine adapted for experimental use at a dose rate of 1.4 to 1.6 Gy/min.

Intracellular glutathione determinations

The GSH status of wild-type CHO, CHO-1000, and C5 cells was determined using the Teitze assay [6] as modified by Bump *et al.* [7].

Cross-resistance experiments

The resistance of the three cell lines to five different agents (2-NLP-3, doxorubicin, colchicine, P3, and Miso) was determined under aerobic conditions in a chronic toxicity assay that was described previously [4].

Involvement of P-glycoprotein in resistance

Survival studies. Acute survival studies under hypoxic and aerobic conditions were run using a known inhibitor of P-glycoprotein, verapamil [8]. Experiments were performed as described above with 2-NLP-3 plus or minus verapamil, prepared in PBS, or verapamil alone. Clonogenic survival was determined in these experiments as already described [4].

Flow cytometry studies. Duplicate samples of the cell lines were prepared, one of which was treated with 30 μM verapamil at 24° for 30 min. Both samples were then treated with the stain Hoechst 33342, a substrate for P-glycoprotein [9], at a concentration of 1 μM for 10 min. The samples were then run through a flow cytometer (Coulter Instruments, Hialeah, FL), and the relative levels of Hoechst 33342 fluorescence were measured. By comparing mean fluorescence levels between cell lines, relative quantitation of the levels of P-glycoprotein function were possible.

RESULTS

Acute toxicity studies

An extracellular drug concentration of 0.5 mM 2-NLP-3 was toxic toward both aerobic and hypoxic wild-type CHO cells with an aerobic/hypoxic differential toxicity of approximately 4 (Fig. 1a). Significant hypoxic cell selective toxicity has also been seen at 0.25 mM 2-NLP-3 over 5 hr [4], while a concentration of 1.0 mM resulted in a very high level of cytotoxicity very rapidly in both aerobic and especially hypoxic cells (Fig. 1a). Sampling began subsequent to drug addition, and, therefore, the toxicity of 2-NLP-3 seen at time zero was not due to drug carry-over but rather to the time required for sampling. The NLP resistant cell line, CHO-1000, displayed a different pattern of survival in response to 2-NLP-3 (Fig. 1b). There was no toxicity due to 2-NLP-3 under aerobic conditions up to a concentration of at least 2 mM (data not shown). Under hypoxic conditions, 0.5 mM 2-NLP-3 showed

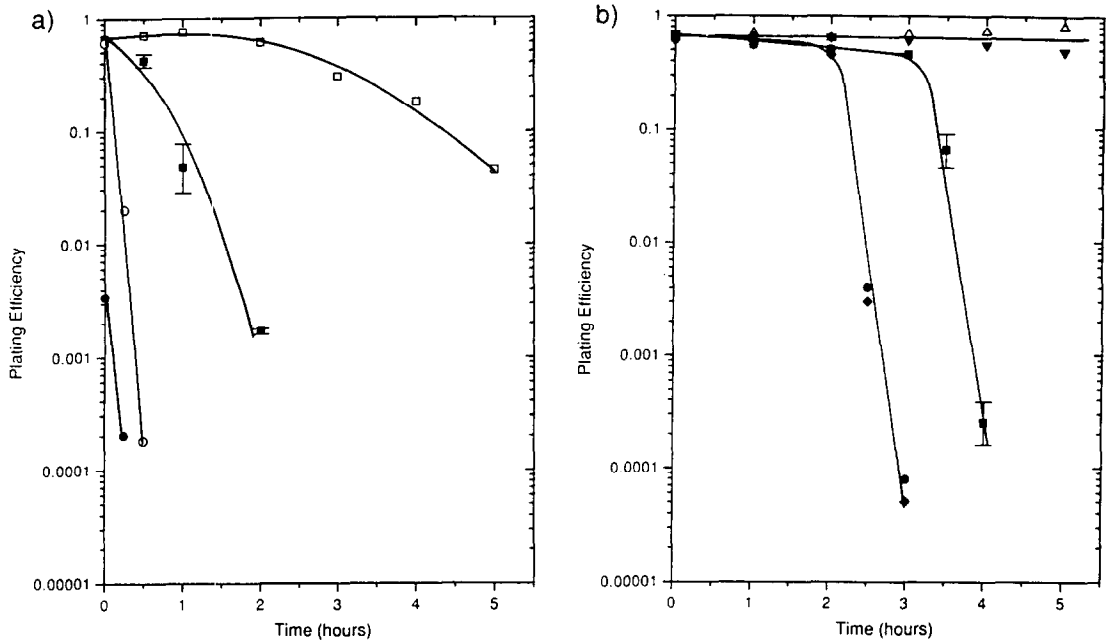

Fig. 1. Cell plating efficiencies after acute drug exposures of 0–5 hr under aerobic (open symbols) and hypoxic (closed symbols) conditions. (a) CHO wild-type cells exposed to 0.5 mM (squares) and 1 mM (circles) 2-NLP-3. (b) CHO-1000 cells exposed to 0.25 (inverted triangles), 0.5 mM (squares), 1.0 mM (circles), and 2.0 mM (diamonds) 2-NLP-3. C5 cells treated with 2.0 mM 2-NLP-3 are shown as upright triangles. Points represent the means ± SD of at least 3 experiments.

no toxicity up to 3 hr followed by a sharp increase in cell killing. When the concentration of 2-NLP-3 was raised to 1.0 mM, a similar pattern was seen with a period where little toxicity was observed, from 0 to 2 hr, followed by rapid cell killing (Fig. 1b). If the concentration was raised further to 2.0 mM, the survival pattern was the same as observed for 1.0 mM, suggesting a saturation of the toxic effects.

The P-glycoprotein overexpressing cell line, C5, a subline of CHO Aux B1 cells originally isolated by chronic exposure to colchicine, was also tested for cross-resistance to 2-NLP-3. No acute aerobic or hypoxic cytotoxicity due to 2-NLP-3 could be seen in the C5 cell line at external drug concentrations up to 2 mM (see Fig. 1b for representative data).

The sensitivity of CHO-1000 and C5 cells to 0.5 mM P3 was also assessed under hypoxic and aerobic conditions, and the results are shown in Fig. 2a. C5 cells were resistant under both aerobic and hypoxic conditions up to 5 hr of exposure. In contrast, although CHO-1000 was resistant to P3 under aerobic conditions, it became sensitive to the drug after a 3-hr hypoxic incubation, showing a rapid loss of viability between 4 and 5 hr of exposure (Fig. 2a). Neither CHO-1000 nor C5 was more resistant to the toxic effects of the untargeted 2-nitroimidazole, Miso, than CHO wild-type cells at a concentration of 5.0 mM under hypoxic exposure conditions (Fig. 2b). No toxicity was seen for any of the cell lines under aerobic exposure conditions (data not shown). These data suggest that resistance to 2-NLP-3 was mediated by the positively charged phenanthridine ring system of the compound.

Radiation survival experiments

The DNA-targeted 2-nitroimidazole, 2-NLP-3, is a potent radiosensitizer of hypoxic wild-type CHO cells *in vitro*, producing almost the full oxygen effect at a concentration of 0.5 mM (Fig. 3 and [4]). The radiosensitizing potency of 2-NLP-3 diminishes when used with CHO-1000 cells. No sensitization was observed at the lowest concentration used (0.25 mM). When the drug level was raised to 0.5 mM, a small amount of sensitization was observed, which increased further as the concentration of the compound was raised to 1.0 and 2.0 mM. A maximum SER of approximately 1.5 at 2.0 mM was obtained at the 10% survival level (Fig. 3). A similar level of sensitization can be achieved in wild-type CHO cells at an extracellular drug level of 50–75 μM 2-NLP-3 (Fig. 3 and [4]). The radioresponse of aerobic CHO-1000 cells was not altered significantly by concentrations of 2-NLP-3 up to 2 mM, and the OER obtained in the CHO-1000 radiation survival experiments was approximately 2 (data not shown). The radiation response of the colchicine resistant C5 cells was not affected by concentrations of 2-NLP-3 up to 2.0 mM under either hypoxic or aerobic conditions (Fig. 3). The OER in these experiments was also approximately 2 (data not shown).

The results for SER versus extracellular drug concentration of 2-NLP-3 for CHO-1000 and C5 cells were compared with previous data for CHO

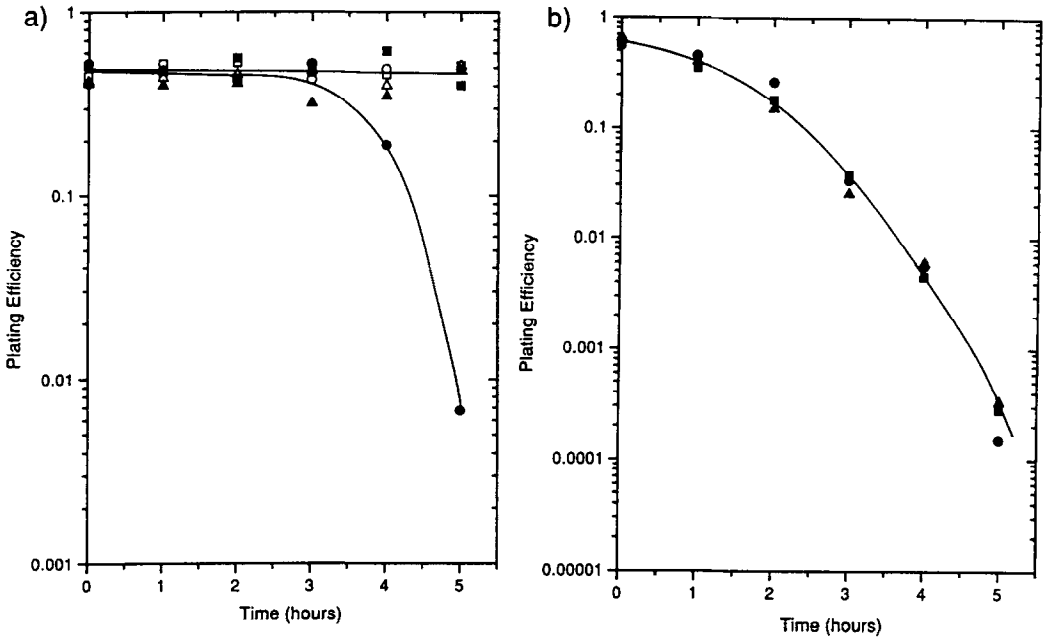

Fig. 2. (a) CHO wild-type (squares), CHO-1000 (circles), and C5 (triangles) cells exposed to 0.5 mM P3 under aerobic (open symbols) and hypoxic (closed symbols) exposure conditions. (b) Wild-type CHO (squares), CHO-1000 (circles), and C5 (triangles) cells exposed to 5.0 mM Miso under hypoxic conditions. Points represent the means of at least 3 experiments.

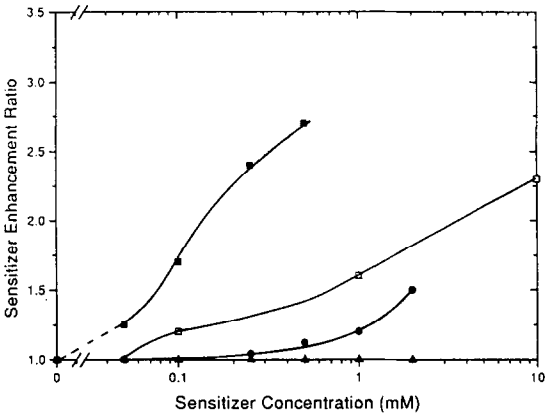

Fig. 3. Sensitizer enhancement ratios versus sensitizer concentration. CHO wild-type: 2-NLP-3 (closed squares); CHO-1000: 2-NLP-3 (circles), Miso (open squares); C5: 2-NLP-3 (triangles). Points are the means of 3 or 4 experiments. CHO wild-type data are taken from Cowan *et al.* [4].

wild-type cells with 2-NLP-3 (Fig. 3). CHO-1000 cells required about 30 times the external 2-NLP-3 concentration to give an SER of 1.5 when compared with wild-type CHO cells. Miso produced identical levels of sensitization in all cell lines studied. The Miso results are represented by data for CHO-1000 cells in Fig. 3.

Intracellular glutathione determinations

When CHO cells were exposed to 0.5 mM 2-NLP-3, GSH levels were depleted rapidly (Fig. 4a). Under hypoxic conditions, levels of GSH were reduced to below 10% of control cells after a 2-hr exposure, an effect that is likely due to metabolic reduction of the parent drug. In aerobic cells, GSH was reduced more slowly. Depletion of GSH was also significant in CHO-1000 cells at a concentration of 0.5 mM 2-NLP-3 (Fig. 4b). Under hypoxic conditions, 10% or less of the control GSH remained after 4–5 hr of drug exposure. Aerobic depletion again occurred more slowly. A similar pattern of depletion was seen with C5 cells with hypoxic exposure levels reaching only 20–30% of control at 4–5 hr (Fig. 4c). The degree of GSH depletion under both hypoxic and aerobic conditions was related to the toxicity of 2-NLP-3 under acute exposure conditions (see Fig. 1).

Cross-resistance studies

The spectrum of resistance to colchicine, doxorubicin, 2-NLP-3, P3, and Miso was investigated with wild-type CHO, CHO-1000, and C5 cells by determining the highest drug concentration that the cells would tolerate under continuous aerobic (chronic) exposure conditions (Table 1). The P-glycoprotein expressing C5 cell line was the most resistant cell line to colchicine and doxorubicin, drugs known to be substrates for P-glycoprotein, as well as P3. CHO-1000 was the cell line most resistant to 2-NLP-3. All three cell lines had similar sensitivities to Miso in these experiments.

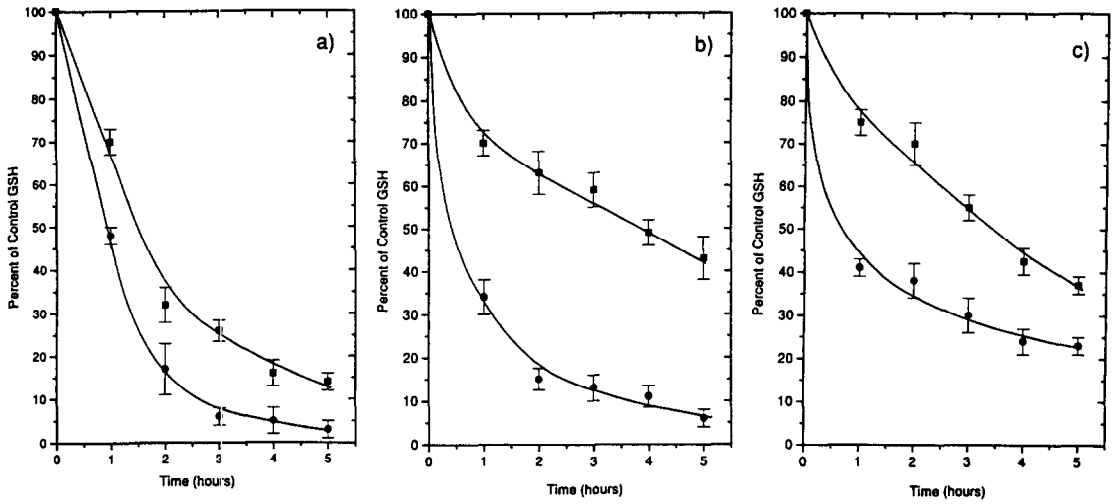

Fig. 4. GSH depletion as a function of time of exposure to 0.5 mM 2-NLP-3 under aerobic (squares) and hypoxic (circles) exposure conditions. (a) CHO wild-type cells. (b) CHO-1000 cells. (c) C5 cells. The absolute values for GSH were 5.7 ± 0.6, 6.1 ± 0.3, 5.2 ± 0.4 nmol/10^6 cells for CHO wild-type, CHO-1000, and C5 cells, respectively. Points are the means ± SD of 3 or 4 experiments.

Table 1. Spectrum of chronic drug toxicity

Compound	C5 vs CHO wild-type	CHO-1000 vs CHO wild-type
Colchicine (0.063)*	63†	16
Doxorubicin (0.014)	31	16
2-NLP-3 (150)	2	8
P3 (80)	33	5
Miso (700)	1	1

* Number in brackets is the highest micromolar drug concentration tolerated by CHO wild-type cells.

† Ratio of highest drug concentrations that gave no toxicity.

Involvement of P-glycoprotein in resistance

Survival studies. To test for the involvement of P-glycoprotein in the resistant phenotypes, acute toxicity experiments were carried out using a known inhibitor of the putative pump, verapamil. The highest non-toxic concentration of verapamil was determined to be 75 μM, using a chronic toxicity assay with wild-type CHO cells, the most sensitive cell line (data not shown).

The use of verapamil at a concentration of 70 μM had no effect on either hypoxic or aerobic CHO wild-type cells when used as a single agent (data not shown). In combination with 1 mM 2-NLP-3, the production of rapid and significant cell kill was observed, similar to that seen in Fig. 1a in the absence of verapamil, with at least 4 logs of cell kill over 2 hr in both aerobic and hypoxic cells (data not shown).

A different situation was seen with the two resistant cell lines. Again, the use of 70 μM verapamil as a single agent had no effect on the survival of hypoxic or aerobic cells in either cell line over a 5-hr exposure (representative data for CHO-1000 cells are shown in Fig. 5). In combination with 1 mM 2-NLP-3, CHO-1000 displayed a toxic response similar to wild-type CHO cells under hypoxic conditions, producing between 3 and 4 logs of cell kill in 30 min (compare Fig. 1a and Fig. 5). This result suggests the involvement of P-glycoprotein in the resistant phenotype of this cell line. Surprisingly, under aerobic conditions, although some increased toxicity due to 2-NLP-3 was observed with verapamil present, it was far less than what would have been predicted if P-glycoprotein was mediating all of the resistance to 2-NLP-3 (see Fig. 1b). In the P-glycoprotein expressing C5 cells, inhibition of the pump by 70 μM verapamil in combination with 1 mM 2-NLP-3 resulted in a high level of toxicity under both aerobic and hypoxic conditions (Fig. 5), similar to that seen with wild-type CHO cells (Fig. 1a). These results suggest that only part of the aerobic resistance of CHO-1000 to 2-NLP-3 can be explained by P-glycoprotein function.

Flow cytometry studies. To test more directly for functional P-glycoprotein in the cell lines, they were incubated in the presence and absence of 30 μM

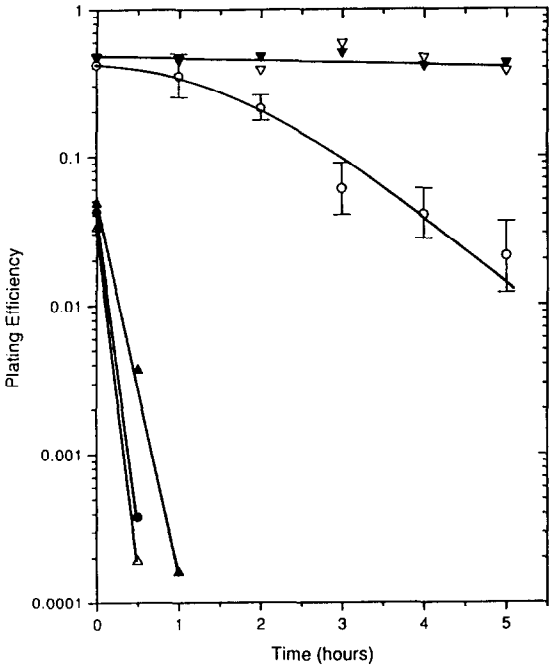

Fig. 5. Effect of 1 mM 2-NLP-3 in the presence 70 μM verapamil on the plating efficiency of CHO-1000 cells (circles) and C5 cells (upright triangles) under aerobic (open symbols) and hypoxic (closed symbols) exposure conditions. The effect of 70 μM verapamil alone on CHO-1000 cells under aerobic and hypoxic conditions is shown by the inverted triangles. Points are the means ± SD of at least 3 experiments.

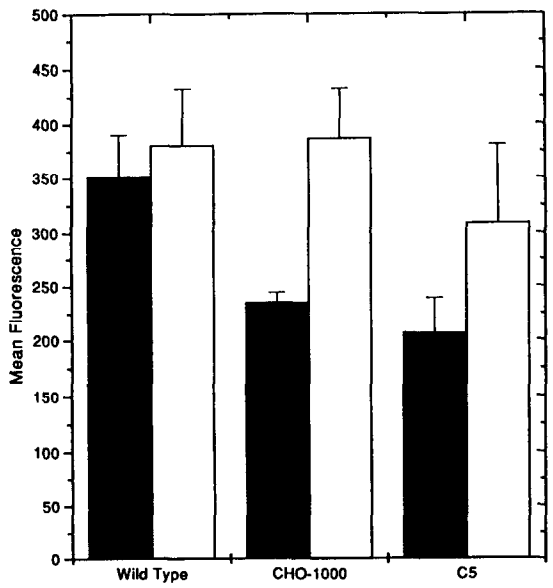

Fig. 6. Mean fluorescence of CHO wild-type, CHO-1000, and C5 cells incubated in the presence of Hoechst 33342 with (open bars) or without (black bars) 30 μM verapamil. Error bars represent the range of the data found in two experiments.

verapamil with the fluorescent stain Hoechst 33342, a substrate for the putative pump [9]. Relative mean fluorescence was then measured by flow cytometry. These results are shown in Fig. 6. Wild-type CHO cells express a low level of P-glycoprotein, and relative fluorescence due to the Hoechst 33342 was increased by only 5–10% in the verapamil-treated cells. CHO-1000 and C5 showed a 40–60% increase in fluorescence in the verapamil-treated cells. By this functional assay, both CHO-1000 and C5 cells have higher levels of P-glycoprotein than CHO wild-type cells. There was no statistically significant difference in the levels of P-glycoprotein in C5 and CHO-1000 cells as measured by this assay.

DISCUSSION

These studies show that it is possible to induce resistance to 2-NLP-3 in CHO cells by growing cells continuously in increasing concentrations of the drug. This has been seen previously for another DNA-intercalating agent, Adriamycin [10], as well as a variety of other chemotherapeutic agents [8]. The resistance induced in the case of 2-NLP-3 was likely due to the phenanthridine-intercalating moiety, since no resistance was seen to an untargeted 2-nitroimidazole, Miso (Fig. 2b). Comparisons were made between wild-type CHO, CHO-1000, and C5 cells in an attempt to elucidate the mechanism of resistance of CHO-1000 cells. P-glycoprotein overexpressing CHO-C5 cells were resistant to acute aerobic and hypoxic exposures of 2-NLP-3, suggesting that 2-NLP-3 is a substrate for the pump. CHO-1000 exhibited somewhat different results when run in the same assay. No toxicity of 2-NLP-3 toward CHO-1000 cells was seen under aerobic conditions. Under hypoxic exposure conditions, 0.25 mM 2-NLP-3 was non-toxic, even though 0.25 mM produces significant toxicity toward wild-type CHO cells [4]. However, when the concentration was raised to 0.5 mM, approximately 3 logs of cell kill was seen after 4 hr. This is in contrast to the toxicity pattern seen with CHO wild-type cells where 2-NLP-3 showed increasing toxicity with time.

The differential behaviour of CHO-1000 cells after hypoxic exposure to 2-NLP-3 compared with C5 and wild-type CHO cells was also reflected in the toxicity of P3 toward these cell lines. Although P3 showed no aerobic toxicity toward any cell line, hypoxic CHO-1000 cells showed a sharp decrease in survival due to P3 that was not seen with the other cell lines (Fig. 2a). This response was very similar to that seen for exposure to 0.5 mM 2-NLP-3 in the same cell lines. These results suggest that the hypoxic toxicity of 2-NLP-3 in CHO-1000 cells is not mediated by the bioreductive activation of the nitroimidazole but by the phenanthridine end of the molecule.

The differences in the aerobic and hypoxic toxicity of 2-NLP-3 and P3 toward CHO-1000 and C5 cells suggest that the mechanisms of resistance in these cell lines are not the same. The P-glycoprotein that C5 produces seems to be able to protect completely against the toxic effects of 2-NLP-3 and P3 under acute exposure conditions. For this mechanism of resistance to be operating in the case of CHO-1000 it is necessary to argue that hypoxic exposure to 2-

NLP-3 and P3 somehow alters the efficient operation of the pump only in CHO-1000. The lack of radiosensitizing potency seen with CHO-1000 after 1 hr of drug exposure is consistent with a lower level of the drug reaching the nucleus, its site of action. An SER of only 1.5 could be achieved at an extracellular concentration of 2.0 mM in CHO-1000 cells. The same effect could be achieved in wild-type CHO cells at a concentration of approximately 50 μM.

A possible explanation for the unusual behaviour of CHO-1000 cells to hypoxic exposure to 2-NLP-3 is that these cells produce a higher level of some protective species, such as the cellular detoxifying agent GSH, whose depletion is toxic to the cell or facilitates the occurrence of toxic events. The depletion of GSH has been shown to mediate the toxic effects of both chemicals [11] and radiation [12]. Previous data have indicated that cellular GSH levels are depleted by over 90% when CHO wild-type cells are exposed to toxic concentrations of a 2-nitroimidazole, 1-CH_3-2-nitroimidazole, under hypoxic exposure conditions [13]. There was, however, little difference in the basal levels of GSH in the three cell lines studied (Fig. 4), but differential effects on GSH levels following exposure to the drug were possible. The depletion of GSH was rapid and significant in both wild-type CHO and CHO-1000 cells, although significantly less in the latter cell line. This depletion occurred under both hypoxic and aerobic conditions with the aerobic effect likely mediated by the futile cycling of the nitro group with concomitant production of superoxide radicals and hydrogen peroxide. Hydrogen peroxide is a substrate for enzymes utilizing GSH as a cofactor [14]. Interestingly, GSH was also depleted in C5 cells in these experiments, but to a significantly lesser amount than in CHO-1000. This result indicates that 2-NLP-3 is capable of getting into these P-glycoprotein expressing cells and that the reaction with GSH can occur before drug efflux occurs. GSH depletion correlated with toxicity particularly under hypoxic exposure conditions, suggesting that depletion of this protective species is correlated with the toxic events observed in the three cell lines.

To determine the spectrum of drug resistance, the CHO-1000 cell line was compared with wild-type CHO cells and the P-glycoprotein expressing C5 line in chronic toxicity experiments with five separate agents (Table 1). The C5 cell line was the most resistant to all the agents with the exception of 2-NLP-3. It was striking that C5 was almost 10 times more resistant to P3 than was CHO-1000 since the structure of P3 is also part of 2-NLP-3, the compound used to select for CHO-1000. All cell lines were equally sensitive to the untargeted 2-nitroimidazole, Miso, under both aerobic (Table 1) and hypoxic (Fig. 2d) exposure conditions. This suggests that the resistance of CHO-1000 cells is not due to a lack of enzymes required for the effective reduction of 2-nitroimidazoles. Further evidence for this was obtained by experiments in which the accumulation of a ^{99m}Tc-labelled 2-nitroimidazole, BMS 181321 [15], was shown to be similar under both hypoxic and aerobic conditions in all three cell lines studied (data not shown).

Studies with verapamil, a known inhibitor of the P-glycoprotein pump, were performed to look for the possible involvement of the pump in CHO-1000 resistance. Verapamil itself had no effect on either hypoxic or aerobic cells at 75 μM, above the highest concentration used in these studies, in any of the cell lines. Use of a 30 μM concentration of the inhibitor in combination with 1 mM 2-NLP-3 under aerobic conditions also resulted in a small increase in toxicity with C5 cells (data not shown). Increasing the verapamil concentration to 70 μM gave results for 2-NLP-3 toxicity in C5 cells (Fig. 5) similar to those seen for wild-type CHO cells (Fig. 1a).

There was an enhancement in toxicity in aerobic CHO-1000 cells treated with 30 μM verapamil plus 2-NLP-3 (data not shown), but toxicity did not increase further when the concentration of the inhibitor was raised to 70 μM (Fig. 5). This result again suggests that the observed resistance of CHO-1000 cells was not due to P-glycoprotein alone. Under hypoxic conditions, inhibition of P-glycoprotein by 70 μM verapamil resulted in very similar levels of 2-NLP-3 cytotoxicity in all three cell lines (Fig. 5). These data suggest that the pump is effectively inhibited under these conditions and that it does play some role in determining the toxic response of both resistant cell lines under hypoxia. Differences in the sensitivity of the P-glycoprotein present in C5 and CHO-1000 cells to verapamil seem unlikely since 70 μM verapamil completely eliminated the resistance of both cell lines to colchicine (data not shown). Further evidence that P-glycoprotein may be involved is obtained from the flow cytometry studies, which indicated the presence of the protein in all three cell lines tested, but at much higher levels in the two resistant cell lines (Fig. 6).

The present data indicate that it is possible to produce cell lines stably resistant to the effects of a DNA-targeted 2-nitroimidazole, and at least part of this resistance is due to the induction of the multidrug resistance phenotype, a common characteristic of which is the presence of increased levels of the P-glycoprotein pump. This pump does seem to play a part in the resistance of CHO-1000, but does not account for all of it. This is particularly true under aerobic exposure conditions where a high level of resistance to 2-NLP-3 was observed that could not be abrogated by verapamil. Other possible mechanisms of resistance, which have been reported before for other compounds, include: alterations in the quantity or activity of topoisomerase II [16], sequestration away from target sites [10], biotransformation of the compound to a non-toxic form [17], and changes in plasma and/or mitochondrial membrane potentials [18]. Whether any of these or other mechanisms are involved in the resistance of CHO-1000 cells is unknown. Further work to characterize the precise mechanism will be necessary.

Acknowledgements—The authors would like to thank Dr. David Hedley for assistance with the flow cytometry studies. Support for this work from The Medical Research Council of Canada and The National Cancer Institute of Canada is greatly appreciated.

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