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CYTOTOXICITY AND ACTIVATION OF CB1954 IN A HUMAN TUMOUR CELL LINE

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Abstract—CB1954 (5-aziridin-1-yl-2,4-dinitrobenzamide) is a monofunctional alkylating agent, to which Walker 256 cells are very sensitive. These cells express a nitroreductase which reduces CB1954 to a bifunctional crosslinking agent 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. *In vitro* testing on the human colon line LS174T showed that the differential cytotoxicities between the monofunctional agent (CB1954), and the active species (generated *in situ* by the addition of NADH and the Walker rat nitroreductase) were smaller than anticipated due to the unexpected toxicity of CB1954 (IC_{50} value for CB1954 on LS174T cells of 78 μM). The toxicity of the chemically synthesised active form was less than if it had been generated *in situ* (on LS174T cells). Further experiments showed that NADH was toxic at the levels used to generate the active species (500 μM). Gel filtration and electrophoresis experiments demonstrated that the human colon carcinoma and choriocarcinoma cell lines MAWI and JAR, as well as LS174T express an enzyme of similar molecular weight to that of the 33 kD Walker cell nitroreductase, which is capable of reducing CB1954 to its toxic metabolite, and reducing MTT to its insoluble formazan salt. The expression of this enzyme presumably accounts for the unexpected toxicity of CB1954.

CB1954 (5-aziridin-1-yl-2,4-dinitrobenzamide) was originally synthesised by Khan and Ross at the Chester Beatty Institute [1]. It displays potent activity against the Walker 256 tumour, a mammary carcinoma in rats [2]. The high degree of sensitivity of the Walker cells is directly attributable to the expression of an intracellular nitroreductase which converts the monofunctional CB1954 into the bifunctional 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. This second species then forms DNA interstrand crosslinks, thereby causing cytotoxicity [3].

The nitroreductase itself is a monomeric protein, of molecular weight 33.5 kDa, containing one molecule of FAD per enzyme [4]. It catalyses the transfer of four or six electrons to CB1954 to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide respectively. To do this it can use both NADH and NADPH as electron donors. This dual specificity for adenine dinucleotides has been the basis for the classification of nitroreductase as a form of NAD(P)H dehydrogenase, or DT diaphorase [4].

Clinical reports on the use of CB1954 are limited to an unpublished study at the Royal Marsden by Dr Wiltshaw, which gave disappointing results with a lack of any tumour regressions. CB1954 has been shown to be ineffective in a range of murine tumours and in the HT-29 human colon carcinoma xenograft [5]. However, Tisdale and Habberford report that EJ, a cell line derived from human bladder cancer, displays sensitivity intermediate between that of Walker 256, which is sensitive, and TLX5, a highly resistant lymphoma [6].

A possible use for CB1954 would be as a prodrug in antibody enzyme directed chemotherapy, the ADEPT system. This is a system whereby a tumour specific monoclonal antibody–enzyme conjugate is localised at the tumour site, and a non-toxic (or inactivated) prodrug is injected, and is converted to an active cytotoxic species at the tumour, thereby delivering potent chemotherapy directly to the tumour [7, 8].

Little is known about the effect of CB1954 on colorectal cancers of human origin, with the exception of the above studies. Our original aim was to use CB1954 in conjunction with an immunoconjugate comprising Walker rat nitroreductase and an anti-CEA (carcino-embryonic antigen) monoclonal antibody in the ADEPT system against colon cancer. We report here the effects of the CB1954/Walker rat nitroreductase system on cell lines derived from human colon tumours.

MATERIALS AND METHODS

Materials. Nitroreductase enzyme (EC 1.6.99.2) isolated from Walker 256 cells [4], CB1954 and its active form were kind gifts from the late Professor John J. Roberts from the Institute of Cancer Research at Sutton. The Walker cell line (W_s) and its alkylating agent-resistant sub-line (W_r) were also provided by Professor Roberts. All chemicals were from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and were analytical grade unless otherwise stated.

Cell culture. Cells were maintained as monolayers in DMEM with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Flow Laboratories) supplemented with 10% foetal calf serum (Northumbria Biologicals Limited [NBL]), 2 mM glutamine (NBL) and penicillin and streptomycin (100 μg/mL and 100 units/mL respectively).

Dose response. Cells were plated at 1×10^5 cells/

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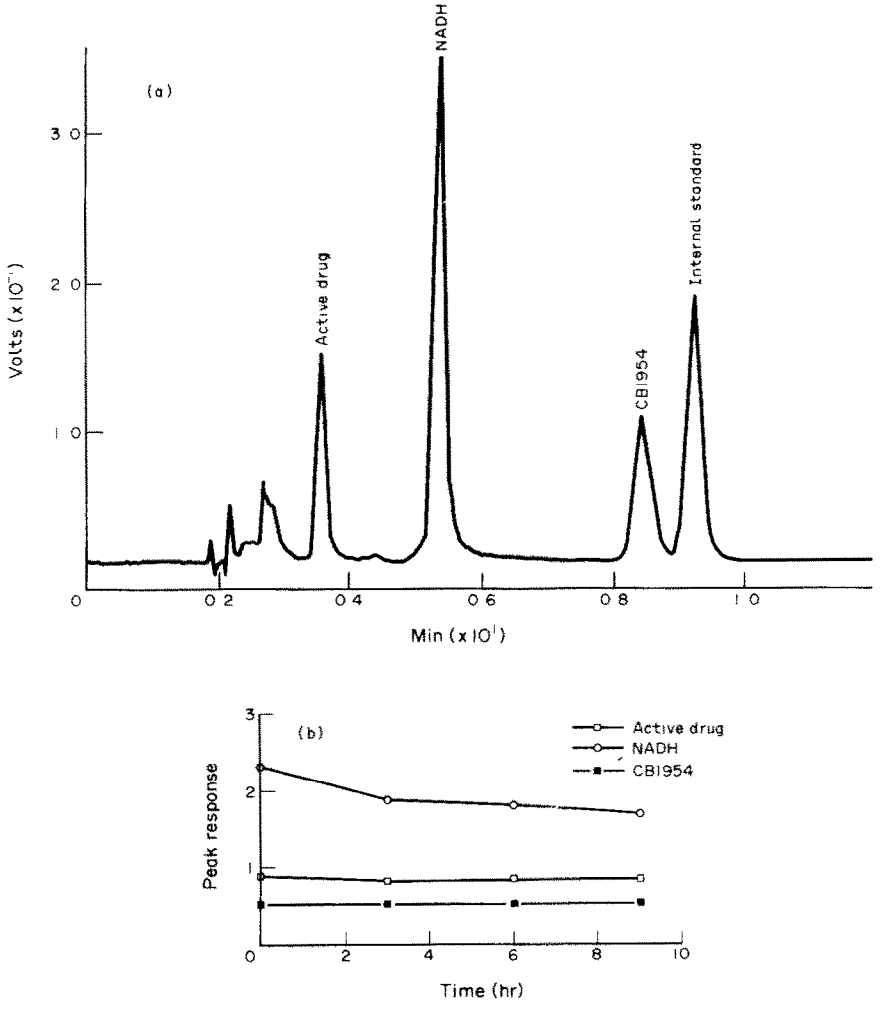

Fig. 1. (a) HPLC chromatogram of a reaction mixture of CB1954 (100 μM), NADH (500 μM) and nitroreductase (25 μg/mL) incubated in DMEM for 30 min at 37°. This shows retention times of CB1954, NADH, the active form of CB1954 and the internal standard. (b) Stability of the reaction mixture when stored at 20° in the presence of internal standard.

well in six well plates (Costar) in 2 mL DMEM as before (except that the buffer was bicarbonate instead of HEPES), and incubated for 24 hr in a 95% air–5% CO_2 incubator at 37°. Cells were treated for a specific time period in a combination of CB1954 (dissolved in 10% dimethylsulfoxide/Dulbecco's modified Eagles medium (DMSO/DMEM), appropriate solvent controls were included), NADH in PBS, or nitroreductase; all solutions were passed through 0.2 μm filters before use. After the cells were exposed to the drugs, they were washed twice in fresh DMEM, and incubated for a total of 7 days post treatment. After the end of the assay, the cells were washed in saline and harvested with trypsin/EDTA (NBL). Once the cells were in a suspension, they were mixed with an equal volume of 0.4% trypan blue, and the viable cells were scored on a haemocytometer.

MTT assay. This assay relies on the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] to a blue formazon salt by succinate dehydrogenase in the mitochondria. This only happens in living cells under the conditions used.

Cells were plated out at a density of 800 cells/well in half area 96 well plates (Costar). The cells were treated as above, and the plates processed as previously detailed [9]. For larger experiments to determine cytotoxicities of many cell lines at one time the MTT assay was used.

Cellular uptake and production of the active drug. The uptake and activation of CB1954 by LS174T cells was compared to Walker 256 (W_s) cells, and an alkylating agent-resistant subline, (W_r) approximately 10-fold more resistant to CB1954. Cells suspended at 10^7 cells/mL in DMEM were incubated at 37° with 50 μM CB1954, and 500 μM NADH. Aliquots of 400 μL were removed, and

vortexed with 500 μL of an oil mixture (silicone oil and "Three In One Oil" in a ratio of 4:1). These were centrifuged at 11,400 *g* for 2 min. The supernatants and cell pellets were mixed with 2 volumes of methanol containing an internal standard. The samples were analysed by HPLC as outlined below.

Cell fractionation. Cells were trypsinised and washed three times in PBS, before being resuspended in 1.5 mL of PBS containing either 1% aprotinin [1], or 1 mM phenylmethylsulphonyl fluoride. Cells were lysed by sonication (3 min on ice at 60% pulse activity) before being centrifuged at 11,400 *g* for 30 min to bring down any debris. The lysate was then filtered to 0.2 μm. Protein concentration was determined by the Pierce protein assay kit.

FPLC. A total of 5 mg of protein in 1 mL sample volume was run on a Superose 12 column connected to a Pharmacia FPLC system. The column was equilibrated and run in Dulbecco's PBS (Flow), at a flow rate of 0.5 mL/min.

SDS and Native PAGE. SDS-PAGE was carried out on the Pharmacia Phast system as a modification of Laemmli's method [10], according to the manufacturer's instructions. Native PAGE was also performed on the Phast system. Briefly, native PAGE is a method of electrophoresis which uses the same discontinuous gel system, but is run in the absence of SDS, in a high pH buffer. This means the proteins which have a low or average pI will have an overall negative charge, and so migrate to the anode. Thus molecular weights of proteins can be estimated, although not as accurately as with SDS-PAGE, and they retain functional activity.

HPLC. Samples were analysed on a Waters isocratic system using a Zorbax C_{18} column (25 × 0.4 cm) and a mobile phase of 20% methanol, 80% aqueous (0.125 M K_2HPO_4, 6.25 mM tetrabutylammonium phosphate adjusted to pH 5.0). All reagents were HPLC grade (FSA). The flow rate was 1 mL/min with UV detection at 325 nm.

Samples were diluted in two volumes of ice-cold methanol containing internal standard (0.05 mg/mL *p*-nitrobenzamide) with 0.1% cysteine hydrochloride to stabilise the active form of the drug. Following centrifugation, 20 μL was injected. A sample chromatogram of the compounds in DMEM is displayed in Fig. 1a. In addition the stability of these components during storage in an HPLC vial at 20° is shown in Fig. 1b.

RESULTS

Differential cytotoxicity

The differential cytotoxicity between CB1954, and its activated form, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (whether generated *in situ* or as an exogenously added agent) was determined. A 2 hr incubation period was chosen as virtually complete turnover of CB1954 was achieved with enzyme (results not shown).

LS174T cells were incubated for 2 hr with a range of CB1954 concentrations either with or without enzyme (25 μg/mL nitroreductase and 500 μM NADH) or with the chemically synthesised active form of the drug (Fig. 2).

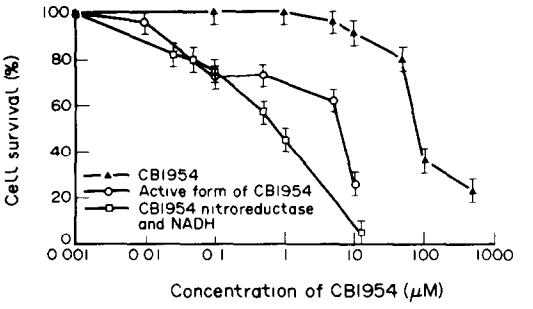

Fig. 2. Differential dose response of LS174T cells to CB1954 prodrug and its active species whether chemically synthesised, or generated in situ by NADH (500 μM) and nitroreductase (25 μg/mL). Cells were exposed to the compounds for a 2 hr period. Values shown include standard error bars (N = 4).

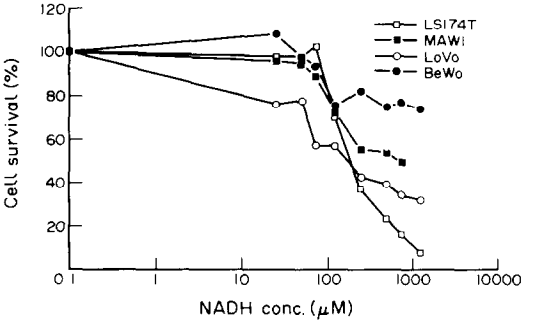

Fig. 3. Dose response of four human cell lines to NADH: LS174T, MAWI, LOVO and BEWO. Assay by MTT method.

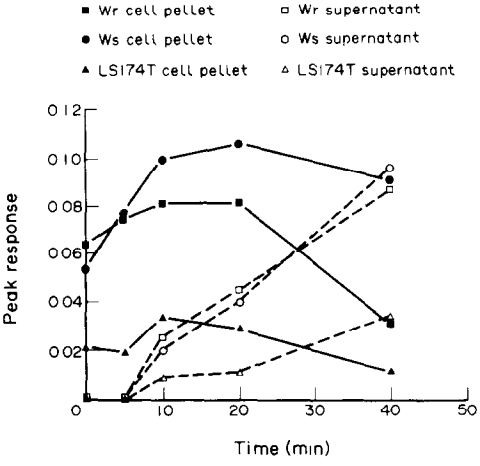

Fig. 4. Relative active drug levels in LS174T, W_1 and W_2 cells incubated at 37° in DMEM containing NADH (500 μM) and CB1954 (50 μM).

Under these conditions an IC_{50} value of 78 μM was obtained for CB1954 on its own, 0.4 μM for the drug activated *in situ*, and 8 μM for the chemically synthesised form. It would appear that the differential in toxicity between *in situ* generation, and exogenous

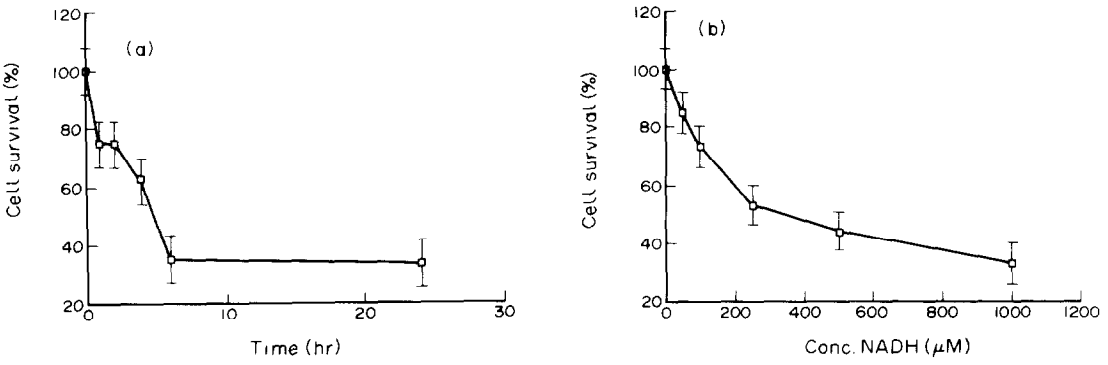

Fig. 5. (a) Time course of NADH cytotoxicity (500 μM) against LS174T cells. (b) Dose response of LS174T cells to 16 hr exposure to NADH.

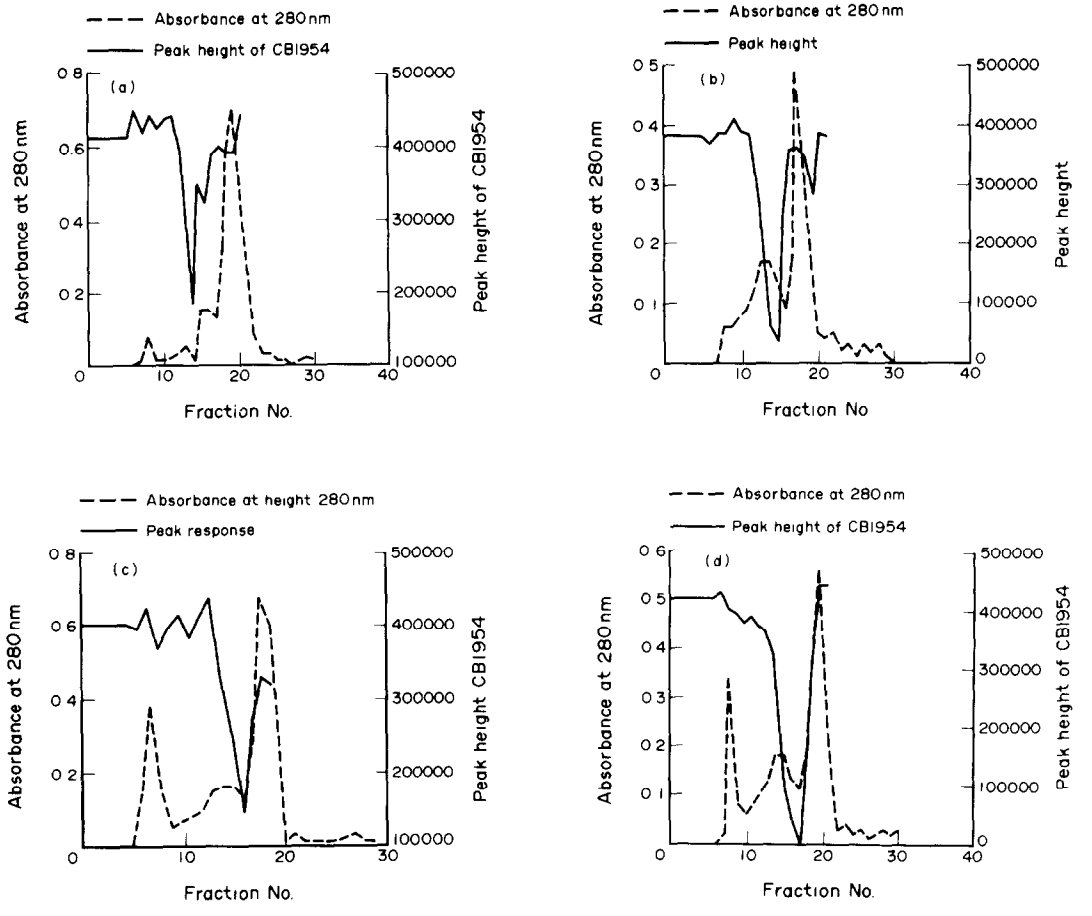

Fig. 6. Enzymically active fractions separated by FPLC and detected by CB1954 HPLC assay: (a) LS174T, (b) MAWI, (c) JAR and (d) W_r cell lines.

addition, could be partially accounted for by NADH, which is itself cytotoxic as detailed below (see Figs 3 and 4 and Ref. 11).

Further experiments were carried out to determine the differential cytotoxicity over a greater exposure time. LS174T cells were treated to three continuous daily consecutive doses of CB1954 either with or without the enzyme and co-factor as before. The data (not shown) indicates that not only is the toxicity of CB1954 increased ($IC_{50} = 4$ μM), but that the NADH exerts a toxic effect, since the growth of one of the control cell groups (NADH-treated) is being inhibited.

The dose response of NADH on LS174T cells is

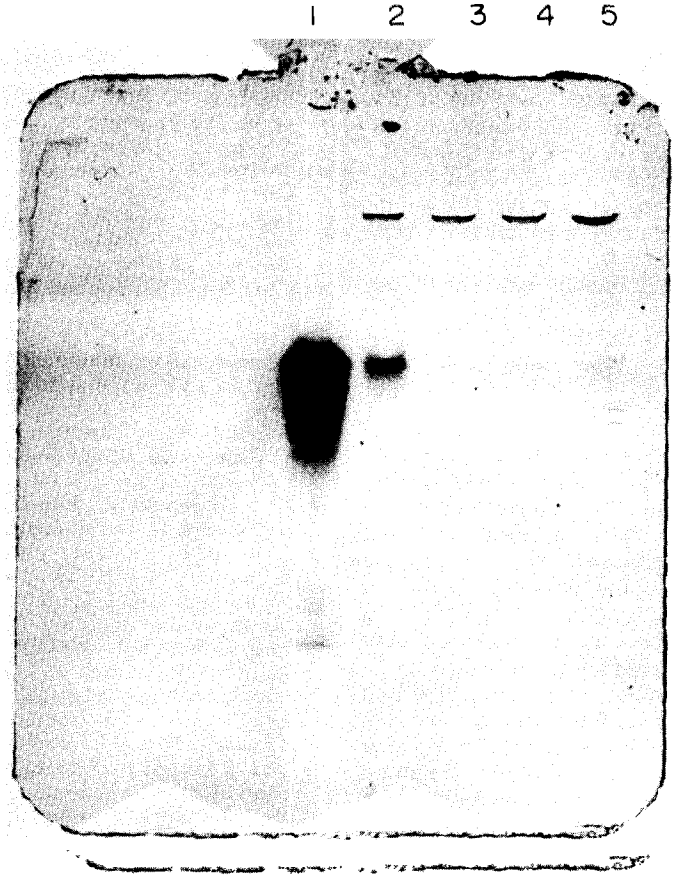

Fig. 7. Native PAGE of cell fractionates stained with MTT. Lane 1, Nitroreductase enzyme; Lane 2, W_r cell lysate; Lane 3, LS174T cell lysate; Lane 4, MAWI cell lysate; Lane 5, JAR cell lysate. One μg of protein applied to each lane.

shown for a sixteen hour exposure time (Fig. 5b), and the time course of cytotoxicity based on a dose of 500 μM NADH, and a variable time of exposure (Fig. 5a).

Since NADH inhibited the growth of LS174T cells, the cytotoxic dose response was repeated using other cell lines (LoVo and MAWI, two colorectal carcinoma lines, and BeWo, a choriocarcinoma line) as well as LS174T (Fig. 3).

All cell lines showed some sensitivity, but LS174T was the most sensitive at a concentration of 500 μM, i.e. the dose used in these experiments. The cytotoxic effect of NADH has been previously documented [11].

Cellular uptake and production of active drug

The results (Fig. 4) show that the active drug is detectable in the cell pellets immediately after administration of CB1954 and NADH. At 10 min the drug is found in the supernatant. The formation of the active drug in the cells reached a plateau at about 10 min, as the NADH levels drop. Active drug levels in the Ws cell pellets are approximately four times those in the LS174T, with the Wr pellets having intermediate concentrations. It is worth noting that at no time are the levels of intracellular NADH or CB1954 much above detectable levels, presumably because they are reacting to form the activated drug. Once the NADH level has dropped significantly (<20 μM) the level of active drug within the cells also falls and that of the supernatant rises. This is presumably because there is less active drug being produced in the cell, and active drug already present in the cell is diffusing out.

Identification of a nitroreductase-like enzyme in human cell lines

An enzymic activity which can reduce CB1954 in a nitroreductase-like manner was found in human tumour cell lines MAWI, JAR, and LS174T. Fractions (1 mL) from cell lysates processed by gel filtration on FPLC were incubated with CB1954 and NADH at a final concentration of 30 and 500 μM, respectively, at 37° for 12 hr, and the amount of CB1954 quantitated by HPLC as described, as a measure of enzyme activity. Activity was found in fractions 14–17 in the human lines, whereas in the Walker cell line, the activity is limited to the 15 to 18 range (Fig. 6). These fractions of maximum CB1954 turnover correspond with peak active drug

levels. The peak of activity derived from Walker W_r cells coincided with the 30,000 Mw range.

Native PAGE

Samples of lysate (1 μL) were loaded onto a 12% native PAGE gel as described and either stained with silver for total protein or with MTT (5 mg/mL) and NADH (500 μM) for 20 min at 37°. Under these conditions MTT is a substrate for the Walker rat nitroreductase enzyme, and any other NADH dehydrogenase [12]. The converted MTT is directly visible as a blue band, so regions with activity can be discerned (Fig. 7). All four lines show a single band, although the human cell lines are much fainter.

DISCUSSION

CB1954 is currently being evaluated as a potential prodrug due to the extreme differential cytotoxicity between the drug and its activated form [4]. However, our results have demonstrated that CB1954 has certain limitations. This is due to the NADH requirement of the enzyme, the drug's apparent toxicity *in vitro*, and the ability of the cells which were tested to convert the prodrug to the active species in the absence of added enzyme.

The toxicity of NADH and the anticipated difficulty of its administration raise the question whether NADH concentrations could be maintained at the tumour site for effective therapy, without causing unpleasant side effects. NADH is less stable at low pH [13], which is typical of the conditions found in solid tumours.

Unexpectedly high toxicity of the prodrug has been explained by the discovery of an enzymic activity which converts CB1954 to its active form. Native PAGE gels, and fractionation studies show that there is a human protein of similar molecular weight to the Walker rat nitroreductase, which reduces MTT and CB1954 in the presence of NADH. The amount of activity in the human cells is much less than that in the rat, either measured by the intracellular production of the active drug, or by the MTT gel staining method.

This activity may be a form of DT diaphorase, resembling the Walker rat nitroreductase, or activation may occur by other nitroreductases, of similar molecular weight, which would merit a further study of its own. The recent paper by Boland *et al.* [14] on the kinetics of the rat and human DT diaphorase shows that the levels of the enzyme are similar in rat and human cell lines. However they show that CB1954 is a much poorer substrate for the human enzyme.

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