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SELECTIVE INHIBITION OF RIBONUCLEOTIDE REDUCTASE BY THE MONOFUNCTIONAL ALKYLATING AGENT 5(1-AZIRIDINYL)-2,4-DINITROBENZAMIDE (CB 1954)

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Abstract—The monofunctional alkylating agent 5-(1-aziridinyl)-2,4-dinitrobenzamide (CB 1954) is a potent and selective inhibitor of the growth of the Walker carcinoma (LD_{50} 0.002 μg/ml). Growth inhibition by CB 1954 is accompanied by a rapid inhibition of DNA synthesis with little effect on RNA and protein biosynthesis. Interference with the biosynthesis of DNA by CB 1954 has been shown to be due to inhibition of ribonucleoside diphosphate reductase. The order of effectiveness of inhibition of the enzyme by CB 1954 in four cell lines parallels the tumour growth inhibitory activity. Furthermore, analogues of CB 1954 with methyl substitution on the amide group were less effective inhibitors of the growth of the Walker carcinoma and also less potent inhibitors of ribonucleotide reductase. Treatment of Walker cells with CB 1954 caused a rapid inhibition of enzyme activity and a corresponding fall in the levels of deoxyribonucleoside triphosphates which corresponded with the effect of the drug on DNA synthesis. A human bladder carcinoma has been shown to be particularly susceptible to growth inhibition by CB 1954. Measurement of the effect of CB 1954 on ribonucleotide reductase from biopsy specimens of human tumours could provide a basis for patient selection for therapy by this agent.

5(1-Aziridinyl)2,4-dinitrobenzamide (CB 1954, Fig. 1) is a potent and selective inhibitor of the growth of the Walker carcinoma in rats, having a therapeutic index (LD_{50}/ED_{90}) of 70, the highest ever recorded against the Walker tumour [1]. This drug is unusual amongst the alkylating agents in having only one alkylating group, since early studies showed that the most cytotoxic and the most potent inhibitors of tumour growth were at least bifunctional, i.e., they should have two alkylating arms, which can combine with two nucleophilic centres in biological macromolecules [2]. Since the primary effect of alkylating agents is inhibition of DNA synthesis, this led to the view that the functionality of the alkylating agents enabled them to cross-link nucleophilic centres occurring in the same (intrastrand) or in adjacent (interstrand) strands of the DNA helix [3]. Such a reaction would be impossible with a monofunctional agent. CB 1954 also differs from the classical cytotoxic alkylating agents in having a minimal effect on the haematopoietic system and being inactive against many experimental animal tumours which normally respond to a range of bifunctional alkylating agents [1]. Another unusual feature is the ability to protect against the cytotoxic effect of CB 1954 by a variety of aminoimidazolecarboxamides and anthranilamide, adenine and 2,4-dinitrophenol [4, 5]. The protection by 4-amino-5-imidazolecarboxamide led to the view that CB 1954 acted as a purine antimetabolite [4]. The most effective protector, 4-amino-2-phenylimidazole-5-carboxamide (2-phenyl AIC), reversed the cytotoxicity of CB 1954 when given before or at the same time, but showed a decreased protecting ability even when given only 30 min after CB 1954. The low chemical reactivity of the aziridine ring and the failure to protect with cysteine [4] led to the suggestion that CB 1954 might react with its site of action in two phases, an initial and reversible binding followed by a slow alkylation [5]. This suggests that both CB 1954 and 2-phenyl AIC interact with the same cellular receptor.

A feature of CB 1954 cytotoxicity is the selective inhibition of the incorporation of [^{3}H]thymidine into DNA with little effect on RNA and protein synthesis [6]. 2-Phenyl AIC also inhibits the incorporation of [^{3}H]thymidine into DNA at concentrations used in the protecting experiments [7]. One common factor in the chemical structures of both CB 1954 and the protecting agents is that they are capable of chelation of metal ions. DNA synthesis requires a balanced supply of deoxyribonucleotides and since deoxyribonucleotides, in contrast to ribonucleotides, are found at extremely low levels in animal cells [8], the reductive conversion of ribonucleotides to deoxyribonucleotides is thought to be a crucial rate-controlling step in the pathway leading to the biosynthesis of DNA. Reduction of ribonucleotides occurs on a multisubunit enzyme complex that requires iron, and reduced disulphydryl protein serves as the reducing agent. Such an enzyme complex could provide a suitable target for CB 1954, and inhibition would account for the selective effect of this agent on DNA synthesis. The present investigation was undertaken to determine the effect of CB 1954 on the ribonucleotide reductase system of tumours with

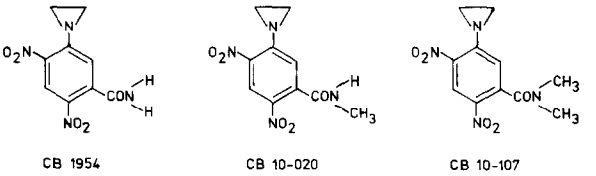

Fig. 1. Structure of drugs used in this study.

a range of sensitivities to its cytotoxic action and to determine if a correlation existed between the ability of CB 1954 and its congeners (Fig. 1) to inhibit ribonucleotide reductase and their tumour growth inhibitory activity. A knowledge of the biochemical mechanism of action of CB 1954 would allow the rational clinical use of this agent.

MATERIALS AND METHODS

Chemicals. 5-[Methyl-^{3}H]thymidine (sp. act. 5 Ci/mmole), [5-^{3}H]cytidine 5′-diphosphate, ammonium salt (sp. act. 11 Ci/mmole), 5-[methyl-^{3}H]thymidine 5′-triphosphate, ammonium salt (sp. act. 60 Ci/mmole), deoxy [8-^{3}H]guanosine 5′-triphosphate, ammonium salt (sp. act. 13.2 Ci/mmole), deoxy [8-^{3}H]adenosine 5′-triphosphate, ammonium salt (sp. act. 25 Ci/mmole), and deoxy [5-^{3}H]cytidine 5′-triphosphate, ammonium salt (sp. act. 25.5 Ci/mmole) were purchased from the Radiochemical Centre, Amersham, U.K. *Micrococcus luteus* DNA polymerase was obtained from Miles Laboratories Ltd., Slough, U.K., and poly [d (A-T). d (A-T)], poly [d (I-C). d (I-C)], nucleosides and nucleotides from Sigma Chemical Co., London, U.K. Culture medium was purchased from GIBCO Bio-Cult, London, U.K. CB 1954 and the analogues CB 10-020 and CB 10-107 (Fig. 1) were kindly supplied by Dr. D. E. V. Wilman, Chester Beatty Cancer Research Institute, London, and the human bladder carcinoma (EJ) by Dr L. M. Franks, Imperial Cancer Research Fund, London, U.K.

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and incubated under an atmosphere of 10% CO_2 in air. For growth experiments cells were grown in duplicate wells (3.5 ml) of a 24-well plastic plate (Flow Laboratories, Irvine, Scotland). Cells were counted daily using a Coulter counter. Growth curves were constructed and the degree of inhibition was calculated from the linear part of the growth curves. Drugs were dissolved in dimethylsulphoxide (DMSO). A resistant line of the Walker tumour was derived from the sensitive line by repeated treatment with increasing doses of chlorambucil. A human bladder carcinoma was treated with 0.025% trypsin, 10 mM EDTA prior to counting.

Nucleic acid synthesis. Walker cells were suspended in medium at 2×10^5 cells per ml and incubated with the concentrations of CB 1954 indicated in Figs. 12 and 13. At intervals, 1-ml portions of the cell suspension were removed and incubated with [methyl-^{3}H]thymidine (5μCi/ml) for 1 hr at 37°. The reaction was stopped with ice-cold 0.9% NaCl and the cells were immediately filtered through a glass fibre filter disc (Whatman GF/C, 2.5 cm) wetted with saline and were washed with ice-cold 5% trichloroacetic acid and absolute ethanol. After drying for 2 hr at 70° the acid-insoluble radioactivity on the filters was determined using a toluene PPO and POPOP scintillation mixture.

Enzyme preparation. Walker cells were obtained either from the peritoneal cavity of Wistar rats or from tissue culture. Cells from animals were washed several times with 0.016 M Tris–HCl, pH 7.2, containing 7.2 g/l NH_4Cl to remove red cell contamination and finally cells from both sources were washed with 0.9% NaCl prior to lysis. The cell pellet was suspended in 10 mM phosphate, pH 7.4, containing 17 mM dithiothreitol and 1.7 mM $MgCl_2$ at 4° and either sonicated for 10 sec with a 20Kc MSE sonic oscillator or homogenized in a Potter–Elvehjem homogenizer with a toothed teflon pestle turning at 900 rpm for 3 min. All operations were performed at 4°. The homogenate was centrifuged at 100,000 *g* for 1 hr and the supernatant fraction was used as a source of enzyme after extensive dialysis against excess homogenizing buffer to remove deoxynucleotides. The protein concentration of the supernatant fraction was determined by the method of Lowry *et al.* [9] using bovine serum albumin as a standard.

Ribonucleotide reductase assay. The reduction of ribonucleotides to deoxyribonucleotides was measured by the conversion of cytosine ribonucleotide to the deoxyribonucleotide by a method similar to that used by Youdale and MacManus [10]. The assay was carried out in a total volume of 60 μl of 20 mM Tris–HCl, pH 7.6, containing 5 μCi [^{3}H]CDP, 17 mM dithiothreitol, 8.5 mM $MgCl_2$, 3.6 mM ATP and 127 μM $(NH_4)_2SO_4 \cdot FeSO_4$. Both the ATP and the acidic drugs were neutralized to pH 7.6 before adding to the assay mixture. Drugs were dissolved in DMSO at 100 × the required concentration and diluted with incubation buffer prior to assay. The assay tubes were incubated for 1 hr at 37° and the reaction was terminated by heating in a boiling water bath for 2 min. After cooling on ice a mixture of dCMP and CMP (50 μg of each) was added as carrier to each sample and the mixture was incubated at 37° for 30 min with apyrase (100 μg) to hydrolyse the diphosphates to monophosphates. The reaction was terminated by boiling for 2 min, cooled and the precipitated protein was sedimented by centrifugation for 5 min at 350 g. A sample (10 μl) of the supernatant fluid was applied to a PEI cellulose plate (Schleicher & Schüll, Dassel, F.R.G.) and developed in 35 ml 2.0 M LiCl/65 ml 2% boric acid. Both the monophosphate and deoxymonophosphate were located under u.v. light, the spots were removed from the plate and extracted with 0.2 ml 1N HCl and the radioactivity was measured in PCS scintillation fluid (Hopkin & Williams, Poole, U.K.) using a Tracer Lab scintillation counter. The total radioactivity in each sample was measured by counting 10 μl of the supernatant fluid applied to a PE I cellulose plate and extracted before chromatography.

Assay for deoxyribonucleoside triphosphate. The assay for deoxyribonucleoside triphosphates was a modification of the method of Walters *et al* [8] except that *Micrococcus luteus* DNA polymerase was used instead of *Escherichia coli* DNA polymerase.

The assay was carried out in a total reaction volume of 0.2 ml and contained the following: 10 μmoles Tris–HCl buffer (pH 8.0 for the dATP and dTTP pools and pH 8.6 for the dCTP and dGTP pools), 2 μmoles $MgCl_2$, 0.2 μmoles 2-mercapthoethanol, 10 μg of the appropriate template (poly [d (A-T). d (A-T)] or poly [d(I-C). d(I-C)] and 4 units of the *M. luteus* DNA polymerase. The standard curve was produced using 200 pmoles of the tritiated dNTP and 1–200 pmoles of the complementary dNTP. For

determination of the unknowns appropriate amounts of neutralized cell extract were added and the mixture was incubated at 37° for 1 hr. The reaction was terminated by chilling in an ice bath, 100 μg of DNA was added followed by 5 ml of 10% trichloroacetic acid and, after mixing, the resulting precipitate was collected by filtration on a Whatman GF/C glass fibre filter. The filters were washed with 3 × 5 ml quantities of cold 5% trichloroacetic acid, dried and the radioactivity was determined in a toluene, PPO scintillation mixture. A standard curve with known amounts of each limiting deoxyribonucleoside triphosphate was established each time the assay was used, as was a blank containing no cell extract.

RESULTS

Drug sensitivity of cell lines. The relationship between the concentration of CB 1954 and the inhibition of cell growth for Walker carcinoma, a human bladder carcinoma (EJ) and the TLX5 lymphoma is shown in Fig. 2. The concentration which gave 50 per cent inhibition of growth (LD_{50}) for Walker carcinoma which was either sensitive or resistant to chlorambucil was 0.002 μg/ml. In contrast, there was a 60-fold difference in the sensitivites of these cell lines to the bifunctional alkylating agent chlorambucil, the LD_{50} values being 0.1 and 6.3 μg/ml, respectively (Table 1). This result differs from that of previous studies [6, 11] in which cross-resistance was observed between CB 1954 and the bifunctional alkylating agents. Although the TLX5 lymphoma is insensitive to CB 1954 (LD_{50} 40 μg/ml), as previously reported [1], the human bladder carcinoma shows a degree of sensitivity (LD_{50} 0.7 μg/ml) comparable with that of the Walker carcinoma. This suggests that bladder carcinomas may be suitable targets for studies *in vivo*. The structural specificity of CB 1954 for an effective cytotoxic action is shown by a comparison with analogues with increasing methyl substitution on the amide group (Table 1). Thus the LD_{50} increases from 0.002 μg/ml for CB 1954 to 0.004 μg/ml for CB 10-020 and 0.07 μg/ml for CB 10-107, paralleling the activity of these agents as antitumour agents *in vivo* [12]. This suggests that the difference in activity is not due to host effects, but probably arises from differences in affinity of these analogues to cellular receptors.

Inhibition of ribonucleotide reductase. The rate of formation of dCMP from CDP by a cytosolic extract of Walker carcinoma was linear with reaction time up to 60 min and was proportional to the amount of protein present (Fig. 3). The effect of increasing concentrations of CB 1954 on the activity of the enzyme prepared from Walker carcinoma sensitive or resistant to chlorambucil, a human bladder carcinoma (EJ) and TLX5 lymphoma is shown in Fig. 4. The order of effectiveness of CB 1954 as an inhibitor of ribonucleotide reductase from the various cell lines *in vitro* parallels the growth inhibitory activity, with the TLX5 lymphoma being less sensitive than EJ, which in turn was less sensitive than the Walker carcinoma. There was no difference in sensitivity to CB 1954 between ribonucleotide reductase isolated from either chlorambucil-sensitive or resistant Walker carcinoma. The order of potency of the analogues against the enzyme isolated from Walker carcinoma also paralleled their cytotoxic potency, with CB 1954 being more effective than CB 10-020 which in turn was more effective than CB 10-107 (Fig. 5). This suggests that the difference in activity of this congeneric series is probably related to differences in binding to the enzyme rather than to differences in uptake by the cell. The degree of

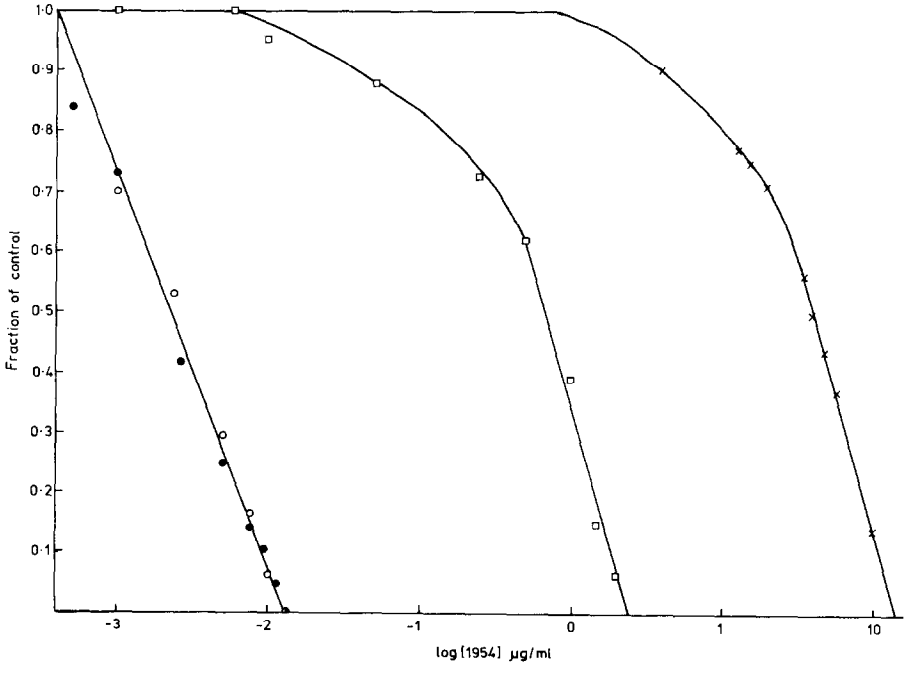

Fig. 2. Dose–response curves for CB 1954 against Walker carcinoma sensitive (○—○) and resistant (●—●) to chlorambucil, human bladder carcinoma (□—□) and TLX5 lymphoma (×—×).

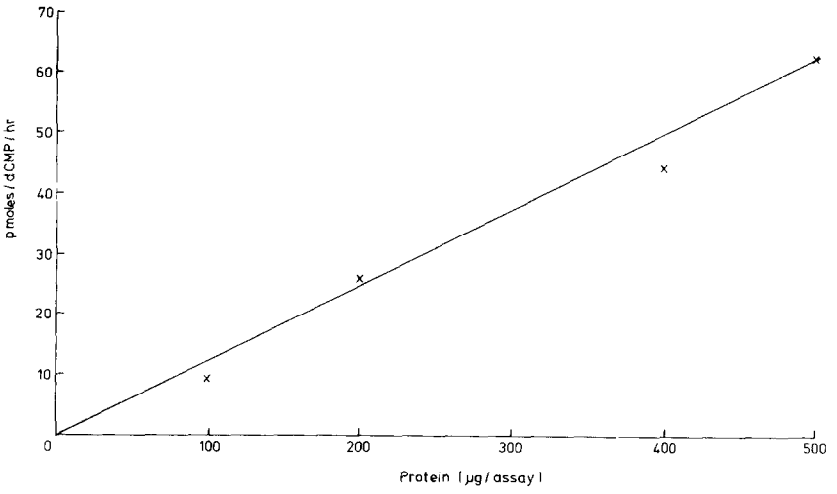

Fig. 3. Rate of reduction of CDP by cytosolic extracts of Walker carcinoma as a function of protein concentration.

Table 1. Sensitivities of cell lines to chlorambucil, CB 1954, CB 10-020 and CB 10-107

	LD_{50}(μg/ml)			
Cell line	Chlorambucil	CB 1954	CB 10-020	CB 10-107
*WS	0.1	0.002	0.004	0.07
*WR	6.3	0.002	—	—

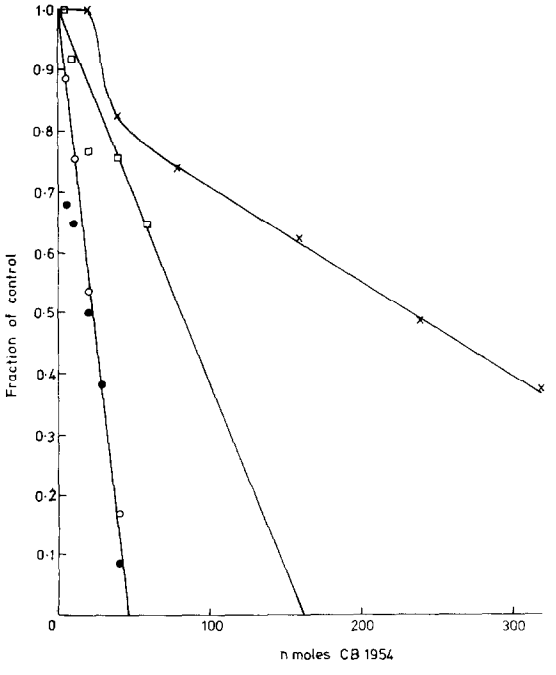

Fig. 4. Effect of increasing concentrations of CB 1954 on ribonucleotide reductase from Walker carcinoma sensitive (○—○) or resistant (●—●) to chlorambucil, human bladder carcinoma (□—□) and TLX5 lymphoma (×—×). The protein concentration was 200 μg per assay.

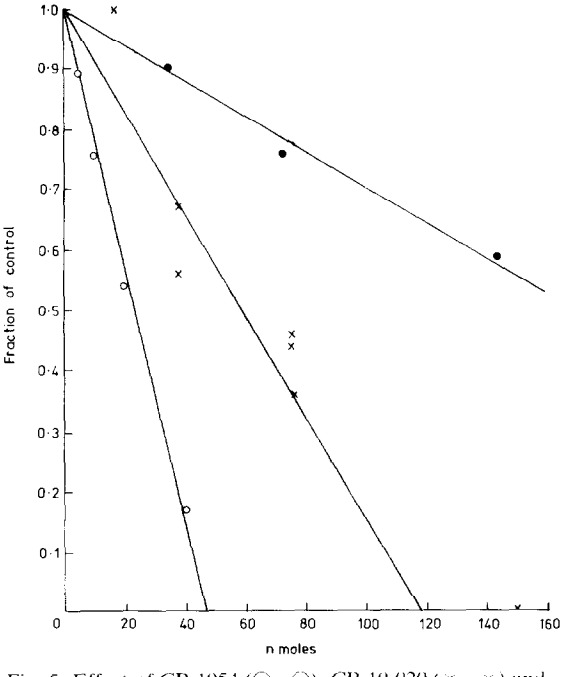

Fig. 5. Effect of CB 1954 (○—○), CB 10-020 (×—×) and CB 10-107 (●—●) against ribonucleotide reductase from Walker carcinoma. The protein concentration was 200 μg per assay.

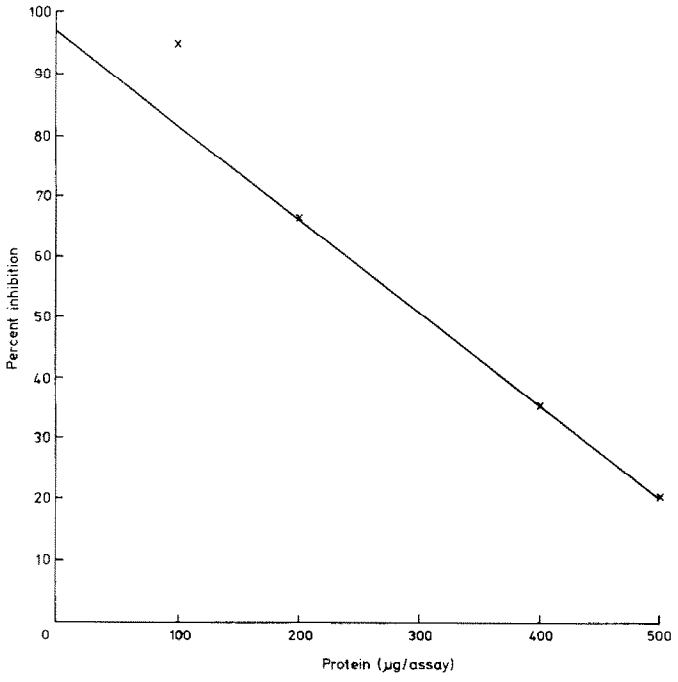

Fig. 6. Effect of protein concentration on the inhibition of ribonucleotide reductase by 20 nmoles of CB 1954.

inhibition of ribonucleotide reductase by CB 1954 was proportional to the enzyme concentration in the assay (Fig. 6). This suggests that the cytosolic protein contains a fixed number of enzyme sites that are titrated out by CB 1954, and indicates that the inhibition is irreversible. This was also confirmed by the results in Fig. 8 in which dialysis of the enzyme from cells treated with CB 1954 did not remove inhibition. Attempts at preincubation of enzyme with CB 1954 for various times prior to the addition of the substrate were unsuccessful due to the rapid loss of enzyme activity in controls incubated under the same conditions. This suggests that ribonucleotide reductase is unstable in the absence of its substrate. Like CB 1954, 2-phenyl AIC also inhibits ribonucleotide reductase, though higher concentrations are required to produce the same degree of inhibition (Fig. 7). Unlike CB 1954, the inhibition produced by 2-phenyl AIC is reversible due to the absence of any group able to form a covalent linkage with nucleophilic sites on the enzyme.

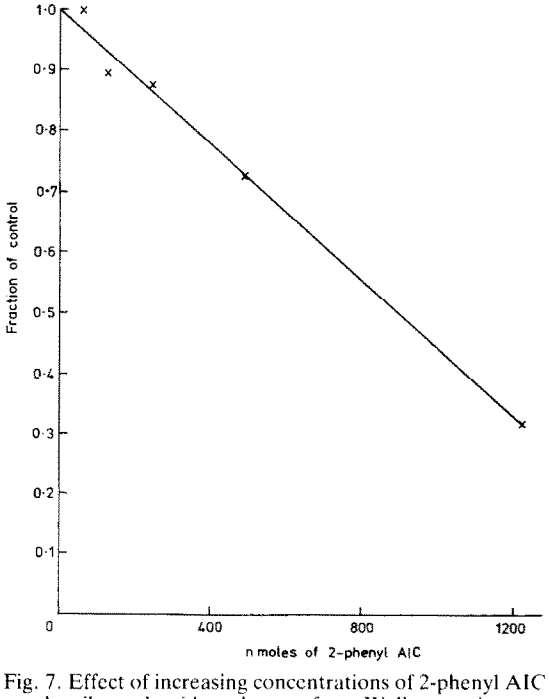

Fig. 7. Effect of increasing concentrations of 2-phenyl AIC on the ribonucleotide reductase from Walker carcinoma.

The effect of treatment of intact Walker cells with 0.05 μg/ml of CB 1954, a concentration just sufficient to cause complete growth inhibition, on the activity of ribonucleotide reductase is shown in Fig. 8. Enzyme levels are very rapidly reduced and reach zero within 4 hr. This is followed by a wave of enzyme synthesis by the cell, either due to derepression of enzyme synthesis by a fall in the product levels (Fig. 9) or due to the progressive accumulation of cells in the S-phase of the cell cycle, a time of increased ribonucleotide reductase synthesis [13]. Possibly due to a lower rate of reaction of the aziridine ring of CB 1954 with cellular nucleophiles compared with the 2-chloroethylamines, the newly synthesized enzyme is also inhibited by CB 1954 and within 24 hr the level is reduced in a dose-related fashion (Table 2). This confirms that pharmacological concentrations of CB 1954 cause an inhibition of ribonucleotide reductase with a time course paralleling the effects on deoxynucleoside triphosphate levels (Fig. 9) and incorporation of [^{3}H]thymidine into DNA (Fig. 10).

Effect on deoxynucleoside triphosphate pool sizes and incorporation of [^{3}H]*thymidine into acid-insolu-*

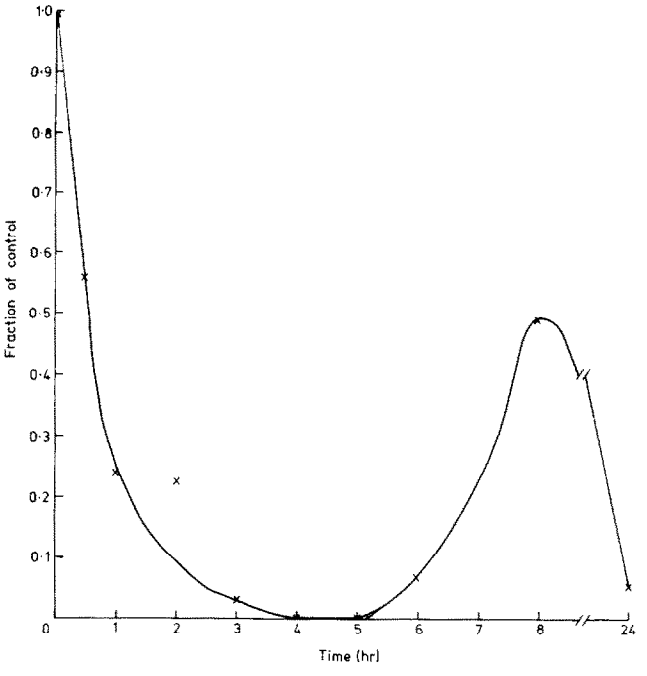

Fig. 8. Effect of incubation of Walker cells with 0.05 μg/ml CB 1954 on the activity of ribonucleotide reductase. The enzyme preparations were dialysed prior to assay.

ble material. The changes in deoxyribonucleoside triphosphate content of Walker cells exposed to 0.05 μg/ml CB 1954 for varying time periods are shown in Fig. 9. As observed in other cell lines [14], the pool size of dTTP is substantially larger than that of dATP, dGTP or dCTP and consequently the effect of inhibition of its synthesis is less pronounced than on the other deoxyribonucleoside triphosphate levels. The pool size of dGTP and dCTP is only one-seventh of that of dTTP and within 4 hr the intracellular concentration of both becomes unmeasurable by the polymerase assay, which is capable of measuring 0.5 pmoles/10^6 cells. This coincides with the time at which the activity of ribonucleotide reductase has been reduced to zero. This suggests that the reduction in the incorporation of [^{3}H]thymidine into acid-insoluble material produced by CB 1954 is due to a lack of dATP, dGTP and dCTP. CB 1954 produces a dose-related linear inhibition of [^{3}H]thymidine incorporation into acid-insoluble material up to 5 hr after its addition, after which the rate of inhibition decreases (Fig. 10). This

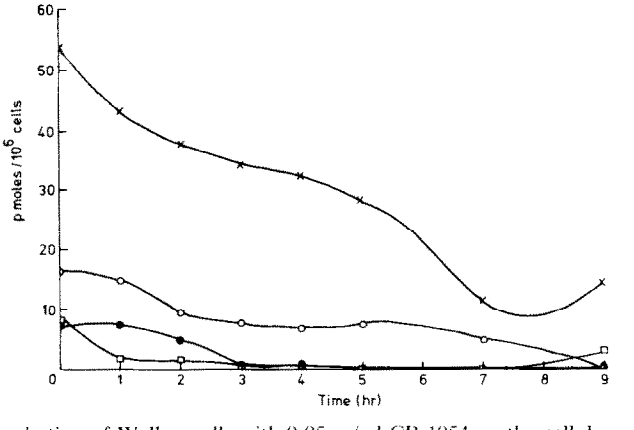

Fig. 9. Effect of incubation of Walker cells with 0.05 μg/ml CB 1954 on the cellular contents of dTTP (×—×), dATP (○—○), dCTP (●—●) and dGTP (□—□). The extreme range of values about each point was not greater than ± 5%. At time intervals after treatment with CB 1954 cells were transferred to centrifuge tubes pre-cooled in ice and centrifuged at 350 *g* for 5 min. The cell pellet was resuspended twice in ice-cold 0.9% NaCl and after centrifugation was suspended in 1 ml of ice-cold 0.5 N perchloric acid for 20 min. The supernatant fraction obtained by recentrifugation at 1000 *g* for 5 min at 0° was made 50 mM with respect to Tris by adding 1 M Tris. HCl, pH 8 and potassium perchlorate was precipitated by adding 5 N KOH to pH 7.5–8.0. The supernatant fraction obtained by removing the potassium perchlorate was stored at −20° until assayed.

Table 2. Effect of incubation of Walker carcinoma cells with CB 1954 for 24 hr on the activity of ribonucleotide reductase

Concentration of CB 1954 (μg/ml)	Specific activity (pmoles. mg protein^{-1}. hr^{-1})	Fraction of control
0	11	1.0
0.005	10.2	0.92
0.01	3.9	0.35
0.05	0.55	0.05

coincides with the time at which an increased ribonucleotide reductase is observed (Fig. 8). If CB 1954 inhibits synthesis of DNA by preventing reduction of ribonucleotides to deoxyribonucleotides the drug should not alter rates of precursor incorporation into DNA when adequate concentrations of deoxyribonucleoside triphosphates are present. A combination of 0.1 mM deoxyadenosine, 0.1 mM deoxyguanosine and 1.0 μM deoxycytidine gave some protection against the inhibitory effects of CB 1954 on uptake of [^{3}H]thymidine into acid-insoluble material (Fig. 11). A similar partial protection was also given against the inhibitory effects of hydroxyurea, also an inhibitor of ribonucleotide reductase, on the incorporation of thymidine into DNA [15]. Addition of the deoxynucleosides caused a depression in DNA synthesis in control cultures up to 2 hr, after which DNA synthesis proceeded linearly. Up to 3.75 hr incorporation of [^{3}H]thymidine into the DNA of cells treated with CB 1954 exceeded that of control cultures, though the extent of reversal decreased with time, paralleling the repression of DNA synthesis in the control. At this time, presumably, the deoxynucleosides were used up and then DNA synthesis paralleled that of control culture, though at a reduced rate.

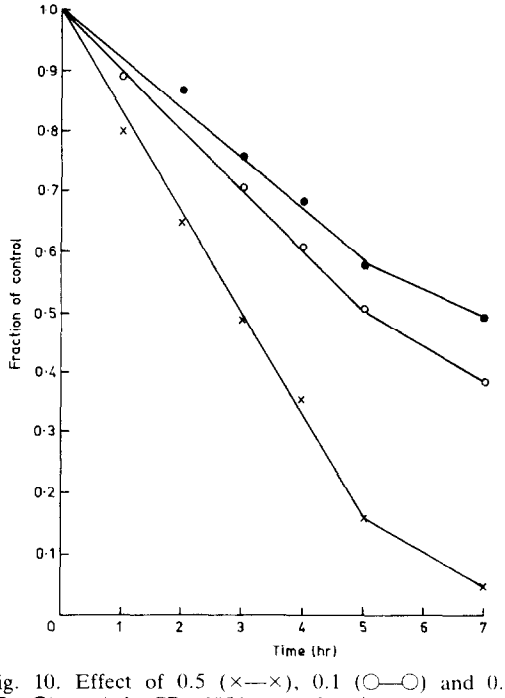

Fig. 10. Effect of 0.5 (×—×), 0.1 (○—○) and 0.05 (●—●) μg/ml CB 1954 on the incorporation of [^{3}H]thymidine into acid-insoluble material.

DISCUSSION

The primary biochemical lesion created by CB 1954 has been shown to be inhibition of DNA synthesis [4, 6] and the evidence presented in this report suggests that this arises as a result of inhibition of ribonucleotide reductase rather than by a direct chemical interaction with DNA. That CB 1954 produces its growth inhibitory effect by a mechanism different from that of the bifunctional alkylating agents is suggested by its inability to cross-link DNA [16], the lack of cross-resistance with chlorambucil,

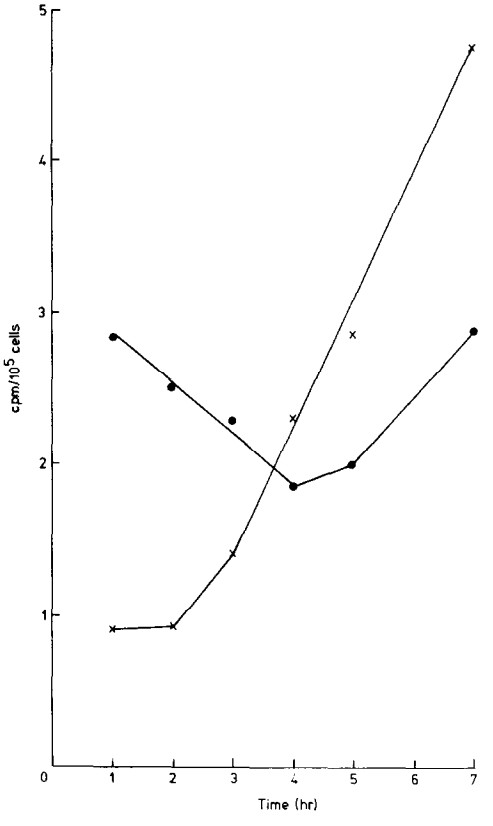

Fig. 11. Effect of 0.1 mM deoxyadenosine and deoxyguanosine and 1.0 μM deoxycytidine on the incorporation of [^{3}H]thymidine into acid-insoluble material of control cultures (×—×) or cells treated with 0.1 μg/ml CB 1954 (●—●).

Table 3. Specific activity of ribonucleotide reductase from different tumours

Tumour	Specific activity (pmoles. mg protein^{-1}. hr^{-1})
Walker carcinoma (chlorambucil-sensitive)	11
Walker carcinoma (chlorambucil-resistant)	10
Bladder carcinoma	36
TLX5 lymphoma	31

the ability to protect against the cytotoxic effect with 2-phenyl AIC [5] and that, unlike resistance to a bifunctional alkylating agent, cellular resistance to CB 1954 is lost on fusion with a sensitive cell [17]. The concentrations of CB 1954 required to inhibit ribonucleotide reductase activity and the synthesis of DNA, as well as the relative potencies of CB 10-020 and CB 10-107, suggests that the interference with the reduction of ribonucleotides to deoxyribonucleotides is sufficient to account for blockage of the biosynthesis of DNA by these agents. The fact that the relative potencies of the compounds as inhibitors of cell proliferation and the tumour selectivity correlate with the relative strength as inhibitors of reductase activity suggests further that blockade of the formation of deoxyribonucleotides is responsible, at least in part, for the anti-neoplastic activity.

A feature of CB 1954 action that has continuously been pointed out [1, 11] is that although it is highly active against the Walker carcinoma it is inactive against a large number of other transplantable tumours, many of which are highly sensitive to difunctional alkylating agents. However, all other tumours on which CB 1954 has been tested are either sarcomas or leukaemias and not carcinomas. This study shows that a human bladder carcinoma shows sensitivity to CB 1954 in a dose range at which it might be expected to be active *in vivo*. Other carcinomas may be equally sensitive. In fact, epithelial tissue and not the haematological system proved most sensitive to CB 1954 at the mimimum toxic dose in rats [1]. CB 1954 tends to concentrate in the urinary tract and the predominance of pathological effects were in the urinary tract in the transitional epithelium from the kidney pelvis to the bladder [1]. This may make bladder carcinomas the tumours of choice for clinical study. Since the relative effectiveness of CB 1954 as a tumour growth inhibitor *in vitro* parallels the effectiveness of this agent as an inhibitor of ribonucleotide reductase, this could prove useful as an *in vitro* test for sensitivity on biopsy specimens of human tumours.

Hydroxyurea is the only drug in general clinical use for which the primary mode of action is inhibition of ribonucleotide reductase [18]. The disadvantage of this drug is that it requires frequent large doses in order to maintain an effective concentration required for activity. The advantage of CB 1954 over hydroxyurea is the enormous tissue selectivity and ability to inhibit the wave of enzyme synthesis which probably arises by a derepression of enzyme synthesis by the decreased pool size of dTTP [19]. A similar increase in enzyme level has been reported after treatment of cells with hydroxyurea [13]. Hydroxyurea and other S-phase-specific anti-tumour agents have been shown to be most effective on an intermittent schedule [20]. This suggests that the activity of CB 1954 against human tumours could be improved under optimized conditions.

CB 1954 appears to be a particularly good inhibitor of the ribonucleotide reductase system of the Walker carcinoma. The reason for this selectivity is unknown, but is not related to a decreased activity of the enzyme in sensitive tissues (Table 3). Tissue selectivity is also shown by other enzyme inhibitors, e.g. inhibitors of prostaglandin synthetase and cyclic nucleotide phosphodiesterase. The inhibition of ribonucleotide reductase by CB 1954 is irreversible and from structure–activity studies [12] it is likely that the amide group becomes bound to the enzyme, possibly by chelation of a metal ion. Increased substitution on the amide, as in the analogues CB 10-020 and CB 10-107, would decrease the acidity of the amide group and reduce the chelating ability. Once bound to the enzyme, the aziridine ring could alkylate appropriate nucleophilic centres such as sulphydryl groups, which are known to be essential for the activity of ribonucleotide reductase [21]. Protection by 2-phenyl AIC and other agents would be explained by competition with CB 1954 for binding to ribonucleotide reductase. The mode of action of hydroxyurea and its analogues has been attributed to their ability to chelate transition metals [22]. However, the best metal-chelating agents are not always the best inhibitors and Elford *et al.* [23] have suggested a possible interference with a free radical that is present during the enzymatic reaction. Such a mechanism may also be appropriate to a nitroaromatic compound such as CB 1954. A study of the mechanism of inhibition of ribonucleotide reductase by CB 1954 is now in progress.

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