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2,4-DINITRO-5-ETHYLENEIMINOBENZAMIDE (CB 1954): A POTENT AND SELECTIVE INHIBITOR OF THE GROWTH OF THE WALKER CARCINOMA 256

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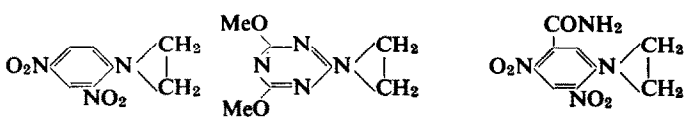
Abstract—The carcinostatic and pharmacological properties of a new cytotoxic agent, 2,4-dinitro-5-ethyleneiminobenzamide (CB 1954) have been investigated. CB 1954 exhibits a highly specific inhibitory effect on the growth of the Walker rat carcinoma 256. Lesser activity is shown against the Novikoff hepatoma and the primary benzpyrene tumour. The compound has little or no activity against the rat Yoshida sarcoma or the ADJ/PC6A mouse plasma cell tumour, the growth of which is considerably inhibited by difunctional alkylating agents of the nitrogen mustard gas type. The effects on the haematopoietic system are not unduly severe and the pathological effects at the minimum toxic dose are manifest in the liver and urinary tract.

2,4-DINITRO-1-ETHYLENEIMINOBENZENE (I) is one of the few compounds containing a single alkylating function that effectively inhibit the growth of the Walker rat carcinoma 256 (Ross and Mitchley;[1] Hendry *et al.*[2]). Recent investigation of this compound has shown that it exhibits a high therapeutic index (T.I.) when assayed against this tumour [T.I. = dose to kill 50 per cent of animals (LD_{50})/dose to produce 90 per cent inhibition of tumour growth (ED_{90}) = 226 mg/kg/22 mg/kg = 10].[3] This index is comparable with the following indices for some clinically useful compounds: chlorambucil (T.I. = 7), melphalan (T.I. = 9), merophan (T.I. = 14) and triethylenemelamine (T.I. = 9·5).



(I) (II) (III)

The only other ethyleneimine derivative of comparable structure which has shown activity against this tumour is 4,6-dimethoxy-2-ethyleneimino-1,3,5-triazine (II)[2] and recent re-examination of this compound has shown that it has a therapeutic index of only 1·4 against the Walker carcinoma 256 (LD_{50}/ED_{90} = 228 mg/kg/160 mg/kg).

In the course of the examination of a large series of compounds structurally related to (I), the preparation and properties of which will be reported later, a derivative carrying an amido substituent (III) was obtained which has shown a therapeutic index considerably higher than that of any compound yet tested against the Walker carcinoma at this Institute.

The effects of this new compound, 2,4-dinitro-5-ethyleneiminobenzamide (CB 1954), against several rat and mouse tumours and preliminary investigations of the pharmacological activity are now described. Whilst the compound exhibits high potency and outstanding specificity of action against the Walker carcinoma 256 it is without effect on some other tumours which are susceptible to the action of many difunctional alkylating agents. CB 1954 also differs from the latter agents in that its effects on the haematopoietic system are relatively slight and the pathological effects at the minimum toxic dose (MTD) in rodents are not on the intestinal epithelium and haematopoietic tissue but on the liver and urinary tract epithelium.

MATERIALS AND METHODS

2,4-Dinitro-5-ethyleneiminobenzamide (III). A solution of 3-chloro-4,6-dinitrobenzamide[4] (5 g) in ethyl acetate (450) ml) was added dropwise with stirring to an ice cooled solution of ethyleneimine (1·02 ml) and triethylamine (3 ml) in ethyl acetate (250 ml). After addition was complete (½ hr) stirring was continued for 4 hr and the temperature was allowed to rise to 20°. The filtered solution was passed through a silica gel column which was further eluted with fresh ethyl acetate. Concentration of the combined eluates under reduced pressure caused the separation of the *amide* as yellow prisms, m.p. 189° (corr.), yield 3·2 g (Found: C, 42·7%; H, 3·2%; N, 22·0%. Calc. for $C_9H_8N_4O_5$: C, 42·8%; H, 3·2%; N, 22·2%).

The solubility of 2,4-dinitro-5-ethyleneiminobenzamide in various solvents, expressed as mg/ml is as follows: water (2), ethanol (10), propylene glycol (12), dimethylacetamide (100) and dimethyl sulphoxide (400).

Antineoplastic assays

Walker rat carcinoma 256. The protocol for testing the compound as an inhibitor of the growth of a one day old subcutaneous Walker carcinoma is described by Rosenoer *et al.*[5] In a preliminary experiment to determine the effect on a more established subcutaneous tumour, CB 1954 was administered 3 days after implantation

TABLE 1. ASSAY OF CB 1954 AGAINST THE WALKER RAT CARCINOMA 256 BY THE STANDARD METHOD[4]

Dose mg/kg (i.p. arachis oil)	Toxicity (No. of rats surviving at end of test: 10 days after tumour implant)	% Inhibition of tumour growth
40	0/3	—
20	3/3	100
10	3/3	100
5	3/3	100
4	3/3	100
2·5	3/3	100
1·0	3/3	99·6
0·5	3/3	97
0·25	3/3	83
0·125	3/3	50
	LD_{50} 28 mg/kg	ED_{90} 0·4 mg/kg

Therapeutic index $LD_{50}/ED_{90} = 70$

when the tumours weighed about 1 g. In both cases the drug was given as a single intraperitoneal (i.p.) injection in arachis oil.

For assay against a 5- to 6-day-old intramuscular Walker carcinoma 256 the technique was essentially that described by Cobb.[6] 2–5 × 10^6 live tumour cells were injected into the muscle of one hind limb of 250-g female Wistar rats. After 5–6 days the tumours were well established (weight about 2 g) and CB 1954 in dimethylacetamide was administered as a single intravenous (i.v.) or intraperitoneal (i.p.) injection. The therapeutic indices recorded in Table 2 were calculated from the expressions LD_{50}/CR_{50} or LD_{50}/CR_{10} as described by Cobb.[6]

TABLE 2. CHEMOTHERAPEUTIC INDICES OF CB 1954 COMPARED WITH THOSE OF HN2 AND MELPHALAN IN THE ASSAY AGAINST THE 5–6 DAY INTRAMUSCULAR WALKER CARCINOMA 256

Compound	Route	LD_{50} mg/kg+	CR_{10} mg/kg+	CR_{50} mg/kg+	Index LD_{50}/CR_{10}	Index LD_{50}/CR_{50}
CB 1954	i.v.	22	0·57	0·9	38·6	24·5
	i.p.	29	0·47	0·6	61·6	48
HN2	i.v.	1·5	0·06	0·12	25	12·5
	i.p.	1·5	0·28	*	5·4	*
Melphalan	i.v.	5·3	0·27	0·6	19·6	8·8
	i.p.	5·1	0·42	0·6	12·1	8·5

+ Single dose in dimethylacetamide.
* Cure rate of 50 per cent not achieved by this route.

Yoshida rat sarcoma. Wistar rats were inoculated subcutaneously (s.c.) in the inguinal region with 2 × 10^6 live Yoshida sarcoma cells and seven days later CB 1954 in dimethyl sulphoxide was administered as a single i.p. injection (doses 32, 16, 8 and 4 mg/kg). Three rats were used for each dose level. Seven days after treatment the animals were killed and the tumours dissected out and weighed.

Bilateral transplants of Walker carcinoma 256 and Yoshida sarcoma. Wistar rats were inoculated with 2 × 10^6 live Yoshida sarcoma cells in one flank and with 4 × 10^6 live Walker carcinoma 256 cells in the other flank. Single injections of CB 1954 (5 mg/kg i.p. in arachis oil) were made 1, 2, 5 and 6 days after inoculation.

Bioassay against Walker carcinoma and Yoshida sarcoma cells. Ascites tumour cells were drawn off from the host rat, washed twice with saline and then added to a mixture of TC 199 cell culture fluid (Glaxo Laboratories, Greenford, England) and horse serum (60:40) giving a final concentration of 10^7 cells/ml. A range of concentrations of CB 1954 in dimethylsulphoxide (0·1 ml) was added to the cell suspensions (10 ml) and the mixtures incubated for 2 hr at 37°. Groups of five rats were injected (i.p.) with the resultant incubation fluids (1 ml) and survival times recorded for those animals in which the tumour became established (Table 3).

ADJ/PC6A Mouse plasma cell tumour. The protocol for this assay is given by Wade *et al.*[7] CB 1954 was administered as a single i.p. injection in arachis oil.

L1210 Mouse leukaemia. The protocol for this assay is essentially that already described[8] except that the C_{57}/DBA2 hybrid strain of mouse was used as host. CB 1954 was administered in arachis oil as an i.p. injection either as a single dose or as five consecutive daily doses starting on the day after tumour inoculation.

Benzo[a]pyrene fibrosarcoma. Fibrosarcomata were induced in the flank of male Wistar rats by the implantation of 10 mg benzo[a]pyrene pellets. When the tumour volume was between 2·5 and 20 ml the animals were treated with CB 1954 in dimethylacetamide (1 mg/kg every fourth day for ten s.c. injections).

Novikoff rat hepatoma. The subline of the Novikoff rat hepatoma[9, 10] used in these experiments was obtained from Dr. D. M. Shepherd in 1966. Although originally

TABLE 3. BIOASSAY OF CB 1954 AGAINST WALKER CARCINOMA 256 CELLS AND AGAINST YOSHIDA SARCOMA CELLS

Walker carcinoma 256 cells			Yoshida sarcoma cells		
Concentration of drug (γ/ml)	% Tumour take	Survival time of animals with tumours (days)	Concentration of drug (γ/ml)	% Tumour take	Survival time of animals with tumours (days)
none	100	9	none	100	8·0
0·25	60	21	3·13	100	7·8
0·5	20	> 30	6·25	100	7·8
1·0	0	—	12·5	100	8·0
2·0	0	—	25	100	8·2
			50	80	10·0
			100	20	> 30
			200	0	—

passaged in Sprague–Dawley rats it is now maintained in male Chester Beatty albino rats by transplantation in the ascites form. The test is carried out as follows:

0·5 m. of ascitic fluid from a 6- to 7-day old peritoneal tumour is injected into each of fourteen rats. Three logarithmically spaced doses of the drug (in arachis oil) are given i.p. to nine rats (three rats per dose) as a single injection 24 hr after inoculation. After 14–18 days the tumours are dissected out and weighed. The tumour growth inhibitory activity is assessed by comparing the mean tumour weights in treated and untreated animals.

Pharmacological effects

Toxicity, body growth inhibition and haemotoxicity. Female C57/DBA2 mice and 8- to 12-week old male Wistar rats were used and CB 1954 was administered by i.p. injection in arachis oil. In all instances where arachis oil was used as solvent the compound was first dissolved in a little acetone and then diluted with arachis oil, the final concentration of acetone being about 10%. The animals were weighed daily. Blood counts were carried out at two day intervals in blood taken from a tail vein. Four rats were used in each experiment and the mean response curve was plotted showing the percentage of the normal count as a function of time after injection of a single dose of CB 1954.

Pathological effects

For histological study, the LD_{50} of CB 1954 in dimethylacetamide (see Table 2) was given as a single i.v. or i.p. injection into 250-g female Wistar rats. The effects at the minimum toxic dose (MTD) were obtained by s.c. injection of an aqueous solution of CB 1954 (1 mg/kg daily for 10 days) into 250-g female Wistar rats.

RESULTS

Antineoplastic effects

Walker rat carcinoma 256. The results obtained in the standard assay against a one-day old subcutaneous tumour are shown in Table 1. The derived chemotherapeutic index of 70 is exceptionally high. The growth of a 3-day old subcutaneous implant was completely inhibited by single doses of 20, 10 and 5 mg/kg. Table 2 shows the chemotherapeutic indices (median lethal dose/the dose to produce cure rates (CR) of 10 or 50 per cent)[6] in the assay against a 5–6 day old Walker tumour. Some corresponding values for HN2 and melphalan are given for the purposes of comparison. CB 1954 has a higher therapeutic index against the established Walker tumour by both i.v. and i.p. routes of administration than the clinically useful compounds also included in the Table. The differential between CB 1954 and melphalan in the freshly implanted tumour assay (see above) is maintained in the case of the established tumour.

Yoshida sarcoma. No significant inhibition of the growth of this tumour was produced by the maximum tolerated dose (16 mg/kg).

Bilateral transplants of Walker carcinoma 256 and Yoshida sarcoma. Complete inhibition of the growth of the Walker carcinoma 256 was produced when CB 1954 was administered as a single 5 mg/kg dose on the days 1, 2 or 5 after inoculation and 99 per cent inhibition was produced when the compound was given on the 6th day. In all cases the growth of the Yoshida sarcoma was not affected.

Bioassay against Walker carcinoma 256 and Yoshida sarcoma cells. In control experiments it was shown that rats inoculated with 10^7, 10^6 and 10^5 Yoshida sarcoma cells died after 8·0, 10·8 and 18·2 days respectively. Table 3 shows the survival time of rats inoculated with 10^7 Yoshida sarcoma cells which had been inoculated with various concentrations of CB 1954. It can be seen that a concentration of 50γ ml CB 1954 was the lowest concentration that significantly affected Yoshida sarcoma cells *in vitro* and 200γ/ml was required to kill all cells.

Table 3 also shows the results of a similar assay using Walker carcinoma 256 cells. In this case a concentration of 0·25γ/ml of CB 1954 had a significant toxic effect and 1γ/ml killed all the tumour cells.

ADJ/PC6A Mouse plasma cell tumour. At a dose of 80 mg/kg (about $\frac{3}{4}$ of the LD_{50}) 76 per cent inhibition of the growth of this tumour was obtained. Thus the chemotherapeutic index is probably less than 1. This low activity may be contrasted with the high activity of many difunctional alkylating agents, for example, aniline mustard (*N*,*N*-di-2-chloroethylaniline) exhibits a therapeutic index of 80 in this system.[11]

L 1210 Mouse leukaemia. No significant increase in survival time was produced when CB 1954 was given as a single dose of 100 mg/kg or as five daily doses of 56 mg/kg starting on the day following inoculation. This result has been confirmed by Professor Mathé of the Institut de Cancerologie et d'Immunogenetique (Paris) and by the Cancer Chemotherapy National Service Center in the United States.

Primary benzo[a]pyrene fibrosarcoma. Of the thirteen rats treated three showed a significant reduction in tumour growth rate when compared with control animals treated with solvent alone. After 28 days of treatment the tumour volume of the three sensitive fibrosarcomata was between 15 and 20 ml, whereas the ten untreated control tumours had all reached a volume of 80 ml.

Novikoff hepatoma. The maximum effect obtainable with CB 1954 on this tumour is 67 per cent inhibition of growth at a single dose of 10 mg/kg. Comparable figures for some typical difunctional alkylating agents are: aniline mustard, 80 per cent at 40 mg/kg, chlorambucil 60 per cent at 12 mg/kg, and merophan 80 per cent at 2 mg/kg.

Pharmacological effects

Toxicity. The LD_{50} in the mouse of a single dose of CB 1954 given by the i.p. route (dissolved in 10% acetone in arachis oil) is approximately 100 mg/kg; deaths occurring within 24 hr. However, a similar daily injection of 80 mg/kg for 8 days, although producing a steady fall in weight for 11 days at which time the animals weighed only 70 per cent of their pretreatment weight, did not produce any deaths up to a period of 12 days. CB 1954 is much more toxic in the rat—the LD_{50} for a single i.p. dose (in acetone/arachis oil) being about 20 mg/kg. When CB 1954 in aqueous solution is given to rats as 8 consecutive daily s.c. injections, doses of 1 and 2 mg/kg are not lethal but a dose of 4 mg/kg produced one death in the group of four rats treated. (See also below.)

Body growth inhibition. In response to a single (i.p. in arachis oil) dose of CB 1954 rats show a prolonged period of weight loss ranging from about 4 days following a dose of 6 mg/kg to as long as 10 days following a dose of 12 mg/kg. The actual weight loss may not be so severe, and not as rapid, as occurs in response to many difunctional alkylating agents.[12] Moreover, it is not accompanied by the diarrhoea which is usually observed in the case of the latter agents.

When given as 8 daily s.c. injections of 1 mg/kg produced a retardation of growth, some decrease in food intake, but no actual weight loss. A similar course of injections of 2 mg/kg however produced a very severe, steady weight loss with progressively marked drop in food intake which persisted—some rats refusing to eat—for a period of up to 7 days after stopping the injections.

Haemotoxicity

Blood counts. Following a single i.p. dose of 6 mg/kg in arachis oil, about $\frac{1}{4}$ of the LD_{50}, both lymphocytes and neutrophils show a slight fall, reaching counts of not less than 60 per cent of normal with a minimum at about 2 days followed by a marked transient neutrophilia. In this respect of CB 1954 shows some resemblance to difunctional alkylating agents.[12] However, doses of 9 and 12 mg/kg do not show a progressively increased depression of leucocytes but give rise to a more extended period of neutrophilia and an increase to above normal values in the number of blood platelets and some increase in haemoglobin.

Bone marrow. Following a single s.c. dose of 10 mg/kg of CB 1954 in arachis oil rat bone marrow shows some depression of myeloid elements—about 50 per cent fall with a minimum at 2 days. After a slight initial fall during the first day after injection there is a stimulation of erythropoiesis which after about 4 days may be followed by a fall in erythroid elements in the marrow before final restoration to normal values. None of the effects of CB 1954 on the haematopoietic system can be considered as unduly severe and it would appear that with this drug the haemotoxicity (normally a major feature of the action of cytotoxic alkylating agents) is overshadowed by its other systemic pathological effects.

Pathological effects

Effects at the LD_{50} *in rats.* Except for minor details the pathological changes were not affected by the route of administration of CB 1954. Death occurred between 3 and 27 days after treatment. The rats showed loss of epithelium from both small and large intestine. In some areas of the small intestine the architecture of the villi was completely lost. In the spleen, the lymphoid follicles were hypoplastic and there was evidence of increased red cell breakdown. There was centrilobular venous thrombosis in the liver with accompanying congestion and occasional portal venous thrombosis. The epithelial lining of the kidney pelvis, ureter and bladder was ulcerated and in parts bore large macronuclear cells.

Effects at the minimum toxic dose in rats. The organs affected by the MTD were the lungs, liver and urinary tract. In the lungs the bronchiolar epithelium became thin and disorientated. The liver showed scattered centrilobular thromboses. The pathological change in the urinary tract was in the transitional epithelium from the kidney pelvis to the bladder. It consisted of scattered replacement of normal epithelium by macronuclear cells. The appearance of these cells was of an increase in nuclear and cytoplasmic constituents with apparent failure of the cells to divide.

DISCUSSION

The high activity of 2,4-dinitro-5-ethyleneiminobenzamide (III) against Walker rat carcinoma 256 is not confined to the new transplant but is also shown against a well established tumour. It is, therefore, unlikely that the high therapeutic index is due to an inhibition of the "take" of the tumour transplant. The *benzo[a]pyrene* tumour, which is resistant to most cytotoxic agents does respond and 67 per cent inhibition of the growth of the Novikoff hepatoma can be achieved. The fact that the ADJ/PC6A mouse plasma cell tumour and the Yoshida rat sarcoma, which are sensitive to a wide range of alkylating agents, are but little affected by CB 1954 suggests that this compound is acting by a mechanism different from that usually associated with difunctional alkylating agents of the mustard gas type. These are believed to act by crosslinking nuclear material but such a mechanism is unlikely to apply to CB 1954 although primary alkylation by the ethyleneimino group followed by reaction of the nitro groups with cellular thiol groups could not be ruled out.

The presence of the reactive ethyleneimino group is essential for biological activity since analogues of (I) in which this group is replaced by a dimethylamino or 2-hydroxyethylamino group are inactive.[2] The sensitivity of the Walker carcinoma 256 cells in culture argues against the activity being due to a metabolite generated in the host as in the case of Endoxan. Endoxan will cause complete inhibition of the growth of the Walker carcinoma 256 implanted in a rat at a single dose of 3 mg/kg (corresponding to 3γ/g of tumour assuming uniform distribution throughout the body whereas in cell culture a concentration of 2000γ/ml is required to kill all the tumour cells. In contrast, CB 1954 will produce complete tumour growth inhibition at 0·5 mg/kg in the rat (0·5γ/g of tumour) which is very close to the concentration required to kill the cells in the bioassay (Table 3).

The possibility that the drug is activated within the sensitive Walker carcinoma cells cannot be ruled out. That the dinitroethyleneiminobenzene derivatives (I) and (III) produce their cytotoxic effects by intracellular hydrolysis to give the oxidative phosphorylation inhibitory dinitrophenols can probably be discounted since dinitro-

phenol does not inhibit the growth of Walker carcinoma cells in culture at a concentration of 400γ/ml.

The increase in the potency of the amide (III) as compared with the parent compound (I) could possibly be associated with intracellular hydrolysis of the amide group to give 2,4-dinitro-5-ethyleneiminobenzoic acid. This strong acid would be almost completely ionised at physiological pH and would not readily pass out through the cellular membrane. If the sensitive Walker carcinoma 256 cells should prove to be able to effect this hydrolysis, the dinitro-benzamide structure would be a useful carrying structure to which other suitable cytotoxic groups might be attached with a view to localising their action. While this factor could account for the high potency of the amide against the Walker tumour it cannot explain the high selectivity of action against this tumour since 2,4-dinitro-1-ethyleneiminobenzene (I) is also effective against the Walker carcinoma 256 but not against the Yoshida sarcoma or ADJ/PC6A plasma cell tumours.

The problem in the clinical usage of the majority of cytotoxic alkylating agents is their lack of specificity, besides neoplastic tissues other proliferating normal tissues are affected. Although many difunctional alkylating agents have a wide spectrum of activity this is associated with excessive damage to normal tissues, such as the haematopoietic system and the intestinal mucosa. CB 1954 is of particular interest in that it has a limited spectrum of activity but exhibits a high therapeutic index against at least one experimental tumour, the Walker 256 rat carcinoma, and shows a relatively low haemotoxicity. If it can be established why this tumour is so susceptible to attack the knowledge gained should be of value in designing agents that are more specific towards particular types of neoplastic tissue.

If CB 1954 is to be of clinical use it has to be established that some human tumours respond to its growth inhibitory activity. Since the specificity of action is also shown in cell culture, Walker tumour cells being much more sensitive than Yoshida tumour cells, it is encouraging that two lines of human tumour cells maintained in culture have proved to be very sensitive to CB 1954. Professor A. Leonardi of the Istituto di Recerche Farmacalogiche "Mario Negri", Milan, has shown that a concentration of 10γ/ml produces 100 per cent inhibition of growth of human KB lymphoma cells and Dr. Easty of this Institute has shown that a concentration of 1γ/ml produces 90 per cent inhibition of growth of HeLa cells.

Another indication for clinical utility is that the drug tends to concentrate in the urinary tract. A spectrophotometric assay indicates that after i.v. injection of 10 mg/kg of CB 1954 to a rat the concentration of active drug in the urine after one hour is 100γ/ml; uniform distribution of the drug in tissue fluids would result in a concentration of 10γ/ml. This concentration is consistent with the predominance of pathological effects in the urinary tract and suggests that CB 1954 would be useful in treating early tumours at this site.

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