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PROTECTION AGAINST THE EFFECTS OF THE ANTITUMOUR AGENT CB 1954 BY CERTAIN IMIDAZOLES AND RELATED COMPOUNDS

JOHN A. HICKMAN and DAVID H. MELZACK
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London SW3 6JB, England

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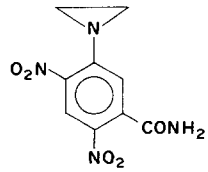
Abstract—A number of compounds including 2,4-dinitrophenol, adenine, indole acetic acid and anthranilamide have been found to protect against the cytotoxicity of CB 1954 [5-(1-aziridinyl)-2,4-dinitrobenzamide] against the Walker tumour. Investigation of a number of anti-inflammatory compounds structurally related to these protectors has shown that only an aryl derivative of a previously described protector, AIC (4-aminoimidazole-5-carboxamide), is a more potent protector. This compound, 2-phenyl AIC (4-amino-2-phenylimidazole-5-carboxamide), has been found to be thirty times as potent a protector as AIC and exhibited a dose reduction factor of almost 90 in an antitumour test. However, it does not reverse (post-protect against) the cytotoxicity of CB 1954. The mechanism of protection does not appear to be via an effect upon cellular energetics nor upon uptake of CB 1954 into the cell.

The monofunctional alkylating agent CB 1954 [5-(1-aziridinyl)-2,4-dinitrobenzamide] is a potent and selective inhibitor of the Walker tumour [1]. This is unusual because the cytotoxic alkylating agents are generally difunctional. CB 1954 has several properties typical of a difunctional alkylating agent, such as a selective effect upon DNA synthesis and cross-resistance with difunctional agents, and some characteristics more like those of an antimetabolite [2]. Thus, high concentrations of the purine precursor AIC, (4-aminoimidazole-5-carboxamide), and several purines could completely protect against the anti-tumour activity of CB 1954 although they could not reverse it [3]. However, an effect of CB 1954 on purine biosynthesis appeared to be unlikely when it was found that anthranilamide was a protector [3] and the absence of any effect of CB 1954 upon the *de novo* synthesis of purines confirmed this [4].



CB 1954

The present studies were to investigate the types of compounds capable of protecting against CB 1954 and to determine their structure activity relationships. Studies on the reversal or protection against cytotoxic compounds have, in many cases, afforded an indication of their mechanism of cytotoxicity [5] and elucidation of the mechanism of the protection against CB 1954 may provide similar information on the unusual mechanism of action of CB 1954.

MATERIALS AND METHODS

Tumour passage

The Walker tumour in ascites form was passaged weekly in 6-week old male Wistar rats by intraperitoneal injection of 2×10^5 ascites cells. An inverse correlation was shown to exist between survival time and the number of tumour cells injected, over the range 10^3–10^7 cells.

Bioassay

The bioassay procedure was performed aseptically. Walker ascites cells were suspended in TC 199 and horse serum (60:40) at a concentration of 10^6 cells cm^{-3} and then incubated with the various agents for 2 hr at 37°. For each incubation 1-cm^3 aliquots were then injected intraperitoneally into groups of five animals. The survival times of the rats receiving the treated cells was compared with those of the controls receiving incubated cells only, to give a measure of cell kill. Compounds were added in 0·1 cm^3 DMSO to 10 cm^3 incubates. Insoluble compounds were sonicated in DMSO before addition.

Studies in vitro

Walker ascites cells were removed from rats on the sixth or seventh day after transplantation and washed in a cell lysis medium [6] until they were free of red cells. The ascites cells were resuspended in TC 199 and horse serum (60:40) at a concentration of $1{\cdot}5 \times 10^6$ cells cm^{-3} and incubated for 1 hr at 37° before the addition of drugs. All drugs were suspended or dissolved in DMSO and added in a vol of 0·1 cm^3. Radioactively-labelled compounds were then added at various times.

Aliquots of 1 cm^3 of the cell suspension were removed, at timed intervals, onto glass fibre filter discs (Whatman GF/C, 2·5 cm) wetted with saline. To measure total cellular uptake of radioactively-labelled compound, the cells were washed with 15 cm^3 of 0·9% saline. For determinations of the incorporation of radioactively-labelled compounds into macromolecules, the cells were washed with 15 cm^3 of 0·9% saline followed by 15 cm^3 of 0·2 N perchloric acid, followed by a further 15 cm^3 0·9% saline. The filters were placed in plastic vials and dried overnight

at 70°. Scintillation fluid (toluene, dioxan, ethanol, naphthalene and butyl-PBD) was added directly to the filters and the samples were counted in a Packard Tri-Carb Model 3375 liquid scintillation counter.

Studies in vivo

1. *Protection against the animal toxicity of CB* 1954 *by 2-Phenyl AIC.* 2-Phenyl AIC (200 mg kg^{-1}) in DMA (dimethylacetamide) 10% in arachis oil was administered to 6-week old female Wistar rats by intraperitoneal injection. One hour later, CB 1954 in acetone 10% in arachis oil was administered by the same route. Doses of CB 1954 ranged from 50 to 400 mg kg^{-1}. Cumulative deaths in each group of five animals per dose of CB 1954 were noted, as well as changes in body wt, over a period of 10 days.

2. *Protection against the antitumour effect of CB* 1954. Female Wistar rats carrying a 72-hr old solid Walker tumour in the inguinal region, were given 2-phenyl-AIC (200 mg kg^{-1}) intraperitoneally in DMA 10% in arachis oil. One hour later serial doses of CB 1954 in acetone (10%) in arachis oil were administered by the same route to groups of five animals. The tumours were dissected out after 7 days and weighed.

Drugs and radioactive precursors

Compounds with BRL serial numbers and 2-phenyl AIC were the gift of Dr. J. Heyes of Beecham Research Laboratories, Brockham Park, Surrey. Indomethacin was the gift of Merck, Sharpe and Dohme, Ltd., Hoddesden, Herts.; flufenamic acid that of Parke Davis Ltd., Pontypool, Gwent, and fenclozic acid that of ICI Pharmaceuticals, Macclesfield, Cheshire. CB 1954 was synthesized at this Institute and all other chemicals were purchased from commercial sources.

[5-*Me*-^{3}H]thymidine was obtained from the Radiochemical Centre, Amersham, at a sp. act. of 5 Ci $mmole^{-1}$. It was added to give 2·5 μCi cm^{-3} of tumour suspension. Radioactivity was incorporated into cells at a linear rate. After 60 min the value was usually 50×10^3 counts/min per 10^6 cells. ^{14}C-CB 1954 with a sp. act. of 6·7 mCi m-$mole^{-1}$ was synthesized by Dr. M. Jarman of this Institute. It was added to give 10 μg cm^{-3} of tumour suspension.

RESULTS

Structure–activity relationships of protecting compounds

The bioassay was used for this study. Determinations of the structural requirements for protection were performed by a study of structural analogues of AIC, of CB 1954 and of anthranilamide.

Tables 1 and 2 show the results of testing various compounds for ability to protect against the cytotoxicity of CB 1954 (1 μg cm^{-3}) against Walker cells

Table 1. Results of testing various compounds for ability to protect against the cytotoxicity of CB1954 against walker cells in the bioassay system

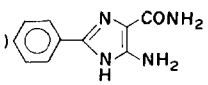
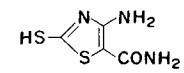
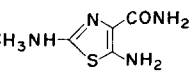
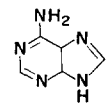
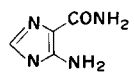
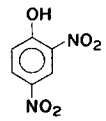
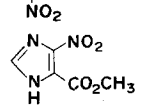
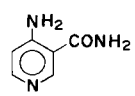
Treatment			Increase in survival time (days)
1 μg cm^{-3} CB 1954			>20
1 μg cm^{-3} CB 1954 + 50 μg cm^{-3}			
	2-Phenyl-AIC	(1) 	0
1 μg cm^{-3} CB 1954 + 500 μg cm^{-3}			
	CB10-251	(2) 	2
	CB10-255	(3) 	2
	Adenine	(4) 	3
	AIC	(5) 	3
	2,4-DNP	(6) 	3
	MNIC	(7) 	3
1 μg cm^{-3} CB 1954 + 1000 μg cm^{-3}			
	4-Aminonicotinamide (8)		0

Table 1 (*cont.*)

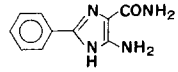
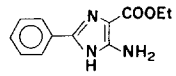
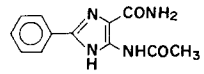
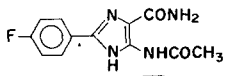
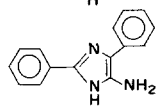
Treatment			Increase in survival time (days)
	Anthranilamide	(9)	0
	2-Aminonicotinamide	(10)	2
	Indole acetic acid	(11)	2
	Benzimidazole	(12)	3
	Tryptophan	(13)	3
	AIC-riboside	(14)	6
	2,4-Dinitrobenzamide	(15)	11
	meta-Dinitrobenzene	(16)	> 20
	Fenclozic acid	(17)	> 20
	Guanine	(18)	> 20
1 μg cm^{-3} CB1954 + 750 μg cm^{-3}	Indomethacin*	(19)	> 20
1 μg cm^{-3} CB1954 + 250 μg cm^{-3}	Flufenamic acid*	(20)	> 20

* Maximum non-toxic dose.

in the bioassay system. CB 1954 alone gave an increase in survival time compared with controls of more than 20 days. In the presence of a good protector, however, this survival time was brought back to that of the controls. In the table, the compounds have been grouped under the lowest concentration at which they gave good protection. Compounds which prevented the cytotoxicity of CB 1954 by reducing the increase in survival time to no more than 3 days have been classified as good protectors while those that gave less reduction have been considered non-protectors.

The first group of compounds (Table 1, compounds 1–5) are structurally analogous to AIC and contain

Table 2. Results of testing derivatives of 2-phenyl AIC for ability to protect against the cytotoxicity of CB1954 against walker cells in the bioassay system

Treatment ($100\ \mu g\ cm^{-3}$)		Increase in survival time (days)
2-Phenyl AIC		0
BRL 2250		3
BRL 10202		4
BRL 8922		11
BRL 4025		>20

changes to both the nucleus (e.g. the thiazoles, compounds 2 and 3) and to the attached groups. Compounds 6, 15 and 16 are structurally analogous to CB 1954 itself. Compounds 11–13 may be metabolically related to anthranilamide.

The failure of AIC riboside as a protector (Table 1, compound 14) suggested that the purine bases rather than the ribosides used in the previous study [3] might be more efficient protectors. This was found to be the case and a clear difference between adenine and guanine was observed.

The observation that aryl derivatives of a number of the protectors were non-steroidal anti-inflammatory compounds (see Discussion) led to an investigation of a number of such compounds as potential protectors. Flufenamic acid, indomethacin and fenclozic acid (Table 1, compounds 20, 19 and 17) are the aryl derivatives of anthranilate, indole acetic acid and a thiazole. They were, however, ineffective as protectors. An aryl derivative of AIC has proved to be the most potent protector and a number of its analogues have been studied for an assessment of their structure–activity relationships. These are shown in Table 2. The high potency of 2-phenyl-AIC (Table 2) was such that all further studies on the protection against CB 1954 were made using this compound.

Protection against the toxicity and antitumour effects of CB 1954 in vivo *by 2-phenyl-AIC*

The dose–response curves for the toxicity and antitumour effect of CB 1954 in 2-phenyl-AIC pretreated and un-pretreated animals are shown in Fig. 1.

A dose reduction factor is given by a comparison of LD_{50}'s and ID_{90}'s for protected and non-protected animals i.e.

$$\text{Dose reduction factor} = \frac{\text{Dose in protected animal}}{\text{Dose in non-protected animal}}.$$

From this the dose reduction factor for the LD_{50}'s is 3·2 and for the ID_{90}'s is 87. The therapeutic index (LD_{50}/ID_{90}) of CB 1954 in the protected animal is 2·3 compared with 60 in the non-protected animal, a reduction factor of 27.

Protection against the effect of CB 1954 *on thymidine incorporation* in vitro *by 2-phenyl-AIC*

The selective effect of CB 1954 (1 $\mu g\ cm^{-3}/10^6$ cells) upon DNA synthesis, as measured by the incorporation of [5-*Me*-^{3}H]thymidine into macromolecules, has previously been shown to be protected against by AIC (1000 $\mu g\ cm^{-3}/10^6$ cells) [3]. 2-Phenyl-AIC affords similar protection at 50 $\mu g\ cm^{-3}$ per 10^6 cells and is thus some twenty times more potent than AIC. The protection is time dependent: simultaneous addition with CB 1954 or preincubation with 2-phenyl-AIC (50 $\mu g\ cm^{-3}$) gives 100% protection, whereas addition 0·5 h after the addition of CB 1954 gives only 75% protection.

It was observed that a concentration of 500 $\mu g\ cm^{-3}$ of 2-phenyl-AIC, which was without toxicity in the bioassay system when incubated for 2 hr, depressed thymidine incorporation to approx 50% of the control level after 1 hr incubation. This is being further investigated.

Attempted reversal of CB 1954 *by a supraprotective dose of 2-phenyl-AIC*

The reversal (post-protection) of CB 1954 by 2-phenyl-AIC was attempted using a concentration in the bioassay which was twenty times that of the minimum dose to give complete protection. 2-Phenyl-AIC (1000 $\mu g\ cm^{-3}$) was added to bioassay incubates of 10^6 Walker cells at time intervals after the addition of 1 $\mu g\ cm^{-3}$ of CB 1954. No reversal of CB 1954

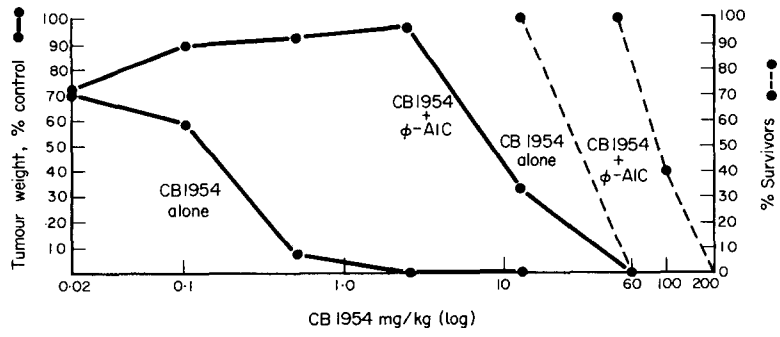


Fig. 1. Dose–response curves of CB 1954 with and without ϕ-AIC pretreatment. ●——●: anti-tumour effect; ●- - -●: animal toxicity effect.

Table 3. Effect of altering the time of addition of the protector 2-phenyl-AIC (1 mg cm^{-3}) relative to CB 1954 (1 μg cm^{-3}) measured in the bioassay system

Time of addition of 2-phenyl-AIC after CB 1954 (min)	Increase in survival time (days)
0	0
30	3
60 and above	>20
2-phenyl-AIC alone	0
CB 1954 alone	>20

was found after CB 1954 had been in contact with the cells for more than 0·5 hr (Table 3).

Effect of certain protectors on the cellular uptake of CB 1954

The total cellular uptake of [^{14}C]CB 1954 was measured *in vitro* after 15 min preincubation with 500 μg cm^{-3} of the protectors 2,4-DNP (2,4-dinitrophenol), adenine and 2-phenyl-AIC. CB 1954 (10 μg cm^{-3}) was added and samples taken at intervals after this. No significant change in the total uptake over a period of 1 hr was found (Table 4).

Table 4. Uptake of [^{14}C]CB 1954 (10 μg cm^{-3}) by Walker cells after 1 hr in the presence of some protectors

Protector	Uptake of [^{14}C]CB 1954 as % of control
2,4-DNP	112
Adenine	104
2-Phenyl-AIC	96

DISCUSSION

In the early stages of this investigation an apparently disparate group of compounds were found to protect against CB 1954. These were AIC, certain thiazoles, adenine, 2,4-DNP, anthranilamide and indole acetic acid (Table 1). Assuming they protected by a single mechanism, an attempt was made to find a common property for these compounds which might suggest a mechanism of protection. The aryl derivatives of AIC [7], certain thiazoles [8], anthranilates [9] and indole acetic acids [10] have all been reported to have non-steroidal anti-inflammatory activity and the last two classes of compounds have also been reported, like 2,4-DNP, to be uncouplers of the mitochondrial oxidative phosphorylation of ADP [11]. These properties, together with the finding that adenine and its nucleotides acted as protectors, suggested that the mechanism of protection might involve a disturbance of cellular energetics. A comparison of the effect of anthranilate and one of its aryl derivatives, flufenamic acid, on the uncoupling of oxidative phosphorylation, showed that the aryl derivative was much more potent [11]. It was therefore considered that the aryl derivatives of the protectors with anti-inflammatory activity might similarly be more potent as protectors. However, indomethacin [(1-*p*-chlorobenzoyl-5-methoxy-2-methylindol-3-yl)acetic acid], flufenamic acid [*N*-($\alpha\alpha\alpha$-trifluoro-*m*-tolyl)anthranilic acid] and fenclozic acid [2-(4-chlorophenyl)-thiazol-4-yl acetic acid] each failed to show any protective activity, while 2-phenyl-AIC proved to be the most potent protector of all the compounds investigated.

This result raised the interesting possibility that 2,4-DNP might be protecting by a mechanism less obvious than its well known effect upon oxidative phosphorylation since the uncoupling anti-inflammatory drugs have failed to protect and also because it appears that 2-phenyl-AIC is not an uncoupler*. Another property shared by 2,4-DNP and the anti-inflammatory compounds is the inhibition of prostaglandin synthesis [12]. This activity has been proposed as a mechanism of action of the non-steroidal anti-inflammatory drugs [13]. However, it is unlikely that prostaglandin metabolism is involved in the mechanism of protection since indomethacin failed to protect and the anti-inflammatory arylimidazoles do not appear to be prostaglandin inhibitors.†

The potency of the protection by 2-phenyl-AIC in the bioassay system led us to carry out all further studies with this compound. In addition our investigation of a number of closely related aryl imidazoles showed that 2-phenyl-AIC was again the most potent protector. These structure–activity studies suggest that a free amino group at the 4-position of the imidazole ring is essential for activity, whilst alteration of the 5-amido group results in a reduction of activity and that a CO-R group is essential at this position. In previous studies on the protection against cytotoxic alkylating agents, dose reduction factors (based on the ID_{90}'s) of less than 10 were obtained [18] so that the reduction factor of almost 90 for 2-phenyl-AIC pretreated animals was therefore exceptional and may suggest the existence of a specific mechanism for reversal. In comparison with AIC, 2-phenyl-AIC had a more selective effect *in vivo* upon the antitumour activity of CB 1954 than upon the whole animal toxicity and this was reflected in the reduction of the therapeutic index (LD_{50}/ED_{90}) by a factor of 30. A concentration of 2-phenyl-AIC, one twentieth of that of AIC was required to protect against the selective effect of CB 1954 upon DNA synthesis.

The low chemical reactivity of the aziridinyl alkylating function of CB 1954 [14] and the failure to protect against CB 1954 with high concentrations of cysteine in the bioassay system [2], suggested that CB 1954 might react with its site of action in two phases: an initial and reversible binding followed by slow alkylation in accordance with its chemical reactivity. It was considered that an excess of 2-phenyl-AIC might be able to reverse the initial binding. However, a 20-fold excess of 2-phenyl-AIC failed to reverse, or post-protect against, the effects of CB 1954 suggesting that, contrary to the evidence of the low activity of the aziridinyl function, the initial binding might bring about an increase in the reactivity of the aziridinyl group.

The mechanism of protection by 2-phenyl-AIC is now under investigation. Certain imidazoles with a

* J. A. Hickman and D. H. Melzack. Unpublished results.

† J. Heyes. Personal communication.

hydrophobic 2-substituent, and the 8-substituted purines derived from them, have been reported to be inhibitors of cyclic nucleotide metabolism [15, 16]. CB 1954, like the difunctional alkylating agents, has been reported to raise cyclic AMP levels in Walker cells and it was shown that a direct relationship existed between the level of cyclic AMP and cell kill [17]. However, unlike the difunctional alkylating agents, CB 1954 did not bring this about by an inhibition of phosphodiesterase. Current investigations include the study of the effects of CB 1954 and 2-phenyl-AIC on adenine nucleotide metabolism and this may explain both the mechanism of action of CB 1954, its cross-resistance with other difunctional alkylating agents and the mechanism of protection.

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