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STUDIES ON THE PROTECTION BY IMIDAZOLES AGAINST THE CYTOTOXICITY OF THE ANTITUMOUR ALKYLATING AGENTS MELPHALAN AND CB 1954

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Abstract—The effect of two imidazoles, the purine precursor 4-amino-imidazole-5-carboxamide (AIC) and an aryl derivative 4-amino-2-phenylimidazole-5-carboxamide (2-phenyl AIC), which protect against the cytotoxicity of the monofunctional alkylating agent CB 1954, have been investigated as protectors against the cytotoxicity of melphalan, a difunctional alkylating agent. The effects of the imidazoles on the melphalan-induced depression of thymidine incorporation into Walker cells has been compared with the effect on the depression produced by CB 1954. In addition, the effects of 2-phenyl AIC alone on precursor incorporation have been studied. It has a rapid and potent depressant effect on adenine incorporation, a moderate depressant effect upon pyrimidine incorporation but no effect on leucine incorporation or upon the uptake of glucose into the Walker cell.

The monofunctional alkylating agent CB 1954 (5-(1-aziridinyl)-2,4-dinitrobenzamide) has many properties which resemble those of the difunctional alkylating agents such as melphalan (*p*-di-(2-chloroethyl)-amino-L-phenylalanine). Thus it brings about a selective depression of thymidine incorporation [1], an increase in the intracellular level of 3',5'-cyclic adenosine monophosphate (cAMP) [2] and a tumour with an induced resistance to melphalan was cross-resistant to CB 1954 [1]. The potent and selective cytotoxicity of CB 1954 to the Walker tumour was shown to be protected against by certain compounds, including the purine precursor 4-amino-imidazole-5-carboxamide (AIC), adenine, indole acetic acid, anthranilamide and 2,4-dinitrophenol [3, 4]. The most potent protector was an aryl derivative of AIC, 4-amino-2-phenylimidazole-5-carboxamide (2-phenyl AIC) [4]. This compound protected against the CB 1954 induced depression of thymidine incorporation into the Walker cell, and against the cytotoxicity of CB 1954 to Walker cells both *in vitro*, in a bioassay system, and *in vivo*. Thus, in a tumour-bearing animal pretreated with 200 mg kg^{-1} 2-phenyl AIC, the dose of CB 1954 required to cause a 90 per cent decrease in tumour weight was increased 90-fold. In the bioassay system, in which ascites cells are incubated with the drugs *in vitro* before the cells are injected into animals, 50 $\mu g\ cm^{-3}$ of 2-phenyl AIC completely protected against the concentration of CB 1954 (1 $\mu g\ cm^{-3}$) which gave the animals a survival time of greater than 30 days compared to six or seven days when the cells were untreated. Thus animals injected with cells which had been treated with a mixture of CB 1954 and 2-phenyl AIC died at the same time as the controls [4]. A study of this protection phenomenon was hoped to give an indication of the site of action of CB 1954 and an explanation of its selective toxicity.

We now report the results of an investigation into the effects of two of the protectors, AIC and 2-phenyl AIC, on the cytotoxicity of melphalan in the bioassay system and upon the melphalan-induced depression of thymidine incorporation into Walker cells. In addition, we have studied the effects of 2-phenyl AIC on precursor incorporation. We reported briefly that this compound has a depressant effect upon thymidine incorporation but was without toxicity in the bioassay [4]. Its effects on the incorporation of other precursors have now been studied and the results are presented here.

MATERIALS AND METHODS

Drugs and radioactive precursors. AIC was purchased from Sigma Ltd. 2-Phenyl AIC was the kind gift of Dr. J. Heyes of Beecham Research Laboratories, Brockham Park, Surrey. Drugs were dissolved or suspended by sonication in dimethylsulphoxide and added to cell suspensions to give a final solvent concentration of 1%. The controls received the solvent alone.

Radioactively labelled precursors were obtained from the Radiochemical Centre, Amersham, England. The following levels of radioactivity were used per cm^3 of cell suspension: adenine [8-^{14}C] 50 mCi m-mole^{-1}, 0.2 μCi cm^{-3}; L-leucine [4,5-^{3}H], 58 Ci m-mole^{-1}, 5 μCi cm^{-3}; uridine [5-^{3}H], 5 Ci m-mole^{-1}, 5 μCi cm^{-3}; thymidine [5-Me-^{3}H], 5 Ci m-mole^{-1}, 2.5 μCi cm^{-3}; D-glucose [U-^{14}C], 281 mCi m-mole^{-1}, 5 μCi cm^{-3}.

Bioassay and incorporation procedures. The Walker tumour was passaged in ascites form as described previously [4]. The cells were removed from the animal on the sixth or seventh day after transplant.

Bioassay. This was carried out under aseptic conditions. The cells were washed with saline and resuspended in TC 199 and horse serum (60:40) at a final cell concentration of $10^6\ cm^{-3}$. The cells were incubated for 1 hr at 37° before the addition of drugs.

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Table 1. Results of the bioassay of melphalan and CB 1954 with or without AIC or 2-phenyl AIC pretreatment of Walker cells compared with the effects on thymidine incorporation

Treatment	Bioassay: day of death (Median)	Inhibition of thymidine incorporation (%)
None	6	0
(A) CB 1954 1 $\mu g\,cm^{-3}$	>30	52 (± 4.0)
(B) Melphalan 1 $\mu g\,cm^{-3}$	>30	55 (± 4.4)*
(C) AIC 1 $mg\,cm^{-3}$	6	0 (± 7.0)
(D) 2-Phenyl AIC 1 $mg\,cm^{-3}$	6	Not done
(E) 2-Phenyl AIC 50 $\mu g\,cm^{-3}$	6	13 (± 1.5)
(A) + (C)	6	0 (± 7.0)
(A) + (D)	6	†
(A) + (E)	6	15 (± 2.6)
(B) + (C)	>30	26 (± 12)*
(B) + (D)	12	†
(B) + (E)	Not done	50 (± 4.4)

The experiments marked † were not performed since this concentration of 2-phenyl AIC had a depressant effect upon thymidine incorporation. Standard deviations shown in parenthesis.

* P = <0.025 by student's *t*-test.

The imidazoles were added 5 min before either CB 1954 or melphalan. At the end of the required incubation period 1 cm^3 of the cell suspension was injected intraperitoneally into groups of five animals. The survival time of the rats receiving the treated cells was compared with those receiving the untreated cells and the assay terminated after 30 days.

Incorporation of precursors and uptake of glucose. Ascites cells were washed with a cell lysis medium [5] until free from red cells. The cells were then suspended at $10^6\,cm^{-3}$ in TC 199 and horse serum (60:40) and incubated for 1 hr at 37° before the addition of drugs. After the required period of incubation, radioactive precursors were added. 1-cm^3 aliquots of the cell suspension were then removed, at timed intervals, and placed on glass-fibre filter discs (Whatman GF/C 2.5 cm) wetted with saline. The cells were washed with 15 cm^3 of 0.9% saline, 15 cm^3 of 0.2 N perchloric acid and finally with 15 cm^3 of 0.9% saline. The total cell uptake of glucose was estimated in the same way except that the wash with perchloric acid was omitted. The filters were placed in plastic vials and dried overnight at 70°. Scintillation fluid (toluene, dioxan, 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. Triplicate samples were taken at each time point.

When a combined bioassay–incorporation experiment was performed, aliquots of cells from the bioassay were removed, radioactive precursors added and the cells sampled in triplicate as described above.

RESULTS

Bioassay of melphalan with AIC or 2-phenyl AIC. The results for the 4 hr bioassay of melphalan with AIC or 2-phenyl AIC are shown in Table 1. For comparison the results for the bioassay of an equitoxic concentration of CB 1954 have been included [3, 4].

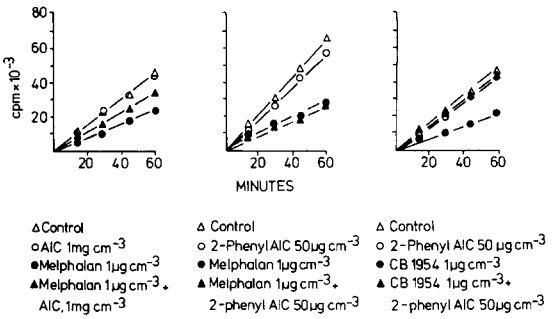

Fig. 1. The effects of 2-phenyl AIC and AIC on the depression of thymidine incorporation induced by melphalan or CB 1954 after 4 hr incubation. The results for CB 1954 have been published previously but are shown for comparison [3, 4].

Effects of AIC or 2-phenyl AIC on the alkylating agent induced depression of thymidine incorporation. The effect of pre-incubation with AIC or 2-phenyl AIC upon the depression of thymidine incorporation induced by either CB 1954 or melphalan after 4 hr incubation is shown in Table 1 and compared with the bioassay result. High concentrations (> 50 $\mu g\,cm^{-3}$) of 2-phenyl AIC could not be used for these experiments since it depressed thymidine incorporation in its own right (see below). In general, the cells had incorporated about 5×10^4 cpm after 1 hr. Representative profiles of these experiments are shown in Fig. 1. The results in Table 1 are the average of three determinations.

Effect of 2-phenyl AIC on precursor incorporation and the uptake of glucose. The effect of 50 and 500 $\mu g\,cm^{-3}$ of 2-phenyl AIC upon the incorporation of precursors into macromolecules is shown in Table 2 and representative profiles are shown in Fig. 2. There was no effect of either 2-phenyl AIC (50 $\mu g\,cm^{-3}$) or CB 1954 (10 $\mu g\,cm^{-3}$) upon glucose uptake into Walker cells. The results in Table 2 are the average of at least three determinations.

DISCUSSION

Although CB 1954 resembles melphalan in a number of its properties, the present results indicate that the protection afforded by AIC and 2-phenyl

Table 2. Effects of 2-phenyl AIC on precursor incorporation after 1 hr (0.25 hr preincubation)

Treatment	Precursor incorporated	Inhibition (%)
50 $\mu g\,cm^{-3}$ 2-Phenyl AIC	Thymidine	13 (± 1.5)
	Uridine	16 (± 6.0)
	Adenine	33 (± 5.0)
	L-Leucine	5 (± 2.5)
500 $\mu g\,cm^{-3}$ 2-Phenyl AIC	Thymidine	50 (± 4.4)
	Uridine	66 (± 15.5)
	Adenine	65 (± 4.6)
	L-Leucine	17 (± 4.6)

Standard deviations shown in parenthesis.

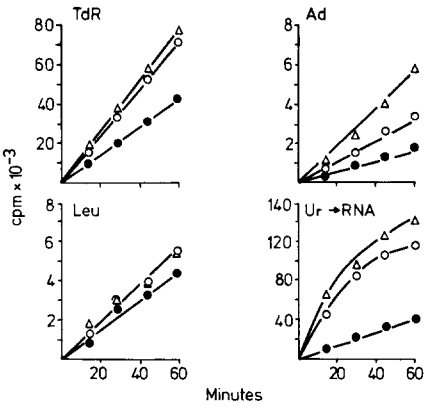

Fig. 2. The effect of a 0.25 hr incubation of 2-phenyl AIC on precursor incorporation into PCA insoluble material of Walker cells. Δ, controls; O, 50 μg cm^{-3} 2-phenyl AIC; ●, 500 μg cm^{-3} 2-phenyl AIC.

AIC against the cytotoxicity of CB 1954 is exclusive to it and does not extend to the difunctional agent. Thus, whilst 50 μg cm^{-3} of 2-phenyl AIC and 1 mg cm^{-3} of AIC completely protects against the cytotoxicity of CB 1954 (1 μg cm^{-3}) in the bioassay system it does not protect against an equivalent concentration of melphalan (Table 1). At a high concentration of 2-phenyl AIC (1 mg cm^{-3}) its protective effect against melphalan was only marginal; the six-day increase in survival time representing a cell kill in the bioassay of 99 per cent [6]. However the parallel study of the effects of the protectors on the alkylating agent induced depression of thymidine incorporation showed some difference to these bioassay results. AIC (1 mg cm^{-3}) reduced by some 50 per cent the depression of thymidine incorporation caused by melphalan. The concentration of 2-phenyl AIC which itself had a marginal effect on thymidine incorporation (50 μg cm^{-3}) and protected against the CB 1954 induced depression of incorporation had no effect on the depression caused by melphalan. The higher concentration of 2-phenyl AIC which had shown marginal protection against the cytotoxicity of melphalan could not be used in the thymidine incorporation experiments because it had an effect of its own.

There is thus a disparity in the results for AIC and melphalan in the two systems. AIC protected against melphalan to some extent in the thymidine incorporation system but gave no protection in the bioassay. This may reflect a large difference in the sensitivities of the two methods in their abilities to measure the cytotoxicity to the Walker cell. This could be because the depression of thymidine incorporation may not be a true measure of the inhibition of DNA synthesis. Recent evidence has shown that early measurements of the effect of alkylating agents show a depression of thymidine incorporation while DNA synthesis may be continuing unimpeded [7, 8]. This suggests that the AIC protection against the melphalan-induced depression of thymidine incorporation might be an effect on thymidine transport rather than on a later event in thymidine utilisation such as DNA synthesis. It has been suggested previously that the effect of CB 1954 on thymidine incorporation may be upon a transport process since the incorporation of [6-^{3}H]uridine into DNA was inhibited to a much lesser degree than that of thymidine [9].

The absence of any effect of AIC on thymidine incorporation suggests that the depressant effects of 2-phenyl AIC on thymidine incorporation may not be related to the protection phenomenon. However, this effect of 2-phenyl AIC was felt to merit further attention mainly because it was without toxicity in the bioassay even in high concentrations. It was also interesting to find whether, like the alkylating agent against which it protected, it had a selective effect on thymidine incorporation. The results (Fig. 2, Table 2) show that the effect was not selective. The effect on adenine incorporation was more marked that that on either thymidine or uridine. The structural similarities of the 2-phenyl AIC to adenine may account for this effect. Unlike the effects of the alkylating agents on precursor incorporation the effects of 2-phenyl AIC were rapid in onset. The lack of any effect upon leucine incorporation or glucose uptake into the cell suggests that 2-phenyl AIC is not a general inhibitor of transport, and would confirm a previous supposition [4] that the mechanism of protection does not involve cellular energetics, since the transport of both leucine and glucose is an energy-dependent process [10]. A study of the selective effect upon purine and pyrimidine incorporation may provide further information on the properties of 2-phenyl AIC which are responsible for its protection against CB 1954. At present under investigation are the transport and incorporation of the precursors, with particular reference to thymidine incorporation and adenine nucleotide metabolism.

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