Biochemical Pharmacology, Vol. 24, pp. 211–217. Pergamon Press, 1975. Printed in Great Britain.

INHIBITION OF CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE—A POSSIBLE MECHANISM OF ACTION OF BIFUNCTIONAL ALKYLATING AGENTS

MICHAEL J. TISDALE and BARRY J. PHILLIPS

Department of Biochemistry, St. Thomas's Hospital Medical School, London, SE1 7EH, England and Ludwig Institute for Cancer Research, Chester Beatty Research Institute, Fulham Road, London, SW3 6JB, England

(*Received*: 14 *May* 1974; *accepted* 11 *July* 1974)

Abstract—The effect of anti-tumour alkylating agents on the adenosine 3′,5′-monophosphate (cyclic AMP) levels of sensitive and resistant Walker carcinoma cells in tissue culture has been investigated. Chlorambucil caused a two-fold elevation of cyclic AMP, in the sensitive line only, 8 hr after treatment with a dose of 5 μg/ml. A comparable increase is produced by the cyclic nucleotide phosphodiesterase inhibitor aminophylline at a dose which produces the same degree of inhibition of cell growth. The therapeutically-inactive monofunctional *N*-ethyl analogue of chlorambucil had no effect on the cyclic AMP level of the sensitive cells at a dose of 250 μg/ml after 8 hr, while the highly selective monofunctional alkylating agent, CB 1954, at 1 μg/ml caused an elevation of cyclic AMP in the sensitive line 24 hr after treatment. This is not a non-specific effect caused by an inhibition of cell growth for the antimetabolite methotrexate had no effect on the intracellular cyclic AMP of sensitive Walker cells at doses which produced complete inhibition of cell growth. The effect of the alkylating agents on cyclic AMP levels is probably due to a specific inhibition of the cyclic nucleotide phosphodiesterase with a low K_m value, since only this form of the enzyme is inhibited, and only in the sensitive cells, when an effect on cell growth and cyclic AMP content is observed. In the case of CB 1954, however, there was no inhibition of either form of the phosphodiesterase at a time when cyclic AMP levels were elevated. A linear relationship exists between the reciprocals of the intracellular cyclic AMP and the percentage growth inhibition produced by a given dose of chlorambucil.

The general consensus of opinion is that the alkylating agents exert their anti-mitotic effects by inter-or intra-strand [1, 2] cross-linking between the N_7 atoms of adjacent guanines of the twin strands of DNA [3, 4]. Thus, while difunctional alkylating agents have been shown to be potent anti-tumour agents in experimental systems, their corresponding monofunctional analogues, while often just as toxic, rarely have any anti-tumour properties.

There are, however, a number of facts that do not fit in with this hypothesis. It is difficult to account for the selectivity of the alkylating agents. Thus, bis-methanesulphonates are more specific inhibitors of the growth of myeloid tissue than are the bis-2-chloroethylamines [5], whereas the latter compounds are more specific for lymphatic tissue [6]. Also, while the general rule that only difunctional alkylating agents show anti-tumour activity is applicable, three mono-functional alkylating agents, all aziridines, show good anti-tumour effects in certain experimental systems. These compounds are of interest since they cannot cross-link DNA in the usual manner and presumably inhibit growth by other mechanisms. One of these agents, 5-aziridinyl-2,4-dinitrobenzamide (CB 1954), proved to be the most selective anti-tumour agent so far tested against the Walker rat carcinoma [7]. Furthermore, the Walker tumour with acquired resistance to CB 1954 is also cross-resistant to the difunctional alkylating agent melphalan, implying that the two drugs have a similar mechanism of action [8].

If the mechanism of the anti-tumour activity of alkylating agents solely involves DNA, then certain differences between sensitive and resistant cells would be expected. While *E. coli* cells resistant to difunctional alkylating agents can excise the difunctionally alkylated guanine moieties from their DNA [9], there appears to be no difference between sensitive and resistant Yoshida sarcoma cells [10, 11], in the level of alkylation of their DNA, the rate of excision of alkylated moieties, and their ability to carry out non-semi-conservative DNA synthesis following treatment with mustard gas.

Adenosine 3′,5′-monophosphate (cyclic AMP) may be the intracellular mediator by which eukaryotic cells control their rate of growth [12, 13]. Cyclic nucleotide phosphodiesterase (adenosine 3′,5′-monophosphate phosphohydrolase, EC 3.1.4.c) converts cyclic AMP to 5′-AMP, and inhibitors of this enzyme induce morphological differentiation of mouse neuroblastoma cells in culture [14]. The phosphodiesterase inhibitor theophylline retards the rate of intradermal growth of Rauscher leukaemia virus-induced tumour in Balb/c mice [15] and suppresses the growth of subcutaneously inoculated Walker rat carcinoma cells [16].

A similarity exists between the anti-mitotic effects of bis-2-chloroethylamines and cyclic AMP. Both inhibit

during the S period of the cell cycle [17, 18], both cause an inhibition of DNA synthesis [1, 18], and both cause cytolysis of mature lymphocytes [6, 19]. Moreover, the anti-tumour action of chlorambucil [4-*p*-(bis-2-chloroethylamino)phenylbutyric acid] against Sarcoma-45 tumours is enhanced when administered in combination with the adenyl cyclase activator adrenaline [20].

In the present study, an investigation has been made of the effect of active and non-active anti-tumour agents on the intracellular cyclic AMP content of sensitive and resistant Walker carcinoma cells in tissue culture, and correlates these effects with an inhibition of the low K_m form of cyclic nucleotide phosphodiesterase.

MATERIALS AND METHODS

8-[^{3}H]Cyclic AMP (sp. act. 20·7 Ci/m-mole) was chased from the Radiochemical Centre, Amersham. Scintillation fluid NE 233 was purchased from Nuclear Enterprises Ltd., Edinburgh. Aminophylline (2 molecules of theophylline and 1 of ethylene diamine) was purchased from Sigma Chemical Co. and unlabelled cyclic AMP from B.D.H. Ltd. Methotrexate was purchased from Lederle Laboratories (Cyanamid) Ltd. All the alkylating agents used in this study were synthesized in the Chester Beatty Research Institute. Beef heart phosphodiesterase was prepared by the method of Goren and Rosen [21].

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, under an atmosphere of 10% CO_2 in air. The methods used to establish and maintain Walker tumour cells in culture have been described previously [22]. A line highly sensitive to alkylating agents, Walker S (WS), was derived from the Walker ascites carcinoma 256. A resistant line (WMI) was established from a tumour line derived from WS by repeated treatment with increasing doses of melphalan. This line has a degree of acquired resistance to other alkylating agents including chlorambucil.

Drug treatment. Cells were taken from rapidly growing cultures and resuspended in fresh medium at 5×10^5 cells/ml. Drug solutions were made up at 100-times the required concentration and 1 ml of each solution was added to aliquots of 100 ml of cell suspension. Aminophylline was dissolved in water and the alkylating agents in dimethyl sulphoxide. Controls received solvent alone. Cells treated with aminophylline were incubated continuously in the presence of the drug. Alkylating agent treatment was limited to 1 hr, after which time the cells were centrifuged at 300 *g* for 3 min, washed, and resuspended in fresh medium. The cell suspensions were then incubated at 37° for the periods stated in the tables.

The effect of drug treatment on cell growth was estimated as previously described [22]. Drugs were added to cells suspended at 10^5/ml in culture medium, and the cells were then either incubated for 1 hr, washed and resuspended at the same density (alkylating agents) or plated out immediately in the presence of aminophylline. In either case the suspensions were dispensed in 200 μl amounts into the wells of a 96-well plastic plate and cell counts were made every 24 hr on several wells of each series. Counts were used to construct growth curves from which percentage inhibition of growth was estimated [22].

Assay of adenosine 3',5'-monophosphate phosphodiesterase. The assay of adenosine 3',5'-monophosphate phosphodiesterase is described in the previous paper. For the assays of both phosphodiesterase and the intracellular cyclic AMP content of Walker carcinoma 256, cells were counted and an appropriate amount of cell suspension was centrifuged at 300 *g* for 2 min. For the phosphodiesterase assay, the cells were resuspended in a large volume of isotonic saline and recentrifuged. The saline was removed and the cells were suspended in 100 mM Tris–HCl, pH 8·1, containing 10 mM $MgSO_4$, and sonicated with a 20-Kc MSE sonic oscillator (5 sec/ml). For the determination of the activity of the high-K_m form of the phosphodiesterase, the cells were initially suspended at a concentration of 10^7/ml of buffer and assayed at 1000 μM cyclic AMP. The activity of the low K_m form of the enzyme was assayed at a substrate concentration of 3·3 μM and the sonicated cell suspension initially contained 10^6 cells/ml of assay buffer. The activity of the phosphodiesterase was determined in each case by the addition of 0·1 ml of sonicated cell suspension to 0·1 ml of the Tris–buffer containing the appropriate concentration of 8-[^{3}H]cyclic AMP. The 5'-AMP formed was quantitated as described in the previous paper. Protein was estimated by the method of Lowry *et al.* [23] using bovine serum albumin as a standard.

Cyclic AMP assay. The culture medium was carefully removed and 1 ml of cold 5% (w/v) trichloroacetic acid was added to it within 30 sec. The cells were then transferred to a tube cooled in ice and the culture dishes were rinsed with 0·5 ml of 5% trichloroacetic acid which was then added to the previous extract. 8-[^{3}H]Cyclic AMP (0·57 pmole; 150,000 cpm) was added and the suspension was centrifuged for 10 min at 10,000 *g*. After the addition of 0·2 ml of 1N HCl, the supernatant was ether extracted and lyophilized. The residue was dissolved in 0·3 ml of 0·086 M Tris–HCl, pH 8·5, containing 4 mM $MgCl_2$ and 150 μg/ml of bovine serum albumin. An aliquot (0·1 ml) of each sample was treated with 10 μg of beef heart phosphodiesterase for 3·5 hr at 35°. After heating at 100° for 5 min, both phosphodiesterase-treated and untreated samples were passed over a column (0·6 × 10 cm) of Amberlite CG-120 Type III 400–600 mesh in 0·1N HCl. The column was rinsed with 6 ml of 0·1N HCl and the cyclic AMP was eluted with dionised water, lyophilized, dissolved in 0·05 M Tris–HCl, pH 7·5, containing 4 mM EDTA (0·3 ml) and the cyclic AMP content was determined by means of an assay kit (Radiochemical Centre, Amersham). A standard curve was performed for each determination. Radioactivity was

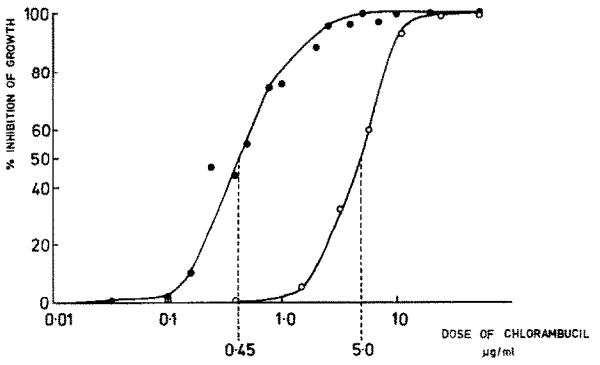

Fig. 1. Dose–response curve for chlorambucil against Walker cells in tissue culture. (●—●) WS; (○—○) WMI.

measured in a modified Bray's solution [24]. The difference between phosphodiesterase-treated and untreated samples was taken to be the cyclic AMP content of the Walker cells.

RESULTS

Drug sensitivity of cells. The relationship between the dose of chlorambucil and inhibition of cell growth for the two cell lines, WS and WMI, is shown in Fig. 1. The doses which gave 50 per cent inhibition of cell growth were 0·45 μg/ml for WS and 5·0 μg/ml for WMI. Fig. 2 shows that the sensitivity of WS to the monofunctional analogue of chlorambucil [(*N*-ethyl-*N*-2-chloroethylamino)-phenylbutyric acid] was much less than its sensitivity to the difunctional compound. In this case 70 μg/ml was required to give 50 per cent inhibition of cell growth. The monofunctional alkylating agent 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) is extremely toxic to WS, the dose for 50 per cent growth inhibition being 0·009 μg/ml. Furthermore the Walker tumour with induced resistance to melphalan (WMI), also shows a considerable degree of cross-resistance to this agent; the dose for 50 per cent growth inhibition being raised to 0·63 μg/ml.

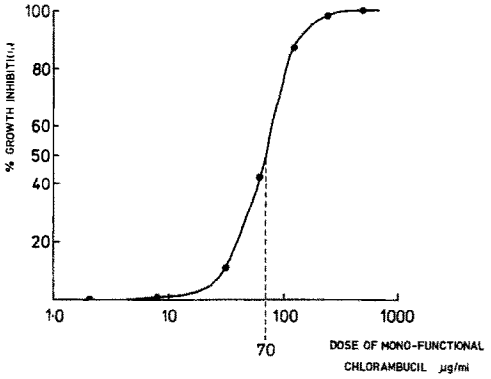

Fig. 2. Dose–response curve for monofunctional chlorambucil against Walker cells.

Effect of alkylating agents on the cyclic AMP levels of sensitive and resistant Walker carcinoma. The levels of cyclic AMP observed in sensitive and resistant Walker cells, 8 hr after treatment with various doses of chlorambucil, are shown in Table 1.

The cyclic AMP of the sensitive cells increases exponentially with increasing drug dose. This relationship is observed whether cyclic AMP levels are expressed in terms either of cell volume or of total cell protein. For this reason all other results are expressed only in terms of cell volume. A double reciprocal plot of the relationship between the intracellular cyclic

Table 1. Cyclic AMP content of sensitive and resistant Walker carcinoma cells 8 hr after treatment with chlorambucil

	Cyclic AMP ± S.E.			
	Sensitive		Resistant	
Culture conditions	(μM)	(pmole/mg protein)	(μM)	(pmole/mg protein)
No additions	1·95 ± 0·1	42·0 ± 2·0	1·35 ± 0·05	41·0 ± 2·0
7·0 μg/ml Chlorambucil	3·66 ± 0·1	100·0 ± 2·0	—	—
5·0 μg/ml Chlorambucil	3·4 ± 0·05	90·0 ± 2·0	1·20 ± 0·05	42·0 ± 2·0
2·5 μg/ml Chlorambucil	3·2 ± 0·05	85·0 ± 2·0	—	—
1·0 μg/ml Chlorambucil	2·8 ± 0·1	72·4 ± 1·8	1·22 ± 0·05	40·0 ± 2·0
0·5 μg/ml Chlorambucil	2·25 ± 0·1	51·0 ± 2·0	1·26 ± 0·05	40·0 ± 2·0

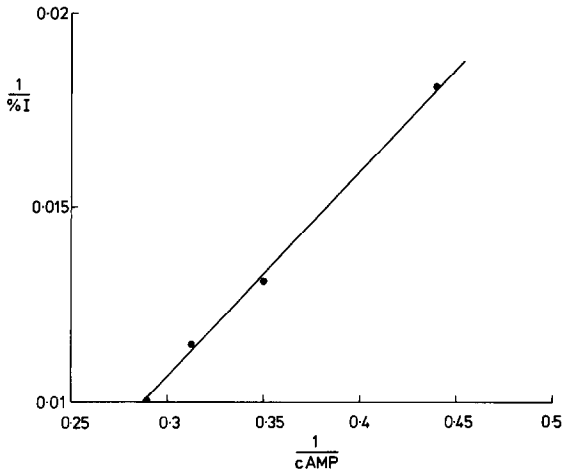

Fig. 3. Double reciprocal plot between the percentage inhibition of growth (% I) of WS in tissue culture by chlorambucil and the intracellular cyclic AMP (cAMP).

AMP levels and the percentage growth inhibition produced by chlorambucil is shown in Fig. 3. The linearity of such a plot suggests that there is either a direct or indirect relationship between percentage growth inhibition and cyclic AMP levels over the range studied. In the case of the resistant line (WMI) the basal cyclic AMP content is somewhat lower than that of the sensitive line when expressed as μM, but not significantly different in terms of total protein. This level is not altered by any of the doses of chlorambucil used, even though a concentration of 5 μg/ml of chlorambucil produces the same degree of growth inhibition in WMI, as does 0·5 μg/ml in WS (Fig. 1).

Table 2 shows that the monofunctional *N*-ethyl analogue of chlorambucil also has no effect on the cyclic AMP content of WS 8 hr after treatment, even at a dose of 250 μg/ml which produces 98 per cent inhibition of cell growth (Fig. 2). Thus inhibition of growth by this monofunctional alkylating agent would appear to be produced by a different mechanism from that of the difunctional analogue.

Table 2. Cyclic AMP content of sensitive Walker carcinoma cells 8 hr after treatment with monofunctional chlorambucil (M.F.C.)

Culture conditions	Cyclic AMP (μM ± S.E.)
No additions	1·94 ± 0·05
250 μg/ml MFC	1·85 ± 0·1
100 μg/ml MFC	1·65 ± 0·1
50 μg/ml MFC	1·80 ± 0·1

The effect of chlorambucil on the cyclic AMP levels in sensitive and resistant Walker carcinoma cells 24 hr after treatment is shown in Table 3. At this time also the resistant line shows no significant elevation of intracellular cyclic AMP at any of the dose levels used. In the sensitive line the concentration of cyclic AMP at the two lower dose levels is not elevated significantly above the control value, whilst the highest dose (5 μg/ml) produces a 34 per cent elevation.

Table 3. Cyclic AMP content of sensitive and resistant Walker carcinoma 24 hr after treatment with chlorambucil

Culture conditions	Cyclic AMP (μM ± S.E.) Sensitive	Resistant
No additions	1·94 ± 0·05	1·2 ± 0·05
5 μg/ml Chlorambucil	2·6 ± 0·1	1·1 ± 0·1
1 μg/ml Chlorambucil	2·1 ± 0·1	1·2 ± 0·1
0·5 μg/ml Chlorambucil	1·92 ± 0·1	1·0 ± 0·1

Table 4 shows that the monofunctional alkylating agent, CB 1954, which is highly effective in inhibiting the growth of the Walker carcinoma *in vivo* [7], also causes a significant elevation of intracellular cyclic AMP in WS 24 hr after treatment. Because of the greater potency of this agent, 1 μg/ml has equivalent growth inhibitory properties to 5 μg/ml of chlorambucil.

Table 4. Cyclic AMP content of sensitive Walker carcinoma 24 hr after treatment with CB 1954

Culture conditions	Cyclic AMP (μM ± S.E.)
No additions	1·95 ± 0·05
1 μg/ml 1954	2·85 ± 0·05
0·4 μg/ml 1954	2·4 ± 0·1
0·05 μg/ml 1954	2·2 ± 0·2

Effect of other cell growth inhibitors on the cyclic AMP levels of sensitive and resistant Walker carcinoma. In order to investigate whether the elevation of intracellular cyclic AMP produced by the alkylating agents is specific or just a general consequence of growth inhibition, the effect of the antimetabolite methotrexate on cyclic AMP levels in WS was determined at doses (1 μg/ml and 0·2 μg/ml) which produce total inhibition of cell growth. No significant increase in cyclic AMP was observed at any dose level tested.

It was also of interest to compare the elevation of cyclic AMP produced by a traditional phosphodiesterase inhibitor such as aminophylline at doses which produced a comparable degree of growth inhibition to the alkylating agents. Aminophylline is more toxic (ID_{50} 150 μg/ml) to WMI than to WS (ID_{50} 250 μg/ml). The effect of 24 hr-treatment of WS and WMI with aminophylline on cyclic AMP levels and growth inhibition is shown in Table 5. At comparable doses the increase in cyclic AMP produced by aminophylline is higher in WMI than in WS, which would account for

Table 5. Cyclic AMP content and percentage growth inhibition of sensitive and resistant Walker carcinoma 24 hr after treatment with aminophylline

Culture conditions	Sensitive		Resistant	
	Cyclic AMP (μM ± S.E.)	% Inhibition of growth	Cyclic AMP (μM ± S.E.)	% Inhibition of growth
No addition	1·77 ± 0·1	0	1·42 ± 0·1	0
500 μg/ml Aminophylline	3·33 ± 0·06	92	4·2 ± 0·05	100
200 μg/ml Aminophylline	2·10 ± 0·1	30	2·8 ± 0·2	75
50 μg/ml Aminophylline	1·78 ± 0·1	0	2·32 ± 0·1	10

its greater toxicity in the former cell line. Also, the intracellular cyclic AMP (4·2 ± 0·05 μM) produced by a dose of aminophylline (500 μg/ml) which causes 100 per cent inhibition of cell growth in WMI, is similar to the level of cyclic AMP (3·66 ± 0·1 μM) produced by a dose of chlorambucil (7 μg/ml) causing a similar degree of growth inhibition in WS (Table 1).

The effect of alkylating agents on cyclic 3',5'-nucleotide phosphodiesterase. In order to investigate the mechanism of elevation of cyclic AMP by alkylating agents in WS the possibility of inhibition of cyclic 3',5'-nucleotide phosphodiesterase was considered. Table 6 shows the specific activity of the enzyme expressed as nmoles of 5'-AMP formed per minute per milligram of protein, measured at 1 mM cyclic AMP (which was considered to measure the "high-K_m enzyme") and at 3·3 μM cyclic AMP (near the physiological substrate level for the "low-K_m enzyme") in total cell sonicated suspensions of WS and WMI. The conditions of incubation with respect to time and enzyme concentration were as described in the previous paper. Total cell suspensions were investigated because of the loss of some of the high affinity enzyme by centrifugation. Culture medium was carefully removed, because of its possible association with some phosphodiesterase activity. When assayed at 1 mM cyclic AMP, the activity of the high-K_m form of the phosphodiesterase in both WS and WMI is similar within the limits of experimental error. Also, the activity of this form of the enzyme is unaffected by any of the doses of chlorambucil used in either cell line. In contrast the activity of the low-K_m form of the enzyme in WMI is only about 50 per cent of that observed in WS. A similar ratio was shown in the previous paper with the 100,000 *g* supernatant fractions of these two cell lines. Also, whilst the specific activity of the low-K_m form of the phosphodiesterase in WMI remains constant at all doses of chlorambucil employed, that in WS is inhibited by 74, 35 and 12 per cent at doses of chlorambucil of 5, 1 and 0·5 μg/ml, respectively. This inhibition is statistically highly significant. Knowing the K_m values of the two enzyme forms, the activities at low substrate concentration can be corrected for the contribution of the high-K_m enzyme and vice versa [25]. At a concentration of cyclic AMP of 3·3 μM the contribution of the low affinity activity to that observed is 25 per cent. Thus the observed percentage inhibition at 5, 1 and 0·5 μg/ml chlorambucil can be corrected for the high-K_m activity, which is not inhibited, to give values of 100, 60 and 37 per cent, respectively. Similarly the contribution of the low-K_m activity at 1000 μM cyclic AMP is 8 per cent. Thus, when the low K_m activity is completely inhibited as with 5 μg/ml of chlorambucil, the specific activity of the high-K_m form would be expected to fall to 1·48 nmole/min/mg protein which is very close to the value given in Table 6. However, since the actual inhibition is only twice the standard error, the high-K_m activity in WS appears to be constant.

Table 7 shows the activity of the two forms of the phosphodiesterase in WS and WMI 24 hr after treatment with chlorambucil. As before, only the low-K_m form of the enzyme in WS is inhibited and the percentage inhibition is not as high as observed 8 hr after treatment. This would explain the decreased elevation

Table 6. Cyclic AMP phosphodiesterase activities of sensitive and resistant Walker carcinoma 8 hr after treatment with chlorambucil

Treatment	cAMP phosphodiesterase activity			
	Resistant		Sensitive	
	High K_m activity (nmole/mg protein /min ± S.E.M.)	Low K_m activity (nmoles/mg protein /min ± S.E.M.)	High K_m activity (nmoles/mg protein /min ± S.E.M.)	Low K_m activity (nmoles/mg protein /min ± S.E.M.)
Control	1·55 ± 0·05	0·25 ± 0·01	1·6 ± 0·05	0·6 ± 0·02
5 μg/ml Chlorambucil	1·54 ± 0·05	0·23 ± 0·01	1·45 ± 0·06	0·16 ± 0·03*
1 μg/ml Chlorambucil	1·53 ± 0·05	0·22 ± 0·01	1·5 ± 0·05	0·39 ± 0·03†
0·5 μg/ml Chlorambucil	1·45 ± 0·05	0·25 ± 0·01	1·55 ± 0·05	0·53 ± 0·03‡

* $P < 0·05$ † $P < 0·01$ ‡ $P < 0·001$

Table 7. Cyclic AMP phosphodiesterase activities of sensitive and resistant Walker carcinoma 24 hr after treatment with chlorambucil

Treatment	cAMP phosphodiesterase % of original			
	Sensitive		Resistant	
	High K_m	Low K_m	High K_m	Low K_m
None	100	100	100	100
5 μg/ml Chlorambucil	100	46	100	100
1 μg/ml Chlorambucil	100	76	100	100
0·5 μg/ml Chlorambucil	100	89	100	100

Table 8. Cyclic AMP phosphodiesterase activity of sensitive Walker carcinoma after treatment with monofunctional chlorambucil (M.F.C.) for 8 hr, and CB 1954 for 24 hr measured at 3·3 μM cyclic AMP

Treatment	cAMP phosphodiesterase activity (nmoles/min/mg protein)	% Inhibition
Control	0·6 ± 0·02	—
250 μg/ml MFC	0·62 ± 0·02	None
100 μg/ml MFC	0·68 ± 0·05	None
50 μg/ml MFC	0·58 ± 0·05	None
1 μg/ml 1954	0·62 ± 0·05	None
0·4 μg/ml 1954	0·61 ± 0·05	None
0·05 μg/ml 1954	0·60 ± 0·02	None

of cyclic AMP observed with chlorambucil after 24 hr. Again neither form of the enzyme is inhibited in WMI.

Table 8 shows the effect of the two monofunctional alkylating agents, (*N*-ethyl-*N*-2-chloroethylamino)-phenylbutyric acid and CB 1954, on the activity of the low-K_m form of the phosphodiesterase 8 hr and 24 hr, respectively, after treatment. Neither agent showed any inhibition of the enzyme which would have been expected on theoretical grounds for a multi-subunit enzyme [26]. Thus elevation of cyclic AMP by CB 1954 appears to be unrelated to an effect on the breakdown of intracellular cyclic AMP.

DISCUSSION

While the present investigation does not prove that the growth inhibitory properties of the bifunctional alkylating agents arise from their ability to elevate intracellular cyclic AMP, there does appear to be a linear relationship between the reciprocals of the intracellular cyclic AMP and the percentage growth inhibition produced by a given dose of chlorambucil. Also the increase in cyclic AMP produced is specific to the antitumour alkylating agents, for the anti-metabolite methotrexate and the monofunctional *N*-ethyl analogue of chlorambucil produce no elevation of cyclic AMP at doses which cause total inhibition of cell growth. Most of the agents that cause an elevation of cyclic AMP, such as isoprenaline or prostaglandin E1, produce a much larger increase [27, 28] (8 to 20-fold) than that observed with the alkylating agents (2-fold). In perfused rat liver, however, adrenaline causes only a 3-fold increase in cyclic AMP in 30 sec and this decreases to 2-fold over the next 90 sec. Since adrenaline stimulates glycogen breakdown in perfused rat liver to about the same extent as does glucagon, which produces a 50-fold increase in cyclic AMP levels within 30 sec, it suggests that only a 2-fold increase in tissue cyclic AMP is necessary to activate phosphorylase fully in the perfused liver. Also the increase in cyclic AMP produced by the phosphodiesterase inhibitor aminophylline is similar to that produced by chlorambucil at a dose which produces a comparable degree of growth inhibition. With the alkylating agents the elevated level of the cyclic nucleotide is observed over a much longer period (8–24 hr) and shows a reduced elevation after 24 hr.

Dibutyryl cyclic AMP is known to cause growth inhibition and cytolysis in cultured mouse lymphosarcoma cells if the agent is allowed to remain in contact with the cells for 48 hr [19]. Although the mechanism by which cyclic AMP induces the killing of sensitive lymphoma cells is unknown it may be related to a decreased penetration of macromolecular precursors [29]. A similar effect on uptake of precursors into nucleic acids has been observed with the alkylating agents [8].

The selectivity of the alkylating agents for certain cell types could be explained by an inhibition of the high affinity form of the cyclic nucleotide phosphodiesterase, since only this form of the enzyme is inhibited and only in the sensitive cells. The contribution of this

form of the enzyme to the total is greater in those cells sensitive to alkylating agents. A mechanism of resistance has been proposed in the preceding paper and the possible reasons for the selective inhibition of this form of the enzyme will also be published [26]. The possibility of considering cyclic nucleotide phosphodiesterase as a target for raising intracellular levels of cyclic AMP specifically in tumour cells is increased by the suggestion that different molecular forms of the enzyme exist in different cell types [30]. Additional evidence for a change in cyclic nucleotide phosphodiesterase in the resistant tumour is provided by the increased sensitivity of this cell line to the growth-inhibitory effects of aminophylline.

Thus in the present investigation a correlation appears to exist between anti-tumour activity, elevation of cyclic AMP levels, and inhibition of the form of the cyclic nucleotide phosphodiesterase with low K_m. The only exception appears to be with the monofunctional alkylating agent CB 1954 which increases cyclic AMP levels without any apparent effect on the high affinity phosphodiesterase. There are several possible explanations for such an effect. If the enzyme is inhibited by a reversible mechanism such as that observed with the methyl xanthines, no apparent inhibition would be observed, since the effect would be diluted out before analysis. Another mechanism by which cells may regulate their intracellular cyclic AMP levels is by leakage to the exterior [28]. As the Walker carcinoma is a fairly rapidly dividing tumour, a low intracellular cyclic AMP level, maintained perhaps by a leakage mechanism, which is specifically inhibited by CB 1954, would be advantageous. This would explain the absolute specificity of this agent towards the Walker carcinoma.

The elevation of intracellular cyclic AMP produced in response to the alkylating agents could explain the suppressing effect that these agents have on antibody production [31]. The interaction of antigen with antigen-sensitive cells can lead to antibody synthesis (induction) or it can render these cells non-inducible (paralysis). It has been suggested that cyclic AMP may be the intracellular mediator of a paralytic signal [32]. Moreover, the delayed hyperglycaemia observed after *in vivo* administration of alkylating agents [33] could be explained by an elevation of intracellular cyclic AMP similar to that observed with glucagon; the delayed effect being the time required to produce sufficient cyclic AMP to stimulate glycogenolysis.

Acknowledgements—The authors wish to thank Professor L. Young for his interest. M. J. Tisdale wishes to acknowledge the receipt of a research grant from the Cancer Research Campaign.

REFERENCES

1. P. Brookes and P. D. Lawley, *Biochem. J.* **80,** 496 (1961).
2. W. G. Flamm, N. J. Bernheim and L. Fishbein, *Biochim. biophys. Acta* **224,** 657 (1970).
3. P. Brookes and P. D. Lawley, *Isotopes in Experimental Pharmacology*, p. 403. University Chicago Press, Chicago (1965).
4. P. D. Lawley and P. Brookes, *Biochem. J.* **109,** 433 (1968).
5. L. A. Elson, *Ann. N.Y. Acad. Sci.* **68,** 826 (1958).
6. L. A. Elson, D. A. G. Galton and M. Till, *Br. J. Haemat.* **4,** 355 (1958).
7. L. M. Cobb, T. A. Connors, L. A. Elson, A. H. Khan, B. C. V. Mitchley, W. C. J. Ross and M. E. Whisson, *Biochem. Pharmac.* **18,** 1519 (1969).
8. T. A. Connors and D. H. Melzack, *Int. J. Cancer* **7,** 86 (1971).
9. P. D. Lawley and P. Brookes, *Nature, Lond.* **206,** 480 (1965).
10. C. R. Ball and J. J. Roberts, *Chem.-Biol. Interactions* **2,** 321 (1970).
11. C. R. Ball, T. A. Connors, J. A. Double, V. Ujhazy and M. E. Whisson, *Int. J. Cancer* **1,** 319 (1966).
12. J. Otten, G. S. Johnson and I. Pastan, *Biochem. biophys. Res. Commun.* **44,** 1192 (1971).
13. B. M. Bombik and M. M. Burger, *Expl Cell Res.* **80,** 88 (1973).
14. K. N. Prasad and J. R. Sheppard, *Expl Cell Res.* **73,** 436 (1972).
15. D. Webb, W. Braun and D. J. Plescia, *Cancer Res.* **32,** 1814 (1972).
16. R. Keller, *Life Sci.* **11,** 485 (1972).
17. S. Gross, *Ph. D. Thesis Univ. of London* (1961).
18. R. V. Wijk, W. D. Wicks, M. M. Bevers and J. V. Rijn, *Cancer Res.* **33,** 1331 (1973).
19. V. Daniel, G. Litwach and G. M. Tomkins, *Proc. natn. Acad. Sci. U.S.A.* **70,** 76 (1973).
20. A. I. Kravchenko and Yu. N. Korobova, *Farmak. Toksiks.* **30,** 737 (1967).
21. E. H. Goren and O. M. Rosen, *Molec. Pharmac.* **8,** 380 (1972).
22. B. J. Phillips, *Biochem. Pharmac.* **23,** 131 (1974).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 262 (1951).
24. G. A. Bray, *Analyt. Biochem.* **1,** 279 (1960).
25. W. J. Rutten, B. M. Schoot, J. J. H. H. M. De Pont and S. L. Bonting, *Biochim. biophys. Acta* **315,** 384 (1973).
26. M. J. Tisdale, *Chem.-Biol. Interactions* **9,** 145 (1974).
27. V. Daniel, H. R. Bourne and G. M. Tomkins, *Nature New Biol.* **244,** 167 (1973).
28. T. J. Franklin and S. J. Foster, *Nature New Biol.* **246,** 119 (1973).
29. P. V. Hauschka, L. P. Everhart and R. W. Rubin, *Proc. natn. Acad. Sci. U.S.A.* **69,** 3542 (1972).
30. D. N. Harris, N. S. Semenuk and S. M. Hess, *Annual Reports in Medicinal Chemistry* Vol. 8, p. 224. Academic Press, New York (1973).
31. M. C. Berenbaum, *Biochem. Pharmac.* **11,** 29 (1962).
32. J. Watson, R. Epstein and M. Cohen, *Nature, Lond.* **246,** 405 (1973).
33. L. A. Elson, *Radiation and Radiomimetic Chemicals*, p. 2. Butterworths, London (1963).