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INHIBITION OF AMINO ACID UPTAKE AND INCORPORATION INTO PROTEINS IN FRIEND ERYTHROLEUKEMIA CELLS BY THE ANTHRACYCLINE ANTITUMOR ANTIBIOTIC ACLACINOMYCIN A

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Abstract—Treatment of Friend erythroleukemia cells with aclacinomycin A caused a concomitant inhibition of the uptake of ^{14}C-α-aminoisobutyric acid (AIB) and of the incorporation of ^{3}H-alanine into proteins. The decrease in amino acid uptake and incorporation into proteins was dose-dependent and reached a maximum of 60% within 3 hours at the concentration of aclacinomycin A, 200 ng/ml. A comparison of the effect on protein incorporation of ^{3}H-alanine and cell proliferation by various anthracycline antitumor antibiotics in a concentration range of 50–200 ng/ml revealed that two other *N*-alkylated anthracyclines, pyrromycin and marcellomycin, are also potent inhibitors of the incorporation of amino acids into proteins. Inhibition of amino acid incorporation into proteins correlated well with the reduction of cell number at a later time. In contrast, adriamycin and daunomycin inhibited the incorporation of ^{3}H-alanine into proteins only weakly, although these substances were highly active at inhibiting cell proliferation. Studies with an inhibitor of RNA synthesis, actinomycin D, suggest that the concomitant inhibition of amino acid uptake and incorporation into proteins observed with aclacinomycin A is not due to a reduced RNA synthesis. In addition, aclacinomycin A, up to a concentration of 10 μg/ml, did not inhibit protein synthesis in a cell-free translational system from rabbit reticulocytes. These results indicate that the reduction of amino acid incorporation into proteins after treatment of Friend erythroleukemia cells with aclacinomycin A may be due to a reduced uptake of amino acids. Inhibition of the transport of ^{14}C-AIB may be indicative for an interaction of aclacinomycin A with the plasma membrane.

The anthracycline antitumor antibiotics belong to the most powerful agents in the chemotherapy of cancer [1]. In addition to the widely-used members of this family, adriamycin and daunomycin, a growing interest is devoted to the new yellow anthracycline antibiotic aclacinomycin A, which was discovered and tested in clinical trials first in Japan [2–5]. This antibiotic is similar in chemical structure to adriamycin and daunomycin, but contains a pyrromycinone aglycone and an *N*-dimethylated sugar moiety, rhodosamine, with an additional disaccharide. Aclacinomycin A is the first of the *N*-alkylated anthracyclines to be used clinically. While adriamycin and daunomycin inhibit DNA and RNA synthesis at approximately equivalent concentrations, aclacinomycin A inhibits RNA synthesis more efficiently than DNA synthesis [6, 7]. Although the antitumor activity of aclacinomycin A is comparable to that of adriamycin, its cardiotoxicity and bone marrow toxicity were reported to be significantly less than those of adriamycin [8–10]. Moreover, aclacinomycin A and related anthracyclines with *N*-alkylated sugar moieties proved to be nonmutagenic (or very weakly mutagenic at best) in bacterial and mammalian cells [4, 11], whereas adriamycin and daunomycin are highly mutagenic and carcinogenic [12, 13]. These findings make aclacinomycin A of considerable clinical interest.

The anthracycline antitumor antibiotics produce a complexity of biochemical effects [1]. However, the exact mechanism(s) of their actions has not been clarified. Adriamycin and daunomycin are known to interact strongly with the DNA and cause DNA fragmentation [14–17]. However, growing evidence suggests that alteration of DNA structure is not a general mechanism of the cytostatic action of anthracyclines [11, 18–20]. Thus, the lack of mutagenicity of aclacinomycin A may be based on another type of interaction with the DNA resulting in preferential inhibition of RNA synthesis [6, 7]. On the other hand, molecular mechanisms which do not affect directly the DNA could also contribute to the antitumor activity of aclacinomycin A: membrane binding and formation of lipid peroxides have been discussed as alternative biochemical mechanisms of anthracyclines to cause specific organ toxicity or tumor-cell killing [1, 21].

The present results show that aclacinomycin A inhibits amino acid uptake and incorporation into proteins in Friend erythroleukemia cells in a concentration range, in that it reduces proliferation, and are indicative of an anthracycline-induced alteration of cell membrane function.

MATERIALS AND METHODS

Chemicals. Aclacinomycin A was kindly provided by Dr. Oki, Sanraku Ocean Ltd. (Tokyo); pyrro-

mycin and marcellomycin were a gift from Dr. G. Lenaz, Bristol-Myers Co. (N.Y.) and adriamycin and daunomycin from Dr. F. Arcamone, Farmitalia-Carlo Erba (Milano, Italy). ^{3}H-Alanine (36 Ci/mmol), ^{3}H-leucine (135 Ci/mmol), ^{3}H-uridine (26 Ci/mmol) and ^{35}S-methionine (1400 Ci/mmol and 1080 Ci/mmol) were purchased from Amersham, U.K.; ^{14}C-α-aminoisobutyric acid (52.6 mCi/mmol) was obtained from New England Nuclear Corp. (Boston, MA). MEM alpha medium without nucleosides and fetal calf serum were obtained from Gibco (Karlsruhe, F.R.G.); HEPES buffer solution from Serva (Heidelberg, F.R.G.). Actinomycin D, cycloheximide, edeine, α-aminoisobutyric acid, bovine serum albumin, creatine phosphate and hemin were purchased from Sigma Chemical Co. (St. Louis, MO); creatinophosphokinase from Boehringer (Mannheim, F.R.G.); dibutylphthalate and dinonylphthalate from Fluka (Buchs, Switzerland); soluene-350 and dimilume-30 from Packard (Downers Grove, IL); amino acids and analytical grade chemicals from Merck (Darmstadt, F.R.G.).

Cell cultures. Friend erythroleukemia cells, line F4-6 [22], were provided by Dr. W. Ostertag, Heinrich-Pette-Institut für Experimentelle Virologie, Hamburg, F.R.G. Cells were grown in suspension cultures in alpha medium without nucleosides plus 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2) supplemented with 10% fetal calf serum.

Assay of amino acid uptake. α-Aminoisobutyric acid (AIB) uptake was measured with 1 ml samples containing 0.7–1.0 × 10^{7} cells. After washing the cells once with Earle balanced salt solution (EBSS), incubations were carried out in EBSS in the presence of 50 μM unlabeled AIB and 0.1 μCi ^{14}C-AIB. Immediately after starting the incubation and after 5 min incubation at 37°, duplicate 200 μl-samples were collected by centrifugation through dibutylphthalate–dinonylphthalate 3:1 oil mixture. Pellets were solubilized in 250 μl of Soluene-350. After overnight incubation at 60°, 5 ml of Dimilume-30 scintillation fluid was added and radioactivity measured in a liquid scintillation counter. O-point values were subtracted in order to correct the results for non-specific trapping by the cell pellet. The uptake of ^{3}H-alanine (12.5 μCi/ml), ^{3}H-leucine (12.5 μCi/ml) and ^{35}S-methionine (10 μCi/ml) was determined similarly with the modification that alpha-medium without nucleosides was used instead of EBSS.

Measurement of amino acid or nucleoside incorporation. For amino acid incorporation, 0.6 ml-samples of cells (5 × 10^{6}) were incubated at 37° for 10 min in alpha medium without nucleosides in the presence of ^{3}H-alanine (7.5 μCi) or ^{3}H-leucine (7.5 μCi) or ^{35}S-methionine (6 μCi). After the incubation, triplicate 100 μl-samples were collected on filters (Whatman 3MM) and a hot trichloracetic acid (TCA) precipitation was performed as described by Mans and Novelli [23]. TCA precipitate-associated radiolabel was determined by liquid scintillation spectrometry.

To measure the incorporation of uridine into RNA, similar samples were incubated for 30 min with 7.5 μCi ^{3}H-uridine. The filters in this case were washed once with ice-cold 10% TCA for 10 min, three times with ice-cold 5% TCA for 5 min, once with ethanol and finally once with diethylether for 5 min.

Assay of protein synthesis in rabbit reticulocyte lysates. Cell free protein synthesis was measured with rabbit reticulocyte lysates as described by Pelhalm and Jackson [24]. Twenty μl of lysate in buffered saline [24] containing 16 μM hemin, 40 μg/ml creatinephosphokinase, 10 mM creatinephosphate amino acids without methionine (each 0.1 mM, valine and leucine 0.15 mM) were incubated with 0.5 μCi ^{35}S-methionine at 37° for 20 min. Incubation was stopped by dilution of the samples with 280 μl distilled water. After decolorization [24], 100 μl aliquots were collected on filters and protein-associated radiolabel was determined as described above.

Estimation of protein contents. Protein contents of the samples were determined by the method of Lowry *et al.* [25] using bovine serum albumin as standard.

RESULTS

Effect of aclacinomycin A on amino acid uptake by F4–6 cells and incorporation into their proteins

Logarithmically-growing F4–6 cells were treated with aclacinomycin A, 200 ng/ml, and at various times after the beginning of treatment the incorporation of ^{3}H-alanine into protein and the uptake of the non-metabolizable amino acid, ^{14}C-AIB was assayed. AIB is not incorporated into protein but it is taken up in the cells by the same mechanisms as alanine [26, 27]. Aclacinomycin A (200 ng/ml) was previously shown to reduce cell proliferation and to induce terminal erythroid differentiation in F4–6 cells [28]. The present results show a concomitant decrease of the ^{14}C-AIB uptake and ^{3}H-alanine incorporation in F4–6 cells following treatment with aclacinomycin A (Fig. 1). The decrease of amino acid uptake and incorporation reached a maximum of 60% at 3 hr after addition of aclacinomycin A.

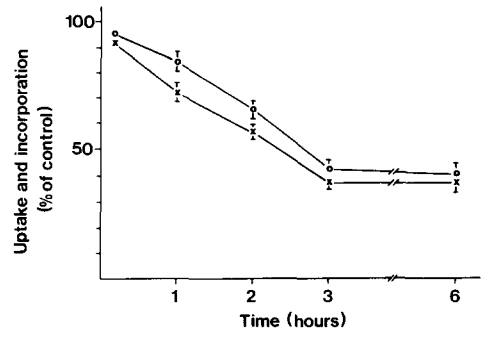

Fig. 1. Effect of aclacinomycin A, 200 ng/ml, on ^{3}H-alanine incorporation into proteins (○) and uptake of ^{14}C-AIB (×) in F4–6 cells. At various exposure times, amino acid uptake and incorporation was measured as described in Materials and Methods. Results are expressed as percent of uptake or incorporation relative to untreated cells and represent the means of three experiments ± standard error. Averages of the ^{3}H-alanine incorporation and ^{14}C-AIB uptake in control cells were: 7.35 pmol/mg protein/10 min and 28.5 pmol/10^{6} cells/5 min, respectively.

Table 1. Effect of aclacinomycin A on uptake of ^{3}H-alanine, ^{3}H-leucine and ^{35}S-methionine in F4–6 cells and on their incorporation into cellular proteins

Amino acid	Aclacinomycin A	Uptake (pmol/10^{7} cells/10 min)	Incorporation (pmol/mg protein/10 min)
^{3}H-Alanine	–	23.50 ± 2.01	7.57 ± 0.80
	+	13.47 ± 1.45	3.22 ± 0.49
^{3}H-Leucine	–	2.99 ± 0.12	1.91 ± 0.19
	+	1.71 ± 0.31	0.94 ± 0.07
^{35}S-Methionine	–	0.295 ± 0.016	0.132 ± 0.011
	+	0.157 ± 0.030	0.066 ± 0.015

F4–6 cells were incubated in the presence and absence of aclacinomycin A (200 ng/ml) for 5 hr. Uptake and incorporation of amino acids in control and aclacinomycin A treated cells were determined in alpha-medium as described in the text. Results represent the means of three experiments ± SE.

Inhibition of incorporation of amino acids into proteins could also be demonstrated when the incorporation of ^{35}S-methionine or ^{3}H-leucine instead of ^{3}H-alanine was measured. Table 1 shows a concomitant decrease of the uptake and incorporation of ^{3}H-alanine, ^{3}H-leucine and ^{35}S-methionine, 5 hr after treatment of the cells with 200 ng/ml aclacinomycin A.

Effect of anthracyclines on alanine incorporation into proteins and on cell proliferation

In order to see whether the early inhibition of amino acid incorporation into proteins is a common characteristic of anthracyclines, we compared the effect on ^{3}H-alanine incorporation into proteins and cell proliferation in logarithmically growing Friend cells of aclacinomycin A with that of adriamycin, daunomycin and two other *N*-alkylated anthracyclines, pyrromycin and marcellomycin. The substances were tested at concentrations of 50, 100 and 200 ng/ml. In this concentration range the *N*-alkylated anthracyclines were shown to be active at inhibiting cell proliferation in F4–6 cells [28].

Incorporation of ^{3}H-alanine in TCA-insoluble material was measured at 5 hr after treatment with the drugs. At 24 hr after addition of anthracyclines, survival of the cells was determined on the basis of trypan blue staining; the number of untreated cells approximately doubled during that time. The results are summarized in Table 2 and show that the *N*-alkylated anthracyclines, aclacinomycin A, pyrromycin and marcellomycin, inhibited ^{3}H-alanine incorporation into proteins in a concentration-dependent manner. Inhibition of amino acid incor-

Table 2. Effect of anthracycline antitumor antibiotics on ^{3}H-alanine incorporation into proteins and on cell proliferation in F4–6 cells

Anthracycline	Concentration (ng/ml)	^{3}H-alanine incorporation after 5 hr of treatment (pmol/mg protein/10 min)	Cell number after 24 hr of treatment (% of control)
Aclacinomycin A	—	7.60 ± 0.22	100.0
	50	5.36 ± 0.24	70.3 ± 3.3
	100	4.55 ± 0.16	57.4 ± 3.1
	200	3.39 ± 0.39	36.8 ± 4.0
Pyrromycin	—	7.26 ± 0.25	100.0
	50	5.35 ± 0.44	80.3 ± 7.2
	100	4.87 ± 0.15	76.4 ± 9.2
	200	4.20 ± 0.38	40.8 ± 14.7
Marcellomycin	—	7.79 ± 0.36	100.0
	50	5.42 ± 0.32	67.7 ± 0.5
	100	4.91 ± 0.20	56.7 ± 3.3
	200	3.47 ± 0.18	33.9 ± 0.3
Adriamycin	—	7.12 ± 0.46	100.0
	50	7.07 ± 0.37	18.0 ± 3.5
	100	5.95 ± 0.32	12.8 ± 2.3
	200	5.93 ± 0.33	5.3 ± 2.5
Daunomycin	—	7.70 ± 0.75	100.0
	50	6.20 ± 0.38	18.8 ± 4.2
	100	6.05 ± 0.62	9.7 ± 3.9
	200	5.31 ± 0.44	2.0 ± 1.7

^{3}H-Alanine incorporation into proteins was measured as described in the text. The cell number of control and anthracycline-treated cells was determined as number of trypan blue negative cells. Within 24 hr the number of untreated cells about doubled. Results represent the means of three experiments ± SE.

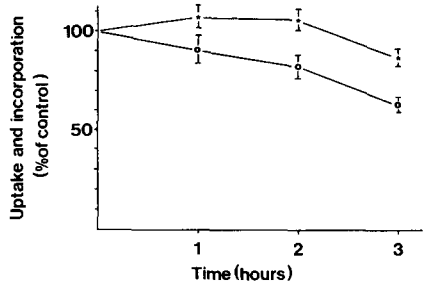

Fig. 2. Effect of actinomycin D, 1 μg/ml, on ^{3}H-alanine incorporation into proteins (○) and uptake of ^{14}C-AIB (★) in F4–6 cells. Results are expressed as percentages of controls and represent the means of three experiments ± standard error. Averages of the ^{3}H-alanine incorporation and ^{14}C-AIB uptake in control cells were 7.50 pmol/mg protein/10 min and 26.0 pmol/10^{6} cells/5 min, respectively.

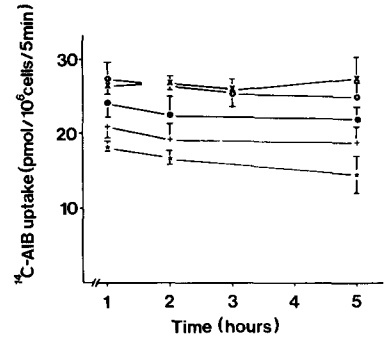

Fig. 3. Effect of aclacinomycin A, 50 ng/ml (●), 100 ng/ml (+), 200 ng/ml (★), and 100 ng/ml adriamycin (○), on the uptake of ^{14}C-AIB in F4–6 cells. ×: ^{14}C-AIB uptake in untreated cells. Results are the means of three experiments ± standard error.

poration into proteins by these substances correlated well with reduction of cell number measured after 24 hr. On the other hand, adriamycin and daunomycin inhibited ^{3}H-alanine incorporation only weakly, although these substances were highly active at inhibiting cell proliferation. These results show that the potent inhibition of amino acid incorporation into proteins by aclacinomycin A and related compounds is a biochemical characteristic of these substances differing from the mechanism(s) of action of adriamycin and daunomycin.

Effect of actinomycin D on amino acid uptake and incorporation

Aclacinomycin A is known to preferentially inhibit RNA synthesis [6, 7]. Therefore, the question arises whether the decrease of amino acid incorporation into proteins and amino acid uptake in the cells after treatment with aclacinomycin A results from the inhibition of RNA synthesis. To investigate this possibility, we studied the effect of an inhibitor of the RNA synthesis, actinomycin D, on ^{3}H-alanine-incorporation into proteins and on uptake of ^{14}C-AIB in F4–6 cells. Actinomycin D was tested at a concentration of 1 μg/ml which was found to inhibit ^{3}H-uridine incorporation into TCA-insoluble material to more than 80% immediately after addition and to cause cell death within 6–7 hours. However, as shown in Fig. 2, up to 2 hr after administration, actinomycin D did not inhibit ^{14}C-AIB uptake and only weakly inhibited protein synthesis in F4–6 cells. These results suggest that inhibition of RNA synthesis does not account for the concomitant decline of amino acid uptake and incorporation into proteins caused by aclacinomycin A (Fig. 1).

Effect of aclacinomycin A on cell-free protein synthesis in rabbit reticulocyte lysates

In order to obtain information on the effect of aclacinomycin A on protein synthesis directly in a cell free system, we studied the effect of aclacinomycin A on the translational activity of rabbit reticulocyte lysates [24, 29, 30]. Aclacinomycin A was tested at the concentrations of 100 ng/ml, 1 μg/ml and 10 μg/ml (Table 3). Cycloheximide, 10 μg/ml, and edeine, 15 μM, which inhibits specifically the initiation of protein synthesis, were used as positive controls. The results indicate that aclacinomycin A up to a concentration of 10 μg/ml is not an inhibitor of the cellular protein synthetizing system.

Concentration-dependence of the effect of aclacinomycin A on AIB uptake

Figure 1 shows a concomitant inhibition of ^{3}H-alanine incorporation into proteins and ^{14}C-AIB uptake after treatment of Friend cells with aclacinomycin A. The results in Fig. 2 and Table 3 suggest that the concomitant decrease of amino acid

Table 3. Effect of aclacinomycin A on the protein synthesis in rabbit reticulocyte lysates

Substance	Concentration	^{35}S-Methionine incorporation (fmol/20 μl lysate/20 min)	
		Determination 1	Determination 2
Control		287.3	281.6
Aclacinomycin A	100 ng/ml	305.1	309.0
	1 μg/ml	342.2	310.8
	10 μg/ml	306.8	268.7
Edeine	15 μM	41.4	42.8

Incorporation of ^{35}S-methionine into proteins in lysates from rabbit reticulocytes was determined as described in the text. Values obtained with cycloheximide (10 μg/ml) have been subtracted as background. Results are the means of triplicate determinations.

uptake and incorporation into proteins by aclacinomycin A treatment is not caused by an inhibition of RNA synthesis or a direct inhibition of cellular protein synthesis. Since a reduced amino acid uptake would also lead to an inhibition of their incorporation into proteins, we investigated whether the inhibition of ^{14}C-AIB uptake by aclacinomycin A is similarly dose-dependent as the inhibition of ^{3}H-alanine incorporation induced by the agent (Table 2). As shown in Fig. 3, ^{14}C-AIB transport into F4–6 cells is, indeed, reduced by aclacinomycin A at concentrations which also inhibit amino acid incorporation into proteins. On the other hand, ^{14}C-AIB uptake was not affected by adriamycin, 100 ng/ml. These results, too, suggest a strong, possibly causal, relationship between the effects of aclacinomycin A on amino acid transport and amino acid incorporation into proteins in F4–6 cells.

DISCUSSION

Our results demonstrate that aclacinomycin A and related anthracyclines are active at inhibiting amino acid incorporation into proteins in Friend erythroleukemia cells. Inhibition of ^{3}H-alanine incorporation by aclacinomycin A began one hour after treatment and occurred parallel to the inhibition of the uptake of the non-metabolisable model amino acid AIB in the cells.

Aclacinomycin A is known to preferentially inhibit the RNA synthesis [7, 31]. In these studies no direct inhibition of protein synthesis was observed. Our results with rabbit reticulocyte lysates, a cell-free system with high translational activity [24, 29, 30], also show that aclacinomycin A is not a direct inhibitor of cellular protein synthesis. Moreover, results of the studies with the RNA-synthesis inhibitor, actinomycin D, indicate that the concomitant inhibition of ^{3}H-alanine incorporation into proteins and AIB uptake by aclacinomycin A is not due to a reduced RNA synthesis. On the other hand, inhibition of AIB uptake with aclacinomycin A exhibited a concentration-dependence similar to that of the inhibition of the ^{3}H-alanine incorporation into proteins caused by this agent (Table 2, Fig. 3). The correlation between inhibition of AIB uptake and inhibition of ^{3}H-alanine incorporation by aclacinomycin A suggests that a reduced transport of amino acids might be responsible for the inhibition of amino acid incorporation into proteins in F4–6 cells after treatment.

The *N*-alkylated anthracycline, aclacinomycin A, inhibited amino acid uptake and incorporation into proteins at concentrations that also induce a reduction of cell proliferation. Therefore these biochemical effects could possibly contribute to the cytostatic activity of aclacinomycin A. On the other hand, however, the parent compounds, adriamycin and daunomycin, are inactive or only weakly active at inhibiting AIB uptake or ^{3}H-alanine incorporation into proteins, although these substances are highly active at inhibiting cell proliferation. Thus, the early inhibition of amino acid uptake and incorporation by *N*-alkylated anthracyclines may be indicative of mechanisms of action differing from those of adriamycin and daunomycin. In accord with this suggestion, aclacinomycin A and related *N*-alkylated anthracyclines—in contrast to the highly mutagenic and DNA-binding agents, adriamycin and daunomycin—are non-mutagenic [4, 11, 13, 20]. Possibly, inhibition of protein synthesis via reduction of amino acid uptake is also responsible for the cytostatic action of *N*-alkylated anthracyclines.

The *N*-alkylated anthracyclines inhibit amino acid incorporation into proteins at concentrations that also induce erythroid differentiation of F4–6 cells [28]. Therefore, the effect of aclacinomycin A on amino acid uptake and incorporation may be of interest also in relation to the differentiation-inducing capacity of the agent. A reduction of overall protein synthesis (by an inhibition of the polypeptide chain initiation) favours the commitment of Friend erythroleukemia cells to terminally differentiate [32, 33]. A reduced amino acid uptake could mimic such an effect, since amino acid starvation also results in a specific inhibition of the initiation phase of protein synthesis [34]. Moreover, similar to aclacinomycin A (present results), the potent differentiation-inducing agent, dimethylsulfoxide [35], also causes a concomitant decrease of amino acid uptake and incorporation into proteins in F4–6 cells [36].

Inhibition of the transport of AIB by aclacinomycin A may be indicative of an interaction of this substance with the cell membrane functions. The plasma membrane has been considered as one of the possible sites of action of anthracyclines [1, 21]. In addition, alteration of membrane functions, such as amino acid uptake, transport of nucleosides and glucose, have been implicated in the cytostatic action of alkylating antitumor substances [37, 38]. Therefore, the present results with aclacinomycin A may provide further support for the concept that changes in the membrane functions, which affect the rate of synthesis of cellular macromolecules, may be involved in the mode of action of at least certain antitumor agents.

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