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MECHANISM OF ACTION OF DIKETOCORIOLIN B

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SUMMARY

Diketocoriolin B, a microbial product with antibacterial and antitumor activity, inhibits hypotonic hemolysis while deoxycholate-induced hemolysis of rat erythrocytes is enhanced. It induces energy-independent swelling of rat liver mitochondria and reduces the stability of rat liver lysosomes.

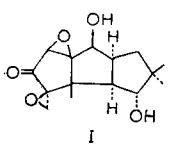
Diketocoriolin B inhibits the transport of glycine and K^+ into Yoshida sarcoma cells and causes a net loss of cellular K^+.

Diketocoriolin B inhibits (Na^+–K^+)-ATPase prepared from Yoshida sarcoma cells and various rat tissues. Other membrane enzymes, such as Mg^{2+}-ATPase, K^+-dependent phosphatase, alkaline phosphatase and 5′-nucleotidase are hardly affected. However, mitochondrial ATPase is inhibited.

The main effect of diketocoriolin B on tumor cells appears to be on the (Na^+–K^+)-ATPase system.

INTRODUCTION

As reported in other papers[1,2], the sesquiterpene compounds coriolin and coriolin B were isolated from the culture broth of *Coriolus consors*. Coriolin B showed no antitumor and antibacterial activity, but its oxidation product, diketocoriolin B, showed antibacterial activity similar to coriolin and stronger activity than coriolin against Yoshida rat sarcoma cells in tissue cultures, mouse leukemia 1210 or Ehrlich ascites tumor. The structures of coriolin (I), coriolin B(II) and diketocoriolin B (III) are as follows:



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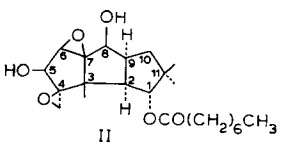

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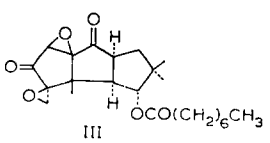

III

The mechanism of action of this type of compound has not been previously studied. In this paper, we report the mode of action of diketocoriolin B. It inhibits

(Na^+-K^+)-ATPase of the cell membrane of tumor cells, causing cessation of growth.

MATERIALS AND METHODS

Growth of Yoshida sarcoma cells

Yoshida sarcoma cells were grown *in vitro* as reported previously[3].

Experiments on hemolysis

For the determination of hemolysis, 0.8 ml of blood was taken from a rat by heart puncture and was mixed with 75 units of heparin. After centrifugation at $1500 \times g$ for 5 min, the erythrocytes were suspended in 10 ml of 154 mM NaCl–10 mM sodium phosphate buffer (pH 7.0). The effect of diketocoriolin B on hypotonic hemolysis was tested by the procedure of Seeman and Weinstein[4]. Experiments on deoxycholate-induced hemolysis were conducted by the method of Sheppard *et al.*[5].

Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared by the method of Dodge *et al.*[6] with a slight modification as follows: red blood cells from 1.5 ml of rat blood were washed twice in 2 ml of 310 mosM sodium phosphate buffer (pH 7.0); the cell paste, 0.8 ml as a whole, was poured into 15 ml of 20 mosM sodium phosphate buffer (pH 7.0)–1 mM EDTA. The ghosts were collected from the suspension by centrifugation at $20000 \times g$ for 40 min and washed twice in 15 mM Tris–HCl (pH 7.4). The final precipitate was suspended in 15 mM Tris–HCl buffer (pH 7.4) and used for the determination of enzymatic activity.

Preparation of mitochondria

Mitochondria were prepared from the liver of overnight-starved rats (Donryu strain) by the method of Stancliff[7], suspended in 0.333 M sucrose–1.33 mM EDTA·Tris (pH 7.4), and used for the swelling experiments within 5 h after preparation.

Transport studies

For transport studies, Yoshida sarcoma cells washed twice with cold phosphate-buffered saline or Krebs–Ringer phosphate were used. For experiments on [^{14}C]glycine flux, the cells were suspended in Krebs–Ringer phosphate and for the study of the $^{42}K^+$ flux, they were suspended in Krebs–Ringer phosphate supplemented with glucose (0.1%). The cells were incubated at 37 °C and intracellular radioactivity of [^{14}C]-glycine or $^{42}K^+$ was determined as follows: a 0.1 ml sample of cell suspension was withdrawn, immediately mixed with 2 ml of ice cold medium, quickly poured over a Millipore filter (25 mm diameter, 3–5 μm pore size[8], presoaked in cold Krebs–Ringer phosphate), and filtered with a negative pressure of 3 cm Hg. The cells on the filter were washed twice with 2 ml of ice-cold Krebs–Ringer phosphate, care being taken to avoid drying of the filter during filtration and washing. After drying, the radioactivity in the filter was determined.

Enzyme preparation

(Na^+-K^+)-ATPase of Yoshida sarcoma cells. (Na^+-K^+)-ATPase was prepared

from Yoshida rat sarcoma cells using deoxycholate by the method of Ahmed and Judah[9]. The deoxycholate-treated preparation was further treated with NaI by the method of Uesugi *et al.*[10]. The activity of the resulting preparation was 5–15 μmoles of ATP hydrolyzed per mg protein per h. In this preparation, the (Na^+-K^+)-ATPase: Mg^{2+}-ATPase ratio, was 5–10.

(Na^+-K^+)-ATPase of rat liver. A crude microsomal fraction was prepared from a homogenate of rat liver in 0.25 M sucrose–1 mM EDTA (pH 7.0) and treated with deoxycholate and NaI in the same way as was the enzyme from Yoshida sarcoma cells. The (Na^+-K^+)-ATPase activity of the preparation was 2–6 μmoles of ATP hydrolyzed per mg protein per h, while its (Na^+-K^+)-ATPase:Mg^{2+}-ATPase ratio was 0.5–1.5.

(Na^+-K^+)-ATPase of rat brain. A microsomal pellet obtained from a homogenate of brain in 0.25 M sucrose was suspended in 0.25 M sucrose–10 mM Tris·HCl buffer (pH 7.4). The suspension was made 1 M with KCl and 0.2% with deoxycholate, and centrifuged at $35000 \times g$ for 30 min[11]. The precipitate was treated with NaI as described above. The (Na^+-K^+)-ATPase activity of the preparation was 20 μmoles of ATP hydrolyzed per mg protein per h, while its (Na^+-K^+)-ATPase:Mg^{2+}-ATPase ratio was 20–25.

Determination of the enzyme activity

The ATPase activity was measured with appropriate concentrations of ATP (as the Tris salt) and $MgSO_4$ with or without NaCl and KCl. The enzyme activity was expressed in terms of the amount of inorganic phosphate liberated. Activity in the absence of Na^+ and K^+ ("Mg^{2+}-ATPase") was subtracted from the total activity in the presence of Na^+ and K^+ to give (Na^+-K^+)-ATPase activity. The activity of K^+-dependent phosphatase was determined by measuring the amount of *p*-nitrophenol produced from *p*-nitrophenyl phosphate. Mitochondrial ATPase activity was measured in terms of the production of inorganic phosphate from ATP in the presence of dinitrophenol. Full details of the individual assays are given in the legends to tables.

DNA, RNA or protein synthesis in Yoshida sarcoma cells

Yoshida sarcoma cells were suspended in Eagle's medium containing 10% bovine serum at a density of 10^6 cells/ml. To test the effect on DNA, RNA or protein synthesis the cells were incubated at 37 °C with [^{3}H]thymidine (0.3 μCi/ml), [^{3}H]uridine (0.5 μCi/ml) or [^{14}C]leucine (0.2 μCi/ml), respectively. Aliquots of 0.1 ml were taken and placed on disks of filter paper (Whatman 3MM, 22 mm diameter). The disks were processed by the method of Byfield *et al.*[36] to determine the acid insoluble radioactivity.

Determination of radioactivity and other analytical methods

The millipore filter carrying a ^{14}C- or ^{3}H-labeled sample was placed in a counting vial with scintillation solution (6 g PPO and 100 g naphthalene in 1 l dioxane) and the radioactivity was determined in a Beckman liquid scintillation system. A filter paper disk containing a radioactive sample was processed likewise except that a scintillation solution consisting of 5 g PPO and 0.3 g POPOP in 1 l toluene was used instead. Samples containing $^{42}K^+$ were placed in counting vials with 1 ml of water and the radioactivity was determined in the same liquid scintillation counter. No scintillation mixture was used for this determination.

The amount of liberated inorganic phosphate was determined by the method of Eible and Lands[12]. *p*-Nitrophenol liberated from *p*-nitrophenyl phosphate was determined by reading the absorbance at 405 nm. Protein concentration was determined by the method of Lowry *et al.*[13] in which bovine serum albumin was used as the standard.

Chemicals

The sodium salt of ATP (Sigma Chemical Co.) was converted to its Tris salt by passing through a Dowex 50 column in the Tris form. [^{3}H]Thymidine, [^{3}H]uridine, [^{14}C]glycine, [^{14}C]leucine and ^{42}KCl were purchased from Daiichi Pure Chemical Co. Coriolin and diketocoriolin B were dissolved in methanol (or ethanol) at high concentrations and added to reaction mixtures in a small volume so that the final concentration of the alcohol did not exceed 0.25%. At a concentration below 0.25%, alcohol did not affect the systems employed in this report.

RESULTS

Effect of coriolins on the growth of Yoshida sarcoma cells in vitro

The effect of diketocoriolin B, coriolin and coriolin B on the growth of Yoshida sarcoma cells is shown in Fig. 1. The ID_{50} values of coriolin and diketocoriolin B were 4.0 μg/ml and 0.75 μg/ml, respectively, while coriolin B was inactive at the concentra-

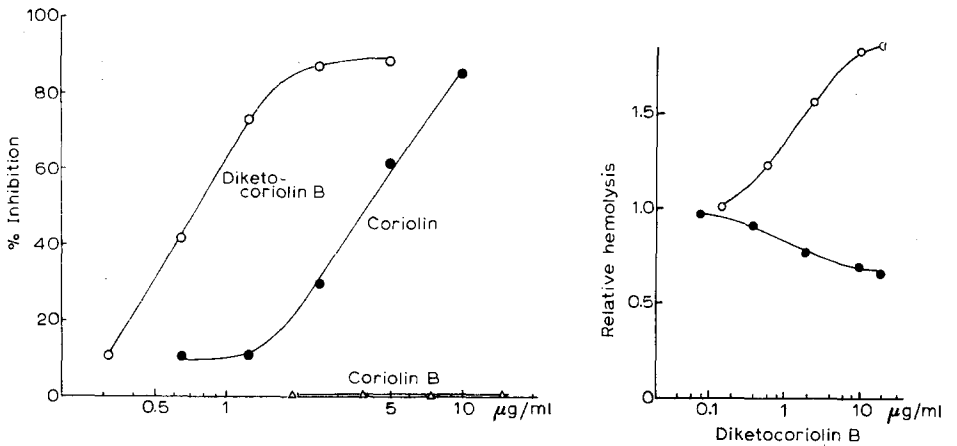

Fig. 1. Effect of coriolin and its derivatives on the growth *in vitro* of Yoshida sarcoma cells. Yoshida sarcoma cells, suspended in lactalbumin medium at a density of 10^5 cells/ml, were inoculated into tubes and various levels of coriolin or its derivatives were added. The cells were grown for 48 h at 37°C and the cell number was determined. The results were expressed as percent inhibition of growth.

Fig. 2. Effect of diketocoriolin B on hemolysis of rat erythrocyte. For hypotonic hemolysis, 68.5 mM NaCl–10 mM sodium phosphate buffer (pH 7.0) was employed, and the deoxycholate hemolysis was induced by 0.02% sodium deoxycholate in the isotonic NaCl solution (154 mM NaCl–10 mM sodium phosphate buffer (pH 7.0).) The concentration of the erythrocytes was $1.6 \cdot 10^7$ cells/ml. Hemolysis was determined by measuring the absorbance of released hemoglobin at 543 nm after centrifugation at 1500 × *g*. The ordinate represents the extent of hemolysis relative to that of the control which received no drug. A relative hemolysis of 1.0 indicates an absolute degree of hemolysis of around 50%. ○, hypotonic hemolysis; ●, deoxycholate hemolysis.

tions tested. These results suggest the importance of the carbonyl on C-5 for activity. Based on this observation, the details of the action were studied with diketocoriolin B. Morphological changes of cells after exposure to diketocoriolin B were observed microscopically: cells were swollen and formed protrusions 20 min after the addition of diketocoriolin B to the cell cultures. This observation suggested that diketocoriolin B could modify the function of the cell membrane, leading to a disturbance in osmotic equilibrium. To confirm this possibility, the effect of diketocoriolin B on hemolysis, mitochondrial swelling and stability of lysosomes was examined. These phenomena are thought to occur following changes in the structure of the membrane.

Effect of diketocoriolin B on hemolysis

Diketocoriolin B alone was not hemolytic to rat erythrocytes, but it inhibited or stimulated the hemolysis induced by hypotonicity or by addition of deoxycholate, respectively, as shown in Fig. 2. In connection with this phenomenon, the studies by Seeman *et al.*[14] are pertinent. A number of compounds, including tranquilizers and local anesthetics, inhibited hypotonic hemolysis, suggesting that these compounds were taken into the lipid layer of the erythrocyte membrane and utilized for the expansion of the cell surface. On the other hand, polyene antibiotics[15] or phosphatidylcholine[16], which induce hemolysis, are thought to react with the cholesterol of the membrane and to distort the structure of the membrane. Disturbance of the cation gradient due to inhibition of (Na^+-K^+)-ATPase, which will be described later, may be another cause of hemolysis.

Effect on mitochondrial swelling

Isolated mitochondria have been reported to undergo conformational changes in response to the changes in the metabolic state, such as those caused by respiratory inhibitors or uncouplers, accompanied by the translocation of ions in some cases. The conformational changes lead to swelling or contraction[17]. On the other hand, these changes are also caused by compounds affecting ion transport. Antibiotics which induce cation translocation, such as valinomycin[18,19] and gramicidin[20,21], are known to cause these changes. When various amounts of diketocoriolin B were added to the suspension of rat liver mitochondria in 0.333 M sucrose solution without any exogenous energy source, rapid swelling of mitochondria was shown by a decrease of the absorbance at 520 nm. As shown in Fig. 3, the swelling became more evident when the concentration of diketocoriolin B was increased. It is known that inorganic phosphate causes mitochondrial swelling in a medium containing an appropriate energy source, such as succinate. Diketocoriolin B was found to work independently from inorganic phosphate under these conditions, since the effects appeared additive. These observations suggest that diketocoriolin B directly interacts with some component of the mitochondrial membrane, resulting in a modification of its structure which leads to the swelling.

Effect of diketocoriolin B on the stability of lysosomes

The lysosomal membrane has several characteristics common to those of the cell membrane[22,23]. It is known that some polyene antibiotics, such as filipin and amphotericin B, cause hemolysis and also make the lysosomal membrane unstable[24]. We examined the effect of diketocoriolin B on a crude preparation of the lysosomes

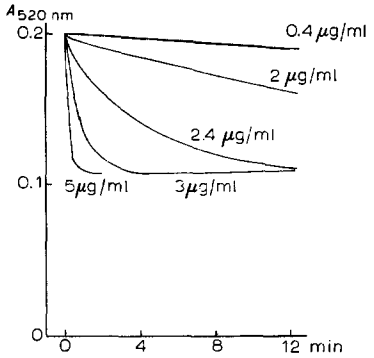

Fig. 3. Induction of mitochondrial swelling by diketocoriolin B. The reaction mixture contained, in a total volume of 0.5 ml, 0.333 M sucrose, 1.33 mM Tris–EDTA (pH 7.4) and mitochondria (35 μg protein). At zero time, an indicated amount of diketocoriolin B was added and the change in absorbance at 520 nm of the mixture was followed at 25 °C.

from rat liver. The lysosomes were incubated in 0.28 M sucrose solution containing diketocoriolin B at various concentrations, and the acid phosphatase and β-glucuronidase activities remaining in the particulate fraction of the mixture were determined later. Diketocoriolin B, at a concentration of 16 or 64 μg/ml, accelerated the release of these enzymes from the lysosomes, indicating that it made the lysosomes unstable. It should be noticed, however, that a larger amount of diketocoriolin B was required for making the lysosomes unstable than for induction of mitochondrial swelling.

The experimental data described above suggest that diketocoriolin B interacts with these membrane structures. Filipin, a polyenic antibiotic, is known[15,24–26] to interact with cholesterol, which is abundant in the erythrocytic and lysosomal membranes but which is poor in the mitochondrial membrane. The mode of action of diketocoriolin B was apparently different from that of polyenic antibiotics, because diketocoriolin B was more effective in the mitochondrial system than in the lysosomal system and by itself it did not cause hemolysis. These observations indicate that diketocoriolin B interacts with some membrane component other than cholesterol.

The effect of diketocoriolin B on glycine and K^+ transport

The transport of ions and amino acids is an important function of the cell membrane. Since diketocoriolin B was thought to affect the membrane structure, we wondered if it affects such processes in the Yoshida sarcoma cells.

Glycine transport. As shown in Fig. 4a, [^{14}C]glycine uptake by Yoshida sarcoma cells was markedly inhibited. Under the conditions of this experiment, only 2% of the total radioactivity taken up into the whole cell was found to be localized in the acid insoluble fraction. Fig. 4b shows the effect on the retention of [^{14}C]glycine in the intracellular amino acid pool. As is apparent in the figure, it accelerated the efflux of [^{14}C]glycine immediately after the initiation of the incubation.

K^+ transport. Yoshida sarcoma cells were suspended in Krebs–Ringer phosphate medium supplement with glucose and ^{42}KCl, and the influx of $^{42}K^+$ into the cells was examined. As shown in Fig. 5a, $^{42}K^+$ influx was markedly suppressed by diketocoriolin B immediately after the initiation of the incubation. In contrast, as will

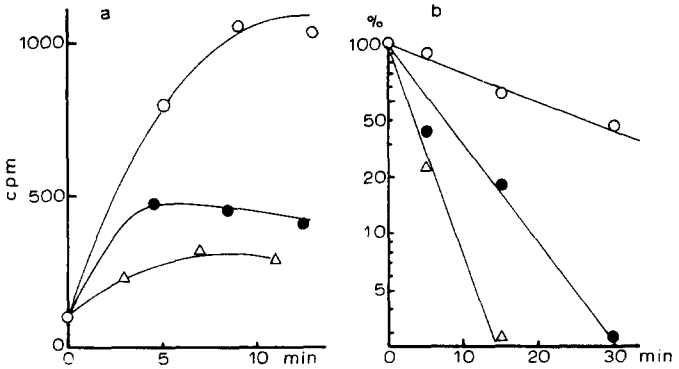

Fig. 4. Effect of diketocoriolin B on the transport of glycine. (a) Effect on influx. Yoshida sarcoma cells were suspended in Krebs–Ringer phosphate at a density of $3 \cdot 10^6$ cells/ml and [^{14}C]glycine was added to make finally 1 mM and 0.2 μCi/ml. The desired amount of diketocoriolin B was added to the suspension and incubation was initiated at 37 °C. At the time indicated, a 0.1 ml sample was taken and the incorporated activity was determined. (b) Effect on efflux. Yoshida sarcoma cells were incubated for 10 min as described above. Cells were chilled in an ice bath and collected by centrifugation (1000 $\times g$, 1 min). After washing twice with cold Krebs–Ringer phosphate, the cells were suspended in cold Krebs-Ringer phosphate at a density of $1.1 \cdot 10^6$ cells/ml. An indicated amount of diketocoriolin B was added to the suspension and the mixture was incubated at 37 °C. 0.1 ml samples were withdrawn at intervals and the intracellular radioactivity was determined. ○, control; ●, diketocoriolin B 2.5 μg/ml; △, diketocoriolin B 10 μg/ml.

be described, the efflux of $^{42}K^+$ was accelerated. Yoshida sarcoma cells were grown for 16 h in a lactalbumin hydrolysate medium containing ^{42}KCl, washed and subsequently suspended in fresh medium including unlabeled KCl. As shown in Fig. 5b, the efflux of $^{42}K^+$ into the medium was markedly enhanced by diketocoriolin B.

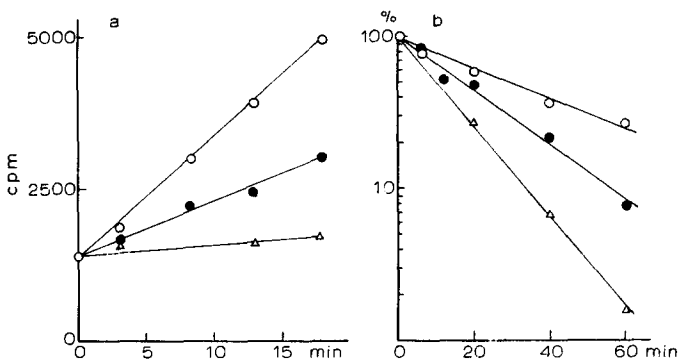

Fig. 5. Effect of diketocoriolin B on the transport of K^+. (a) Effect on influx. Yoshida sarcoma cells were suspended in Krebs–Ringer phosphate, which contained ^{42}KCl at 2.4 μCi/ml and was supplemented with 0.1% glucose, at a cell density of $2 \cdot 10^6$ cells/ml. The influx of $^{42}K^+$ was determined as described in the legend to Fig. 4. (b) Yoshida sarcoma cells were suspended in lactalbumin–Tyrode medium supplemented with 10% bovine serum and 0.5 μCi/ml of ^{42}KCl ($1 \cdot 10^6$ cells/ml) and incubated for 16 h at 37 °C. The suspension was chilled in an ice bath and the cells were collected by centrifugation (1000 $\times g$, 1 min), washed twice with the fresh medium which had no ^{42}KCl, and suspended in the same medium ($1 \cdot 10^6$ cells/ml). Otherwise, the experiments were conducted as in the study of glycine efflux. ○, control; ●, diketocoriolin B 2.5 μg/ml; △, diketocoriolin B 10 μg/ml.

In mammalian cells, the transport of amino acids is closely associated with Na^+ transport across the membrane. The rate of transport of certain amino acids is dependent on the extracellular concentration of Na^+ or on the difference between intra- and extracellular concentrations of Na^+ (ref. 27). On the other hand, the transport of Na^+ or K^+ across the membrane of mammalian cells is thought to be mediated by (Na^+–K^+)-ATPase localized in the plasma membrane[28]. Ouabain, a specific inhibitor of this enzyme, blocks the transport of amino acids[29]. In view of our previous observations and of these facts, we assumed that diketocoriolin B might be another inhibitor of (Na^+–K^+)-ATPase. It should be remembered that the stimulation of K^+ efflux (or exchange), shown in Fig. 5b, is not solely caused by inhibition of (Na^+–K^+)-ATPase. Other mechanisms, such as induction of the "potassium channel" proposed for the action of phalloidin[30], should also be taken into consideration.

Effect of diketocoriolin B on the (Na^+–K^+)-ATPase of Yoshida sarcoma cells

A particulate fraction obtained from Yoshida rat sarcoma cells by treatment with deoxycholate and NaI was used as the enzyme. The enzymatic activity of this preparation required Mg^{2+} and was activated several-fold by adding Na^+ and K^+. This Na^+- and K^+-dependent activity was sensitive to ouabain, and accordingly was regarded as a (Na^+–K^+)-ATPase. As shown in Table I, diketocoriolin B inhibited (Na^+–K^+)-ATPase but not Mg^{2+}-ATPase. It inhibited (Na^+–K^+)-ATPase from other organs as well but did not inhibit Mg^{2+}-ATPase in any case, as shown in Table II.

TABLE I

EFFECT OF DIKETOCORIOLIN B ON THE (Na^+–K^+)-ATPase OF YOSHIDA SARCOMA CELLS

The reaction mixture contained, in a total volume of 0.1 ml, 2 mM ATP (Tris salt), 3 mM $MgSO_4$, 10 mM Tris–HCl buffer (pH 7.4) and the enzyme preparation (20 μg protein) with or without 120 mM NaCl and 10 mM KCl. It was incubated for 15 min at 37 °C and then the reaction was terminated by the addition of ice-cold 10% $HClO_4$. After diluting to 1.0 ml or 1.5 ml, the liberated P_i was determined as described in Materials and Methods. Activity in the absence of Na^+ and K^+ (Mg^{2+}-ATPase) was subtracted from the total activity in the presence of Na^+ and K^+ to give the (Na^+–K^+)-ATPase activity.

	Concn of inhibitors	*P_i liberated (nmoles/assay tube)*			*% Inhibition of (Na^+–K^+)-ATPase*
		In presence of		*(Na^+–K^+)-ATPase activity*	
		Mg^{2+}	*Mg^{2+},Na^+,K^+*		
Control	0	9.1	35.2	26.1	–
Diketocoriolin B	1	9.1	32.7	23.6	9.6
(μg/ml)	2	9.6	27.9	18.3	29.9
	4	8.5	18.7	10.2	61.0
Control	0	11.2	36.4	25.2	–
Ouabain (M)	10^{-6}	10.9	33.6	22.7	9.9
	10^{-5}	14.9	27.7	12.8	49.2
	10^{-4}	33.1	41.1	8.0	68.3

TABLE II

EFFECT OF DIKETOCORIOLIN B ON THE (Na^+–K^+)-ATPase OF VARIOUS TISSUES

ATPase activity of the preparations from rat liver (10 μg protein) or rat brain (14 μg protein) was determined as described in the legend to Table I. ATPase activity of the erythrocyte ghost (14 μg protein) was determined by a similar procedure but with incubation for 30 min with a mixture of 115 mM NaCl and 20 mM KCl for total ATPase activity.

Source of enzyme	*Diketocoriolin B (μg/ml)*	*P_i liberated (nmoles/assay tube)*			*% Inhibition of (Na^+–K^+)-ATPase*
		In presence of		*(Na^+–K^+)-ATPase activity*	
		Mg^{2+}	*Mg^{2+},Na^+,K^+*		
Brain	0	1.9	38.8	36.9	–
	2	1.9	24.1	22.2	39.9
	4	1.9	19.5	17.6	52.3
	8	1.9	10.9	9.0	75.6
Liver	0	16.2	24.5	8.3	–
	2	18.4	27.0	8.6	0
	10	18.5	21.3	2.8	66.3
Erythrocyte ghost	0	4.9	13.2	8.3	–
	0.4	7.4	12.0	4.6	44.6
	2	5.8	9.2	3.4	59.0
	10	4.8	5.0	0.2	97.5

Effect of diketocoriolin B on the K^+-dependent phosphatase activity of (Na^+–K^+)-ATPase

(Na^+–K^+)-ATPase involves a K^+-dependent phosphatase activity which is thought to be one of a series of reaction steps catalyzed by this enzyme[31]. As Table III shows, diketocoriolin B did not inhibit the phosphatase activity with *p*-nitrophenyl phosphate. The lack of inhibition of K^+-dependent phosphatase activity, as against the rather strong inhibition of (Na^+–K^+)-ATPase activity, suggests that the latter is involved with the phosphorylation step. However, this is unlikely because a kinetic study indicated that the latter inhibition was competitive with respect to ATP (Kunimoto, T. and Umezawa, H., unpublished).

Effect of diketocoriolin B on other enzymes which are localized in the plasma membrane

To determine the effect of diketocoriolin B on other enzymes which are also localized in the plasma membrane, the effects on the alkaline phosphatase and 5′-nucleotidase[32] of the rat erythrocyte ghost were examined. The substrates for the former and latter were *p*-nitrophenyl phosphate and 5′-AMP, respectively. Diketocoriolin B did not inhibit either enzyme.

These observations strongly support the conclusion that diketocoriolin B is a specific inhibitor of (Na^+–K^+)-ATPase among the enzymes localized in the plasma membrane.

TABLE III

EFFECT OF DIKETOCORIOLIN *B* ON K^+-DEPENDENT PHOSPHATASES

The reaction mixture contained 2 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$, 10 mM KCl, 50 mM Tris–HCl buffer (pH 7.4) and the enzyme preparations (about 30 μg protein) in a total volume of 0.1 ml. The mixture was incubated at 37 °C for 30 or 50 min for determining the activity of the enzyme preparation from rat brain or erythrocyte ghost, respectively, and the reaction was stopped by adding 0.4 ml of 0.2 M Na_2CO_3. The absorbance of the resulting solution was read at 405 nm. Activity in the absence of K^+ was measured concurrently, and was substracted from the total activity to give the K^+-dependent activity.

Source of enzyme	*Diketocoriolin B* (μg/ml)	*p-Nitrophenol liberated* (*nmoles/assay tube*)	
		Total activity	*K^+-dependent activity*
Brain	0	20.4	8.8
	0.4	20.6	9.2
	2	22.0	10.9
	10	23.1	10.1
Erythrocyte ghost	0	16.5	3.0
	0.4	17.5	4.0
	2	17.5	3.0
	10	15.5	2.8

Effect of diketocoriolin B on mitochondrial functions

As stated previously, diketocoriolin B induces mitochondrial swelling, suggesting that it possibly affects mitochondrial functions. In the presence of an uncoupler such as 2,4-dinitrophenol, mitochondria hydrolyze ATP to yield ADP and phosphate.

TABLE IV

EFFECT OF DIKETOCORIOLIN B ON MITOCHONDRIAL ATPase

The reaction mixture contained, in 0.1 ml, 2 mM ATP, 80 mM KCl, 10 mM Tris–HCl buffer (pH 7.4), 0.05 mM 2,4-dinitrophenol and the mitochondrial preparation (30 μg protein). The incubation was carried out at 37 °C for 15 min and the reaction was terminated by the addition of 0.1 ml of cold 10% $HClO_4$. The released P_i was determined as described in Materials and Methods. Activity in the absence of added 2,4-dinitrophenol was measured concurrently.

Diketocoriolin B (μg/ml)	*P_i liberated* (μmoles/*mg protein per h*)			*% of control*
	−Dinitrophenol	*+Dinitrophenol*	*Dinitrophenol-stimulated activity*	
0	3.6	9.0	5.4	100
0.4	3.2	6.7	3.5	65
2	2.4	3.4	1.0	19
10	2.4	3.1	0.7	13

This reaction is thought to be the reverse of the ATP generating reaction involved in oxidative phosphorylation. As shown in Table IV, diketocoriolin B counteracted dinitrophenol by inhibiting this ATPase activity.

Effect of diketocoriolin B on cellular macromolecular synthesis

Cellular DNA, RNA and protein syntheses were examined by determining the incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{14}]leucine into the acid-insoluble fraction, respectively. As shown in Fig. 6, protein synthesis was rapidly and most severely inhibited while DNA and RNA synthesis were incompletely inhibited. The discrimination was especially obvious at lower concentrations of diketocoriolin B. This discrimination was thought to be due to the fact that leucine is taken up into the cell through an active transport mechanism closely linked to (Na^+–K^+)-ATPase, a target of diketocoriolin B, while thymidine and uridine are incorporated through a facilitated diffusion mechanism[33,34].

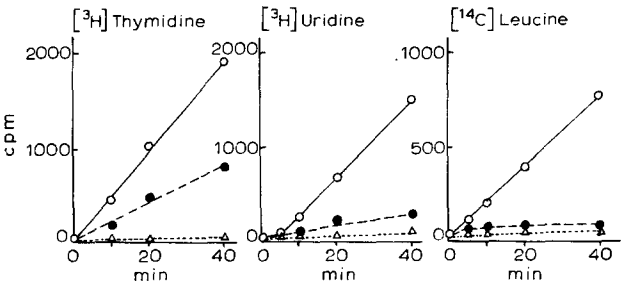

Fig. 6. Effect of diketocoriolin B on macromolecular synthesis in Yoshida sarcoma cells. The incorporation of [^{3}H]thymidine, [^{3}H]uridine or [^{14}]leucine into the acid-insoluble fraction was determined as described in Materials and Methods, ○, control; ●, diketocoriolin B 2.5 μg/ml; △, diketocoriolin B 10 μg/ml.

DISCUSSION

The (Na^+–K^+)-ATPase of the plasma membrane and the dinitrophenol-stimulated ATPase of the mitochondrion were found to be specific sites of action of diketocoriolin B. Emmelot and Bos[35] have reported that Mg^{2+}-ATPase, (Na^+–K^+)-ATPase, alkaline phosphatase and K^+-dependent phosphatase were all inactivated by removal of phospholipid, but 5′-nucleotidase was not. However, the specific inhibition of (Na^+–K^+)-ATPase by diketocoriolin B cannot be accounted for simply by a possible interaction of this compound with phospholipids. Dissociation of a particular protein–phospholipid complex by diketocoriolin B might be a plausible model, as will be reported in our next paper (Kunimoto, T. and Umezawa, H., in preparation).

(Na^+–K^+)-ATPase controls the balance of Na^+ and K^+ concentrations across the cell membrane[28], which regulates a number of biological events; the transport of amino acids, sugars[27] and biologically active substances such as noradrenalin[37], protein biosynthesis[38], glycolysis[39], respiration[40], and possibly cell division[41]. It is of great interest that diketocoriolin B, a specific inhibitor of (Na^+–K^+)-ATPase, is selectively toxic to tumor cells, especially leukemia cells.

Only a few antitumor substances, such as oligomycin and related macrolide antibiotics[42–44] and hellebrigenin[45], a steroid from the plant *Bersama abyssinica*,

have been reported to inhibit this enzyme. Compared with these antitumor substances, diketocoriolin B is unique in view of its structure and biochemical activity. The mode of action of diketocoriolin B resembles that of oligomycin: both antibiotics inhibit (Na^+–K^+)-ATPase and mitochondrial ATPase but not K^+-dependent phosphatase. However, there are distinct differences between them in that diketocoriolin B by itself causes mitochondrial swelling and does not inhibit but rather stimulates the respiration of cells as well as of mitochondria*. Hellebrigenin, another inhibitor of the (Na^+–K^+)-ATPase of the plasma membrane, is similar in structure to ouabain. As will be reported, the inhibition of (Na^+–K^+)-ATPase by diketocoriolin B is reversed by phosphatidylserine. It is known that some organic solvents, phenethyl alcohol, anesthetics, polyene antibiotics and some peptide antibiotics react with various membrane systems. However, no membrane enzyme has been identified yet as their target except (Na^+–K^+)-ATPase, which was reported very recently to be inhibited by the antibiotic Dio 9 (ref. 46).

Diketocoriolin B might provide new insights concerning the function of the cell membrane, especially the function and the regulation of the (Na^+–K^+)-ATPase of the plasma membrane.

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