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TRANSPORT-DEPENDENT MEMBRANE DAMAGE AND THE IRREVERSIBLE INACTIVATION OF NUCLEOSIDE TRANSPORT BY SHOWDOMYCIN

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Abstract—The initial rate and maximal level of uptake of the nucleoside antibiotic, showdomycin, by L1210 cells were concentration dependent. These were inhibited by both adenosine and 6-[(2-hydroxy-5-nitrobenzyl)thio]purine-9-β-D-ribofuranoside (HNBMPR), a potent, specific nucleoside transport inhibitor. When showdomycin accumulation reached 1–2 nmoles/10^6 cells, its association with the cells decreased rapidly and acid-insoluble material containing reacted antibiotic was released. Under these conditions, severe plasma membrane damage occurred, as indicated by extensive cell lysis and trypan blue staining. This damage was inhibited by HNBMPR, suggesting that the attack on membrane integrity took place from the cytoplasmic side. Treatment of cells with showdomycin reduced both its own uptake as well as that of adenosine and the binding of [^{3}H]nitrobenzylthioinosine. The magnitude of binding of this specific nucleoside transport inhibitor was many orders less than the uptake of showdomycin, indicating that considerable amounts of antibiotic can enter the cells prior to its inactivation of the transport system.

Showdomycin is a C-nucleoside antibiotic whose activity is due to the alkylating property of its maleimide moiety [1, 2]. It is a competitive inhibitor of nucleoside transport in *Escherichia coli* [3, 4] and slowly inactivates the carrier in rabbit lung macrophages [5] and L1210 murine leukemia cells [6]. The antibiotic was twice as toxic to L1210 cells *in vitro* than it was to bone marrow progenitor cells of the granulocyte–macrophage series [6, 7]. This differential was not observed with maleimide and was dependent upon transport via the nucleoside carrier [6]. Cytidine protected the bone marrow cells preferentially and increased the differential toxicity [7], forming the basis of a chemotherapeutic regimen (Y. Uehara, J. M. Fisher and M. Rabinovitz, manuscript in preparation). Such transport-dependent cytotoxicity prompted an investigation of the relationship between carrier-dependent uptake of showdomycin and the interaction of the antibiotic with cellular constituents.

MATERIALS AND METHODS

Materials

Bovine serum albumin was obtained as serum fraction V from Miles Laboratories, Elkhart, IN. Fetal calf serum was purchased from Flow Laboratories, Rockville, MD. RPMI 1630 medium and Dulbecco's phosphate buffered saline were supplied by the NIH Media Unit. 6-[(2-Hydroxy-5-nitrobenzyl)thio]purine-9-β-D-ribofuranoside (HNBMPR) was obtained from CalBiochem, San Diego, CA. Adenosine was obtained from the Sigma Chemical Co., St. Louis, MO. Showdomycin was provided by Dr. John Douros, of the Natural Products Branch, DTP, NCI, and was prepared as a stock solution of 20–40 mM in distilled water, immediately before use.

[1-^{14}C]Showdomycin (50 mCi/mmole) was obtained from Research Products International, Elk Grove Village, IL. It was purified by thin-layer chromatography on cellulose by development with *n*-butanol–acetic acid–water (50:25:25, by vol.), dissolved in water and stored frozen. It was stable as as a frozen solution for the period during which the experiments were performed. [^{3}H]Nitrobenzylthioinosine (NBMPR) was obtained from Moravek Biochemicals, City of Industry, CA as a solution of 20 Ci/mmole in methanol. [2,8-^{3}H]-Adenosine (36.7 Ci/mmole) was purchased from the New England Nuclear Corp., Boston, MA. Trypan blue (0.4%) in normal saline was obtained from the Grand Island Biological Co., Grand Island, NY. The silicone oil, Versilube F-50 (sp. g. 1.045 at 25°, viscosity 70 centistokes at 25°), was obtained from the Harwick Chemical Corp., Cambridge, MA.

Methods

Showdomycin and adenosine uptake. L1210 cells were grown to the logarithmic phase in RPMI 1630 medium, supplemented with 16% heat-inactivated fetal calf serum. They were harvested by centrifugation at 300 *g* for 5 min and washed twice with medium composed of Dulbecco's phosphate buffered saline containing 0.1% glucose (pH 7.4) except where otherwise indicated. The cells were then suspended at a concentration of 10^6 cells/ml, brought to 37°, and [^{14}C]showdomycin or [^{3}H]adenosine was added and incubation continued as indicated in individual experiments. Aliquots (200 μl) of the incubation mixture were layered on

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Versilube F-50 silicone oil in a microcentrifuge tube and uptake was terminated by centrifugation of the cells through the oil at 12,000 *g* for 1 min in an Eppendorf microcentrifuge. Tips containing the cell pellet were cut off, mixed with liquid scintillation fluor, and counted on a Beckman liquid scintillation counter. Uptake estimates were performed in duplicate.

Nitrobenzylmercaptopurine riboside (NBMPR) binding to cells. Cells were prepared as described above and binding assays were initiated by addition of [^{3}H]NBMPR (20 Ci/mmole, 0.2 μCi/ml) and terminated by centrifuging 200 μl aliquots of the incubation mixture through silicone oil at 12,000 *g* for 1 min. Tips containing the cell pellet were cut off and assayed for radioactivity as described above.

Determination of showdomycin reactivity with acid-insoluble material. The extent of the reaction between showdomycin and trichloroacetic acid (TCA)-insoluble material released from cells was determined by the method of Mans and Novelli [8] with minor modifications. Samples (50 μl) of each supernatant fraction left above the silicone after the cells were pelleted were absorbed onto filter paper disks (Whatman 3 mm, 2.4 cm dia.). These were immersed in ice-cold 10% TCA, 5 ml or more per disk, for 30 min. They were then washed in fresh cold TCA for an additional 30 min and then either heated at 90° for 15 min in 5% TCA or washed in cold TCA for the same period. They were then washed again in 5% TCA, incubated in a 1:1 ether–ethanol mixture for 5 min at 37°, and suspended in ether at room temperature. The disks were dried and incorporated radioactivity was estimated in a liquid scintillation counter.

Cell lysis and trypan blue exclusion. Cells were incubated with showdomycin with or without transport inhibitors in medium consisting of Dulbecco's phosphate buffered saline and 0.1% glucose. An aliquot of the suspension, 0.9 ml containing 0.9×10^6 cells, was mixed thoroughly with 0.1 ml of a trypan blue solution. Cell lysis and drug uptake were estimated after 5 min in a hemocytometer.

RESULTS

Uptake and release of showdomycin and associated cell damage

The rate of uptake of showdomycin was concentration dependent (Fig. 1). At concentrations up to 5 μM, maximum accumulation occurred after 30 min of incubation and remained at that level for an additional 30 min. At higher concentrations, however, maximum accumulation was reached within 15 min and then the showdomycin label associated with the cells decreased rapidly. The loss of accumulated showdomycin was due to its release into the medium, and a substantial portion was protein bound (Fig. 2). Under these conditions cells were lysed or stained with trypan blue, indicating extensive membrane damage (Table 1).

Inhibition of showdomycin uptake, release and associated cell damage by adenosine and HNBMPR

Showdomycin uptake was inhibited by adenosine and HNBMPR, a potent nucleoside transport inhibitor [9], indicating that the antibiotic was transported via the nucleoside transport system (Fig. 3). This nucleoside transport inhibitor reduced the initial rate of showdomycin uptake to 10 percent of control and the maximum accumulation to 50 percent (Fig. 4). Under these conditions showdomycin release was blocked (Fig. 4) and associated cell damage retarded (Table 2).

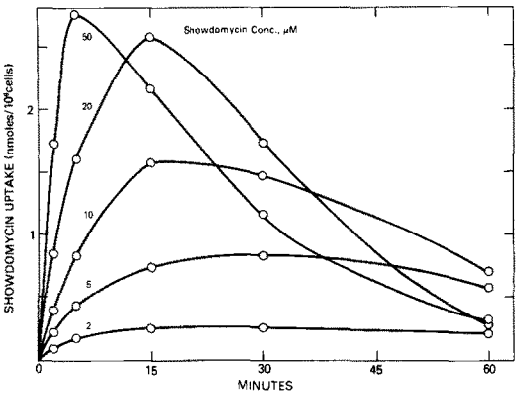

Fig. 1. Showdomycin uptake by L1210 cells. L1210 cells (10^6 cells/ml) were incubated at 37° with the indicated concentrations of [^{14}C]showdomycin. Duplicate 200 μl aliquots of the incubation mixture were then removed at various times, and uptake was terminated by centrifugation of the cells through silicone oil at 12,000 *g*. Radioactivity associated with the cell fraction was estimated as described in Materials and Methods.

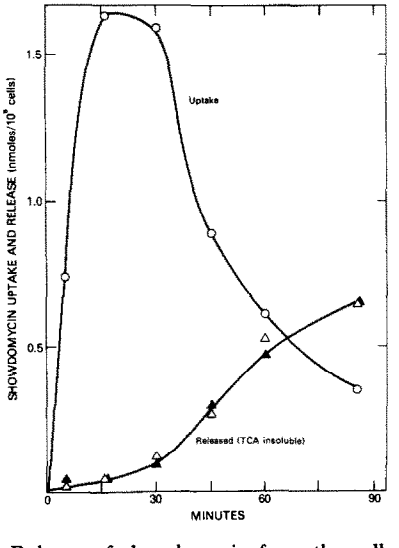

Fig. 2. Release of showdomycin from the cells as acid-insoluble material. [^{14}C]Showdomycin was added to L1210 cells (10^6 cells/ml) at a concentration of 10 μM, and at indicated times 200 μl aliquots were removed and uptake was terminated by centrifugation of the cells through silicone oil. Tips containing the cell pellet were prepared for estimation of showdomycin content (○) and aliquots of the incubation medium above the oil were prepared for estimation of released cold (△) and hot (▲) acid-insoluble material as indicated in Materials and Methods.

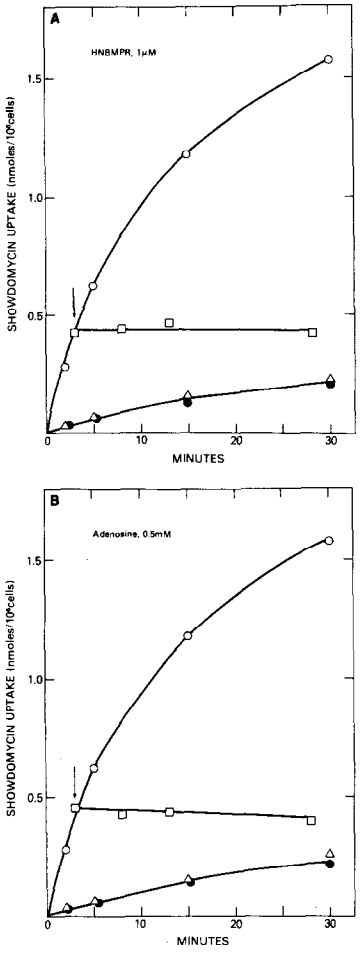

Fig. 3. HNBMPR and adenosine inhibition of showdomycin uptake. HNBMPR (panel A) or adenosine (panel B) was added at the indicated final concentration either 15 min before (△), simultaneously with (●), or 3 min after (□) the addition of [^{14}C]showdomycin (10 μM). Open circles (○) indicate control incubation in the absence of inhibitor. Showdomycin uptake was estimated as described in Materials and Methods.

Table 1. Plasma membrane damage by showdomycin*

Showdomycin concentration (μM)	Cells		
	Lysed	Stained	Intact
	(Percent of initial inoculum)		
0	0	1	99
10	0	4	96
20	13	25	62
50	38	45	17

* L1210 cells, at a concentration of 10^6 cells/ml, were incubated with the indicated concentrations of showdomycin for 30 min at 37° and then examined as described in Materials and Methods.

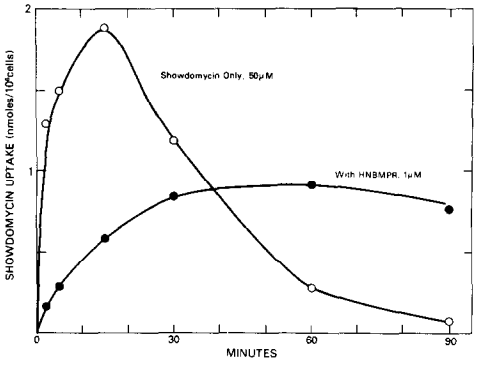

Fig. 4. Inhibitory effect of HNBMPR on showdomycin uptake, release, and cytotoxicity. L1210 cells (10^6 cells/ml) were incubated with [^{14}C]showdomycin (50 μM) with or without HNBMPR (1 μM), and at indicated times aliquots of the incubation medium were removed and assayed for (a) showdomycin uptake and (b) cell number and trypan blue exclusion as shown in Table 2.

Table 2. Hydroxynitrobenzylmercaptopurine riboside (HNBMPR) inhibition of plasma membrane damage by showdomycin*

Incubation time (min)	Showdomycin (μM)	HNBMPR (μM)	Cells		
			Lysed	Stained	Intact
			(Percent of initial inoculum)		
15	0	0	0	1	99
	50	0	39	24	37
	50	1	0	2	98
30	0	0	0	1	99
	50	0	46	38	16
	50	1	10	3	87
60	0	0	6	2	92
	50	0	42	49	9
	50	1	6	29	65

* At indicated times, cells incubated as described in the legend of Fig. 4 were examined for lysis and trypan blue exclusion.

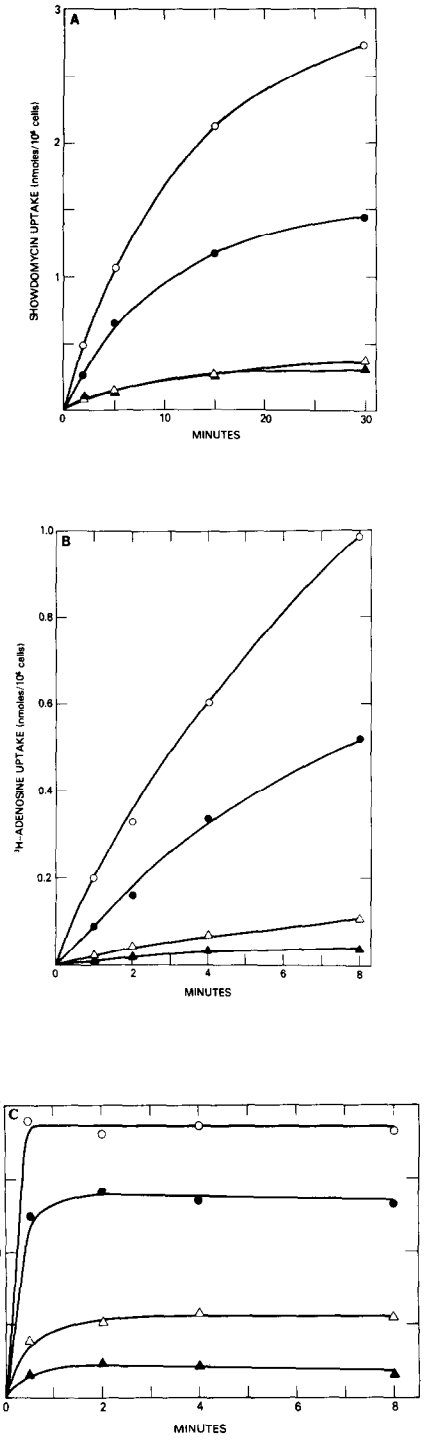

Fig. 5. Inactivation of showdomycin and adenosine uptake activities and HNBMPR binding in showdomycin-pretreated cells. L1210 cells (10^6 cells/ml) were incubated without showdomycin (○) or with 10 μM (●), 20 μM (△) or 50 μM (▲) showdomycin for 30 min at 37°. The cells were then washed twice with medium composed of Dulbecco's phosphate buffered saline (pH 7.4) containing bovine serum albumin (0.1 mM) and glucose (0.1%), resuspended at 10^6 cells/ml, and assayed for (A) [^{14}C]showdomycin uptake, (B) [^{3}H]adenosine uptake, and (C) [^{3}H]NBMPR binding. All labeled substrates were at a 10 μM concentration.

Irreversible inactivation of the nucleoside transport system by showdomycin

Cells incubated with showdomycin and then washed free of drug had a defective nucleoside transport system. This was documented with regard to showdomycin uptake (Fig. 5A), adenosine uptake (Fig. 5B), and nitrobenzylthioinosine (NBMPR) binding (Fig. 5C). The latter compound binds specifically to the nucleoside transport carrier [10]. If cells were first incubated with adenosine or HNBMPR and washed, their uptake of showdomycin was not reduced (Fig. 6). These results indicate that showdomycin caused irreversible damage to the nucleoside transport site, but that inhibitions by adenosine and HNBMPR were reversible.

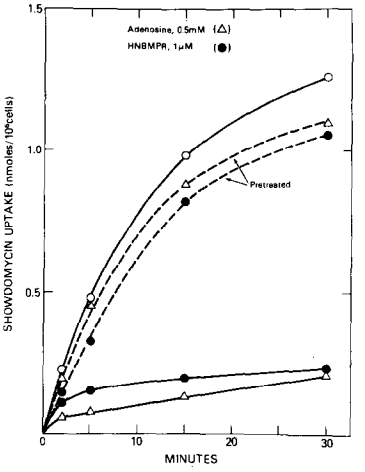

Fig. 6. Effect of preliminary adenosine and HNBMPR treatments on showdomycin uptake. L1210 cells (10^6 cells/ml) were incubated with adenosine (0.5 mM) or HNBMPR (1 μM) for 15 min at 37°. Then they were washed as in Fig. 5, resuspended at 10^6 cells/ml, and [^{14}C]showdomycin was added to a final concentration of 10 μM. Key: (---) cells pretreated as described above; and (——) adenosine and HNBMPR added at zero time. Showdomycin uptake was estimated as described in Materials and Methods.

DISCUSSION

Previous studies have indicated that sulfhydryl reagents can cause extensive plasma membrane damage to tumor cells as indicated by cell lysis [11] or bleb formation [12]. Such studies could not differentiate between damage to the external or cytoplasmic portions of the membrane. By the use of a transport specific sulfhydryl reagent, showdomycin, we conclude that damage is principally at the cytoplasmic site. It appears unlikely that cell damage is the result of alkylation of the nucleoside transport system. The amount of showdomycin taken up by L1210 cells prior to inactivation of cellular transport sites was orders of magnitude greater than NBMPR binding sites (0.2 pmole/10^6 cells), a value closely approximating that reported for HeLa cells [10]. The results suggest that plasma membrane damage may be due to interaction of showdomycin with a stabil-

izing sulfhydryl group at the cytoplasmic side of the plasma membrane [13], and they indicate that showdomycin toxicity is directly dependent upon its rate of transport into the cytoplasm and is inversely dependent upon its rate of inactivation of the nucleoside carrier sites and resulting protection from further intracellular damage.

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