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Evidence that ribavirin is transported in *Aedes albopictus* cells by the nucleoside transport system

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Summary

Aedes albopictus cells (CAE-3-6) deficient in the transport of nucleosides showed a markedly impaired ability to transport the antiviral compound, ribavirin. Furthermore, in order to inhibit either cell growth or viral replication, the transport negative CAE-3-6 cells required significantly higher concentrations of ribavirin than were needed for a similar degree of inhibition in the parental C7 cells. Although ribavirin did lower the level of GTP in CAE-3-6 cells the effect was slower and less striking than in the parental cells. These results indicate that ribavirin is transported across the plasma membrane of *Aedes albopictus* cells by the same transport system which functions to transport naturally occurring nucleosides.

ribavirin; nucleosides; *Aedes albopictus* cells; transport system

Introduction

Many of the known compounds with antiviral properties are nucleoside analogues [1]. In general these compounds are not active as such but must be modified within the cell, usually phosphorylated [1]; even before such modifications they must, of course, be transported into the cell.

Nucleoside transport has been studied in many different animal cells and has been shown to occur by a process termed facilitated diffusion. This type of transport is concentration dependent but energy independent; furthermore no enzymatic modifications of nucleosides occur during the course of transport [2].

The nucleoside carrier in those cells which have been studied (mainly mammalian cells) clearly has a low specificity for substrate, recognizing both purine and pyrimidine ribosides as well as many nucleoside analogues. There are, however, differences in the specificity of the carrier observed from cell type to cell type. For example, nitrobenzylthioinosine (NBTI) is a potent and specific inhibitor of nucleoside trans-

port in many vertebrate cells including RPMI 6410, Hela and P388 cells but does not inhibit transport in Novikoff hepatoma cells [6,7].

Relatively little work has been done on nucleoside transport in mosquito or other insect cells. However, the isolation of *Aedes albopictus* cell mutants unable to transport any of the naturally occurring nucleosides suggests, as is the case with mammalian cells, that mosquito cells use a single transport system to transport all nucleosides [8].

The antiviral compound, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole carboxamide; Rbv) is a guanosine analogue with an incomplete purine ring. Its mechanism of action is believed to involve the inhibition of inosine monophosphate dehydrogenase, and thus the prevention of GMP synthesis [3]. Previous work in this laboratory has shown that treatment with Rbv markedly reduced yields of Sindbis virus (SV) in *Aedes albopictus* cells, and depending on specific conditions also in BHK and CHO cells [4]. In all cases the effect on viral replication correlated with the depletion of GTP pools.

It is clear from earlier studies that in order to be active Rbv must be phosphorylated, likely by adenosine kinase, to the monophosphate [5]. Little, however, is known about how Rbv enters cells. Because of its unusual structure and its lack of a complete purine ring (see Fig. 4) we wished to know whether it would be recognized and transported by the same carrier that cells use to transport naturally occurring nucleosides.

The observation that the cytostatic effects of Rbv in 6410 cells can be prevented by pretreatment with NBTI led to the suggestion that Rbv is transported by the nucleoside carrier in these cells [6]. We have used a different approach to examine the question of Rbv transport, taking advantage of a mutant of *Aedes albopictus* cells, CAE-3-6, which is deficient in nucleoside transport [8].

Based on both direct measurements of transport and on the effects of Rbv in the nucleoside transport-deficient cells we conclude that the major mode of entry for Rbv into *Aedes albopictus* cells is via the nucleoside transport system.

Materials and Methods

Cells, virus and media

The C7 clone of *Aedes albopictus* mosquito cells [9], a subclone of C7 cells designated C7-10, and the nucleoside transport deficient CAE-3-6 cells [8] were all grown at 28°C in E medium (MEM containing non-essential amino acids [9]) supplemented with heat inactivated (56°C, 30 min) fetal calf serum. Dialyzed serum was used in certain experiments as indicated. The preparation of primary chick embryo fibroblasts (CEF) [10] and stocks of Sindbis virus has been described elsewhere [11]. Cells were infected with virus at an input multiplicity of approximately 10 pfu per cell. After adsorption (45 min 34.5°C, 5% CO_2), the inoculum was removed, the monolayers were washed with phosphate buffered saline and the cultures were refed with E medium containing 10% dialyzed fetal calf serum (E-10). Infected cells were incubated at 34.5°C. Virus titers were measured by plaque assay at 34.5°C [12] on CEF.

Transport assay

The assay for Rbv transport was as described for adenosine transport [8]. Cells grown on coverslips were immersed in phosphate-buffered saline (PBS) containing [^{3}H]Rbv (10 μCi/ml, 30 μg/ml) for various lengths of time as indicated. After removal from the Rbv the cells were quickly washed, treated as described previously [8] and then assayed for radioactive counts.

Analysis of nucleotide pools

Perchloric acid extracts were prepared from rapidly growing cells as described previously [4]. Nucleotides were resolved on a Whatman partisil SAX anion exchange column using a Waters HPLC [4]. The data were analyzed using an HP3000A integrator.

Chemicals and isotopes

[5-^{3}H]ribavirin (14 Ci/mM) and unlabeled ribavirin were purchased from ICN Pharmaceuticals. The mycophenolic acid (MPA) was a generous gift of Dr. K.F. Kosh, Lilly Research Laboratories.

Results

Transport of [^{3}H]Rbv

As shown in Fig. 1 the nucleoside transport deficient CAE-3-6 cells showed a

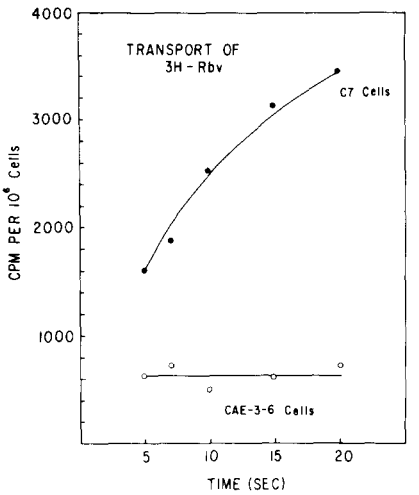


Fig. 1. Transport of Rbv in C7 and CAE-3-6 cells. Cells were grown on glass coverslips (22×22 mm, #1-1/2) to a density of about 1 × 10^6 cells per coverslip. Each coverslip was rinsed in PBS at 28°C and incubated in transport medium (PBS containing 10 μCi [^{3}H]Rbv/ml and sufficient unlabeled Rbv to bring the final concentration to 30 μg/ml) also at 28°C. At the times indicated, coverslips were removed from the transport medium and rinsed consecutively in four 100-ml baths of ice-cold PBS. Coverslips were processed for counting in a scintillation counter and cell counts were determined as previously described [8].

markedly reduced capacity to transport [^{3}H]Rbv. Whereas the amount of [^{3}H]Rbv transported into the parental C7 cells increased rapidly between 5 and 20 s after immersion in the transport medium, with CAE-3-6 cells no increase in intracellular [^{3}H]Rbv was observed over the same time period.

In other experiments, however, we did observe that Rbv does cross the plasma membrane of transport deficient cells after incubation for longer periods of time, but only with a low efficiency. For example, CAE-3-6 cells had to be incubated with [^{3}H]Rbv for 90 min to accumulate the same amount of radioactivity as C7 cells could take μp in 5 min. As pointed out elsewhere [2], however, to accurately measure transport per se as opposed to other processes, e.g. uptake, it is necessary to use very short incubation periods as illustrated in Fig. 1.

The demonstration that CAE-3-6 cells failed to transport Rbv supports the idea that Rbv is transported into cells by the same carrier used to transport naturally occurring nucleosides. The fact that some ribavirin enters the CAE-3-6 cells after longer labeling periods suggests either (1) that Rbv can get into these cells, albeit not very efficiently, by some means other than the nucleoside transport system, or (2) that the nucleoside carrier in these cells is capable of a low level of activity.

Effects of Rbv on cell growth and virus replication

Another way to test the entry of Rbv into CAE-3-6 cells is to compare its effects on these cells with its effects on the parental C7 cells.

The effects of Rbv on cell growth are shown in Fig. 2. Whereas a concentration of 5

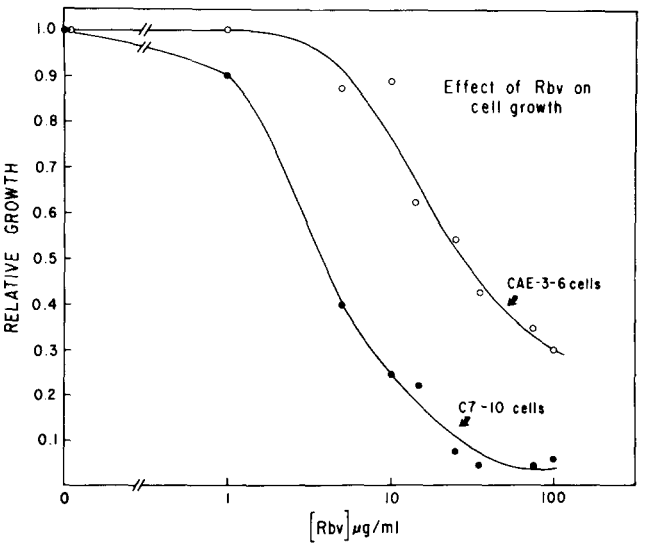


Fig. 2. Effect of Rbv on cell growth. Cells were plated in E5 medium in 60-mm plates and allowed to attach for 2 h at 20°C. The original medium was removed and replaced with 4 ml of E medium (supplemented with 10% dialyzed serum) and the indicated concentrations of Rbv. Cells were counted after refeeding (0 time) and again after 3 days. For each cell type the counts were normalized to the number obtained in the absence of Rbv. The untreated C7 cells grew from a density of 0.192×10^6 to 3.88×10^6 cells per plate. The CAE-3-6 cells grew from 0.212×10^6 to 2.28×10^6 cells per plate in the absence of Rbv.

μg Rbv per ml reduced the growth of C7 cells to 50% of that observed for the untreated cultures, in the CAE-3-6 cells 45 μg per ml was required to achieve a similar effect.

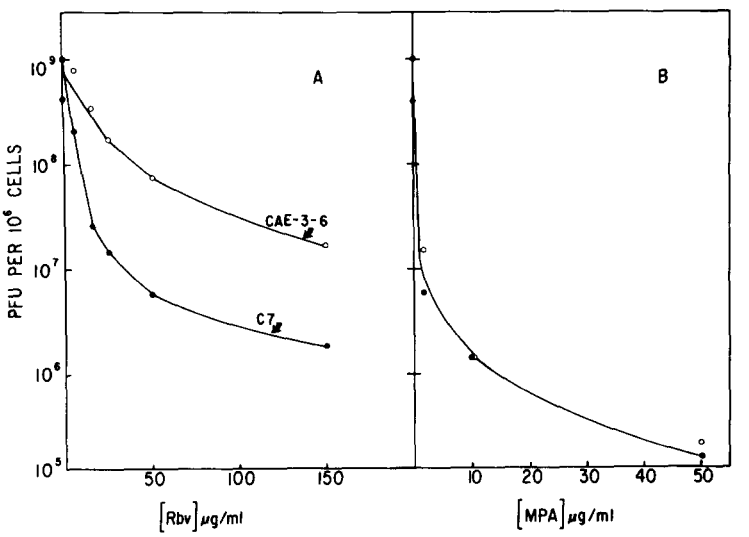
Fig. 3A illustrates a comparable difference in the concentration of Rbv necessary to reduce the yield of Sindbis virus in the two cell types. In C7 cells, 15 μg Rbv per ml reduced the titer of SV 40 fold but had little effect on the yield from the CAE-3-6 cells. Although the titer of SV in the CAE-3-6 cells was reduced 30 fold at a concentration of 150 μg Rbv per ml, this same concentration of Rbv reduced the viral yield from C7 cells 200–300-fold.

Since CAE-3-6 cells were selected in a multistep process, the possibility existed that more than one genetic alteration may have occurred in these cells. Although MPA, like Rbv, is an IMP dehydrogenase inhibitor, it is not a nucleoside analogue (see Fig. 4) and would not be expected to use the nucleoside transporter. Since the effects of MPA on virus replication (Fig. 3B) and on cell growth (not shown) are similar in C7 and CAE-3-6 cells, the possibility that the reduced sensitivity of CAE-3-6 cells to Rbv resulted from an altered IMP dehydrogenase is unlikely.

Effect on nucleotide pools

The effects of Rbv on cell growth and virus replication are thought to result from a decrease in the size of the GTP pool [4]. Although, as shown above, the CAE-3-6 cells were less sensitive to Rbv than the C7 cells, at sufficiently high concentrations inhibition of cell growth and virus replication did occur.

Table 1 shows that for C7-10 cells treated with 150 μg Rbv/ml, about 35 min was required to reduce the relative GTP level by 50%. In CAE-3-6 cells, assuming that the



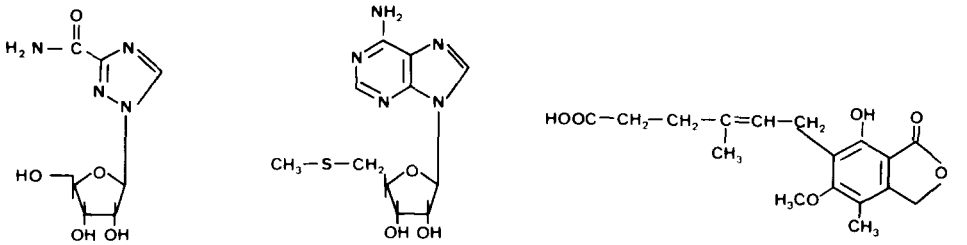


Fig. 4. Structural formulae of ribavirin, 5′-methylthioadenosine and mycophenolic acid.

TABLE 1

ATP and GTP concentrations in C7 and CAE-3-6 cells after exposure to Rbv for different times

Cells	Time (min in Rbv)	ATP (pmol/10^6 cells)	GTP (pmol/10^6 cells)	Relative GTP
C7-10	0	500	220	1.0
	35	700	120	0.54
	80	700	80	0.36
	180	600	36	0.16
CAE-3-6	0	600	130	1.0
	35	700	120	0.92
	80	750	110	0.85
	180	740	80	0.66

Cells were grown for 3 days in E medium (supplemented with 5% serum) to a density of 7–8 × 10^6 cells per 100 mm petri dish. The original medium was then replaced with E medium without serum. At 2 h after refeeding, Rbv was added to a final concentration of 150 μg/ml and cells were incubated for the times indicated. The cell extracts were prepared as described in Materials and Methods. Relative GTP was determined for each cell type by normalizing the pmol/10^6 cells at each time point to the concentration measured in the untreated (0 time) samples.

rate of reduction shown in Table 1 was continued, approximately 260 min would be needed to achieve the same effect. We conclude that although Rbv did reduce the size of the GTP pool in both cell types the effect was clearly less marked and occurred significantly more slowly in the CAE-3-6 cells than in the C7 cells.

Discussion

Three lines of evidence support the idea that most nucleosides enter animal cells by means of a common carrier. (1) Nucleosides compete with each other for entry into cells [2,13]. (2) Compounds which inhibit transport of one nucleoside also inhibit transport of other nucleosides [2,6]. (3) Somatic cell mutants have been described which are deficient in the transport of many nucleosides [8,13].

The fact that the nucleoside carrier is able to transport both purine and pyrimidine nucleosides as well as analogues with modifications of the purine or pyrimidine base or the sugar moiety is consistent with the low specificity of this transport system.

In general, changes in either moiety of the nucleoside can alter the efficiency of but do not necessarily prevent transport [6]. One exception is the naturally occurring nucleoside 5'-methylthioadenosine (see Fig. 4), which on the basis of competition experiments involving mouse lymphoma cells is thought to be transported not by the nucleoside carrier but by the carrier which transports the purine base, adenine [14].

In this work we have used nucleoside transport deficient cells to determine whether the guanosine analogue Rbv is recognized and transported by the nucleoside carrier. We have shown reduced transport of Rbv in these cells and we have demonstrated that the effects of Rbv on cell growth and virus replication are reduced in these cells compared with the parental cells. These findings indicate that in spite of the fact that Rbv has an incomplete purine ring structure, it is nevertheless transported across the cell membrane by the nucleoside carrier.

Understanding how antiviral compounds get inside cells is essential for the rational understanding of how such compounds act as well as for the development of more efficient therapeutic agents. Somatic cell mutants deficient in the transport of antiviral compounds should prove extremely useful for such purposes.

Acknowledgements

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