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NITROBENZYLTHIOINOSINE-INSENSITIVE URIDINE TRANSPORT IN HUMAN LYMPHOBLASTOID AND MURINE LEUKEMIA CELLS[1]

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SUMMARY: Both human lymphoblastoid (RPMI 6410) and murine leukemia (L1210) cells were found to have a component of uridine transport which is insensitive to the nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR). In both cell lines NBMPR-insensitive uridine transport is inhibited by other nucleosides and by the sulfhydryl reagent p-chloromercuribenzenesulfonate. In RPMI 6410 cells NBMPR-insensitive transport accounts for only 2% of the initial rate of uridine transport. In contrast, 20% of the initial rate of transport of L1210 cells is insensitive to NBMPR, and uridine uptake over longer periods (10 min) is completely insensitive to NBMPR.

The uptake of physiological nucleosides and their cytotoxic analogs by mammalian cells involves the mediated transport of the nucleoside across the plasma membrane followed by metabolism, primarily phosphorylation, of the nucleoside within the cell (2,3). The transport process appears to be quite similar in a number of cultured cell lines in that it is very rapid, has a broad substrate specificity and has very high K_m and V_{max} values (2,3).

p-Nitrobenzylthioinosine (NBMPR)[2] and other S^6-derivatives of 6-mercaptoinosine and 6-mercaptoguanosine have proven to be useful and specific probes of nucleoside transport (4). NBMPR binds reversibly to cultured cells with a K_D of 10^{-9} to 10^{-10} M (5,6), and occupancy of these high affinity sites by NBMPR is associated with loss of nucleoside transport activity (7-9). How-

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[2]Abbreviations: NBMPR (p-nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; pCMBS, p-chloromercuribenzenesulfonate.

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ever, in some cells the relationship between NBMPR binding and inhibition of transport is complex. The dose response curve in HeLa cells is biphasic with ≈40% of the uridine transport activity remaining when the high affinity NBMPR-binding sites are saturated (8). In other cell lines it has been observed that even in the presence of high concentrations of NBMPR (10^{-7} to 10^{-5} M) nucleoside uptake continues at a slow rate (6,10). The mechanism by which NBMPR-insensitive uptake occurs, and the role this process may play in the uptake of physiological and cytotoxic nucleosides is unknown. In some cases, NBMPR-insensitive uptake has been attributed to non-mediated diffusion of the nucleoside across the plasma membrane (6). In other studies it has been proposed that both NBMPR-sensitive and -insensitive uptake are properties of a single complex transporter, or possibly two separate transport systems (10).

The present study examines NBMPR-sensitive and -insensitive uridine uptake in human lymphoblastoid (RPMI 6410) and murine leukemia (L1210) cells and provides evidence that NBMPR-insensitive uptake occurs via a carrier-mediated process.

METHODS

Cells

RPMI 6410 and RPMI 6410/MP/DU/AU cells were maintained as described previously (11). L1210 cells were maintained as static suspension cultures in RPMI1640 medium supplemented with 10% horse serum.

Uridine Uptake

Cells were harvested during log growth, washed and resuspended in Buffer A (25 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaP_i and 10 mM glucose). Short term uptake of [5-^{3}H]uridine (10-20 μCi/μmol) was determined as described (11) except that in experiments with RPMI 6410 cells uptake was stopped by addition of 10 μM dipyridamole followed immediately by centrifugation of the cells through silicone oil. For L1210 cells the "inhibitor stop" was omitted and the uptake period terminated by centrifugation of the cells through silicone oil. In long term uptake experiments cells were incubated with [5-^{3}H]uridine in a shaking water bath (22°) and at the times indicated aliquots were removed and the cells collected by centrifugation through silicone oil. The cell pellets were processed and counted as described (11).

RESULTS AND DISCUSSION

We have previously reported (11) that uridine transport in RPMI 6410 cells is similar to that of other mammalian cells in that it has a high K_m

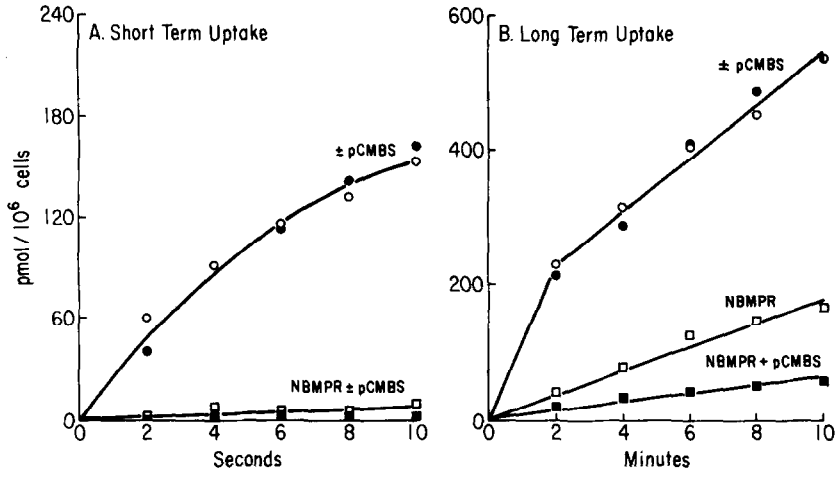

Figure 1: Effect of NBMPR and pCMBS on Uridine Uptake in RPMI 6410 Cells. Cells (2×10^7/ml) were incubated for 20 min at 22° with the indicated inhibitors. Uridine (100 μM) uptake was determined over a 10 sec time course (A) or a 10 min time course (B) as described under Methods. ○, no additions; ●, 200 μM pCMBS; □, 1 μM NBMPR; ■, 1 μM NBMPR and 200 μM pCMBS.

(250 μM) and V_{max} (30 pmol/10^6 cells-sec) and is sensitive to NBMPR (IC_{50} = 2 nM). As shown in Fig. 1A, the initial rate of uridine transport appears (within the limits of detection) to be completely inhibited by 1 μM NBMPR. However, when uptake is examined over a longer period of time (Fig. 1B) it is clear that there is a significant rate of uridine uptake in the presence of NBMPR. This NBMPR-insensitive uptake of uridine is significantly inhibited by the addition of pCMBS, whereas pCMBS alone has no effect on uptake over 10 min or on the initial rate of transport (Fig. 1A). To determine whether inhibition by pCMBS is due to an effect on permeation or on the intracellular metabolism of uridine, uptake was examined in a uridine kinase-deficient mutant, RPMI 6410/MP/DU/AU. These cells are unable to phosphorylate uridine or uridine analogs (12), but are unaltered in their nucleoside transport capacity (11). As would be expected in the absence of metabolism, uridine uptake reaches a plateau within the first minute (Fig. 2). Similar to the results obtained with the wild type cells, there is a significant rate of NBMPR-insensitive uptake which can be inhibited by the addition of pCMBS. These results suggest that uptake in the presence of NBMPR is not simply free diffusion of uridine across the plasma membrane, but rather that it is a mediated process

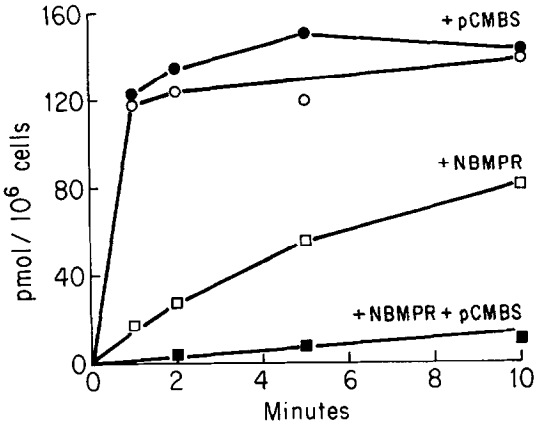

Figure 2: Effect of NBMPR and pCMBS on Uridine Uptake in Uridine Kinase Deficient Cells (RPMI 6410/MP/DU/AU).
Incubations and uptake measurements were as described in Figure 1B. ○, no additions; ●, 200 μM pCMBS; □, 1 μM NBMPR; ■, 1 μM NBMPR and 200 μM pCMBS.

which can be inhibited by pCMBS. This conclusion is further supported by the observation that NBMPR-insensitive uridine uptake is inhibited by other nucleosides (Table 1).

NBMPR-insensitive uridine transport can also be observed in L1210 cells (Fig. 3). In this case the NBMPR-insensitive process appears to make a greater contribution to the total transport rate, and can even be observed in the short term uptake experiments (Fig. 3A). In addition, pCMBS alone inhibits the initial rate of uridine transport and the effects of NBMPR and

Table 1

Inhibition of NBMPR-Insensitive Uridine Transport by Other Nucleosides

Nucleoside	Concentration μM	% Inhibition RPMI 6410	L1210
Adenosine	100	37	45
Adenosine	1000	84	90
2'-Deoxyadenosine	100	44	37
2'-Deoxyadenosine	1000	86	88
Thymidine	100	22	24
Thymidine	1000	72	60

Cells (2 x 10^7/ml) were incubated with 1 μM NBMPR for 20 min at 22° and then assayed for uridine uptake as described under Methods. ^{3}H-Uridine (100μM) and unlabeled nucleoside were added simultaneously to initiate the uptake period. 5 min uptake assays were used with RPMI 6410 cells and 20 sec assays with L1210 cells.

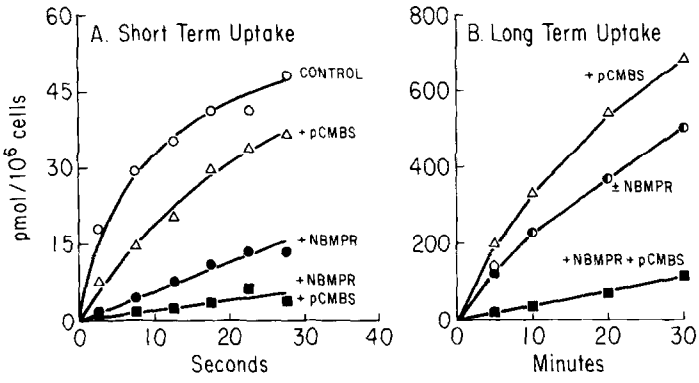

Figure 3: Effect of NBMPR and pCMBS on Uridine Uptake in L1210 Cells. Incubations and uptake measurements were as described in Figure 1. ○, no additions; ●, 1 μM NBMPR; △, 200 μM pCMBS; ■, 1 μM NBMPR and 200 μM pCMBS.

pCMBS together appear to be additive, suggesting that these two inhibitors may act independently. Similar results were obtained with ATP-depleted cells (data not shown), and therefore effects of pCMBS on uridine metabolism can again be ruled out. As was observed in RPMI 6410 cells, NBMPR-insensitive uridine transport in L1210 cells is also inhibited by other nucleosides (Table 1).

When uridine uptake in L1210 cells is examined over a longer period of time, it is not inhibited by either NBMPR or pCMBS when added individually (Fig. 3B). In fact, pCMBS alone produces a slight stimulation of uptake. However, the addition of both NBMPR and pCMBS together results in profound inhibition of uridine uptake. The rate-limiting step in uridine uptake over this long period of time is presumably the intracellular phosphorylation by uridine kinase rather than the transport of uridine into the cell (2,3). The lack of inhibition by either NBMPR or pCMBS alone suggests that the rate of transport by either mechanism is faster than the rate of uridine metabolism in these cells. Accordingly, uptake can only be inhibited when both transport processes are blocked (i.e. +NBMPR and pCMBS in Figure 3B).

The mechanism for stimulation of uridine uptake by pCMBS is not clear. Heichal et al. (10) have also observed a stimulation of uridine and cytosine-arabinoside uptake by pCMBS in transformed hamster fibroblasts, and have proposed that pCMBS acts as an allosteric stimulator of nucleoside transport in

these cells. It is unlikely that this is the mechanism of stimulation of uridine uptake in L1210 cells since the initial rate of uridine transport is inhibited by pCMBS (Figure 3A), and stimulation is only apparent when uptake is measured over longer periods of time (Figure 3B). These results suggest that pCMBS may stimulate uridine metabolism.

The results presented here suggest that in both RPMI 6410 and L1210 cells there is a component of nucleoside transport which is insensitive to NBMPR. The relative rates of NBMPR-sensitive and -insensitive uridine transport differ considerably between the two cell lines. In L1210 cells, NBMPR-insensitive transport accounts for about 20% of the total transport rate and is easily detected in the short term uptake experiments (30 sec). The rate of NBMPR-insensitive transport appears to be sufficient to support the continued uptake of uridine over long periods of time even when the major transporter is blocked by NBMPR. In contrast, the rate of NBMPR-insensitive uridine transport in the RPMI 6410 cells is only 2% of the total rate of transport and is not detectable in the short assays (Fig. 1A). Consistent with this, pCMBS has no effect on the rate of transport determined in the short assays. In these cells the second transport process can only be detected when measurements are made over longer periods of time and the major transporter is blocked by NBMPR.

The physiological significance of NBMPR-insensitive nucleoside transport is not clear. It is not yet known whether there are any differences in substrate specificity between the two types of transport. However, the existence of these two types of transport activity in mammalian cells may have important implications in the chemotherapeutic use of nucleoside transport inhibitors in combination with cytotoxic nucleosides (13) or inhibitors of de novo pyrimidine biosynthesis (14,15).

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