Sodium-dependent and Equilibrative Nucleoside Transport Systems in L1210 Mouse Leukemia Cells: Effect of Inhibitors of Equilibrative Systems on the Content and Retention of Nucleosides

Lina Dagnino and Alan R. P. Paterson

Cancer Research Group (McEachern Laboratory) and Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

ABSTRACT

The presence of 10 μM dipyridamole in incubation media of L1210/C2 cells decreased initial rates of zero-trans influx of formycin B (FB, 50 μM), a poorly metabolized inosine analogue, from 4.84 pmol/μl cell water/s to 0.87 pmol/μl cell water/s. However, after a 5-min interval of uptake, free FB levels in dipyridamole-treated cells were 165 pmol/μl cell water, 2.3-fold greater than in dipyridamole-free cultures. This indicated the presence of a concentrative, dipyridamole-insensitive nucleoside transport (NT) system in L1210 cells, in addition to the equilibrative NT systems known to be expressed in these cells. The concentrative system was demonstrable only in the presence of NT inhibitors and required extracellular Na+. The presence of 8 μM 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine or 15 μM dilazep also induced an accumulation of free FB above steady-state levels, although of a lesser magnitude than that observed with dipyridamole. It appears that NT inhibitors induced nucleoside accumulation by inhibiting bidirectional nucleoside movements mediated by the equilibrative component of nucleoside transport in L1210/C2 cells without interfering with inward FB fluxes mediated by the Na+–dependent transporter. The presence of NT inhibitors also enhanced the cellular accumulation and retention of arabinosyladenine and its 5′-triphosphate in these cells. The increased cellular accumulation of 9-β-D-arabinofuranosyladenine and 9-β-D-arabinofuranosyladenine triphosphate by dipyridamole was associated with enhanced antiproliferative activity of 9-β-D-arabinofuranosyladenine towards the leukemia cells.

INTRODUCTION

Nucleoside permeation across the plasma membrane of mammalian cells is mediated by equilibrative and by concentrative, ion-driven systems (1–6). Inhibitors of equilibrative systems include nucleoside analogues such as NBMPR (7, 8) and nonnucleoside drugs such as dipyridamole and dilazep (1, 9, 10). Equilibrative NT systems present in human erythrocytes and S49 mouse lymphoma cells have high sensitivity to NBMPR; concentrations of that agent in the 10–100 nM range are sufficient to virtually abolish NT processes in these cells (11–13). In contrast, equilibrative NT systems expressed in Walker 256 rat carcinosarcoma cells (14) and in Novikoff rat hepatoma cells (15) exhibit low sensitivity to NBMPR. Concentrations of the latter agent in the vicinity of 10 μM only partially inhibit the transport of nucleosides in these cells. Nucleoside transport systems of high and of low sensitivity to NBMPR have been identified in HeLa cells (16) and in mouse leukemia L1210 cells (17).

Dipyridamole and NBMPR have been shown to protect various types of cultured cells from the antiproliferative activity of toxic nucleoside analogues (18–20). This protection has been attributed to inhibition of cellular NT systems with consequent decrease in the influx of the nucleoside drugs. By contrast, the presence of dipyridamole or NBMPR in culture medium containing 2′-deoxyadenosine increased the antiproliferative activity and cellular content of that nucleoside in L1210 cells (21, 22). Hitherto, this effect of the NT inhibitors has not been explained.

Concentrative NT systems have been described in a number of cells of epithelial (3–5) and nonepithelial origin (6). The present report describes the operation of L1210/C2 cells of a Na+-dependent, concentrative NT system, insensitive to inhibitors of equilibrative NT processes. This study also examined the enhanced accumulation of nucleosides and their metabolites induced in L1210 cells by inhibitors of equilibrative transport. Such accumulation appears to result from the inhibition of bidirectional nucleoside movements via equilibrative systems without impairment of concentrative nucleoside influx via the Na+-dependent system. Portions of this study have been described in preliminary reports (23, 24) and a detailed characterization of the Na+-dependent system present in L1210 cells is described elsewhere.

MATERIALS AND METHODS

Chemicals. 2′-Deoxycoformycin was provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. NBMPR was prepared in this laboratory (25). ara-A was purchased from Pfannstiel Laboratories, Waukegan, IL. Tricaprylylamine (Alamine 336) and 1,1,2-trichlorotrifluoroethane (Freon TF) were obtained, respectively, from the Henkel Co., Kankakee, IL, and Dupont Canada, Maitland, Ontario, Canada. Dilazep was a gift from Hoffmann-LaRoche, Basel, Switzerland. Cell culture materials were purchased from GibCO Canada Inc., Burlington, Ontario, Canada. PEI-cellulose TLC sheets were purchased from Brinkmann Instruments, Westbury, NY. PEI (borate)-cellulose TLC sheets were prepared as described (26). After storage, 1H-labeled nucleosides (Moravek Biochemicals, Brea, CA) were purified by HPLC (27). [1,2-14C]Polyethylene glycol (2 mCi/g) was obtained from New England Nuclear, Lachine, Quebec, Canada. Paraffin oil (Saybolt viscosity 125–135) was obtained from Fisher Scientific, Fair Lawn, NJ. Silicone 550 oil was obtained from Dow Corning, Mississauga, Ontario, Canada. FB was a gift from the late Professor Hamao Umezawa, Institute for Microbial Chemistry, Tokyo, Japan.

Cell Culture. Mouse leukemia L1210/C2 cells, a cloned line, were obtained from Dr. C. E. Cass, University of Alberta, and cultured as described previously (28). In growth inhibition studies, replicate 10-ml cultures initially contained 105 cells/ml. Influx Measurements. Time courses of nucleoside influx in leukemia

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1 This study was supported by the National Cancer Institute of Canada.

2 Recipient of a Studentship award from the Alberta Heritage Foundation for Medical Research. Present address: Institut de Recherches Cliniques de Montréal, 110 Avenue des Pins Ouest, Montréal, Quebec, Canada H2W 1B7.

3 Senior Research Scientist of the National Cancer Institute of Canada. To whom requests for reprints should be addressed, at Department of Pharmacology, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada.

4 The abbreviations are: NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; ara-A, 9-β-D-arabinofuranosyladenine; ara-ATP, 9-β-D-arabinofuranosyladenine 5′-triphosphate; dCF, 2′-deoxycoformycin; FB, formycin B; HPLC, high-performance liquid chromatography; NT, nucleoside transport; PEI, polyethylenimine; TLC, thin-layer chromatography.

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L1210 cells were measured as in a previously described procedure (27). The cells were exposed to permeant for graded intervals initiated by rapid addition of 0.1-ml portions of [3H]-labeled permeant solution to 0.1-ml portions of cell suspension. The assay mixtures were contained in 1.5-ml microcentrifuge tubes over 0.1 ml of oil [Silicone 550 oil:paraffin oil 85:15 (v/v); 1.03 g/ml]. Intervals of nucleoside uptake were initiated with microcentrifuge tubes placed in the rotor of an Eppendorf 5412 centrifuge and were ended by centrifugal pelleting of cells under the oil. Cellular uptake of [3H]-labeled permeant during pelleting was previously estimated to be equivalent to permeant influx during an interval of 2 s (29). Intracellular water volumes were estimated by subtracting the extracellular space (determined with [3H]-polyethylene glycol used in place of permeant) from total pellet water space, measured with [1H]2O (29). In the influx assays, cells were suspended in serum-free Fischer’s medium containing 20 mM (2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4, 22°C) in place of the NaHCO3 buffer, or in phosphate-buffered solutions (1.0 mM CaCl2, 2.6 mM KCl, 1.4 mM KH2PO4, 138 mM NaCl, 8 mM Na2HPO4, 5 mM glucose, pH 7.4, 22°C). In some experiments, Na+ was replaced by equivalent concentrations of K+ or N-methyl-d-glucammonium. To obtain time courses, triplicate measurements for each point were obtained in a single experiment, each experiment was conducted 2–4 times.

ara-A Metabolites. Cell suspensions were incubated in growth medium containing 1.1 mM DCF for 1 h at 37°C and then [3H]ara-A (20 μM, final) was added to each suspension, with or without a NT inhibitor. After specified intervals of culture at 37°C with these agents, the L1210/C2 cells were collected by centrifugation (150 x g, 2 min, 4°C) and were washed once with ice-cold growth medium. Cell pellets were extracted at 4°C with 0.4 M trichloroacetic acid. After neutralization [0.5 M Alamine 336 in Freon TF (30)], the extracts were chromatographed together with carrier compounds in a HPLC system [C18 reversed-phase column eluted with a water-methanol gradient (27)], or on PEI-cellulose thin layers, as described by Lauzon et al. (31). For assay of [3H] content, compounds on the chromatograms were located by the carrier compounds. In the TLC or HPLC assays, at least 95% of the applied [3H] activity was accounted for on the chromatograms. [3H]-9-β-D-Arabinofuranosylhypoxanthine arising from [3H]ara-A deamination did not exceed 4% of total [3H] activity in any of the cell extracts prepared.

Extracellular Metabolites of ara-A. After culture with [3H]ara-A as described in the preceding section, cells were washed once with ice-cold growth medium and incubated at 37°C in nucleoside-free medium for 3 h. To identify extracellular products of [3H]ara-ATP catabolism, samples of culture medium were freeze-dried and the residues were extracted with 0.4 M trichloroacetic acid. After neutralization, the extracts were analyzed by TLC or HPLC. The following TLC analysis systems were used: (a) PEI (borate)-cellulose developed with water, or with acetic acid:2-propanol:water (65:23:12, v/v/v); (b) PEI-cellulose developed with water; and (c) silica gel developed with methanol:chloroform:3% acetic acid (2:3:1, v/v/v). HPLC analysis was performed as described (27).

RESULTS

Influx of FB in L1210/C2 Cells. Transport of FB (50 μM) into L1210/C2 cells in the presence or absence of 10 μM dipyridamole was measured in the experiment of Fig. 1. The influx of FB was progressive and cells achieved steady-state FB concentrations of about 70 μM within 1 min. Although initial rates of FB influx were substantially reduced in the presence of 10 μM dipyridamole (from 4.84 pmol/μl cell water/s to 0.87 pmol/μl cell water/s; Fig. 1A), the cellular content of FB increased with time and reached about 170 μM after 5 min of uptake (Fig. 1B). The concentrative character of FB influx in

* During experiments that measured FB uptake, conversion of FB in L1210/C2 cells to other products (possibly FB phosphates) was less than 5%, as shown by TLC analysis of cell extracts.
The presence of 10 μM dipyridamole in the culture medium during incubation of L1210/C2 cells with [3H]ara-A increased the cellular content of ara-A and ara-ATP by as much as 12- and 5.6-fold, respectively (Fig. 4). The maximum concentrations of ara-A and ara-ATP observed in cell suspensions containing dilazep were similar to those reached in dipyridamole-containing cultures. However, the high levels of ara-A and ara-ATP achieved in dilazep-containing medium were of short duration (Fig. 4). By the end of the 21-h incubation period, ara-ATP concentrations in cells cultured with dilazep were not significantly different from those in inhibitor-free cultures.

Cellular levels of ara-A and ara-ATP attained in culture medium containing 8 μM NBMPR were much lower than in the presence of dilazep or dipyridamole. The maximum cellular concentrations of ara-A achieved in NBMPR-containing medium were about 3-fold greater than in inhibitor-free cultures. Cellular ara-ATP concentrations in the presence and in the absence of NBMPR were similar (Fig. 4).

Effect of NT Inhibitors on Cellular Retention of ara-ATP. L1210/C2 cells were “loaded” with [3H]ara-ATP by incubation at 37°C with 1.1 μM dCF for 1 h, followed by culture with 20 μM [3H]ara-A at 37°C for 3 h. After a single wash with cold medium, cells were resuspended in ara-A-free medium and changes with time in the 3H content of the cells and the medium were followed. Under those conditions, cellular concentrations of ara-A and ara-ATP declined with a half-life of 73 min (Fig. 5); the half-life was unchanged when the resuspension medium contained 8 μM NBMPR. When cells containing [3H]ara-ATP were resuspended in ara-A-free medium with 10 μM dipyridamole, the half-life of cellular ara-ATP increased 2.6-fold, to 190 min (Fig. 5). The presence of 15 μM dilazep in the resuspension medium

Na+-independent NT component, evidently the equilibrative, inhibitor-sensitive nucleoside transporter known to be expressed in L1210 cells (17). When cells were suspended in Na+-containing medium, the initial influx rates of FB decreased from 4.7 pmol/μl cell water/s in the absence of inhibitors to 3.0, 1.5, or 0.9 pmol/μl cell water/s, in the presence of NBMPR, dilazep, or dipyridamole, respectively (data not shown). However, as influx of FB continued over a 7-min interval, the FB content of cells treated with NBMPR, dilazep, or dipyridamole was 1.2-, 1.6-, or 2-fold greater than in untreated cells (68 pmol/μl cell water; Fig. 3B). Thus, inhibition of equilibrative NT systems mediating FB transport was followed by FB accumulation in cells suspended in Na+-containing medium. Of the 3 inhibitors assayed, dipyridamole induced the strongest inhibition of FB fluxes mediated by the equilibrative NT system, together with the largest cellular accumulation of FB when the Na+-dependent NT system was operative.

Effect of NT Inhibitors on Accumulation of ara-A and ara-ATP in L1210/C2 Cells. Since incubation of L1210/C2 cells with dipyridamole, dilazep, and NBMPR resulted in the accumulation of FB, the effects of these NT inhibitors on the accumulation of ara-A and its metabolites were investigated. As seen in Fig. 4, cells cultured in medium containing 20 μM [3H]ara-A (without NT inhibitors) attained ara-A and ara-ATP concentrations of about 6 nmol/10⁶ cells and 17 nmol/10⁶ cells. These values are equivalent to about 20 and 60 μM, respectively, since the intracellular water space in L1210/C2 cells under these conditions was about 0.3 ml/10⁶ cells. 9-β-D-Arabinofuranosyladenine 5'-monophosphate and 9-β-D-arabinofuranosyladenine 5'-diphosphate accounted for less than 9% of the 3H content of cell extracts.

The presence of 10 μM dipyridamole in the culture medium during incubation of L1210/C2 cells with [3H]ara-A increased the cellular content of ara-A and ara-ATP by as much as 12- and 5.6-fold, respectively (Fig. 4). The maximum concentrations of ara-A and ara-ATP observed in cell suspensions containing dilazep were similar to those reached in dipyridamole-containing cultures. However, the high levels of ara-A and ara-ATP achieved in dilazep-containing medium were of short duration (Fig. 4). By the end of the 21-h incubation period, ara-ATP concentrations in cells cultured with dilazep were not significantly different from those in inhibitor-free cultures.

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Fig. 3. Effect of NT inhibitors on inward fluxes of FB in L1210/C2 cells. A, influx of FB (80 μM) was measured in cells suspended in medium containing N-methyl-d-glucammonium (NMG+) in place of Na+ without NT inhibitors (O), or with 10 μM dipyridamole (Δ), 15 μM dilazep (●), or 8 μM NBMPR (C). B, influx of FB (60 μM) was measured in cells suspended in medium with Na+ containing 10 μM dipyridamole (Δ), 15 μM dilazep (●), 8 μM NBMPR (C), or without NT inhibitors (O).

Fig. 4. Accumulation of ara-A and ara-ATP in L1210/C2 cells cultured with dCF and NT inhibitors. Cells were incubated at 37°C in growth medium containing 1.1 μM dCF for 1 h prior to the addition of [3H]ara-A (20 μM, final) with or without an NT inhibitor. Cellular ara-A and ara-ATP levels were measured at the intervals specified. O, 8 μM NBMPR; ●, 15 μM dilazep; Δ, 10 μM dipyridamole; C, without an NT inhibitor.
resulted in a 1.7-fold increase in ara-ATP half-life (124 min; Fig. 5). The decline in cellular [3H]ara-ATP content was accompanied by the appearance in the extracellular medium of corresponding amounts of 3H-labeled material that coeluted with ara-A during HPLC analysis (data not shown).

Cellular formation of ara-ATP has been shown to be involved in ara-A cytotoxicity towards L1210 cells (42). When the proliferation rates of cells cultured in the presence of graded concentrations of ara-A and 1.1 μM dCF for 48 h was measured, the concentration of ara-A that inhibited cell proliferation by 50% was 8 μM. The presence of 10 μM dipyrindamole in cell cultures containing dCF and ara-A, shown to increase the cellular accumulation and retention of ara-ATP (Figs. 4 and 5), decreased the concentration of ara-A that inhibited cell proliferation by 50% to 2.3 μM. This concentration of dipyrindamole by itself was without effect on the proliferation rates of the leukemia cells (Fig. 6).

DISCUSSION

The influx of FB and ara-A in L1210/C2 cells is mediated by nonconcentrative (equilibrative) and by Na+-dependent transport systems. In the absence of NT inhibitors, the poorly metabolized nucleoside FB entered the cells to reach steady-state concentrations that reflect the relative rates of the inward and outward FB fluxes. In the presence of inhibitors of equilibrative NT activity, inward fluxes of FB were reduced but, with time, cellular FB levels exceeded steady-state levels. Such accumulation would be consistent with inhibition of FB fluxes, both inward and outward, via equilibrative NT systems, without effect on Na+-dependent FB influx.2 In a similar manner, ara-A (and, consequently, ara-ATP) became concentrated in L1210/C2 cells during culture with inhibitors of equilibrative NT processes.

The experiment of Fig. 3 showed that 8 μM NBMPR, 15 μM dilazep, and 10 μM dipyrindamole inhibited to different extents FB permeation processes (approach to equilibrium, 5–7 min) in Na+-deficient medium. Dipyrindamole was the strongest inhibitor of FB influx mediated by the Na+-independent component of nucleoside influx, and also induced the largest accumulation of FB when the Na+-dependent system operated in the cells. Conversely, NBMPR inhibited Na+-independent FB influx the least and induced the lowest accumulation of FB in cells suspended in Na+-containing medium (Fig. 3). It is not known whether higher concentrations of NBMPR or dilazep induce cellular nucleoside accumulation to levels similar to those observed in the presence of 10 μM dipyrindamole. The cellular accumulation of FB following inhibition of equilibrative nucleoside transport suggests that the latter mediates substantial leakage from nucleoside pools in these cells. Earlier reports have described permeant accumulation in IEC-6 cells (cultured intestinal epithelial cells that express equilibrative and concentrative NT activities) in medium containing FB and 10 μM NBMPR (5), and during uptake of 3-O-methylglucose by chicken intestinal cells treated with cytochalasin B, an inhibitor of equilibrative glucose transport (36, 37).

Although the time-dependent decreases in levels of ara-A and ara-ATP that occurred in L1210/C2 cells incubated in the presence of dilazep (Fig. 4) probably reflected ara-A efflux from the cells, such decreases were not attributable to degradation of dilazep during the experiment, since parallel experiments with [3H]dilazep showed that 90% of the 3H activity comigrated with authentic dilazep on silica gel thin-layer chromatograms after 20 h of incubation at 37°C (data not shown). Dilazep has 2 basic nitrogen atoms and is a pH-related reduction in dilazep inhibition of ara-A efflux may have influenced ara-A and ara-ATP retention in those cultures (Figs. 4 and 5).

The measurement of ara-A movement into and out of L1210/C2 cells is complicated by the formation of phosphorylated metabolites of ara-A in the cells. The decline of ara-ATP levels in cells resuspended in medium without ara-A was associated with ara-A efflux. It appears that loss of cellular ara-A was followed by dephosphorylation of ara-ATP, presumably via 9-β-D-arabinofuranosyladenine 5’-monophosphate and 9-β-D-arabinofuranosyladenine 5’-diphosphate, and caused further outward movement of ara-A. When L1210/C2 cells were “loaded” with [3H]ara-ATP by incubation with 20 μM [3H]ara-A, the presence of 10 μM dipyrindamole or 15 μM dilazep, but not that

2 The sodium-dependent NT component in L1210 cells is insensitive to NBMPR and dipyrindamole at concentrations of 10 and 20 μM, respectively (40).
of 8 μM NBMPR, significantly prolonged ara-ATP retention (Fig. 5). Dipyridamole has been shown to have similar effects on the decay of cellular levels of 1-β-d-arabinofuranosylcytosine 5′-triphosphate in RPMI 6410 cells and HeLa cells, and the addition of dipyridamole to cell suspensions significantly prolonged ara-ATP retention in L1210/C2 cells (21, 40). Thus, the NT inhibitor-induced increase in cellular retention of ara-ATP was consistent with blockade by such inhibitors of ara-A exit from the cells, although this interpretation does not preclude the possibility of NT inhibitor effects on ara-A phosphorylation-dephosphorylation processes. The enhancement in the content and retention of ara-A and ara-ATP in L1210 cells has potential therapeutic implications, since ara-ATP synthesis and incorporation of ara-A residues into DNA are mechanisms involved in ara-A cytotoxicity (41–43).

In summary, this study demonstrated the operation in L1210/C2 cells of a concentrative, Na⁺-dependent NT system insensitive to inhibition by 10 μM dipyridamole, 8 μM NBMPR, or 15 μM dilazep. Inhibition of equilibrative fluxes in cells suspended in Na⁺-containing media resulted in the accumulation of FB, ara-A, and ara-ATP, and the retention of ara-ATP in L1210/C2 cells. The addition of dipyridamole to cell suspension media containing ara-A and dCF synergistically increased the antiproliferative activity of ara-A.

REFERENCES


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