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The Role of Nucleoside/Nucleotide Transport and Metabolism in the Uptake and Retention of 3'-Fluoro-3'-Deoxythymidine (FLT) in Human B-Lymphoblast Cells

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Abstract

Introduction—Recent studies in the human adenocarcinoma cell line A549 have identified cell growth dependent equilibrative nucleoside transporter-1 (hENT1) as a modifier of 3'-fluoro-3'-deoxythymidine (FLT) uptake and retention. In the present study, we used the ability to isolate human lymphoblastoid clones deficient in thymidine kinase 1 (TK1) to study how metabolism and nucleoside transport influence FLT uptake and retention.

Methods—Transport and metabolism of FLT were measured in the human lymphoblastoid cell line TK6 and in 8 clones isolated from TK6. Four clones were TK1-proficient while four were TK1-deficient. Both influx and efflux of FLT were measured under conditions where concentrative and equilibrative transport could be distinguished.

Results—Sodium-dependent concentrative FLT transport dominated over equilibrative transport mechanisms and while inhibition of hENT1 reduced FLT uptake, there were no correlations between clonal variations in hENT1 levels and FLT uptake. There was an absolute requirement of TK1 for concentration of FLT in TK6 cells. FLT uptake reached a peak after 60min incubation with FLT after which intracellular levels of FLT and FLT metabolites declined. Efflux was rapid and was associated with reductions in FLT and each of its metabolites. Both FLT and FLT-monophosphate were found in the efflux buffer.

Conclusions—Initial rates of FLT uptake were a function of both concentrative and equilibrative transporters. TK1 activity was an absolute requirement for the accumulation of FLT. Retention was dependent on nucleoside/nucleotide efflux and retrograde metabolism of FLT nucleotides.

Keywords

FLT Transport; FLT Metabolism; Nucleoside Transporters; Nucleotide Transporters

1. Introduction

The thymidine analog FLT (3'-fluoro-3'-deoxythymidine) has been extensively studied for its potential to detect changes in tumor proliferation [1–5]. Its use as a surrogate for tumor

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proliferation is based on the assumption that thymidine kinase-1 (TK1), a cell cycle dependent cytosolic enzyme, is the primary factor driving nucleoside uptake and retention in tumors [6]. Recent studies have shown that variations in nucleoside transporters can influence intracellular FLT levels and therefore need to be considered when interpreting FLT scans [7–10].

Previous work with the human adenocarcinoma cell line A549 demonstrated that FLT was transported into cells primarily by the equilibrative transporter hENT1 and that inhibition of hENT1 reduced overall FLT levels [8, 10]. We also reported that hENT1 levels increased as cells moved from plateau-phase culture to exponential growth [10]. In the present work, we used the ability to easily isolate individual clones deficient in TK1 activity from the human B-lymphoblast cell line TK6 to examine how FLT transport and metabolism influence uptake and retention of FLT. Unlike A549 cells, we observed no relationship between hENT1 levels and either FLT uptake or growth related changes in TK6 cells. However, we did identify other important FLT transport processes active in TK6 but not in A549, and we identified passive and active mechanisms that led to a rapid loss of FLT and FLT metabolites from these cells under exponential growth conditions.

2. Materials and Methods

2.1 Reagents

[methyl-³H]-3'-Deoxy-3'-fluorothymidine (³H]-FLT; 351.5GBq/mmol) and [³H]-S-(p-nitrobenzyl)-6-thioinosine (³H]-NBMMP; 740GBq/mmol) were purchased from Moravex Biochemicals (Brea, CA). High-purity nitrobenzylmercaptapurine ribonucleoside (NBMMP), and other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). Antibodies were purchased from Abcam (Cambridge, MA).

2.2 Cell Culture Conditions

The cell line TK6 was derived from a single human lymphoblastoid line, WIL2, which was isolated in 1968 [11]. Individual TK1-proficient and TK1-deficient TK6 clones were isolated as described in [12]. To select for TK1-deficient clones, cells were isolated in medium containing 2 µg/mL trifluorothymidine. Each trifluorothymidine-resistant clone was expanded in drug-free medium. Each clone was also analyzed for sensitivity to aminopterin, which is toxic to TK1-deficient cells [12]. Multiple independent clones were analyzed to reduce the possibility of any clone-specific effect influencing results. Cells were maintained in suspension at 37°C in an atmosphere of 5% CO₂/95% air in RPMI 1640 medium containing 10% fetal bovine serum.

2.3 Cell Doubling Time, BrdU Incorporation, and Ki67 Index

Cell doubling time was determined by counting cell numbers at daily intervals in flasks seeded at a concentration of 2×10^5 cells/mL. Cell line doubling times were calculated using Graphpad Prism Software (version 5.01). Cell cycle phase distributions were determined by BrdU-labeling and flow cytometry as previously described [10]. Ki67 indices were determined on parallel samples by staining with FITC-conjugated anti-Ki67 antibody and propidium iodide. Samples were analyzed on a Becton-Dickinson LSR2 cytometer using FACSDiva software (version 6.0). 20,000 events were collected for each sample, and data were analyzed with FlowJo software (version 9.0, Tree Star Inc., Ashland, OR, USA).

2.4 TK1 Activity Determination

10^7 cells were removed from culture media, washed once in ice-cold PBS, then incubated for 30min in ice-cold lysis buffer as described by Sherley and Kelly [13]. Cells were

vortexed, then centrifuged for 15min at $13,000 \times g$. Supernatants were kept on ice and assayed immediately or snap-frozen in liquid nitrogen and stored at -80°C for up to 2 weeks prior to analysis. Protein content in lysates was determined with a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL). Reactions were initiated by adding $15\mu\text{L}$ of reaction mix to $25\mu\text{L}$ of lysate and incubating at 37°C . Reaction mix was as described by Rasey et. al. [6] and Hruby et. al [14], and contained 10MBq/mL [methyl- ^3H]-thymidine (specific activity – 740GBq/mM). TK1-specific activity was distinguished from total TK activity by using reaction mix with or without 10mM deoxycytidine triphosphate as described by van der Wilt, et. al [15]. Reactions were terminated at 0, 5, 10, and 15 minutes by removing $8\mu\text{L}$ aliquots of reaction mixture to tubes containing $40\mu\text{L}$ of ice-cold 6% trichloroacetic acid (TCA). Samples were then vortexed for 20s, centrifuged at $13,000 \times g$ for 10min, and supernatants were removed to new tubes. Acidified supernatants were neutralized with saturated potassium bicarbonate, and relative levels of ^3H -thymidine and ^3H -thymidine-monophosphate were determined by HPLC analysis as described below, and as described by Huang et. al. [16].

2.5 FLT Transport Studies

FLT uptake and loss were carried out in either sodium-containing or sodium-free HEPES-buffered Ringer's solution as previously described [10]. For tracer influx, cells were removed from culture media, washed once with buffer, and assayed in a Coulter counter (Z2, Beckman Coulter). Aliquots of counted cells were incubated in buffer containing 74kBq/mL ($2\mu\text{Ci/mL}$) of ^3H -labeled FLT, with gentle rocking at 37°C . After incubating for times ranging from 30–120min, cells were pelleted by centrifuging for 1min at $13,000 \times g$. Pelleted cells were washed twice in ice-cold PBS, incubated in 5% Triton X-100 for 2 hours, and then radioactivity measured in a Tri-Carb 1900 liquid scintillation counter using Ultima Gold scintillation cocktail (Perkin Elmer) with appropriate corrections for background and quenching.

For measuring initial rates of FLT transport, the protocol was modified as follows: washed and counted cells were incubated in $200\mu\text{L}$ of buffer containing 74KBq/mL ^3H -FLT for 60s at 37°C , then influx was terminated by the addition of $1000\mu\text{L}$ of ice-cold PBS containing $100\mu\text{M}$ NBMPR and 10mM thymidine. Cells were pelleted, washed, and radioactivity was measured as described above. To study ENT-mediated transport, reactions were carried out in buffer containing $100\mu\text{M}$ NBMPR.

For efflux studies, cells were incubated for 1h in ^3H -FLT, washed twice in ice-cold PBS, and placed in buffer with or without Na^+ and/or $100\mu\text{M}$ NBMPR. Samples were incubated at 37°C for up to 120 min. Aliquots of cells were placed immediately into 5% Triton X-100 at the beginning of efflux to determine the starting amount of activity in the cells. Samples of efflux buffer were removed from other aliquots for scintillation counting. Activity remaining in the cells after efflux was also measured by solubilizing cell pellets in 5% Triton X-100 for 2 hours and taking samples for scintillation counting.

2.6 HPLC Analysis of Tracer Metabolites

Cells were treated as described above and then suspended in ice-cold 6% trichloroacetic acid (TCA) to measure FLT metabolites. Acidified cells were vortexed for 20s, incubated on ice for 10min, vortexed again, and centrifuged at $14,000 \times g$ for 10 min. The resulting supernatants were stored at -80°C for up to 2 weeks and neutralized with saturated potassium bicarbonate prior to HPLC analysis. Samples were analyzed using a Hewlett-Packard HP1050 HPLC system equipped with an in-line degasser (Alltech) and a Radiomatic 625TR flow scintillation analyzer (Perkin Elmer). A Waters Symmetry C_{18} $3.5\mu\text{m}$ ($150 \times 4.6\text{mm}$) column equipped with a NovaPak C_{18} guard column was used, and

maintained at 27°C for all assays. Mobile phase was delivered at 1.0 mL/min using the following gradient program: A–B (100:0) 0–4 min → (60:40) at 15 min → (40:60) at 35–45 min. Buffer A consisted of 10mM tetrabutylammonium hydroxide, 10mM KH₂PO₄, and 0.25% methanol, pH 6.9. Buffer B consisted of 6mM tetrabutylammonium hydroxide, 50mM KH₂PO₄, and 50% methanol, pH 7.0. Sample injection volume was 50µL, and UV absorbance was at 254nm. A standard mixture containing TdR, TMP, TDP, TTP, FLT, and FMAU was run prior to each assay to validate metabolite retention times. Details on the methods are described in [10].

3. Results

3.1 Variations in hENT1 levels were unrelated to growth characteristics

The parent TK6 cell line, four independently isolated clones of TK6, and four independently isolated trifluorothymidine-resistant clones were analyzed for a variety of cell growth characteristics as well as TK1 activity and hENT1 content (Table 1). There were only minor differences in cell doubling times, although the percentages of S phase cells were significantly lower in the trifluorothymidine-resistant clones ($P < 0.0001$). As expected, the trifluorothymidine-resistant clones had little or no measurable TK1 activity. There was also little or no BrdU incorporation in these clones. Ki67 levels varied from 58% to 85%. There were significant variations in hENT1 levels in the eight TK6 clones ($P < 0.0001$). The hENT1 values ranged from 3.3 to 11.5×10^7 NBMPR binding sites/µg protein. There was no obvious relationship between these variations and any of the growth characteristics of the clones. There was also no significant correlation between hENT1 abundance and TK1 activity ($P=0.24$; $r^2=0.54$).

3.2 Absolute requirement of TK1 for concentration of FLT in cells

FLT uptake following 1min incubation in buffer was used to estimate initial rate of FLT uptake in the 8 TK6 clones. Trifluorothymidine-resistant (TK1-deficient) clones had lower levels of uptake at 1min as compared to the TK1-proficient clones (Fig 1A). This was likely due to the rapid metabolism in TK1-proficient cells (Table 2). There was no statistically significant ($P=0.98$, $r^2=0.10$) correlation between the number of hENT1 sites and 1min FLT uptake levels for the 8 clones characterized. When the four TK1-proficient clones were evaluated separately, the relationship between the number of hENT1 sites and 1min FLT uptake levels, while not significant ($P=0.09$), showed a stronger correlation ($r^2 = 0.75$) suggesting a possible relationship that might be revealed by analyzing a larger data set. Therefore an additional 3 clones were isolated and evaluated. There was no correlation between hENT1 and FLT uptake in this larger group ($P=0.84$, $r^2=0.11$).

One hour incubation led to 8- to 15-fold concentration of FLT in TK1-proficient clones as compared to buffer levels (Fig 1B). Trifluorothymidine-resistant clones did not concentrate FLT (Fig 1B). There was no significant correlation between hENT1 levels and 60min uptake values for TK1-proficient and deficient clones evaluated together ($P=0.46$, $r^2=0.31$) or the TK1-proficient clones evaluated separately ($P=0.92$, $r^2=0.20$).

3.3 Na-dependent transport dominated in TK6

In separate experiments, 1min FLT uptake was measured in buffer containing no sodium in order to measure the contribution of concentrative transporters, and in buffer containing 100µM NBMPR to measure the contribution of equilibrative transporters to overall transport. Only TK6 and TK6-2, a TK1-deficient clone, were analyzed. Elimination of sodium inhibited 52% of FLT uptake in TK6 while 100µM NBMPR reduced FLT transport by 39%. For the TK1-deficient clone, TK6-2, elimination of sodium from the buffer inhibited FLT uptake by 33% while NBMPR inhibited uptake by 23% (Fig 2A). The

difference between the two clones likely reflected the metabolism that occurs rapidly in TK6 cells. The results were consistent with a greater contribution of concentrative transporters in defining initial rates of FLT uptake in TK6 cells. Incubation in sodium-free buffer supplemented by 100 μ M NBMPR reduced FLT uptake by 76% in TK6 cells and by 30% in TK6-2 consistent with FLT diffusion or residual transporter activity.

The transport inhibition studies were expanded to address the role of nucleoside transporters in modulating peak FLT intracellular concentrations by analysis of uptake after 1h incubation with FLT (Fig 2B). Here the magnitude of inhibition in TK6 cells associated with eliminating sodium or incubating with NBMPR was similar to the 1min time points (58% and 41%, respectively). Incubation in sodium-free buffer supplemented by 100 μ M NBMPR reduced FLT uptake by 69%. In the TK6-2 clones, elimination of sodium reduced FLT uptake by 25% in while incubation in NBMPR had no measurable effect on 1h FLT levels. Incubation in sodium-free buffer supplemented by 100 μ M NBMPR reduced FLT uptake in TK6-2 clones by less than 10%. Presumably, the smaller effect of transport inhibition in TK6-2 clones were a reflection of the inability of FLT to concentrate in these TK1-deficient clones; Diffusion over 1h reduced the effect of transporter inhibition.

3.4 Transporter mediated efflux of FLT

For TK6, there was an initial phase of increased intracellular tracer uptake for 1h (Fig 3A). Longer incubation in buffer containing FLT was associated with a reduction in intracellular radioactivity. After 2h incubation, the intracellular radioactivity was nearly 30% lower than the 1h concentration. The decline was not associated with any loss of membrane integrity as measured by Trypan blue exclusion studies and there was no reduction in activity for the TK1-deficient TK6-2 clones (Fig 3B). In contrast to the FLT observations, levels of radiolabeled thymidine and metabolites continued to increase at exponential rates (Fig 3A).

Efflux was measured in cells incubated in FLT for 1h and then maintained in nucleoside-free buffer for up to 2h. Under these conditions, the intracellular radioactivity in TK6 cells declined exponentially to reach levels close to the initial buffer by 2h after incubation in FLT-free buffer (Figure 3A). With the TK6-2 clone (Fig 3B) intracellular levels of FLT declined at a much slower rate.

Part of the efflux was mediated by NBMPR-sensitive transport. Incubating TK6 cells in FLT-free buffer containing NBMPR lead to increased FLT concentrations remaining in cells (Fig 2C). Incubation of TK6 cells in buffer containing no sodium increased FLT efflux slightly. Larger relative effects were observed in the TK6-2 cells. NBMPR doubled the FLT remaining in cells after a 1h efflux time.

3.5 Nucleoside metabolism

There was rapid metabolism of FLT to FLT-MP in TK6 cells (Table 2); half of the intracellular radioactivity after 1min incubation was FLT-MP. From 30min-120min, the relative proportion of intracellular FLT levels increased slowly from 2% to 8%. The proportion of intracellular activity associated with FLT-DP mirrored the increase in FLT while FLT-TP proportions increased in parallel to FLT-DP. The ratio of FLT-TP to FLT-DP was constant at 6.94 ± 0.15 over the 30–120min incubation. The ratio of FLT-TP to FLT was constant at 10.14 ± 2.48 over the same time. In contrast, FLT-MP proportions declined during this time from 56% to 25%.

Changes in the levels of different metabolites were measured in TK6 cells preloaded with FLT for 1h prior to incubation in FLT-free buffer (Table 2). Over the 120min incubation in FLT-free buffer, the total activity in cells declined by 85%. During this time, there was essentially no change in the relative proportions of intracellular FLT and its metabolites. The

mean (\pm sem) ratio of FLT-TP to FLT-DP at 6.13 ± 0.72 was not significantly different from the influx values ($p=0.35$). The corresponding ratios for FLT-TP to FLT-MP and FLT were 1.89 ± 0.16 and 17.13 ± 4.57 , respectively.

The buffer was analyzed for different FLT species that came out of the cells during the 120min efflux. About 50% of the total recovered activity was FLT and the remainder was FLT-MP. Incubation of cells with cyclosporine A, an inhibitor of P-glycoprotein [17], reduced the relative proportion of FLT-MP in buffer to 29% of the total activity recovered from the buffer with the remaining activity being FLT.

4. Discussion

While TK1 activity is considered to be the rate limiting factor driving FLT uptake and retention in tumors, recent publications suggest a role for nucleoside transporters as well [7, 10, 18]. Nucleoside transporter levels vary across different tissues and between different tumors [19–22]. They also can differ in cycling and non-cycling cells [10, 23, and 24]. In a previous publication [10], we reported that hENT1 levels increased as cells from the human tumor cell line A549 moved from a non-proliferative state into active cell growth. In the present study we examined whether there might be a general link between hENT1 levels and different cell growth parameters in actively growing cells. In our study, we took advantage of clonal variation in hENT1 levels to test this relationship. We observed no correlation between hENT1 levels and any cell growth characteristics suggesting no strong link between hENT1 levels and cell growth characteristics in actively-growing cells. Differences noted by us and others [10, 23, 24] might reflect movement of cells from a G0-state into active growth rather than changes in overall growth rate.

We also examined the relationship between hENT1 levels and FLT uptake in TK6 clones. Paproski et al. [8] had reported that reducing hENT gene expression in A549 cells reduced FLT uptake. We observed no correlation between hENT1 levels and either 1min or 60min uptake levels (Fig 1). The difference between our results and those of Paproski et al. [8] were probably due to the different repertoire of transporters used by A549 and TK6 cells. The predominant mechanism for FLT transport in A549 cells was reported to be by hENT1 [10] while that for TK6 was hCNT (Fig 2). As Paproski et al. [8] suggested, variations in hENT1 levels should have less effect on FLT uptake in cells where concentrative transport predominates. Our studies confirmed the absolute requirement of TK1 in concentrating FLT in cells. TK1-deficient clones did not concentrate FLT above the buffer level (Figs 1, 2).

The kinetics of uptake was qualitatively different in the TK6 cells from previously reported results in A549 human tumor cells [10, 25]. In A549 cells under exponential growth conditions, FLT activity levels increased rapidly up to about 30min and then increased more slowly for the second hour. In contrast, FLT activity in TK6 cells peaked after 60min in FLT-containing buffer (Fig 3A) and then declined slightly over the next 60min. The decline was due to a loss in activity associated with FLT-MP without compensatory changes in FLT or other FLT metabolites (Table 2). The effect was not observed with thymidine where intracellular activity associated with thymidine continued to increase over the 2h incubation.

To understand the basis for the loss in FLT-MP, we examined efflux of activity from cells incubated for 1h in buffer containing FLT, followed by incubation in FLT-free buffer for up to 2h. Removing FLT from the buffer led to a rapid loss of activity from cells. There was about a 70% loss of radioactivity from cells 60min after the transfer of cells to FLT-free buffer (Figure 3). By 2h, about 85% of the activity had been lost from cells. For comparison, Grierson et al. [25] reported a 50% drop in intracellular radioactivity after a 60min incubation of A549 cells in FLT-free medium.

In A549 cells, the loss of activity when cells were transferred to FLT-free medium was associated with reductions in FLT and FLT-MP and increases in FLT-TP [25]. About 80% of the activity recovered in the buffer was FLT with the remaining 20% being FLT-MP. In that publication [25], Grierson et al suggested that the primary factor underlying loss of activity was retrograde metabolism of FLT-MP back to the nucleoside, followed by its diffusion out of the cell. As for the observation of FLT-MP in the efflux material, Grierson et al. presented evidence to suggest that it might reflect some nucleotide transport process.

For TK6, the proportions of intracellular FLT and FLT metabolites remained constant over the 2h washout (Table 2), consistent with a loss of all four species over time. The primary species observed in the efflux buffer were FLT (49%) and FLT-MP (51%). The results are consistent with what Grierson et al. observed in A549 cells; there is both a retrograde metabolism of FLT-MP back to the nucleoside, followed by its diffusion out of the cell and a nucleotide transporter to pump FLT-MP out of cells. This diffusion of FLT out of TK6 cells was mediated in part by hENT since NBMPR inhibited FLT loss (Figure 2C). Guo et al. [26] reported that the multi-drug resistance protein MRP8, an ATP-binding cassette (ABC) transporter associated with p-glycoprotein, mediated the efflux of 5'-fluoro-2'-deoxyuridine monophosphate. Incubation of TK6 cells with cyclosporine A, an inhibitor of the ATPase activity of p-glycoprotein [17] reduced the relative levels of FLT-MP in buffer suggesting that MRP8 or a similar protein might mediate efflux of FLT-MP out of cells. Interestingly, cyclosporine A had little effect on overall activity lost from cells which would be consistent with retrograde metabolism of FLT-MP into FLT compensating for reduced FLT-MP efflux.

While the intracellular activity declined in TK6 cells incubated in FLT-free buffer, the relative proportions of FLT and its metabolites did not change. This observation supports retrograde metabolism of FLT-TP and FLT-DP in TK6 cells. There are a number of enzymatic activities that have been reported to catabolize deoxyuridine and thymidine triphosphates [27–29] that could account for the observed loss of FLT-TP. As the ratios of FLT-TP to FLT-DP were constant and the same both in the presence and absence of FLT in buffer, regulation of nucleotide pool concentrations might underlie the retrograde metabolism of these two nucleotide species.

The measurements of FLT uptake, metabolism, and efflux from TK6 cells suggest that it might not be easy to generalize FLT results from one tumor to another. While TK1 is an absolute requirement for FLT uptake, there are other activities such as nucleoside and nucleotide transporters as well as retrograde metabolism that should influence the kinetics of FLT uptake and loss. They might explain some of the FLT uptake variation reported for different tumor cell lines [30, 31]. Understanding the role of these additional non-TK1-related variables on FLT levels in vivo is needed to build confidence in our interpretation of clinical scans.

5. Conclusions

Unlike A549 cells, initial rates of FLT uptake in TK6 cells were a function of both concentrative and equilibrative transporters. Concentration of FLT was primarily dependent on TK1 activity. However, retention of FLT and FLT metabolites was a function of nucleoside and nucleotide efflux as well as retrograde metabolism of FLT nucleotides.

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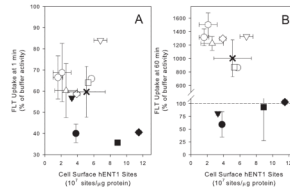


Figure 1. Comparison between cell surface hENT1 sites and intracellular concentration of FLT after (A) 1min and (B) 60min incubation with buffer containing [H-3] FLT. Uptake is expressed as a percentage of the activity in an equivalent volume of buffer. TK6 parent (x), TK1-proficient clones of TK6 (○), TK1-deficient TK6 clones (●).

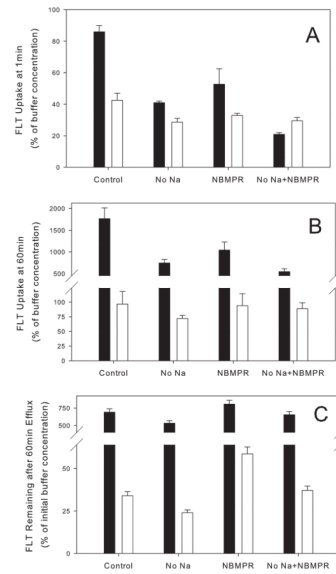


Figure 2. Inhibition of [H-3]FLT uptake in TK6 cells (filled bar) and in TK6-2, a TK1-deficient TK6 clone (open bar) by sodium-free buffer \pm 100 μ M NBMPR. Results are expressed as a percentage of the radioactivity normalized to the initial FLT levels in an equivalent volume of buffer. (A) 1min uptake values. (B) 60min uptake values. (C) FLT remaining in cells after a 60min efflux into FLT-free buffer.

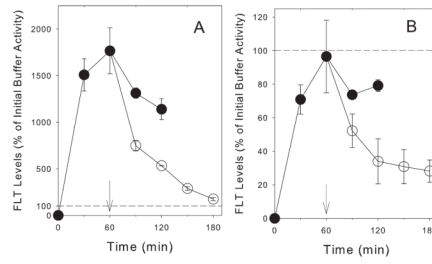


Figure 3.

Time-dependent changes in intracellular levels of radioactivity in (A) TK6 cells and (B) TK1-deficient TK6-2 cells incubated for up to 120min in [H-3]FLT (●), cells preloaded by incubation in [H-3]FLT for 60min, then washed and cultured in [H-3]FLT-free buffer for up to an additional 120 min (○), and cells incubated with [H-3]TdR (x). Solid line: Average initial [H-3]FLT activity in equivalent volume of buffer \pm 1sd around average (dashed lines).

Table 1

Characteristics of Cell Lines¹

Cell Line	Cell Doubling Time (h)	%S Phase Cells	% Ki67-Labeled Cells	% BrdU-Labeled Cells	TKI Activity (fmol dTMP/30 ² /ug protein)	hENT1 Sites (10 ⁷ sites/ug protein)
TK6 (parent)	20.4 ± 0.7	45.9 ± 2.3	90.7 ± 2.1	46.9 ± 2.9	193.9 ± 9.7	7.0 ± 1.5
Control Clones						
TK6-Clone T02	19.1 ± 0.3	55.1 ± 3.6	70.4 ± 5.1	46.4 ± 2.4	298.1 ± 46.1	5.7 ± 0.1
TK6-Clone T03	19.0 ± 0.3	57.1 ± 2.8	75.0 ± 7.0	46.0 ± 2.3	345.6 ± 17.1	6.8 ± 0.6
TK6-Clone T04	20.3 ± 0.5	57.7 ± 2.9	73.1 ± 6.3	39.2 ± 0.5	379.9 ± 34.8	5.3 ± 0.1
TK6-Clone T05	19.2 ± 0.4	59.7 ± 2.3	73.1 ± 2.8	43.4 ± 1.2	289.3 ± 23.6	3.9 ± 0.2
Mean ± sem	19.4 ± 0.3	57.4 ± 1.0	72.9 ± 1.0	43.8 ± 1.7	328.2 ± 21.2	5.43 ± 0.6
Trifluorothymidine-Resistant Clones						
TK6-Clone T2	21.3 ± 0.7	42.8 ± 4.0	84.8 ± 2.5	0.5 ± 4.0	5.0 ± 1.8	3.8 ± 0.1
TK6-Clone T4	18.3 ± 0.0	47.9 ± 1.8	87.3 ± 0.0	0.8 ± 1.8	NMA	3.3 ± 0.2
TK6-Clone T13	21.8 ± 0.2	43.6 ± 1.7	62.8 ± 10.1	2.4 ± 0.8	NMA	8.9 ± 0.3
TK6-Clone T14	20.6 ± 0.1	42.5 ± 1.7	58.4 ± 6.5	2.4 ± 0.9	NMA	11.5 ± 0.3
Mean ± sem	20.5 ± 0.8	44.2 ± 1.3	73.3 ± 7.4	1.5 ± 0.5	1.3 ± 1.3	6.9 ± 2.0

¹ TK6 is the parent cell line, TK6-T2 and TK6-T4 are independent TRIFLUOROTHYIMIDINE-resistant clones, and the remaining cells are independently isolated TK6 clones.² fmol dTMP/30³/μg protein³ 10⁷ sites/ug protein⁴ No measurable activity

Table 2

Metabolites of FLT

Influx min	Percent of Total Activity in Cells				Total Activity in Cells (DPM/10 ⁶ Cells)
	FLT	FLT-MP	FLT-DP	FLT-TP	
1	51.4	48.6	0	0	5663.6 ± 321.4
30	2.1	56.0	5.0	36.9	42358.4 ± 12023.3
60	5.3	44.4	6.5	43.7	48628.0 ± 11411.8
90	7.0	32.2	7.7	53.2	25003.0 ± 13803.4
120	8.1	25.4	8.5	58.0	34593.5 ± 7660.4
Efflux min	FLT	FLT-MP	FLT-DP	FLT-TP	
0	5.3	44.4	6.5	43.7	45689.9 ± 13501.1
30	2.1	31.0	10.2	56.6	22098.3 ± 3870.3
60	2.8	26.6	7.9	62.7	16547.4 ± 5189.7
90	7.1	31.3	11.2	50.4	10560.4 ± 3749.7
120	4.6	31.4	8.5	55.5	6816.9 ± 1521.7
Percent of Total Activity in Buffer					Total Activity in Buffer
Efflux min	FLT	FLT-MP	FLT-DP	FLT-TP	
120	49.0	51.0	0	0	89955.6 (89.6) ¹
120 ²	71.0	29.0	0	0	76463.2 (75.8) ¹

¹ Percentage of total activity recovered from cells and efflux buffer.² Efflux measured in FLT-free buffer containing Cyclosporine A.