Etoposide increases equilibrative nucleoside transporter 1 activity and fluorothymidine uptake: Screening of 60 cytotoxic agents

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Abstract. Equilibrative nucleoside transporter 1 (ENT1) is a major regulator for the uptake of [^{18}F]fluorothymidine ([^{18}F]FLT), a promising positron emission tomography tracer for treatment monitoring. Various antimetabolites such as 5-fluorouracil often increase ENT1 activity and [^{18}F]FLT uptake (flare) in spite of cell death. However, it has not yet been defined which agents induce [^{18}F]FLT flare and what is the role of [^{18}F]FLT flare in cell viability. Sixty cytotoxic agents from the LOPAC1280 library were screened for ENT1 activity in HeLa cells which predominantly express ENT1, and topoisomerase inhibitors (i.e., aurintricarboxylic acid, idarubicin, camptothecin and etoposide) were identified as potent inducers of ENT1 activity. The changes in ENT1 activity were closely correlated with [^{3}H]FLT uptake (Spearman's correlation coefficient, r=0.66; P<0.01). Etoposide significantly increased ENT1 activity and [^{3}H]FLT uptake accompanying cell cycle arrest at S/G2/M phase and the increase in TK1 expression and activity in both ENT1-low expressing HT29 and ENT1-high expressing MDA-MB-231 cells. The inhibition of ENT1 activity by dipyridamol or S-(p-nitrobenzyl)-6-thioinosine repressed the etoposide-induced cell death in HeLa cells, whereas it induced no changes in the other cell lines. In conclusion, etoposide is identified as a potent inducer for ENT1 activity and [^{3}H]FLT uptake. The role of ENT1 activity by etoposide was cell-type dependent, which requests caution for the application of ENT1-mediated [^{18}F]FLT flare for treatment monitoring.

Introduction

[^{18}F]Fluorothymidine ([^{18}F]FLT) is a promising positron emission tomography (PET) tracer for monitoring tumor size (1). As [^{18}F]FLT uptake is regulated by the salvage pathway for DNA synthesis involving equilibrative nucleoside transporter 1 (ENT1) and thymidine kinase 1 (TK1), the decrease in cell proliferation by drugs eventually reduces [^{18}F]FLT uptake. Yet, recent studies have reported that some cytotoxic agents induce an initial increase in [^{18}F]FLT uptake (known as [^{18}F]FLT flare) during cell death. These chemotherapy agents include antimetabolites, such as gemcitabine, 5-fluorouracil (5-FU), methotrexate and capecitabine (2-6). The mechanism underlying chemotherapy-induced [^{18}F]FLT flare may differ based on treatment schedules. Acute exposure to 5-FU induces the redistribution of ENT1 to the plasma membrane, whereas prolonged treatment with 5-FU increases the activity of ENT1 and TK1, along with S phase arrest (7,8). However, not all cytotoxic agents targeting DNA or inducing S phase arrest provoke [^{18}F]FLT flare. For example, cisplatin induces DNA damage by crosslinking, which does not increase FLT uptake (2,9,10). To date, no individualized evaluation of cytotoxic agents for [^{18}F]FLT flare has been undertaken.

ENT1 is the main nucleoside transporter responsible for FLT uptake, as it is widely expressed in mammalian cells and tissues (11). It bi-directionally transports purine and pyrimidine nucleosides in a sodium-independent manner (12). ENT1 differs from ENT2 in that it is sensitive to the inhibitor S-(p-nitrobenzyl)-6-thioinosine (NBTI) analogues. ENT1 activity is increased at the transcriptional level during S phase (13). It is also rapidly regulated at the post-transcriptional level via PKCδ/ε, which converts it to the active and available form at the plasma membrane (14). Studies on the pharmacological regulation of ENT1 have focused on the downregulation of ENT1 activity by cardioprotective agents, such as dilazep, draflazine and dipyridamole, and by cytotoxic agents, including immunosuppressive, anticancer, and antiviral compounds (15,16). Screening with protein kinase inhibitors showed that inhibitors of tyrosine kinases, protein kinase C, cyclin-dependent kinase, mTOR and p38 MAPK selectively decrease ENT1 and inhibit cell proliferation. The pharmacological mechanisms that increase ENT1 activity and lead to [^{18}F]FLT flare have not yet been examined.

Here, we aimed to identify pharmacologically active agents that increase ENT1 activity, and to investigate their effects on FLT uptake and cell proliferation. We screened 60 cytotoxic compounds from the LOPAC1280 library, which regulate apoptosis, cell cycle progression, DNA intercalation and DNA

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metabolism, and which may be involved in the regulation of cell viability or FLT metabolism (Table I). We also investigated the role of ENT1 activation for cytotoxic agent-induced cell death through screening. The induction of ENT1 activity and its role in cell death was confirmed in three types of cell lines with etoposide which was identified in this screening for the first time.

Materials and methods

Materials. HeLa, HT29 and MDA-MB-231 cells were obtained from the American Type Culture Collection (Rockville, MD). [^{3}H]S-(p-Nitrobenzyl)-6-thioinosine ([^{3}H]NBTI) (10 Ci/mmol) and [*methyl*-^{3}H]FLT (9.5 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). [*methyl*-^{3}H]thymidine (9.5 Ci/mmol) was from Perkin-Elmer (Waltham, MA). The LOPAC1280 library and other reagents were purchased from Sigma-Aldrich (St. Louis, MO) for screening ENT1 activity. Etoposide (E1383) used in confirmatory experiments of ENT1 activity was additionally purchased from Sigma-Aldrich.

Cell culture, treatment and preparation. Cells were maintained in RPMI-1640 containing 10% fetal bovine serum, 10 U/ml penicillin and 10 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. For screening, all of the agents were used initially at a concentration of 10 μM, but some agents that induced dramatic cell death within 24 h at 10 μM were used at a reduced concentration (1 μM for β-lapachone, CHM-1 and mitoxantrone; 0.5 μM for idarubicin). Cell lysates were prepared as described previously (7). Protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA).

ENT1 activity in the plasma membrane. The number of [^{3}H]NBTI binding sites/cell was determined according to Perumal *et al* (8). To determine the Kd and B_{max} values for each cell line, 1x10^6 cells were incubated with Hank's buffered salt solution containing [^{3}H]NBTI at a concentration of 0.12, 0.2, 0.47, 0.94, 1.88, 3.75, 7.50 or 15 nM for 1 h under gentle agitation. In a parallel series of samples, 20 μM S-(4-nitrobenzyl)-6-thioguanosine (NBTG) was included for the calculation of specific binding. After washing with Na^+-containing buffer, cells were resuspended in lysis buffer. The radioactivity of the lysates was measured in a liquid scintillation counter (Perkin-Elmer). Screening was performed with [^{3}H]NBTI at 4.6-fold higher than the Kd value of HeLa cells (3.75 nM) to economize the amount of isotope. Confirmatory studies were conducted for etoposide using 10 nM [^{3}H]NBTI, which is 6- to 15-fold higher than the Kd value of all the cell lines. Screening was repeatedly performed in five sets, with 10 agents/set (n≥4). In every set, the vehicle and 5-FU were included as internal controls (n=15).

Cell viability. Cells were plated at a density of 2x10^3 cells/well in 96-well plates, incubated overnight, and exposed to each drug for 72 h at the same concentration used for the screening of ENT1 activity. Cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay according to the manufacturer's instructions (Promega, Madison, WI). Three independent experiments were performed, each of which was performed in triplicate.

[^{3}H]FLT uptake assay. Twenty-four hours after seeding in 6-well plates at 3x10^5 cells/well, exponentially growing cells were exposed to agents for 24 h. The media were then removed and replaced with 1 ml fresh medium containing [^{3}H]FLT and incubated for a further 2 h. The radioactivity in the cells and the supernatants was measured in a liquid scintillation counter (Perkin-Elmer). An aliquot of each cell fraction was used for cell counting by trypan blue exclusion. [^{3}H]FLT uptake was calculated as 100 x CPM_{cells}/(CPM_{cells} + $CPM_{supernatants}$/1x10^5 viable cells. All experiments were independently repeated four times.

Flow cytometric analysis. Cells treated with vehicle or etoposide for 24 h were trypsinized, fixed in 70% ethanol, stained with propidium iodide, and subjected to flow cytometry (Becton Dickinson, San Jose, CA). Data obtained from the Particle Analysis System were processed with ModFit LT software (Verity Software House). All experiments were independently repeated three times.

Measurement of TK1 activity. TK1 activity was measured as described previously with slight modifications (7). Briefly, phosphate-buffered saline-washed cells were trypsinized, lysed, and differentially centrifuged to isolate the cytosolic fractions. Samples were mixed with an equal volume of reaction buffer containing [*methyl*-^{3}H]thymidine followed by incubation at 37˚C with gentle stirring. The slope of the time-activity curve was used to calculate the number of picomoles of phosphorylated thymidine/min/μg protein (pmol/min/μg).

Immunoblot analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed as described (7). Multiple analyses were performed with different sets of samples. Changes in expression levels were determined by scanning densitometry using Universal Hood II (Bio-Rad) and Quantity One software and normalized relative to the level of β-actin expression.

Statistical analysis. Data are expressed as the means ± SD. The Wilcoxon's signed-rank test and repeated measured ANOVA were used to compare treatments, and the Spearman's correlation coefficient was used to analyze ENT1 activity and [^{3}H]FLT uptake data using the software package SPSS 12.0 ($r \geq 0.70$, strong correlation; $0.5 \leq r < 0.7$, moderately strong correlation; $0.3 \leq r < 0.5$, weak-to-moderate correlation; and $0.1 \leq r < 0.3$, weak correlation) (17). Statistical significance was set at $P<0.05$ or $P<0.01$ as indicated in the Results section. Prism software (version 4.03; GraphPad, San Diego, CA) was used for calculation of the B_{max} and Kd by curve fitting according to the equation: $Y = B_{max} \times X/(Kd + X)$, where Y and X are the specific binding and [^{3}H]NBTI concentration in nmol/l, respectively.

Results

Screening of 60 cytotoxic agents for ENT1 activity. Screening of 60 cytotoxic agents known to induce apoptosis, inhibit cell cycle progression, increase DNA intercalation, or inhibit DNA metabolism for ENT1 activity was performed using HeLa cells (Table I). As HeLa cells predominantly express the ENT1 rather

Table I. The cytotoxic agents and their representative targets.

Agent	Target
Altretamine	Antineoplastic
3-Aminobenzamide	Inhibits poly(ADP-ribose) synthetase
4-Amino-1,8-naphthalimide	Inhibits poly(ADP-ribose) polymerase
Amsacrine	Inhibits topoisomerase II
Ancitabine	Antineoplastic, metabolized to cytarabine
Apigenin	G2/M phase arrest
Aurintricarboxylic acid	Inhibits topoisomerase II
5-Azacytidine	Inhibits DNA methyltransferase
Azelaic acid	Inhibits mitochondrial oxidoreductases
Bay 11-7085	Inhibits NFκB
Benzamide	Inhibits poly(ADP-ribose) synthetase
O^6-Benzylguanine	Inhibits O6-alkylguanine-DNA alkyltransferase
β-Lapachone	Induces apoptosis
5-Bromo-2'-deoxyuridine	Incorporates into DNA
Caffeic acid phenethyl ester	Inhibits NFκB
(S)-(+)-camptothecin	Inhibits topoisomerase II
Carboplatin	Antineoplastic
Carmustine	Alkylates DNA; causes interstrand crosslinks
CB 1954	Antineoplastic
CCT007093	PPM1D inhibitor
Chlorambucil	Crosslinks DNA
Chloroquine	Binds strongly to double-stranded DNA
CHM-1 hydrate	Binds tubulin and inhibits tubulin polymerization
Cisplatin	Crosslinks DNA
Cyclophosphamide	Crosslinks DNA
Cytosine-1-β-D-arabinofuranoside	Inhibits DNA synthesis
Daidzein	Inhibits mitochondrial aldehyde dehydrogenase; G1-phase arrest
Ellipticine	Inhibits topoisomerase II
Emetine	Inhibits RNA-protein translation
Etoposide	Inhibits topoisomerase II
10058-F4	Inhibits c-Myc-Max interaction; prevents c-Myc target gene transactivation
5-Fluoro-5'-deoxyuridine	Inhibits DNA synthesis
5-Fluorouracil	Inhibits thymidylate synthetase; S phase arrest
Fusidic acid	Inhibits protein synthesis
Ganciclovir	Pro-drug nucleoside analogue
Gossypol	Inhibits PKC
Hydroxyurea	Inactivates ribonucleoside reductase
Idarubicin	Inhibits topoisomerase II
1,5-Isoquinolinediol	Inhibits poly(ADP-ribose) synthetase
L-Mimosine	Iron chelator that inhibits DNA replication; G1-phase arrest
MDL 28170	Inhibits calpain I and II
Melphalan	Crosslinks DNA
Methotrexate	Folic acid antagonist
Minocycline	Inhibits basement membrane protease
m-Iodobenzylguanidine	Inhibits ADP ribosylation, mitochondrial membrane potential
Mitoxantrone	Inhibits DNA synthesis
Mizoribine	Inhibits inosine monophosphate dehydrogenase; G/S arrest
Nimustine	Alkylates DNA
PAC-1	Activates caspase-3
p-Benzoquinone	Inhibits topoisomerase II
Phosphonoacetic acid	Inhibits DNA polymerase
Pifithrin-μ	Inhibits p53 binding to mitochodria, Bcl-xL, and Bcl-2
Pyrocatechol	Causes DNA strand breakage
Retinoic acid	Induces caspase-dependent apoptosis
Retinoic acid p-hydroxyanilide	Vitamin A acid analogue
Ribavirin	Inhibits of inosine monophosphate dehydrogenase
Se-(methyl)selenocysteine	Chemopreventive agent
SU 9516	Inhibits cyclin-dependent kinase-2
TG003	Inhibits cdc2-like kinase

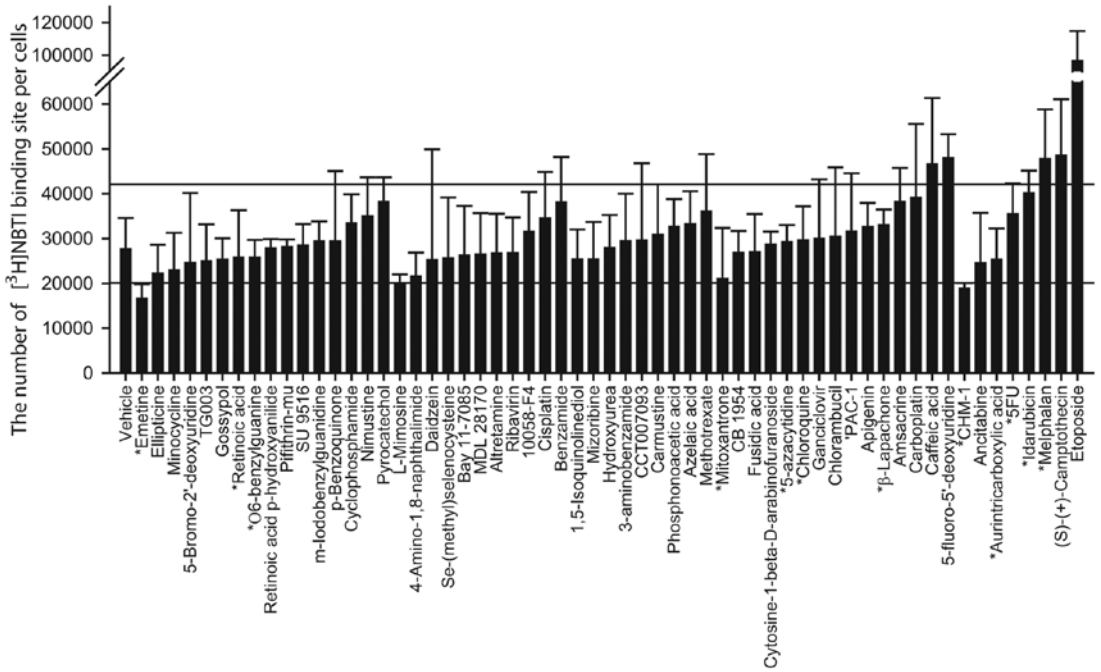

Figure 1. Screening of 60 cytotoxic agents for ENT1 activity and viability in HeLa cells. Cells were exposed to agents that induce apoptosis, inhibit cell cycle progression, increase DNA intercalation, or inhibit DNA repair (Table I) for 24 h at 10 μM (with the exception of 1 μM for β-lapachone, CHM-1 and mitoxantrone; and 0.5 μM for idarubicin). The number of [^{3}H]NBTI binding sites/cell was independently measured using vehicle and 5-FU as internal controls. Data are expressed as the means ± SD, and the lines indicate ± 2 SD from vehicle-treated samples. *Agents used in Fig. 3.

than other nucleoside transporter isotypes (14), ENT1 activity is expected to reflect nucleoside dynamics. In general, it is recommended that [^{18}F]FLT-PET not be performed immediately after treatment to avoid off-target effects. Thus, the equilibrative response to agents was evaluated after 24 h of treatment. Most of the agents studied had a minimal effect on ENT1 activity (Fig. 1). Emetine, ellipticine, and minocycline decreased ENT1 activity by 40%. The agents that increased ENT1 activity by >2 standard deviations were 5-FU (DNA metabolism inhibition), melphalan (DNA alkylation), camptothecin (topoisomerase I inhibition), idarubicin (topoisomerase II inhibition), and etoposide (topoisomerase II inhibition).

Changes in the cell viability induced by the 60 cytotoxic agents. Cell viability changes in HeLa cells in response to the screened agents were measured to determine whether the changes in ENT1 activity correlated with cell death. All of the agents were used at the same concentrations as indicated in Fig. 1, and were incubated with the cells for 72 h. Of the compounds tested, 12% reduced the relative cell viability by >60%, 24% reduced the relative viability by between 20 and 60%, and 64% reduced relative viability by <20% (Fig. 2A). Plotting the relative cell viability vs. ENT1 activity revealed that the two parameters showed no correlation (Fig. 2B).

Changes in [^{3}H]FLT uptake correlate with ENT1 activity. Uptake changes for [^{3}H]FLT were examined to investigate whether the changes in ENT1 activity by pharmacologically active agents influence [^{18}F]FLT-PET. We randomly selected 14 agents and measured [^{3}H]FLT uptake after 24 h of exposure (Fig. 3A). Drug treatment changed [^{3}H]FLT uptake from -16 to 111% compared with vehicle treatment. [^{3}H]FLT uptake was highly correlated with the relative changes in ENT1 activity (Spearman's correlation coefficient, r=0.66; P<0.01) (Fig. 3B).

Confirmation of etoposide-induced ENT1 activity and uptake of [^{3}H]FLT in HeLa, HT29 and MDA-MB-231 cells. As etoposide effectively increased ENT1 activity in the screening (Fig. 1), we confirmed the effect of etoposide in HT29 cells (a ENT1-low expressing cell line) and MDA-MB-231 cells (a ENT1-high expressing cell line) (18). We confirmed the concentration of ligand required to reach half maximal binding (Kd, affinity) and the maximal binding (B_{max}, the number of transporters) in our systems and calculated these values as 1.626 and 188,587 for MDA-MB-231 cells, 0.808 and 60,996 for HeLa cells, and 0.643 and 46,111 for HT29 cells, respectively (Fig. 4A). The number of [^{3}H]NBTI binding sites/cell increased 3.45 and 2.83 times after etoposide treatment in HT29 and MDA-MB-231 cells, respectively (Fig. 4B) (P<0.05). In parallel, the basal level (vehicle-treated) of [^{3}H]FLT uptake was higher in MDA-MB-231 cells than in HT29 cells. Treatment with etoposide for 24 h induced the increase in [^{3}H]FLT uptake by 49% in HT29 cells and 38% in MDA-MB-231 cells (Fig. 4C) (P<0.05), which was correlated with a change in ENT1 activity.

Etoposide-induced cell cycle changes in HeLa, HT29 and MDA-MB-231 cells. In order to investigate the molecular mechanism for the increase in ENT1 activity by etoposide, we measured the etoposide-induced effect on the cell cycle. Etoposide significantly increased the fraction of HeLa, HT29 and MDA-MB-231 cells in the S and G2M phases by ~2-fold after 24 h of treatment (Fig. 5A) accompanied by an increase in TK1 activity (Fig. 5B) (P<0.05 for HT29 cells; P<0.01 for

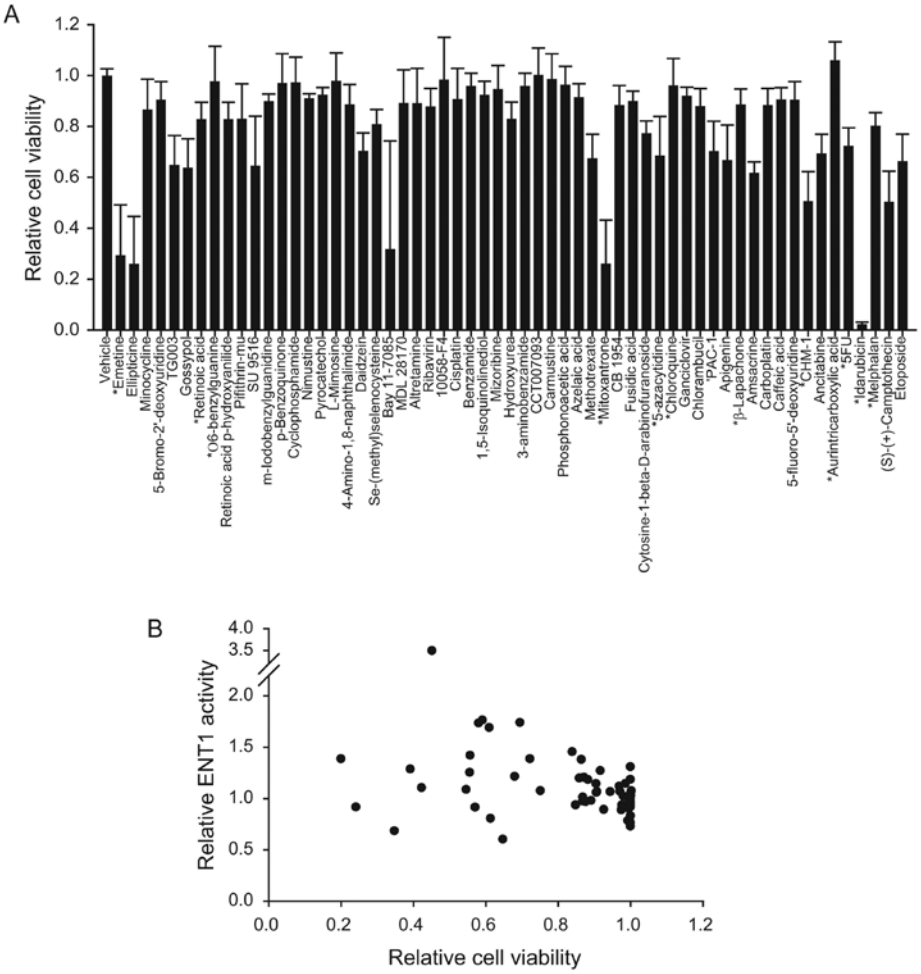

Figure 2. Screening of 60 cytotoxic agents for changes in the viability of HeLa cells. (A) Cells were exposed to the indicated agents for 72 h and cell viability was measured using an MTS assay (n=3). Data are expressed as the means ± SD. (B) Relative viability was plotted against relative ENT1 activity at 24 h using data from Fig. 1.

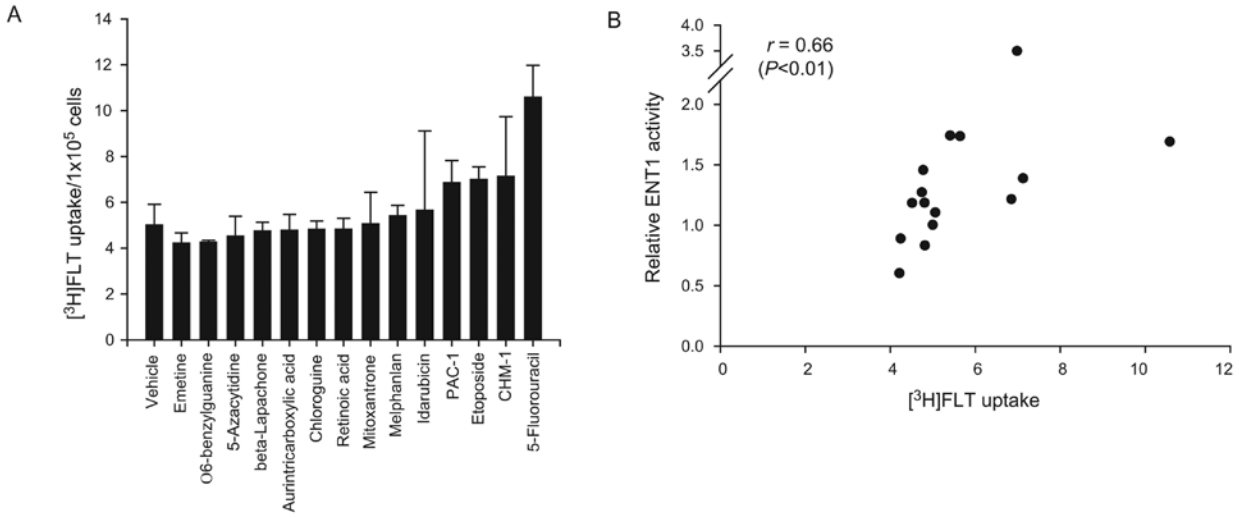

Figure 3. Changes in [^{3}H]FLT uptake in relation to ENT1 activity. (A) Cells were exposed to 14 cytotoxic agents from Fig. 1 for 24 h. After incubation with [^{3}H]FLT in fresh media for an additional 2 h, the uptake of [^{3}H]FLT was determined and normalized to 1x10^6 cells (n=4). Data are expressed as the means ± SD. (B) [^{3}H]FLT uptake was plotted against relative ENT1 activity from Fig. 1. Spearman correlation coefficient (r) and P-value are shown.

MDA-MB-231 cells). Time-dependent analysis showed that TK1 expression significantly increased at 24 h in HeLa and HT29 cells, and remained elevated until 48 h post-stimulation (Fig. 5C). The expression of cyclin A was slightly increased by

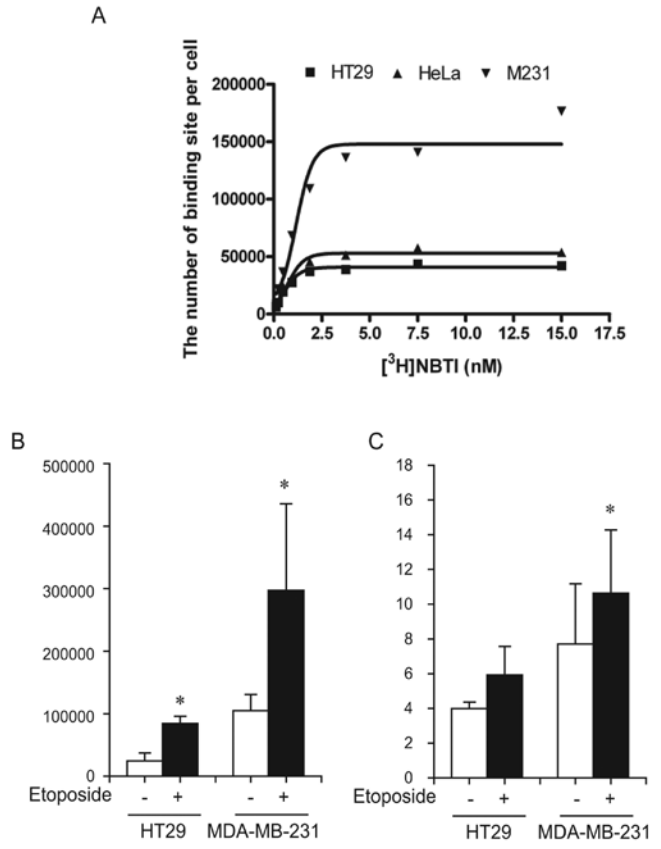

Figure 4. Etoposide-induced changes in ENT1 activity and [^{3}H]FLT uptake in HeLa, HT29 and MDA-MB-231 cells. (A) Exponentially growing cells were trypsinized and incubated with various concentrations of [^{3}H]NBTI either with or without 20 μM NBTG. The number of specific binding sites for [^{3}H]NBTI/cell was determined. Results were independently confirmed and a representative one is shown. (B and C) Cells were exposed to vehicle or 10 μM etoposide for 24 h and were subjected to measurement of ENT1 activity with (B) 0 nM [^{3}H]NBTI or (C) [^{3}H]FLT uptake. Data are expressed as the means ± SD. *P<0.05 compared with vehicle-treated samples.

etoposide in HeLa cells, whereas it was significantly increased in HT29 cells.

Combination effect of ENT1 inhibitors on etoposide-induced cytotoxicity in HeLa, HT29 and MDA-MB-231 cells. In order to apply ENT1-mediated [^{18}F]FLT flare to monitor therapeutic response, the mechanism for ENT1 activation should be related with the cell death pathway. We hypothesized that the inhibition of ENT1 activity may affect etoposide-induced cytotoxicity when the increase in ENT1 activity by etoposide contributes to the increase in salvage capacity and resistance mechanism. We monitored the etoposide-induced cell viability with dipyridamol or NBTI, inhibitors for the nucleoside transporter, after a 72-h treatment (Fig. 6). Etoposide dose-dependently decreased cell viability in three cell lines (P<0.05). The maximum cytotoxic effect was highest in HT29 cells with 41.3%. The HeLa cells treated with dipyridamol or NBTI alone showed a slight decrease in viability. The HeLa cells that were co-treated with dipyridamol or NBTI did not show a dose-dependent cytotoxic effect for etoposide, suggesting the possibility that the inhibitors repress the dose-dependent cytotoxic effect of etoposide or that the cytotoxic effect of the inhibitor is dominant over that of etoposide. However, dipyridamol or NBTI did not exhibit any changes in etoposide-induced cell death in the HT29 and MDA-MB-231 cells. These results indicate that the role of ENT1 activation by etoposide is cell type-dependent and could be downstream from the pathway that is subordinate or irrelevant to cell death.

Discussion

A recent study showed that various cytotoxic drugs induce [^{18}F]FLT flare, which raises caution concerning the schedule of dosing and imaging. ENT1 is a part of the salvage pathway for DNA synthesis and is an indispensable mediator of FLT transportation. However, extensive screening for the pharmacological regulation of ENT1 activity in relation to [^{18}F]FLT flare has not yet been performed. In this study, we identified ENT1 modulators by screening 60 cytotoxic agents, and showed that pharmacological agent-induced changes in ENT1 activity correlate with [^{3}H]FLT uptake. We specifically confirmed that etoposide-induced increases in ENT1 activity were due to cell cycle arrest in the proliferation phase.

To date, no adequate assay system for evaluating ENT1 activity on a large scale has been established, which has limited the availability of information regarding the regulation of ENT1. A previous study of ENT1 activity induced by 22 protein kinase inhibitors conducted by the Graves group used a [^{3}H]uridine uptake assay (16). This assay has advantages in that it is simple and only a small number of cells are required. However, it was not suitable for our screening, as the incubation time for [^{3}H]uridine uptake assay is approximately 30 sec and a large number of samples could provoke prolonged washout time leading to [^{3}H]uridine efflux into the media. In this study, we adopted a [^{3}H]NBTI binding assay as NBTI inhibits selective [^{3}H]NBTI binding to ENT1 in the plasma membrane and we could stably analyze up to 10 agents at once. In all sets, vehicle and 5-FU were included as internal controls. However, the method still has some limitations in that the standard deviation was relatively high. The values for vehicle- and 5-FU-treated samples were 27,606.4±6,948.4 and 46,595.8±14,776.0, respectively. Given that the ideal assay for screening has a Z factor >0.5 (19), the overlap between the positive and the negative controls makes it difficult to identify agents that induce ENT1 activity to a lesser extent than 5-FU. This may be due to the fact that the [^{3}H]NBTI binding assay requires multiple steps for each individual sample. The development of a more accurate assay system, such as a 96-well plate-based ligand binding assay, would allow confirmation of the present findings.

Our data demonstrated for the first time that topoisomerase I/II inhibitors such as aurintricarboxylic acid, idarubicin, camptothecin and etoposide potently increase ENT1 activity at a level comparable with, or higher than, 5-FU. The increase in ENT1 activity mediated by etoposide was confirmed in MDA-MB-231 and HT29 cells. Yet, not all of the topoisomerase inhibitors altered ENT1 activity (amsacrine, ellipticine, and p-benzoquinone). Similarly, some antimetabolites minimally changed ENT1 activity within a 2 SD variation (ribavirin, methotrexate and ganciclovir) in contrast to 5-FU. In addition, the inducers for cell cycle at S phase such as hydroxyurea (21) and mizoribine (22) did not increase ENT1 activity in this system. It is thought that the potency or duration of ENT1

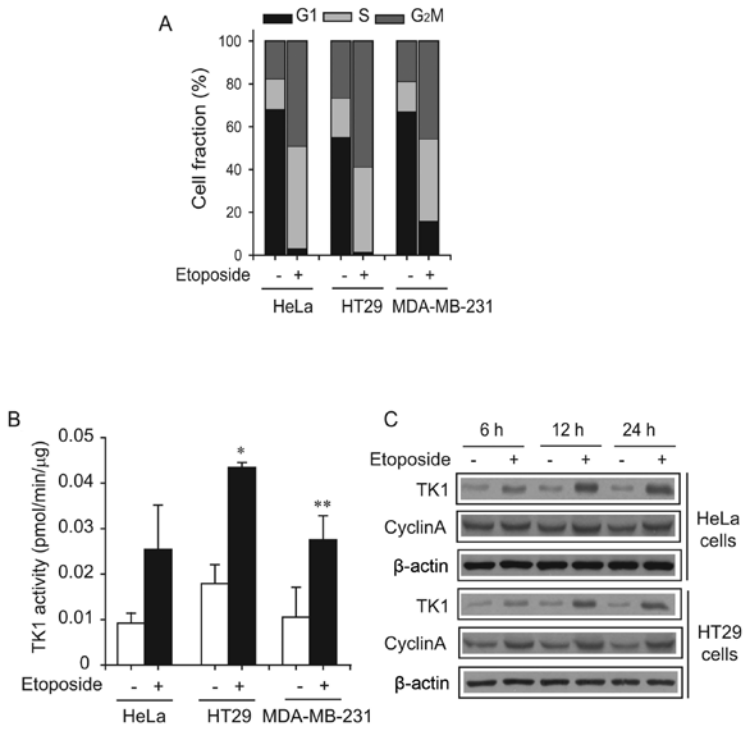

Figure 5. Cell cycle changes in HeLa, HT29 and MDA-MB-231 cells by etoposide. Cells were treated with vehicle or 10 μM etoposide for 24 h and subjected to the measurement of the cell cycle distribution by propidium iodide staining (A) and TK1 activity (B). Time-dependent changes in TK1 and cyclin A expression were monitored after incubation of cells with vehicle (-) or 10 μM etoposide (+) for 6, 24 or 48 h by immunoblot analysis (C). All experiments were independently repeated 3 times. Data are expressed as the mean ± SD. *P<0.05; **P<0.01 compared with vehicle-treated samples.

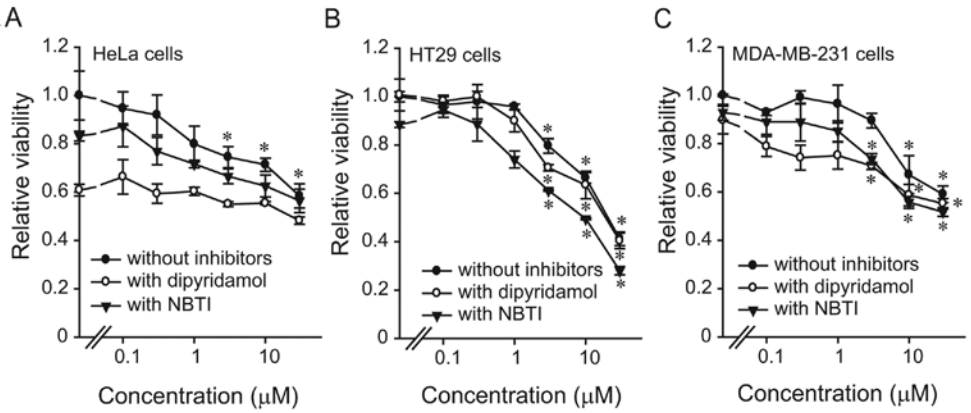

Figure 6. Effect of the combination of etoposide with ENT1 inhibitors on the cell viability of HeLa, HT29 and MDA-MB-231 cells. Cells were exposed to etoposide at a concentration of 0, 0.1, 0.3, 1, 3, 10, or 30 μM, without inhibitors (black circle), with 10 μM dipyridamol (white circle) or 10 μM NBTI (black triangle) for 72 h. The viability was measured with MTS assay. All experiments were repeatedly confirmed. Data are expressed as the means ± SD. *P<0.05 compared with the samples treated with 0 μM etoposide (DMSO only) for each treated group.

activation by each agent would be different as we used a fixed concentration for screening and obtained various ranges of cytotoxicity (Fig. 2). When agents are used at the isotoxic dose in each cell lines it is expected that they produce a similar tendency for ENT1 activation.

The possibility has been raised that [^{18}F]FLT flare could be used for the treatment monitoring of the thymidylate synthase inhibitor (3,20). However it has never been examined whether [^{18}F]FLT flare reflects the degree of treatment response. Our results showed that ENT1 activity did not correlate with cell viability measured by MTS, but highly correlated with [^{3}H]FLT uptake based on the data from 14 randomly selected agents (Figs. 2 and 3). The increase in ENT1 activity may contribute to the influx of thymidine into the cytoplasm, the increase in salvage capacity, and eventually treatment failure. Our study on etoposide revealed that etoposide-induced ENT1 activation was cell-type dependent (Fig. 6). In HeLa cells, the blocking of ENT1 activity repressed etoposide-induced cell death in HeLa cells, indicating ENT1 activation contributes to an etoposide-induced mechanism for HeLa cell death. In contrast, ENT1 inhibitors had no effect on etoposide-induced cell death in HT29 and MDA-MB-231 cells, suggesting that the pathway

for ENT1 activation may be irrelevant to cell death. If anticancer agents may affect ENT1 activity independent of the cell death pathway, FLT uptake may not reflect anticancer agent-induced changes in cell viability. Therefore, it would be better to monitor the effect of anticancer agents after a prolonged period of exposure to avoid the ENT1-mediated effect on FLT uptake. Additionally, the kinetics of ENT1 activity induced by anticancer agents should be carefully investigated.

Our studies found that the modulation of ENT1 activity by pharmacological agents is closely related to [^{3}H]FLT uptake. Furthermore, we demonstrated that etoposide is a potent inducer of ENT1 activity, which is accompanied by an increase in TK1 activity and [^{3}H]FLT flare. This study provides valuable information regarding the possible application of FLT-PET for etoposide-based chemotherapy.

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