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Tracking cellular stress with labeled FMAU reflects changes in mitochondrial TK2

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Abstract

Purpose—Fluoropyrimidines like 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-thymine (FMAU) and 3′-deoxy-3′-fluorothymidine (FLT) accumulate in tumors and are being used as positron emission tomography tumor-imaging tracers. Proliferating tissues with high thymidine kinase 1 (TK1) activity retain FLT; however, the mechanism of selective accumulation of FMAU in tumors and certain other tissues requires further study.

Methods—Retention of [^{3}H]FLT and [^{3}H]FMAU was measured in prostate cancer cell lines PC3, LNCaP, DU145, and the breast cancer cell line MD-MBA-231, and the tracer metabolites were analyzed by high-performance liquid chromatography (HPLC). FMAU retention, thymidine kinase 2 (TK2) activity, and mitochondrial mass were determined in cells stressed by depleted cell culture medium or by treating with oxidative, reductive, and energy stress, or specific adenosine monophosphate-activated protein kinase activator, or eIF2 inhibitor. TK1 and TK2 activities and mitochondrial mass were determined by FLT phosphorylation, 1-β-D-arabinofuranosylthymine (Ara-T) phosphorylation, and flow cytometry, respectively.

Results—FMAU retention in rapidly proliferating cancer cell lines was five to ten times lower than FLT after 10 min incubation. HPLC analysis of the cellular extracts showed that phosphorylated tracers are the main retained metabolites. Nutritional stress decreased TK1 activity and FLT retention but increased retained FMAU. TK2 inhibition decreased FMAU retention and phosphorylation with negligible effects on FLT. Oxidative, reductive, or energy stress increased FMAU retention and correlated with mitochondrial mass (r^2= 0.88, p=0.006). FMAU phosphorylation correlated with increased TK2 activity (r^2=0.87, p=0.0002).

Conclusion—FMAU is preferably phosphorylated by TK2 and can track TK2 activity and mitochondrial mass in cellular stress. FMAU may provide an early marker of treatment effects.

Keywords

FMAU; Positron emission tomography; Thymidine kinase 2; Stress; Cancer

Introduction

Positron emission tomography (PET) is used to image the distribution of radiolabeled compounds within the body. One group of compounds used in PET is fluoropyrimidines. While [^{11}C]thymidine can be used for PET imaging, the short half-life of [^{11}C] (approximately 20 min) and rapid metabolism by thymidine phosphorylase in the blood limit

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its applications. Thymidine phosphorylase-resistant fluoropyrimidines have been developed to overcome those limitations. These compounds can be labeled with [^{18}F] (with a half-life of about 110 min) making them more appealing agents for imaging purposes. An example of fluoropyrimidines in PET imaging is 3′-deoxy-3′-fluorothymidine (FLT), which is successfully used in oncology [1–3]. It is a specific substrate of thymidine kinase 1 (TK1), and its tissue uptake is correlated with cellular proliferation [2, 4, 5], which makes it a useful tool to measure the tumor proliferation rate. However, this limits its application in detecting metastatic foci in normal tissues with a high proliferation rate, such as bone marrow [6]. Unlike FLT, [1-(2′-deoxy-2′-fluoro-beta-D-arabinofuranosyl)-thymine (FMAU)] was developed to image tumor proliferation, but it has low baseline marrow uptake in patients [7, 8]. A more complete understanding of the mechanisms of FLT and FMAU retention is needed to understand these differences. Previous studies on thymidine kinase activities in the cytosolic and mitochondrial cell fractions and recombinant human TK1 imply that FMAU is a better substrate for thymidine kinase 2 (TK2) compared to TK1 in humans (2.7 times faster phosphorylation by mitochondrial fraction and 4.7 times smaller Km compared to recombinant human TK1 [9, 10]. It has been previously demonstrated that TK1 can phosphorylate thymidine and FLT more efficiently compared to FMAU 40 and 20 times, respectively [11]. However, due to the high levels of TK1 in proliferating tissues, the relative contribution of TK2 in retaining FMAU in proliferating cells has not been studied. The availability of the TK1-specific substrate (FLT) TK2-specific substrate (Ara-T), and TK2 inhibitor [bromovynil deoxyuridine (BVDU)] [12–14], provided the needed tools to measure the role of TK2 in FMAU retention. Previous experiments with H_2O_2 and certain antineoplastic treatments have shown to increase mitochondrial DNA and mass [15–19]. These changes were assumed to be due to oxidative stress. In the current study, it was hypothesized that since TK2 is a mitochondrial enzyme, its activity might be increased in cellular stress. The results revealed that both FLT and FMAU are metabolically retained in cells primarily in the phosphorylated forms, but unlike FLT, FMAU retention is primarily due to TK2 activity even in the proliferating cells with high TK1 activity. It also shows that FMAU retention increases not only after oxidative stress but also after different types of stress involving various pathways. Furthermore, increased FMAU phosphorylation is correlated with the activity of mitochondrial thymidine kinase (TK2) and mitochondrial mass, indicating the potential for tracking cellular stress and mitochondrial mass with [^{18}F]FMAU PET imaging.

Materials and methods

Cell culture

Human prostate cancer cell lines PC3 and LNCaP were obtained from the American Tissue and Cell Culture (Manassas, VA, USA). The human prostate cancer cell line DU145 and the human breast cancer cell line MD-MBA-231 are gifts from Dr. Q. Ping Dou (Karmanos Cancer Institute, Detroit, MI, USA). Cells were grown in monolayers with Roswell Park Memorial Institute (RPMI)-1640 with 10% fetal bovine serum and 1% penicillin and streptomycin (Invitrogen, Grand Island, NY, USA) and grown at 37°C with 5% CO_2. Cell number was determined after trypsinizing (Invitrogen, St. Louis, MO, USA), trypan blue staining, and counting on a hemocytometer and then counting the number of viable (unstained) and dead (stained) cells. Mycoplasma infections were ruled out by Mycotect Kit™ (Invitrogen, St. Louis, MO, USA).

Uptake measurements

Cells were incubated in six-well cell culture plates with 1,600 Bq [^{3}H]FMAU [96% pure by high-performance liquid chromatography (HPLC), specific activity 2.1 Ci/mmol; Moravek, Brea, CA, USA] or [^{3}H]FLT (purity of 98% by HPLC, specific activity 34 Ci/mmol;

Moravek) at 37°C for the stated time period of each test. To measure total cellular uptake, the cells were washed with a solution containing 50% ethylene glycol (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS; Invitrogen, NY, USA) three times to stop further transmembrane transport and phosphorylation quickly. To slow down the transport and reactions, the washing solution was kept as cold as possible by keeping it on dry ice until it started to freeze (−34°C). After washing, the cells were lysed by 1 M KOH, mixed with ten times the lysate volume with Hionic-Fluor scintillation cocktail (Perkin-Elmer, Waltham, MA, USA), and the activity of samples was measured by a Packard Tricarb 2100TR liquid scintillation analyzer (Perkin-Elmer). To measure the retention of trapped tracers, the cells were washed with 37°C RPMI-1640 three times, allowing the tracers that are not trapped to leave the cells. To measure DNA incorporation, 1 M perchloric acid was added to the cells and centrifuged for 20 min at 14,000×*g* (Eppendorf centrifuge 5417R, Westbury, NY, USA). The pellet was washed with 100% 0°C ethanol (Sigma-Aldrich) three times, spun after each wash for 20 min at 14,000×*g*, and dissolved in 1 M KOH; and then the activity was measured by the method above. To block FMAU incorporation into the DNA by inhibiting DNA polymerase gamma, dideoxycytidine (ddC) was used [20]. Cells were incubated for 2 h with 200 μM ddC (Sigma-Aldrich), washed two times with 5 ml 37°C, and then were incubated with FMAU for cellular uptake and DNA incorporation after 2 h.

HPLC analysis

HPLC was used to determine the relative amounts of parent compounds and their metabolites in cells. Analysis was based on the method developed by Grierson et al. [5]. Briefly, 200 μl of sample was loaded onto a Hamilton PRP-X100 10 μm 150×4.1 mm column (Hamilton, Reno, NV, USA). The column was eluted at 1 ml/min with mobile phases A (10% acetonitrile in water) and B [0.5 M $(NH_4)_2HPO_4$, pH 7.5] according to the following gradient: 0–10 min gradient %B=(100)*t*5/105, 10–15 min 100% B, 15–16 min 100% B to 100% A (linear), 16 to 20 min 100% A. Peaks were identified by comparing them with the peak of the original compounds and equivalent thymidine phosphate standards and were proved to be phosphates by incubating with 4 U/ml of calf intestinal alkaline phosphatase (New England Biolabs, Ipswich, MA, USA) at 37°C for 4 h in RPMI-1640 with 0.5 mM MgSO4 and 50 μl/ml 10XNE Buffer 3 (New England Biolabs, Ipswich, MA, USA) and then reanalyzed by HPLC.

Testing the effects of nutritional stress

To put cells under nutritional stress, they were split to approximately 200,000 cells per well in six-well cell culture plates and were kept in the same medium for 5 days, and then retention of the tracers was measured. To determine the effect of nutritional stress on tracer uptake into the cells and its phosphorylation, total cellular uptake including both retained and non-retained tracers was analyzed by HPLC. Cells were incubated with either [^{3}H]FLT or [^{3}H]FMAU, washed with −34°C 50% ethylene glycol in PBS three times, and were frozen rapidly by putting the plate on a mixture of methanol and dry ice. The cell lysate was then analyzed by HPLC.

Testing the effects of TK2 inhibitor on FMAU and FLT

To inhibit TK2, 0.34 μM BVDU (Sigma-Aldrich) [13], was incubated with [^{3}H]FMAU in cell culture studies. To confirm that BVDU decreases FMAU retention by TK2 inhibition, rather than by inhibiting FMAU transport into the cells, PC3 cell lysates were also co-incubated with tracers and BVDU, and the generated compounds were analyzed by HPLC.

Testing the effects of cellular stress on FMAU retention and TK2

Cells were put under oxidative stress with 0.03% H_2O_2 (Sigma-Aldrich), reductive stress with 1 μM dithiotreitol (DTT; Bio-Rad, Hercules, CA, USA), and energy stress with 1 μM dinitrophenol (DNP; Sigma-Aldrich). To stimulate adenosine monophosphate-activated protein kinase (AMPK), cells were treated with 0.2 mM 5-amino-4-imidazolecarboxamide riboside (AICAR; Berry & Associates, Dexter, MI, USA). To inhibit elongation initiation factor 2 (eIF2), cells were treated with 10 nM salubrinal (Calbiochem, CA, USA). To determine if FMAU phosphorylation is accompanied by increased activity of TK2, control and treated cells (for 24 h) were lysed by freezing and thawing the cells three times. Cells treated in parallel were counted, and the amount of lysate used for incubation was adjusted according to cell number and was incubated with [^{3}H]FMAU or TK2-specific substrate, [^{3}H]Ara-T (purity of 99% by HPLC, specific activity 4 Ci/mmol; Moravek) for 4 h at 37°C and then analyzed by HPLC. The HPLC counts were adjusted for the total loaded counts of 100,000 disintegrations per minute (DPM), and the phosphorylated fractions were determined.

Flow cytometry

After 24 h treatments, cells were washed, trypsinized, and then washed twice with PBS. To eliminate factors affecting permeability to mitotracker green and its integration into mitochondrial membrane, cells were fixed in 0°C 70% ethanol for 20 min and then incubated in 20 nM mitotracker green (Invitrogen, St. Louis, MO, USA) for 20 min at 37°C. Cells were kept on ice while being transferred to a FACScan flowcytometer (BD Biosciences, San Jose, CA, USA) equipped with CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). The mean fluorescence intensity was measured with the FL1 detector. Cell clumps and cellular debris were excluded from the analysis using conventional dot-plot gating.

Statistic analysis

The relationship between parameters was evaluated with linear regression models, fit by least-squares estimation of the slope and intercepts. The goodness of fit was assessed by r^2 value. GraphPad Prism version 4b (Graphpad Software, San Diego, CA, USA) was used for regression models.

Results

FLT retention is higher than FMAU in proliferating cells

To compare the amount of trapped FMAU and FLT in proliferating cells, after incubation with tracers, the cells were washed with their culture media (warm RPMI-1640) to remove untrapped tracers from the cells and extracellular space. Results showed that PC3 cells had trapped about 11 times more FLT than FMAU in 10 min (control FLT vs. control FMAU in Table 1). This ratio between FLT and FMAU remains high (about 15 times more FLT than FMAU) over 1 h of incubation. There was also a high ratio of retained FLT to FMAU at 10 min in other cell lines as well (sevenfold in LNCaP cells, eightfold in DU-145, and fivefold in breast cancer cell line MDA-MB-231; Fig. 1a).

A comparison between total cellular uptake of FMAU and FLT demonstrated about 7.9% difference between their uptakes during the first minute of incubation in PC3 cells (Fig. 1b). One-minute incubations did not lead to trapping, and the tracers could be almost completely washed out of the cells at this time (trapped tracers in Fig. 1c and d at 1 min). The difference between trapped FLT and FMAU became visible only after 2 min, when trapped FLT accumulated 11 times more than FMAU over the period of 10 min (trapped curves in Fig. 1c and d). It was also noted that less than 10% of trapped FMAU was incorporated into DNA

by 10 min (Fig. 1d). Pre-incubation with ddC (to inhibit DNA polymerase gamma) did not have a significant effect on FMAU retention (1,445±21) compared to (1,428±10) DPM per million cells in control at 10 min. Pre-incubation with ddC did cause a 78% decrease in DNA incorporation after 2 h (606±10 vs. 2,750±69 DPM per million cells) in treated and control cells, respectively.

Phosphorylated FMAU and FLT are the main trapped forms

To identify the main metabolic retained forms of the tracers in the cells, extracts were analyzed by HPLC. Phosphorylated FMAU and FLT were the main retained forms trapped inside the cells after 10 min of incubation (44% and 86% of total activity, respectively; Fig. 2a and b). The rest of the uptake was distributed among small peaks, barely detectable above the background. Therefore, the rate of phosphorylation is the limiting factor in trapping these tracers.

Nutritional stress causes opposite changes in FMAU and FLT retentions

Measuring tracer retention after incubating PC3 cells in depleted medium resulted in approximately 73% decrease in trapped FLT but caused 92% increased trapped FMAU (Table 1). To measure the effect of nutritional stress on TK1 activity, HPLC analysis of the total cellular uptake was performed. The results showed that the control and nutritionally stressed cells had similar amounts of FLT in the parent form [mean value 2,410 (range, 2,140–2,560) vs. 2,556 (range, 2,352–2,672) DPM per million cells in control and stressed cells, respectively] after 10 min of incubation, but the amount of phosphorylated FLT had decreased significantly in stressed cells [mean value 13,811 (range, 12,825–14,625) vs. 2,675 (range, 2,387–2,801) DPM per million cells in control and stressed cells, respectively; Fig. 3].

Blocking TK2 inhibits FMAU retention and phosphorylation

To determine the role of TK2 in metabolic retention and phosphorylation of FMAU, BVDU, an inhibitor of TK2, was incubated with FMAU. The TK2 inhibitor, BVDU, decreased trapped FMAU by 71% (Table 1) but did not decrease trapped FLT in cell culture studies with PC3 cells. HPLC analysis of the cell lysates confirmed that BVDU inhibition of TK2 decreases FMAU phosphorylation by more than 73% (Fig. 4a,b), without significant effects on FLT (Fig. 4c,d), signifying the role of TK2 activity in FMAU retention and phosphorylation.

Various types of cellular stress increase FMAU retention and TK2 activity

All PC3 cells treated with H_2O_2, DTT, DNP, AICAR, and salubrinal had increased intracellular trapped FMAU (Table 1). After 24 h of treatment with stressors, cell numbers decreased (AICAR 67%, DNP 34%, DTT 20%, and H_2O_2 57% compared to control; Table 1). Similar tests showed that treating LNCaP cells with H_2O_2 and DTT for 24 h increased trapped cellular uptake of FMAU by 125% and 24%, respectively. Since Ara-T is a specific substrate for TK2, phosphorylated Ara-T was used to determine the activity of TK2. HPLC analysis of cell lysates showed a correlation between phosphorylation of Ara-T, as an indicator of TK2 activity, and FMAU phosphorylation (r^2= 0.87, p=0.0002; Fig. 5a).

Various types of cellular stress increase mitochondrial mass

Flow cytometry using mitotracker green staining demonstrated increased mitochondrial mass after starvation, AICAR, DTT, H_2O_2 or salubrinal treatments of PC3 cells (Fig. 5b). There was also a correlation between intracellular trapped FMAU and mitochondrial mass (r^2=0.88, p= 0.006) in stressed cells.

Discussion

Mammalian cells have two known thymidine kinases, TK1 and TK2. TK1 is a cytosolic, cell cycle-dependent enzyme with highest levels of activity during S phase [21–23]. TK2 is independent of the cell cycle and localized in the mitochondria [24–26]. Thymidine analogs, FLT and FMAU, have high retention in tumor tissue and are used for PET tumor imaging [6, 7]. Compared to FLT, FMAU has a faster clearance, shorter imaging time, and low uptake in the bone marrow [7]. Previous work has shown that FLT is phosphorylated specifically by TK1 [1, 2, 5], and its uptake in tumors correlate with TK1 activity and proliferation rate [27]. Unlike FLT, FMAU uptake did not correlate with tumor proliferation in animal studies [28]. Furthermore, the mechanism of increased FMAU retention in tumors with little retention in proliferating bone marrow was not known. As shown in the present and previous studies, uptake of FLT in the fast proliferating cells in culture is much higher than FMAU [7, 29]. One potential reason for higher FLT uptake could be the higher transmembrane transport of FLT compared to FMAU. In the case of FMAU and FLT, 1-min incubation leads to rapid intracellular accumulation, but both tracers can be washed out of the cells, as demonstrated by curves of trapped FMAU and FLT. This suggests that 1 min is early enough to measure their transport into the cells. Similar levels of FMAU and FLT uptake in the first minute (Fig. 1a) suggested comparable transport of both tracers into the cells. Furthermore, tracers are metabolically trapped inside the cells primarily in the phosphorylated forms, as shown by HPLC analysis (Fig. 2a,b). Keeping cells in their medium for an extended length of time induces nutritional stress, decreases proliferation, and causes cell death. As expected, such a stress decreased the amount of trapped FLT; however, it demonstrated opposite effects on trapped FMAU, suggesting a different mechanism for its metabolic retention. The similar level of FLT in the cells under nutritional stress and control cells indicates that FLT can enter the cells at a comparable rate in both cases; however, decreased FLT phosphorylation is an indication of lower TK1 activity after nutritional stress. Increased trapped FMAU, despite decreased amounts of trapped FLT, suggests that FMAU phosphorylation is not due to TK1 activity.

Decreased TK1 activity could not explain increased FMAU retention; therefore, TK2 was tested as the second candidate. Since BVDU inhibits TK2 and not TK1 [12–14], it was used to determine the role of TK2 in FMAU retention and phosphorylation. BVDU co-incubation, at a concentration that did not have significant effects on FLT retention and phosphorylation, decreased trapped FMAU. Furthermore, decreased FMAU phosphorylation in cell lysates in the presence of BVDU confirmed the preferential phosphorylation of FMAU by TK2 (trapped FMAU and FLT with BVDU in Table 1 and Fig. 4). It also confirmed the previous studies on thymidine kinase activities in cytosolic and mitochondrial cellular fractions and recombinant human TK1, which had suggested FMAU as a weaker substrate for TK1 than TK2 in humans [9, 10], especially compared to thymidine and FLT [11]. Decreasing trapped FMAU in the presence of BVDU in proliferating human cells with high FLT uptake demonstrates the level of contribution of TK2 in metabolic retention of FMAU and the fact that high levels of TK1 activity in these cells does not efficiently trap FMAU.

Due to the lack of 3′ hydroxyl group, FLT incorporation into the DNA leads to termination of DNA elongation. Unlike FLT, FMAU can incorporate into the DNA. FMAU retention in human tumors was shown to occur in about 10 min, which is the window of human PET tumor imaging [30]. Low incorporation of FMAU into the DNA after 10 min of incubation indicated that DNA incorporation does not have a significant role in the FMAU PET images. Blocking DNA incorporation of FMAU by ddC, without significant effects on FMAU retention, ruled out DNA incorporation as a necessary step in its cellular retention as well.

Considering that FMAU is mainly captured in the phosphorylated form, its correlation with TK2 activity was tested. As a specific substrate of TK2, Ara-T phosphorylation was used to measure TK2 activity [4, 31]. Correlation between phosphorylation of FMAU and Ara-T, as well as correlation between FMAU retention and mitochondrial mass, suggest that FMAU can be used to track TK2 activity and mitochondria, which is in agreement with high FMAU retention in mitochondrial rich tissues, like heart, in PET imaging studies in human subjects [7].

Increased FMAU retention during cellular stress, correlation between FMAU retention and mitochondrial mass and TK2, low incorporation of FMAU into the DNA, and finally, unchanged FMAU retention despite blocked DNA incorporation distinguish FMAU retention and imaging from proliferation and DNA synthesis. These also explain the low uptake in the human bone marrow in PET imaging studies [7] and weak correlation with tumor proliferation [28]. However, these data should be interpreted in the context of variations in pharmacokinetics and pharmacodynamics in different species. For example, rodents have much higher levels of thymidine in the blood than levels found in dogs and humans, and this may interfere with tracer uptake of FMAU and FLT in rodents. A second issue is that the affinities of TK1 and TK2 for thymidine, FMAU, and FLT may vary between species. Thus, although the present study shows that TK2 plays the main role in FMAU retention in humans, this tracer might be a better substrate for TK1 in some other species and show high uptake and rapid incorporation into the DNA in proliferating tissues [32]. A third factor that may play a role in selective tissue accumulation is blood clearance. Rapid clearance from blood in human subjects [7] limits the access of the tissues to FMAU to about 11 min. In dogs and mice, however, the compound remains in the circulation much longer [32, 33]. This prolonged access may give time for TK1-rich tissues to accumulate high levels of FMAU and change the pattern of retention and relative tissue specificity of this tracer compared to humans.

Increased captured FMAU after oxidative, reductive, and energy stress, by treating cells with H_2O_2, DTT, and DNP, suggested that different types of stress may increase TK2 activity. Two main known stress pathways are AMPK activation in energy stress [34–36] and eIF2 inhibition in endoplasmic reticulum (ER) stress and hypoxia [37–39]. Furthermore, increased TK2 activity in reducing conditions suggests a connection between ER stress and mitochondria. It can also be postulated that treating cells with reducing agents may increase reactive oxygen species and oxidative stress [40, 41]. However, DTT reverses S-nitrosation and its inhibition of mitochondrial complex I [42]. Therefore, keeping cells in a reducing condition with DTT weakens such a possibility. An opposite mechanism can also be hypothesized, where oxidative stress or mitochondrial damage can lead to the generation of ER stress [43–45]. It has also been suggested that mitochondria play a role in ER stress and ER-induced apoptosis [46–48]. Regardless of the mechanisms involved, these data indicate that increased TK2 activity and mitochondria are general phenomena observed after different types of stress. Further studies are needed to clarify the pathways leading to increased TK2 activity and mitochondria.

Although FMAU accumulates more slowly than FLT in the fast proliferating cells, its intracellular capture is close to FLT in stressed cells. Pilot clinical studies show that tumor uptake of FMAU and FLT are similar [7, 29]. AMPK activation and eIF2 inhibition have been shown in tumors [37, 49, 50]. Together, these suggest that unlike fast proliferating cells in the lab, tumor cells in the body are under stress. Therefore, measuring FMAU retention may offer important new information about tumor metabolism that complements data from other sources, including PET imaging with FLT. It may also prove to be a useful tool to measure for the effect of treatment by imaging stress or for imaging apoptosis. Identifying

the mechanisms involved in vivo in patients and their potential diagnostic and therapeutic applications will require further studies.

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References

1. Rasey JS, Grierson JR, Wiens LW, Kolb PD, Schwartz JL. Validation of FLT uptake as a measure of thymidine kinase-1 activity in A549 carcinoma cells. J Nucl Med. 2002; 43:1210–7. [PubMed: 12215561]
2. Toyohara J, Waki A, Takamatsu S, Yonekura Y, Magata Y, Fujibayashi Y. Basis of FLT as a cell proliferation marker: comparative uptake studies with [3H]thymidine and [3H]arabinothymidine, and cell-analysis in 22 asynchronously growing tumor cell lines. Nucl Med Biol. 2002; 29:281–7. [PubMed: 11929696]
3. Mankoff DA, Shields AF, Krohn KA. PET imaging of cellular proliferation. Radiol Clin North Am. 2005; 43:153–67. [PubMed: 15693654]
4. Munch-Petersen B, Cloos L, Tyrsted G, Eriksson S. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. J Biol Chem. 1991; 266:9032–8. [PubMed: 2026611]
5. Grierson JR, Schwartz JL, Muzi M, Jordan R, Krohn KA. Metabolism of 3′-deoxy-3′-[F-18]fluorothymidine in proliferating A549 cells: validations for positron emission tomography. Nucl Med Biol. 2004; 31:829–37. [PubMed: 15464384]
6. Shields AF, Grierson JR, Dohmen BM, Machulla HJ, Stayanoff JC, Lawhorn-Crews JM, et al. Imaging proliferation in vivo with [F-18] FLT and positron emission tomography. Nat Med. 1998; 4:1334–6. [PubMed: 9809561]
7. Sun H, Sloan A, Mangner TJ, Vaishampayan U, Muzik O, Collins JM, et al. Imaging DNA synthesis with [18F]FMAU and positron emission tomography in patients with cancer. Eur J Nucl Med Mol Imaging. 2005; 32:15–22. [PubMed: 15586282]
8. Bading JR, Shahinian AH, Bathija P, Conti PS. Pharmacokinetics of the thymidine analog 2′-fluoro-5-[(14)C]-methyl-1-beta-D-arabinofuranosyluracil ([(14)C]FMAU) in rat prostate tumor cells. Nucl Med Biol. 2000; 27:361–8. [PubMed: 10938471]
9. Cheng YC, Dutschman G, Fox JJ, Watanabe KA, Machida H. Differential activity of potential antiviral nucleoside analogs on herpes simplex virus-induced and human cellular thymidine kinases. Antimicrob Agents Chemother. 1981; 20:420–3. [PubMed: 6272634]
10. Wang J, Eriksson S. Phosphorylation of the anti-hepatitis B nucleoside analog 1-(2′-deoxy-2′-fluoro-1-beta-D-arabinofuranosyl)-5-iodouracil (FIAU) by human cytosolic and mitochondrial thymidine kinase and implications for cytotoxicity. Antimicrob Agents Chemother. 1996; 40:1555–7. [PubMed: 8726039]
11. Schwartz JL, Tamura Y, Jordan R, Grierson JR, Krohn KA. Monitoring tumor cell proliferation by targeting DNA synthetic processes with thymidine and thymidine analogs. J Nucl Med. 2003; 44:2027–32. [PubMed: 14660729]
12. Franzolin E, Rampazzo C, Perez-Perez MJ, Hernandez AI, Balzarini J, Bianchi V. Bromovinyl-deoxyuridine: A selective substrate for mitochondrial thymidine kinase in cell extracts. Biochem Biophys Res Commun. 2006; 344:30–6. [PubMed: 16630572]
13. Balzarini J, Hernandez AI, Roche P, Esnouf R, Karlsson A, Camarasa MJ, et al. Non-nucleoside inhibitors of mitochondrial thymidine kinase (TK-2) differentially inhibit the closely related herpes simplex virus type 1 TK and Drosophila melanogaster multifunctional deoxynucleoside kinase. Mol Pharmacol. 2003; 63:263–70. [PubMed: 12527796]

14. Johansson M, van Rompay AR, Degreve B, Balzarini J, Karlsson A. Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of Drosophila melanogaster. J Biol Chem. 1999; 274:23814–9. [PubMed: 10446143]
15. Lee CF, Liu CY, Hsieh RH, Wei YH. Oxidative stress-induced depolymerization of microtubules and alteration of mitochondrial mass in human cells. Ann N Y Acad Sci. 2005; 1042:246–54. [PubMed: 15965069]
16. Kluza J, Marchetti P, Gallego MA, Lancel S, Fournier C, Loyens A, et al. Mitochondrial proliferation during apoptosis induced by anticancer agents: effects of doxorubicin and mitoxantrone on cancer and cardiac cells. Oncogene. 2004; 23:7018–30. [PubMed: 15273722]
17. Lee HC, Yin PH, Lu CY, Chi CW, Wei YH. Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. Biochem J. 2000; 348(Pt 2):425–32. [PubMed: 10816438]
18. Mancini M, Anderson BO, Caldwell E, Sedghinasab M, Paty PB, Hockenbery DM. Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis in a human colon carcinoma cell line. J Cell Biol. 1997; 138:449–69. [PubMed: 9230085]
19. Reipert S, Berry J, Hughes MF, Hickman JA, Allen TD. Changes of mitochondrial mass in the hemopoietic stem cell line FDCP-mix after treatment with etoposide: a correlative study by multiparameter flow cytometry and confocal and electron microscopy. Exp Cell Res. 1995; 221:281–8. [PubMed: 7493625]
20. Gray NM, Marr CL, Penn CR, Cameron JM, Bethell RC. The intracellular phosphorylation of (−)-2′-deoxy-3′-thiacytidine (3TC) and the incorporation of 3TC 5′-monophosphate into DNA by HIV-1 reverse transcriptase and human DNA polymerase gamma. Biochem Pharmacol. 1995; 50:1043–51. [PubMed: 7575660]
21. Wintersberger E, Rotheneder H, Grabner M, Beck G, Seiser C. Regulation of thymidine kinase during growth, cell cycle and differentiation. Adv Enzyme Regul. 1992; 32:241–54. [PubMed: 1496920]
22. Hannigan BM, Barnett YA, Armstrong DB, McKelvey-Martin VJ, McKenna PG. Thymidine kinases: the enzymes and their clinical usefulness. Cancer Biother. 1993; 8:189–97. [PubMed: 7804359]
23. Wang N, He Q, Skog S, Eriksson S, Tribukait B. Investigation on cell proliferation with a new antibody against thymidine kinase 1. Anal Cell Pathol. 2001; 23:11–9. [PubMed: 11790855]
24. Koch J, Storstad EL. Incorporation of[3H]thymidine into nuclear and mitochondrial DNA in synchronized mammalian cells. Eur J Biochem. 1967; 3:1–6. [PubMed: 6079770]
25. Berk AJ, Meyer BJ, Clayton DA. Mitochondrial-specific thymidine kinase. Arch Biochem Biophys. 1973; 154:563–5. [PubMed: 4632422]
26. Gentry GA. Viral thymidine kinases and their relatives. Pharmacol Ther. 1992; 54:319–55. [PubMed: 1334563]
27. Yamamoto Y, Nishiyama Y, Ishikawa S, Nakano J, Chang SS, Bandoh S, et al. Correlation of $^{(18)}$F-FLT and $^{(18)}$F-FDG uptake on PET with Ki-67 immunohistochemistry in non-small cell lung cancer. Eur J Nucl Med Mol Imaging. 2007; 34:1610–6. [PubMed: 17530250]
28. Bading JR, Shahinian AH, Vail A, Bathija P, Koszalka GW, Koda RT, et al. Pharmacokinetics of the thymidine analog 2′-fluoro-5-methyl-1-beta-D-arabinofuranosyluracil (FMAU) in tumor-bearing rats. Nucl Med Biol. 2004; 31:407–18. [PubMed: 15093810]
29. Shields AF, Briston DA, Chandupatla S, Douglas KA, Lawhorn-Crews J, Collins JM, et al. A simplified analysis of [18F]3′-deoxy-3′-fluorothymidine metabolism and retention. Eur J Nucl Med Mol Imaging. 2005; 32:1269–75. [PubMed: 15991018]
30. Tehrani OS, Muzik O, Heilbrun LK, Douglas KA, Lawhorn-Crews JM, Sun H, et al. Tumor imaging using 1-(2′-deoxy-2′-18F-fluoro-{beta}-D-arabinofuranosyl)thymine and PET. J Nucl Med. 2007; 48:1436–41. [PubMed: 17785728]
31. Arner ES, Spasokoukotskaja T, Eriksson S. Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues. Biochem Biophys Res Commun. 1992; 188:712–8. [PubMed: 1359886]

32. Sun H, Mangner TJ, Collins JM, Muzik O, Douglas K, Shields AF. Imaging DNA synthesis in vivo with 18F-FMAU and PET. J Nucl Med. 2005; 46:292–6. 46/2/292 [pii] 46/2/292 [pii]. [PubMed: 15695789]
33. Wang H, Oliver P, Nan L, Wang S, Wang Z, Rhie JK, et al. Radiolabeled 2′-fluorodeoxyuracil-beta-D-arabinofuranoside (FAU) and 2′-fluoro-5-methyldeoxyuracil-beta-D-arabinofuranoside (FMAU) as tumor-imaging agents in mice. Cancer Chemother Pharmacol. 2002; 49:419–24. [PubMed: 11976837]
34. Polekhina G, Gupta A, Michell BJ, van Denderen B, Murthy S, Feil SC, et al. AMPK beta subunit targets metabolic stress sensing to glycogen. Curr Biol. 2003; 13:867–71. [PubMed: 12747837]
35. Hardie DG, Carling D. The AMP-activated protein kinase–fuel gauge of the mammalian cell. Eur J Biochem. 1997; 246:259–73. [PubMed: 9208914]
36. Winder WW, Thomson DM. Cellular energy sensing and signaling by AMP-activated protein kinase. Cell Biochem Biophys. 2007; 47:332–47. [PubMed: 17652779]
37. van den Beucken T, Koritzinsky M, Wouters BG. Translational control of gene expression during hypoxia. Cancer Biol Ther. 2006; 5:749–55. [PubMed: 16861930]
38. Araki E, Oyadomari S, Mori M. Endoplasmic reticulum stress and diabetes mellitus. Intern Med. 2003; 42:7–14. [PubMed: 12583611]
39. Harding HP, Ron D. Endoplasmic reticulum stress and the development of diabetes: a review. Diabetes. 2002; 51(Suppl 3):S455–61. [PubMed: 12475790]
40. Kadara H, Lacroix L, Lotan D, Lotan R. Induction of endoplasmic reticulum stress by the pro-apoptotic retinoid *N*-(4-hydroxyphenyl) retinamide via a reactive oxygen species-dependent mechanism in human head and neck cancer cells. Cancer Biol Ther. 2007; 6 (5):705–11. [PubMed: 17404501]
41. England K, Driscoll CO, Cotter TG. ROS and protein oxidation in early stages of cytotoxic drug induced apoptosis. Free Radic Res. 2006; 40:1124–37. [PubMed: 17050166]
42. Borutaite V, Budriunaite A, Brown GC. Reversal of nitric oxide-, peroxynitrite- and S-nitrosothiol-induced inhibition of mitochondrial respiration or complex I activity by light and thiols. Biochim Biophys Acta. 2000; 1459:405–12. S0005-2728(00)00178-X [pii] S0005-2728(00)00178-X [pii]. [PubMed: 11004457]
43. Lee GH, Kim HK, Chae SW, Kim DS, Ha KC, Cuddy M, et al. Bax inhibitor-1 regulates endoplasmic reticulum stress-associated reactive oxygen species and heme oxygenase-1 expression. J Biol Chem. 2007; 282:21618–28. [PubMed: 17526500]
44. Zhang Y, Soboloff J, Zhu Z, Berger SA. Inhibition of Ca2 + influx is required for mitochondrial reactive oxygen species-induced endoplasmic reticulum Ca2 + depletion and cell death in leukemia cells. Mol Pharmacol. 2006; 70:1424–34. mol.106.024323 [pii]. 10.1124/mol.106.024323 [PubMed: 16849592]
45. Hayashi T, Saito A, Okuno S, Ferrand-Drake M, Dodd RL, Nishi T, et al. Oxidative damage to the endoplasmic reticulum is implicated in ischemic neuronal cell death. J Cereb Blood Flow Metab. 2003; 23:1117–28. [PubMed: 14526222]
46. Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene. 2003; 22:8608–18. [PubMed: 14634622]
47. Masud A, Mohapatra A, Lakhani SA, Ferrandino A, Hakem R, Flavell RA. Endoplasmic reticulum stress-induced death of mouse embryonic fibroblasts requires the intrinsic pathway of apoptosis. J Biol Chem. 2007; 282:14132–9. [PubMed: 17371867]
48. Chin TY, Lin HC, Kuo JP, Chueh SH. Dual effect of thapsigargin on cell death in porcine aortic smooth muscle cells. Am J Physiol Cell Physiol. 2007; 292:C383–95. [PubMed: 17218371]
49. Fels DR, Koumenis C. The PERK/eIF2alpha/ATF4 module of the UPR in hypoxia resistance and tumor growth. Cancer Biol Ther. 2006; 5:723–8. [PubMed: 16861899]
50. Bi M, Naczki C, Koritzinsky M, Fels D, Blais J, Hu N, et al. ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. Embo J. 2005; 24:3470–81. [PubMed: 16148948]

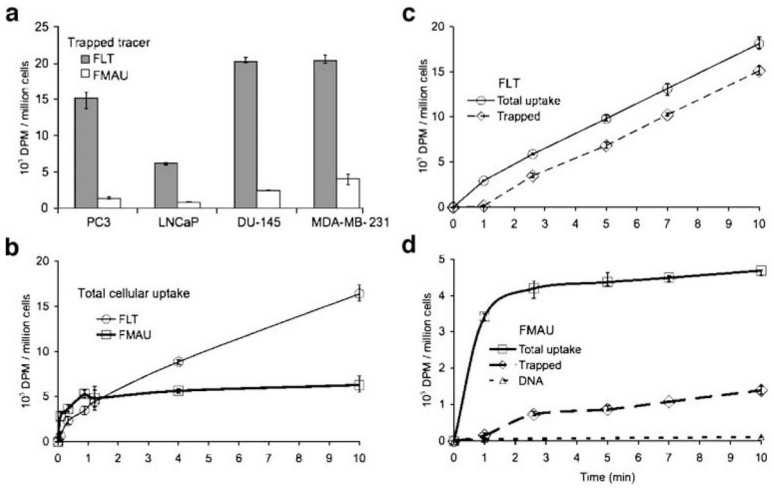

Fig. 1.

Uptake of FMAU and FLT in cell lines. **a** Trapped FMAU and FLT were measured in prostate cancer cell lines PC3, LNCaP, and DU145, and the breast cancer cell line MDA-MB-231. **b**–**d** Time course of total tracer uptake, trapped tracer, and DNA incorporation in PC3 cells incubated with [^{3}H]FMAU or [^{3}H]FLT, measured in three separate sets of experiments, are shown. **b** Total cellular uptake of FLT and FMAU over 10 min incubation. **c** Comparison between total FLT uptake and trapped FLT. **d** Comparison between total FMAU uptake, trapped FMAU, and DNA-incorporated FMAU in PC3 cells. Note: FMAU curves are illustrated *thicker*. The scale in **d** differs from the other two. All measurements were done in triplicate, and *error bars* indicate the range of values

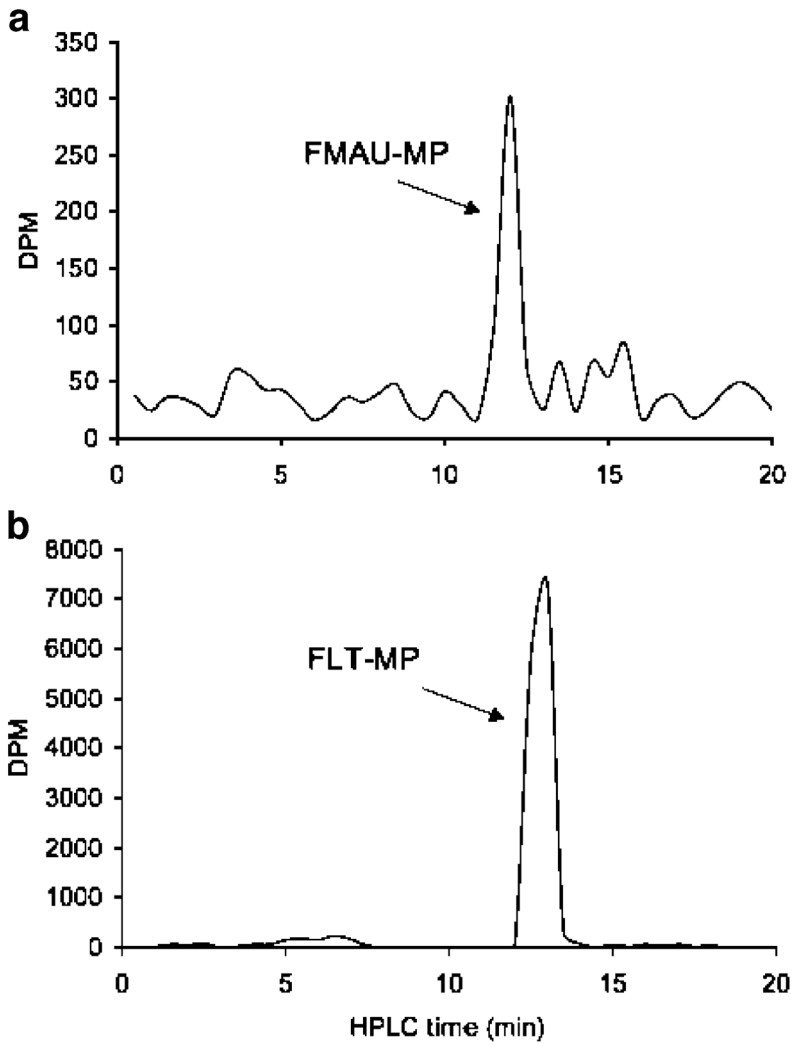

Fig. 2.

HPLC analysis of trapped FLT and FMAU. Cell lysates were loaded on the HPLC column after incubating PC3 cells with [^{3}H]FLT or [^{3}H]FMAU for 10 min. **a** HPLC results of trapped FMAU. **b** HPLC results of trapped FLT. *FMAU-MP* FMAU monophosphate; *FLT-MP* FLT mono-phosphate

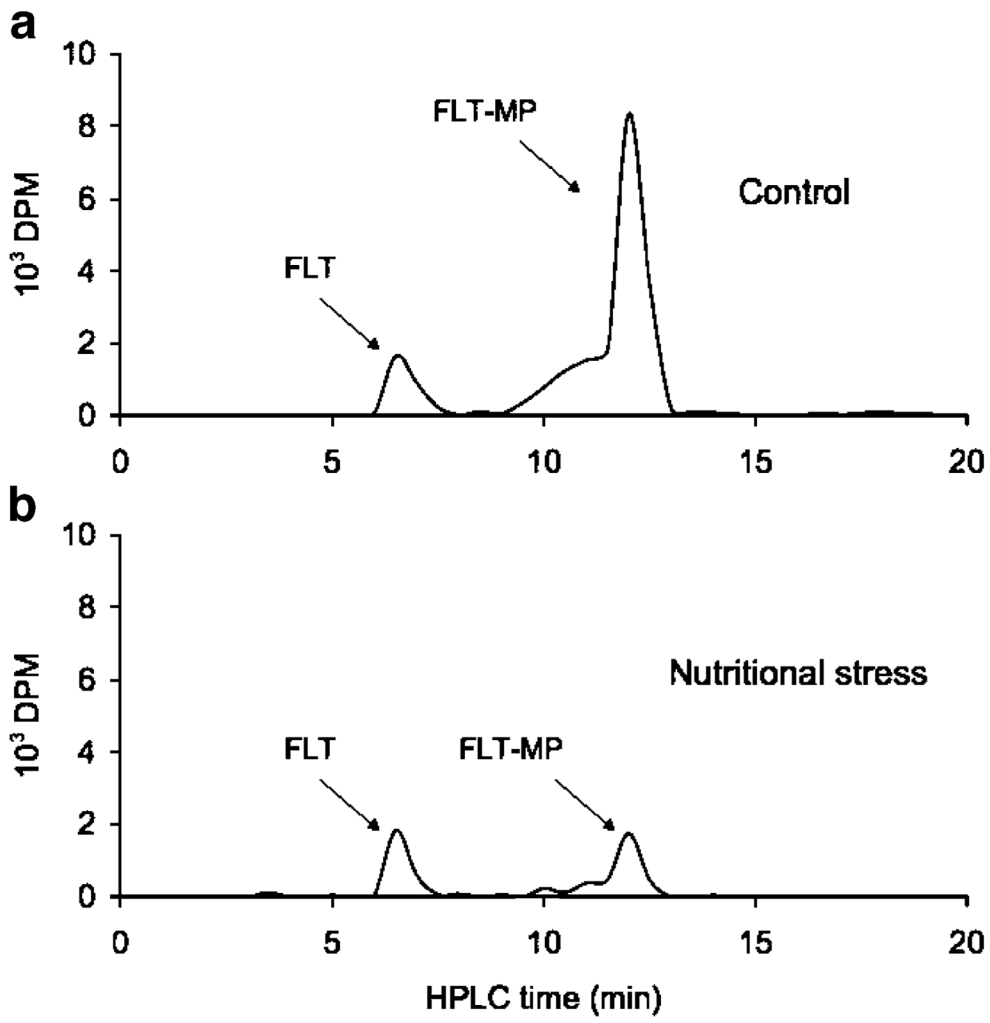

Fig. 3.

HPLC of extracts of cells incubated with [^{3}H]FLT with or without nutritional stress. **a** Cells kept in fresh medium (control). **b** Cells kept in depleted medium (nutritional stress). *FLT-MP* FLT monophosphate

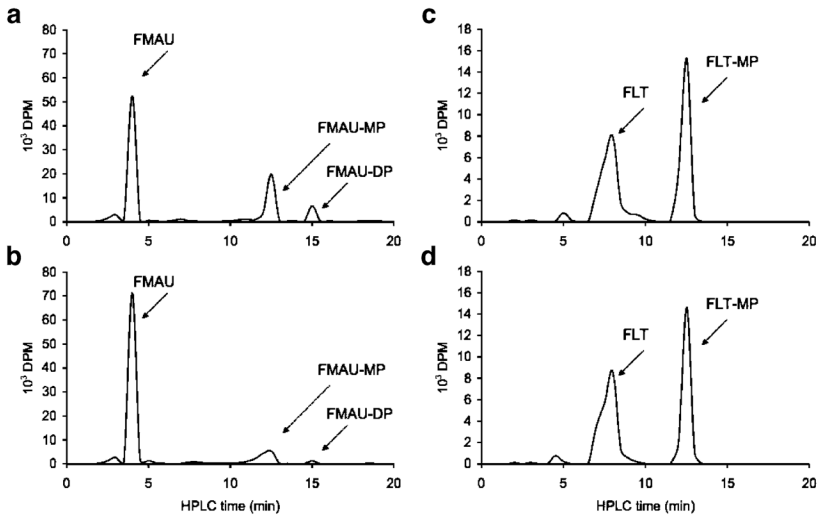

Fig. 4.

Effect of BVDU on phosphorylation of FMAU and FLT in PC3 cell lysates. PC3 cell lysates were incubated with FLT or FMAU, in the presence or absence of BVDU, and then analyzed by HPLC. **a** HPLC analysis of cell lysate incubated with FMAU. **b** HPLC analysis of cell lysate incubated with FMAU and BVDU. **c** HPLC analysis of cell lysate incubated with FLT. **d** HPLC analysis of cell lysate incubated with FLT and BVDU. Note: *scales* in **c** and **d** are different from **a** and **b**. *FMAU-MP* FMAU monophosphate; *FMAU-DP* FMAU diphosphate; *FLT-MP* FLT monophosphate

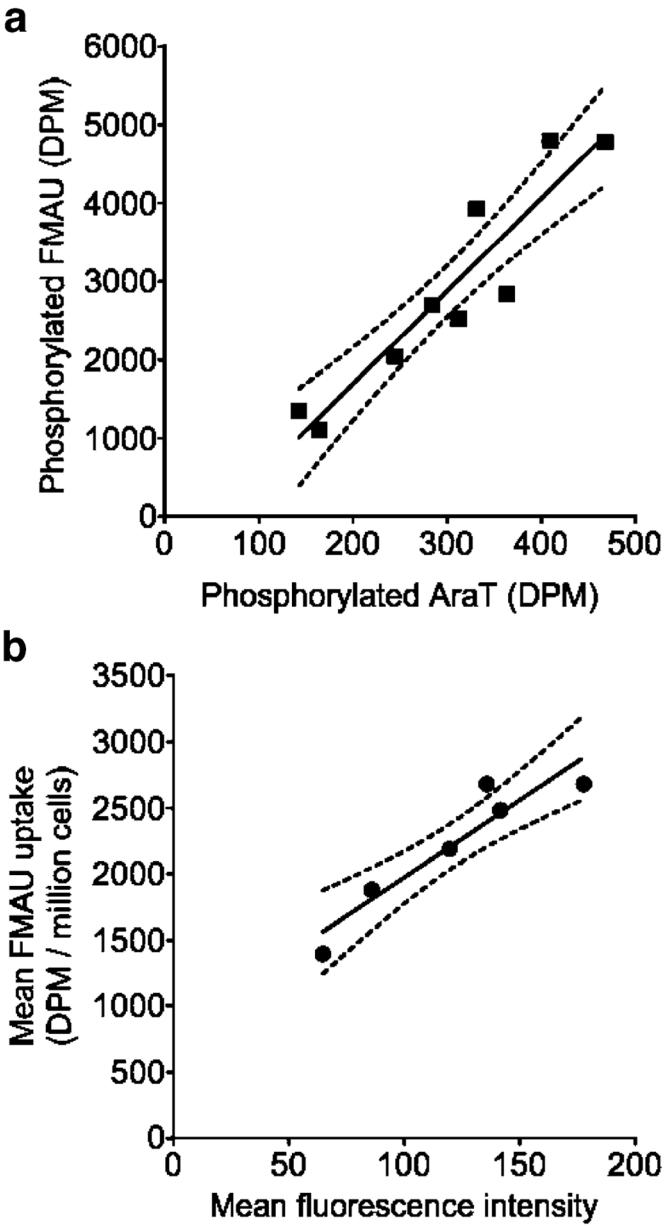

Fig. 5.

Effect of cellular stress on TK2 activity, FMAU phosphorylation and mitochondrial mass. **a** The scatter-plot of phosphorylated FMAU against phosphorylated Ara-T measured by HPLC analysis in PC3 cell lysates. Phosphorylated Ara-T was used as an indicator of TK2 activity. **b** The scatter-plot of trapped FMAU and mitochondrial mass in cells treated with different stressors for 24 h. Mean FMAU uptake was measured as the average trapped FMAU (DPM per million cells) in three samples. Mean fluorescence intensity of cells was measured by fluorescence intensity per incidence in cells stained with mitotracker green to measure mitochondrial mass. *Dashed lines* show 90% confidence limits of mean trapped FMAU for any given mean fluorescence intensity

Table 1

Effects of different treatments on trapped FMAU and FLT in PC3 cells

	Retention of FLT			Retention of FMAU							
	Control	Starved	With BVDU	Control	Starved	With BVDU	24 h treatments				
							AICAR	DNP	DTT	H2O2	DMSO
Retention (DPM per million cells)	15,356 (±768)	4,121 (±359)	17,163 (±616)	1,396 (±255)	2,681 (±118)	406 (±25)	2,484 (±544)	2,800 (±71)	2,650 (±69)	2,189 (±325)	1,457 (±346)
% Change		−73	12		92	−71	78	101	90	57	4

Cells were incubated for 10 min with ^{3}H-FLT or ^{3}H-FMAU and then washed with cell culture medium to remove untrapped tracers. For the BVDU study, cells were incubated with tracers in the presence of BVDU (“with BVDU” column). To observe the effect of nutritional stress on the uptake, cells were kept in the same medium for 5 days (“Starved” column). To see the effect of other stressors, cells were treated for 24 h with mentioned compounds.