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^{18}FDG a PET tumor diagnostic tracer is not a substrate of the ABC transporter P-glycoprotein

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Running title: ^{18}FDG is not a substrate of Pgp

Abstract

2-[^{18}F]fluoro-2-deoxy-D-glucose (^{18}FDG) is a tumor diagnostic radiotracer of great importance in both diagnosing primary and metastatic tumors and in monitoring the efficacy of the treatment. P-glycoprotein (Pgp) is an active transporter that is often expressed in various malignancies either intrinsically or appears later upon disease progression or in response to chemotherapy. Several authors reported that the accumulation of ^{18}FDG in P-glycoprotein (Pgp) expressing cancer cells (Pgp^+) and tumors is different from the accumulation of the tracer in Pgp nonexpressing (Pgp^-) ones, therefore we investigated whether ^{18}FDG is a substrate or modulator of Pgp pump.

Rhodamine 123 (R123) accumulation experiments and ATPase assay were used to detect whether ^{18}FDG is substrate for Pgp. The accumulation and efflux kinetics of ^{18}FDG were examined in two different human gynecologic (A2780/A2780AD and KB-3-1/KB-V1) and a mouse fibroblast (3T3 and 3T3MDR1) Pgp^+ and Pgp^- cancer cell line pairs both in cell suspension and monolayer cultures.

We found that ^{18}FDG and its derivatives did not affect either the R123 accumulation in Pgp^+ cells or the basal and the substrate stimulated ATPase activity of Pgp supporting that they are not substrates or modulators of the pump. Measuring the accumulation and efflux kinetics of ^{18}FDG in different Pgp^+ and Pgp^- cell line pairs, we have found that the Pgp^+ cells exhibited significantly higher ($p \leq 0.01$) ^{18}FDG accumulation and slightly faster ^{18}FDG efflux kinetics compared to their Pgp^- counterparts. The above data support the idea that expression of Pgp may increase the energy demand of cells resulting in higher ^{18}FDG accumulation and faster efflux.

We concluded that ^{18}FDG and its metabolites are not substrates of Pgp.

Abbreviations

CSA	cyclosporin A
^{18}FDG	2-[^{18}F]fluoro-2-deoxy-D-glucose
FDG	2-[^{19}F]fluoro-2-deoxy-D-glucose
^{18}FDG-6-P	2-[^{18}F]fluoro-2-deoxy-D-glucose-6-phosphate
FDG-6-P	2-[^{19}F]fluoro-2-deoxy-D-glucose-6-phosphate
GLUT	glucose transporter
HK-II	hexokinase enzyme type II
MDR	multidrug resistance
PET	positron emission tomography
Pgp	P-glycoprotein
Pgp^+	P-glycoprotein positive
Pgp^-	P-glycoprotein negative
R123	Rhodamine 123

Chemical compounds studied in this article:

2-Chloro-2-deoxy-D-glucose	PubChem CID: 151933
Cyclosporin A	PubChem CID: 5284373
2-deoxy-D-glucose	PubChem CID: 108223
Doxorubicin	PubChem CID: 31703
2-Fluoro-2-deoxy-D-glucose	PubChem CID: 170049
[^{18}F]Fluoro-2-deoxy-2-D-glucose	PubChem CID: 3232583
Rhodamine 123	PubChem CID: 65217
Verapamil hydrochloride	PubChem CID: 62969
Vinblastine	PubChem CID: 241903

1. Introduction

In routine clinical trials, the introduction of ^{18}FDG-PET/CT has lead to a significant improvement in diagnostic accuracy and exerted a considerable impact on patient management including detecting the primary and metastatic tumors, staging, restaging, therapy monitoring, optimization of treatment, follow up of the efficacy of the treatment and prognosis prediction of different malignant tumors (Vallabhajosula, 2007; Kitajima et al., 2011). ^{18}FDG is the most commonly used PET tumor diagnostic radiotracer that allows visualization of the changes in the glucose metabolic rate in tumors (Wahl, 1996; Weber, 2005; Kitajima et al., 2011). ^{18}FDG is transported into the cells by glucose transporters where it becomes phosphorylated by hexokinases to 2-[^{18}F]fluoro-2-deoxy-D-glucose-6-phosphate (^{18}FDG-6-P). ^{18}FDG-6-P is entrapped in the cells immediately after phosphorylation. Glucose 6-phosphatase dephosphorylates ^{18}FDG-6-P to ^{18}FDG, which allows slight efflux of ^{18}FDG from the cells (Wahl, 1996; Southworth et al., 2003; Ong et al., 2008).

Pgp mediated multidrug resistance (MDR) seems to be the most widely observed mechanism in clinical cases of chemotherapy resistance and is known to protect the different tissues from a variety of exogenous and endogenous substances (Goda et al., 2009; Breier et al., 2013). Generally, Pgp substrates are organic amphipathic molecules ranging in size from about 200 to almost 1900 Daltons. The first identified Pgp substrates were chemotherapeutic drugs used in cancer chemotherapy, such a taxanes, anthracyclines, vinca alkaloids. However, many commonly prescribed drugs from various chemical and pharmacological classes are now known to be Pgp substrates (Endres et al., 2006; Eyal et al., 2009). Consequently, the detection of Pgp expression as well as overcoming the Pgp mediated active efflux of chemotherapeutic drugs from the tumor cells are important prerequisites of successful chemotherapy.

Positron emission tomography (PET) and single-photon emission tomography (SPECT) seems to be the most useful methods to study multidrug resistance in tumour tissues *in vivo* (Mairinger et al., 2011). Several authors reported different ^{18}FDG accumulation in multidrug resistant and sensitive cancer cells and/or tumors. A number of them accounted decreased accumulation of ^{18}FDG in different cancer cells which express high level of Pgp (Lorke et al., 2001; Higashi et al., 2004; Yamada et al., 2005; Seo et al., 2009; Smith, 2010; Yu et al., 2012). It was also reported that certain Pgp modulators e.g. verapamil or cepharanthine restored the uptake of ^{18}FDG and this finding was interpreted to suggest that ^{18}FDG might be a substrate of Pgp (Seo et al., 2009). In contrary with the above observations, we measured higher ^{18}FDG uptake in the Pgp^+ cells compared to their Pgp^- counterpart in case of the A2780/A2780AD and KB-3-1/KB-V-1 human gynecologic cancer cell pairs (Márián et al., 2003; Krasznai et al., 2006).

Since ^{18}FDG is one of the most commonly used tumordiagnostic tracer, in the present work we aimed to determine whether ^{18}FDG is a substrate or inhibitor of Pgp. The accumulation and the efflux kinetics of ^{18}FDG were measured in two different human gynecologic cell pairs (A2780/A2780AD and KB-3-1/KB-V-1) and in a mouse fibroblast (NIH 3T3 and NIH 3T3 MDR1) cell line pair. In order to define whether a quantitative relationship exists between the expression levels of GLUT-1, GLUT-3, hexokinase-II (HK-II) and ^{18}FDG uptake, the expression levels of the above proteins were also measured parallel to the ^{18}FDG accumulation measurements using flow cytometry.

2. Materials and methods

2.1. Cell Lines

Drug-resistant (Pgp^+) cell lines and their non-resistant (Pgp^-) counterparts were used in the experiments. The human cervix carcinoma cell line pair (KB-V1 (Pgp^+) and KB-3-1 (Pgp^-); Akiyama et al., 1985; Shen et al., 1986), the human ovarian carcinoma cell lines (A2780AD (Pgp^+) and A2780 (Pgp^-); Louie et al., 1986) and the NIH 3T3 mouse fibroblast cell line and its human mdr1-transfected counterpart (NIH 3T3 MDR1 G185; Brugemann et al., 1992) were grown as monolayer cultures at 37°C in a 95% humidified air, 5% CO2 atmosphere. The cell lines were maintained in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 25 µM/ml gentamycin. The KB-V1 cells were cultured in the presence of 180 nM vinblastine. The A2780AD and NIH 3T3 MDR1 cells were cultured in the presence of 2 µM and 670 nM doxorubicin, respectively. The cells were trypsinized two-three days before the experiments and maintained without drugs until harvesting them at the upper 2/3 part of the log phase. The cell viability was always higher than 90%, as assessed by the trypan blue exclusion test.

2.2. Membrane preparation

The NIH 3T3 MDR1 G185 human mdr1-transfected mouse fibroblast cells obtained from Michael Gottesman's laboratory (National Institutes of Health, Bethesda, MD) were harvested by scraping them into phosphate buffered saline (PBS, pH=7.4) and washed twice. Membrane preparation was carried out according to (Sarkadi et al., 1992) with minor modifications. The cells were lysed and homogenized using glass-Teflon tissue homogenizer in TMEP (50 mM Tris, pH 7.0, 50 mM mannitol, 2 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma P2714)), then the undisrupted cells and nuclear debris were removed by centrifugation at 500 × g for 10 min.

The supernatant was centrifuged for 60 min at 28,000 × g and the pellet containing the membranes was resuspended in TMEP. All procedures were carried out at 4 °C, and the membranes were stored at -70 °C until use.

2.3. ATPase assay

ATPase activity of the isolated NIH 3T3 MDR1 cell membranes was estimated by measuring inorganic phosphate liberation (Sarkadi et al., 1992). Membrane suspensions containing 5 µg membrane protein were preincubated in 60 µl ATPase assay mix (50 mM MOPS, 65 mM KCl, 6.5 mM NaN_3, 2.6 mM DTT, 1.28 mM oubain, 0.65mM EGTA, adjusted to pH 7.0 with Trisma-base) in the presence or absence of 100 µM Na_3VO_4 (vanadate) at 37 °C. The assay mix also contained the studied agent at different concentrations and 40 µM verapamil as stimulator agent in the stimulated samples. The reaction was started by adding 10 µl ATP/Mg^{2+} to a final concentration of 3.2 mM. After 30 minutes incubation at 37 °C, the reaction was stopped by 40 µl 5 % SDS, then the samples were incubated with 105 µl color reagent (10 volume of reagent A, 6 volume of reagent B and 5 volume of reagent C) at room temperature for 30 min. The composition of the reagents was the following; reagent A: 1.5 M H_2SO_4, 1% ammonium molybdate, and 0.014% antimony potassium tartrate, reagent B: 20 % acetic acid, reagent C: 1% ascorbic acid), After the incubation the absorbance of the samples was determined at 700 nm by Biotek Synergy HT plate reader and the amounts of the liberated phosphate (P_i) were calculated. The differences between ATPase activities measured in the absence and the presence of vanadate (100 µM) are considered as Pgp dependent ATPase activity, since the ATPase activity of ABC transporters is inhibited by vanadate and this cell line does not express other drug transporting ABC proteins at significant level (our unpublished data and Ambudkar et al., 1998).

2.4. Radiotracers

The glucose analog 2-deoxy-2-[18F]fluoro-D-glucose (^{18}FDG) was synthesized and labeled with the positron-decaying isotope ^{18}F according to Hamacher et al. (Hamacher et al., 1986). The synthesis was performed on a commercially available GE Tracerlab FX_{FDG} module. The radiochemical purity of ^{18}FDG was better than 99 %. The specific activity of ^{18}FDG was 27±6.5 GBq/μmol.

2.5. log *P* (octanol/water partition coefficient) determination

The determination of log *P* value was performed as we described earlier (Márián et al., 2005). Half milliliter of octanol was added to a centrifuge tube containing 0.5 ml of PBS + 5 μCi (0.185 MBq) ^{18}FDG. The mixture was shaken intensively for 20 minutes and the tubes were centrifuged at 1000 × g for 20 min. The radioactivities of the octanol and water phases were measured with a calibrated Canberra Packard gamma counter. The log ratio of the ^{18}FDG concentrations in the octanol and water phases was calculated.

2.6. *In vitro* radiotracer uptake and efflux studies of cancer cells measured in cell suspension

The cells were washed and resuspended in PBS containing 1 mM glucose (gl-PBS). The samples were preincubated at 37 °C for 10 min at a cell concentration of 1×10^6 ml^{-1} in PBS. 10 μCi (0.37 MBq)/ml ^{18}FDG was then added to each sample. After the addition of the radioligand, cells were further incubated at 37 °C for 30 min. After these steps the control samples (^{18}FDG accumulation for 30 min) were washed 3 times with ice-cold PBS and resuspended in 1 ml of cold PBS, and the radioactivity was measured.

For the investigation of ^{18}FDG efflux the samples were first loaded with ^{18}FDG (at 37 °C for 30 min) and then washed with gl-PBS at room temperature. After centrifugation the supernatant was removed and the cells were resuspended in 2 ml 37°C gl-PBS and further incubated at 37 °C for 10-30 min. The efflux was terminated by the addition of ice-cold PBS. The cells were then washed twice with ice cold PBS and the radioactivity was measured in a Canberra Packard gamma-counter for 1 min within the ^{18}F-sensitive energy window. Decay-corrected radiotracer uptake was expressed as counts min^{-1} $(10^6$ cells$)^{-1}$ (cpm). The displayed data are the means of at least three independent experiments (± SD) carried out using different cell passages, and each experiment was performed in triplicate.

2.7. *In vitro* ^{18}FDG uptake and efflux of cancer cells measured in monolayer, using a miniPET camera

The Pgp^+ and Pgp^- cell pairs were cultured (0.4-0.5 × 10^6 cells in 2 ml culture medium) in Petri dishes (40 × 11mm) for 24-30 hours. By the end of the culture period the cells were grown in monolayer and covered 70-80% of the Petri dishes. Before the ^{18}FDG uptake studies the cell cultures were washed with 2 ml DMEM containing 1mM glucose (gl-DMEM). After washing, the cells were incubated with 100 μCi (3.7 MBq)/2 ml ^{18}FDG for 30 minutes in gl-DMEM at 37°C in a 95% humidified air, 5% CO_2 atmosphere. Thereafter the samples were washed twice at room temperature. Following that 2 ml gl-DMEM was added to each sample and the ^{18}FDG uptake was determined by the MiniPET-II scanner (Lajtos et al., 2013). 2 min acquisition time was used. After the first PET scans the samples were incubated for other 30 or 60 minutes at 37°C in a 95% humidified air, 5% CO_2 atmosphere. After the incubation time the supernatant (medium) was discarded and 2 ml gl-DMEM was added to each sample. The retained ^{18}FDG was determined by the MiniPET-II scanner, using the same acquisition time (2 min). After the PET scans the cells were trypsinized, resuspended in PBS and the cell

numbers were determined. The viability of the cells used in our experiments was always higher than 90%, as assessed by the trypan blue exclusion test. All experiments were carried out using different cell passages and the means (±SD) of at least three independent experiments are given.

2.8. ^{18}FDG-PET data analysis

Ellipsoidal 3-dimensional regions of interest (ROI) were manually drawn around the edge of the tissue culture dish using BrainCad software (http:/www.minipetct.hu). The ^{18}FDG uptake was expressed in Bq/ml.

2.9. Indirect immunofluorescence

Formaldehyde (1% in PBS) prefixed cells were centrifuged at 500 × g for 5 min and washed twice with 1% PBS/BSA. To visualize the Pgp expression, cells (1×10^6 cells/ml) were incubated with 10 µg/ml of UIC2 mAb in PBS containing 1% BSA (PBS/BSA) at 37°C for 40 min. The UIC2 mAb was purified from the supernatant of a hybridoma (purchased from the American Type Culture Collections, Manassas, VA, USA) using affinity chromatography. The anti-Pgp mAb preparations were >97 % pure by SDS/PAGE. After two washes with ice-cold PBS, the cells were incubated with rabbit anti-mouse Alexa 488 secondary antibody (10 µg/ml A488-GaMIgG, Invitrogen, CA) at 4 °C for 30 min.

For the detection of glucose transporters and hexokinase expression, the cells were incubated with the primary antibodies: Rabbit polyclonal to glucose transporter GLUT-1, GLUT-3 (Abcam, diluted 1:250) and Rabbit anti type II hexokinase polyclonal (Chemicon, Millipore, diluted 1:500) antibodies at room temperature for 60 min. After two washes with

ice-cold PBS the cells were incubated with goat anti-rabbit Alexa 488 secondary antibody (10µg/ml A488-GaRIgG, Invitrogen, CA) at 4 °C for 30 min. Negative controls were obtained by omitting the primary antibody.

2.10. Rhodamine 123 accumulation experiments

To demonstrate the function of Pgp in ^{18}FDG accumulation or efflux experiments and to study the effect FDG and deoxy-glucose metabolites on the transport activity of Pgp rhodamine 123 (R123) accumulation measurements were carried out. The cells were washed and re-suspended in PBS containing 5 mM glucose. The samples (1×10^6 cells ml^{-1} in PBS) were pre-incubated at 37 °C for 5 min in the presence or absence (control) of CSA (Cyclosporin A, 10 µM), verapamil (50 µM), FDG, DG, $DG6PO_4$ or ClDG (1, 100 and 1000 µM) and then further incubated at 37 °C for another 20 min in the presence of 0.5 µM R123. R123 uptake was terminated by the addition of ice-cold PBS. The means of the fluorescence intensity histograms were calculated on the basis of 10 000 individual cells, collected in list mode.

2.11. Flow cytometry

A Becton Dickinson FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) was used to determine the fluorescence intensities. Alexa 488 and R123 were excited by the 488 nm line of a solid state laser and the emitted light was detected using a 502 nm dichroic mirror and a 530/30 nm band-pass filter. Cytofluorimetric data were analyzed by BDIS CELLQUEST (Becton-Dickinson) software.

2.12. Data analysis

The data were expressed as means ± S.D. Tracer accumulation data were compared with those measured under control conditions by using Student's *t*-test (two-tailed) and the level of significance was set to $p \leq 0.05$. When ^{18}FDG accumulation or expression of glucose transporters and hexokinase-II were examined in the Pgp^+ and Pgp^- cell pairs we applied z-test comparing the mean of the related Pgp^+/Pgp^- ratios to 1 as a constant and the significance level was set to 0.05 as previously.

3. Results

3.1. Distribution of ^{18}FDG between the octanol and water phase

We determined the distribution of the ^{18}FDG between the aqueous and octanol phases and found that the log *P* value was -2.018±0.03 (n=9) supporting its hydrophilic character.

3.2. Effect of FDG and its metabolites on the R123 uptake of Pgp expressing cells

To test whether FDG and its metabolites interact with Pgp as a substrate or inhibitor we carried out substrate accumulation experiments using rhodamine 123 (R123), a high affinity fluorescent substrate of Pgp. To simulate the *in vivo* conditions and prevent the starvation of the cells the extracellular glucose concentration was held at 5 mM. The R123 uptake of the Pgp^+ and Pgp^- cells was measured in the presence or absence of different concentrations of FDG or deoxy-glucose derivatives (between 1μM to 1 mM). As it is shown in Table 1., Pgp^- cells accumulated R123 at high level, while Pgp^+ cells extruded R123 resulting in much lower R123 fluorescence intensity. Competitive inhibitors of Pgp strongly increased the R123 uptake of Pgp^+ cells, while did not affect that of the Pgp^- cells. FDG and

the applied deoxy-glucose derivatives did not have any effect on the R123 uptake of either cell lines suggesting that these agents are not substrates or modulators of Pgp.

3.3. Effect of FDG on the ATPase activity of Pgp

R123 accumulation assays carried out with intact cells imply that the exact intracellular concentration of FDG and its derivatives is determined by glucose transport mechanisms and consequently is not equal to the extracellular concentration. Thus, we switched to ATPase activity measurements using membrane samples (containing predominantly inside-out vesicles or unsealed membranes), where the concentration of FDG and its derivatives in the aqueous phase is equal to the applied concentration. Since Pgp is an ATP driven active transporter, ATPase activity is required to fuel transport activity. In addition, similarly to several other ABC transporters Pgp also has a basal ATPase activity which can be observed in the absence of treatments with Pgp substrates (black circles), while addition of transport substrates e.g. verapamil stimulates the ATPase activity of the pump in a concentration dependent manner (Fig. 1a). The maximal stimulation of the ATPase activity is between 2 to 10 fold depending on the substrate. Co-treatment with competitive Pgp inhibitors e.g. cyclosporin A decreases the verapamil stimulated ATPase activity in a concentration dependent manner (Fig. 1b). Applying the same method we measured the effect of different 2-deoxy-glucose derivatives 2-fluoro-deoxy-glucose (FDG, Fig. 1c), 2-chloro-2-deoxy-glucose (Fig. 1d), 2-deoxy-D-glucose (Fig. 1e) and 2-deoxy-D-glucose-phosphate (Fig. 1f) on the basal and the substrate stimulated ATPase activity of Pgp. None of these agents had any effect on the basal or the 50 μM verapamil stimulated ATPase activity of Pgp, suggesting that these agents are not substrates or inhibitors of Pgp.

3.4. Pgp, glucose transporter and hexokinase-II expression

Pgp expression was detected by means of indirect immunofluorescence using a monoclonal antibody (UIC2) that recognizes an extracellular epitope of the protein. The mean fluorescence intensity of the UIC2 labeled cells was normalized to that of the isotype control to calculate a signal to background ratio.

The ratio of the relative mean fluorescence intensities of the Pgp^+/ Pgp^- ovarian carcinoma A2780AD/A2780, the epidermoid adenocarcinoma KB-V-1/KB-3-1 and mouse fibroblast cells were 14.7±3.3, 12.0±2.2 and 13±2, respectively (n=3). The above data demonstrate that the Pgp^+ cells express Pgp at high level. The R123 accumulation studies shown in Table 1 demonstrate that the applied Pgp^+ cell lines express functional transporter molecules. The expression of glucose transporters and hexokinase-II enzyme in Pgp^+ and Pgp^- cell lines were also determined (Fig. 2). The glucose transporter expression was detected by means of indirect immunofluorescence using monoclonal antibodies and normalized to that of the isotype control. The ratios of the mean fluorescence intensities of the Pgp^+/Pgp^- were calculated. All of the examined protein expressions were slightly higher in the Pgp^+ cells, but these differences were not significant at $p \leq 0.05$.

3.5. ^{18}FDG accumulation and washout kinetics measured in cell suspension and monolayer

The ^{18}FDG uptake and washout kinetics of the Pgp^+ and Pgp^- cell line pairs were measured both in suspension and in monolayer (Fig. 3). The ^{18}FDG uptakes of all three Pgp^+ cells were significantly higher ($p \leq 0.01$) measured both in suspension and in monolayer than that of their Pgp^- counterparts after 30 min incubation time, except the 3T3MDR1/3T3 cell pairs measured in monolayer (Fig. 3a). The ^{18}FDG accumulation rate in the KB-V-1 and A2780AD cell lines was significantly higher ($p \leq 0.01$) compare to their Pgp^- counterparts (KB-3-1, A2780) when measured in suspension than when it was measured in monolayer, except the 3T3MDR1/3T3 cell pairs measured in suspension and monolayer (Fig. 3a).

Although the washout kinetics of ^{18}FDG in the Pgp$^+$ cell lines were moderately faster, than that of their Pgp$^-$ pairs measured in suspension (both after 10 and 30 min. incubation time) the differences generally were not significant. However, significant differences were found in the retained ^{18}FDG fractions measured in suspension after 30 min washout time (see Fig. 3b) between the Pgp$^+$ and Pgp$^-$ KB cells (p = 0.04), and in case of the A2780 cell line pair (p = 0.065).

Similar tendency was obtained using adherent monolayer cultures but the differences between the Pgp$^+$ and Pgp$^-$ cell lines were even less (Fig. 3c), and were not statistically significant.

4. Discussion

Currently ^{18}FDG is the most important PET radiotracer used in routine clinical cancer imaging, from diagnosing the malignancy to measuring its response to chemotherapy and/or radiation therapy in a wide range of human cancers including gynecologic tumors (Vallabhajosula, 2007; Kitajima et al., 2011).

Several authors reported that the accumulation of ^{18}FDG differs between Pgp expressing and non-expressing cancer cells and tumors (Lorke et al., 2001; Márián et al., 2003; Higashi et al., 2004; Yamada et al., 2005; Krasznai et al., 2006; Seo et al., 2009; Krasznai et al., 2010; Smith, 2010). A number of them accounted decreased accumulation of ^{18}FDG in cancer cells and tumors expressing Pgp at high level (Lorke et al., 2001; Higashi et al., 2004; Yamada et al., 2005; Seo et al., 2009; Smith, 2010, Yu et al., 2012). The most obvious interpretation of this observation would be that ^{18}FDG is a substrate of Pgp. However, there are no exact experiments available in the literature up till now supporting this assumption. The aim of this study was to investigate whether the ^{18}FDG and its metabolites are substrates or modulators of Pgp.

Pgp is an ATP-dependent drug efflux pump exhibiting extremely wide substrate spectrum. It exports structurally unrelated compounds out of cells at the expense of ATP hydrolysis, keeping intracellular levels of chemotherapeutics below a toxic threshold. Pgp is referred as a "hydrophobic vacuum cleaner", because it is believed to extract its substrates directly from the inner leaflet of the plasma membrane (Homolya et al., 1993; Shapiro and Ling, 1997, 1998). In accordance with it Pgp substrates are hydrophobic substances (Sarkadi et al., 1994) with logP values in the range of 2 to 5. For instance the logP value of verapamil is 3.78 (Buchwald and Bodor, 1998), and a competitive Pgp inhibitor cyclosporin A has a log P value of 2.92 (el Tayar et al., 1993). We found that the logP value of ^{18}FDG is -2.02, which

demonstrates its hydrophilic character and does not support its direct interaction with Pgp as a substrate or modulator.

In R123 accumulation assay competitive inhibitors of Pgp (verapamil and CSA) increased the dye uptake of the Pgp^+ cells, while FDG and its derivatives did not have any effect (Table 1). To further test whether FDG is a substrate or modulator of Pgp we carried out ATPase activity measurements. Pgp exhibits a high-capacity substrate-dependent ATP hydrolytic activity that is a direct reflection of its substrate transport capability (Scarborough, 1995). Substrates readily stimulate the ATPase activity of Pgp, while competitive inhibitors bind tightly to the drug-binding site inhibiting substrate transport as well as ATP hydrolysis.

We found in our ATPase assay that FDG does not affect the ATPase activity of Pgp (Fig. 1) further supporting that it is not a substrate or inhibitor of the transporter. FDG is converted to FDG-6-P very rapidly (Kaarstad et al. 2002). FDG-6-P is not commercially available, therefore we used the 2-deoxy-glucose-6-phosphate in our experiments, which has very close chemical characters to that of the FDG-6-P molecule and found that it does not have any influence on the ATPase activity of Pgp. Collectively, these results strongly suggest that ^{18}FDG and the derivatives of 2-deoxy-D-glucose are hydrophilic substances and do not interact with Pgp as a substrate or inhibitor. Thus, it seems likely that other mechanisms are behind the decreased ^{18}FDG accumulation by the Pgp^+ cells or tumors experienced in several previous studies.

The increased energy demand of the cancer cells manifests in higher glucose metabolisms that can be followed by measuring ^{18}FDG accumulation. ^{18}FDG is a glucose analog that is taken up by facilitated diffusion through glucose transporter molecules (GLUT) and phosphorylated by hexokinases in the cells similarly to the glucose molecule. Thus the expression and activity of the GLUT transporters and hexokinases may affect the glucose metabolism of tumor cells and consequently the ^{18}FDG uptake into the tumors (Wahl, 1996;

Vallabhajosula, 2007; Smith, 2010; Breier et al., 2013). In line with the above assumptions numerous studies compared the level of GLUT transporters, the hexokinase activity and the ^{18}FDG accumulation of different tumor cells. Several authors reported strong positive correlation between the GLUT transporter expression, the ^{18}FDG uptake and the malignancy of tumors, while others found only weak correlations (Lorke et al., 2001; Zhao et al., 2005; Smith et al., 2007; Vallabhajosula, 2007; Ong et al., 2008; Paudyal et al., 2008; Seo et al., 2009; Smith, 2010;). Zaho et al. (2005) and Yu et al. (2012) demonstrated that the intra-tumoral ^{18}FDG distribution correlates well with the expression levels of GLUT-1, GLUT-3, and Hexokinase-II. In line with the above Southworth et al. (2003) suggested tissue specific differences in the phosphorylation rate of ^{18}FDG to ^{18}FDG -6-phosphate. The elevated expression levels of GLUT-1, GLUT-3, and HK-II, induced by hypoxia (HIF-1α), may be also be contributing factors to the higher ^{18}FDG accumulation in the tumor region. Hypoxic conditions linked to an increase in HIF-1α expression may affect the expression and transport function of Pgp as well (Breier et al., 2013). In contrary, Seo et al. (2009) measuring the ^{18}FDG accumulation in hepatic cells and tumors found that the GLUT-2 and hexokinase II expression and the glucose-6-phosphatase activity did not correlate with tumor differentiation and ^{18}FDG SUV value.

In our present work, we also examined the correlation between the ^{18}FDG uptake and the expression level of GLUT transporter molecules, and hexokinase II molecules in three cancer cell line pairs (Pgp^+ and Pgp^-) with different tissue origin. However, we did not find strong correlation between the ^{18}FDG accumulation and the expression level of GLUT transporters and hexokinase molecules (compare Fig. 2 and Fig. 3a).

Interestingly, we measured significantly higher ^{18}FDG accumulation in the Pgp^+ cell lines compared to their Pgp^- counterparts (Fig. 3a), in agreement with our previous results (Márián et al., 2003, 2005; Krasznai et al., 2006). Pgp is a transport ATPase with high basal

ATPase activity that is further increased in the presence of substrates (see Fig. 1). Thus, expression of Pgp at high level may increase the ATP demand of cells explaining our observations.

Yu et al. also (2012) measured an increased *in vitro* uptake of ^{18}FDG into the Pgp^{+} Bcap37/MDR1 human breast carcinoma cell line compared to its Pgp^{-} counterpart (Bcap37) upon short (10 min) incubation. Similarly, in their *in vivo* ^{18}FDG uptake experiments they also measured higher ^{18}FDG accumulation in the Bcap37/MDR1 tumors compared to the Bcap37 tumors. It is fully consistent to our result reported in this study. Interestingly, this trend reversed upon long (60 to 120 min) exposure in their experiments, where higher ^{18}FDG uptake was measured in the Bcap37 cell line compared to the Bcap37/MDR1 cell line, however there is no exact explanation for this observation. In addition, induction of other yet unknown mechanisms could also behind this virtual discrepancy.

We obtained higher values of ^{18}FDG accumulation measured in cell suspension than in monolayer, supporting, that the experimental conditions may cause remarkable differences in the ^{18}FDG accumulation in case of the same cell lines (Márián et al., 2000).

In efflux measurements we observed slightly faster ^{18}FDG efflux from the Pgp^{+} KB-V-1 and A2780AD cells compared to their Pgp^{-} counterparts (Fig. 3b and c). Since the elevated efflux rate was measured in those cells that accumulated the highest amount of ^{18}FDG (Fig. 3a), it is probably explained by an increased production of the dephosphorylated ^{18}FDG by the glucose-6-phosphatase enzyme and its concomitant transport by glucose transporter molecules.

Taken together our results ^{18}FDG is not a substrate or modulator of Pgp, thus its uptake into either Pgp^{+} or Pgp^{-} cells seems to be a true reflection of the glucose metabolism of cells and tissues. Consequently, ^{18}FDG uptake is determined by factors affecting the energy demand and glucose metabolism of cells e.g. cell viability, tumor perfusion, hypoxia,

inflammation, chemotherapy, radiation therapy, necrosis and expression level of active transporters including Pgp.

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Conflict of interest None.

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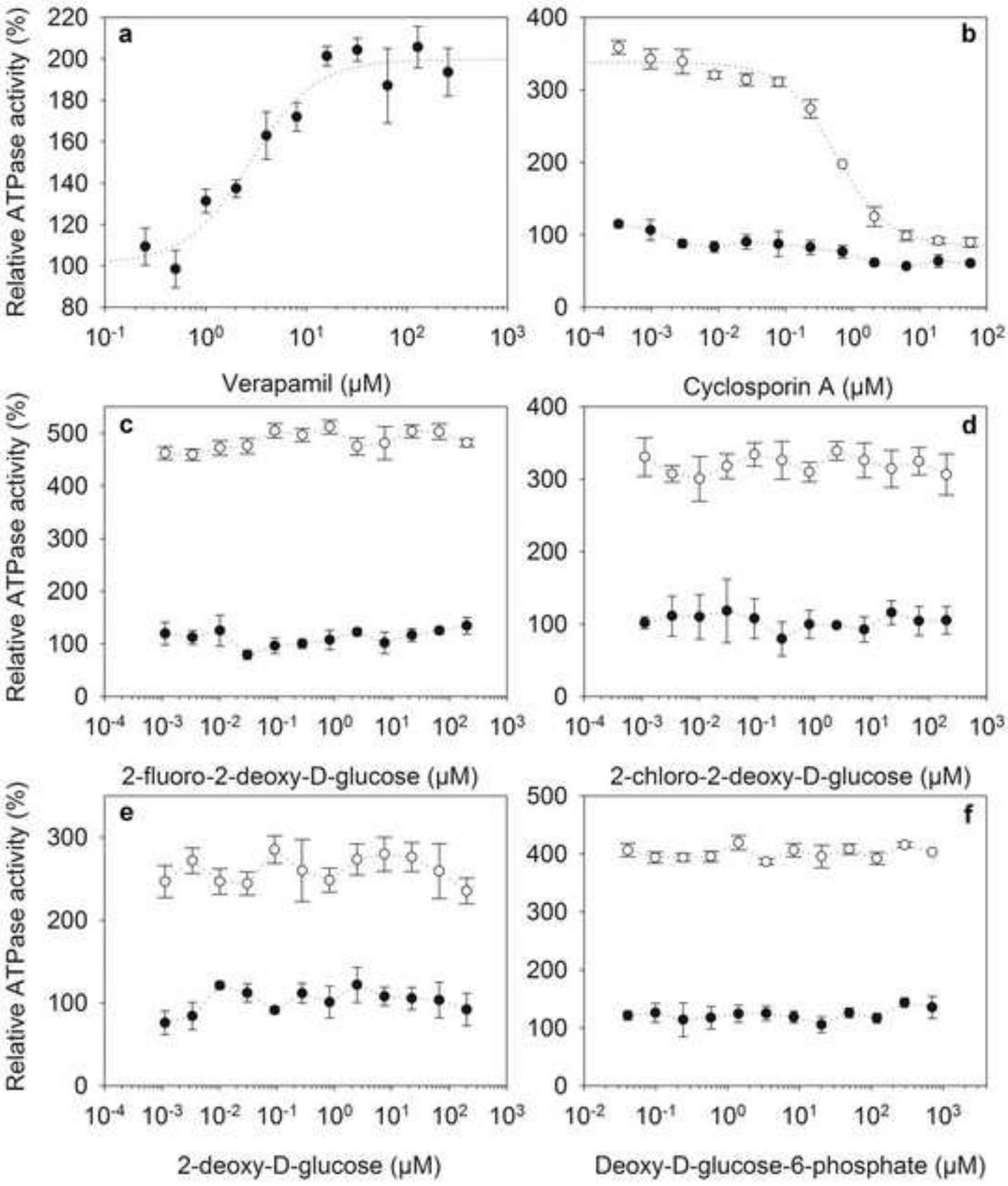
Figure legends

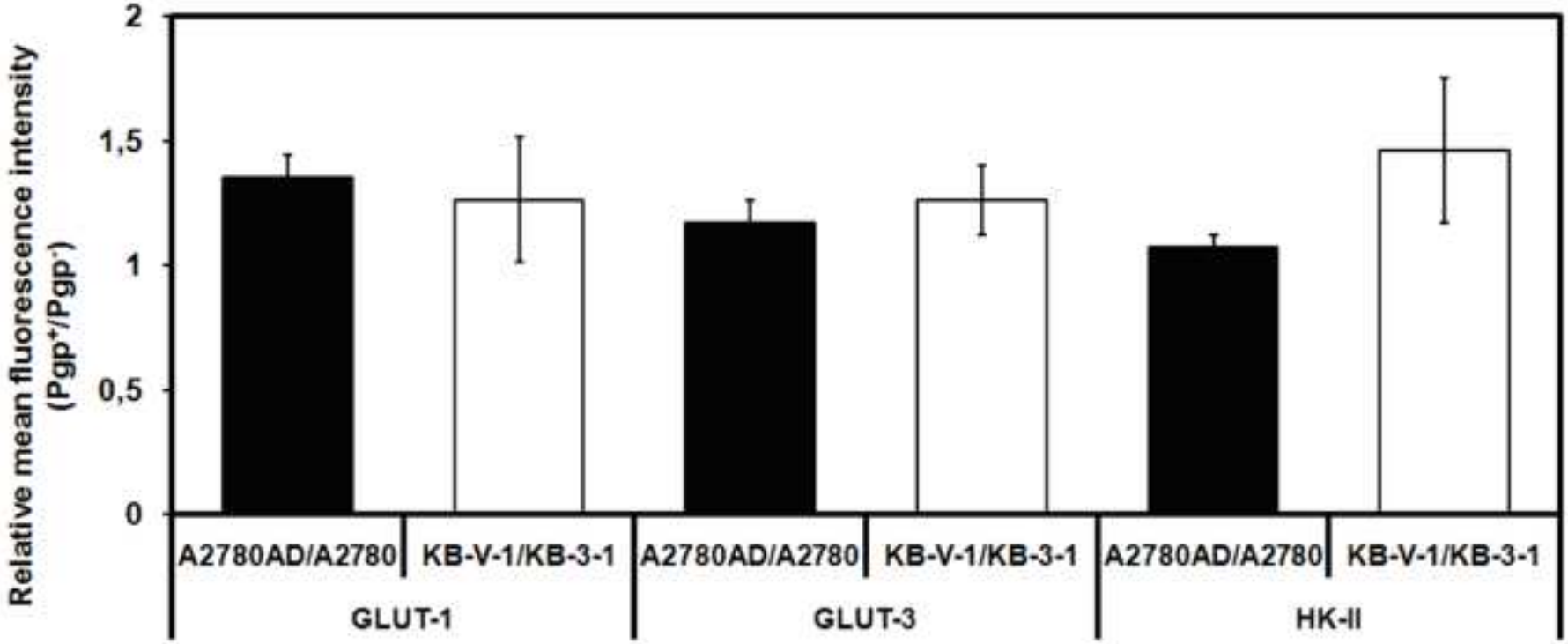
Fig. 1. Effects of cyclosporin A (**b**), 2-fluoro-deoxy-glucose (FDG, **c**), 2-chloro-2-deoxy-glucose (**d**), 2-deoxy-D-glucose (**e**) and 2-deoxy-D-glucose-phosphate (**f**) on the basal (filled symbol) and on the 50 µM verapamil stimulated (empty symbol) ATPase activity of Pgp in membrane samples prepared from NIH 3T3 MDR1 cells. Panel **a** shows the concentration dependent stimulation of the ATPase activity by verapamil. The data points are means of three parallel samples (± SD). The concentrations of the tested agents were varied as shown on the abscissa. The data points are means of three parallel samples (± SD). Each experiment was repeated twice with similar results.

Fig. 2. Flow cytometric analyses of glucose transporters (GLUT-1, GLUT-3) and HK-II expression in tumor cells. Figure shows the ratios of relative mean fluorescence intensities of Pgp^+ and Pgp^- human epidermoid and ovarian carcinoma cell lines. Data are presented as mean ± SD of the results of three independent experiments.

Fig. 3. ^{18}FDG accumulation and washout kinetic of Pgp^+ and Pgp^- cancer cell lines measured in suspension and monolayer. **a** ^{18}FDG uptake ratio of the Pgp^+ and Pgp^- cell line pairs measured in suspension (open square) and monolayer (filled square). Significant differences at $p \leq 0.01$ are indicated with symbol *. The data points are means ± SD of 4 independent experiments measured in triplicates. **b** One million cells were incubated in PBS containing 5 µCi (0.185 MBq)/ml ^{18}FDG for 30 min than the incubation solution was changed for ^{18}FDG free solution, and farther incubated for 10 or 30 min. respectively. The remained ^{18}FDG content of the cells was normalized to the 30 min. ^{18}FDG accumulation value of the cells (control=100%). The data points are means ± SD of 4 independent experiments measured in

triplicates. **c** Cell monolayers were incubated with 50 μCi (1.85 MBq)/ml ^{18}FDG and the ^{18}FDG accumulation and efflux was measured with miniPET camera as described in the Material and methods. The remained ^{18}FDG content (after 30 and 60 min. incubation with ^{18}FDG free incubation solution) of the cells was normalized to the 30 min ^{18}FDG accumulation value of the cells (control=100%). The data points are means ± SD of 4 independent experiments.





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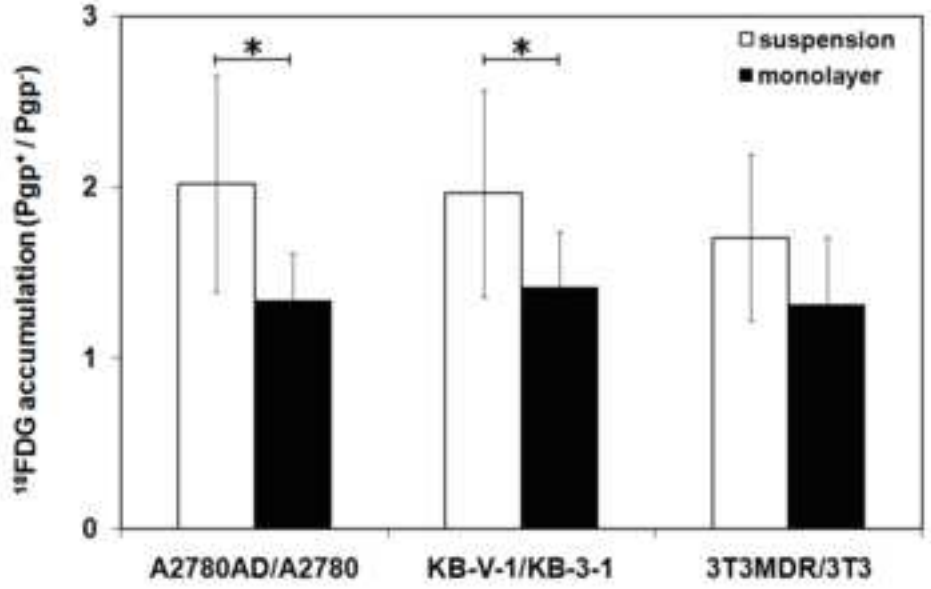

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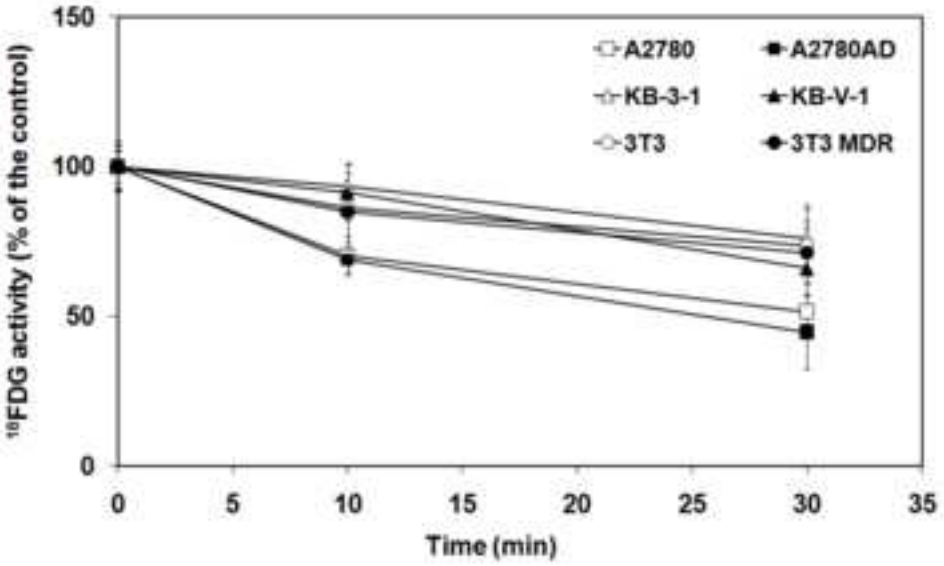

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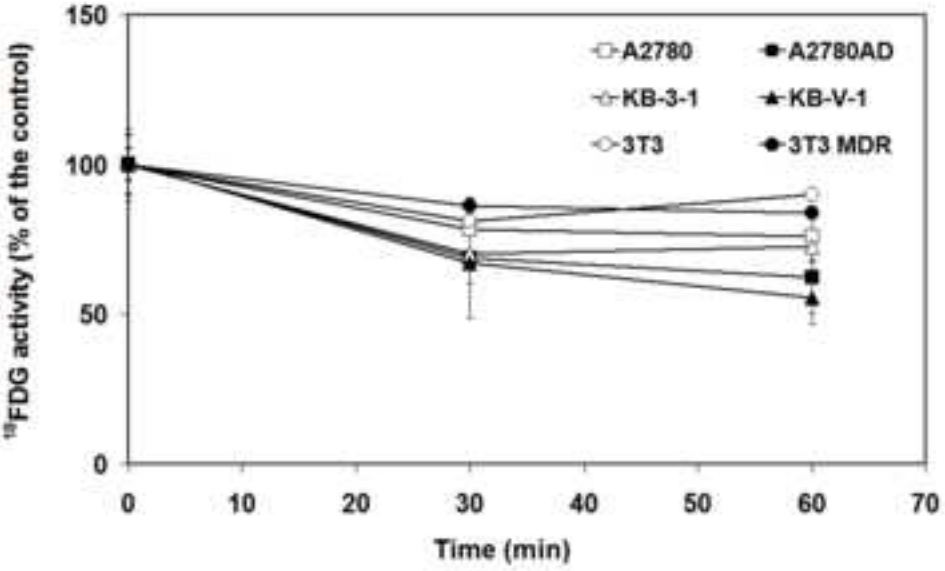

Table 1. Effect of FDG and its metabolites on the R123 uptake of Pgp expressing cells

(mean±SD of 4 independent experiments)

Treatment (μM)	**KB-V-1**	**KB-3-1**	**A2780AD**	**A2780**	**3T3 MDR1**	**3T3**
	R-123 uptake (fluorescence intensity arb. unit)					
untreated	507±97	25059±2512	295±41	10660±1480	2378±404	33535±1496
2-fluoro-deoxy-glucose						
1	509±89	24634±2452	276±27	9984±1167	2379±184	32643±1612
100	498±79	24277±2122	293±36	10159±1008	2417±233	33039±3294
1000	528±78	25990±1801	317±43	10176±1148	2289±356	32691±2036
2-deoxy-D-glucose						
1	542±49	27444±1477	392±28	9739±992	2360±287	28961±3062
100	587±44	24211±1841	334±42	9546±804	2309±195	31828±2667
1000	510±49	23895±1791	405±46	9996±978	2249±263	30740±3568
2-deoxy-D-glucose-phosphate						
1	478±69	24747±2009	319±37	10016±1194	2404±262	34337±1984
100	490±60	24765±2247	311±32	10217±1865	2428±211	32938±2939
1000	510±65	23036±2795	319±46	9940±1461	2355±225	30873±2214
2-chloro-2-deoxy-glucose						
1	486±58	24544±2864	296±39	10239±1234	2378±306	32896±2249
100	500±62	23986±2312	280±32	9978±1062	2275±283	31702±1950
1000	497±48	24391±2989	314±34	9928±951	2297±193	33596±2214
Cyclosoprin A						
10	12806±1165	24316±1058	6453±637	9734±1177	23211±2005	32602±3581
Verapamil						
50	4442±436	20200±355	1749±81	9184±90	8253±1119	29788±104

Graphical Abstract

^{18}FDG is not a substrate of the Pgp pump