Glut-1 and Hexokinase Expression: Relationship with 2-Fluoro-2-deoxy-d-glucose Uptake in A431 and T47D Cells in Culture

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ABSTRACT

Uptake of 2-[18F]-2-deoxy-D-glucose (FDG) has been used as a marker of increased glucose metabolism to visualize, stage, and monitor progression of human cancers with positron emission tomography. Many human tumors have been shown to overexpress the Glut-1 glucose transport protein. The aim of this study is to define whether a quantitative relationship exists between the amount of Glut-1 expressed by cells and their ability to accumulate FDG. We characterized the expression of the known facilitative and sodium-dependent glucose transporter isoforms in six different cancer cell lines used in our laboratory (A431, MDA-MB-231, T47D, CaCo II, MCF7, and HepG2). A431 and T47D cells express, respectively, the highest and lowest amount of Glut-1 mRNA by Northern blot of all of the cells analyzed, and no other glucose transporter isoforms were detectable by this method in both cell lines. Both total and plasma membrane-associated Glut-1 protein levels were higher in A431 than in T47D cells, consistent with the higher Glut-1 mRNA levels. However, T47D cells accumulate FDG more rapidly than do A431 cells, 3-O-Methyl-glucose transport is higher in A431 cells. Although hexokinase I and II mRNA levels are higher in A431 cells than in T47D cells, the ability of mitochondrial preparations to phosphorylate FDG is higher in T47D cells.

Our data indicate that in these cultured cells, FDG uptake correlates better with FDG phosphorylating activity of mitochondrial preparations rather than the level of expression of the Glut-1 or hexokinase I and II genes.

INTRODUCTION

Cancer cell growth is heavily dependent on increased glucose metabolism. The association between neoplastic growth and increased glucose metabolism has been known for many years (1). This property of tumors has also promoted the development of drugs aimed at blocking glucose metabolism for therapeutic purposes (2). The molecular mechanisms by which increased glucose metabolism is sustained, however, are still not fully understood and are the subjects of very intense research efforts.

In recent years, the development of PET4 has provided a tool to study increased glucose metabolism in vivo by measuring uptake of the glucose analogue FDG. Di Chiro et al. (3) first demonstrated that FDG is more avidly accumulated in human brain tumors than in surrounding brain and that tumor recurrence is associated with increased FDG uptake, whereas posttreatment changes, such as radiation necrosis, are not (4). The concept was rapidly extended and applied to tumors in other areas of the body and has been used in many studies to diagnose, monitor, and evaluate treatment response of cancers (5).

Two steps are required to accumulate FDG in cancer cells: (a) facilitated diffusion through a glucose transport protein; and (b) subsequent phosphorylation by one of the hexokinase isozymes, FDG-6-P. FDG-6-P is not transported out of cells nor undergoes glycolytic breakdown; it is metabolically trapped inside cells. Identification of which of these two steps is rate limiting in the process of FDG uptake should provide a better understanding of how glucose metabolism is regulated in cancer cells. This information may also be useful for developing therapeutic strategies aimed at blocking increased glucose consumption in tumors.

Several studies have focused on the expression of glucose transporters and hexokinase activity to define the role of these two classes of genes in the regulation of FDG uptake. Five subtypes of the human facilitative glucose transporters have been described (6). Although most of these subtypes have, to some extent, been detected in different human cancers and cancer cell lines, Glut-1 is the only subtype that has been detected in nearly all cell lines tested (6) and has also been shown to be overexpressed in many human cancers (7). The overexpression of Glut-1 in cancers has lead to speculation that this protein may be regulating glucose metabolism and FDG uptake in cancer cells. On the other hand, several hexokinase subtypes have also been described. Hexokinase I and II have been found to be expressed in tumors. Some authors suggest that these proteins, hexokinase II in particular, are indeed regulating glucose metabolism in cancer cells (8), and some clinical studies show that the FDG phosphorylation step may be rate limiting in the FDG uptake of cancer (9). Some studies also suggest that the lack of glucose-6-phosphatase activity in tumors plays a role in determining FDG retention by preventing dephosphorylation of FDG-6-P to FDG (10).

To shed more light on this issue, we have taken a comprehensive approach to studying the role of the Glut-1 protein and of hexokinase activity in FDG uptake in cultured cancer cells. We have characterized the expression of all of the known facilitative and sodium-dependent glucose transporters in six different cell lines. Among these, we have chosen two cell lines expressing only the Glut-1 isoform; one cell line has severalfold higher levels of Glut-1 mRNA than the other. These two cell lines have been tested for their ability to: (a) express Glut-1 protein as determined by Western blot; (b) accumulate DG when grown in nude mice as xenografts; (c) accumulate FDG in culture in the presence or absence of glucose; (d) transport 3-O-MeG, a glucose analogue that is transported into the cell but not appreciably phosphorylated; (e) synthesize mRNA for hexokinase subtypes I and II; and (f) phosphorylate FDG in mitochondrial extracts. Such an approach has not been used in previous studies on this issue. Furthermore, experiments conducted in cultured cancer cells allow us to control potentially confounding variables, such as blood flow and necrosis, which are present when studying these biochemical parameters in animal tumor models or in human tumors.

MATERIALS AND METHODS

Cell Culture. A431 cells were originally obtained from Dr. G. Todaro (NIH). This is a human epidermoid carcinoma cell line that has been shown to yield s.c. tumors with high efficiency in immunologically incompetent mice (11). T47D cells were obtained from Dr. T. Moody (NIH). This cell line is derived from a pleural effusion of a patient with metastatic breast cancer (12).

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4 The abbreviations used are: PET, positron emission tomography; FDG, 2-fluoro-2-deoxy-D-glucose; FDG-6-P, FDG-6-phosphate; DG, 2-deoxyglucose; 3-O-MeG, 3-O-methyl-D-glucose; RT-PCR, reverse transcription-PCR; SGLT, sodium-dependent glucose transporter; %IDg, percentage of the injected dose per gram of tissue.
Both of these cell lines were grown in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C in DMEM with 4.5 g/l glucose, supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). HepG2, CaCo II, MCF7, and MDA-MB-231 were originally obtained from the American Type Culture Collection (Rockville, MD) and were cultured according to the instructions provided by the supplier. For all cells, the medium was exchanged every 2 to 3 days, and cells were passaged after trypsinization when confluent. Cells that were overconfluent (i.e., confluent for more than 2 days) were discarded, and fresh cultures were reestablished by thawing cells stored in liquid nitrogen. All experiments herein described were repeated on at least three batches of frozen cells to ensure reproducibility.

Radioactive Glucose Analogues. ¹¹¹I-FDG was prepared in the PET Department by standard means (13). All batches were quality controlled to assess purity of the compound and were suitable for patient use (≈1000 Ci/mmol, end of synthesis). [¹⁴C]3-OMeG (50 mCi/mmol) and [³H]DG (30 Ci/mmol) were obtained from DuPont NEN (Boston, MA).

DG Uptake in A431 and T47D Xenografts. Before being implanted in nude mice, cells were trypsinized, washed in culture medium, and resuspended in PBS at a density of 2 × 10⁸/ml. Approximately 100 μl of the cell suspensions were s.c. injected in the flank of Balb-c nu/nu nude mice, cells were trypsinized, washed in culture medium, and resuspended in PBS at a density of 1–200,000 cells/well in 12-well multiwell plates (Costar, Cambridge, MA) 2 or 3 days prior to the uptake experiments. These conditions allowed for the cells to be almost confluent at the time of the assay. On the day of the experiment, the wells were rinsed once in cold uptake medium; subsequently, 1 ml of uptake medium containing 3–5 μCi of [¹¹¹I]FDG was added to each well. Uptake medium consisted of DMEM supplemented with 0.5% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). After 15 min of incubation, the wells were rinsed twice in PBS, the uptake medium was removed, and the cells were solubilized in 1 ml of Solvable (Packard, Meriden, CT) overnight at 50°C. Twenty ml of scintillation fluid (Acquasol; Packard, Meriden, CT) were then added to each vial. Radioactivity in the tumor samples and in aliquots of the injection solution were then quantitatively determined in a Packard β-counter that had been calibrated with quenched ¹³H standards. Uptake was expressed as %ID/g of tumor tissue normalized to a 20-g mouse.

RNA Analysis. Total RNA was extracted from cells grown in culture and, in the case of A431 and T47D cells, xenografted tumors using RNAzol B (Tel-Test, Inc., Friendswood, TX), following the manufacturer’s suggested procedure. Fifteen μg of total RNA were denatured by heating at 65°C for 15 min and loaded onto a 1% agarose formaldehyde gel containing ethidium bromide (14). The samples were electrophoresed for 3 h at 100 V. The ethidium signal was captured using a Molecular Dynamics 595 FluorImager (Sunnyvale, CA), and the gel contents were subsequently transferred to a nylon membrane using a TurboBlotter apparatus (Schleicher and Schuell, Keene, NH). After UV cross-linking, the membrane was hybridized with ³²P-labeled probes obtained by random priming of the following cDNA templates. Plasmids containing the sequences for the human Glut-1, -2, -3, -4, and -5 isoforms were kindly provided by Dr. Charles Burant (University of Chicago, Chicago, IL). The human SGLT 1 and 2 isoform cDNA plasmids (15) were kindly provided by Dr. Matthias Hediger (Harvard University, Boston, MA). The human β-actin and hexokinase I cDNA plasmids were purchased from the American Type Culture Collection (Rockville, MD). A 461-bp fragment of the human hexokinase II mRNA sequence, GenBank accession number Z46376, was amplified by RT-PCR from commercially available poli-A RNA of human myocardium (Clontech, Palo Alto, CA) and cloned into a pCR2 vector (Invitrogen, San Diego, CA). After hybridization, the membranes were washed twice in 2× sodium chloride-sodium citrate with 0.1% SDS at 70°C, exposed overnight on imaging plates, and analyzed using a Fuji BAS-1500 PhosphorImager (Stamford, CT). All probes were tested on commercially available Northern blots (Clontech) prior to use in the experiments reported. The intensities of the different bands were normalized to the β-actin signal or to the 18S band values of the ethidium stain.

Protein Analysis. Cells grown in 15-cm diameter plates were rinsed five times in ice-cold PBS, scraped off the plate, and collected in centrifuge tubes. After centrifugation, the pellet was resuspended in 1 ml of 20 mM Hepes, 1 mM EDTA, and 255 mM sucrose (HES, pH 7.4) containing protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN). The suspension was homogenized with 20 strokes in a Teflon homogenizer on ice, and aliquots were either processed to obtain plasma membrane fractions or total extracts were stored at −70°C prior to use. Plasma membrane preparations were obtained by centrifugation over a sucrose cushion as described (16). Protein concentrations were determined using a Coomassie-Plus kit (Pierce Chemical Co., Rockford, IL). SDS-PAGE was performed as described (17) on precast 10% acrylamide Tris-glycine gels (Novex, San Diego, CA). Samples were not boiled prior to loading. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes using a Novex X-cell II apparatus. The membranes were incubated with dilutions of the following antibodies, according to the technical sheets supplied with the products, in Tris buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h. Polyclonal antibodies raised against the NH₂ terminus of the human Glut-1 protein were purchased from Alpha Diagnostics (San Antonio, TX). A mouse monoclonal antibody (M7PB-E9) against the α1 subunit of the human Na⁺/K⁺-ATPase (Affinity Bioreagents, Golden, CO) was used as a control. After several washes in TBS-T, the membranes were incubated with a dilution of an appropriate fluorescein-labeled secondary antibody (Amersham, Arlington Heights, IL) for an additional hour, and washed several times in TBS-T; finally, the fluorescent signal was detected with a Molecular Dynamics 595 FluorImager.

Cell Uptake Experiments. For experiments with FDG, cells were plated at a density of 1–200,000 cells/well in 12-well multwell plates (Costar, Cambridge, MA) 2 or 3 days prior to the uptake experiments. These conditions allowed for the cells to be almost confluent at the time of the assay. On the day of the experiment, the wells were rinsed once in cold uptake medium; subsequently, 1 ml of uptake medium containing ~20 μCi of ¹¹¹I-FDG was added to each well. Uptake medium consisted of DMEM supplemented with 0.5% FBS with or without 5.5 mM glucose (100 mg/ml) in the medium. FDG was allowed to accumulate into cells in the incubator, with times ranging from 10 to 40 min. Triplicate wells were rinsed twice in cold PBS, and the cells were lysed with 1 ml NaOH to collect cell-associated radioactivity. Uptake of FDG and 3-OMeG between 5 s and 3 min was performed under similar conditions. Cells were plated in 35-mm-diameter dishes. Each plate received ~20 μCi.
[18F]FDG and 2–4 μCi of [14C]3-OMeG, and uptake was evaluated for different times. Radioactivity not incorporated into cells was washed away with five washes in ice-cold PBS with 100 mM phloretin as described (18). Cell-associated counts were recovered in 1 mM NaOH and measured. In some samples, the glucose transport inhibitor cytochalasin-B (Sigma) was added to the uptake medium at a concentration of 50 μM. Fluorine-18 radioactivity was immediately determined using a Packard 5600 gamma counter (Packard, Meriden, CT). After 48 h, 20 ml of scintillation fluid (Acquasol; Packard) were added to the same vials, and 14C radioactivity was quantitatively determined in a Packard β-counter that had been calibrated with quenched 14C standards. Protein concentrations for all samples were determined. The uptake of the two compounds was expressed as the cytochalasin-B-inhibitable percentage of available tracer/mg of cell protein.

**Mitochondrial Phosphorylating Activity**. Mitochondrial preparations from A431 and T47D cells were obtained as described (19). Briefly, cells grown in 15-cm-diameter plates were rinsed several times with ice-cold PBS and recovered by scraping. After centrifugation at 770 × g, cells were resuspended in 40 mM potassium phosphate buffer (pH 7.6) containing 255 mM sucrose. Cells were disrupted using a Polytron homogenizer for 20 s at high speed. The suspension was centrifuged at 770 × g once more to separate undissociated cells. The supernatant was centrifuged at 8800 × g, and the resulting pellet was resuspended in 40 mM potassium phosphate buffer-sucrose and centrifuged once more at 8800 × g. This final pellet was resuspended in a small volume of 40 mM potassium phosphate buffer (pH 7.6), and protein concentration was subsequently determined. The ability of the mitochondrial preparations to phosphorylate FDG was assessed in a total volume of 50 μl of 40 mM potassium phosphate buffer containing 5 mM ATP, 5 mM MgCl2, and 0.1 mM of mitochondrial preparation. Samples containing cold FDG concentrations ranging from 5 μM to 50 μM were tested. Tubes were incubated at 33°C, aliquots were taken at times ranging from 5 to 30 min, and the reaction was stopped by adding 500 μl of 0.3 m ZnSO4. FDG-6-P was precipitated by adding 500 μl of a saturated solution of Ba(OH)2 by mixing and centrifuging for 5 min at 14,000 rpm in an Eppendorf microcentrifuge. Radioactivity in the pellets and in aliquots of the supernatants was then determined to calculate the phosphorylated fraction as described previously (20). In control tubes, mitochondrial preparations were substituted with buffer alone to measure and correct for nonspecific phosphorylation. Initial reaction velocities were determined for the different substrate concentrations, and Michaelis-Menten kinetics were calculated using PRISM software (Graphpad Software, Inc., San Diego, CA).

**RESULTS**

Expression of mRNA for Glut-1, -2, -3, -4, and -5 and for the SGLT types I and II was assessed by Northern blot. A431, T47D, and HepG2 cells expressed detectable levels of Glut-1 only. Glut-1 and Glut-3 mRNAs were detected in MDA-MB-231 and MCF-7 cells. In CaCo II cells, Glut-1, Glut-3, Glut-5, and SGLT-2 were detectable. A431 cells expressed the highest amount of Glut-1 mRNA of all cells tested. The intensity of the Glut-1 band was 11.8 ± 5.6 (mean ± SD, n = 3) times higher when normalized to the β-actin signal and 15.7 ± 7.6 times higher when normalized to the 18S rRNA band on the ethidium stain than in T47D cells, which had the lowest level. These two cell lines were, therefore, selected for further analysis (Fig. 1). The level of Glut-1 protein expression was determined in total extracts and in plasma membrane preparations of A431 and T47D cells (Fig. 2). The Glut-1 band shows higher intensity in A431 than in T47D in both total extracts and plasma membrane-enriched fractions. In plasma membrane fractions, the cell surface marker α1 subunit of the human Na+K+ -ATPase is enriched for both cells lines, and levels of this protein appear higher in A431 in both fractions. The total amount of protein loaded is very similar for all samples, as shown by the amido black staining of the blot. Total Glut-1 protein levels in A431 were 4.4 ± 1.2 (mean ± SD, n = 3) times higher than in T47D cells, similar to the results obtained for the mRNA. Plasma membrane-enriched fractions showed similar differences in Glut-1 protein content, with A431 extracts containing 3.8 ± 1.0 times higher levels than T47D.

FDG uptake in the two cell lines is shown in Fig. 3. Both in the absence (Fig. 3a) or presence (Fig. 3b) of 5.5 mM glucose in the medium, uptake of FDG between 10 and 40 min after addition of the compound is linear for both cell lines and higher in T47D cells than in A431. Absolute uptake values are 17–20 times higher when cells are incubated in medium without glucose as expected. Uptake of FDG

![Fig. 3. FDG uptake in A431 and T47D cells in the presence of no glucose (a) or with 5.5 mM glucose (b) in the uptake medium (n ≥ 3 per time point; bars, SD). Under both conditions, FDG uptake is higher in T47D cells. Uptake values are 17–20 times higher in the absence of glucose in both cell lines.](image1)

![Fig. 4. Initial uptake of FDG in A431 and T47D cells. Values are higher in A431 cells up to the 40-s time point (n ≥ 3 per time point; bars, SD).](image2)
in the two cell lines at early time points and in the presence of 5.5 mM glucose are shown in Fig. 4. A431 cells show more rapid initial uptake of FDG, as values are higher for these cells up to the 40-s time point. T47D cells show higher uptake at later times. Time uptake curves for 3-OMeG are shown in Fig. 5. Both cell lines show uptake of this compound that rapidly reaches equilibrium.

Initial transport rates of 3-OMeG in A431 and T47D cells. Uptake of 3-OMeG rapidly reaches equilibrium in both cell lines. Initial rates are higher in A431 cells (n ≥3 per time point; bars, SD).

DISCUSSION

Among the cell lines tested, A431 and T47D cells were chosen because: (a) both cell lines only express Glut-1 mRNA at detectable levels and no other facilitative or SGLTs, therefore Glut-1 appears to be the isoform mainly being used by these cells to transport FDG; and (b) the level of expression of Glut-1 is very different in these two cells, with the A431 expressing 10–20 times more Glut-1 mRNA than T47D, a condition that should favor uptake of FDG in A431 cells if Glut-1 levels were indeed the rate-limiting step. The level of Glut-1 protein detected by Western blot (Fig. 2) is consistent with results obtained at the mRNA level, with A431 cells expressing ~4 times more total protein than T47D. Furthermore, the amount of Glut-1 associated with the plasma membrane preparations, which should be

Fig. 6. a, Northern blot analysis of A431 and T47D cells grown in culture (c) and as xenografts (x). b, relative levels of β-actin normalized Glut-1 mRNA (n = 3; bars, SD) in A431 and T47D cells grown in culture and as xenografts, as well as DG uptake values 60 min after injection in xenografts (n ≥13 xenografts; bars, SD).
the only fraction of active protein, also shows higher levels in A431 than in T47D, indicating that these two cell lines adequately represent a high (A431)- and a low (T47D)-level plasma membrane Glut-1-containing cell.

The rate of incorporation of FDG in the two cell lines is higher in T47D cells than in A431, both in medium containing physiological glucose concentrations and in the absence of added glucose (Fig. 3), suggesting that the amount of Glut-1 protein is not rate limiting under either of these conditions and that the lower levels of the transporter present in T47D cells are adequate to provide sufficient flux of FDG across the plasma membrane. Another indication that Glut-1 levels are not regulating FDG uptake is given by the results shown in Fig. 4. Intracellular FDG levels, shortly after addition of the uptake medium, are slightly higher in A431 than in T47D cells, indicating that in the very early phase, the amount of transporter present allows more rapid flux into the cells. This situation, however, is rapidly resolved, and by 90 s after addition of radioactivity, T47D cells have a higher accumulation of FDG, suggesting that events following transport into cells, presumably the ability to phosphorylate and trap FDG, rapidly come into play to control how much of the compound is retained. Further evidence is provided by the data on the incorporation into cells of 3-OMeG that shows that this compound, which is not appreciably phosphorylated by cells, very rapidly reaches equilibrium between extracellular and intracellular space in both cell lines. A431 cells show initial transport rates of 3-OMeG that are slightly higher than in T47D cells (0.96 ± 0.30 versus 0.42 ± 0.12% of available tracer/min/mg protein; Fig. 5), consistent with the different amounts of Glut-1 expressed, again indicating that the ability to transport is not the rate-limiting step in FDG retention.

Another interesting aspect of the differences in transport rates between the two cell lines is that they are not as different as one would expect, given the very different levels of the protein in the two cells. Although we did not measure kinetic properties of glucose transport in A431 and T47D cells, our results suggest that the Glut-1 protein may, under different conditions (i.e., in the two different cell lines), be able to transport glucose analogues at different rates. Differences in the intrinsic activity of Glut-1 under some experimental conditions have been demonstrated in other cell systems (22).

The extracellular tracer concentration in the experiments in cells in culture is virtually constant for the duration of the experiment. This situation is very different from that found in the setting of a PET study, where tracer availability (i.e., the concentration of tracer in the blood) decreases with time. The experiments in xenografts show that under experimental conditions, which are similar to those found in human studies, uptake of the glucose analogue DG still appears to be independent of the level of Glut-1 expression (Fig. 6). Under these conditions of tracer delivery, membrane transport does not appear to be rate determining, and uptake of DG is significantly higher in T47D xenografts than in A431.

Thus far, all of the evidence presented points away from Glut-1 levels as the controlling factor on FDG uptake. It appears that flux of FDG, 3-OMeG, and DG across the plasma membrane is not the rate-limiting factor in determining how much FDG is retained in A431 and T47D cells. Indeed, it appears that even with relatively low levels of Glut-1 protein in the plasma membrane, T47D cells are able to sustain higher FDG retention values than A431 cells. Both hexokinase I and II mRNA levels are lower in T47D cells (Fig. 7); however, mitochondrial extracts of these cells, where active hexokinase is presumed to be located (23), show that T47D cells have a higher activity than A431 cells, indicating that: (a) mRNA levels of the two hexokinase isoforms do not necessarily correlate with the actual ability to phosphorylate; and (b) phosphorylating activity in the mitochondria plays a more important role in determining how much FDG is retained by cells and perhaps glucose metabolic rates.

Other studies have focused on the relationship of glucose transporter expression and the uptake of FDG. Most data gathered thus far have focused on vast evidence that the Glut-1 protein is overexpressed in many human tumors (7). In some instances, there appears to be a correlation between the level of expression of Glut-1 and FDG uptake (24); however, there is no direct evidence linking the amount of

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\begin{align*}
K_m &= 109 \pm 49 \\
V_{max} &= 62 \pm 4
\end{align*}
\]

\[
\begin{align*}
K_m &= 328 \pm 145 \\
V_{max} &= 30 \pm 3
\end{align*}
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Glut-1 protein to the level of FDG uptake. The idea that glucose metabolism in tumors may be controlled by the level of Glut-1 expression is, therefore, appealing because it may provide a target for the development of alternative therapeutic strategies. Recently, Torizuka et al. (25) have shown, by kinetic modeling of clinical PET-FDG studies, that the phosphorylation step appears to be rate determining in the uptake of FDG in primary breast cancers but not so in primary lung cancers, suggesting that there may be differences among cancers derived from different tissues in how FDG uptake and perhaps glucose metabolism is controlled by the cells. Our data, although limited to the comparison of two cell lines, indicate that the amount of Glut-1 does not regulate how much FDG, a surrogate marker for glucose metabolism, is retained by cancer cells.

In principle, higher levels of glucose transporter protein do not guarantee increased FDG uptake by cancer cells, and in our two cell systems, metabolic trapping via phosphorylation of FDG appears more likely as the rate-determining step in FDG uptake.

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