

# Influence of resistance to 5-fluorouracil and tomudex on [ $^{18}\text{F}$ ]-FDG incorporation, glucose transport and hexokinase activity

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**Abstract.** Drug resistance is a major obstacle to cancer cure and may influence [ $^{18}\text{F}$ ]-fluorodeoxyglucose (FDG) incorporation. In this study, glucose transport, hexokinase activity and [ $^{18}\text{F}$ ]-FDG incorporation were measured in drug-resistant tumour cells generated by exposing H630 colon and MCF7 breast cancer cells to increasing concentrations of tomudex (raltitrexed) or 5-fluorouracil (5FU). Drug sensitivity was determined using the XTT assay: Tomudex-resistant (H630<sub>TDX</sub> and MCF7<sub>TDX</sub>) cells were more than 40,000-fold less sensitive to tomudex than were the parental wild-type, H630<sub>WT</sub> and MCF7<sub>WT</sub> cells, respectively. 5FU-resistant (H630<sub>R10</sub>) cells were 100-fold less sensitive than parental H630<sub>WT</sub> cells to 5FU. As previously reported for 5FU-resistant MCF7 breast cancer cells, [ $^{18}\text{F}$ ]-FDG incorporation was decreased in H630<sub>R10</sub> colon cancer cells compared to the parental line. By contrast, both tomudex-resistant cell lines exhibited increased [ $^{18}\text{F}$ ]-FDG incorporation compared with the parental lines. H630<sub>R10</sub> and MCF7<sub>TDX</sub> cells exhibited higher rates of glucose transport, measured as the initial rate of O-methylglucose (OMG) uptake, compared to wild-type cells; however, glucose transport was not significantly different between H630<sub>TDX</sub> cells and the parental cells. Hexokinase activity was lower in H630<sub>R10</sub> and MCF7<sub>TDX</sub> cells compared with sensitive parental cells but unchanged in H630<sub>TDX</sub> cells. In conclusion, our results show that [ $^{18}\text{F}$ ]-FDG incorporation is influenced by resistance to antifolate and fluoropyrimidine-based anti-cancer drugs in a drug-dependent manner and the underlying mechanisms appear to be cell- and drug-dependent. Glucose transport may be a useful marker of resistance to 5FU.

## Introduction

Antifolate and fluoropyrimidine-based anti-cancer drugs including 5-fluorouracil (5FU) are commonly utilised for

treating a range of cancer types including breast, colorectal, as well as head and neck tumours. Resistance, either intrinsic or acquired during the course of treatment by selection of resistant tumour cells, is a major basis of response failure to these drugs.

5FU enters cells by facilitated diffusion and is then either catabolised (over 80%) to dihydroxyfluorouracil (1) or converted to a number of different molecules with anti-cancer activity: 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) which is an inhibitor of thymidylate synthase (TS); 5-fluorouridine triphosphate which is incorporated into RNA and 5-fluoro-2'-uridine triphosphate which is incorporated into DNA causing strand termination.

The enzyme TS, which is an essential component in *de novo* pyrimidine synthesis and believed to be the primary target of 5FU therapy, is associated with resistance to 5FU via altered expression of this enzyme (2,3). Peters *et al* (3) reported an inverse relationship between TS expression levels in tumour tissue and response to 5FU in patients with colorectal cancer. Consequently studies have attempted patient stratification according to tumour TS expression as an avenue to combat 5FU resistance. However, the expression of TS in primary tumours has been found to be a poor prognostic biomarker after 5FU chemotherapy in patients with metastatic disease (4). Discordant TS expression between primary and secondary tumours has been reported (5).

S-phase specific anti-cancer drugs, including tomudex and 5FU, exploit the increased proliferation rate and hence the higher percentage of cells in the S-phase to selectively target tumour cells. This mechanism of action of cytotoxic agents underpins their toxicity profile in normal tissues. Resistance to 5FU has been reported to be associated with cell cycle arrest and consequently, to increased numbers of cells in G<sub>1</sub>/G<sub>0</sub> or at the G<sub>0</sub>/S boundary resulting in slower growth (6). Expression of glucose-handling proteins, including the Na-independent family of glucose transporters, GLUTs, has been shown to correlate with proliferation (7,8).

Glucose transport can be measured *in vivo*, non-invasively, using dynamic [ $^{18}\text{F}$ ]-fluorodeoxyglucose (FDG)-positron emission tomography (PET) (9,10) from which K<sub>1</sub> and K<sub>2</sub>, rate constants for FDG transport into and out of the cell, respectively, can be measured. K<sub>3</sub> which is the rate constant for phosphorylation of FDG can also be determined. During the course of chemotherapy, drug-sensitive cells can be replaced by drug-resistant tumour cell populations. We previously reported

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that 5FU-resistant MCF7 breast cancer cells exhibited greatly enhanced glucose transport (11) compared with parental MCF7 cells, suggesting that enhanced glucose transport may be a feature of resistance to 5FU. In this study, we extended our research to the 5FU-resistant colorectal cell line, H630<sub>R10</sub>, and in view of the importance of TS inhibition on the mechanism of action of 5FU, we also measured glucose transport, hexokinase activity and [<sup>18</sup>F]-FDG incorporation in 2 cell lines resistant to the specific TS inhibitor, tomudex (raltitrexed).

## Materials and methods

**Cell lines.** The 5FU-resistant (H630<sub>R10</sub>) colorectal cancer and tomudex-resistant colorectal (H630<sub>TDX</sub>) and breast cancer cell lines (MCF7<sub>TDX</sub>) which had been generated by continuously culturing the drug-sensitive parental wild-type cell lines, H630<sub>WT</sub>, H630<sub>WT</sub> and MCF7<sub>WT</sub>, respectively in medium containing increasing concentrations of the drugs in a stepwise procedure, were a generous gift from Professor P.J. Johnstone (Department of Oncology, The Queen's University of Belfast). Cell lines were cultured in RPMI-1640 (Invitrogen, Paisley, UK) supplemented with 10% FCS, 50 U/ml penicillin and 50 µg/ml streptomycin (complete medium). To avoid an influence of the chemotherapy drug, all resistant cell lines were cultured in drug-free medium for 3 passages before any assays.

**Cytotoxicity (XTT) assay.** In order to determine the sensitivity of resistant and wild-type cells to 5FU and tomudex, cells were seeded in 96-well plates and cultured in the presence of the respective drug concentrations (see below) for 72 h after which the relative cell content of each well was determined using the XTT assay. Briefly, cells were grown to 80% confluence in 75 cm<sup>2</sup> tissue culture flasks and detached using 0.05% trypsin and EDTA and seeded at a density of 2x10<sup>5</sup> cells per well following centrifugation and resuspension in complete medium. After 24-h incubation at 37°C and 5% CO<sub>2</sub>, the drug was added and cells were incubated for 72 h. The range of drug concentrations was: 5FU: H630<sub>WT</sub>, 1 to 100 µM; H630<sub>R10</sub>, 0.05 to 3 mM; tomudex: H630<sub>WT</sub>, 0.1 to 50 nM; H630<sub>TDX</sub>, 2.5 to 500 µM; MCF7<sub>WT</sub>, 0.25 to 50 nM; and MCF7<sub>TDX</sub>, 2.5 to 500 µM. Medium only (background) and vehicle controls were included on each plate following drug incubation, 50 µl of XTT/PMS (4.9 ml sodium 3-[1-phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic hydrate/0.1 ml 1.25 mM phenazine methosulfate) was added to each well and incubated for 2 h at 37°C. Absorbance was measured at 450 and 690 nm in a 96-well plate spectrophotometer (Synergy HT; Biotek). IC<sub>50</sub> values were calculated using GraphPad Prism software. Triplicate independent experiments were performed with triplicate data points on each plate.

**[<sup>18</sup>F]-FDG incorporation.** Flasks (75 cm<sup>2</sup>) of confluent cells were trypsinised, neutralised with complete medium and cell density was adjusted to 1.0x10<sup>6</sup> cells per ml. Five flasks (25 cm<sup>2</sup>) of cells were seeded with 1.0x10<sup>6</sup> cells and incubated in 7 ml of complete medium for 96 h at 37°C and 5% CO<sub>2</sub>. The medium was renewed after 72 h. To determine the rate of [<sup>18</sup>F] FDG incorporation, the medium was replaced with 1 ml of Dulbecco's Modified Eagle's medium (DMEM) containing

5 mM glucose and [<sup>18</sup>F]-FDG (37 KBq/ml) and cells were incubated at 37°C for 20 min. The medium was removed and cells washed 5 times in 5 ml of PBS. Cells were trypsinised with 0.4 ml of 0.05% trypsin/EDTA, neutralised with 0.4 ml of complete medium and the cell suspension transferred to a microcentrifuge tube. Cell associated activity was measured in a well counter with a Scintillation Detector Interface (Oakfield Instruments Ltd., Eynsham, UK). After centrifugation at 400 x g for 5 min, the pellet was resuspended in 0.1 ml of NaOH (1 M) for 24 h at 37°C. After neutralising with 0.1 ml of HCl (1 M), protein content was determined using the bicinchoninic acid protein assay kit (Sigma, Poole, UK).

**[<sup>3</sup>H-methyl]O-methyl-glucose ([<sup>3</sup>H]-OMG) uptake (glucose transport).** O-methyl-glucose (OMG) is transported by glucose transporters but is not metabolised. Thus, its initial rate of uptake after being added to cells is considered to be a measure of glucose transport. Equilibration of OMG between the outside and inside of the cell is complete within seconds. To prevent the loss of OMG uptake into the cell during the incubation period, rapid washing with ice-cold PBS containing the glucose inhibitor, phloretin (100 µM), is required. Flasks of cells were seeded as described above ([<sup>18</sup>F]-FDG incorporation assay). The medium was replaced 30 min prior to the OMG uptake assay with 1 ml of DMEM containing 5 mM glucose. The medium was discarded and replaced with 1 ml of DMEM containing 5 mM glucose and 37 KBq of [<sup>3</sup>H]-OMG (GE Healthcare Life Sciences, Little Chalfont, UK). The medium was rapidly removed after 5 or 10 sec of incubation with [<sup>3</sup>H]-OMG. Cells were washed rapidly 6 times with 5 ml of the ice-cold PBS. The cells were trypsinised with 0.4 ml of trypsin and neutralised with 0.4 ml of complete medium. Cell suspension (0.6 ml) was added to 5 ml of scintillation fluid (Ultima Gold, PerkinElmer, Wokingham, UK). The remaining cell suspension (0.2 ml) was retained for the bicinchoninic acid protein assay (Sigma), after dissolution in NaOH. [<sup>3</sup>H]-OMG uptake was determined on a PerkinElmer Tri-Carb 2100 scintillation counter. Uptake after 5-sec incubation with [<sup>3</sup>H]-OMG was regarded as a measure of glucose transport provided that the uptake after 10 sec was significantly higher than after 5 sec indicating that the equilibrium in the [<sup>3</sup>H]-OMG concentration between the inside and the outside of the cell had not been reached after 5 sec.

**Hexokinase activity.** Flasks of cells were seeded as described above ([<sup>18</sup>F]-FDG incorporation assay). Cells were trypsinised and neutralised with complete medium and centrifuged at 400 x g for 5 min at room temperature. Cells were washed in 1 ml PBS at 4°C, centrifuged at 10,000 x g for 1 min, the supernatant was discarded and the cell pellet was snap-frozen in liquid nitrogen and stored at -80°C.

Cell pellets were homogenised immediately prior to the assay. Cells were thawed then kept on ice and resuspended in 0.2 ml of homogenisation buffer (sucrose 0.25 mM, dithiothreitol 0.5 mM, aminohexanoic acid 1 mM, PMSF 1 mM, Tris-HCl 10 mM, pH 8.0) at 4°C. The cell suspension was transferred to a 1 ml Dounce homogeniser (Fisher Scientific Ltd., Loughborough, UK) and homogenised on ice. After centrifugation for 10 min at 1,000 x g to pellet cell debris, hexokinase activity was measured in the supernatant.

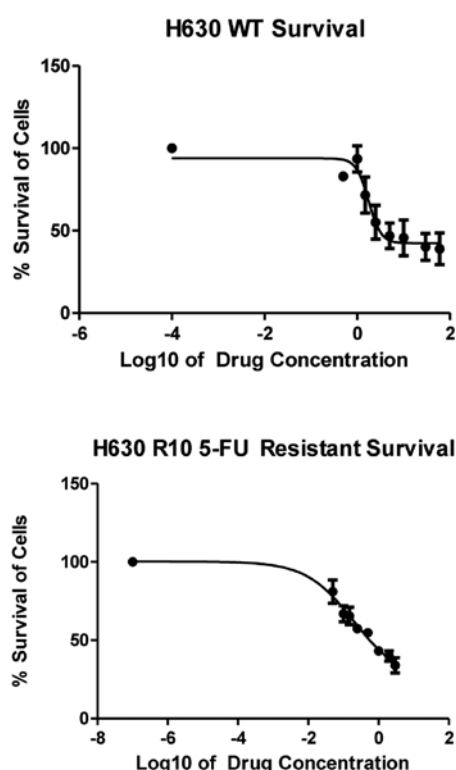


Figure 1. Percentage of H630 wild-type and 5FU-resistant H6305 cell survival after 72-h exposure to a range of doses of 5FU determined using the XTT assay. All experiments were carried out in triplicate each with triplicate replicates.

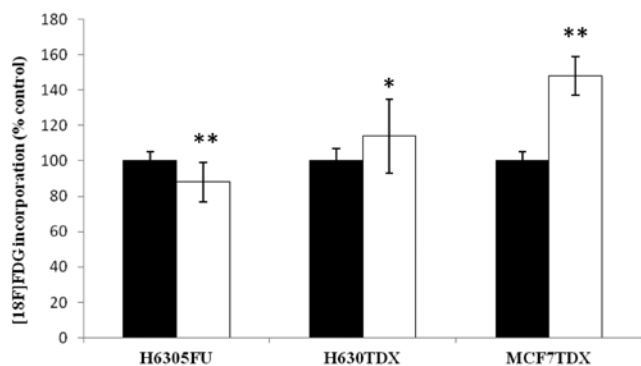


Figure 2. Incorporation of [ $^{18}\text{F}$ ] FDG by 5FU-resistant (H630<sub>R10</sub>) cells, tomudex-resistant (H630<sub>TDX</sub>) cells and tomudex-resistant (MCF7<sub>TDX</sub>) cells (white columns) as a percentage of incorporation by parental (black columns) cells. Significant differences between resistant and parental cells at the \* $P < 0.05$  and \*\* $P < 0.01$  levels (based on the Student's unpaired t-test). All experiments were carried out in triplicate each with at least 3 replicates (units, cpm/mg protein).

Hexokinase activity was measured by adding 100  $\mu\text{l}$  of supernatant to 0.9 ml of hexokinase assay buffer (dextrose 10 mM, NADPH<sup>+</sup> 0.4 mM,  $\text{MgCl}_2$  10 mM, ATP 5 mM, Tris-HCl 100 mM, pH 8.0 and glucose-6-phosphatase 0.15 U) in a 1 ml cuvette at 37°C. The change in absorbance at 340 nm due to the formation of NADPH was measured. The concentration of NADPH was determined from absorbance using the extinction co-efficient for NADPH of 6,220  $\text{mol}^{-1}\text{cm}^{-1}$ .

Table I. Drug sensitivity ( $\text{IC}_{50}$  value) of H630<sub>R10</sub>, H630<sub>TDX</sub> and MCF7<sub>TDX</sub> cells and the respective parental cells.

Drug	Cell line		
	H630 <sub>R10</sub>	H630 <sub>TDX</sub>	MCF7 <sub>TDX</sub>
Tomudex	-	255 $\mu\text{M}$ (5.55 nM)	103 $\mu\text{M}$ (2.02 nM)
5FU	196 $\mu\text{M}$ (1.77 $\mu\text{M}$ )	-	-
Fold resistance	111	46,442	50,790

$\text{IC}_{50}$  values were derived from the dose-response curves shown in Fig. 1 using GraphPad Prism software. The  $\text{IC}_{50}$  values of the parental cell lines are in parenthesis.

**Statistical analysis.** The Student's t-test was used to demonstrate significant differences between the means.

## Results

### Drug sensitivity of wild-type and drug-resistant cell lines.

An example growth inhibitory curve is shown in Fig. 1 (effect of 5FU on H630<sub>R10</sub> cells and their parental controls). Table I shows the  $\text{IC}_{50}$  of each resistant cell line and their parental controls. The  $\text{IC}_{50}$  of 5FU in H630<sub>R10</sub> cells was 111-fold higher than the parental H630 cells. The  $\text{IC}_{50}$  of tomudex in H630<sub>TDX</sub> and MCF7<sub>TDX</sub> cells was more than 40,000-fold higher than the  $\text{IC}_{50}$  for the respective parental cells.

**[ $^{18}\text{F}$ ]-FDG incorporation.** Fig. 2 demonstrates the incorporation of [ $^{18}\text{F}$ ]-FDG during a 20-min incubation period with 37 KBq/ml of [ $^{18}\text{F}$ ]-FDG by 5FU-resistant and tomudex-resistant cells and their respective parental lines. [ $^{18}\text{F}$ ]-FDG incorporation was significantly ( $t=3.7$ ,  $P < 0.01$ ) decreased by 5FU-resistant H630<sub>R10</sub> cells to 88% of the incorporation by parental cells. Incorporation of [ $^{18}\text{F}$ ]-FDG by H630<sub>TDX</sub> cells was significantly ( $t=2.33$ ,  $P < 0.05$ ) increased by 13% compared with H630<sub>WT</sub> cells. MCF7<sub>TDX</sub> cells also exhibited a significantly ( $t=3.61$ ,  $P < 0.001$ ) higher incorporation of [ $^{18}\text{F}$ ] FDG compared with MCF7<sub>WT</sub> cells.

**Glucose transport ([ $^3\text{H}$ ]-OMG incorporation).** Table II shows the uptake of [ $^3\text{H}$ ]-OMG 5 and 10 sec after the addition of [ $^3\text{H}$ ]-OMG-containing medium to drug-resistant and parental cells. OMG uptake after 10-sec incubation of each cell line with [ $^3\text{H}$ ]-OMG was significantly increased compared with uptake after 5 sec, indicating that the equilibrium had not been reached by 5 sec. Therefore, the incorporation of [ $^3\text{H}$ ]-OMG at 5 sec was considered to be an accurate measure of glucose transport. The incorporation of [ $^3\text{H}$ ]-OMG was significantly ( $t=11$ ,  $P < 0.0001$ ) increased by over 100% in H630<sub>R10</sub> cells compared with parental H630 cells. The incorporation of [ $^3\text{H}$ ]-OMG by H630<sub>TDX</sub> cells was not significantly different ( $t=1.98$ ,  $P > 0.05$ ) to that of H630<sub>WT</sub> cells. By contrast, the incorporation of [ $^3\text{H}$ ]-OMG by tomudex-resistant MCF-7 cells was significantly ( $t=4.8$ ,  $P < 0.001$ ) increased compared with the MCF7<sub>WT</sub> cells.

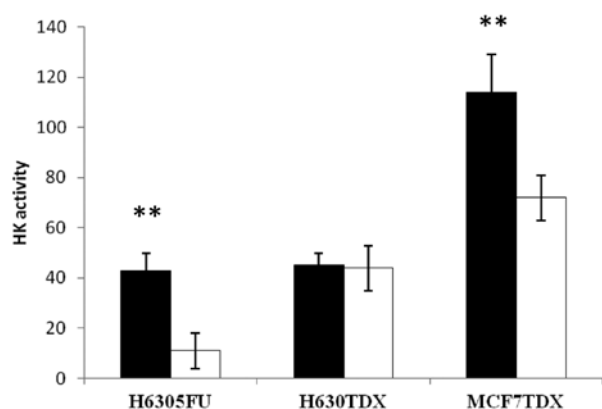


Figure 3. Hexokinase (HK) activity ( $\mu\text{mol NADPH formed/min/mg protein}$ ) in homogenates from parental (black) and drug-resistant (white) (5FU-resistant H630<sub>R10</sub>, tomudex-resistant H630<sub>TDX</sub> and MCF7<sub>TDX</sub> cells) ( $n=6$  replicates per cell line). Significant differences between resistant and parental cells at the  $^{**}P<0.01$  level (based on the Student's unpaired t-test).

**Hexokinase activity.** Hexokinase activity (Fig. 3) in H630<sub>R10</sub> cells was significantly ( $t=8.0$ ,  $P<0.001$ ) lower than in parental H630 cells. Hexokinase activity in the H630<sub>TDX</sub> cells was not significantly ( $t=0.24$ ,  $P>0.05$ ) different from the H630<sub>WT</sub> cells. By contrast, hexokinase activity in MCF7<sub>TDX</sub> cells was significantly ( $t=6.1$ ,  $P<0.001$ ) decreased compared with the parental MCF7 cells.

## Discussion

A rapid means of determining drug response status of tumours to chemotherapy agents has been recognised as an essential clinical tool for decades (12). Methods with the potential of determining potential responders include fresh tumour cell culture, predictive molecular biomarkers in biofluids prior to or at early treatment time-points and imaging biomarkers, such as those obtained with functional imaging modalities, for example PET. PET has the advantage that it is non-invasive and can be carried out serially. However, the relationship between [ $^{18}\text{F}$ ]-FDG-PET measurements and tumour characteristics including drug resistance remains unclear (13).

In this study, we demonstrate, using a colorectal cancer cell line, that resistance to 5FU is associated with decreased incorporation of [ $^{18}\text{F}$ ]-FDG and greatly increased glucose transport. The results are in agreement with our previous study on 5FU-resistant breast cancer cells. Conversely, resistance to tomudex is accompanied by increased [ $^{18}\text{F}$ ]-FDG incorporation whilst the correlation between glucose-transport and resistance to tomudex was found to be cell line-dependent.

Tomudex is a folate analogue which specifically inhibits TS activity. It enters the cell via folate transporters and is rapidly polyglutamated by the enzyme folylpolyglutamate synthase (FPGS) preventing its cellular efflux and increasing its affinity for TS. In common with resistance to 5FU, mechanisms of tomudex-resistance include increased TS expression, but also distinct mechanisms which include the down-regulation of the reduced folate transporter (14) and of FPGS activity (15). 5FU also has distinct TS-independent mechanisms of resistance such as increased dUTPase, cell cycle perturbations and

Table II. [ $^3\text{H}$ ]-OMG uptake by sensitive, 5FU-resistant and tomudex-resistant colon and breast cancer cells.

Cell line	5 sec	10 sec	<sup>a</sup> t, P-value
5FU			
H630 <sub>WT</sub>	785 $\pm$ 146	1,218 $\pm$ 147	5.4, <0.001
H630 <sub>R10</sub>	1,848 $\pm$ 260	2,821 $\pm$ 298	6.5, <0.001
Tomudex			
H630 <sub>WT</sub>	757 $\pm$ 128	1,196 $\pm$ 82	6.4, <0.001
H630 <sub>TDX</sub>	628 $\pm$ 175	966 $\pm$ 220	3.4, <0.01
MCF7 <sub>WT</sub>	367 $\pm$ 88	590 $\pm$ 72	4.9, <0.001
MCF7 <sub>TDX</sub>	550 $\pm$ 88	828 $\pm$ 159	4.4, <0.001

<sup>a</sup>t-test values and significance (Student's unpaired t-test) of difference in OMG uptake by cells after the 5 and 10 sec incubation times. Data represent the means  $\pm$  SD of at least 10 replicates (units, cpm/mg protein) incubated for 5 and 10 sec.

alterations in survival pathways such as NF- $\kappa$ B. We examined biochemical parameters that are measurable during a dynamic [ $^{18}\text{F}$ ]-FDG-PET examination i.e., [ $^{18}\text{F}$ ]-FDG incorporation, glucose transport and hexokinase activity. There are many other factors that may influence [ $^{18}\text{F}$ ]-FDG incorporation, including the concentration of reduced NADPH (16), cytochrome C expression (17) and the rate of flux through the pentose phosphate pathway (18). These mechanisms could underlie changes in glucose utilisation by 5FU and Tomudex-resistant tumour cells and these may be chemotherapy agent and tumour specific.

Although hexokinase activity was decreased in the 2 resistant cells, MCF7<sub>TDX</sub> and H630<sub>5FU</sub>, compared with the parental H630<sub>TDX</sub> cells (present study) and 5FU-resistant MCF7 cells (11) did not exhibit any significant change in hexokinase activity. The lack of consistency in the change in hexokinase activity and type of drug resistance suggest that this is not a useful indicator of drug resistance. The difference in glucose transport by 5FU-resistant MCF7 breast cancer cells (11) and by H630<sub>R10</sub> colorectal cancer cells (present study) compared with wild-type cells is high; therefore, the rate constants,  $K_1$  and  $K_2$ , which measure glucose influx and efflux in dynamic FDG-PET, may be a particularly sensitive indicator of resistance to 5FU but not of resistance to tomudex. Recent studies (9,10) have shown that  $K_1$  can be a useful parameter demonstrating increased glucose transporter expression.

A number of previous studies (19-21) have examined the influence on tumour cell [ $^{18}\text{F}$ ]-FDG accumulation of the overexpression of the P-glycoprotein (P-gp/MDR1), which is a plasma membrane protein that increases the rate of efflux of diverse anti-cancer drugs, including anthracyclines and taxanes. These studies have shown that [ $^{18}\text{F}$ ]-FDG is a substrate for P-gp and consequently tumour cell P-gp expression is associated with low [ $^{18}\text{F}$ ]-FDG accumulation. However, although reduced FDG uptake via enhanced P-gp efflux may be a mechanism of altered FDG dynamics in anthracycline- or taxane-resistant tumours, 5FU is not a P-gp substrate and therefore this is unlikely to be a mechanism in tumours resistant to this agent.

In summary, in this study, we show that resistance to tomudex is associated with increased [ $^{18}\text{F}$ ]-FDG incorpora-

tion. By contrast, tumour cells resistant to other drug types (13), including 5FU, generally exhibit decreased [ $^{18}\text{F}$ ]-FDG incorporation. Resistance of colorectal tumour cells to 5FU was also shown to be accompanied by increased glucose transport and decreased [ $^{18}\text{F}$ ]-FDG incorporation in common with 5FU-resistant breast tumour cells (11).

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