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Two-deoxyglucose as an Anti-metabolite in Human Carcinoma Cell Line RPMI-2650 and Drug-resistant Variants

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Abstract. *The accumulation of 2-deoxyglucose (2-DG), a glycolytic inhibitor, was investigated in a human nasal carcinoma cell line, RPMI-2650 and two of its drug-resistant variants (selected with taxol and melphalan) to assess manipulation of glycolytic potential as a selective means of reducing resistance. 2-DG uptake was increased 3-fold and 9.9-fold in taxol- and melphalan-resistant variants of RPMI-2650, respectively. Two of the principal factors associated with increased 2-DG uptake, namely glucose transporters and hexokinase activity, were increased in the resistant variants. Other changes in glucose metabolism that may affect 2-DG as an antimetabolite were observed, including increases in glucose-6-phosphate dehydrogenase of 10-fold and 100-fold for taxol- and melphalan-resistant variants, respectively, suggesting higher pentose phosphate activity; increased glutamine utilisation and greater sensitivity to iodoacetic acid-induced depletion of ATP levels in the parent relative to the resistant variants.*

A common characteristic of cancer cells is their propensity for high rates of glucose uptake and glycolysis; highly aggressive and poorly-differentiated cancers have the highest rates. A better understanding of the altered pathways of glucose metabolism in cancer cells, and in particular in chemotherapy-resistant cancer cells, may lead to the identification of new targets (perhaps with some degree of cancer- or drug resistance- specificity) for therapeutic intervention (1-3). As many drug-resistant mechanisms are ATP-dependent (especially efflux pumps such as p-glycoprotein (PGP) and multidrug resistance associated proteins (MRPs)), the use of glycolytic inhibitors like 2-deoxyglucose (2-DG) and fluorodeoxyglucose (FDG) metabolised only as far as the initial phosphorylation step in glycolysis, may provide a means of reducing ATP levels and circumventing drug resistance selectively. A combination of increased transport across the cell membrane facilitated by glucose transporters (4,5) and/or increased phosphorylation of those analogues by hexokinases (6), together with low levels of glucose-6-phosphatase (G-6-Pase) in cancer cells in general (7), trap the glucose analogues within cancer cells to a greater degree than normal cells. FDG and 2-DG have been used for cancer imaging, prognosis and evaluation of therapies (8-10) and antimetabolite activity (11,12) respectively in a variety of cancers.

In this paper, we have analysed the human nasal carcinoma line RPMI-2650 and two drug-resistant variants to ascertain the impact of drug resistance on 2-DG accumulation and glucose metabolism and to establish if these characteristics could be exploited to further the detection and treatment of drug-resistant cancers.

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Materials and Methods

All chemicals (unless otherwise stated), FBS, glutamine and lactate kits were obtained from Sigma (Poole, UK). Cell culture media was supplied by Gibco BRL (Paisley, UK). Hexokinase, glucose strips and protease inhibitors were obtained from Boehringer Mannheim (Roche, UK). Radiolabelled 2-deoxy-glucose was obtained from New England Nuclear (Perkin Elmer, UK). GLUT antibodies 1 and 2 were obtained from Chemicon (Chemicon Europe, Hampshire, UK) and GLUTs 3, 4 and 5 were obtained from Santa Cruz (Heidelberg, Germany). ATP determination kits were obtained from Promega (Southampton, UK) and Labsystems (Basingstoke, UK).

Cell lines. The human RPMI-2650 cell line (squamous cell carcinoma of the nasal septum) was obtained from the ATCC (Bethseda, MD, USA). Taxol (Tx) and melphalan (Ml)-resistant variants were established in the centre by Y. Liang (1999). Cells were grown in MEM with 5% FBS, 1% NEAA and L-glutamine.

^{3}H-deoxyglucose uptake. Exponentially growing cells (at 37˚C and 5% CO_2) were washed gently twice with sterile prewarmed PBS A, incubated in serum-free medium for 2 hours, washed again and

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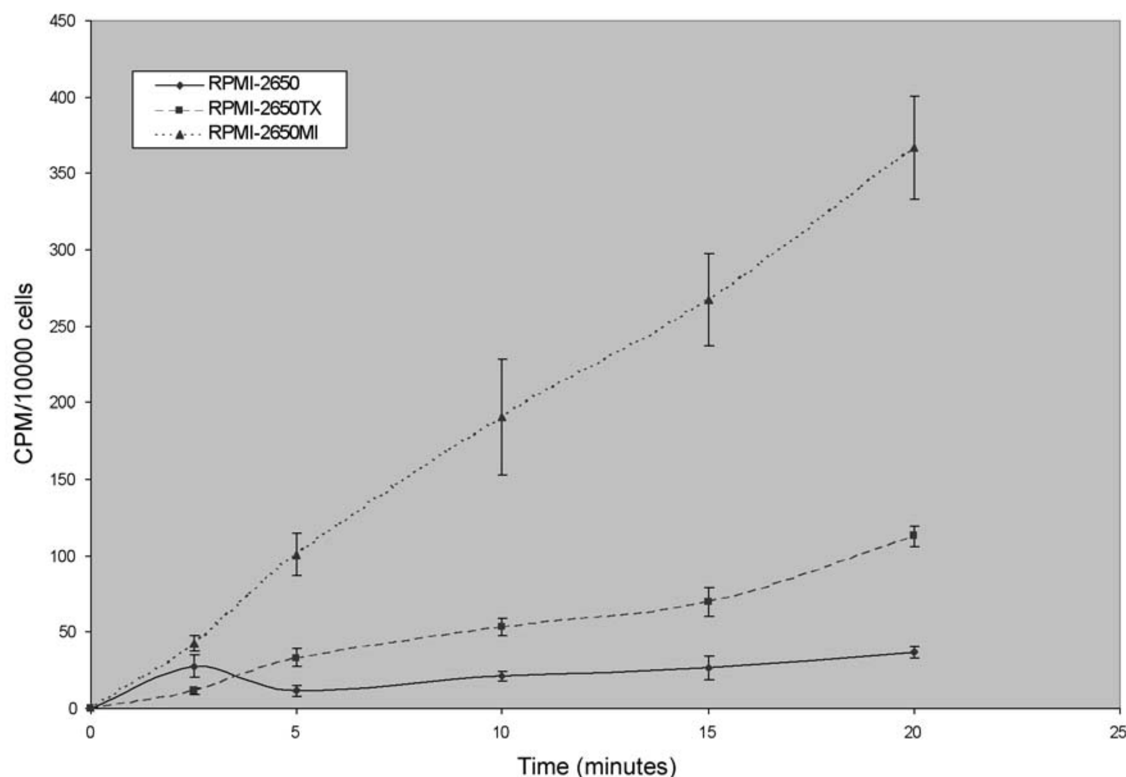

Figure 1. *2DG uptake for RPMI-2650 and variants. Results are expressed as the average ± standard deviation CPM/10^4 cells where N≥3 (3 separate assays with three repeats per assay). Results have been adjusted to take non-specific binding to the cells into account (i.e. CPM at T0 were taken away from CPM at T20). Cell counts were performed in triplicate with coefficients of variation less than 10%.*

exposed to sterile KRB at 50μM glucose at 37˚C for 20 minutes. The cells were washed as before and exposed to ^{3}H-2-deoxyglucose in PBS (0.25μl per ml; activity 1mCi/ml). After a specific incubation time, media was removed and cells were washed twice in ice-cold PBS to stop the reaction. Cells were solubilised in 1% SDS and radioactive tritium detected using a scintillation counter. Cell counts were made on control wells at 0 and 20 minutes.

Western blotting. Western blotting for detection of GLUTs 1-5 was performed on sonicated cell lysates that were centrifuged at 1000rpm to remove nuclear material. Protein determination was made using the Biorad method. Samples were boiled for 2 minutes and separated on a 10% SDS gel (13) with 50μg protein loaded per well. After Western blotting (14), blots with primary antibodies were incubated overnight at 4˚C. Secondary antibody conjugated to HRP (Sigma) was detected by enhanced chemiluminesence (ECL, Amersham, UK). Positive controls were used for each GLUT (HL60 for GLUT1; BHK for GLUT 2; GLUT 3 control; 3T3-LI for GLUT 4 and MCF-7 for GLUT-5).

Enzymatic determinations. Activity of HK (15) and glucose-6-phosphate dehydrogenase (G6PDH) (16) were determined by the increase in absorbance of NADPH at 340nm. Cell suspensions in TEA buffer (50mM, pH7.6) with protease inhibitors were sonicated on ice and centrifuged at 1000rpm to remove nuclear material. The supernatant was centrifuged at 13,000rpm to separate out cytosolic (supernatant) and mitochondrial fractions (pellet). Both fractions were aliquoted and frozen at -80˚C until analysis. Protein determination was by the Biorad method.

Glucose, lactate and glutamine measurements. Exponentially growing cells were washed and exposed to fresh media for 2, 4 or 24 hours and cells were counted. Glucose levels were measured using a glucose meter and strips (Refloflux S and BM-TEST 1-44). Lactate production was measured using an enzymatic kit (Sigma 735-10). Glutamine levels were measured by an enzymatic colormetric kit (Sigma GLN-2).

ATP determinations. Exponentially growing cells were exposed to metabolic inhibitors for 4 hours (1). Cells were washed, solubilised and the ATP levels determined by luminometry. Cell counts were carried out to normalise ATP levels per cell number. The inhibitors used were 2,4, Dinitrophenol (DNP) at 200μM, 10μM Iodoacetic acid (IAA) and 10mM Oxamic acid (Ox).

Toxicity testing. Toxicity was assessed in 96-well plates with acid phosphatase activity as the end point (17). Assays were conducted with 2X, 1X and 0.5X of the IC_{50} for melphalan (ML) or taxol (TX) for each cell line for long-term assays and at 6X, 4X and 2X of the IC_{50} of each drug for short-term assays with or without 4mM 2-DG

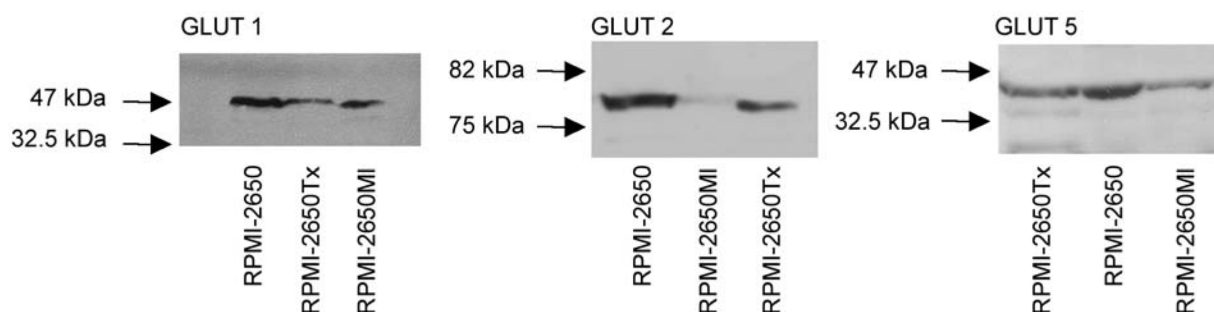

Figure 2. *Western blots of GLUTs for RPMI-2650 and resistant variants.Results of Western blotting based on equal protein loading. One of three separate repeats is shown for GLUTs 1, 2 and 5. No bands were detected for GLUTs 3 and 4 (figures not shown).*

Table I. *Signal-to-volume ratio.*

	RPMI-2650	RPMI-2650Tx	RPMI-2650Ml
Fold increase at 20 minutes	1	3.06	9.96
Cell diameter (μm)	1.05	1.7	1.6
Cell volume (μm^3)	0.606	2.572	2.145
Signal-to-volume ratio compared to RPMI-2650	1	0.715	2.81

Signal to volume ratio is based on the equivalent volume occupied by RPMI-2650.

Table II. *Densitometric analyses of GLUT blots and adjustments for cell number.*

Glucose transporter levels based on equal protein loading (compared on percentage basis to levels in RPMI-2650) (50μg per well)			
Cell line	GLUT 1 (%)	GLUT 2 (%)	GLUT 5 (%)
RPMI-2650	100	100	100
RPMI-2650Tx	53.6 ± 5.1	62.4 ± 25.5	55.9 ± 8.45
RPMI-2650Ml	86.1 ± 10.11	45.3 ± 6.87	48.41 ± 5.49
Glucose transporter levels based on cell number (per 10^5 cells)			
Cell line	GLUT 1	GLUT 2	GLUT 5
RPMI-2650	100	100	100
RPMI-2650Tx	307 ± 29.2	358.6 ± 146	321.3 ± 48.6
RPMI-2650Ml	489.7 ± 57.5	257.6 ± 39.1	275.6 ± 31.1

For each gel, (3 separate gels for each GLUT), the OD/mm readings obtained by densitometric analysis were taken with results for all variants expressed as a percentage of the control (RPMI). The average and standard deviation for 3 separate gels was determined and the results expressed on the basis of equal protein loading. Protein concentration per cell number was determined in triplicate with average concentrations of 0.873, 5.014 and 4.966mg/10^7 cells for RPMI-2650, RPMI-2650Tx and RPMI-2650Ml, respectively.

(in 4mM glucose), a level previously determined as the maximum tolerated clinical dose (19). The IC_{50} for taxol was previously determined (20) as 2.3, 519 and 3.9nM for RPMI-2650, Tx- and Ml-resistant variants, respectively. The IC_{50} for melphalan (20) is 582, 2621 and 6135nM for RPMI-2650, Tx- and Ml-resistant variants, respectively. Cells were set up at $1x10^3$ or $2x10^3$ per well for long and short-term assays, respectively, and allowed to incubate overnight at 5% CO_2 and 37˚C. For long-term assays, drug and/or 2-DG were added and cells incubated for 6 days before analysis. In short-term assays, cells were exposed to 2-DG and /or drug for 4 hours after which time, drug-containing media was removed. The cells were washed twice with basal media and fed with 4mMol glucose-containing media and incubated for a further 4 days before analysis.

Results

Measurement of glucose uptake: ^{3}H-deoxyglucose uptake. Uptake of ^{3}H-deoxyglucose over a 20-minute period revealed differences between the parental and resistant variants (Figure 1). Uptake in the parent was not steady. At 2.5 minutes, uptake was intermediate between Tx- and Ml-resistant variants, but fluctuated at 5 minutes and only slowly rose again thereafter. For Tx- and Ml- resistant variants, uptake increased steadily with 3.06-fold and 9.96-fold increases, respectively, over the parent at 20 minutes. As 2-DG is quite similar to FDG used clinically, we assessed the signal-to-volume ratio (for 2-DG) for these cells as the parental cell line is smaller than the resistant variants (Table I). The Tx- and Ml- resistant variants are 4.2 and 3.5 times larger, respectively, than the parent, suggesting the Ml-resistant variant would be easily distinguished *in vivo* with a 2.8-fold higher signal-to-volume ratio than the parent. The lower signal-to-volume ratio for the Tx-resistant variant (0.715) would make it more difficult to distinguish from the parent.

Table III. *Biochemical analysis on RPMI-2650 and resistant variants.*

Table IIIa. *Hexokinase and glucose-6-phosphate dehydrogenase activity.*

Cell line (U/107 cells)	RPMI-2650 (U/10^7 cells)	RPMI-2650Tx (U/10^7 cells)	RPMI-2650Ml
HK cytosolic activity	0.0252 ± 0.003	0.089 ± 0.0009	0.082 ± 0.02
HK mitochondrial activity	0.0033 ± 0.001	0.022 ± 0.0001	0.024 ± 0.006
Total HK activity	0.0285	0.111	0.106
G6PDH cytosolic activity	0.089 ± 0.017	0.726 ± 0.08	9.256 ± 1.26
G6PDH mitochondrial activity	0.0003 ± 0.0001	0.018 ± 0.0018	0.155 ± 0.037
Total G6PDH activity	0.0893	0.744	9.411

Results are expressed as the average U/10^7 cells ± standard deviation. There were three separate experiments with 2 samples per experiment measured.

Table IIIb. *Glucose, lactate and glutamine analysis at 24 hours.*

Cell line	RPMI-2650 (μM/10^5 cells)	RPMI-2650Tx (μM/10^5 cells)	RPMI-2650Ml (μM/10^5 cells)
Glucose depletion	0.551 ± 0.07	0.632 ± 0.03	1.185 ± 0.16
Glutamine depletion	0.079 ± 0.01	0.4 ± 0.02	0.165 ± 0.03
Lactate production	0.627 ± 0.1	0.429 ± 0.02	1.5 ± 0.06

Results are expressed as average ± standard deviation for 3 separate experiments with two flasks per time point per experiment. Cell counts were made in triplicate. Glucose and glutamine depletion refer to the reduction in cell supernatant levels due to uptake and utilisation by the cells.

Table IIIc. *ATP analysis.*

Cell line	RPMI-2650 (μM/10^4 cells)	RPMI-2650Tx (μM/10^4 cells)l	RPMI-2650M (μM/10^4 cells)
Total cellular ATP	0.62 ± 0.01	0.98 ± .005	1.19 ± 0.07
+DNP	0.36 ± 0.04	0.69 ± 0.05	0.60 ± 0.07
+IAA	0.08 ± 0.03	0.52 ± 0.06	0.45 ± 0.08
+Ox	0.49 ± 0.03	0.60 ± 0.08	1.25 ± 0.20

Results are expressed as average ± standard deviation for 3 separate experiments with four repeats per experiment. Results were obtained as relative light units (in quadruplicate) and converted to Moles ATP from a standard curve. Cell counts were made in triplicate

Western blotting. Analyses of cell lysates based on equal protein loading by Western blotting revealed the presence of GLUTs 1, 2 and 5 at about 40kDa, 62.5kDa and 47kDa, respectively, as would be expected and little or no GLUT 3 and 4 in parental and resistant variants (Figure 2). Based on equal protein loading (Table II), RPMI-2650 had the highest levels of the three GLUTs with the variants expressing about half the level of protein except for GLUT 1 in the Ml-resistant variant (86.1% of that observed in the parent). With protein levels per cell of 5.69-fold and 5.75-fold higher in the Ml- and Tx-resistant variants as compared to the parent, both resistant variants had higher levels of GLUTs on a per cell basis. For RPMI-2650Ml increases of 4.8-fold, 2.6-fold and 2.75-fold levels per cell above the parent for GLUTs 1, 2 and 5, respectively, are seen. For RPMI-2650Tx, there was a 3-fold, 3.58-fold and 3.2-fold increase per cell in GLUTs 1, 2 and 5, respectively.

Biochemical analysis. Consistent with increased 2-DG uptake, both resistant variants have increased levels of HK activity (Table IIIa). The Tx- and Ml-resistant variants have, respectively, a 3.9-fold and 3.7-fold higher total HK activity than RPMI-2650. Activity in the mitochondrial fraction (6.22-fold and 7.24-fold increase, respectively) was increased more significantly than in the cytosolic fraction (3.53-fold and 3.26-fold increase, respectively) for Tx- and Ml-resistant variants. The increase in HK activity alone could be sufficient to explain the increased 2-DG uptake in the Tx- but not in the Ml- resistant variant. Interestingly, results for G6PDH are most significant with a 10-fold and 105-fold increase in activity for the Tx- and Ml-resistant variants, respectively, over the parent, suggesting high flow through the pentose phosphate pathway. Uptake of glucose was doubled in RPMI-2650Ml and unaltered in RPMI-2650Tx as compared to the control over 2, 4 and 24 hours. The discrepancy between glucose and 2-DG uptake in the parent and resistant variants may be due to a number of factors. Higher levels of mitochondrial-bound HK and total cellular ATP in the resistant variants make them less susceptible to end-product inhibition from 2-DG-P. Regulatory control exerted on glucose but not 2-DG uptake from glycolysis (phosphofructokinase and pyruvate kinase) limit glucose but not 2-DG uptake. The possible induction of glucose-6-phosphatase activity on the part of the parent (as suggested by the change in 2-DG uptake at 5 minutes) may allow 2-DG-P to be dephosphorylated to 2-DG and transported out of the parent.

The Tx-resistant variant produced less lactate and utilised greater amounts of glutamine than the parental cell line, suggesting a greater reliance on oxidative phosphorylation and glutaminolysis, respectively.

Analysis of total ATP showed Tx- and Ml-resistant variants to have 1.6-fold and 2-fold higher levels than that

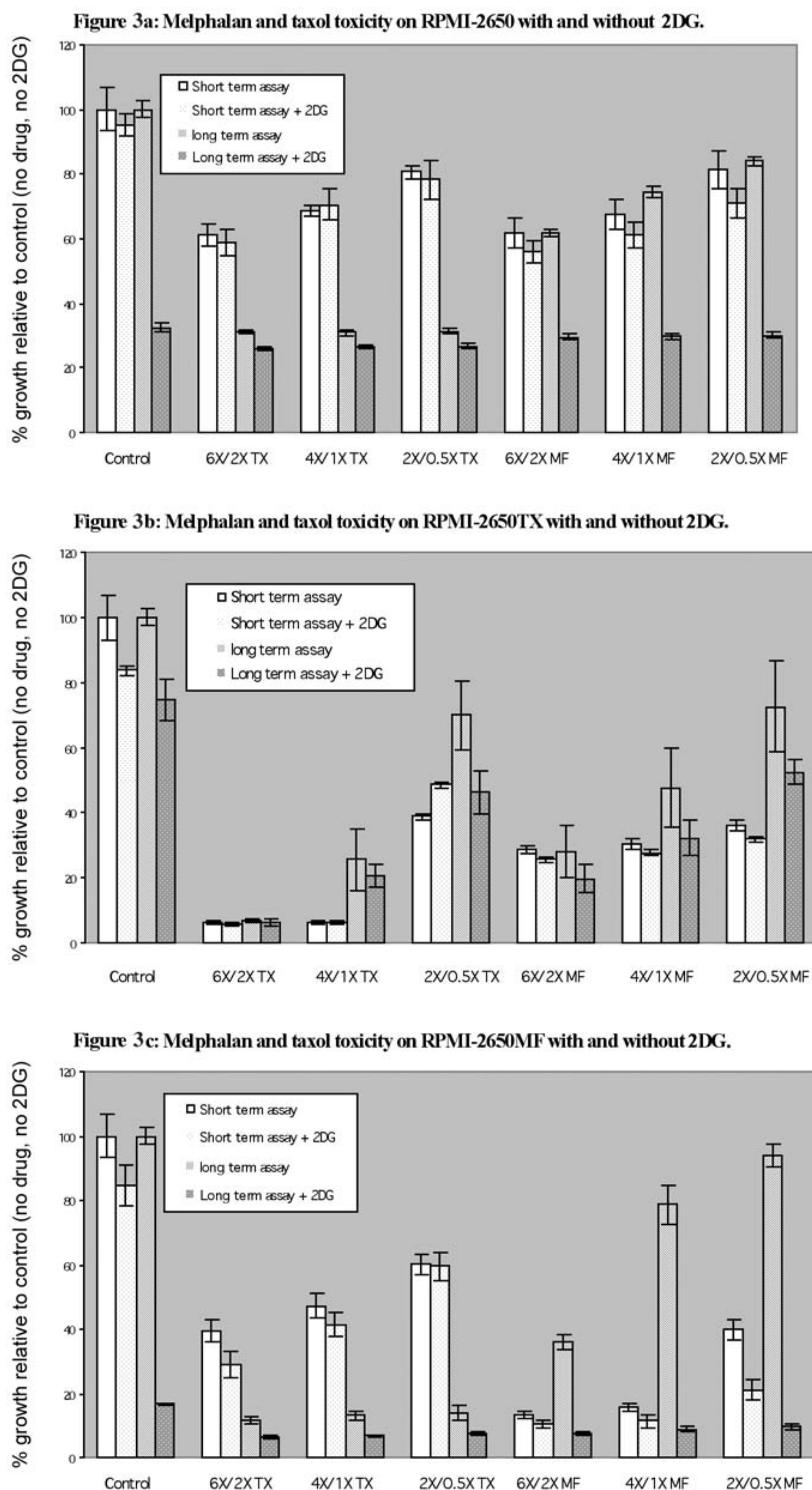

Figure 3. *Effect of 2-DG on drug toxicity. Results are expressed as the average percentage growth relative to control (no drug) ± standard deviation where N=18 (3 separate assays with 6 repeats per assay). 6X/2X refers to 6X IC_{50} for short term assay and 2X IC_{50} for long term assay.*

found in the parent (Table IIIc). Of the metabolic inhibitors, Iodoacetic acid (Glyceraldehyde-3-phosphate dehydrogenase inhibitor) was most effective, reducing ATP levels much more significantly in the parent (by 86.4%) than in Tx-resistant (47.2%) and Ml-resistant (62.4%) variants. Dinitrophenol (mitochondrial inhibitor) were similarly inhibitory on the parent and Ml-resistant variant. Oxamic acid (lactate dehydrogenase inhibitor) was least inhibitory, reducing ATP levels in RPMI-2650 and Tx-resistant variant by 21% and 31.25%, respectively, without affecting the Ml-resistant variant.

Toxicity assays with 2-DG. As the major mechanism of drug resistance in the RPMI-2650Ml and RPMI-2650Tx, ATP-dependent drug efflux, the ability of 2-DG to deplete ATP levels and so reduce resistance in toxicity assays, was investigated.

The toxicity of 2-DG as an anti-metabolite in combination with taxol and melphalan was tested in short and long-term assays for RPMI-2650, Tx- and Ml-resistant variants (Figure 3 a, b and c, respectively). In short-term assays (4 hours), 4mM 2-DG showed little effect alone and did not significantly enhance the toxicity of taxol or melphalan for the parent or Tx- and Ml-resistant variants (Figure 3 a, b and c). In long-term assays (6 days), 4mM 2-DG inhibited growth by 68%, 35% and 83% alone for RPMI-2650, Tx- and Ml-resistant variants, respectively. In the case of the parental cells in long-term assays, addition of taxol or melphalan to 2-DG only reduced growth by 20% and 10%, respectively, with no synergistic or additive effects seen in combination. Similar to parent, the Ml-resistant variant, taxol or melphalan reduced growth to 40% and 50%, respectively, of that seen with 2-DG alone. For the Tx-resistant variant, 2-DG did not significantly increase toxicity of either melphalan or taxol.

Discussion

The characteristics of increased glucose uptake and metabolism in cancer cells has provided a very useful tool for tumour imaging and prognostic evaluation through FDG-PET (9,10,18), while 2-DG been used to a limited extent to enhance traditional chemotherapy or radiotherapy (7,19,20).

The altered glucose metabolism associated with chemotherapeutic resistance (25-27) could potentially provide a target for circumventing resistance, especially where increased glucose metabolism fuels resistance through ATP-driven pumps *e.g.* PGP and MRPs, as is the case for Tx- and Ml-resistant variants, respectively (20). The results presented here indicate a substantial increase in 2-DG uptake in drug-resistant variants, Ml (especially) and Tx compared to the parental cell line RPMI-2650 consistent with findings for MCF-7 and drug-resistant variants (28), while the opposite was found in HT-29 and its drug-resistant variant (29).

For imaging purposes, the signal-to-volume ratio for RPMI-2650Ml is clearly larger than the parent and would be easily detected by FDG-PET, suggesting the possibility of monitoring the development of drug resistance *in vivo*. However, the lower signal-to-volume ratio for RPMI-2650Tx suggests limitations to monitoring drug-resistant variants with FDG-PET.

Increases in glucose and analogue uptake in cancer cells has been attributed to increased transport by glucose transporters (GLUTs) or increased phosphorylation by hexokinases. GLUTs are cytoplasmic membrane proteins (40-70kDa) that exhibit tissue-specific patterns of expression with distinct kinetic and regulatory properties (30,31). Of the 7 GLUTs, GLUT 1 is most often elevated in cancer (32) although changes in GLUTs 2, 4 and 5 are also found (33,34). Hexokinase, the initial and rate-limiting step in glycolysis phosphorylates glucose to glucose-6-phosphate. Of the four isozymes, I and II are most commonly over-expressed in cancer cells (35). The increased 2-DG uptake in the resistant variants was consistent with increases in both GLUT and HK activity on a per cell basis, either of which could account for the activity in the Tx- but not the Ml-resistant variant. The GLUT profile shows expression of GLUT 1, 2 and 5 in the RPMI-2650 and drug-resistant variants. For the Tx-resistant variant there is a general increase in the three GLUTs, while for the Ml- resistant variant, GLUT 1 expression is almost twice as high as GLUTs 2 and 5. Both Tx- and Ml-resistant variants showed similar increases in total HK activity (3.8-fold and 3.7-fold, respectively) and mitochondrial HK activity (6.6-fold and 7.3-fold, respectively). This could facilitate increased glycolytic rates by providing HK with an abundant supply of ATP (direct from mitochondria) and protecting HK from end-product inhibition by glucose-6-phosphate (36) and 2-DG-P. The higher ATP levels found in the Tx- and Ml-resistant variants (1.6-fold and 1.92-fold, respectively) may also facilitate increased 2-DG uptake as previously shown for FDG (37).

Metabolic inhibitors showed that the three cell lines were capable of aerobic and anaerobic glycolysis. Interestingly, RPMI-2650 was inhibited significantly more by IAA than either of the resistant variants. This may be due to the smaller cell size of the parent and presumably smaller amounts of glyceraldehyde-3-phosphate dehydrogenase as IAA inhibits in a stoichiometric fashion.

The striking 10 and 105-fold increase in G6PDH for Tx- and Ml-resistant variants, respectively, is consistent with reports of melphalan resistance resulting in elevated GLUTs and G6PDH (38). Increased pentose phosphate pathway (PPP) activity including G6PDH provide high levels of reduced glutathione and NADPH to facilitate detoxification of drug and drug metabolites.

While 2-DG uptake was related to the proliferative index of some cancers (39), no difference in the doubling time of RPMI-2650 and resistant variants (27) was seen.

Toxicity studies with 2-DG as an anti-metabolite showed little affect either alone or in combination with taxol or melphalan in short term assays suggesting that the 4 hour incubation was insufficient to block glucose metabolism, reduce ATP levels and render the cells more sensitive to the drugs used. Combination of taxol or melphalan with 2-DG enhanced inhibition in long-term assays, all showed increasing toxicity with increasing drug concentration but the dose-dependent increase was only significant in RPMI-2650Tx. In long-term assays, 2-DG alone was more inhibitory to RPMI-2650 (by 68%) and RPMI-2650Ml (by 83%) than RPMI-2650Tx (by 35%) and may correlate with the ability of RPMI-2650Tx to rely on glutaminolysis. The extent of inhibition by 2-DG in the long-term assays is most likely compounded by the reduction in metabolisable glucose in the medium over the 7-day period.

Overall, from these studies we have found that the acquisition of drug resistance in the cell lines studied resulted in increased 2-DG uptake. The biochemical changes facilitating the increase appear not only related to alterations in GLUT expression and hexokinase activity, but also to changes in glutaminolysis and energy requirements. While increased 2-DG uptake (and presumably therefore FDG uptake) provide an opportunity for assessing drug resistance *in vivo* and allowing appropriate modification of the treatment regime, altered metabolism (as seen with glutaminolyis in the Tx-resistant variant) may present some limitations to the system. In addition, the changes in metabolism, and particularly in the use of alternative carbon sources, will have implications for the use of 2-DG as an antimetabolite.

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