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[^{18}F]2-Fluoro-2-deoxy-D-glucose incorporation by AGS gastric adenocarcinoma cells *in vitro* during response to epirubicin, cisplatin and 5-fluorouracil

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Decreased tumour [^{18}F]2-fluoro-2-deoxy-D-glucose (^{18}FDG) incorporation is related to response however its significance at the cell level in gastro-oesophageal cancer and how it relates to cell death is unknown. Here human gastric adenocarcinoma (AGS) cells were treated with lethal dose 10 and 50 (LD_{10} and LD_{50}), determined by using the MTT assay, of the three drugs, epirubicin, 5-fluorouracil and cisplatin, commonly used in the treatment of patients with gastro-oesophageal cancer. ^{18}FDG incorporation was determined after 48 and 72 h of treatment with each drug and related to drug-induced changes in glucose transport, hexokinase activity, cell cycle distribution and annexin V-PE binding (a measure of apoptosis). Treatment of cells for 48 and 72 h with LD_{50} doses of cisplatin resulted in reductions in ^{18}FDG incorporation of 27 and 25% respectively and of 5-fluorouracil reduced ^{18}FDG incorporation by 34 and 33% respectively: epirubicin treatment reduced incorporation by 30 and 69% respectively. Cells that had been treated for 72 h with each drug were incubated in drug-free media for a further 6 days to determine their ability to recover. Comparison of the ability to recover from the chemotherapy agent, with ^{18}FDG incorporation before the recovery period allowed an assessment of the predictive ability of ^{18}FDG incorporation. Cells treated with either 5-fluorouracil or cisplatin demonstrated recovery on removal of the drug. In contrast, cells treated with epirubicin did not recover corresponding with the greatest 72 h treatment decrease in ^{18}FDG incorporation. In contrast to adherent cells treated with cisplatin or 5-fluorouracil, adherent epirubicin-treated cells also exhibited very high levels of apoptosis. Glucose transport was decreased after each treatment whilst hexokinase activity was only decreased after 72 h of treatment with each drug. There was no consistent relationship observed between ^{18}FDG incorporation and cell cycle distribution. Our results show that at the tumour cell level in gastric tumour cells, decreased ^{18}FDG incorporation and glucose transport, accompanies therapeutic growth inhibition. ^{18}FDG incorporation is particularly diminished in cells exhibiting apoptosis.
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Combination chemotherapy may improve the length and quality of survival in a proportion of patients with advanced gastro-oesophageal cancer. With currently available chemotherapy regimens, only 40 – 60% of patients will respond to treatment (Gilbert *et al*, 2002). Present chemotherapy regimens for gastro-oesophageal cancer include epirubicin, cisplatin and 5-fluorouracil (5-FU). If patients are to avoid unnecessary treatment with particularly toxic agents, it is important that non-responders are identified at an early stage.

A number of studies (Couper *et al*, 1998; Weber *et al*, 2001; Kroep *et al*, 2003; Ott *et al*, 2003) have demonstrated that a reduction in tumour uptake of the glucose analogue, [^{18}F]2-fluoro-2-deoxy-D-glucose (^{18}FDG), is observed during and upon completion of chemotherapy in gastro-oesophageal cancer. Although decreased tumour ^{18}FDG uptake has been shown to be associated with response (and may in fact predict tumour response), its significance at the gastro-oesophageal tumour cell level is not known. Response to chemotherapy, as seen on ^{18}FDG-PET with a corresponding reduction in uptake in solid tumours, may be related to a decrease in tumour cellularity or a decrease in ^{18}FDG uptake per cell, or a combination of both. In solid tumours, tumour cells can recover and it is not known if this decreased ^{18}FDG uptake during response is due to modulation in ^{18}FDG uptake by cells that will not recover. This reduction in cellular uptake of ^{18}FDG may occur prior to reduction in tumour volume and therefore may reflect apoptosis. To further confuse matters, a flare phenomenon has been recorded in the early period following exposure to chemotherapy (Basu and Alavi, 2007). In addition chemotherapy may alter ^{18}FDG cellular uptake by hexokinase (HK) and/or glucose transport modulation.

The fluorinated glucose analogue, ^{18}FDG, is transported into tumour cells via a family of glucose transporter proteins, then phosphorylated by the enzyme HK to ^{18}FDG-6-phosphate after which it undergoes little further metabolism. Due to low levels of G-6-Pase in tumour cells this is considered an irreversible reaction (Weber and Cantero, 1955; Warburg, 1956; Gallagher *et al*, 1978).

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Higher levels of HK, namely, HK2 (Torizuka *et al*, 1995; Mathupala *et al*, 1997) and GLUT, especially GLUT1 (Mueckler, 1994; Younes *et al*, 1996; Smith, 1999) and low levels of G-6-Pase (Nelson *et al*, 1996) have been reported in tumour tissue compared with corresponding normal tissue. Both glut proteins and HK activity have been implicated as the rate limiting step in cellular ^{18}FDG uptake (Higashi *et al*, 1997; Waki *et al*, 1998; Brown *et al*, 1999; Kurokawa *et al*, 2004; Tohma *et al*, 2005; Zhao *et al*, 2005), although plasma (or media) glucose concentrations may have more of an impact (Aloj *et al*, 1999; Zhao *et al*, 2002; Burrows *et al*, 2004).

We have determined the effect of three commonly used chemotherapeutic agents (epirubicin, cisplatin and 5-FU) in the treatment of upper gastro-intestinal tumours on the cellular incorporation of ^{18}FDG and on steps associated with its incorporation, that is, glucose transport and HK activity. As tumour cells within solid tumours are likely to be exposed to different drug concentrations, we have treated cells with both lethal dose 50 (LD_{50}) and drug doses that have a low cell growth inhibition (LD_{10}, 5–10% cell death). Previous work on AGS (human gastric adenocarcinoma) cells has shown that they can recover from treatment with cytotoxic doses of combinational regimes of 5-FU and cisplatin but not when epirubicin is included in the combination (Couper and Park, 2003). However, the design of Coupers' study meant that this effect could not be specifically related to epirubicin rather than triple combination chemotherapy. Our study evaluated the effect of each individual chemotherapeutic agent. Growth curves for AGS cells were established after exposure to each epirubicin, cisplatin and 5-FU, with LD_{10} and LD_{50} established for each agent at 48 h. Growth curves were taken to 216 h to determine the extent to which cells were irreversibly damaged by the chemotherapeutic agents. All subsequent experiments on ^{18}FDG uptake and its relationships to glucose transport, HK activity, cell viability, cell cycling and apoptosis were undertaken at 48 and 72 h.

The aims of this study were to elucidate the effect, on cellular (AGS cells) ^{18}FDG incorporation, of exposure to three commonly used chemotherapeutic agents in the treatment of gastro-oesophageal cancer and to determine the mechanisms behind these changes in ^{18}FDG cellular incorporation and their relationship with apoptosis.

MATERIALS AND METHODS

Cell line

A human gastric adenocarcinoma cell line (AGS) (ECACC, Porton Down, Salisbury, UK) was cultured in HAM F-12 media (Sigma, Dorset, UK) (supplemented with sodium hydrogen carbonate, penicillin G, streptomycin sulphate and L-glutamine and 10% fetal calf serum (Labtech International, East Sussex, UK)) at 37°C in a humidified incubator with 5% CO_2 : 95% air.

The cells were subcultured in vented 80 cm^2 flasks (Nunclon Delta Surface, Roskilde, Denmark). Cell suspensions were obtained by trypsinisation (ethylenediaminetetraacetic acid (EDTA)/trypsin) (Sigma) of the adherent cell monolayer, with 5 ml of EDTA/trypsin, when cells reached 70–80% confluence. Cell counts were performed using a haemocytometer (Bright-Line Haemocytometer, Sigma).

A seeding density of 7500 cells per 0.35 cm^2, based on previous work (Couper and Park, 2003), was used for all experiments.

Cytotoxicity assays

These were performed to identify the LD_{10} (5–10% cell death) and LD_{50} doses of three chemotherapeutic agents, commonly used in the treatment of gastro-oesophageal cancers, using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983; Carmichael *et al*, 1987).

Cells were plated in HAM F-12 media, at optimal seeding density, and returned to the incubator for 24 h to allow cell adherence to the plate. Media supplemented with varying concentrations of the chemotherapeutic agents epirubicin (Pharmacia and Upjohn Ltd., Milton Keynes, UK) (0.0039–0.5 $\mu g\,ml^{-1}$), cisplatin (Faulding Pharmaceuticals, Warwickshire, UK) (0.156–20 $\mu g\,ml^{-1}$) and 5-FU (Faulding Pharmaceuticals) (0.156–80 $\mu g\,ml^{-1}$) was then added and the plate returned to the incubator for either 48 or 72 h. A background of media and a control of media and cells were also set up.

After 48 or 72 h of exposure to the chemotherapy agents, MTT (Sigma) was added and then incubated for a further 4 h at 37°C. Using a scanning multi-well spectrophotometer (Dynatech MR5000, Dynatech Labarotaries Inc., Chantilly, VA, USA) measuring spectrophotometric absorbance at 570 nm, the plates were analysed using Biolinx 2.0 software (Biolinx 2.0, Dynatech Labarotaries Inc.). All experiments were repeated three times with six replicates per experiment. All subsequent cell work was based on the LD_{10} and LD_{50} concentrations of the chemotherapy agents at 48 h so that time was the only variable.

Quantifying ^{18}FDG uptake

Cells were seeded and incubated for 24 h, with control flasks were seeded at half the optimum seeding density so that cell density was similar in the control and treated flasks at the time of the ^{18}FDG uptake measurement. Control, LD_{10} and LD_{50} flasks were set up in triplicate and the corresponding concentrations of each chemotherapeutic agent and returned to the incubator for either 48 or 72 h. The incorporation of ^{18}FDG (obtained from the John Mallard PET Centre, Aberdeen) was determined by incubation of treated and control cells with ^{18}FDG (1 $kBq\,ml^{-1}$) for 20 min at 37°C followed by rapid washing with phosphate buffered saline (PBS) as described previously (Smith *et al*, 2006). ^{18}FDG uptake was expressed relative to protein content (milligram of cellular protein) and per treated flask.

Protein assay

Protein content was assessed by the bicinchoninic acid protein assay kit according to the manufacturers' instructions (Sigma).

Glucose transport

Initially the linear phase of [^{3}H]*O*-methylglucose (^{3}H-OMG) uptake, a measure of glucose transport (Cloherty *et al*, 2002), was determined by incubating cells with ^{3}H-OMG for 1, 2, 3, 5, 15 and 30 s. ^{3}H-OMG uptake in control AGS cells was very rapid, with the linear part of the time activity curve for ^{3}H-OMG uptake at 37°C complete within 2 s of beginning incubation. Experiments were, therefore, conducted at 25°C with an exposure to ^{3}H-OMG of 1 s.

Glucose transport rates were determined at both 48 and 72 h following the addition of required dose of chemotherapeutic agent, by incubation with media and ^{3}H-OMG (0.5 $\mu Ci\,ml^{-1}$ (specific activity 111GBq $mmol^{-1}$)), at 25°C as described previously (Smith *et al*, 2006) except that the incubations were performed for 1 s. ^{3}H-OMGuptake was expressed in terms of protein content (milligram of cellular protein).

Hexokinase activity assay

AGS cells were set up and treated with chemotherapy as per ^{18}FDG uptake. Following the incubation for 48 or 72 h with chemotherapy the media was removed, and each flask was washed twice with

ice-cold PBS. Cells were then trypsinised and centrifuged with the cell pellet washed a further two times with ice-cold PBS. The resulting cell pellet was then stored at −70°C until the HK activity was assessed based on a modification to the methods by Miccoli *et al* (1996) as described previously (Smith *et al*, 2006).

Enzyme activity was expressed as mU mg^{-1} cellular protein using the extinction coefficient for NADPH of 6.3×10^3 mol^{-1} cm^{-1}.

Flow cytometry DNA quantification

AGS cells were set up and incubated in chemotherapy as previously described for ^{18}FDG uptake. After the required incubation period the media was discarded and the cells were harvested and cell cycle distribution was determined as described previously (Al-Saeedi *et al*, 2005).

Cell regrowth following exposure to chemotherapy

Cells were plated as for MTT assay and treated for 72 h with LD_{50} and LD_{10} doses of each chemotherapy agent. Following this, the media was removed and the cells were washed with warm (37°C) PBS to remove any traces of residual chemotherapy. Fresh media (without chemotherapy) was then added and the plates were returned to the incubator. After 24, 48, 72, 96, 120 and 144 h incubation, MTT assays were performed to determine the latent cytotoxicity of the chemotherapeutic agents. Each experiment was repeated in triplicate with six replicates for each agent and time point.

Annexin V-PE flow cytometry

Annexin V flow cytometry was used to discriminate between intact cells, early apoptotic and late apoptotic or necrotic cells.

AGS cells were set up as per ^{18}FDG uptake. Control and cells treated for 72 h with chemotherapy agents were detached by incubating the cells in non-enzymatic cell dissociation solution in PBS (Sigma) and added to fresh media. The cell concentration was then adjusted to 3×10^5 cells ml^{-1}, and 1 ml of this cell suspension was then transferred to FACS tubes and centrifuged. The cell pellet was re-suspended in 1 ml of binding buffer (140 mM sodium chloride, 25 mM calcium chloride, 10 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid) hemisodium salt), 500 ml distilled water; Sigma). Following further centrifugation and removal of the supernatant, 15 μl of annexin V staining buffer (10 μl Via-Probe (7-N actinomycin D), 5 μl annexin V-PE) (BD Biosciences Pharmingen, Oxford, UK) was added to each cell sample. The samples were stored in the dark at room temperature for 15 min after which 400 μl of binding buffer was added and annexin V flow cytometry was performed within 1 h, analysing 10 000 cell events.

Annexin V flow cytometry was performed on a Becton Dickinson FACS Calibur (San Jose, CA, USA), and results analysed using CellQuest software (Becton Dickinson).

Statistics

Results are expressed as means, ± s.e.m.. ^{18}FDG, ^{3}H-OMG uptake and HK activity were expressed as a percentage of the control. Significance of difference between means was determined by using the paired *t*-test (Statistical Package for the Social Sciences V13.1, SPSS Inc., Chicago, IL, USA), with a *P*-value of <0.05 indicating statistical significance.

RESULTS

Cytotoxicity assay

Cytotoxic doses resulting in a 5–10% decrease in cell number (LD_{10}) and LD_{50} doses of chemotherapy after 48 h exposure were for cisplatin 0.156 and 5 μg ml^{-1}, 5-FU 0.156 and 20 μg ml^{-1} and epirubicin 0.0039 and 0.125 μg ml^{-1} respectively (Table 1).

Effect of chemotherapy on ^{18}FDG uptake

^{18}FDG uptake per untreated control flask (expressed as cpm per 20 min per flask) was 8714 ± 1182 and 14 753 ± 986 respectively after 48 and 72 h incubation periods. ^{18}FDG uptake, expressed as counts per milligram of cells per minute in untreated control flasks, was 783 ± 94 and 984 ± 35 (cpm per 20 min per mg protein) respectively following 48 and 72 h incubation periods.

All three chemotherapeutic agents caused a reduction in ^{18}FDG uptake per flask of treated cells (Figure 1A and B), with the greatest reduction occurring following 72 h exposure to epirubicin. Upon 48 h exposure to LD_{10} epirubicin, there was a non-significant reduction in ^{18}FDG uptake per flask ($P = 0.104$), with a significant reduction upon exposure to LD_{50} ($P = 0.006$) compared with

Table 1 Chemotherapy dose–time relationship and cell death

		Cell death, % (± s.e.m.)	
Chemotherapy	**Dose**	**48 h**	**72 h**
Epirubicin	LD_{10}	4 (4)	5 (2)
	LD_{50}	50 (1)	69 (10)
Cisplatin	LD_{10}	5 (7)	5 (4)
	LD_{50}	56 (7)	77 (12)
5-Fluorouracil	LD_{10}	9 (8)	13 (9)
	LD_{50}	43 (1)	55 (6)

Results of MTT assay performed at 48 and 72 h.

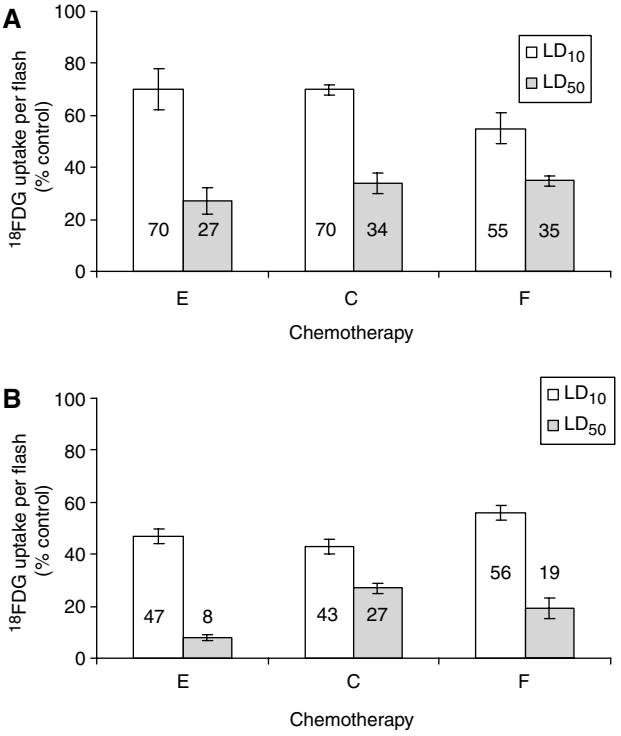

Figure 1 Cellular [^{18}F]2-fluoro-2-deoxy-D-glucose (^{18}FDG) incorporation, per treated flask, after 48 (**A**) and 72 (**B**) h treatment with lethal dose 10 (LD_{10}) (white) and LD_{50} (grey) doses of epirubicin, 5-FU or cisplatin expressed as a percentage of incorporation by untreated controls (E = epirubicin, C = cisplatin, F = 5-fluorouracil).

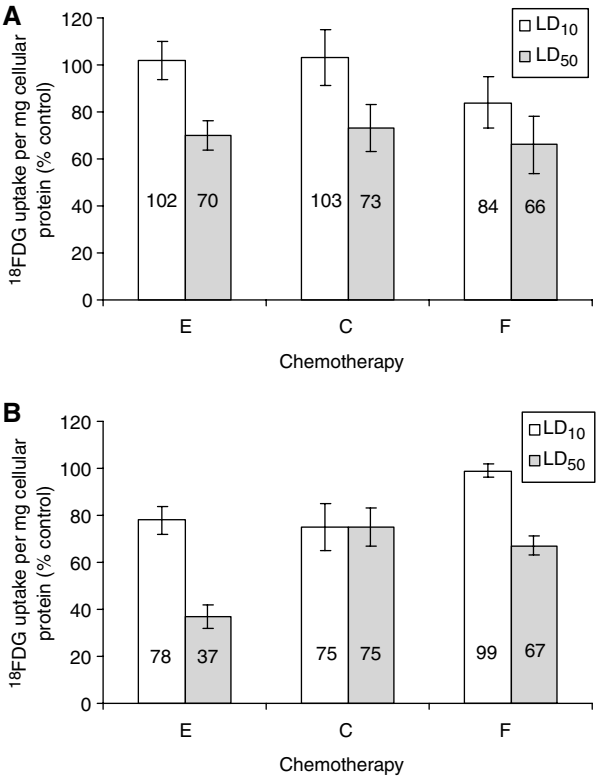

Figure 2 Cellular [^{18}F]2-fluoro-2-deoxy-D-glucose (^{18}FDG) incorporation, per milligram cellular protein, after 48 (**A**) and 72 (**B**) h treatment with lethal dose 10 (LD_{10}) (white) and LD_{50} (grey) doses of epirubicin, 5-FU or cisplatin expressed as a percentage of incorporation by untreated controls (E = epirubicin, C = cisplatin, F = 5-fluorouracil).

untreated controls. Both 48 h LD_{10} and LD_{50} exposure to cisplatin and 5-FU resulted in a significant reduction in ^{18}FDG uptake per flask (cisplatin, $P = 0.037$ and $P = 0.001$; 5-FU, $P = 0.001$ and $P = 0.001$). Compared with untreated controls there was a significant reduction in ^{18}FDG uptake per flask following 72 h LD_{10} and LD_{50} exposure to epirubicin ($P = 0.001$ and $P = 0.001$ respectively), cisplatin ($P = 0.001$ and $P = 0.001$ respectively) and 5-FU ($P = 0.001$ and $P = 0.001$ respectively).

Exposure to all three chemotherapeutic agents resulted in a reduction in cellular ^{18}FDG uptake, with the greatest reduction (63%) occurring on exposure to LD_{50} epirubicin after 72 h exposure (Figure 2A and B). This dose also produced a high level of cell death (69%). Compared with untreated controls there was a significant reduction in ^{18}FDG uptake following 72 h LD_{10} ($P = 0.003$) and both 48 and 72 h exposure to LD_{50} epirubicin ($P = 0.001$ and $P = 0.011$ respectively), with a non-significant reduction in ^{18}FDG uptake following 48 h incubation at LD_{10} levels ($P = 0.789$). ^{18}FDG uptake was significantly decreased in cells exposed to LD_{10} ($P = 0.01$) and LD_{50} ($P = 0.001$) doses of 5-FU for 48 h exposure. Exposure to 5-FU for 72 h resulted in a significant decrease in ^{18}FDG uptake at LD_{50} concentration ($P = 0.001$) whilst there was little difference between control and LD_{10} ($P = 0.839$). Exposure for 48 h to cisplatin, LD_{10} had little effect on ^{18}FDG uptake ($P = 0.636$) in contrast to exposure of LD_{50}, which resulted in a significant reduction in tracer uptake ($P = 0.001$). After 72 h exposure to cisplatin there was little further reduction in ^{18}FDG cellular uptake compared with exposure to 48 h LD_{50}.

Effect of chemotherapy on cell cycle

Exposure to LD_{50} cisplatin and epirubicin resulted in cell cycle arrest in G_2 phase, with LD_{10} doses resulting in G_1 arrest at both 48 and 72 h incubation periods. LD_{10} dose of 5-FU resulted in S phase arrest, whilst the LD_{50} caused G_1 arrest, irrespective of incubation periods (Table 2).

Table 2 Cell cycle analysis following exposure to chemotherapeutic agents

			Cell cycle, % (± s.e.m.)		
Chemotherapy	**Time (h)**	**Treatment**	**G_1**	**S**	**G_2**
Control	48		50 (1)	31 (1)	19 (1)
	72		35 (1)	30 (2)	27 (6)
5-Fluorouracil	48	LD_{10}	36 (2)	48 (0)	14 (1)
		LD_{50}	77 (1)	9 (0)	16 (1)
	72	LD_{10}	14 (1)	64 (3)	19 (1)
		LD_{50}	54 (2)	14 (3)	30 (2)
Cisplatin	48	LD_{10}	57 (0)	24 (5)	19 (6)
		LD_{50}	30 (1)	22 (0)	47 (1)
	72	LD_{10}	48 (1)	27 (2)	24 (1)
		LD_{50}	30 (0)	16 (0)	61 (0)
Epirubicin	48	LD_{10}	59 (0)	21 (1)	21 (0)
		LD_{50}	31 (1)	13 (3)	59 (2)
	72	LD_{10}	46 (1)	25 (2)	28 (1)
		LD_{50}	26 (2)	10 (1)	63 (2)

Flow cytometry was performed on a Becton Dickinson FACS Calibur (San Jose, CA, USA), using blue light (488 nm), detecting forward and 90° angle light scatter. Cell cycle analysis was performed using FlowJo v4.5.2 analysis software (Tree Star Inc., OR, USA), utilising the Dean–Jett–Fox model, analysing 10 000 events.

Effect of chemotherapy on glucose transport

^{3}H-OMG cellular uptake rate was reduced upon exposure to epirubicin, cisplatin and 5-FU, with the greatest reduction (35%) in ^{3}H-OMG uptake resulting from 72 h exposure to LD_{50} epirubicin (Figure 3A and B). Forty-eight hours exposure to LD_{50} epirubicin, cisplatin and 5-FU resulted in a significant reduction in cellular ^{3}H-OMG uptake compared to controls ($P = 0.037$, $P = 0.005$ and $P = 0.002$ respectively) as did 72 h exposure ($P = 0.002$, $P = 0.035$ and $P = 0.001$ respectively). LD_{10} 48 h exposure resulted in a non-significant decrease in ^{3}H-OMG uptake for epirubicin, cisplatin and 5-FU ($P = 0.064$, $P = 0.539$ and $P = 0.05$ respectively). LD_{10} exposure at 72 h caused a significant reduction in uptake for epirubicin ($P = 0.033$) but not for cisplatin or 5-FU ($P = 0.304$ and $P = 0.212$ respectively).

Effect of chemotherapy on cellular HK activity

LD_{10} 48 h exposure to epirubicin, cisplatin and 5-FU resulted in a non-significant increase in cellular HK activity (Figure 4A and B) in comparison to controls ($P = 0.575$, $P = 0.982$ and $P = 0.465$ respectively). Exposure to 48 h LD_{50}, cisplatin and 5-FU also caused a non-significant increase in cellular HK activity ($P = 0.727$ and $P = 0.282$ respectively) whereas with epirubicin there was a non-significant reduction in HK activity ($P = 0.451$).

Exposure to cisplatin and epirubicin for 72 h decreased HK activity, with the greatest reduction caused by exposure to LD_{50} cisplatin (65%). Hexokinase activity was significantly reduced by exposure for 72 h to cisplatin and 5-FU (cisplatin $P = 0.047$ and $P = 0.011$, 5-FU $P = 0.016$ and $P = 0.018$ respectively for LD_{10} and LD_{50}). Exposure for 72 h to epirubicin caused a significant reduction in HK activity with the LD_{50} dose ($P = 0.02$) but not the LD_{10} dose ($P = 0.202$).

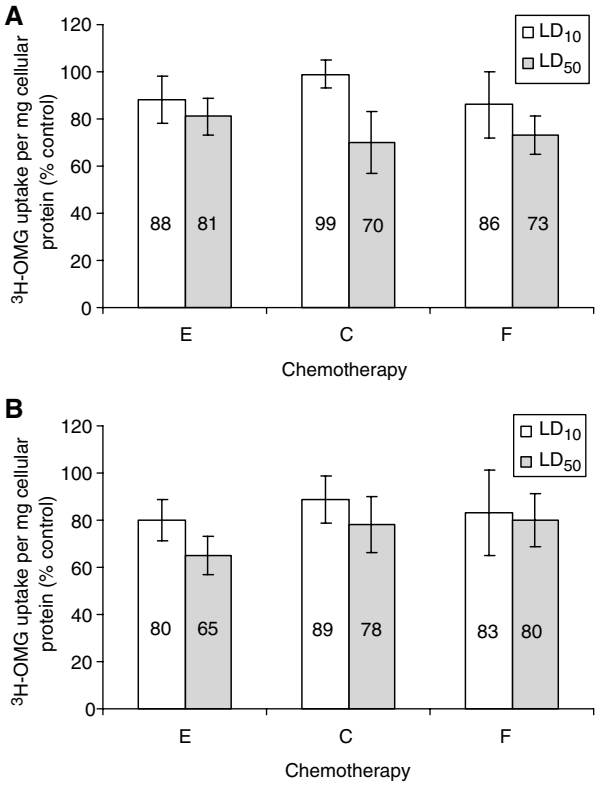

Figure 3 Cellular [^{3}H]*O*-methylglucose (^{3}H-OMG) uptake, per milligram of cellular protein, after 48 (**A**) and 72 (**B**) h incubation with lethal dose 10 (LD_{10}) (white) and LD_{50} (grey) expressed as a percentage of the control (E = epirubicin, C = cisplatin, F = 5-fluorouracil).

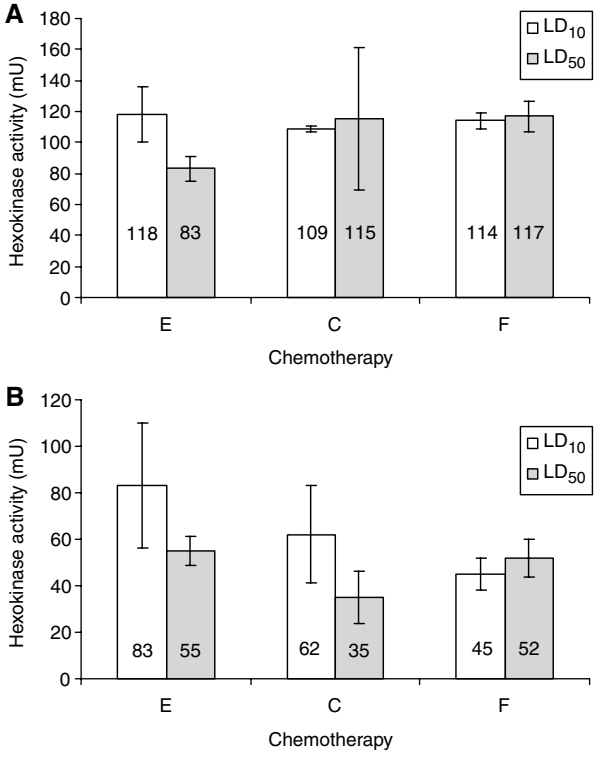

Figure 4 Cellular hexokinase (HK) activity, expressed as milliunits per milligram of cellular protein as a percentage of control (E = epirubicin, C = cisplatin, F = 5-fluorouracil), after 48 (**A**) and 72 (**B**) h exposure to lethal dose 10 (LD_{10}) (white) and LD_{50} (grey) doses of epirubicin, cisplatin and 5-FU (E = epirubicin, C = cisplatin, F = 5-fluorouracil).

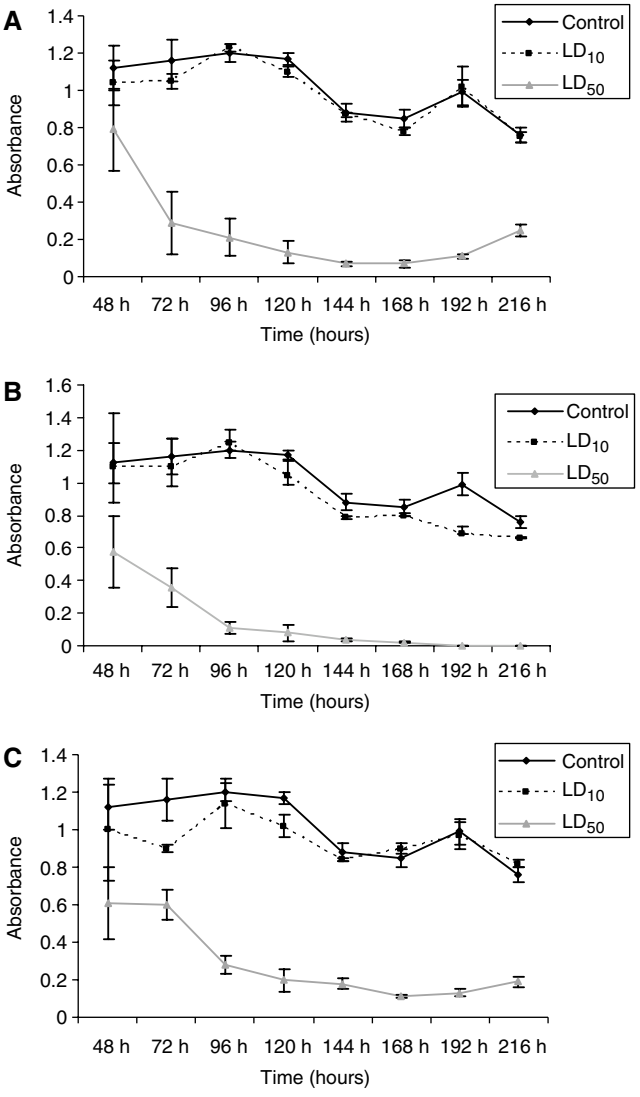

Figure 5 Determination of the growth inhibitory effect of each agent by performing MTT measurements 6 days after treatment with cisplatin (**A**), epirubicin (**B**) and 5-FU (**C**) for 72 h. Cell survival is expressed as absorbance as measured on spectrophotometer.

Latent cytotoxicity of chemotherapeutic agents

Following 72 h exposure to both LD_{10} and LD_{50} of each individual chemotherapeutic agent, cells were washed and fresh media was added followed by further incubation for up to 6 days. Cells were able to recover from exposure to 5-FU and cisplatin when the drug was removed. However, epirubicin-treated cells did not recover even after 6 days of incubation in fresh media (Figure 5A–C). Annexin V-PE flow cytometry revealed that AGS cells exposed to 72 h LD_{50} epirubicin resulted in 69% cell death, 39% of surviving cells were annexin V-PE positive and 7-AAD negative, indicating this group of cells were in the early stages of apoptosis (Table 3).

DISCUSSION

Chemotherapy for tumours at or around the gastro-oesophageal junction is varied, with most regimes including one of epirubicin, cisplatin or 5-FU (Cunningham *et al*, 2006). All three chemotherapeutic agents caused a reduction in ^{18}FDG uptake by AGS cells with epirubicin having the greatest effect, followed by 5-FU then

Table 3 Results of annexin V-PE flow cytometry upon 72 h exposure to chemotherapy

		Cells, % (± s.e.m.)		
Chemotherapy	**Dose**	**Annexin V-PE and 7-AAD negative**	**Annexin V-PE positive and 7-AAD negative**	**Annexin V-PE and 7-AAD positive**
Control		93 (1)	4 (1)	2 (0)
Epirubicin	LD_{10}	94 (1)	2 (1)	4 (0)
	LD_{50}	23 (1)	39 (1)	38 (0)
Cisplatin	LD_{10}	95 (0)	3 (0)	2 (0)
	LD_{50}	80 (2)	15 (2)	6 (0)
5-Fluorouracil	LD_{10}	89 (0)	7 (0)	4 (0)
	LD_{50}	93 (2)	3 (1)	4 (2)

Annexin V-PE FACS, 10 000 events counted per sample with results displayed as a percentage of valid counts (annexin V-PE and 7-AAD negative = healthy cells; annexin V-PE positive and 7-AAD negative = early apoptosis; annexin V-PE and 7-AAD positive = necrosis or late apoptosis).

cisplatin. Previous work on the AGS cell line using the same three chemotherapeutic agents and exposure times identified similar LD_{50} concentrations of each agent, 5-FU 10 μg ml^{-1}, cisplatin 10 μg ml^{-1} and epirubicin 0.25 μg ml^{-1} (Couper and Park, 2003). In common with previous studies, exposure to epirubicin and cisplatin induced dose-dependent G_1 (LD_{10}) and G_2 (LD_{50}) cell cycle arrest (Barry *et al*, 1990; Sorenson *et al*, 1990; Shapiro *et al*, 1998; Zoli *et al*, 2004). 5-FU exposure resulted in a build up of cells in S phase (LD_{10}) and G_1 (LD_{50}). Epirubicin, an anthracycline derivative of doxorubicin, exerts its anti-tumour effects via its action as a DNA intercalating agent and as an inhibitor of topoisomerase II (Cersosimo and Hong, 1986; Bartkowiak *et al*, 1992; Zoli *et al*, 2004). The arrest of AGS cells at higher concentrations of epirubicin may be related to peak activity of topoisomerases occurring during the G_2 phase (Chow and Ross, 1987). Exposure to cisplatin, an alkylating agent, results in the binding of cisplatin to DNA, forming cisplatin-DNA adducts which causes an alteration in the conformation of DNA leading to cell cycle arrest and apoptosis (Jordan and Carmo-Fonseca, 2000; Gonzalez *et al*, 2001; Wang *et al*, 2004). Cell cycle arrest following exposure to cisplatin occurs mainly within G_2 (Eastman, 1990; Sorenson *et al*, 1990), although this may be tumour type dependent (Sekiguchi *et al*, 1996; Shapiro *et al*, 1998). The main mechanism of action of 5-FU is related to its conversion to 5-fluoro-2′-deoxy-5′-monophosphate (via the pyrimidine pathway) leading to the inhibition of thymidylate synthase and hence DNA synthesis (Pinedo and Peters, 1988). Studies have revealed an increase S-phase fraction in tumour cells, caused by 5-FU (Barry *et al*, 1990; Yamane *et al*, 1999; Park *et al*, 2004), including gastric carcinomas (Inada *et al*, 1997).

Dittmann *et al* (2002), evaluating ^{18}FDG uptake in a squamous cell carcinoma oesophageal cell line, reported that 24 h incubation periods in concentrations of 5-FU and cisplatin, resulting in 22.8 and 60.6% cell death respectively, had no effect on cellular ^{18}FDG uptake, following a 24 h period of incubation in chemotherapy-free media prior to uptake experiments. Furthermore, after 24 h exposure to these same concentrations of drugs the S-phase fraction was elevated considerably, yet had no impact on ^{18}FDG uptake. Smith *et al* (2000) investigating tomudex, which is a more specific thymidylate synthase inhibitor than 5-FU, and oxaliplatin (a platinum agent similar to cisplatin) on a colonic tumour cell line found that exposure to tomudex for 24 and 48 h resulted in increasing levels of cellular ^{3}H-DG uptake with increasing exposure to the agent. This increase in uptake was paralleled with an increase in the S-phase fraction. The contrasts between these studies and ours may be in part explained by Yamane *et al* (1999), who revealed that although increasingly lengthy exposure to 5-FU resulted in S-phase accumulation of colorectal cancer cells with increased apoptosis, the Ki-67 labelling index decreased. Therefore, this S-phase accumulation is not proliferative but lethal. Exposure to oxaliplatin (Smith *et al*, 2000) resulted in decreased levels of cellular ^{3}H-DG uptake compared to controls with a varied cell cycle distribution. Although ^{18}FDG uptake in this study was found to be decreased after treatment with each agent, the effect on cell cycle was agent-specific suggesting that the changes in ^{18}FDG uptake are not cell cycle-specific, a finding previously reported by others (Higashi *et al*, 1993; Haberkorn *et al*, 1994).

Recently an analysis (Barros *et al*, 2005) based on the relationship between HK, glucose transport and intracellular glucose concentration shows that, at least in neuronal cells, the flux of glucose through the cell is dependent both on HK and glucose transport and that appreciable increases in flux require increases in both of these activities, whereas decreased flux can be brought about by a reduction in glucose transport or HK activity or both. Qualitatively, in our study, in each case where a decrease in ^{18}FDG incorporation is observed there is a corresponding decrease in glucose transport. By far the greatest decrease in ^{18}FDG incorporation is observed with the LD_{50} dose of epirubicin for 72 h, which also marginally shows the greatest reduction in glucose transport. This treatment also causes an appreciable decrease in HK activity, which may augment the effect of glucose transport on ^{18}FDG incorporation. However, the lack of effect of the LD_{10} dose of 5-FU for 72 h on ^{18}FDG incorporation or glucose transport, which caused a significant decrease in HK activity, suggests that glucose transport is the most important parameter for ^{18}FDG incorporation in this cell line. Further, regression analysis performed on pooled 48 and 72 h data from each treatment shows a strong correlation between the changes in FDG incorporation and glucose transport ($t = 0.863$, $n = 12$, $P < 0.001$) but not between changes in FDG incorporation and HK activity ($t = 0.31$, $n = 12$, $P > 0.1$).

One possible reason for the closer association of glucose transport with ^{18}FDG incorporation compared with HK activity may be that the glucose transport assay uses intact cells so is a true measure of the glucose transport of the AGS cell, whereas the HK assay is performed in cell homogenates. Since HK activity *in vivo* is compartmentalised and highly regulated (Smith, 2000), cell breakage is likely to disrupt these regulatory systems.

We found that ^{18}FDG incorporation was consistently diminished by treatment with epirubicin, 5-FU or cisplatin. We did not see any evidence of increased incorporation. Although response to therapy is generally associated with decreased ^{18}FDG incorporation, a number of studies (Basu and Alavi, 2007) have reported that ^{18}FDG incorporation can increase in some responding tumours. In cases where this 'metabolic flare' phenomenon has been observed in breast tumours treated with anti-oestrogen treatment it has been attributed to the stimulatory effect of the anti-oestrogen at low blood concentrations (the situation when the patient begins therapy) on their cancer cells. A flare phenomenon occasionally found after radiotherapy generally corresponds with influx of inflammatory cells into the tumour (Kostakoglu and Goldsmith, 2004). Kubota *et al* (1992) studying ^{18}FDG uptake in a malignant murine model discovered that ^{18}FDG accumulated not only within the tumour cells, but also in the inflammatory components which appear with growth or tumour necrosis; however, the major source of ^{18}FDG was still tumour cells. Clearly neither of these situations is relevant to our treatment/model type.

Another type of hypermetabolism associated with ^{18}FDG has been reported in both solid tumours (Maruyama *et al*, 1999) and cell lines (Fujibayashi *et al*, 1997). This is likely to reflect biochemical changes within the cells in response to initial damage. We determined ^{18}FDG incorporation at times corresponding to

appreciable cell death so the initial response would have been complete.

Comparing ^{18}FDG incorporation after 48 and 72 h of treatment, when expressed relative to cellular protein, the decrease in ^{18}FDG incorporation is seen to plateau for treatment with cisplatin and 5-FU but not with epirubicin in which ^{18}FDG incorporation continues to decline. This may be explained by the high proportion (77%) of apoptotic cells in cell populations treated with LD_{50} epirubicin for 72 h compared with cells exposed to cisplatin and 5-FU, suggesting that ^{18}FDG incorporation by apoptotic cells is decreased compared with viable cells. Following on from this, determination of the growth inhibitory effect of each agent by performing MTT measurements 6 days after treatment with each agent showed that AGS cells can recover from treatment with 5-FU and cisplatin but not after treatment with epirubicin. In assessing two chemotherapy regimens for gastro-oesophageal cancer, Couper and Park (2003) noted that AGS cells exposed to a combination of LD_{50} of both cisplatin and 5-FU were able to recover following clearance of the chemotherapy. The addition of epirubicin to the combination of cisplatin and 5-FU resulted in a continual growth inhibitory effect (Couper and Park, 2003). The nature of Coupers' study meant that this effect may be due to the combined effect of three chemotherapeutic agents rather than specifically related to epirubicin. Engles *et al* treated MCF-7 breast carcinoma cells for 24 h with doxorubicin (an anthracycline similar to epirubicin) and 5-FU then re-incubated the cells in chemotherapy-free medium for a further 72 h. They found that cell number in populations treated with doxorubicin continued to decrease during the 72 h in doxorubicin-free medium (Engles *et al*, 2006), but addition of 5-FU-free media to MCF-7 cells treated for 24 h with 5-FU was associated with an increase in cell number indicative of recovery. It appears that the efficacy of epirubicin is associated with reduced cellular glycolytic rate (Zhou *et al*, 2002).

One of the limitations of our study is the extrapolation from *in vitro* to *in vivo*. Our studies are performed on well-perfused cells with a good nutrient and oxygen supply in an environment with neutral pH. Within solid tumours *in vivo* there are regions with compromised blood flow and consequent nutrient deprivation, lactic acid production and acidic pH. These are all factors that may influence ^{18}FDG incorporation. Burgman *et al* (2001) showed that induction of hypoxia resulted in increased ^{18}FDG incorporation by MCF-7 cells whilst HK activity is influenced by environmental pH (Miccoli *et al*, 1996). However, the region of tumour growth will have a good blood supply and is where most of the ^{18}FDG is likely to reach.

To simulate the uptake of ^{18}FDG within solid tumours, ^{18}FDG uptake was also expressed as activity per flask. The uptake of ^{18}FDG by detached cells could not be determined as steps to wash away non-incorporated ^{18}FDG would involve centrifugation, which would be a problem with detached cells as these are generally late dying/dead cells with fragile or damaged cell membranes. However, cells that were undergoing early apoptosis, for example, cells treated with epirubicin, were still attached and would be included in the analyses. Furthermore, *in vivo*, dead cells are rapidly removed by macrophages. So the contribution of dead cells *in vivo* is likely to be small.

In summary, treatment of gastric adenocarcinoma cells with cisplatin, 5-FU and epirubicin results in decreased ^{18}FDG incorporation. The greatest reduction in ^{18}FDG uptake per cell is induced by epirubicin. In contrast to cisplatin and 5-FU treated cells, epirubicin-treated cells did not recover when the drug was removed from the medium, corresponding with the annexin V-PE results, suggesting that the level of change in ^{18}FDG incorporation is predictive of tumour cell response. Each chemotherapeutic agent decreased glucose transport suggesting that glucose transport is the rate-limiting step for ^{18}FDG incorporation by AGS cells.

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