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Original Paper

Effect of γ-linolenic Acid on Cellular Uptake of Structurally Related Anthracyclines in Human Drug Sensitive and Multidrug Resistant Bladder and Breast Cancer Cell Lines

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This study investigated the effect on drug uptake in multidrug resistant cells by the incorporation of the essential fatty acid γ-linolenic acid (GLA). The cell lines used were the MCF-7/R resistant human breast cancer and MGH-U1/R bladder cancer. Uptake of drug (doxorubicin, epirubicin, mitoxantrone and idarubicin) after the incorporation of GLA was investigated quantitatively by flow cytometry and qualitatively by confocal microscopy. There was no observable overall increase in drug uptake due to GLA incorporation into the cells as shown by flow cytometry. However, an increase in uptake of the chemotherapeutic agent idarubicin was observed in GLA-treated resistant cells compared with untreated cells using the confocal microscope. This overall increase in cellular drug uptake was not accompanied by a change in cellular drug distribution. Only one drug, mitoxantrone, displayed a change in intracellular drug distribution due to GLA incorporation into MCF-7/R cells. This suggests that essential fatty acid incorporation into the cellular membranes of some resistant cells may cause a shift in the intracellular distribution of certain chemotherapeutic drugs.

Key words: multidrug resistance, GLA, anthracycline, bladder cancer, breast cancer
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INTRODUCTION

MULTIDRUG RESISTANCE (MDR) defines a phenotype of cellular cross-resistance to a broad spectrum of structurally and functionally unrelated cytotoxic drugs. There are two fundamental types of resistance, (1) inherent resistance; where there is no initial significant response to chemotherapy by the cancer; and more commonly, (2) acquired resistance where the cancer initially responds to chemotherapy but then acquires resistance during the course of therapy. Both lead to a lowering of chemotherapeutic drug levels within the cancer cells and hence cell death.

The chemotherapy agents of interest in this study are all structurally related. Doxorubicin (DOX) is a naturally occurring anthracycline antibiotic, originally isolated from the fungus *Streptomyces peucetius var. caesius*. Epirubicin (EPI) is a stereoisomer of DOX. Mitoxantrone (MTX) is an anthraquinone very closely related to the anthracyclines. Idarubicin (IDA), a synthetic anthracycline, is much more lipophilic than the other anthracyclines. As with all anthracyclines, these drugs display natural fluorescence that can be detected within the cells. This endogenous fluorescence is in the red/far red band of the spectrum allowing easy detection and quantification of intracellular drug localisation using established techniques, such as flow cytometry and fluorescence microscopy.

The essential fatty acid γ-linolenic acid (GLA), a polyunsaturated fatty acid (PUFA) has been known for many years to have a preferential cytotoxicity towards cancer cells. When essential fatty acids (EFAs) are added to cancer cells being treated with chemotherapy, increases in cell death have been reported [1–6]. This increase may be through additive or synergistic cell killing. Cellular incorporation of GLA can modulate membrane fluidity which may lead to increased drug uptake. Membrane fluidity is determined by the degree of unsaturation of the fatty acid residues in the component

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phospholipids and by the cholesterol content. Lipid fluidity increases with the degree of unsaturation of the component fatty acids. This increased fluidity influences membrane permeability which is particularly relevant to many cytotoxic drugs, such as EPI, which enter the cells via passive diffusion.

Hence the aim of these studies was to investigate drug uptake and intracellular distribution within drug sensitive and drug resistant cell lines which have had a non-cytotoxic dose of GLA incorporated into their cell membranes.

MATERIALS AND METHODS

The cell lines used in the studies were the MCF-7 breast cancer cell line and the MGH-U1 urothelial cancer cell line. Both these parental cell lines are anthracycline sensitive and were used as a comparison to their MDR counterparts. Both resistant cell lines were raised by exposing the parental cell lines to increasing doses of doxorubicin. The MGH-U1/R cell line has been characterised by McGovern [7] and the MCF-7/R line by Fairchild [8]. All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 2 μM L-glutamine, fetal bovine serum (FBS at 10% v/v), and 100 μg/ml each penicillin/streptomycin, at 37°C, 5% CO_2.

The essential fatty acid used in all experiments was GLA. It was kindly provided by Scotia Pharmaceuticals (Stirling, U.K.) as the clinical formulation Lithium GLA (LiGLA). This came as a solution in 5 ml vials at a concentration of 140 mg/ml GLA and all subsequent dilutions were made using complete DMEM.

The chemotherapy drugs studied were anthracyclines or related compounds exhibiting endogenous fluorescence in the red/far-red band of the visible light spectrum. DOX, EPI and IDA were obtained in dry pellet form from Pharmacia (Milton Keynes, U.K.). MTX is an anthraquinone and was obtained in solution from Lederle Laboratories (Gosport, U.K.). All subsequent dilutions of these four drugs were made using complete DMEM.

Flow cytometry

Flow cytometry was used for studying dynamics of drug uptake in sensitive and resistant cells. The flow cytometer used in these studies was the Becton Dickinson FACScan. Cells were grown to 90% confluence in flasks and were incubated with a set dose of GLA (20 μg/ml), which did not result in cell toxicity, for 24 h before the addition of anthracycline. This time period is sufficient for maximum incorporation of GLA (data not shown). Negative controls were not exposed to GLA. The effect of GLA incorporation on drug uptake was studied for two different drug concentrations: (1) a dose taken from the linear uptake part of the dose–response curve (5 μg/ml) and (2) a dose taken from the saturation region of the dose–response curve (10 μg/ml). Controls had GLA, but no drug. The anthracycline-spiked medium was left for 1 h, while the cells were adherent to the plastic (dose–response data not shown).

Confocal microscopy

Confocal microscopy was used for visualising the intracellular distribution of drugs. The confocal system implemented was the Leica TCS 4D system. The microscope used was the Leitz DMR BE research microscope. The cells were grown on 15 mm easy grip style petri dishes. They were incubated with or without GLA and with or without medium containing drug (same concentrations as above) and viewed whilst in the dishes. Semi-quantitation of the amount of fluorescence, which corresponds to the amount of drug within the cells, in a field of view was recorded. The amount of fluorescence within cellular compartments, i.e. nuclei, cytoplasm and whole cell were also recorded (arbitrary fluorescence of pixels per area). Careful viewing of the cells under phase contrast allowed detection of cell boundaries, the relative size and location of intracellular compartments prior to drawing around them. This promoted confidence in our assessment of the different cellular compartments and the semi-quantification of fluorescence intensity in each when viewing the same field under epifluorescence. To check the reproducibility of the results two independent operators individually took multiple counts of the mean fluorescence intensity per cellular compartments of the same cells. When the results were pooled a 95% confidence of 2–3% around the mean was consistently achieved.

RESULTS

Flow cytometry

The hypothesis behind this work was that the incorporation of GLA in cells will result in increases in overall drug uptake and therefore lead to more toxicity. All four cell lines cells were incubated with GLA, and then the GLA treated cells were exposed to different drug doses and the amount of intracellular drug recorded. These were compared with the results gained, using the same method, from untreated cells exposed to the same drug doses at the same time. DOX, EPI and IDA were tested on bladder cancer cells and DOX, MTX and IDA on breast cancer cells because these drugs are frequently found in treatment regimes for these cancers.

The difference in drug uptake (i.e. detected fluorescence) between sensitive and resistant cell lines is shown in Table 1: an order of magnitude difference between MGH-U1 and MGH-U1/R at both drug doses, (e.g. 30.8 ± 7.0 versus 2.8 ± 0.5, respectively, at 5 μg/ml of DOX) and approximately 2-fold between MCF-7 and MCF-7/R (e.g. 9.2 ± 0.5 versus 4.7 ± 0.2, respectively at 5 μg/ml of DOX). However, there was no significant change in drug uptake by any cell line due to the incorporation of GLA (Table 1). There were variations in drug uptake but these were not significant and occurred between experimental repeats.

Confocal microscopy

As the overall drug uptake was not affected significantly by GLA incorporation into cells the possible effect of GLA on changes to intracellular drug localisation was investigated using the confocal microscope. Both GLA treated and untreated cells were incubated with drug in the same manner and the distribution patterns of the drug viewed and compared between cells with and without GLA incorporation.

If after visualisation on the confocal microscope there appeared to be changes in the amount of or location of intracellular fluorescence due to GLA incorporation then semi-quantitation was performed on these picture files. This analysis consisted of freehand drawing around the cellular compartments, including nucleus, cytoplasm and whole cell, and than recording the mean fluorescence of each compartment (Figure 1).

Bladder cancer cells (Figures 2–4)

Both GLA treated and untreated sensitive cells showed a nuclear pattern of DOX distribution. There were bright spots

Table 1. *Intracellular drug uptake by MGH-U*1 *& MGH-U*1*/R and MCF-*7 *& MCF-*7*/R cell lines after incorporation of GLA into the cells, as shown by flow cytometry. Numbers are means (*± *standard deviation) of arbitrary fluorescent units for at least four experimental repeats. No significance was found in any experimental variation.*

	Sensitive	Sensitive + GLA	Resistant	Resistant + GLA
MGH-U1 & MGH-U1/R				
DOX				
0	0	0	0	0
5	30.8 ± 7.0	14.8 ± 5.0	2.8 ± 0.5	4.0 ± 0.9
10	64.1 ± 6.0	50.0 ± 6.0	4.0 ± 0.8	6.0 ± 0.8
EPI				
0	0	0	0	0
5	42.8 ± 8.0	63.1 ± 13.0	2.4 ± 0.6	3.7 ± 0.7
10	67.1 ± 8.0	70.5 ± 4.0	3.9 ± 0.7	4.7 ± 0.5
IDA				
0	0	0	0	0
5	78.0 ± 15.0	80.6 ± 9.0	3.4 ± 0.6	2.7 ± 0.9
10	130.0 ± 10.0	123.8 ± 11	6.8 ± 1.2	5.2 ± 1.2
MCF-7 & MCF-7/R				
DOX				
0	0	0	0	0
5	9.2 ± 0.5	15.2 ± 5.0	4.7 ± 0.2	4.6 ± 0.6
10	19.5 ± 3.0	27.0 ± 5.0	10.0 ± 0.7	8.8 ± 1.0
MTX				
0	0	0	0	0
5	19.7 ± 5.6	24.0 ± 6.0	15.2 ± 0.5	14.8 ± 1.6
10	32.4 ± 2.0	23.6 ± 5.0	18.2 ± 1.2	19.9 ± 2.8

of fluorescence observed within the nuclei that may correspond to drug binding to chromatin or the nucleoli. The pattern of intracellular DOX distribution was mainly cytoplasmic with lesser amounts entering the nucleus and was visible in both sets of resistant cells, GLA treated and untreated (Figure 2).

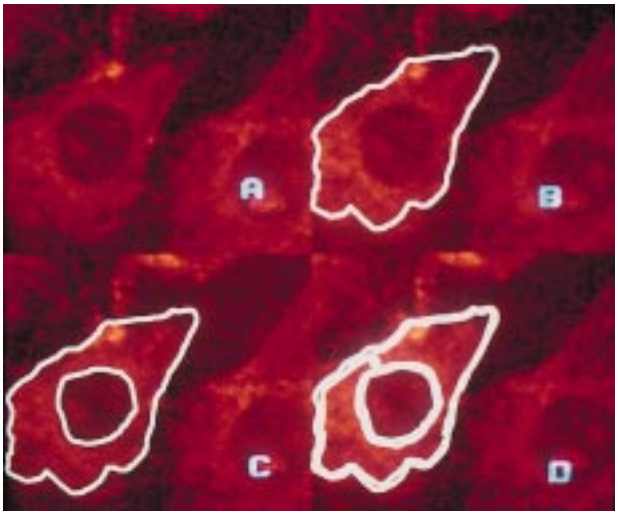

Figure 1. Analysis of drug uptake in intracellular compartments, using the confocal microscope (×900).

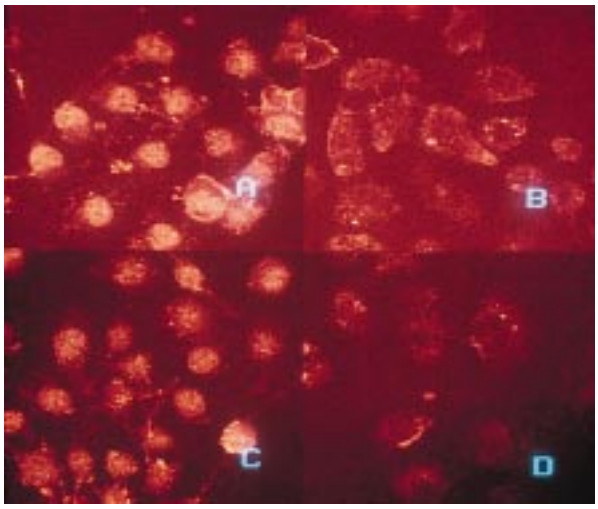

Figure 2. Patterns of intracellular DOX (10 μg/ml) in GLA (20 μg/ml) treated and untreated MGH-U1 and MGH-U1/R cells, detected using the confocal microscope. (A) MGH-U1 cells, untreated with GLA; (B) MGH-U1/R cells, untreated with GLA; (C) MGH-U1 cells, GLA treated; (D) MGH-U1/R cells, GLA treated cells, (×300).

GLA treated and untreated MGH-U1 cells had similar levels of fluorescent intensity and similar patterns of intracellular localisation of EPI which was mainly in the nuclei with some cytoplasmic distribution. As with DOX localisation, intense spots of fluorescence were observed within the nuclei. There was also no observable effect of GLA on EPI uptake by the MGH-U1/R cells. Both GLA treated and untreated cells appeared to take up similar levels of EPI and both showed the drug located in the cytoplasm (Figure 3).

Unlike the two drugs described above, IDA had similar patterns of intracellular distribution within the sensitive and resistant cell lines. There was very little nuclear distribution of the drug with most of the IDA staying in the cytoplasm. The cytoplasmic fluorescence was more granular in the resistant

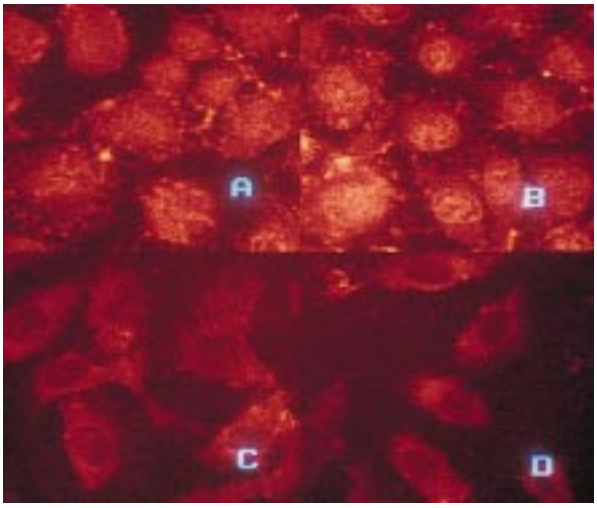

Figure 3. Patterns of intracellular EPI (10 μg/ml) in GLA (20μg/ml) treated and untreated MGH-U1 and MGH-U1/R cells, detected using the confocal microscope. (A) MGH-U1 cells, untreated with GLA; (B) MGH-U1/R cells, untreated with GLA; (C) MGH-U1 cells, GLA treated; (D) MGH-U1/R cells, GLA treated cells (×600).

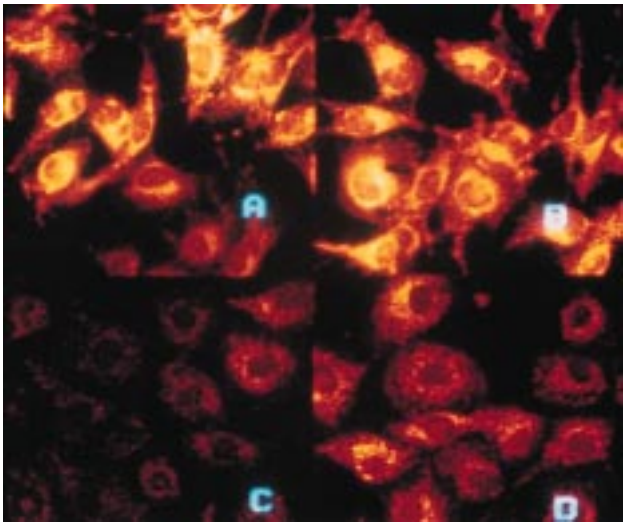

Figure. 4. Patterns of intracellular IDA (10 μg/ml) in GLA (20 μg/ml) treated and untreated MGH-U1 and MGH-U1/R cells, detected using the confocal microscope with corresponding intensity graphs. (A) MGH-U1 cells, untreated with GLA; (B) MGH-U1/R cells, untreated with GLA; (C) MGH-U1 cells, GLA treated; (D) MGH-U1/R cells, GLA treated cells, (× 300).

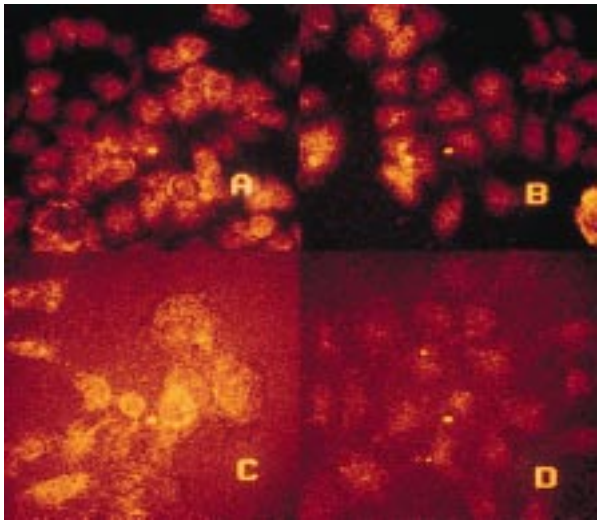

Figure. 5. Patterns of intracellular DOX (10 μg/ml) in GLA (20 μg/ml) treated and untreated MCF-7 and MCF-7/R cells, detected using the confocal microscope. (A) MCF-7 cells, untreated with GLA; (B) MCF-7/R cells, untreated with GLA; (C) MCF-7 cells, GLA treated; (D) MCF-7/R cells, GLA treated cells (× 300).

cell line than that seen in the sensitive cells which may indicate vesicles with drug (Figure 4A,B). The confocal picture showed that an increase in IDA uptake occurred after incorporation of GLA into resistant cells (Figure 4B,D). Semi-quantitation revealed a significant increase in drug uptake in GLA treated resistant cells compared with untreated resistant cells. This increase occurred in all cellular compartments, that is the nuclei, cytoplasm and whole cells (Table 2). This could be regarded as being due to overall higher level of drug uptake in GLA treated cells compared with untreated cells. Sensitive cells showed similar fluorescent intensity and intracellular drug distribution, in the cells with or without GLA treatment (Figure 4A,C).

Breast cancer cells (Figures 5–7)

There was little difference in DOX uptake or intracellular localisation between sensitive cells with or without GLA (Figure 5A,C). Both had nuclear DOX accumulation with very little in the cytoplasm. In resistant cells there was a difference in DOX localisation; the untreated cells displayed nuclear 'holes' with mainly cytoplasmic fluorescence and very strong perinuclear drug localisation. The GLA treated cells had taken up the DOX mainly into their nuclei, producing a pattern of intracellular drug distribution similar to that observed in sensitive cells. However, semi-quantitation of the different cellular compartments within non-treated and GLA treated cells showed no significant difference in amounts of drug taken up by the two cell sets (data not shown).

Both sets of sensitive cells showed nuclei lacking fluorescence with perinuclear rings of MTX uptake and very punctate bright points of fluorescence (Figure 6A,B). GLA treated resistant cells had a different pattern of intracellular MTX distribution than untreated resistant cells (Figure 6D). The untreated cells had little nuclear fluorescence, with the MTX

Table 2. Semi-quantitation by confocal microscopy of the changes in intracellular drug localisation in different cellular compartments, i.e. nuclei, cytoplasm and whole cell, in MGH-U1, MGH-U1/R and MCF-7/R cells, due to the incorporation of GLA into these cells. Numbers are means (± standard deviation) of fluorescent pixels/area, over a minimum of ten fields. Significance shown in tables.

	Without GLA			With GLA		
	Nuclei	Cytoplasm	Whole cell	Nuclei	Cytoplasm	Whole cell
MGH-U1 + DOX	41.7 ± 4.3	24.8 ± 3.4 NS	30.9 ± 5.5	28.3 ± 10.9 NS	12.0 ± 10.2 $P<0.04$	20.1 ± 6.7
MGH-U1/R + DOX	40.3 ± 7.4	49.8 ± 7.95 NS	47.6 ± 5.4	29.1 ± 8.6 NS	32.3 ± 8.9 $P\approx 0.04$	30.2 ± 12.7
MGH-U1/R + IDA	10.5 ± 1.5	11.8 ± 1.9 All display significance $P=<0.01$	12.0 ± 2.0	16.6 ± 3.4 $P=0.0002$	19.3 ± 3.86 $P=0.0006$	19.5 ± 3.7 $P=0.0004$
MCF-7/R + MTX	24.9 ± 7.4	22.18 ± 4.6 Nuclear uptake showed significance	22.7 ± 3.4	37.2 ± 5.2 $P<0.001$	18.4 ± 3.4 NS	24.2 ± 3.0 NS
MCF-7/R + IDA	51.4 ± 5.1	4.5 ± 10.6 Nuclear uptake showed significance	47.1 ± 10.3	71.2 ± 12.7 $P=0.002$	43.9 ± 8.3 NS	49.2 ± 9.6 NS

NS, non-significant.

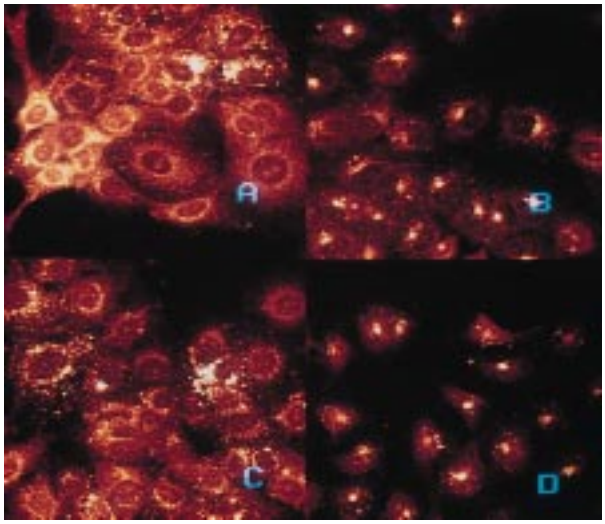

Figure. 6. Patterns of intracellular MTX (10 μg/ml) in GLA (20 μg/ml) treated and untreated MCF-7 and MCF-7/R cells, detected using the confocal microscope. (A) MCF-7 cells, untreated with GLA; (B) MCF-7/R cells, untreated with GLA; (C) MCF-7 cells, GLA treated; (D) MCF-7/R cells, GLA treated cells (× 300).

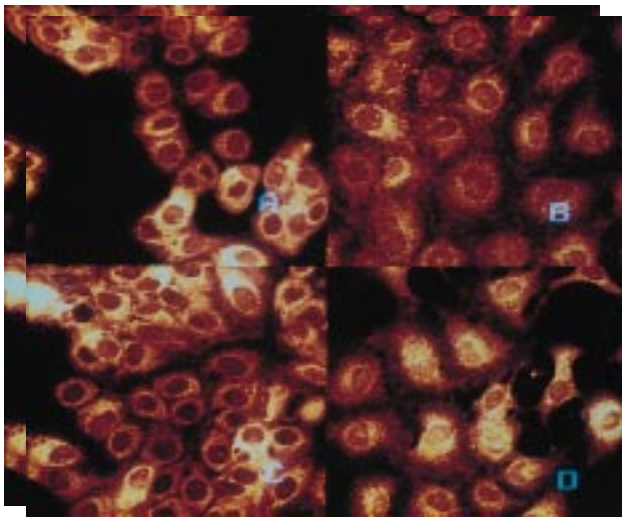

Figure. 7. Patterns of intracellular IDA (10 μg/ml) in GLA (20 μg/ml) treated and untreated MCF-7 and MCF-7/R cells, detected using the confocal microscope. (A) MCF-7 cells, untreated with GLA; (B) MCF-7/R cells, untreated with GLA; (C) MCF-7 cells, GLA treated; (D) MCF-7/R cells, GLA treated cells (× 300).

mainly distributed in the cytoplasm especially in the perinuclear region (Figure 6B). GLA treated resistant cells had nuclear MTX accumulation with less MTX in the cytoplasm whilst still retaining an area of very high perinuclear fluorescence (Figure 6D). This shift in drug distribution into the nuclei was significant (Table 2). However, there was no significant increase in drug uptake by the whole cells. It should be noted that it was rather difficult to define clearly and precisely the exact compartmentalisation of this particular drug in any of the confocal slices all through these cells. It was believed there was a non-partitioning of the MTX into cytoplasmic intracellular compartments in MCF-7/R cells after GLA incorporation.

GLA treated sensitive cells showed similar intracellular patterns and levels of IDA uptake compared with the untreated sensitive cells (Figure 7). The fluorescence was mainly cytoplasmic. The resistant cells showed similar patterns of intracellular IDA localisation regardless of whether they had been incubated with GLA or not (Figure 7B,D). The pattern of IDA uptake in resistant cells was very similar to the pattern of uptake in the sensitive cells (Figure 7A,B). However, those resistant cells with GLA incorporated into them produced a higher level of fluorescent intensity with the additional IDA located mainly in the perinuclear region (Figure 7D). The perinuclear rings of fluorescence in these GLA treated cells were brighter than those observed in untreated resistant cells. There was a significant increase in IDA uptake by the nuclei of the GLA treated resistant cells compared with the untreated cells but this increase did not occur in other cellular compartments (Table 2). That there was no change in drug uptake by the whole cell was not surprising, considering that the fluorescence was mainly in the perinuclear region of the cells. This area was difficult to define in terms of cellular compartments which were nuclear or cytoplasmic. The actual sensitivity of the machine is not good enough to visualise any smaller cellular compartments, such as the Golgi apparatus which are located in the perinuclear area.

DISCUSSION

It has been suggested that pre-treatment of cells with EFAs would lead to increased cancer cell kill by increased cytotoxic drug uptake. This increased cell death could be due to either increased drug uptake, with the EFA changing the membrane fluidity [4], or to the additive effects of the cytotoxicity of the EFA and the drug [9].

GLA is known to incorporate into the cell lines used here, presumably via the plasma membrane, from previous experiments using radioactive LiGLA (results not shown). This incorporation should have, in turn, led to an increase in membrane fluidity and increased drug uptake. However, there was no increase in cellular uptake of the chemotherapeutic agents studied using flow cytometry. Other studies have reported increased cellular uptake of various cytotoxic drugs (or increased death) due to prior incubation of the cells with an EFA [1–6], including GLA [3] on MCF-7 cells resulting in an increase in the uptake of DOX [6]. This latter study demonstrated that sub-lethal doses of GLA (20 μg/ml) can enhance sensitivity of MCF-7 cells to DOX *in vitro*. Also murine leukaemia cells grown in medium supplemented with the EFA docosahexaenoic (DHA) increased their sensitivity to DOX compared with unsupplemented cells. There was no change in the growth rate or cloning ability of the cells when only DHA was present [1]. However, none of these reports used flow cytometry but rather various types of cell viability assays or spectrophotometry and the majority of the EFAs were in the form of diethyl esters or other non-clinical salts, such as sodium GLA rather than the clinically applicable lithium GLA.

In our experiments, the use of GLA to enhance the cytotoxic action of anthracyclines by increasing drug uptake was investigated using concentrations of GLA which were non-cytotoxic to the cells. Although no increased drug uptake was recorded for any drug in any of the four cell lines using flow cytometry, when confocal microscopy was employed one drug (IDA) appeared to have increased uptake in the resistant cell lines due to GLA incorporation. For DOX, MTX and

EPI no significant change in drug uptake was visible in any of the cell lines due to GLA treatment. However, a significant increase in IDA uptake was observed in both resistant cell lines MGH-U1/R and MCF-7/R after 24 h incubation with GLA, but this was not accompanied by a change in intracellular IDA distribution, which remained perinuclear. Why equivalent experiments using flow cytometry and confocal microscopy should record differing results is uncertain. Out of the two techniques, flow cytometry may be more accurate at recording the amount of intracellular drug as it records amount of drug fluorescence per cell whereas the confocal microscope can only semi-quantitate the amount of drug per cell. However, for flow cytometry the cells have to be trypsinised from the flasks after drug incubation prior to being put through the machine, an unnecessary step for the confocal microscope. This trypsination may have some effect on the cells causing a change in the intracellular IDA content. For the other drugs there was no change in drug uptake recorded by either method.

For DOX, EPI and IDA there was no change in the intracellular drug distribution patterns as a result of GLA treatment in any of the cell lines as detected by confocal microscopy, although small changes could have occurred that the machine was not sensitive enough to detect. DOX and EPI displayed similar patterns of drug distribution in both the sensitive cell lines, with intense fluorescence in the nuclei with little in the cytoplasm. This progressive decrease of drug localisation from nucleus through the perinuclear area to the cytoplasm has previously been reported [10]. The intracellular localisation of IDA was mainly perinuclear. Also, there was also no change in the intracellular drug distribution patterns as a result of GLA for the MCF-7 cell line and MTX. However, there was a change in the intracellular MTX localisation pattern in GLA treated MCF-7/R cells compared with untreated cells. This was observed as an increase in the amount of nuclear fluorescence but there was no overall increase in MTX uptake. MTX is believed to be sequestered in cytoplasmic pools of drug so it could be that GLA releases this sequestering. GLA may also affect the nuclear membrane allowing MTX to enter the nucleus. Changes in intracellular drug localisation have been observed using different reversal agents, such as verapamil and estramustine, in bladder cancer multidrug resistant cells (MGH-U1/R) with EPI. Again on reversal of resistance the drug shifted from the cytoplasmic distribution, usually associated with resistant cells, to accumulate predominantly in the nucleus [11]. The shift of EPI from the cytoplasm to the nucleus in resistant cells was also observed with the reversal agent estramustine. This study concluded that the nuclear concentration of EPI correlated with increased cell death, corresponding with the more important mechanisms of action of the anthracyclines [12]. Another study addressed increased sensitivity of MGH-U1 and MGH-U1/R cells to EPI post treatment with differing doses of GLA [13].

Enhancing intracellular anthracycline uptake, probably by alteration in cell membrane fluidity is not believed to be the only mechanism by which fatty acids are able to modulate drug resistance or enhance sensitivity to cancer drugs [14]. One mechanism by which tumour cells show resistance to the cytotoxic action of anticancer drugs includes increased expression of anti-oxidant enzymes. These anti-oxidant defences have been shown to be suppressed by addition of *cis*-unsaturated fatty acids (c-UFAs), the metabolites of EFAs, alpha-linolenic acid and eicosapentaenoic acid [15]. This study also suggested a close relationship between c-UFAs, anticancer drugs and their action on phosphate kinase C (PKC) with regard to the sensitivity of tumour cells to drug [15]. Drug accumulation in MDR cells can be regulated by protein kinases in particular PKC-mediated phosphorylation of Pgp [16]. Inhibition of PKC activity might, therefore, offer another avenue to counteract MDR [14].

Cell death by the action of fatty acids is believed to be mediated by free radicals and lipid peroxides [14]. There is also evidence that free radicals generated by the anthracyclines, within the cell, account for part of the cytotoxicity observed with these drugs [17]. Hence increased cancer cell killing could be through augmented drug-induced free radical production and heightened lipid peroxidation due to the EFAs [18].

Another mechanism by which EFAs and c-UFAs may modulate MDR is by upregulation of the tumour supressor gene *p53* expression. p53 is an essential component of the apoptotic process induced by anticancer drugs [19]. p53 deficient tumours show resistance to chemotherapy using doxorubicin or gamma-radiation, with few apoptotic cells produced and little regression, compared with tumours expressing p53 that were sensitive to treatment and had a high proportion of apoptotic cells [19]. Exposure of rat mammary tumour (NMU) cells to the c-UFA docosahexaenoic acid (DHA) led to an increase in the expression of p53 and was associated with suppression of cell proliferation. However, addition of the EFA linolenic acid (LA) enhanced the growth of NMU cells and decreased levels of p53 in these cells [20].

Whether the enhanced cytotoxicity reported is due to increased drug uptake alone or the additive effects of drug and EFA on the cell is unknown. Many of the reports of increased cell death are equivocal on whether the PUFA used was at concentrations that were directly cytotoxic. As yet, there are no reports of tumours resistant to the cytotoxic effects of EFAs. Therefore, the clinical use of EFAs at high-doses cytotoxic to cancer cells with or without other agents may be of potential benefit.

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