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ORIGINAL ARTICLE

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Ifosfamide metabolism and DNA damage in tumour and peripheral blood lymphocytes of breast cancer patients

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Abstract *Purpose*: This study was designed to determine individual variation in the metabolism of ifosfamide (IF) and any influence this may have on the degree of DNA damage produced in both peripheral blood lymphocytes (PBL) and in tumour tissue. *Methods*: The pharmacokinetics and metabolism of IF and also of doxorubicin (DOX) were determined in patients receiving IF/DOX neoadjuvant chemotherapy for the treatment of advanced breast cancer. The DNA-damaging effects of this regimen were measured using the comet assay in PBL and in breast tumour tissue obtained by fine needle aspirate. Parallel in vitro studies were carried out in order to establish if DNA damage caused by IF metabolites or DOX was predictive of cytotoxicity in breast cancer cell lines. *Results*: The median AUC, half-life and clearance of IF were found to be 291 $\mu M \cdot$ min, 5.2 h and 66 ml/min per m^2, respectively. A high degree of interpatient variability (up to sevenfold) was observed in the metabolism of both IF and DOX and also in their metabolites. Treatment-related changes in the amount of DNA damage were observed in both PBL and tumour cells. That in PBL peaked 48 h after the end of IF infusion (median 17% damaged cells at 48 h compared to 4% damaged before treatment). DNA damage in tumour cells was not elevated above low pretreatment values (median 1.5% damaged cells) until 3 weeks after IF and DOX treatment (median 30% damaged cells), by which time damage in PBL showed almost complete resolution to basal levels. The DNA damage in PBL determined 24 h after the start of chemotherapy was found to be related to the AUC of 4-hydroxyifosfamide (4OHI; $P = 0.05$). The amount of damage in either tissue did not significantly correlate with clinical response or toxicity, but lower amounts of damage were observed in the tumour cells 3 weeks after treatment in those patients that subsequently relapsed, compared to those that remained disease free. DNA damage (more than 20% damaged cells) was observed after exposure to active IF metabolites at concentrations equal to or greater than the IC_{50} in MCF-7 and MDA-MB231 cell lines. At concentrations of 4OHI similar to those determined in vivo, an equivalent level of DNA damage was observed in PBL and in cell lines and was associated with significant growth inhibition. DNA damage induced by DOX was not predictive of cytotoxicity. *Conclusion*: Systemic DNA damage appeared to be related to levels of the active metabolite, consistent with the results of in vitro investigations of DNA damage. Further studies are warranted to substantiate this observation and to explore the relationship between metabolism, DNA damage and antitumour activity.

Key words Ifosfamide · Comet assay · Clinical · Breast cancer

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Introduction

Ifosfamide (IF), the structural isomer of cyclophosphamide, has been used clinically for three decades. It has demonstrated single-agent activity against breast cancer [29], and is also used in combination with anthracyclines for the treatment of advanced breast cancer and sarcoma. There has been recent interest in the use of neoadjuvant chemotherapy to downstage large primary tumours, reduce the need for mastectomy and render inoperable tumours operable [9]. Those patients who achieve a good response may enjoy prolonged control of their disease and improved survival rates. The success of treatment depends on a number of factors including age and stage of disease at diagnosis [8]. There is wide interindividual variation in the clinical response to treatment with IF and doxorubicin (DOX) chemotherapy [24]. Interindividual variation in the pharmacology of the chemotherapeutic agents, systemically and within tumour cells, may be an important underlying factor.

IF, as a prodrug, requires metabolic activation before it can exert an antineoplastic effect. The metabolism of IF is well documented. It is first hydroxylated at the C4 position by hepatic cytochrome P450 enzymes, including CYP3A4 [5, 33]. Subsequent β-elimination of acrolein from 4-hydroxyifosfamide (4OHI) yields isophosphoramide mustard (IPM), a bifunctional alkylator [6, 7]. This electrophilic mustard covalently crosslinks DNA in a cell cycle-independent manner [23]. IPM is ionized at physiological pH and it is likely that 4OHI is the active transport form of IF which enters cells more readily and releases IPM intracellularly [32]. Deactivation of IF can occur by enzymatic conversion of aldoifosfamide (which exists in equilibrium with 4OHI) to carboxyifosfamide, mediated by aldehyde dehydrogenases (ALDH). However, the major route of deactivation of IF is via dechloroethylation, at the endo- or exocyclic nitrogen, again mediated by CYP3A4 [33].

DNA damage is a measurable downstream effect of IF chemotherapy, both systemically in the peripheral blood lymphocytes (PBL) and in the tumour. The amount of damage due to IF treatment will depend in part upon the metabolism. Higher systemic concentrations of 4OHI may result in higher intratumoral concentrations of IPM, which may lead to greater DNA damage and more effective cytotoxicity. The amount of DNA damage may thus be related to the metabolism and pharmacokinetics of IF, and may provide useful information in the prediction of clinical response or toxicity.

DNA damage was determined in the current study using single-cell gel electrophoresis (the comet assay) [31]. This technique has the advantage of direct visualization of DNA damage in individual cells, important when studying a heterogeneous population of cells such as that within a tumour. It is also compatible with in vivo work, needing only a few thousand cells, and its sensitivity is comparable to that of other methods when an average degree of damage across a population of cells is studied [27].

The aims of this study were to determine the inter-individual variation in the metabolism of IF and DOX in patients with advanced breast cancer who received IF/DOX neoadjuvant chemotherapy. DNA damage in the PBL and in the tumour cells of these patients was also measured. The relationship between drug pharmacology and DNA damage, and the impact of these parameters on clinical endpoints such as response or relapse were evaluated. These clinical studies were complemented by in vitro investigations into the relationship between DNA damage and cytotoxicity.

Materials and methods

The following substances were a gift from Asta-Werke (Frankfurt, Germany), and were authenticated in their laboratories: IPM, carboxyifosfamide (CXI), 3-dechloroethylifosfamide (3DCI), 2-dechloroethylifosfamide (2DCI) and 4OHI. The purity of these compounds was also confirmed by thin-layer chromatography (TLC). DOX, daunorubicin and cyclophosphamide were obtained from Sigma (Poole, UK) and doxorubicinol was a gift from Dr Elena Strocchi, Bologna, Italy.

Clinical study

Patient characteristics

This study was approved by the Joint Ethics Committee of the local health authority and universities. The selection criteria for patients on this study were diagnosis of primary advanced or metastatic breast cancer suitable for neoadjuvant treatment, no prior chemotherapy and a tumour size above 3 cm. Eight patients in total were recruited onto study. All eight were diagnosed with invasive ductal carcinoma of the breast. The patients' ages at treatment ranged from 25 to 50 years. Patients were to receive four courses of neoadjuvant treatment, after which they underwent surgery, followed by adjuvant chemotherapy. The time from first course to surgery was 3–4 months.

DOX was administered as a bolus dose of 40 mg/m^2. This was immediately followed by a 24-h continuous infusion of IF at a dose of 5 g/m^2. Mesna (5 g/m^2) was administered with the IF and an additional 1 g/m^2 over 8 h after the end of the IF infusion.

Blood samples were taken for pharmacokinetic analysis before treatment, and 1, 2, 4, 8, 12 and 24 h during and after infusion until 24 h after the end of infusion. For DNA damage measurements, PBL were isolated from blood samples taken before treatment, 24 h after the start of IF infusion, 24 h after infusion, and 3 weeks after treatment. Tumour cells were acquired by fine needle aspirate (FNA), from accessible tumour sites, before treatment, 24 h after the start of IF infusion and 3 weeks after treatment. The aspirates were subjected to cytological examination which confirmed that the majority of cells present were from the tumour.

Analysis of metabolites

Ifosfamide and inactive metabolites

Quantification of IF, CXI, 2DCI and 3DCI was performed by TLC [1]. Each plate included a standard curve and two tracks for quality assurance samples. The linearity was evaluated by regression analysis and an r^2 of 0.94 or above was seen over the range 5–50 μg/ml.

4-Hydroxyifosfamide

The detection of 4OHI by HPLC was also based on a published method [34]. In brief, acrolein released by 4OHI in whole blood was derivatized with 3-aminophenol (10 mg/ml) to form 7-hydroxyquinoline which can be determined fluorometrically. The internal standard methylvinylketone reacts with 3-aminophenol to form 4-methyl-7-hydroxyquinoline, also a fluorescent product. This reaction was performed at the bedside for clinical samples due to the instability of 4OHI.

The concentrations of 7-hydroxyquinoline in derivatized patient samples were quantitated using a Spherisorb phenyl 5-μm column with dimensions of 150 × 4.6 mm (Jones Chromatography, Glamorgan, UK) with an isocratic mobile phase of 25% (w/w) methanol in 0.45 *M* acetate buffer (pH 4). The chromatographic system used for analysis consisted of a Perkin Elmer autosampler (model ISS-101), with an injection volume of 75 μl, a Waters model 510 pump, with a flow rate of 1 ml/min, and an Applied Biosystems 980 programmable fluorescence detector with an excitation wavelength of 350 nm and a cut-off filter at 470 nm. Light protection at all stages throughout the analysis was important to prevent sample decay.

Chromatographic data were acquired and analysed using Minichrom (Version 1.6, VG Data Systems, UK) and quantitation based on a standard curve was generated from control blood samples spiked with concentrations of 4-hydroperoxyifosfamide (0–25 μ*M*).

The standard curve generated encompassed concentrations of 4-OHI found in the patient samples (1–10 μ*M*). The standard curve was linear over the range 1–25 μ*M* with an r^2 of 0.98.

Doxorubicin

A method based on that of Camaggi et al. [4] was used for determination of DOX and doxorubicinol in the plasma of patients on this study using HPLC. The internal standard (0.05 ml of 1 μg/ml daunorubicin) was added to a 1-ml plasma sample from which DOX and daunorubicin were extracted using a mixture of methanol and 0.1 *M* KH_2PO_4 (plasma/methanol/KH_2PO_4 1:1:1 v/v/v). This was followed by solid-phase extraction of the two anthracyclines using preconditioned cartridges (Bond Elut LRC C_{18}; Varian, Surrey, UK). The eluate was then evaporated to dryness under oxygen-free nitrogen, and the residue reconstituted in 200 μl mobile phase of which 100 μl was injected onto the column.

Quantitation of DOX was by HPLC using an Apex Cyano 3-μm column (150 × 4.6 mm) with an isocratic mobile phase of 2.5:1 (w/w) phosphate buffer 0.02 *M* KH_2PO_4 (pH 3.5)/MeCN. The chromatographic system was as described above, but with an excitation wavelength of 475 nm and a cut-off filter at 550 nm. Quantitation was based on a standard curve generated from control plasma samples spiked with DOX and doxorubicinol at concentrations from 2 to 200 ng/ml.

In vitro cytotoxicity studies

Drug sensitivity

The breast cancer cell lines MCF-7 and MDA-MB231 (both derived from human breast adenocarcinomas) were maintained at 37 °C in an atmosphere containing 5% CO_2, and routinely subcultured in Dulbecco's modified Earle's medium (DMEM) and RPMI, respectively, both supplemented with 10% fetal calf serum. The cytotoxicity of active metabolites of IF, and of DOX and doxorubicinol was assessed by growth inhibition of exponentially growing cells after three doubling times (DT) (DTMCF-7 40 h, DTMDA-MB231 23 h) using a Coulter counter. The stabilities of each drug meant that actual exposure times differed. 4OHI was stable in tissue culture medium for approximately 2 h (data not shown), whilst DOX was stable in medium for up to 10 days at 37 °C [21]. The IC_{50} value (the initial drug concentration that inhibited the growth of cells by 50% of control) was determined from the sigmoidal growth inhibition curve.

Comet assay

Cell preparation. For in vitro investigations, each of the cell lines was incubated with IF, DOX or metabolites for 48 h and was processed immediately upon removal of drug. This time-point was chosen in order to enable approximate comparison with the in vivo data. Cells were trypsinized before proceeding with the comet assay (as described below).

PBL from patients were isolated using Lymphoprep (Nycomed, Norway) lymphocyte isolation solution. This produces a very pure lymphocyte separation, with only 1–5% contamination. The PBL were suspended in a mixture of RPMI medium, plasma and dimethylsulphoxide (7:2:1 v/v/v) at a concentration of 5 million cells per millilitre. The lymphocytes were frozen in aliquots (approximately 50,000 cells) at −80 °C until analysis within 3 weeks of collection. The DNA damage induced by storage of PBL at −80 °C for 3 weeks was investigated in lymphocytes isolated from a control subject. No DNA damage due to freezing and thawing was detected (data not shown).

Tumour cells were harvested by FNA. Two aspirates were obtained at each time-point, pooled and kept on ice. Routine cytology was performed on each of the pooled aspirates to confirm the presence of tumour cells. A portion of the cell suspension in agarose was used to form a second layer on the slides prepared below. An internal control was included in the preparation with analyses of patient tumour cells at each time-point (pretreatment, 24 h and 3 weeks) and analysed on a separate slide. The control consisted of an aliquot of MDA-MB231 cells that had been irradiated with 10 Gy and immediately frozen at −80 °C (as above) until required for analysis. The interassay variability was assessed within each patient by calculating the mean of the three internal standards for each time-point. The deviation from the mean was below 10% within each patient.

Measurement of DNA damage. The comet assay was performed as described by Singh et al. [31] with minor modifications. Slides were pretreated to aid agarose binding. Normal agarose (0.75% in Dulbecco's modified PBS) was placed on the slide, then immediately scraped off with a coverslip and left to dry. Briefly, cells (approximately 50,000 in 10 μl), either prepared in vitro or from patients, were embedded in agarose, lysed in a solution containing 2.5 *M* NaCl, 100 m*M* EDTA, 10 m*M* Tris, 1% *N*-lauroyl sarcosine, 1% Triton X-100 and 10% DMSO (the last two components added immediately prior to use) followed by immersion in alkali (300 m*M* NaOH, 10 m*M* EDTA, pH 13.5) for 30 min, before electrophoresis at 22 V for a further 30 min. Slides were neutralized (with 0.4 *M* Tris, pH 7.5) and the DNA stained with 50 μl of an ethidium bromide solution (20 μg/ml in distilled water). Throughout the assay, cells and solutions were kept on ice to minimize any effect of DNA repair enzymes.

All slides from any given experiment were coded and analysed blind. Images of damaged or control cells were captured using a confocal microscope linked to COMOS software (Biorad). DNA damage was quantified in each individual cell by moment analysis (see definition 3 below) using Komet software version 3.1 (Kinetic Imaging, Liverpool, UK). The distributed tail moment was quantified for each of 60 cells per sample (two duplicate slides each having 30 cells analysed). These were then log-normalized to account for the distribution of a typical damaged cell intensity profile. The average of the control cell population was determined and any cell with a distributed tail moment greater than two standard deviations above this value was classed as damaged. The number of damaged cells was expressed as a percentage of the total number of cells studied (see definition 4 below).

Definition of terms

The terms used in the comet analysis are defined as follows:

1. Tail moment: product of tail length and tail intensity
2. Individual pixel moment: product of distance from edge of head and pixel intensity
3. Distributed tail moment: sum of all individual pixel moments in the tail
4. Percent damaged cells: (number of cells > mean + 2SD of the control cells/total number of cells) × 100

Pharmacokinetic methods

The exposure of each patient to IF or metabolite or to DOX or metabolite is expressed as the area under the plasma concentration time curve (AUC). The AUC for parent drugs and metabolites was estimated using the trapezoidal rule, with extrapolation to infinity. The half-life was calculated from the log-concentration time data.

Statistical analyses

In vitro. The distributed tail moments (usually $n = 60$ cells) of untreated cells were compared with those of treated cells using the two-tailed Mann-Whitney nonparametric test. This was also used for testing the effect of different doses of drug. The amounts of

DNA damage caused by equitoxic doses of drug (Fig. 1) were compared using a chi-squared analysis on the mean percentage of damaged cells in the drug-treated population (from three individual analyses).

In vivo. The amounts of DNA damage (in terms of percent damaged cells) in patient tumour and PBL samples were compared using the Wilcoxon matched pairs test. The correlations between IF metabolism and DNA damage were assessed using the Pearson correlation.

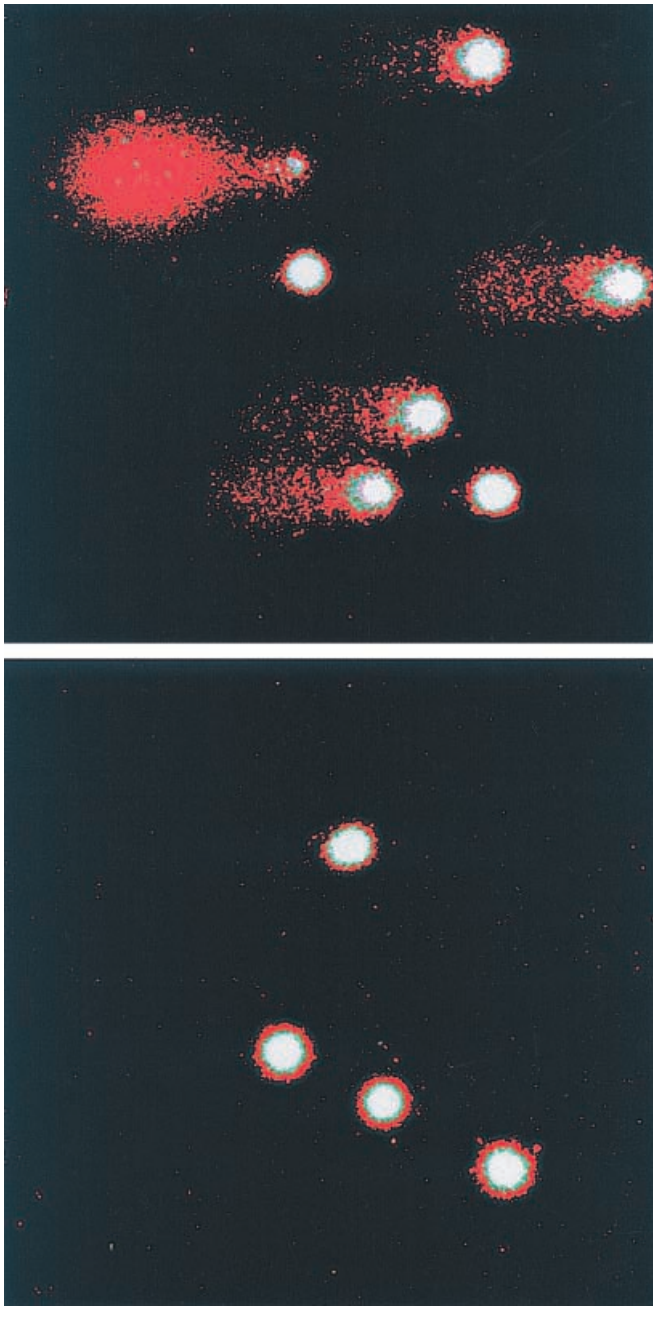

Fig. 1A,B Comets produced from typical PBL from a patient undergoing IF/DOX chemotherapy before treatment (**A**) and 48 h after the start of treatment (**B**)

Results

Clinical and toxicity data

After four courses of treatment, given at 3-week intervals, one of eight patients studied had a good clinical response and five showed a partial response. Response was undetermined in one patient (patient 6) who was referred for further surgical opinion. At the time of evaluation six patients were still alive, four of whom had not relapsed (Table 1). Chemotherapy was generally well tolerated, with only one patient exhibiting greater than grade one toxicity (patient 4 experienced grade 2 mucositis), excluding alopecia. All patients were also given prophylactic dexamethasone and ondansetron.

Metabolism studies

The pharmacokinetics and metabolism of IF were similar to those described previously [2] and are shown in Tables 2 and 3. The median peak plasma concentration was 155 μ*M* (range 124–210 μ*M*) for IF, 54 μ*M* (40–162 μ*M*) for 3DCI, 42 μ*M* (17–72 μ*M*) for 2DCI and 25 μ*M* (9–53 μ*M*) for CXI. Concentration-time curves for 4OHI showed the same trend towards rapidly

Table 1 Patient characteristics, clinical response, and status of disease (*S* smoker, *NS* non-smoker, *U* unknown, *SD* stable disease, *CR* complete response, *PR* partial response, *W* well and disease-free, *R* relapse, *D* death)

Patient	Date of birth	Smoking	Response	Status at review (months since studied)
1	21/10/71	S	SD	R (17), D (18)
2	01/11/46	NS	CR	R (20)
3	15/05/60	NS	PR	W (14)
4	28/07/53	S	PR	R (5), D (5)
5	09/02/64	NS	PR	R (11)
6	01/06/53	U	U	W (13)
7	28/09/56	U	PR	W (26)
8	25/08/54	U	PR	W (16)

Table 2 Pharmacokinetic parameters of unchanged ifosfamide in 7 patients after a 24 hour infusion at a dose of 5 g/m^2 (*ND* not determined)

Patient	AUC (μ*M* · min)	$t_{1/2}$ (h)	Cl (ml/min/m^2)
1	346	14.6	55
2	291	2.6	66
3	279	5.0	69
4	295	7.7	65
5	189	5.2	102
6	982	11.2	19
7	276	5.0	69
8[a]	ND	ND	ND

[a] Patient 8 samples lost

Table 3 Area under the concentration time curve (AUC) of four ifosfamide metabolites in seven patients after a 24-h infusion at a dose of 5 g/m^2 (*ND* not determined due to loss of samples (patient 8) or assay failure (others); *UD* undetected, concentrations below the limit of quantitation)

Patient	AUC (μM · min)			
	3DCI	2DCI	CXI	4OHI
1	258	291	39	10
2	62	40	7[a]	ND
3	91	76	UD	17
4	49	55	13	ND
5	58	154	49	ND
6	280	UD	UD	9
7	109	39	34	11
8	ND	ND	ND	10

[a] Metabolite detectable at single time-point

increasing levels of this metabolite, again to a maximum at 24 h. The levels of 4OHI declined steeply from 24 to 48 h, but were still detectable up to 26 h after the end of IF infusion in four of the five patients for whom data were available. The median peak plasma concentration of 4OHI was 6.1 μ*M* (range 2.0–7.5 μ*M*).

The median AUC across patients was 291 μ*M* · min (range 189–982 μ*M* · min) for IF, 91 μ*M* · min (49–280 μ*M* · min) for 3DCI, 65 μ*M* · min (39–291 μ*M* · min) for 2DCI, 23 μ*M* · min (7–49 μ*M* · min) for CXI and 10 μ*M* · min (9–17 μ*M* · min) for 4OHI. The median half-life of IF was 5.2 h (2.6–14.6 h) and the median clearance of IF was 66 ml/min per m^2 (19–102 ml/min per m^2) (Table 2). The fivefold variation in AUC of IF was skewed by one outlier (patient 6). Excluding this patient, the AUC of IF shows only a twofold variation. The variation in AUC of metabolites was 5-fold for 3DCI, 7.5-fold for 2DCI, 7-fold for CXI, and 2-fold for 4OHI.

DOX concentrations exhibited biexponential elimination. The plasma concentration-time profile of doxorubicinol closely followed that of the parent drug. The median peak plasma concentration of DOX across patients was 21.7 ng/ml (range 11.6–28.8 ng/ml), and that of doxorubicinol was 9.3 ng/ml (4.4–18.1 ng/ml).

The pharmacokinetic data of DOX (Table 4) showed a 2.5-fold variation in AUC, and a median of 27.2 μg/ml · min (range 14.4–59.2 μg/ml · min). The AUC of doxorubicinol showed a 4.5-fold variation and a median of 13.4 μg/ml · min (6.4–29 μg/ml · min). The median half-life and clearance of DOX were 9.6 h (4.4–60.9 h) and 1314 ml/min per m^2 (675–2779 ml/min per m^2), respectively.

DNA damage in vivo

The DNA damage in the PBL of each individual patient was determined at four different time-points. Before treatment, cells exhibited a low level of damage (range 0–10%, median 3.5% damaged cells; Fig. 1A and 2A). The frequency of cells exhibiting DNA damage was increased (12–37% damaged cells, median 12.5%, $P = 0.016$ compared to pretreatment) at 24 h in six of the eight patients, corresponding to the end of the IF infusion. This increase in DNA damage was maintained or further increased at 48 h (10–37% damaged cells, median 21.5%, $P = 0.019$) after the start of treatment in all six of these patients showing the initial increase (Fig. 2A). In addition, one patient (patient 8), in whom the amount of DNA damage at 24 h (5%) was no different from that before treatment, showed an increased number of cells with DNA damage at 48 h (37%). Resolution of damage to pretreatment levels was seen in five of seven patients 3 weeks after treatment (median 17%, $P > 0.05$). Patient 4 showed no increase in DNA damage above basal levels at any time-point.

DNA damage was also assessed in patient tumour cells at three time-points in six patients (patients 2–8; Fig. 2B). In the other two patients there were insufficient tumour cells available to carry out DNA damage analysis using the comet assay. Again the cells analysed before treatment exhibited the lowest amount of damage (median 3%, range 0–3%). However, in some patients the basal damage was slightly higher in PBL than in the corresponding tumour cells (patients 4 and 6). There was no significant difference in the amount of DNA damage measured in the tumour cells before treatment and at 24 h (median 2%, range 0–23%). However, a shift towards increased numbers of cells with DNA damage occurred 3 weeks after treatment (median 30%, range

Table 4 Pharmacokinetic data for unchanged DOX after a bolus dose of 40 mg/m^2 (*ND* not determined)

Patient	AUC (μg/ml · min)		$t_{1/2}$ DOX (h)	Cl DOX (ml/min/m^2)	AUC ratio (Doxorubicinol/DOX)
	DOX	Doxorubicinol			
1	59.2	25.9	60.6	675	0.44
2	18.4	13.9	11.9	2177	0.75
3	14.4[a]	7.1[a]	ND	2779	0.53
4	37.5	29.0	4.4	1066	0.77
5	24.7	14.2	7.2	1840	0.57
6	31.4	12.8	21.4	1276	0.41
7	16.5	6.4	4.8	1276	0.38
8	29.6[a]	12.9[a]	ND	1352	0.44

[a] AUC to 48 h

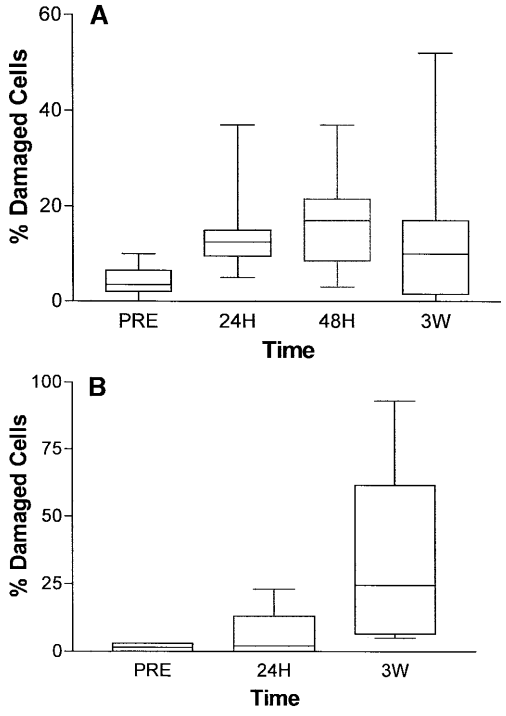

Fig. 2A,B Summary of DNA damage in the PBL (**A**) and tumour cells (**B**) of all patients. The box extends from the 25th to the 75th percentile with the median represented by a line. The whiskers show the range of the data

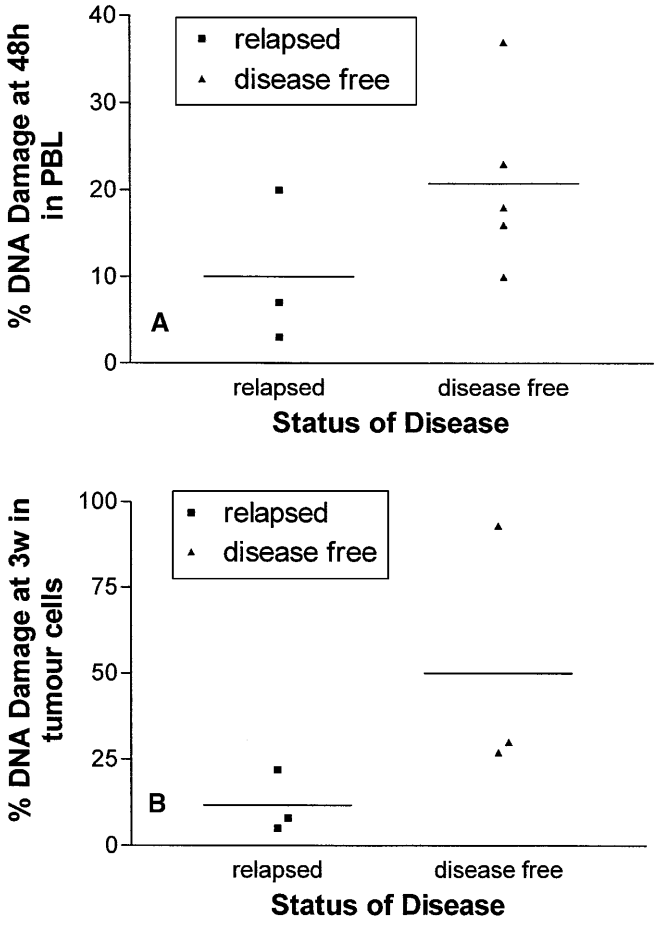

Fig. 3 Relationship between the percent damage in the PBL 48 h after treatment (**A**) and in the tumour cells 3 weeks after treatment (**B**) and status of disease. Each point represents one patient and the mean is represented by a line

5–93%). This was observed in four of five patients with tumour material available at that time (Fig. 3B) and represented a significant increase over pretreatment and 24-h values ($P = 0.015$). There was no consistent or predictive relationship between the maximum amount of DNA damage in PBL and that in tumour cells in these patients (data not shown).

Relationship between the pharmacology of IF and DOX, DNA damage in PBL and tumour and clinical response

Potential relationships were investigated between levels of IF (and its active and inactive metabolites) or levels of DOX (and its major metabolite) and DNA damage in PBL and tumour cells of patients. A positive relationship was observed between the AUC of 4OHI and the percentage DNA damage in PBL measured at 24 h and 48 h, but these relationships reached only borderline significance ($P = 0.05$) and were based on limited data ($n = 4$).

No relationship was found between the concentrations of DOX or doxorubicinol and the amount of DNA damage observed in PBL at any time-point. Neither were any relationships found between the AUC of IF or metabolites or DOX (and its metabolite) and the DNA damage seen in the tumour cells at any time-point. No relationships were found between the amount of damage seen at any time-point in either the PBL or tumour, and the clinical response although in one patient the response was undetermined.

With respect to disease status (relapsed or disease-free), the median percent DNA damage at 48 h in the PBL was higher in those patients who were disease-free at the time of evaluation (Fig. 3A). This greater amount of DNA damage in disease-free patients was more apparent in the tumour cells at 3 weeks after treatment (Fig. 3B), but in neither case was the difference statistically significant ($P > 0.05$ in both cases).

In vitro studies of growth inhibition and DNA damage

The relative growth-inhibitory concentrations of 4OHI, IPM and DOX were determined in two human breast cancer cell lines (Table 5). IF, inactive as the parent drug, has an IC_{50} of approximately 1 m*M*. 4OHI was found to be 500-fold more potent than IF and also 2–3-fold more potent than IPM. DOX was found to be more potent than both 4OHI (at least 200-fold) and IPM in both cell lines.

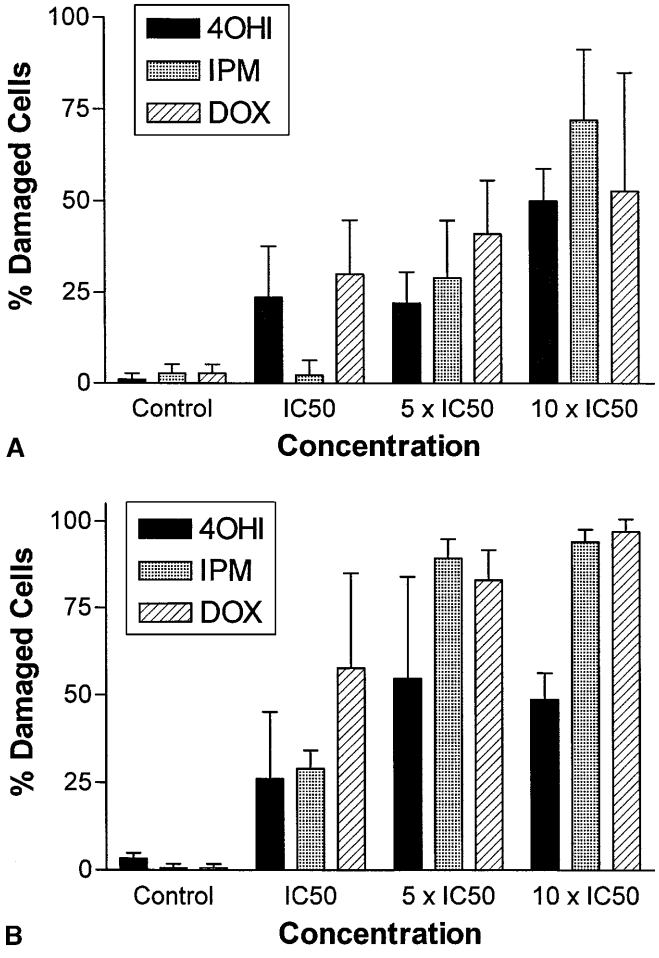

Fig. 4A,B Comparison of the amount of DNA damage induced by exposure to 4OHI, IPM mustard and DOX in MCF7 cells (**A**) and MDA-MB231 cells (**B**). The bars represent the mean percent damaged cells and the standard deviation from three separate experiments

Table 5 IC_{50} values for two breast cancer cell lines after exposure to 4OHI, IPM and DOX

	MCF7		MDA-MB231	
	IC_{50} ± SEM (μM)	Number of observations	IC_{50} ± SEM (μM)	Number of observations
4OHI	2.2 ± 0.2	3	4.2 ± 0.3	3
IPM	6.1 ± 0.8	3	8.7 ± 0.7	3
DOX	0.011 ± 0.001	4	0.009 ± 0.001	4

DNA damage in vitro

Figure 4 illustrates DNA damage induced by cytotoxic concentrations of 4OHI, IPM and DOX in the two breast cancer cell lines. In general, cellular DNA damage was increased with increasing growth-inhibitory concentrations of each drug. In MCF7 cells (Fig. 4A) the pattern of induced damage varied between drugs. After exposure to IC_{50} concentrations in vitro, greater than 20% damage was observed after exposure to 4OHI and DOX. However, no IPM-induced damage was observed at this concentration. At concentrations above the IC_{50}, on treatment with all three agents the cell populations exhibited greater than 20% damage. This compares with the median DNA damage observed in vivo in both PBL and in tumour cells (20–30%).

In MDA-MB231 cells (Fig. 4B) the amount of DNA damage induced by all three agents was generally much higher than that induced in MCF7 cells. In these cells, in contrast to the pattern of damage in MCF7 cells, IPM-induced damage was observed after exposure to the IC_{50}, equivalent to that induced by 4OHI at the same degree of growth inhibition. DOX-induced damage in MDA-MB 231 cells was greater than 50% at all concentrations tested.

The IC_{50} values of 4OHI used to treat cells in vitro (2 μM for MCF7 cells, 4 μM for MDA-MB 231 cells) are encompassed in the range of peak concentrations of 4OHI measured in vivo (range 2–7.5 μM, $n = 5$). Although the time of exposure to these concentrations of 4OHI in vitro may have differed from those in vivo, the amount of DNA damage produced in both cases was broadly similar.

Discussion

There is a wide interindividual variation in the clinical response to treatment with oxazaphosphorines [2, 12, 20], and insights into the determinants of sensitivity of these drugs may help to improve efficacy and limit the toxicity of these agents. This variation in response could be due to the intermediate stages between administration of drug and the antineoplastic effect, including transformation into active metabolites (and also deactivation of parent drug in tissues), drug transport into the tumour microenvironment, probably as 4OHI, and cellular penetration. Once inside the cell the breakdown to IPM followed by interaction of IPM with its target of DNA and formation of DNA adducts may occur.

The AUC of IF (Table 2) varied over a fivefold range and the median AUC was comparable to that found in similar studies [2, 12]. Individual differences in the expression of activating enzymes, in particular CYP3A4, will influence the amount of 4OHI produced, and wide variation in the hepatic expression of cytochrome P450 enzymes (including CYP3A4 and CYP2B6) has been frequently reported [5, 35]. Concentrations of 4OHI were determined during and after a 24-h infusion and found to be in the range 0–10 μM in all patients studied (Table 3).

3DCI was the major metabolite of IF and exhibited a fivefold variation amongst individuals. The exceptionally high AUC of IF in one patient (patient 6) could be attributed to a markedly delayed formation of the 3DCI metabolite. The variation in 2DCI was approximately sixfold (Table 3). Another important mechanism of inactivation is via ALDH, a family of enzymes which

irreversibly deactivate aldoifosphamide to CXI. ALDH isozymes are expressed at relatively high levels in some normal tissues, for example liver, spleen and bone marrow [11]. A sevenfold variation in the AUC of CXI was determined between individuals in this clinical study (Table 3).

The reaction of IPM with DNA is assumed to proceed in a similar manner to that of cyclophosphamide-derived phosphoramide mustard, via highly unstable aziridinium intermediates [14, 22]. All nitrogen mustards react preferentially at the N7 position of guanine on DNA to form monofunctional adducts as well as N7-N7 interstrand crosslinks [28]. The number of crosslinks formed in a cell [15], the genomic site at which the crosslinking occurs [16] and also the ability of the cell to repair these lesions [17] will determine the extent of cytotoxicity, but excessive strand scission due to the instability of these guanine adducts [18] could also add to the cytotoxic effect.

DNA damage in the tumour cells of patients receiving IF/DOX chemotherapy was determined in this study. The DNA damage detectable by comet analysis in tumours showed a degree of individual variation in the amount, but the not the time course (Fig. 3B). No significant correlations were found between the amount of damage and concentrations of active IF metabolites in the peripheral circulation. Only a borderline correlation was observed between the AUC of the active metabolite 4OHI and the levels of damage in the PBL, measured 48 h after the start of treatment (Pearson correlation $P = 0.05$, $n = 4$). There was also an apparent difference between those patients that relapsed and those that remained disease-free, in the amount of DNA damage observed in the tumour cells and in the PBL (Fig. 4), but again this was not statistically significant.

The unmodified comet assay used in this study detects single stand breaks and alkali labile sites, but not crosslinks. The comet assay may be modified to specifically measure IF-induced crosslinks in patients after treatment with single-agent IF [13]. However, the overall measurement of DNA damage in the form of strand lesions appears also to be a useful measure of how cells respond to alkylating agents. In a recent study using the comet assay in PBL of patients treated with dacarbazine, DNA damage has been found to be related to excretion of methylated adenine and to the degree of toxicity observed [3].

The response to DNA damage induced by active metabolites of IF in vitro was compared using one cell line that expresses wild-type p53 (MCF7) and another that expresses mutant p53 (MDA-MB231). The latter cells required a slightly higher concentration of 4OHI (IC_{50} 4.2 μM vs 2.3 μM) and IPM (8.7 μM vs 6.1 μM) to achieve 50% growth inhibition than MCF7 cells (Table 5). DNA damage after exposure to 4OHI in vitro was similar in both cell lines, but after exposure to IPM there was more damage observed in the MDA-MB231 cells (mutant p53) after an IC_{50} or fivefold IC_{50} dose, implying a cell line-specific effect (Fig. 1). This differential effect of IPM may have been masked after exposure to 4OHI by the confounding DNA-damaging effects of acrolein.

DNA damage was used as a surrogate endpoint for cytotoxicity in this study, both clinically and in vitro. The situation in vivo is clearly complex, but a dose response relationship between cytotoxicity and DNA damage has been established in human breast cancer cell lines. DNA strand lesions (in the form of single strand breaks and alkali labile sites) are observed in vitro at concentrations of 4OHI consistent with those measured in vivo (Fig. 1; Tables 3 and 4). Therefore, information about the amount and time-course of DNA damage in individual patients in vivo may be useful in predicting response to chemotherapy and optimizing chemotherapy regimens. This hypothesis was not confirmed in the current study, but the study was limited by the small number of patients available.

As the chemotherapy regimen used in this study was IF given in combination with DOX, it was important to consider the contribution of DOX to the DNA damage in vivo, measured in the PBL and the tumour cells. The AUC of DOX had a 2.5-fold variation, whilst that of doxorubicinol had a 4.5-fold variation (Table 4). Concentrations of DOX in tissues are higher than those measured in plasma [19], whilst tissue uptake of doxorubicinol does not occur to the same extent as the parent drug [30].

Growth inhibition of human breast cancer cells by DOX occurred at much lower concentrations (IC_{50} 10 nM for both cell lines) than that by active IF metabolites (Table 5). DOX-induced DNA damage has previously been assessed using the comet assay [25], but both DOX and another topoisomerase II inhibitor, etoposide, induce damage that is not predictive for cytotoxicity in vitro [26]. A lack of relationship between DNA damage and the antiproliferative effects of DOX has been previously reported [10]. Our results show that DNA damage was not concentration-dependent in MCF7 cells, but in the MDA-MB231 cell line, the amount of damage, which was concentration-dependent, was much more pronounced. The DOX-induced DNA damage in these human breast cancer cell lines was not predictive of cytotoxicity, as the IC_{50} values for DOX in the two cell lines were equivalent.

Although the DNA-damaging effects of 4OHI and IPM have been proven to be related to cytotoxicity in human breast cancer cells in vitro, no relationship between concentrations of metabolites and DNA damage, or any relationship between the amount of DNA damage observed in an individual (in the PBL or in the tumour cells) and clinical endpoints such as relapse or response were firmly established in this study. However, the low number of patients, and the actual number of measurements taken in each patient restricted this kind of analysis. These results are not inconsistent with such a correlation and, in particular, do suggest a relationship between lower amounts of DNA damage in the tumour cells and earlier relapse.

In conclusion, these studies have demonstrated that the metabolism of IF varies widely amongst individuals and that this is matched by, if not causally related to, variation in the downstream effect of cellular DNA damage.

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