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Relationship between doxorubicin cell sensitivity, drug-induced DNA double-strand breaks, glutathione content and P-glycoprotein in mammalian tumor cells

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We have measured the cytotoxic effect of 1 h exposure to doxorubicin (DOX) on a panel of tumor cell lines. Cellular effects were measured by monolayer colony-forming assay and a colorimetric cytotoxicity assay. As parameters of chemosensitivity we used two different end-points: the dose of DOX that reduces to 50% the number of colonies (ID_{50}) and the dose of DOX that reduces the final optical density to 50% of the control value (IC_{50}). There was a significant correlation between both chemosensitivity indices ($r = 0.886$, $p = 0.0034$). DOX-induced DNA double-strand breaks (dsb) were evaluated using pulsed-field gel electrophoresis (PFGE) and compared with cellular effects, P-glycoprotein expression (P-170) and intracellular glutathione (GSH) levels. Our results showed a relationship between the slope of DNA dsb dose–response curves and the percentage of cells that express P-170 ($r = -0.957$, $p = 0.0002$). Our study also detects a positive relationship between cellular chemosensitivity parameters and GSH content [ID_{50} versus GSH ($r = 0.794$, $p = 0.0186$), IC_{50} versus GSH ($r = 0.790$, $p = 0.0198$)] in our panel of cell lines.

Key words: Cell sensitivity, doxorubicin, doxorubicin-induced DNA double-strand breaks.

Introduction

The major reason for the limited clinical results of chemotherapy is that the drugs available do not have sufficiently large 'therapeutic indices'. This is easy to say, dose-limiting toxicities occur at drug doses which are insufficient to eliminate all malignant cells.[1] Tumor cell resistance to drugs inducing DNA damage [e.g. doxorubicin (DOX)] is therefore an important factor of consideration. It is likely that some mechanisms of resistance are specific for each drug, being related to the types of DNA damage and their repair mechanism. Some others are more general and shared by several types of DNA damaging agents, leading to various types of multidrug resistant phenotypes. One of these phenotypes is related to an altered expression of the MDR-1 gene whose product influences drug transport. Recent reviews of this problem have been published by Gottesman and Biedler.[2,3] Other mechanisms of resistance to DOX concern topoisomerase II alterations,[4] detoxification through the glutathione[5] and intracellular glutathione (GSH) levels.[6]

If the response to cytostatic therapy could be predicted with confidence, not only would it recommend drugs with a high probability of patient benefit, but it would also reduce unnecessary toxicity associated with ineffective therapeutic modalities. Numerous attempts have been made to design *in vitro* assay systems to predict chemosensitivity *in vivo*,[7] but most of these have failed. Inherent chemoresistance of tumor cells can be best measured by assays for clonogenicity. However, such tests are comparatively time consuming and the results are obtained only after several weeks. For treatment of individual cancer patients it is necessary to develop a more rapid assay system in order to plan the treatment on the basis of such assays.

The main objective in this work was to study the possibilities to develop a DNA damage assay as a predictor of cellular sensitivity to DOX (Farmitalia) using pulsed field gel electrophoresis (PFGE). We have evaluated DNA damage induction using PFGE and compared it with cell survival data [clonogenic assay (CA) and colorimetric cytotoxic assay (CCA)], P-glycoprotein expression and intracellular GSH levels.

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Materials and methods

Cells and standard culture conditions

Eight human tumor cell lines have been studied in this work. Most of them have been described previously.[8] Six of them were derived from human breast cancer (MCF-7 clones BB, BUS and GS, T47D clones B1 and B8 and EVSA-T), RT-112 from a human bladder carcinoma and A2780 cp from a human ovarian cancer.[9] Cell lines were grown in 5% fetal bovine serum (FBS)-supplemented Dulbecco's modified Eagle's medium (DME) (PAA-Labor, Austria) with penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Freedom from mycoplasma contamination was checked regularly by testing with Hoeschst 33528.

Cellular chemosensitivity assays

Cells were harvested with trypsin–EDTA (0.05–0.02%) and suspended in full culture medium. CA were performed in monolayers in 25 cm^2 plastic flasks (Nunc, Denmark). Cells were treated 24 h after seeding with DOX dilutions made in DME for 1 h, range 0.001–1 μg/ml, each dose being used in three replicate flasks. At the end of treatment the DOX solution was removed and cells were reincubated in complete culture medium for 14–21 days. Colonies of at least 50 cells were scored as surviving cells. From the plots of surviving fraction against DOX dose, on semilogaritmic coordinates, it was possible to calculate the ID_{50} as a measure of chemosensitivity. Three separate experiments were done for each cell type.

To set up the CCA assay,[10,11] cells were trypsinized from monolayer and diluted to 4×10^4 cells/ml. Cells were in exponential phase of growth during the whole experiment. Aliquots of 1 ml were pipetted into wells of 24-well tissue culture plates (Falcon) and the plates were incubated for 24 h. DOX was then added to the wells in a volume of 1 ml per well at a range of concentrations (0.01–10 μg/ml), each dose being used in at least four replicate wells. After 1 h incubation, DOX solutions were removed and complete culture medium was added to let the cells grow for a further 3 days. At the end of this time the cultures were washed with PBS prior to fixation with 10% trichloroacetic acid (TCA) (4°C) for 30 min and then washed with tap water to remove TCA. Plates were air dried and then stored until use. TCA-fixed cells were stained for 20 min with 0.4% (w/v) sulforhodamine B (SRB) dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were rinsed with 1% acetic acid to remove unbound dye. The cultures were air dried and bound dye was solubilized with 10 mM Tris base (pH 10.5). Optical density (OD) was read in a Titertek multiscan plate reader at 492 nm. The photometer response was linear with dye concentration and it was proportional to cell numbers counted in parallel with a hemocytometer. From the data it was possible to calculate the IC_{50} (and hence cell number). Five independent experiments were performed for each cell type.

DNA damage assays

To measure DNA dsb, cells seeded in 25 cm^2 plastic flasks were labeled with methyl-[^{14}C]thymidine (Amersham; 2.11 GBq/mmol), at a concentration of 0.05 μCi/ml for 48 h, and chased with non-radioactive medium for 18 h. To determine the time-dependence of DNA dsb level after treatment with DOX, the drug (1 μg/ml) was added for 1 h. Following drug exposure the drug solution was replaced by DME + FBS and then cells were incubated for 0–24 h. Two flasks were used for each point. DNA damage was stopped by the addition of excess ice-cold medium and transfer of the flask to 4°C. At the end of this time the cells were harvested, resuspended in DME and centrifuged. The cell pellet was mixed with 0.8% ultralow melting point agarose (ULMP-agarose; Sigma) in PBS-A at 37°C at a concentration of 10^6–10^7 cells/ml. The suspension was pipetted into plug moulds (250 μl; BioRad). These were kept at 4°C until the agarose had set. The plugs were removed and suspended in ice-cold lysis buffer [1 mg/ml proteinase K (Sigma) in 2% lauryl-sarkosine (Sarkosyl, Sigma)–0.5 M EDTA at pH 7.6]. Lysis was on ice for 1 h and then incubated at 37°C for 24 h.[12] Cell plugs were divided into samples of approximately 25 μl and loaded into the wells of an 0.8% agarose gel (LMP-agarose; Sigma). DNA dsb measurements were performed on a clamped homogeneous electric field pulse-field unit (CHEF-DR II; BioRad) as described previously.[8,13] PFGE in its various forms has become one of the most widely used methods of DNA dsb measurement in mammalian cells.[14] On the basis of the time course data, we chose 4 h after DOX treatment to determine the relationship between DNA dsb and DOX doses. The cells, labeled as above, were treated with different DOX concentrations prepared in DME (range 0.5–20 μg/ml) for 1 h, then removed and cells were kept at 37°C for 4 h in complete culture medium. Cell plugs, lysis procedure and

PFGE were performed as above. Three independent experiments were done for each cell type and appropriate untreated control points were included in all experiments.

GSH determination

All the cultures were analyzed during exponential growth on whole cells attached to the plastic layer. We have used a modification of Tietze's assay,[15,16] based on the principle that GSH can be measured by an enzymatic recycling procedure in which it is sequentially oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of GSH reductase to 5-thiobenzoic acid (TNB). For this assay cultures were plated in 24-well plates containing 0.4–2 × 10^5 cells per well. After 18–24 h the monolayer was washed twice with PBS prior to GSH extraction with 0.5 ml of 0.6% 5-sulfosalicilic acid (5-SA) (30 min in the dark, 4°C). After this treatment the cells remain attaced on the wells and it is possible to stain them using SRB to give the cell number.[10,11] Glutathione was measured mixing 25 μl of sample in the wells of a 96-well plate and adding 200 μl of a solution with three components (175 μl of 0.3 mM NADPH, 25 μl of 6 mM DTNB and 2.5 μl of 50 unit/ml of GSH reductase). The rate of formation of TNB was followed measuring the OD at 405 nm (Titertek MultiScan plate reader) at 0, 1, 2, 3 and 4 min, these values were plotted against time and the slope of TNB formation kinetics was calculated by linear regression. Standards of known GSH content (0–12 nmol/100 μml) were made up by serial dilution in 0.6% 5-SA. The values of OD were plotted against time. In order to determine the calibration line we plotted TNB formation slopes againt GSH concentration and calculated a linear regression. Results were expressed as nmol of GSH/10^6 cells.

P-glycoprotein assay

The P-glycoprotein expression was determined by flow cytometry. Cells were harvested, centrifuged and washed twice with PBS. The cell pellet was resuspended to a cell density of 5 × 10^5 cells/ml in 70% ice-cold ethanol and kept at −20°C for 15 min. To remove the ethanol, the suspension was centrifuged and the pellet was mixed with 2 ml of cold 0.1% BSA in PBS. The final cell density was adjusted to 5 × 10^5 cell/100 μl in 0.1% BSA/PBS. Aliquots of this cell suspension were processed in parallel, one with a dilution 1/10 of MDR/JSB-1 monoclonal antibody (Sanbio, Netherlands) and the other one was used as negative control (non-specific fluorescence). JSB-1 antibody reacts with a conserved cytoplasmatic epitope of the plasma membrane-associated 170–180 kDa glycoprotein. All tubes were incubated for 30 min at 4°C. Excess of antibody was removed by washing with BSA/PBS and the cells were spun down again. Then pellets from test and negative control tubes were resuspended into 100 μl FITC (Biomeda) diluted 1/80 in PBS and incubated for 30 min at 4°C. Finally, the cells were washed twice and mixed with 700 μl of BSA/PBS and run on an Ortho Cyteron Absolute flow cytometer (excitation 488 nm, emission 560 nm). Determination of the percentage of cells that are positive for this protein was made using a cut-off of the crossover point between total fluorescence and background fluorescence. Specific mean fluorescence uptake (SMFU) of monoclonal antibodies against P-170 as defined by Kute and Quadry[17] was the difference in mean channel number for total and non-specific uptake histograms.

Results

CA

The measurement of the cytotoxic effect of 1 h exposure to DOX was determined by the colony-forming assay. All the cell lines used showed a biphasic dose–response pattern with flattening of the curves above 0.01 μg/ml (Figure 1). Values at

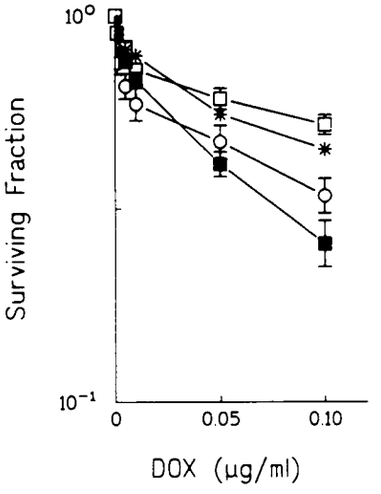


Figure 1. Cell survival curves for MCF-7 GS cells (□), EVSA-T (*), MCF-7 BUS (○) and T47D-B8 (■) after 1 h DOX treatment (μg/ml). Colonies of at least 50 cells were scored as surviving cells.

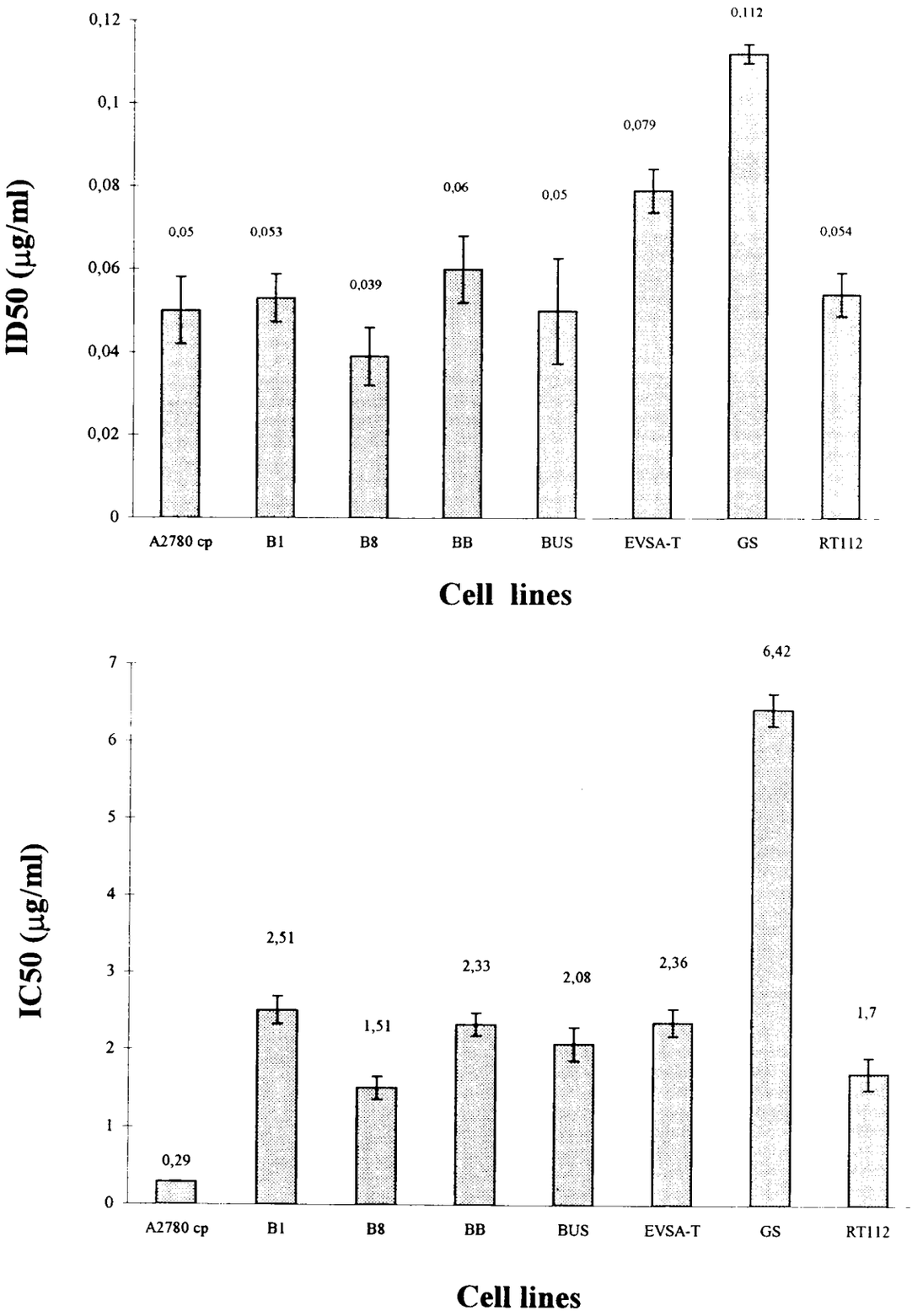


Figure 2. Doxorubicin cellular chemosensitivity response: (A) ID_{50}: dose of DOX (µg/ml) that reduces to 50% the surviving fraction. (B) IC_{50}: dose of DOX (µg/ml) that reduces to 50% the cell growth. Values are mean of at least three experiments performed using three flasks per experiment.

the ID_{50} were calculated from the curves and these are shown in Figure 2(a).

CCA

The CCA after 1 h exposure to DOX allows us to calculate the IC_{50} with respect to the control wells (Figure 2b). These values span a wider range (0.29–6.42 μg/ml) than the ID_{50} values and are quantitatively different (Figure 3), but there is a significant relationship between IC_{50} and ID_{50} (Figure 4) ($r=0.886$, $p=0.0034$). Therefore with this set of cell lines both assays can be used to measure the cellular DOX sensitivity. To design the CCA we also let the cells grow for 1, 2, 3, 4, 5, 6 and 7 days after the treatment. We found a similar IC_{50} value after 3, 4 and 5 days but not at the other intervals. It appears that in the first and second days the consequences of DOX exposure had not been fully expressed and by day 6 the control wells had reached the plateau phase of growth (data not shown).

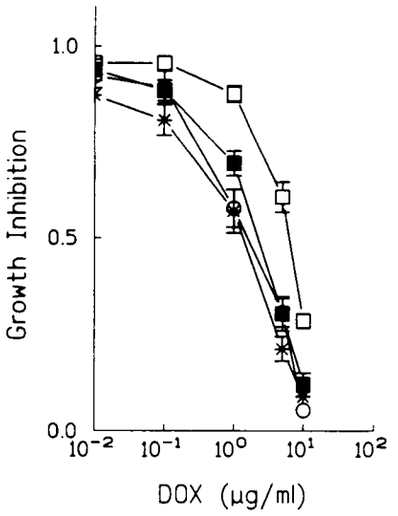


Figure 3. Growth inhibition curves for MCF-7 GS (□), EVSA-T (*), MCF-7 BUS (○) and T47D-B8 (■) cells measured by the colorimetric cytotoxicity assay (SRB) after 3 days of 1 h DOX treatment (μg/ml).

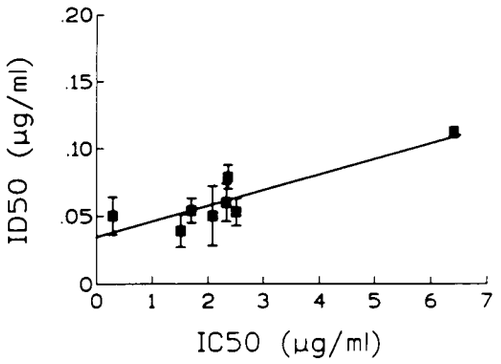


Figure 4. Relationship between ID_{50} and IC_{50} values. $ID_{50}=(0.012\pm0.002)\times IC_{50}-(0.034\pm0.007)$; $r=0.886$, $p=0.0034$.

Time-dependent DOX DNA dsb induction

The time-dependence of DOX induced DNA dsb was measured by PFGE. All the cell lines revealed an initial increase in the amount of DNA extracted in the first 5–7 h (Figure 5a). In an attempt to clarify whether the rate of formation of DNA dsb has any relationship with the parameters obtained from the cellular chemosensitivity assays, we have fitted a straight line to the experimental points corresponding to that first phase (Figure 5b). The rate of DOX DNA dsb induction span the interval 1.15–2.8% DNA extracted/h (Table 1). We have not found any relationship between this measurement of molecular damage and the cellular response to DOX.

Dose–response curves

To establish the dose–response relationships we have chosen to assess damage in the initial phase of the time courses shown in Figure 5(a). We have

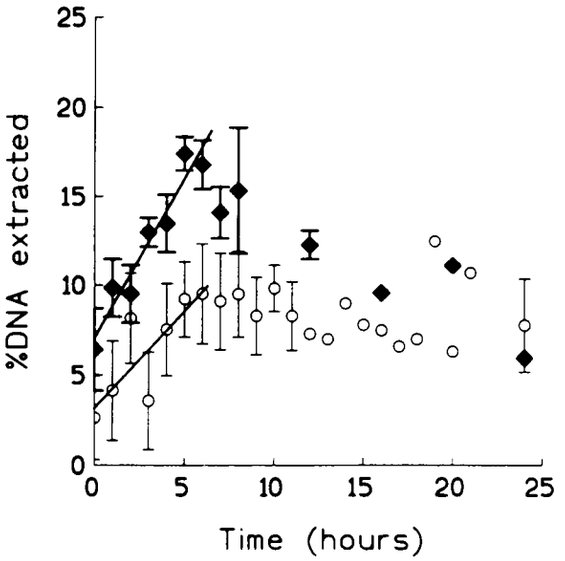


Figure 5. (A) Time-dependent DOX DNA dsb induction curves for MCF-7 BUS (○) and MCF-7 BB ◆.

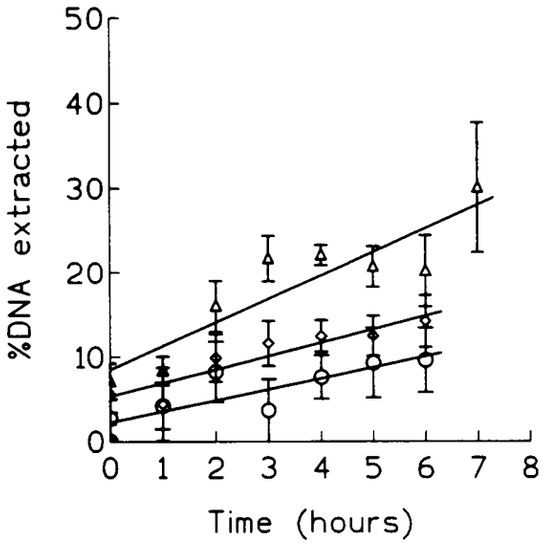


Figure 5. (B) Time-dependent DOX DNA dsb induction curves for MCF-7 BUS (○), RT112 (◇)

Table 1. DNA assays as a measure of molecular DOX-induced damage

Cell line	Time–response curves		Dose–response curves	
	slope[a]	*p*	slope[b]	*p*
A2780 cp	1.70 ± 0.35	0.0103	0.057 ± 0.001	0.047
B1	2.80 ± 0.55	0.0022	0.016 ± 0.005	0.027
B8	1.15 ± 0.28	0.0061	0.013 ± 0.003	0.036
BB	1.79 ± 0.21	0.0004	0.008 ± 0.002	0.008
BUS	1.08 ± 0.35	0.0260	0.015 ± 0.003	0.008
EVSA- T	2.00 ± 0.48	0.0088	0.023 ± 0.002	0.002
GS	1.95 ± 0.54	0.0148	0.019 ± 0.005	0.015
RT112	1.59 ± 0.29	0.0029	0.027 ± 0.004	0.029

[a] Slope of the initial increase in DNA dsb (% DNA extracted/h).
[b] Slope of dose–response curves [DNA extracted/(μg/ml) DOX].

therefore used the 4 h time point for the comparison of different doses of DOX. The pattern of DNA dsb as a function of drug concentration showed an initial increase in the percentage of DNA extracted followed by a plateau and then by a decline in DNA lesions (Figure 6). We have used the slope of the initial phase of this dose–response curve as a parameter of molecular damage. The slopes, expressed as the fraction of DNA extracted per μg of DOX, ranged from 0.008 to 0.149 (Table 1). We have not found any relationship between this measurement of DNA damage and the cellular response to DOX.

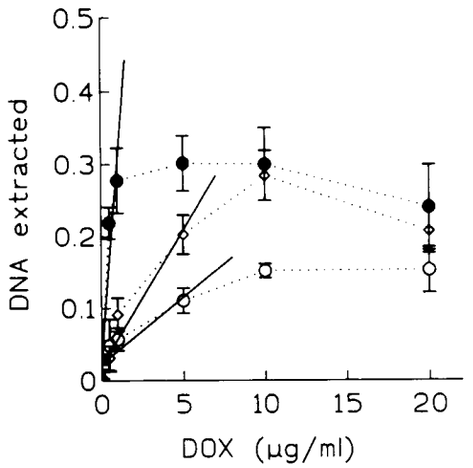


Figure 6. Dose–response curves for A2780 cp (●), RT112 (◇) and MCF-7 BUS (○).

Intracellular GSH levels

We have measured the GSH content of the cells in the same growth conditions as used for the survival assays and we have used these values for comparison with the chemosensitivity of our cell lines. The results obtained are shown in Table 2. There is a relationship between chemosensitivity and GSH content (Figure 7) but these are heavily dependent on the data for MCF-7 GS [ID_{50} versus GSH ($r=0.794$; $p=0.0186$), IC_{50} versus GSH ($r=0.790$; $p=0.0198$)]. There was no significant correlation between GSH content and the DNA damage parameters that we have chosen.

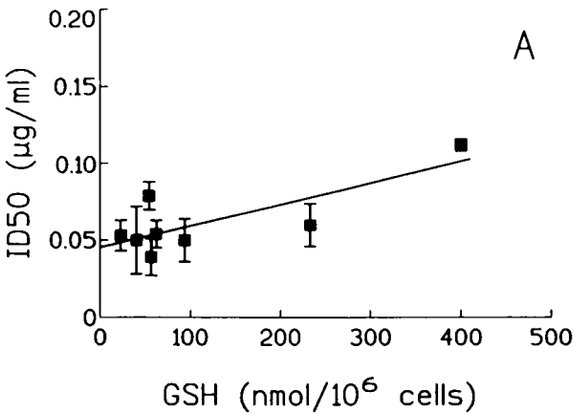
P-glycoprotein expression

The results obtained for the measurement of P-glycoprotein expression of the cells used in this work

Table 2. Glutathione and P-glycoprotein expression

Cell line	GSH content (nmol/10^6 cells)	P-170 expression (%)	SMFU[a]
A2780 cp	93.6 ± 7.01	83.7 ± 8.6	33.4 ± 4.2
B1	22.6 ± 6.03	92.2 ± 3.4	40.0 ± 2.1
B8	56.2 ± 12.10	93.6 ± 7.7	32.6 ± 4.7
BB	233.4 ± 43.84	88.4 ± 3.7	26.1 ± 1.4
BUS	40.2 ± 3.61	69.4 ± 14.6	15.9 ± 1.9
EVSA- T	54.0 ± 4.66	93.9 ± 5.6	43.6 ± 14.8
GS	400.0 ± 45.19	85.3 ± 11.6	25.4 ± 7.7
RT112	62.0 ± 15.92	75.9 ± 3.2	39.0 ± 6.3

[a] Specific mean fluorescence uptake of monoclonal antibodies against P-170.



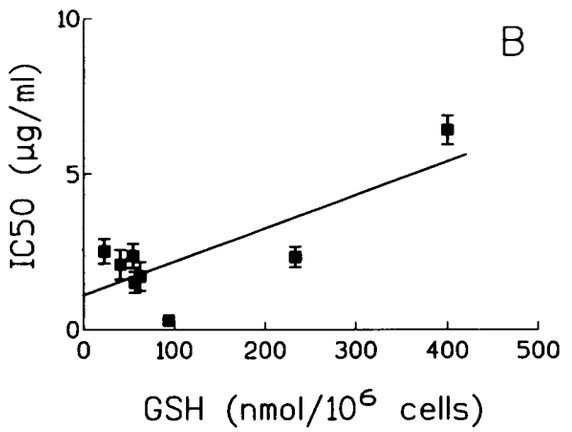


Figure 7. (A) Relationship between GSH content and ID_{50}. $ID_{50} = (0.011 \pm 0.003) \times [GSH] + (1.10 \pm 0.06)$; $r = 0.794$, $p = 0.0186$. (B) Relationship between GSH content and IC_{50}. $IC_{50} = (1.4 \times 10^{-4} \pm 4.0 \times 10^{-5}) \times [GSH] + (0.051 \pm 0.008)$; $r = 0.790$, $p = 0.0198$.

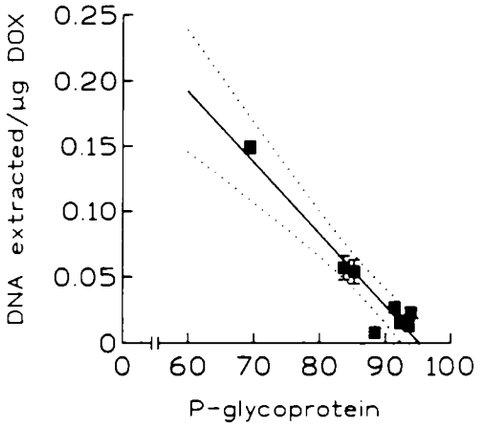


Figure 8. Relationship between P-glycoprotein and DNA extracted (µg/ml) DOX. $DNA_{ext} = -(5.6 \times 10^{-3} \pm 7 \times 10^{-4}) \times [\%P\text{-}170] - (0.52 \pm 0.01)$; $r = -0.957$, $p = 0.0002$.

are shown in Table 2. These values were compared with the cellular and molecular parameters of chemosensitivity, and we have found a linear relationship between both the percentage of cells that express P-170 and the SMFU values measured by flow cytometry and the fraction of DNA extracted per µg of DOX (Figure 8) [DNA_{ext} versus %P-170: $r = -0.957$; $p = 0.0002$; DNA_{ext} versus SMFU: $r = -0.717$; $p = 0.0455$].

Discussion

It has been realized for some time that human tumor cell lines differ widely in their survival characteristics after treatment with DOX. These differences have been explained mainly by pleotropic multidrug resistance (MDR-1) including increased rate of drug efflux[18] or by alteration in glutathione metabolism.[5,6] There is currently increasing interest in the molecular mechanisms underlying these variations; not only to explore novel means to control tumors but also to allow rapid predictive assays of tumor response.

In seeking to determine the molecular basis of cellular chemosensitivity we have compared various elements of DNA damage with measurements of cell survival (CA and CCA), GSH content and P-170 expression. This is an empirical approach focused to improve the knowledge about the tumor cell response to DOX.

Our first aim in this work was to determine whether the CA and the CCA could be used to estimate cellular sensitivity to DOX treatment. Although there are clear differences among the results given by each assay, there was a significant correlation ($p = 0.0034$) between these chemosensitivity indices. Therefore within this group of human cell lines treated with DOX the SRB assay provides a colorimetric end-point that is useful in quantitating clonogenicity as was described by Skehan.[10] Interpreted correctly, growth assays can provide reproducible measurement of survival which compares properly with clonogenic cell survival measurements. The colorimetric assay can be used to obtain survival curves faster than clonogenic assays and has advantages when measuring drug response in those cells which do not form colonies. Its most serious disadvantage is that if the assay is misinterpreted or survival measured when the cells are not in exponential growth then survival can be overestimated.[19] In a comparison of clonogenic assays and different monolayer assays, Wilson *et al.*[20] showed for two cell lines that although the assays

did not produce identical dose–response data they each showed a dose-dependent response and did correlate with colony formation. This indicates that the chemosensitivity of the total proliferating cell population shares the same profile as the clonogenic population.[21]

In an effort to understand the differences in chemosensitivity we have focused on the identification of determinants involved in resistance to DOX in human cancer cells. These studies included the rate of formation of DNA dsb and dose–response curves. Our study shows that there is no quantitative relationship for DOX between the cytotoxic effect and the formation of DNA dsb, perhaps because of differences in dsb repair among the cell lines or because of the influence of other types of drug-induced DNA lesions (e.g. topoisomerase II alterations) on cell killing.

There are few data on this subject but the relationship between DNA ssb or DNA dsb kinetics and dose–response curves after cell treatment with DOX showed similar patterns using alkaline elution,[3,22] and it has been suggested that the biphasic behavior when DNA dsb were investigated as a function of drug concentration is probably due to the occurrence of a change in the DNA structure when a high level of intercalation of the drug is present in DNA.[3] With the PFGE assay we cannot distinguish whether the change in DNA extracted is the result of a reduced efficiency of strand break production at high doses or whether the change in DNA conformation alters the mobility in the gel.

The dsb induction kinetic pattern after DOX treatment could be explained by the occurrence of two different processes: dsb induction and rejoining. In the first phase, DNA damage induced seems to have a predominance whilst DNA rejoining is the dominant process in the final step of the kinetic curves (Figure 5a).

Our results showed a close relationship between the slope of DNA dsb dose–response curves and the percentage of cells that express P-170. Studies of cellular drug pharmacokinetics revealed that cells with P-glycoprotein expression showed reduced drug uptake compared with the drug-sensitive parental cells and this was mainly due to increased efflux. We have found that in the 4 h after treatment, which seemed to be dominated by damage induction rather than repair, the amount of DOX was higher in those cells in which the percentage of P-170 expression was less. Ultimately this is not related to cell survival so it is likely that repair levels may also be different in these cells.

Our study has also depicted a positive relationship between cellular chemosensitivity parameters (ID_{50} and IC_{50}) and glutathione content in our panel of cell lines. As large fluctuations in the day-to-day glutathione content have been reported,[23,24] we have used GSH content values in 24 h old cultures as our standard for comparison with the DOX sensitivity of our cell lines since that is when we performed the chemosensitivity experiments. The results obtained imply that chemoresponsiveness to DOX treatment can be determined by estimating cellular thiols levels, as has been reported by other authors,[4] although it is also important to consider GSH detoxification systems.

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