Cytotechnology **39:** 15–25, 2002.

Intracellular localisation studies of doxorubicin and Victoria Blue BO in EMT6-S and EMT6-R cells using confocal microscopy

S.M. Burrow[1], Prof. D.A. Phoenix[1,*], M. Wainwright[1] and M.J. Tobin[2]
[1]Department of Forensic Science, University of Central Lancashire, Preston PR1 2HE, UK; [2]CLRC Daresbury Laboratory, Warrington WA4 4AD, UK; [*]Author for correspondence (e-mail: daphoenix@uclan.ac.uk)

Received 3 July 2001; accepted in revised form 5 March 2002

Abstract

The subcellular localisation of doxorubicin and Victoria Blue BO (VBBO) in a murine mammary tumour cell line EMT6-S, and the resistant sub-line EMT6-R was studied, using confocal microscopy, in order to investigate their sites of action. In cells treated with doxorubicin (10 μM) for 90 min, the pattern of intracellular drug distribution differed between the two cell lines. Doxorubicin was found to localise mainly in the nucleus of the sensitive cell line, whereas weak fluorescence was observed in the cytoplasm of the resistant cells, in a punctuate pattern, with no nuclear involvement. The drug also appeared to be effluxed more rapidly by the resistant cell line. The accumulation of doxorubicin at various time intervals over 1 h in EMT6-S cells showed that the drug clearly interacted with both the plasma membrane and the nucleus. In contrast to doxorubicin, the intracellular distribution of VBBO in both EMT6-S and EMT6-R was similar, VBBO was clearly localised throughout the cytoplasm, in a punctuate pattern, which may be consistent with the widespread distribution of mitochondria. A more apical pattern of accumulation was noted in the EMT6-R cell line. No interaction with the plasma membrane was evident. These results indicate that the main modes of action for the two drugs differ markedly, suggesting involvement of both the membrane and the nucleus in the case of doxorubicin, but mitochondrial involvement for VBBO.

Key words: Confocal, Doxorubicin, EMT6-R, EMT6-S, MDR, Victoria Blue BO

Introduction

Multiple drug resistance (MDR) is a major problem in cancer chemotherapy. In many cases the initial response to treatment is encouraging, with tumour shrinkage due to the elimination of drug-sensitive cells, however, when relapse occurs, it is often associated with the development of drug resistance. There remains a constant challenge to identify novel anticancer agents to try to overcome the problem of MDR. Acquired resistance occurs after exposure to chemotherapeutic agents, and is often associated with a decreased intracellular concentration of the drug. Tumour cells displaying the 'classic' MDR phenotype are resistant to anthracyclines, *Vinca* alkaloids, epidophyllotoxins, taxol and actinomycin D (Gottesman and Pastan 1993), and have been found to over express P-glycoprotein (Pgp), a 170–180 kDa glycoprotein which acts as an energy-dependent efflux pump, in their cell membrane. Pgp has also been found in the Golgi apparatus (Molinari et al. 1994) and the nucleus (Baldini et al. 1995) of MDR cells. Other studies suggest that Pgp may act as an intracellular pH regulator, possibly sequestering drugs into specific subcellular compartments which may mediate efflux or affect their pH-dependent binding to cellular targets (Thiebault et al. 1990; Roepe 1992).

MDR appears to be multifactorial, with many different mechanisms contributing to the MDR phenotype. One important phenomenon associated with MDR is an altered subcellular drug distribution (Schuurhuis et al. 1991). Various fluorescence studies have demonstrated that the development of MDR is associated with a relative shift of doxorubicin or

daunorubicin fluorescence from the nucleus to the cytoplasm (Schuurhuis et al. 1989; Broxterman et al. 1990; Gervasoni et al. 1991; Schuurhuis et al. 1991). Schuurhuis et al. (1991) suggest that this phenomenon may make an important contribution to the resistance displayed against anthracyclines by MDR cells.

The intracellular distribution of the anthracycline, doxorubicin, is of interest due to its widespread use in the treatment of cancer and to extensive studies into its mode of action (Carter 1975; Tewey et al. 1984; Tritton 1991; Gewirtz 1999). The mechanisms by which doxorubicin exerts its cytotoxicity have proved to be very complex and several theories have been proposed. It is known to act mainly by intercalation with DNA and interaction with nucleic acids and nuclear components, such as DNA topoisomerase II (Gabbay et al. 1976; Tritton 1991; Gewirtz 1999). In addition, doxorubicin has been shown to be cytotoxic without entering the cell and this cytotoxicity does not necessarily correlate with DNA damage or inhibition of DNA synthesis (Tritton and Yee 1982). Other studies have shown that the mechanism of action may be directly related to drug-membrane interactions and particularly to drug lipid-interactions (De Wolf et al. 1991, 1993).

Phospholipids are extremely important in transmembrane signalling. Much attention has been focussed on the role of phosphatidylinositols (in particular, phosphatidylinositol *bis*-phosphate (PIP_2)) in this process (Nishizuka 1984); however, there is growing evidence that phosphatidylcholine, sphingomyelin and their metabolites are also important mediators of signal transduction (Zeisel 1993). Tritton (1991) postulates that doxorubicin exerts its cytotoxicity by interacting with, and damaging the functions of, both the plasma membrane and nuclear DNA, and that for cytotoxicity to occur, the activation of the protein kinase C (PKC) pathway, following membrane perturbation, is crucial for signal transduction between the cell surface and the nucleus.

Doxorubicin has been shown to interact with other subcellular targets, such as the cytoskeleton (Molinari et al. 1990) and studies using cultured tumour cells displaying multiple drug resistant (MDR) characteristics have also demonstrated alterations in intracellular drug accumulation and distribution (Gervasoni et al. 1991; Schuurhuis et al. 1991; Coley et al. 1993), further adding to the complexity of the drug's action.

A novel area of study involves the use of photodynamic therapy employing agents based on commercial cationic dyes with photodynamic potential, such as the triarylmethane dye Victoria Blue BO (VBBO). Studies comparing the cytotoxicity of VBBO with that of the more conventional anti-cancer drug, doxorubicin, in a murine mammary tumour cell line EMT6-S, and the resistant sub-line EMT6-R, have shown the MDR cell line to be considerably more susceptible to VBBO than to doxorubicin (Wainwright et al. 1999). In addition, pre-treatment of EMT6 cells with VBBO, prior to exposure to doxorubicin, increased doxorubicin toxicity in both cell lines two-fold, suggesting that VBBO is independent of the Pgp efflux pump. This was confirmed by the addition of verapamil, which increased the cytotoxicity of doxorubicin against EMT6-R cells 18-fold, and against EMT6-S cells 2-fold, but had no effect on the cytotoxic effect of VBBO against these cell lines (Burrow et al. 2000).

The aim of this study was to use confocal microscopy to examine the intracellular localisation of doxorubicin and VBBO in EMT6 cells, and to compare their subcellular distribution.

Materials and methods

Intracellular localisation of doxorubicin in treated EMT6-S and EMT6-R cells, following recovery in drug-free medium

2 ml aliquots of EMT6-S and EMT6-R cells were seeded at a cell density of 1×10^4 cells ml^{-1} into 35 mm petri dishes (Falcon, Fahrenheit Laboratories, Rotherham, UK) in RPMI 1640 medium, supplemental with 10% (v/v) foetal calf serum, 200 mM L-glutamine (Sigma, Poole, UK), and penicillin/streptomycin solution at 1×10^4 units ml^{-1} and 10 mg ml^{-1}, respectively, in 0.9% (w/v) Sigma). A sterile quartz coverslip (suprasil, 0.5 mm diameter $\times$ 0.2 mm thick, Heraeus Silica & Metals Ltd., Byfleet, UK) was placed into each petri dish and the cells were allowed to attach for 3 days (EMT6-S cells) or 4 days (EMT6-R cells) whilst incubating at 37 °C, 5% CO_2: 95% air. A coverslip with attached EMT6-S cells was placed in a flow cell which was adapted for use with the confocal microscope by fixing to a microscope slide. RPMI 1640 medium was added to the cells by the use of an attached syringe and the cells were examined for autofluorescence.

The incubating medium was aspirated from the petri dishes, replaced with medium containing doxorubicin (10 μM), and the cells were incubated for 90 min, under conditions described above. The cells were

rinsed with RPMI 1640 medium, incubated for a further 40 min and examined with a scanning laser confocal fluorescence microscope (objective lens Zeiss 40× Ultafluar, numerical aperture (N.A.) 0.7) fitted with an argon ion laser at 488 nm + assay maximum projection.

Time course studies

EMT6-S and EMT6-R cells were grown as described above. The incubating medium was aspirated from the petri dishes, replaced with medium containing VBBO at a concentration of 5 μM, and images were taken at various intervals over the period of 1 h using a scanning laser confocal fluorescence microscope (objective lens Zeiss 40× Ultafluar, numerical aperture (N.A.) 0.7) fitted with a helium neon laser at 633 nm. This procedure was repeated for EMT6-S cells using doxorubicin at a concentration of 10 μM, and using an argon ion laser at 488 nm for the confocal microscopy.

Results

Intracellular localisation of doxorubicin in treated EMT6-S and EMT6-R cells, following recovery in drug-free medium

Nuclear localisation of doxorubicin in the EMT6-S cell line was clearly visible 40 min after rinsing the cells with RPMI 1640 (Figures 1a – 1f) and some cytoplasmic distribution was also seen (Figures 1b – 1e). No evidence of localisation in the plasma membrane was noted. In EMT6-R cells weak fluorescence was seen in the cytoplasm, in a punctuate pattern (Figures 2a – 2f) but no nuclear accumulation was noted. The intensity was increased × 10 to image the EMT6-R cells compared to that used with EMT6-S cells.

Time course studies

Localisation of VBBO in EMT6-S cells was markedly different to that of doxorubicin. The drug was taken up rapidly by the cells and could clearly be seen after 4 min (Figure 3a). The concentration of drug in the cell increased marginally over the next 11 min (Figures 3b – 3c) and remained constant until 60 min when a slight decrease was noted (Figures 3d – 3g). VBBO was clearly localised throughout the cytoplasm in a punctuate pattern, which may be consistent with the widespread distribution of mitochondria. Some diffuse fluorescence was evident in the nuclear region after 8 min(Figure 3b); however, this did not appear to increase over time.(Figures 3b – 3g). Interestingly, there was no evidence of interaction with the plasma membrane.

Uptake of VBBO by the resistant cells appeared to be slower than in the sensitive cells (Figures 4a – 4g)) with an increase in accumulation of the drug seen over 40 min(Figures 4a – 4f). This concentration did not appear to change between 40 and 60 min(Figures 4f & 4g). VBBO was again seen to be localised throughout the cytoplasm, however, there did appear to be an apical accumulation of the drug in these cells, which may indicate localisation within the Golgi apparatus or mitochondria. Weak, diffuse fluorescence appeared in the nuclear region after 8 min (Figure 4b) increasing somewhat over 25 min(Figures 4b – 4e). However, the main area of localisation was the cytoplasm. In common with EMT6-S cells no interaction with the plasma membrane was seen.

Doxorubicin was also taken up rapidly by EMT6-S cells and could be seen in the plasma membrane and nucleus after only 2 min(Figure 5a). Localisation in the plasma membrane and nucleus increased with time up to 10 min(Figures 5a – 5d), however, very little drug was seen in the cytoplasm. There also appeared to be apical concentration of doxorubicin in the plasma membrane of the cell (Figures 5a – 5g). Figures 5c & 5d show considerable accumulation in the plasma membrane, but subsequent images show this concentration to diminish (Figures 5e – 5h). The nuclear accumulation appeared to be slightly reduced after 15 min (Figure 5e), and continued to diminish over 40 min (Figure 5h).

Discussion

A clear difference was seen in intracellular doxorubicin accumulation between EMT6-S and EMT6-R cells. Doxorubicin was shown to localise predominantly in the nucleus of the parental cell line, whereas weak fluorescence was observed in the cytoplasm of the EMT6-R cells, in a punctuate pattern, but no nuclear accumulation was observed. Meschini et al. (1994) also found a lack of nuclear accumulation in the resistant strain of the human breast cancer cell line, MCF-7 DX; however, in contrast to our studies these authors showed extensive cytoplasmic localisation. Other studies have also found distinct differ-

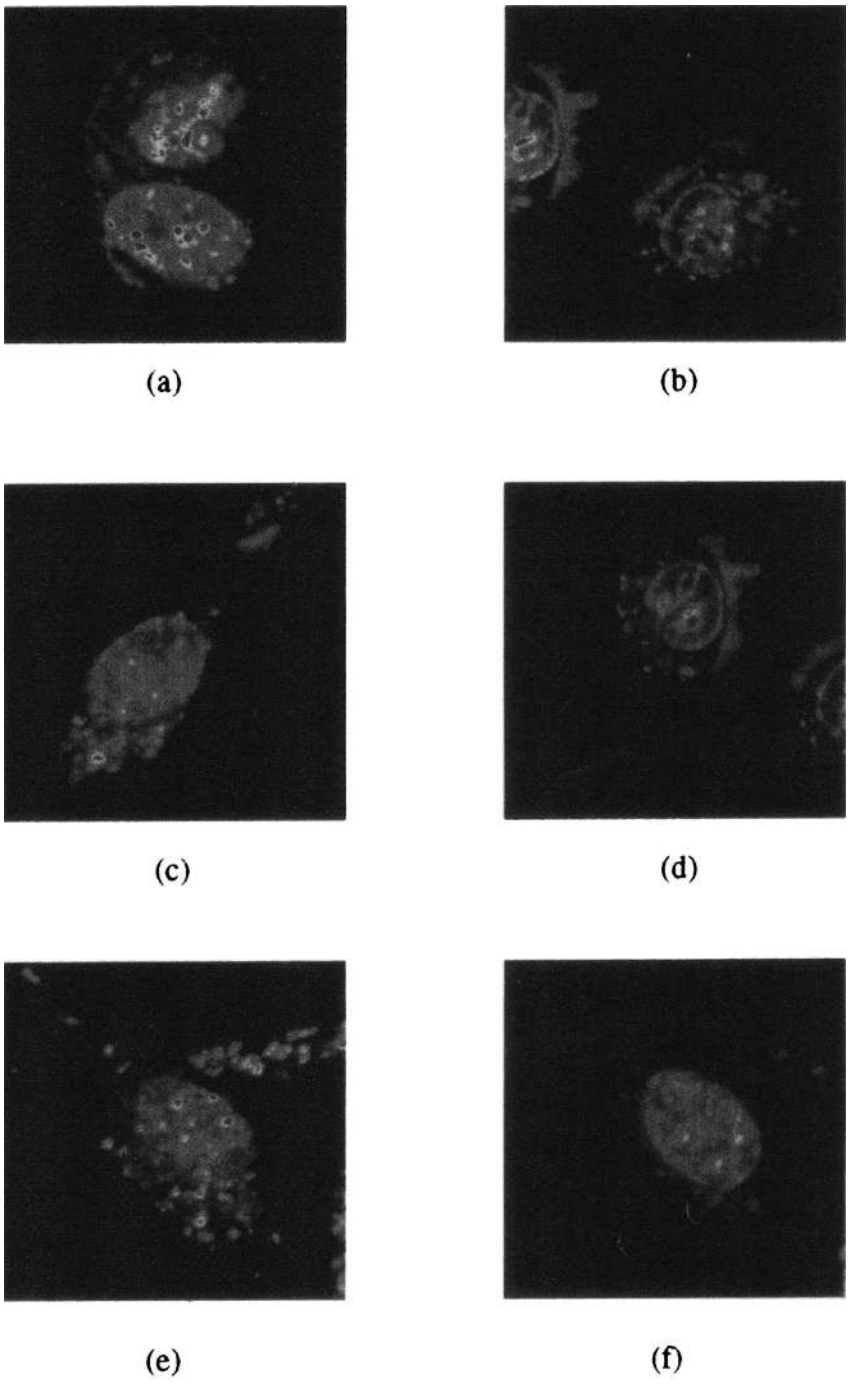

Figure 1. Intracellular distribution of doxorubicin in EMT6-S cells following exposure to doxorubicin for 90 min, prior to rinsing with RPMI 1640 medium. Cells were imaged by confocal fluorescence microscopy 40 min after rinsing. Figures (a–f) show different cells following a single scan with the laser beam. * Scale of fluorescence intensity (gray – 255, maximum; black – 0, minimum)

ences between localisation of anthracyclines in a variety of sensitive and resistant cell lines (Coley et al. 1993; Duffy et al. 1996; Davies et al. 1996). Meschini et al. (1994) examined the effect of the resistance modifier, verapamil, on the subcellular distribution of doxorubicin in MCF-7 DX (resistant)

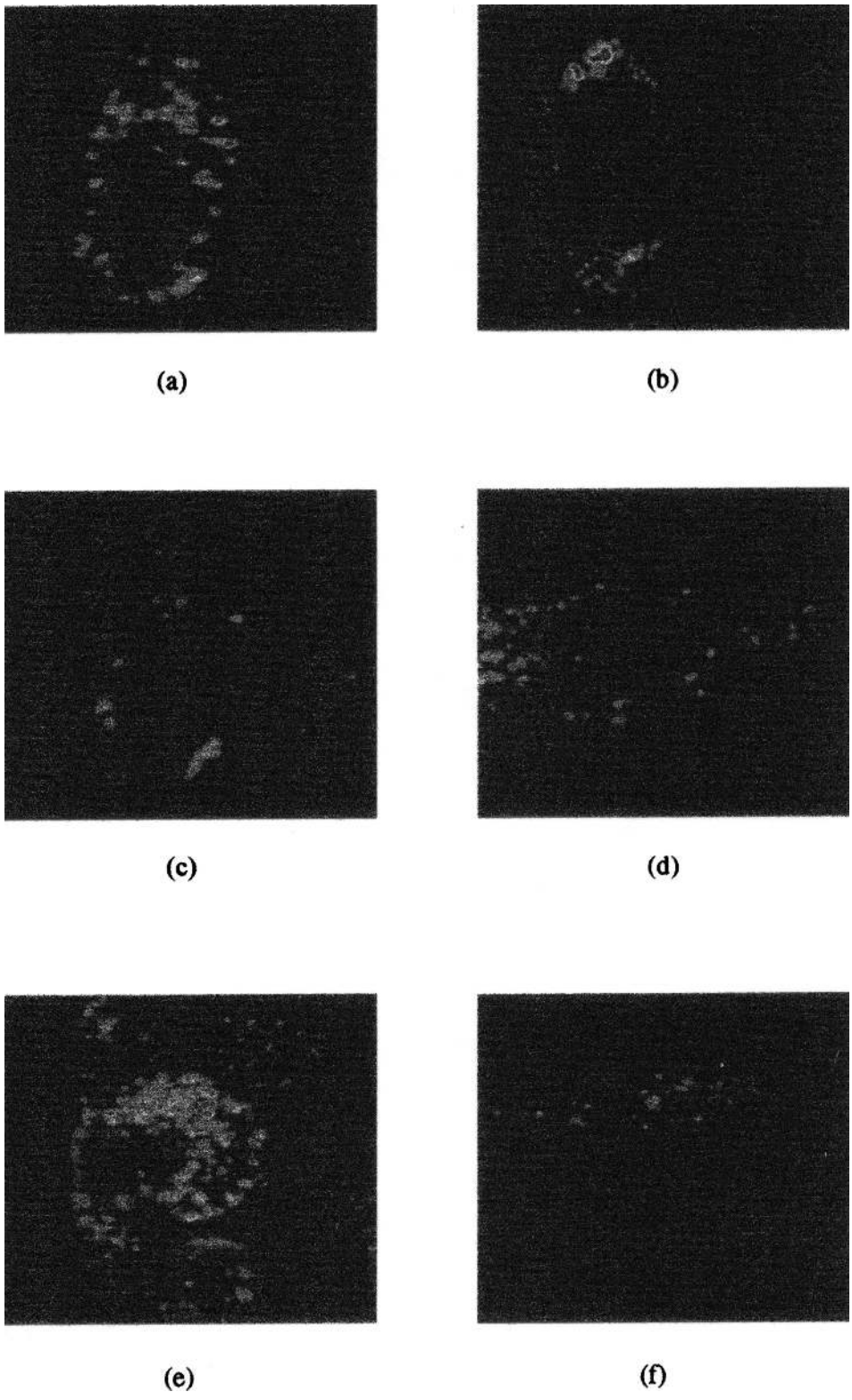

Figure 2. Intracellular distribution of doxorubicin in EMT6-R cells following exposure to doxorubicin for 90 min, prior to rinsing with RPMI 1640 medium. Cells were imaged by confocal fluorescence microscopy 40 min after rinsing. Figures (a–f) show different cells following a single scan with the laser beam. * Scale of fluorescence intensity (gray – 255, maximum; black – 0, minimum)

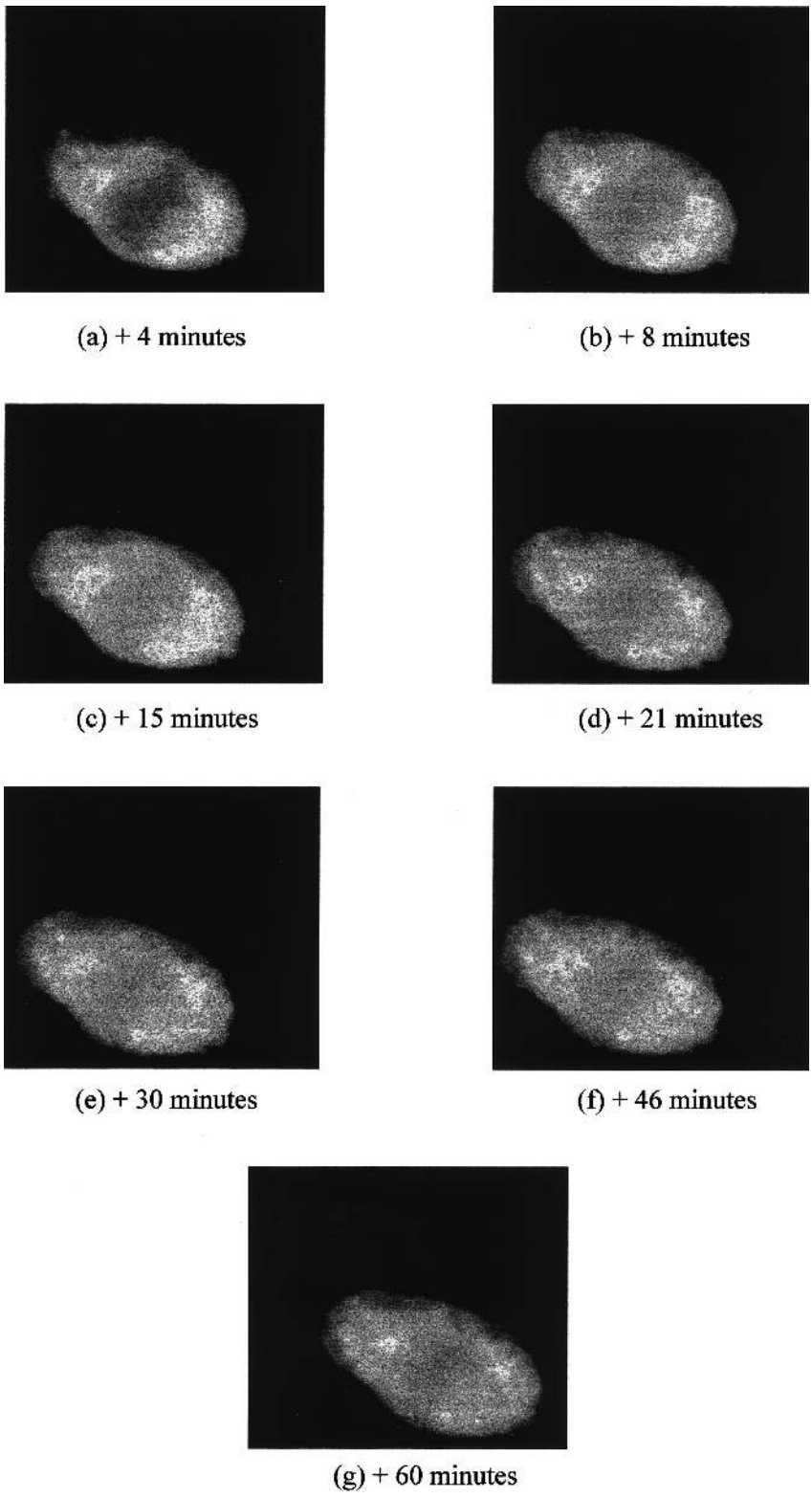

Figure 3. Intracellular distribution of doxorubicin (10 μM) in EMT6-S cells imaged by confocal fluorescence microscopy. A single cell was imaged at various time intervals (indicated below each figure) following the addition of doxorubicin. * Scale of fluorescence intensity (gray – 255, maximum; black – 0, minimum)

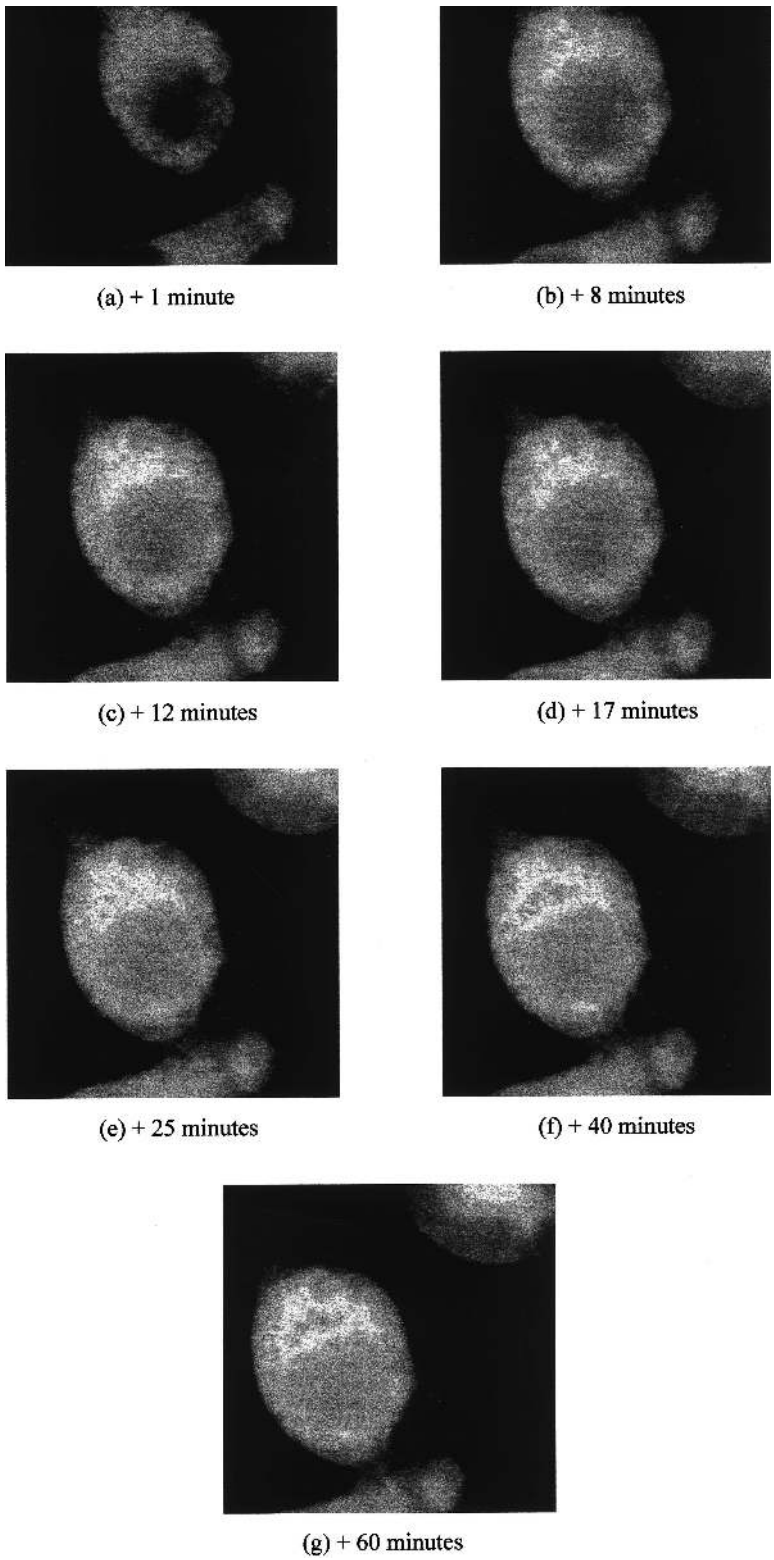

Figure 4. Intracellular distribution of Victoria Blue BO (VBBO) (5 μM) in EMT6-S cells, imaged by confocal fluorescence microscopy. A single cell was imaged at various time intervals (indicated below each figure) following the addition of VBBO. * Scale of fluorescence intensity (gray – 255, maximum; black – 0, minimum)

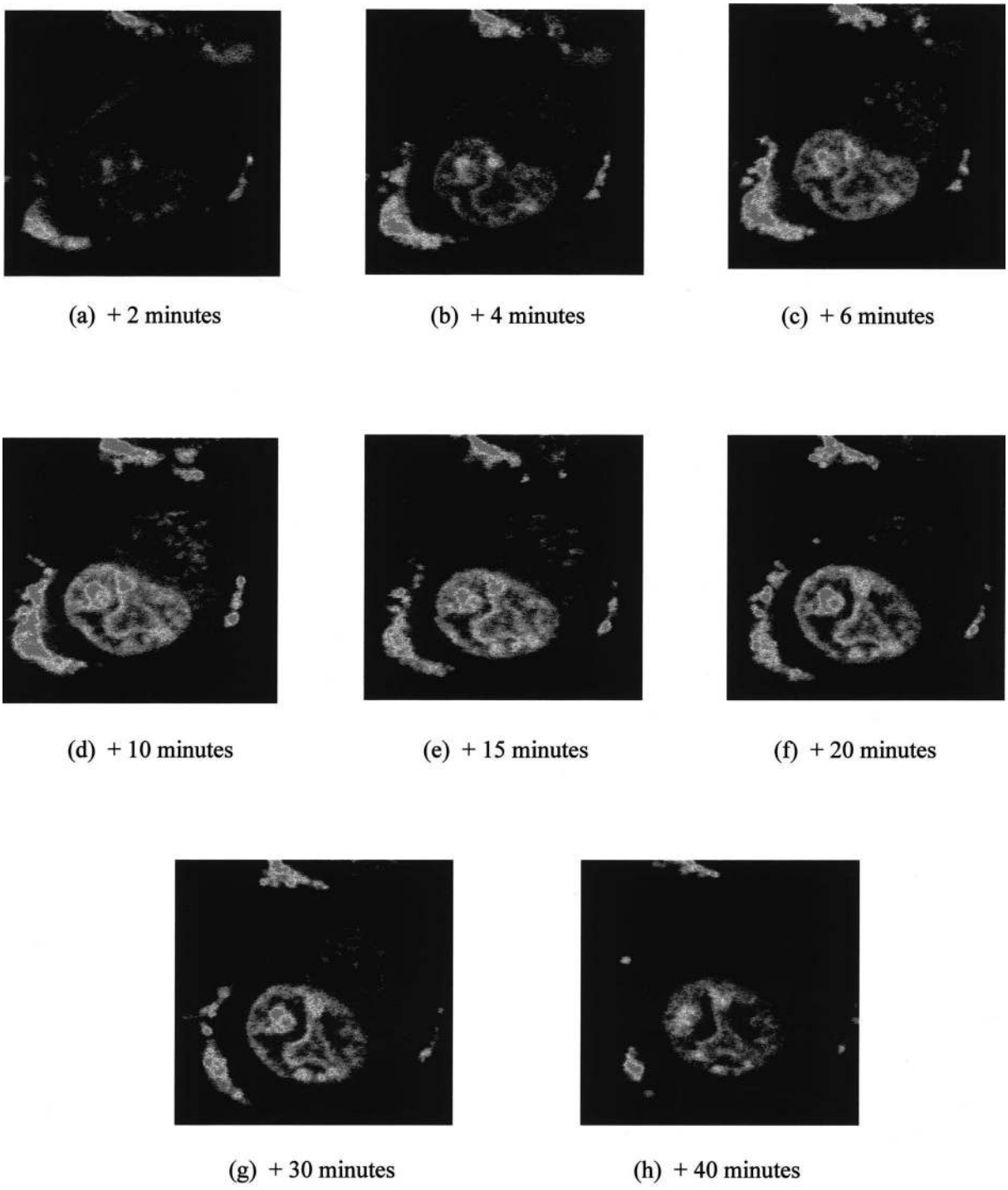

Figure 5. Intracellular distribution of Victoria Blue BO (VBBO) (5 μM) in EMT6-R cells, imaged by confocal fluorescence microscopy. A single cell was imaged at various time intervals (indicated below each figure) following the addition of VBBO. * Scale of fluorescence intensity (gray – 255, maximum; black – 0, minimum)

cells. The intracellular concentration of the drug was increased and it appeared to Iocalise in a specific area close to the nucleus. This supports a recent study by Rutherford and Willingham (1993) who identified the accumulation of anthracycline molecules in the *trans*-Golgi system of resistant cells which were also treated with verapamil.

Several workers have shown that MDR can be associated with altered intracellular drug accumulation and Iocalisation (Fojo et al. 1985; Willingham et al. 1986; Coley et al. 1993). Schuurhuis et al. (1991) have suggested that, in addition to drug efflux, Pgp may be involved in the relocalisation of drugs by pumping them into other cellular organelles away from their cytotoxic targets. Other studies suggest that a pH shift in some cytoplasmic organelles may contribute to the redistribution of anticancer drugs in MDR cells (Thiebault et al. 1990; Schindler et al. 1996). Anthracyclines accumulate in their protonated form on the side of the membrane at which the pH is lower, due to their basic character, suggesting that cationic molecules become trapped in acidic cytoplasmic vesicles (Millot et al. 1997).

Doxorubicin was rapidly taken up by EMT6-S cells, with the drug clearly localising in the plasma membrane and nucleus after only 2 mm. The drug continued to accumulate in the plasma membrane and nucleus for up to 10 min; however, very little fluorescence was noted in the cytoplasm. This supports the mechanism of action suggested by Tritton (1991) who proposed that perturbation of the membrane induces subsequent signal transduction via diacylglycerol and protein kinase C. In these studies Tritton was able to emphasise a cytotoxic, nuclear response which did not involve drug – nucleus interaction by binding to polymers to prevent uptake. There also appeared to be apical concentration of doxorubicin in the plasma membrane of the sensitive cells. Considerable accumulation of doxorubicin in the plasma membrane was evident up to 10 min, although after this time the localisation changed. The nuclear accumulation did not appear to increase, therefore it must be assumed that the doxorubicin diffused out of the cell into the surrounding medium. Less nuclear accumulation was seen after 15 min and very little fluorescence was noted in the plasma membrane or cytoplasm.

Meschini et al. (1994) studied the intracellular localisation of doxorubicin in Ml4 human melanoma cells and in MCF-7 human breast cancer cells (both sensitive and resistant cell lines). Following treatment over 1 h with 1 μgml^{-1} doxorubicin, the drug localised in the nuclei of the parental cell lines, with only weak cytoplasmic fluorescence seen in some cells. This is in good agreement with the findings of the present study. Meschini et al. (1994) also examined cells which had previously been exposed to doxorubicin but were then allowed to recover for 71 h in a drug-free medium. Complete efflux of doxorubicin from the nucleus was shown, with occasional fluorescent vesicular structures localised to perinuclear regions in the cytoplasm. Other workers (Peterson and Truet 1978; Hindenburg et al. 1987; Vale 1987) have suggested that doxorubicin diffuses across the membrane and binds to anionic vesicles which are transported back to the cell surface via microtubules. Meschini et al. (1994) support this hypothesis, and suggest that the accumulation of doxorubicin in the perinuclear region may indicate binding to pre-lysosomes and the Golgi apparatus since these organelles are associated with the transport of secretory vesicles to the cell surface. Doxorubicin is also known to localise in mitochondria and to exert some cytotoxicity via damage to the electron transfer chain in mitochondria (Goormatigh and Russchaert 1984; Ellis et al. 1987).

The intracellular distribution of VBBO was found to be markedly different to that of doxorubicin. VBBO was taken up extremely rapidly, with intense fluorescence evident after only 4 min, increasing only marginally up to 15 min. The concentration of the drug appeared to be constant up to 60 min when a slight decrease was noted. VBBO was clearly distributed widely throughout the cytoplasm, in a punctuate pattern, which may be consistent with the distribution of mitochondria. Very little evidence of nuclear localisation was seen. In contrast to doxorubicin, no interaction between VBBO and the plasma membrane was demonstrated. The resistant cell line initially appeared to show slower uptake of VBBO than the parental line, with a more gradual increase in cellular accumulation. Equilibration was seen at 40 min, compared to 15 min in EMT6-S cells. Although similar distribution of the drug was seen throughout the cytoplasm in each cell line, there appeared to be an apical concentration of doxorubicin in the resistant line. Very little nuclear association was observed and there was no apparent interaction with the plasma membrane. The punctuate pattern of distribution within the cytoplasm suggests that the intracellular localisation of VBBO may be consistent with mitochondrial distribution in EMT6 cells, in agreement with other workers who have previously shown localisation of

the dye in the mitochondria of tumour cells (Modica-Napolitano et al. 1990; Fiedorowicz et al. 1993). Interestingly, little difference was found between the intracellular distribution of VBBO in EMT6-S and EMT6-R cells, in contrast to that of doxorubicin. This supports previous studies comparing the cytotoxic effect of VBBO with doxorubicin which showed that EMT6-R cells required almost 100-fold more doxorubicin to overcome the resistance compared to EMT6-S cells, whereas only a 10-fold increase in concentration was required for VBBO (Wainwright et al. 1999). These studies also showed that VBBO does not appear to be effluxed by, or interact with, Pgp, which supports the lack of membrane localisation shown by VBBO in EMT6 cells. Even so whilst VBBO is thought to initiate its effect via free radical generation the level of cellular Glutatione has been found to be independent of cytotoxicity and this agent has been postulated to require a specific sub-cellular localisation for effect (Burrow et al. 2000)

These results indicate that the main modes of action for the two drugs, VBBO and doxorubicin, differ markedly, demonstrating interaction with both the membrane and the nucleus in the case of doxorubicin, but mitochondrial involvement for VBBO.

References

Baldini N., Scotlandi K., Serra M., Shikita T., Zini N., Ognibene A. et al. 1995. Nuclear immunolocalisation of P-glycoprotein in multidrug resistant cell lines showing similar mechanisms of doxorubicin distribution. *Eur. J. Cell. Biol.* 68: 226–239.

Broxterman H.J., Schuurhuis G.J., Lankelma J., Baak J.P.A. and Pineda H.M. 1990. Towards functional screening for multidrug resistant cells in human malignancies. In: Mihich E. (ed.), *Drug Resistance: Mechanism and Reversal*. John Libbey, CIC, Roma, pp. 309–319.

Burrow S.M., Phoenix D.A., Wainwright M. and Waring J.J. 2000. Reduced cellular glutathione or verapamil do not affect the cytotoxicity of the PDT drug VBBO on drug sensitive and drug resistant EMT6 cells. *Cell Mem. Biol.* 14: 357–367.

Carter S.K. 1975. Adriamycin – a review. *J. Natl. Cancer Inst.* 55: 1265–1274.

Coley H.M., Amos W.B., Twentyman P.R. and Workman P. 1993. Examination by laser scanning confocal fluorescent imaging microscopy of the subcellular localisation of anthracyclines in parent and multidrug resistant cell lines. *Br. J. Cancer* 67: 1316–1323.

Duffy P.M., Hayes M.C., Gatrell S.K.E., Cooper A. and Smart C.J. 1996. Determination and reversal of resistance to epirubicin intravesical chemotherapy. A confocal imaging study. *Br. J. Urol.* 77: 824–829.

Davies C.L., Duffy P.M., MacRobert A.J., Loizidou M.C., Cooper A.J. and Taylor I. 1996. Localization of anthracycline accumulation in sensitive and resistant urothelial tumor cell lines. *Cancer Detect. Prevent.* 20: 625–633.

De Wolf F.A., Maliepaard M., van Dorsten F., Berguis I., Nicolay K. and de Kruiff B. 1991. Comparable interaction of doxorubicin with various acidic phospholipids results in changes of lipid order and dynamics. *Biochim. Biophys. Acta* 1096: 67–80.

De Wolf F.A., Staffhorst R.W.H.M., Smits H.P., Onwezen M.F. and de Kruijff B. 1993. Role of anionic phospholipids in the interaction of doxorubicin and plasma membrane vesicles: drug binding and structural consequences in bacterial systems. *Biochemistry* 32: 6688–6695.

Ellis C.N., Ellis M.B. and Blakemore W. 1987. Effect of adriamycin on heart mitochondrial DNA. *Biochem. J.* 245: 309–312.

Fiedorowicz F., Galindo J.R., Julliard M., Mannoni P. and Chanon M. 1993. Efficient photodynamic action of Victoria Blue BO against the human leukemic cell lines K-562 and TF-1. *Photochem. Photobiol.* 58: 356–361.

Fojo A., Akiyama S., Gottesman M.M. and Pastan I. 1985. Reduced drug accumulation in multiply drug-resistant human K.B. carcinoma cell lines. *Cancer Res.* 45: 3002–3007.

Gabbay E.J., Grier D., Fingerle R.E., Reimer R., Levy R., Pearce S.W. et al. 1976. Interaction specificity of the anthracyclines with deoxyribonucleic acid. *Biochemistry* 15: 2062–2070.

Gewirtz D.A. 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem. Phamzacol.* 57: 727–741.

Gervasoni J.E., Fields S.Z., Krishna S., Baker M.A., Rosado M., Thuraisamy K. et al. 1991. Subcellular distribution of daunorubicin in P-glycoprotein-positive and -negative drug-resistant cell lines using laser-assisted confocal microscopy. *Cancer Res.* 51: 4955–4963.

Goormatigh E. and Russchaert J.M. 1984. Anthracycline glycoside membrane interactions. *Biochim. Biophys. Acta* 779: 271–288.

Gottesman M.M. and Pastan I. 1993. Biochemistry of Multidrug Resistance Mediated by the Multidrug Transporter. *Ann. Rev. Biochem.* 62: 385–427.

Hindenburg A.A., Baker M.A., Gleyzer E., Stewart V.J., Case N. and Taub R.N. 1987. Effect of verapamil and other agents on the distribution of anthracyclines and on the reversal of multidrug resistance. *Cancer Res.* 47: 1421–1425.

Meschini S., Molinari A., Calcabrini A., Citro G. and Arancia G. 1994. Intracellular localisation of the antitumour drug adriamycin in living cultured cells: a confocal microscopy study. *J. Microsc.* 176: 204–210.

Millot C., Mikllot J.-M., Morjani M., Desplaces A. and Manfait M. 1997. Characterization of acidic vesicles in multidrug-resistant and sensitive cancer cells by acridine orange staining and confocal microspectrofluorometry. *J. Histochem. Cytochem.* 45: 1255–1264.

Modica-Napolitano J.S., Joyal J.L., Ara G., Oseroff A.R. and Aprille J.R. 1990. Mitochondrial toxicity of cationic photosensitizers for photochemotherapy. *Cancer Res.* 50: 7876–7881.

Molinari A., Calcabrini A., Crateri P. and Arancia G. 1990. Interaction of anthracycline antibiotics with cytoskeletal components of cultured carcinoma cells (CG5). *Exp. Mol. Pathol.* 53: 11–33.

Molinari A., Cianfriglia M., Meschini S., Calcabrini A. and Arancia G. 1994. P-glycoprotein expression in the golgi apparatus of multidrug resistant cells. *Int. J. Cancer* 59: 789–795.

Nishizuka Y. 1984. Turnover of inositol phospholipids and signal transduction. *Science.* 225: 1365–1369.

Peterson C. and Truet A. 1978. Transport and storage of daunorubicin and doxorubicin in cultured fibroblasts. *Cancer Res*. 38: 4645–4649.

Roepe P.D. 1992. Analysis of the steady-state and initial rate of doxorubicin efflux from a series of multidrug-resistant cells expressing different levels of P-glycoprotoen. *Biochemistry* 22: 12555–12564.

Rutherford A.V. and Willingham M.C. 1993. Ultrastructural localisation of daunomycin in multidrug resistant cells with modulation of the multidrug transporter. *J. Histochem. Cytochem*. 41: 1573–1577.

Schindler M., Grabski S., Hoff E. and Simon S.M. 1996. Defective pH regulation of acidic compartments in human breast cancer cells (MCF-7) is normalized in adriamycin-resistant cells (MCF-7adr). *Biochemistry* 35: 2811–2817.

Schuurhuis G.J., Broxterman H.J., Cervantes A., van Heijningen T.H.H.M., de Lange J.H.M., Baak J.P.A. et al. 1989. Quantitative determination of factors contributing to doxorubicin resistance in multidrug-resistant cells. *J. Natl. Cancer Inst*. 81: 1887–1893.

Schuurhuis G.J., Broxterman H.J., de Lange J.H.M., Pinedo H.M., van Heijningen T.H., Ruiper C.M. et al. 1991. Early multidrug resistance defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein. *Br. J. Cancer* 64: 857–861.

Tewey K.M., Rowe T.C., Yand L., Halligan B.D. and Liu L.F. 1984. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* 226: 466.

Thiebault F., Currier S.J., Whitaker J., Haugland R.F., Gottesman M., Pastan I. et al. 1990. Activity of the multidrug transporter results in alkalinization of the cytosol; measurements of cytosolic pH by rnicroinjection of a pH-sensitive dye. *J. Histochem. Cytochem*. 38: 685–690.

Tritton T.R. and Yee G. 1982. The anticancer agent adriamycin is actively cytotoxic without entering cells. *Science* 217: 248–250.

Tritton T.R. 1991. Cell Death in Cancer Chemotherapy: The Case of Adriamycin. In: Tomei D. and Cope F. (eds), *Apoptosis: The Molecular Basis of Cell Death*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Wainwright M., Phoenix D.A., Burrow S.M. and Waring J.J. 1999. Cytotoxicity and adjuvant activity of cationic photosensitizers in a multidrug resistant cell line. *J. Chemother*. 11: 61–68.

Willingham M.C., Cornwell M.M., Cardarelli C.O., Gottesman M.M. and Pastan I. 1986. Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and -sensitive KB cells: Effects of verapamil and other drugs. *Cancer Res*. 46: 5941–5946.

Vale R.D. 1987. Intracellular transport using microtubule-based motors. *Ann. Rev. Cell Biol*. 3: 347–378.

Zeisel S.H. 1993. Choline phospholipids: signal tranduction and carcinogenesis. *FASEB J*. 7: 551–557.