Pergamon

European Journal of Cancer Vol. 30A, No. 5, pp. 687–690, 1994

0959-8049/94 $7.00 + 0.00

0959-8049(94)E0054-8

Paclitaxel-induced Cytotoxicity—the Effects of Cremophor EL (Castor Oil) on Two Human Breast Cancer Cell Lines With Acquired Multidrug Resistant Phenotype and Induced Expression of the Permeability Glycoprotein

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Paclitaxel (Taxol®) is a new cytotoxic agent with considerable activity in phase II studies on metastatic breast cancer. Paclitaxel for clinical use is dissolved in the solvents cremophor EL and ethanol. In this study, we added paclitaxel, formulated either in cremophor EL and ethanol or only in ethanol, in increasing concentrations to two parental human breast cancer cell lines (ZR 75–1 and HS 578T) and their corresponding sublines with acquired doxorubicin resistance and P-glycoprotein expression. Paclitaxel dissolved either in ethanol or ethanol plus cremophor EL, resulted in steep and almost identical dose–response curves for the parental lines ZR 75–1 and HS 578T, respectively, independent of the solvent used. When paclitaxel was formulated only in ethanol the effects on the corresponding doxorubicin-resistant sublines were significantly reduced compared with paclitaxel dissolved in ethanol plus cremophor EL. These effects by cremophor EL may partly explain some of the antitumoral effects observed by paclitaxel in anthracycline failing patients.

Key words: human breast cancer, cell lines, multidrug resistance, paclitaxel (Taxol®), cremophor EL (castor oil)
Eur J Cancer, Vol. 30A, No. 5, pp. 687–690, 1994

INTRODUCTION

PACLITAXEL (TAXOL®) IS a natural cytotoxic compound derived from the bark of Western yew [1]. The drug has attracted much attention during the last 2 years due to high response rates even in doxorubicin-resistant breast cancers, and cisplatinum-resistant ovarian cancers [2, 3]. Responses in the breast cancer group were noteworthy, while the resistance pattern for paclitaxel has been described to belong to the multidrug-resistant phenotype [4–6]. However, the resistance mechanisms for paclitaxel are complex and may also involve alterations of tubulin [7].

Paclitaxel works via promotion of the assembly of microtubules and stabilisation of the tubulin polymers [8]. The high *in vivo* activity of paclitaxel is partly in contrast to the preclinical data which revealed poor, modest, or high activity of paclitaxel depending on the models used [9]. Paclitaxel used in humans is dissolved in polyethoxylated castor oil (cremophor EL) and ethanol. Cremophor EL has been described to revert the vinblastine resistance in K562 cells with a multidrug resistance phenotype [10]. The effect by cremophor EL has lately also been studied on daunorubicin-resistant cells [11].

In accordance with this, we suspected that part of the effects by paclitaxel in breast cancer patients with doxorubicin resistance may partly be related to cremophor EL.

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Revised 6 Dec. 1993; accepted 17 Dec. 1993.

MATERIALS AND METHODS

Cell lines and tissue culture

In order to test this hypothesis, we used two human breast cancer cell lines with acquired doxorubicin (dox) resistance, ZR 75-1-dox and HS 578T-dox, together with their corresponding parental cell lines ZR 75-1 and HS 578T. ZR 75-1 is a 'receptor positive' cell line, i.e. it contains high levels of oestrogen and progesterone receptors, and HS 578T is a 'receptor negative' cell line, i.e. it contains low levels of these receptors [12–14]. The dox sublines showed immunohistochemical expression of the P-glycoprotein using two different antibodies recognising different epitopes [15–17]. The parental lines were negative for the P-glycoprotein [15]. The used sublines, ZR 75-1-dox and HS 578T-dox in this study, were adapted to growth in the presence of 40 and 400 ng/ml of doxorubicin, respectively. The dox sublines maintained their dox resistance and growth rate after at least 4 months removal of dox, followed by re-exposure of dox. ZR 75-1-dox and HS 578T-dox were both cross resistant to etoposide and vincristine, thus fulfilling the criteria of multidrug resistance related to increased expression of the P-glycoprotein [15].

The monolayer growing cell lines, both parental and dox sublines, were cultured in Eagle's minimal essential medium (MEM) (Northumbria Biological Limited, NBL, U.K.) sup-

plemented with 10% fetal calf serum (FCS) (from NBL) and 2 mM glutamine at 37°C in humidified atmosphere containing 95% air and 5% CO_2. No antibiotics were added to the cultures. The cell lines were screened negative with a DNA probe technique for mycoplasma [18].

Paclitaxel

Paclitaxel (6 mg/ml) (30 mg/vial in 50% v/v cremophor EL and dehydrated alcohol) from Bristol-Myers-Squibb (PRI, batch F92 A 004M, New York, U.S.A.) and paclitaxel from Sigma Chemical Co. (St Louis, Missouri, U.S.A.) were used. We dissolved and used paclitaxel in identical concentrations of ethanol and cremophor EL, according to the above, and paclitaxel dissolved only in ethanol.

Paclitaxel was added in the following final concentrations: 0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 2.0, 4.0, 8.0 and 10.0 μg/ml. This corresponds to a maximal ethanol and cremophor EL concentration of 0.23% v/v, respectively (Figures 1 and 2).

As a control, we added only cremophor EL and ethanol to the studied cell lines in the same concentrations as they were investigated together with paclitaxel.

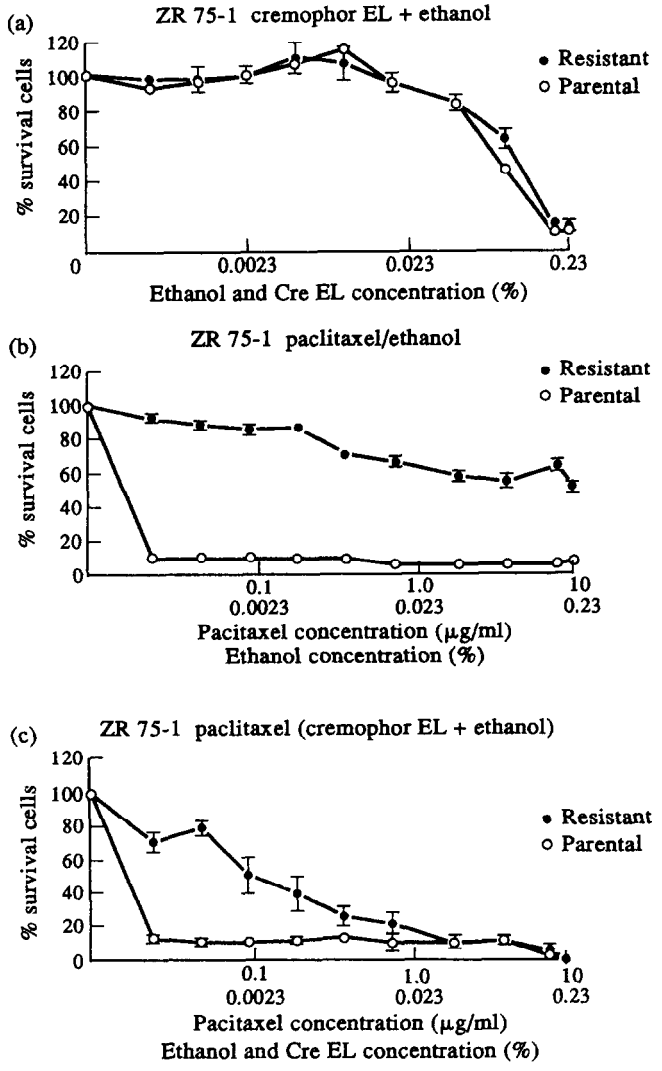

Figure 1. ZR 75-1 and the multidrug-resistant subline ZR75-1-dox. Dose–response curves: (a) ethanol and cremophor EL alone in the same concentrations as when used together with paclitaxel; (b) paclitaxel dissolved in dehydrated ethanol; (c) paclitaxel dissolved in dehydrated ethanol/cremophor EL (1/1).

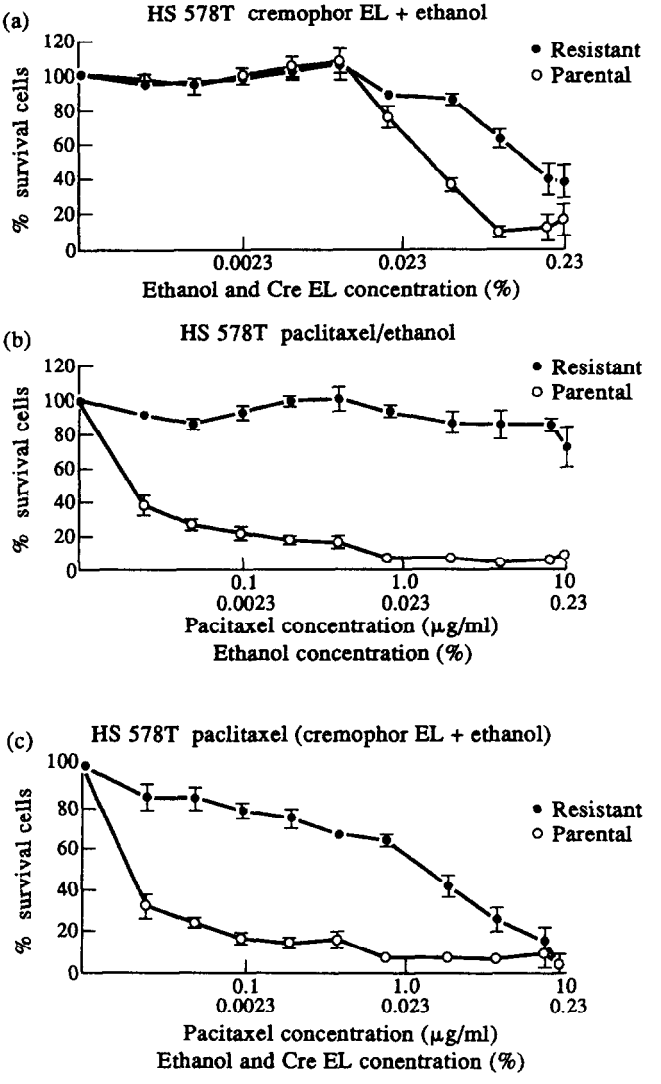

Figure 2. HS 578T and the multidrug-resistant subline HS 578T-dox. Dose–response curves: (a) ethanol and cremophor EL alone in the same concentrations as when used together with paclitaxel; (b) paclitaxel dissolved in dehydrated ethanol; (c) paclitaxel dissolved in dehydrated ethanol/cremophor EL (1/1).

Measurement of cellular cytotoxicity by taxol

A semiautomatic fluorometric method for determination of cytotoxicity and viability was used [19]. Briefly, 10 μl of paclitaxel-containing solution, from the sources outlined above, were added at the final calculated concentrations (increasing paclitaxel concentrations from 0 to 10.0 μg/ml) into the wells of a flat-bottomed 96-well microtitre plate (Nunc, Roskilde, Denmark). The corresponding ethanol and cremophor EL concentrations were 0–0.23% v/v, respectively. The microtitre plates were kept frozen at −20°C until used. Cells were trypsinised into single cell suspension on day 0 from optimal growing stock cultures according to the above. Thirty microlitres of cells (15 000 cells) were seeded into 160 μl of medium (Eagle's with 10% FCS) in each well (first medium, then cells). One empty column (blank wells) received only culture medium. One column contained culture medium and tumour cells. The cells were then incubated for 3 days at 37°C in a humidified atmosphere with 95% air and 5% CO_2. On day 3, the plates were centrifuged, the medium was removed, and cells washed in phosphate-buffered

saline (PBS). Two hundred μl of fluorescein diacetate (FDA) (Sigma Chemical Co.) dissolved in PBS were added to experimental and control wells [19]. The plates were incubated for 30 min [19], and then read in the 96-well scanning fluorometer (Fluoroscan 2) at 538 nm. The fluorometer was blanked against wells containing assay medium and fluorescent dye without cells [19]. The effects of paclitaxel dissolved in ethanol ± cremophor EL and ethanol + cremophor EL alone were related to the viability status in the control wells, medium and cells. The results were expressed as a per cent of viable cells of corresponding control cultures. The results were transferred into a Macintosh SE computer for graphical and statistical processing utilising the Excel and Cricket softwares.

Three independent experiments were conducted, and three wells were analysed per concentration and time. For statistical analysis, we used the unpaired and two-tailed *t*-test (Statview).

RESULTS

Parental cell lines

Cremophor EL and alcohol, without the addition of paclitaxel, resulted in flat dose–response curves for the lines ZR 75-1 and HS 578T (Figures 1 and 2a). The 50% inhibitory concentration (IC_{50}) and IC_{90} values, respectively, were 0.09 and >0.23% for ZR 75-1 (Table 1). The corresponding values for HS 578T were 0.04 and >0.23%, respectively (Table 1).

Both ZR 75-1 and HS 578T demonstrated steep dose–response curves for the studied paclitaxel formulations (Figures 1 and 2b,c). Paclitaxel dissolved in ethanol demonstrated almost identical dose–response curves compared with the paclitaxel formulation in cremophor EL/ethanol. The IC_{50} values were identical (< 0.025 μg/ml) for both formulations and both cell lines (Table 1). The IC_{90} values were also similar (Table 1). The parental line ZR 75-1 was significantly more sensitive ($P < 0.0001$ to $P = 0.0002$) to paclitaxel dissolved in ethanol compared with the dox subline (Figure 1b). ZR 75-1 was significantly ($P = 0.0001$ to $P = 0.02$) more sensitive than the dox subline of ZR 75-1 in the concentration interval from 0.025 to 0.1 μg/ml with the solvents ethanol and cremophor EL together with paclitaxel (Figure 1c).

Table 1. IC_{50} *and* IC_{90} *values in μg/ml and %, respectively, for ZR 75-1 and HS 578T and their corresponding sublines*

	Cr EL/Eth	Pacl/Eth	Pacl/CR EL/Eth
ZR 75-1			
IC_{50} μg/ml			
ZR 75-1	0.09%	<0.025	<0.025
ZR 75-1-dox	0.12%	>10	0.1
IC_{90} μg/ml			
ZR 75-1	>0.23%	0.025	0.1
ZR 75-1-dox	>0.23%	>10	2
	Cr EL/Eth	Pacl/Eth	Pacl/Cr EL/Eth
HS 578T			
IC_{50} μg/ml			
HS 578T	0.04%	<0.025	<0.025
HS 578T-dox	0.15%	>10	1.6
IC_{90} μg/ml			
HS 578T	>0.23%	8	0.8
HS 578T-dox	>0.23%	>10	9.5

dox, subline with acquired doxurubicin resistance. Cr EL, cremophor EL; Eth, ethanol; Pacl, paclitaxel. IC_{50}/IC_{90}, the concentration required to cause a 50 and 90% cell kill, respectively.

HS 578T was significantly ($P < 0.0001$ to $P = 0.005$) more sensitive than the corresponding dox subline when paclitaxel was dissolved only in alcohol (Figure 2b). This was also the case ($P < 0.0001$ to $P = 0.04$) for the combined solvents, except for the two highest paclitaxel concentrations (Figure 2c).

Dox sublines

Cremophor EL and ethanol alone resulted in a flat dose–response curve for ZR 75-1-dox, little different from the parental cell line ZR 75-1 (Figure 1a). The subline HS 578T-dox was significantly ($P = 0.0004$ and $P = 0.001$) less sensitive to cremophor EL and ethanol at the concentrations 0.046 and 0.092% v/v, respectively (Figure 2a).

Generally, ZR 75-1-dox showed a steeper dose–response curve (significance level from $P < 0.0001$ to $P = 0.04$) for the paclitaxel formulation in cremophor EL/ethanol (IC_{50} 0.1 μg/ml and IC_{90} 2.0 μg/ml) compared with paclitaxel dissolved in only ethanol (IC_{50} and IC_{90} > 10.0 μg/ml) (Figures 1b, c, Table 1). The addition of paclitaxel to cremophor EL/ethanol compared with the solvents alone was significantly ($P = 0.0002$ to $P = 0.02$) more effective except at 0.05 μg/ml paclitaxel (Figures 1a, c).

HS 578T-dox demonstrated a significantly ($P = 0.0006$ to $P = 0.01$) higher sensitivity to paclitaxel in the solvents ethanol/cremophor EL at the concentrations ≥ 0.2 μg/ml (IC_{50} 1.6 μg/ml and IC_{90} 9.5 μg/ml) compared with paclitaxel dissolved only in ethanol (IC_{50} and IC_{90} > 10.0 μg/ml)(Figures 2b, c, Table 1). The effect of cremophor EL plus ethanol on the dox subline of HS 578T was significantly inferior ($P = 0.0007$ to $P = 0.02$) to paclitaxel/ethanol/cremophor EL, except at 0.025, 0.05 and 8 μg/ml paclitaxel concentrations (Figures 2 a, c).

DISCUSSION

Paclitaxel has been described to be effective even in breast cancer patients who had failed on anthracycline combinations [2]. This was unexpected because paclitaxel resistance has been described as belonging to the multidrug-resistance phenotype [4–6]. However, other resistance mechanisms related to tubulin changes have also been described [7].

These contradictions in previous reports on paclitaxel therapy and the reversion potential of the multidrug-resistance phenotype by cremophor EL were the main objectives for carrying out the present study [10, 11]. The clinical significance of our observations is further stressed by the recent report, after completing this study, on measured plasma levels of cremophor in the range from 0.09 to 0.20% v/v in patients with ovarian cancer treated paclitaxel [20]. In our study, we used cremophor EL from 0 to 0.23% v/v (Figures 1 and 2). Clinical cremophor EL concentrations may thus be able to cause the effects we observed in this study.

Our data showed that cremophor EL enhanced the cytotoxic effect of paclitaxel on the human breast cancer cell lines with the multidrug-resistant phenotype. The effects by paclitaxel on these dox sublines were significantly hampered when the drug was dissolved in ethanol. The corresponding parental cell lines ZR 75-1 and HS 578T were equally very sensitive to paclitaxel dissolved in either ethanol or in ethanol/cremophor EL. This strong argument suggests that cremophor EL had a drug reversion potential on the dox sublines. Furthermore, the combination of cremophor EL and ethanol alone at higher concentrations had a cytotoxic effect but this was significantly inferior to that with paclitaxel. However, ethanol alone at the same concentrations (data not shown) had no effect on any of the

parental or dox sublines. There was no observable difference between commercially produced paclitaxel and our own paclitaxel preparation in ethanol and cremophor EL (data not shown).

These data indicate that the high response rate reported on breast cancer patients with doxorubicin-resistant tumours using paclitaxel may not, *per se*, be due to the cytotoxic effect of paclitaxel alone but rather is caused by the combination of paclitaxel and the solvent cremophor EL.

Treated breast cancers tend to express high levels of P-glycoprotein/the *mdrl* gene compared with corresponding untreated cases [21–23]. The two dox sublines used in this study fit well to this model. However, contradictory results have also been reported [24, 25].

Furthermore, cyclosporine A has recently been described as reverting drug resistance in patients with VAD-resistant multiple myeloma [26], and it should also be noted that the solvent for cyclosporine A is cremophor EL. This is further indirect evidence supporting the theory that doxorubicin-failing breast cancer patients may have responded just by adding cremophor EL to their doxorubicin-containing regimen.

Thus, these data strongly suggest that all described antitumoral effects by paclitaxel may not exclusively be related to the drug itself, but rather to the combination of paclitaxel and cremophor EL.

1. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from taxus brevifolia. *J Am Chem Soc* 1971, **93**, 2325–2327.
2. Holmes FA, Walters RS, Theriault RL, *et al.* Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. *J Natl Cancer Inst* 1991, **83**, 1797–1805.
3. McGuire WP, Rowinsky EK, Rosenhein NB, *et al.* Taxol: a unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. *Ann Intern Med* 1989, **111**, 273–279.
4. Gupta RS, Cross-resistance of vinblastine and taxol resistant mutants of Chinese hamster ovary cells and other anticancer drugs. *Cancer Treat Rep* 1985, **69**, 515–521.
5. Roy SN, Horwitz SB. A phosphoglycoprotein associated with taxol resistance in J744.2 cells. *Cancer Res* 1985, **45**, 3856–3863.
6. Bradley G, Juranka PF, Ling V. Mechanism of multidrug resistance. *Biochim Biophys Acta* 1988, **948**, 87–128.
7. Horwitz SB. Mechanism of taxol resistance. The second national cancer institute workshop on taxol and taxus. Alexandria, Virginia. *Bristol-Myers-Squibb Oncol* 1992, 9–10.
8. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by taxol. *Nature* 1979, **277**, 665.
9. NCI. *Clinical Brochure: Taxol.* Bethesda, MD, NSC 125973, NCI 1983, revised 1991, p. 11.
10. Woodwock DM, Jefferson S, Linsenmeyer Crowther PJ, *et al.* Reversal of multidrug resistance phenotype with cremophor EL, a common vehicle for water-insoluble vitamins and drugs. *Cancer Res* 1990, **50**, 4199–4203.
11. Friche E, Demant EJF, Schestad M, Nissen NI. Effect of anthracycline analogs of photolabelling of p-glycoprotein by 1251-iodomycin and 3hazidopine: relation to lipophilicity and inhibition of daunorubicin transport in multidrug resistant cells. *Br J Cancer* 1993, **67**, 226–231.
12. Hackett AJ, Smith HS, Sprionger EL, *et al.* Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578st) cell lines. *J Natl Cancer Inst* 1977, **58**, 1795–1806.
13. Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce JJ. Human breast carcinoma cells in culture: establishment and characteristics of three new cell lines. *Cancer Res* 1978, **38**, 3352–3364.
14. Horwitz KB, Zava DT, Thilager AK, Jensen EM, McGuire WL. Steroid receptor analysis of nine breast cancer cell lines. *Cancer Res* 1978, **38**, 2434–2437.
15. de la Torre M, Larsson R, Nygren P, Bergh J. Characterization of 4 doxorubicin resistant human breast cancer cell lines. *Anticancer Res* 1993, **13**, 1425–1430.
16. Kartner S, Evernden-Porelle D, Bradley G, Ling V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature* 1985, **316**, 820–823.
17. Hamada H, Tsuruo. Functional role for the 170 to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc Natl Acad Sci U.S.A.* 1986, **83**, 7785–7789.
18. Johansson KE, Johansson I, Göbel UB. Evaluation of different hybridization procedures for the detection of mycoplasma contamination in the cell cultures. *Mol Cell Probes* 1990, **4**, 33–42.
19. Nygren P, Larsson R. A rapid fluorometric method for semiautomated determination of cytotoxicity and cellular proliferation of human tumor cell lines in microculture. *Anticancer Res* 1989, **9**, 1111–1120.
20. Webster L, Linsenmeyer M, Millward M, Morton C, Bishop J, Woodcock D. Measurement of cremophor EL following taxol: plasma levels sufficient to reverse drug exclusion mediated by the multidrug-resistant phenotype. *J Natl Cancer Inst* 1993, **85**, 1685–1690.
21. Salmon SE, Grogan TM, Miller T, Scheper R, Dalton WS. Prediction of doxorubicin resistance *in vitro* in myeloma, lymphoma, and breast cancer by P-glycoprotein staining. *J Natl Cancer Inst* 1989, **81**, 696–701.
22. Schneider J, Bak M, Efferth Th, Mattern J, Volm MP. P-glycoprotein expression in treated and untreated human breast cancer. *Br J Cancer* 1989, **60**, 815–818.
23. Sanfilippo O, Ronchi E, De Marco C, di Fronzo G, Silvestrini R. Expression of P-glycoprotein in breast cancer tissue and *in vitro* resistance to doxorubicin and vincristine. *Eur J Cancer* 1991, **27**, 155–158.
24. Merkel DE, Fuqua SAW, Tandon AK, Hill SM, Bazdar AU, McGuire WL. Electrophoretic analysis of 248 clinical breast cancer specimens for P-glycoprotein overexpression or gene amplification. *J Clin Oncol* 1989, **7**, 1129–1136.
25. Ro J, Sahin A, Ro JY, Fritsche H, Hortobagyi G, Blick M. Immunohistochemical analysis of P-glycoprotein expression correlated with chemotherapy resistance in locally advanced breast cancer. *Human Pathol* 1990, **21**, 787–791.
26. Sonneveld P, Durie BGM, Lokhurst HM, *et al.* Modulation of multidrug-resistant multiple myeloma by cyclosporin. *Lancet* 1992, **340**, 255–259.

Acknowledgements—We are grateful to Drs P. Nygren and R. Larsson for access to the Fluoroscan II machine with surrounding facilities and for fruitful discussions of the results. The study was supported by grants from the Swedish Cancer Society.