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Zhirong Zhan · Stefania Scala · Anne Monks
Curtis Hose · Susan Bates · Tito Fojo

Resistance to paclitaxel mediated by P-glycoprotein can be modulated by changes in the schedule of administration

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Abstract *Purpose*: Increasing use of paclitaxel in clinical oncology has stimulated interest in its mechanisms of resistance and ways to overcome these. Studies were performed with paclitaxel to determine the role of P-glycoprotein in drug sensitivity, and the effect of schedule on relative resistance. We have previously reported that prolonged exposure to P-glycoprotein substrates decreases relative resistance in multidrug resistant cells. *Methods*: Using both unselected and drug-selected cell lines, cross-resistance and cytotoxicity reversal studies using cyclosporin A were performed. In multidrug-resistant cells, cross-resistance was evaluated after 3-, 24-, and 96-h exposures to paclitaxel. *Results*: Cross-resistance to paclitaxel in P-glycoprotein-expressing sublines was shown to be comparable to that of other drugs transported by P-glycoprotein. Sensitivity to paclitaxel could be modulated by cyclosporin A in unselected cell lines expressing P-glycoprotein and not in P-glycoprotein-negative cell lines. Resistance to paclitaxel was reduced tenfold by increasing the duration of exposure in P-glycoprotein-expressing cells. This effect was not observed in a paclitaxel-resistant cell line which does not express P-glycoprotein. *Conclusions*: These studies extend observations on the schedule dependence of paclitaxel cytotoxicity and the role of P-glycoprotein in mediating paclitaxel sensitivity. The schedule dependence of relative resistance suggests that infusional paclitaxel may help in overcoming P-glycoprotein-mediated resistance.

Key words Paclitaxel · Drug resistance · Resistance reversal · P-glycoprotein · Scheduledependence

Z. Zhan · S. Scala · S. Bates · T. Fojo (✉)
Medicine Branch, Clinical Oncology Program,
Division of Cancer Treatment, National Cancer Institute,
NIH, 9000 Rockville Pike, Bethesda, MD 20892, USA
Fax (301) 402-0172

A. Monks · C. Hose
Program Resources, Inc./Syn Corp.,
NCI-Frederick Cancer Research and Development Center,
Frederick, MD 21702, USA

Introduction

Originally isolated from the western yew, *Taxus brevifolia* [29], paclitaxel has been shown to be an active agent in a wide range of malignancies, especially breast and ovarian cancer [4, 7, 16, 18, 20, 24, 27]. Although early studies suggested that paclitaxel acts as a mitotic inhibitor in a similar manner to the vinca alkaloids, subsequent studies have demonstrated that instead of inhibiting the polymerization of tubulin, paclitaxel stabilizes microtubules and promotes their assembly [21–23]. Microtubules arise from the polymerization of α and β tubulin (and possibly γ tubulin), in the presence of GTP and various microtubule-associated proteins (MAPs). Paclitaxel lowers the critical concentration for microtubule assembly and prevents disassembly under conditions that result in dissolution in the absence of paclitaxel [11, 21–23]. Although the reasons for cell death are unclear, interference with the function of a normal mitotic spindle is likely to play an important role [3, 9].

As a drug that interferes with the organization and function of microtubules, the cytotoxicity of paclitaxel can be expected to demonstrate a schedule dependence [3, 13]. The extent to which this schedule dependence can be exploited clinically remains to be determined, with available evidence indicating that it may be beneficial in some tumor types, and of no added benefit in others [5, 25, 33].

In vitro selections with paclitaxel have been reported to result in the overexpression of P-glycoprotein and the acquisition of a multidrug resistance phenotype [8, 15, 19]. P-glycoprotein, a membrane phosphoglycoprotein originally described by Ling, has been shown in numerous in vitro models to mediate the multidrug resistance phenotype [6, 10, 28]. Resistance occurs as a result of reduced drug accumulation, secondary to increased drug efflux mediated by P-glycoprotein. Clinical interest has been stimulated by the demonstration that a number of compounds can reverse resistance by

inhibiting P-glycoprotein-mediated drug efflux. These agents include the phenothiazines, verapamil and other antiarrhythmic agents, and the cyclosporines, including the cyclosporin D analog, PSC 833 [1, 26].

With the increasing use of paclitaxel clinically, we sought to compare the relative tolerance of multidrug-resistant cell lines to paclitaxel, and the degree to which unselected cell lines expressing P-glycoprotein could be sensitized to paclitaxel by the addition of cyclosporin A. Because numerous experiments had demonstrated a schedule dependence of adriamycin and vincristine in P-glycoprotein-expressing cell lines, suggesting that relative resistance is dependent on the duration of exposure, we sought to determine whether a similar schedule dependence exists for paclitaxel (unpublished observations; [12]). The results of these studies are described in the present report.

Materials and methods

Cell lines

The cell lines used in the present study were obtained or derived from several sources. All cells were maintained in either DMEM or RPMI with 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. Four unselected cell lines were used including two expressing P-glycoprotein [HCT-15 (colon) and CAKI-1 (renal cell)] and two without P-glycoprotein expression [OVCAR-5 (ovarian) and NCI-H23 (lung)]. The relative *MDR*-1 levels in these cell lines have been previously established by quantitative PCR: HCT-15 457; CAKI-1 177; OVCAR-5 <0.1; and NCI-H23 <0.1 [2]. Four drug-resistant cell lines were compared, including three multidrug-resistant cell lines expressing P-glycoprotein and one paclitaxel-resistant cell line without P-glycoprotein. Parental MCF-7 (breast), MDA-MB-231 (breast) and ZR75B (breast) cells were used to isolate the three multidrug-resistant sublines. All three cell lines were isolated from parental cells as populations following continuous drug exposure. For all three cell lines, the concentration of drug was increased gradually. MCF-TH cells were selected with adriamycin; as were ZR-Ad300 cells. They are maintained in 200 and 300 ng/ml adriamycin, respectively. MDA-Vb20 cells were isolated with stepwise increases in vinblastine and are carried in 20 ng/ml vinblastine. All three cell lines express high levels of *MDR*-1 mRNA (by quantitative PCR) and P-glycoprotein (by immunoblotting), and can be sensitized to adriamycin or vinblastine by verapamil or cyclosporin. In addition to these three multidrug-resistant cell lines, a paclitaxel-resistant cell line designated 1A9 PTX(10) was also utilized. These cells were selected from parental ovarian A2780 cells (clone 1A9) with continuous paclitaxel in the presence of 5 μg/ml verapamil, and do not have increased levels of P-glycoprotein, as measured by quantitative PCR (unpublished observations). In the initial step of the selection, clones surviving 5 ng/ml paclitaxel were isolated and expanded. The cells used in this study were derived from one clone and are maintained in 15 ng/ml paclitaxel and 5 μg/ml verapamil. Evidence indicates these cell have alterations in tubulin (unpublished observations).

Cytotoxicity studies

All drugs were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Cytotoxicity was determined using a sulforhodamine B (SRB) assay to determine growth inhibition/cell kill or by performing cell counts using six-well plates. The SRB assay was performed as previously described [17]. Adherent cell cultures were fixed in situ by adding 50 μl cold 50% (wt/vol) trichloracetic acid (TCA) (final concentration 10% TCA) and incubating for 60 min at 4 °C. The supernatant was then discarded, and the plates were washed five times with deionized water and dried. SRB solution (100 μl, 0.4% wt/vol in 1% acetic acid) was added to each well, and the culture was incubated for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid. Then the plates were air dried. Bound stain was solubilized with Tris, and the optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 515 nm. Alternately, some studies were carried out in six-well plates with cell counts performed on a Coulter counter. This approach was utilized in the schedule dependence experiments because it allowed for the removal and addition of medium at the necessary times. All experiments were done in triplicate, and all were performed independently at least three times.

In all cases, drug was initially added 24 h after plating, and when drug-resistant cell lines were examined, cells that had been carried in drug-free medium for 1 week were used. The data are presented as percent growth inhibition, where 100% represents the value in wells to which no cytotoxic drug had been added. In the experiments examining sensitization by cyclosporin A with the unselected cell lines, cyclosporin A was added 3 h before the cytotoxic agents, and cell survival/growth was determined 48 h after drug addition. In the schedule-dependence experiments in which different exposure times were compared, drug was removed after 3, 24 or 96 h, and drug-free medium added. Cell survival/growth was determined after 7 days. Furthermore, cells exposed to drug for 96 h had fresh drug-containing medium added after the first 48 h of drug treatment.

With the exception of paclitaxel, which for some experiments was dissolved in Cremophor EL, all cytotoxic drugs were dissolved in DMSO. The concentration of DMSO did not exceed 0.05% in any of the schedule-dependence experiments and did not exceed 0.5% in any of the reversal experiments. The stock solution of paclitaxel in Cremophor EL was 6 mg/ml and this was dissolved in medium to achieve the final concentration. The highest concentration of paclitaxel used in a cytotoxicity study was 300 ng/ml (a 1:20 000 dilution); consequently, the highest Cremophor EL concentration was 0.0005%. Similarly, in reversal of resistance studies a final cyclosporin concentration of 1 μg/ml was used, using a stock solution of 50 mg/ml and a dilution of 1:50 000, for a final Cremophor EL concentration of 0.00002%. In numerous experiments, these concentrations have never been demonstrated to have any effect on drug sensitivity (unpublished observation). These concentrations are also lower than those reported to modulate cytotoxicity [14, 30]. Because of the poor solubility of paclitaxel in other solvents, and since the concentrations of Cremophor EL used were felt to be insignificant, no other solvents were utilized.

Results

The principal goal of this study was to determine the schedule dependence of paclitaxel resistance, but first we sought to determine how paclitaxel compared with other natural products as a P-glycoprotein substrate. To do this, we utilized both drug-selected cell lines overexpressing P-glycoprotein and unselected cell lines with a range of P-glycoprotein levels. To be certain that multidrug resistance associated protein (MRP) overexpression would not confound the results, the levels of MRP and *MDR*-1 mRNA were measured by quantitative PCR in the three selected human breast sublines [2]. These results are summarized in Table 1. The values shown are in arbitrary units relative to the mRNA level in the SW620 human colon carcinoma cell line, which was used as the reference cell line. Relative levels of *MDR*-1 expression compared with the respective

Table 1 *MDR*-1 and MRP expression determined by quantitative PCR. Values are in arbitrary units. Quantitative PCR was performed as previously described [2]. SW-620 colon carcinoma cells were used as the reference cell line, and β_2-microglobulin and 28S were used as internal controls

	MDR-1	MRP
SW-620	10	10
MCF-7	0.05	4.9
MCF-7TH	3320	27.5
MDA-MB231	0.26	4.9
MDA-Vb20	2110	3.8
ZR75B	0.67	6.8
ZR-Ad300	6920	16.8

Table 3 Enhancement of paclitaxel cytotoxicity by cyclosporin A. Values are IC_{50} without cyclosporin A/IC_{50} with cyclosporin A

	HCT-15	CAKI-1	OVCAR-5	NCI-H23
Adriamycin	9	20	2	2
Actinomycin-D	35	36	1	1
Vinblastine	20	16	1	2
Vincristine	155	172	2	3
Paclitaxel	38	90	1	1
5-Fluorouracil	1	1	1	1

parental line could not be accurately derived because the three parental cell lines express very low levels of *MDR*-1 mRNA or P-glycoprotein. The levels of MRP mRNA in the resistant cells are similar to or slightly higher than those of their respective parents, suggesting that resistance in these sublines is a consequence of overexpression of P-glycoprotein, rather than MRP. Table 2 summarizes the results of cytotoxicity assays in the drug-selected cell lines. As can be seen, cross-resistance to paclitaxel occurred in all, at levels comparable to those of the other drugs.

These observations are extended in Table 3 which summarizes the degree of sensitization by cyclosporin A in four unselected cell lines including two without P-glycoprotein (NCI-H23 and OVCAR-5) and two expressing P-glycoprotein (HCT 15 and CAKI-1). Table 3 summarizes the degree of sensitization for the six drugs listed. This value was obtained by dividing the IC_{50} in the absence of cyclosporin A by that in the presence of 1 μg/ml cyclosporin A. In all the cell lines, no significant sensitization was observed for 5-fluorouracil, which is not a P-glycoprotein substrate. However, with five other drugs known to be P-glycoprotein substrates (adriamycin, actinomycin-D, vinblastine, vincristine, and paclitaxel) sensitization was observed primarily in the P-glycoprotein-expressing cell lines. The degree of sensitization observed for paclitaxel was comparable to or greater than that of all the other agents.

Having established paclitaxel as a drug whose activity is mediated by P-glycoprotein, we next examined the extent to which resistance to paclitaxel was affected by the duration of drug exposure. We used for these studies paclitaxel-resistant cell lines expressing P-glycoprotein and a paclitaxel-resistant cell line without P-glycoprotein expression. The pairs (parental and resistant) have similar doubling times in cell culture when maintained in drug-free medium, and all studies were performed after the resistant cells had been in drug-free medium for at least 1 week.

Figure 1 shows the results of the relative resistance as a function of drug exposure using parental MCF-7 cells, and a P-glycoprotein-expressing adriamycin-selected subline, MCF-7TH. With a 3-h exposure, the relative resistance to paclitaxel was 2813, which fell to 385 after 24 h and to 275 after 96 h. This schedule dependence of resistance was independent of the schedule dependence of cytotoxicity, which in this experiment was most marked as the exposure time was increased from 3 to 24 h. When similar studies were performed using ovarian carcinoma A-2780 cells (clone 1A9) and the 1A9 PTX(10) paclitaxel-resistant subline without P-glycoprotein expression, the relative resistance was independent of the duration of exposure as shown in Fig. 2 (4 h 7.05 vs 72 h 10). So that in contrast to the results in the cell lines expressing P-glycoprotein, relative resistance did not decrease with increasing drug exposure.

Discussion

The widespread use of paclitaxel in clinical oncology has increased interest in its mechanisms of resistance and ways to overcome this. Selections with paclitaxel were originally reported to result in overexpression of P-glycoprotein, suggesting that paclitaxel resistance was mediated by P-glycoprotein [8, 15, 19]. Paclitaxel activity is schedule dependent, with increased efficacy observed with more prolonged administration [13]. In the present

Table 2 Multidrug-resistant human breast cancer cells IC_{50} values and relative resistance (*IC*$_{50}$ the concentration of drug (in ng/ml) inhibiting cell growth/survival 50%, *PTX* paclitaxel, *ADR* adriamycin, *VBL* vinblastine, *ACT-D* actinomycin-D, *RR* relative resistance: resistant cell line IC_{50}/parental cell line IC_{50})

Cell line	PTX	RR	ADR	RR	VBL	RR	ACT-D	RR
MCF-7	2		10		0.03		0.25	
MCF-7TH	145	73	2500	250	14.5	480	55	220
MDA-MB231	0.07		1		0.03		0.13	
MDA-Vb20	150	2140	160	160	9	300	8.8	68
ZR75B	0.5		0.7		0.06		0.25	
ZR-Ad300	200	400	700	1000	10	16	50	200

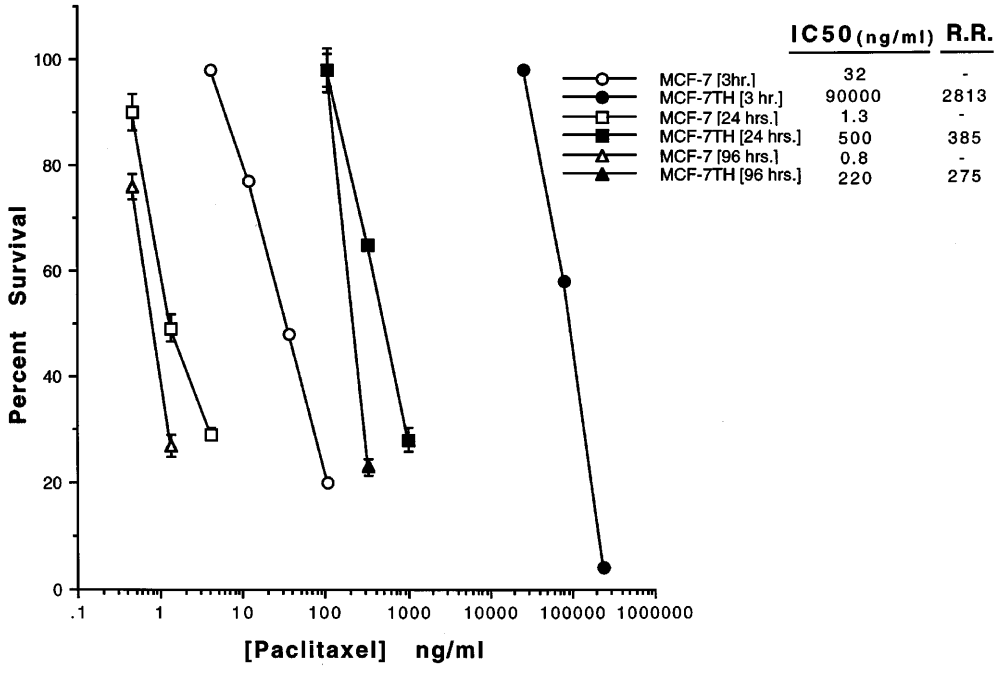

Fig. 1 Schedule dependence of relative resistance to paclitaxel. Resistance was determined in MCF-TH cells which were selected with adriamycin and express high levels of P-glycoprotein relative to parental MCF-7 cells. Relative resistance is seen to be schedule dependent, with the highest crossresistance seen following a 3-h drug exposure. Percent growth/survival is plotted and represents the value relative to that in control wells, which were not treated with cytotoxic drug. Standard deviations are shown where they exceed the size of the symbol

study, we confirmed the previous observations, and found that relative resistance was also schedule dependent in P-glycoprotein-expressing cells, as previously observed with other drugs transported by P-glycoprotein [12]. That the schedule-dependent resistance is a function of P-glycoprotein transport was supported by results in a paclitaxel-resistant cell line which does not express P-glycoprotein and in which relative resistance was independent of schedule.

The ultimate goal of the present study was to address the role of infusional paclitaxel as a maneuver to overcome P-glycoprotein-mediated resistance. In both drug-selected cell lines expressing P-glycoprotein and in unselected cell lines with a broad range of P-glycoprotein expression, paclitaxel sensitivity was affected by P-glycoprotein to an extent as great as or greater than other well-known P-glycoprotein substrates. In the drug-selected cell lines, high levels of cross-resistance to paclitaxel were observed. In the unselected cell lines, sensitization by cyclosporin A was observed in the P-glycoprotein-expressing cell lines. These results support previous observations demonstrating that

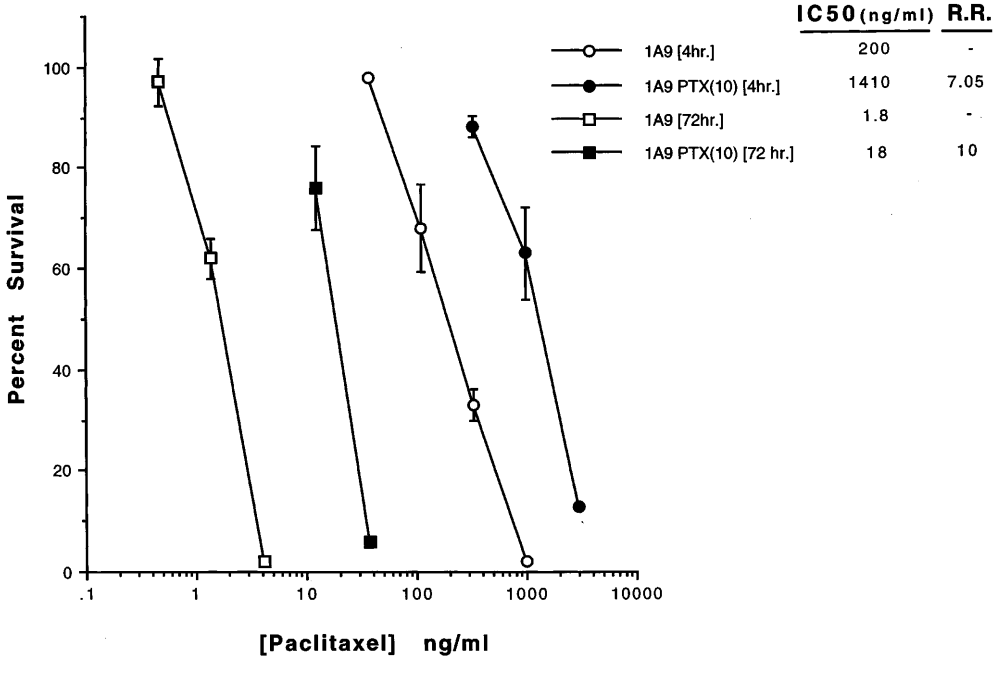

Fig. 2 Schedule independence of relative resistance to paclitaxel. The resistance of 1A9 PTX(10) cells, which were selected with paclitaxel in the presence of verapamil and do not express P-glycoprotein, relative to parental 1A9 cells was determined (1A9 is an A2780 clone). In contrast to the results with P-glycoprotein-expressing cells, relative resistance was schedule independent. Percent growth/survival is defined as in Fig. 1. Standard deviations are shown where they exceed the size of the symbol

paclitaxel sensitivity is influenced by P-glycoprotein and prompted an examination of the role of schedule in reversing paclitaxel resistance. Previous studies with adriamycin, and similar unpublished results with vincristine and actinomycin-D (unpublished observations; [12]) have indicated that longer infusions can overcome P-glycoprotein-mediated resistance. Similar observations were made with paclitaxel when the relative resistance of P-glycoprotein-expressing cells was determined. Although the mechanism(s) mediating this effect is (are) not fully understood, one can speculate that at the lower concentrations that result from continuous exposure, preferential binding to a drug target may occur over binding/transport by P-glycoprotein. This could result if the affinity or binding constants for targets were higher than those for P-glycoprotein. That this effect is not a general phenomenon is supported by the results in paclitaxel-resistant cells which do not express P-glycoprotein and in which increased sensitivity with prolonged administration was not observed. This effect is to be distinguished from the schedule dependence of paclitaxel observed in other experiments, and observed with the cell lines utilized in the present study. It suggests that with tumors expressing P-glycoprotein, dual benefits might be derived from continuous infusion regimens.

Schedule dependence of paclitaxel has previously been reported [13], and was not unexpected for a drug which interferes with microtubules. In the cell lines utilized in these studies, the largest effect on cytotoxicity was observed when the 3-h results were compared with the 24-h results (not shown). Differences between 24-h and longer treatments were not as marked. This is to be expected since the doubling time of the cell lines used in this study, as for many cell lines, is less than 24 h. Consequently, a 24-h treatment period is sufficient to expose all (or nearly all) cells to drug during a defined vulnerable period, and longer exposures do not provide much added benefit. These observations support a schedule dependence for paclitaxel, but the differences with varying times apply to the in vitro models investigated and should not be extrapolated to clinical oncology. That is, these data do not indicate that 24-h infusions are optimal in the treatment of patients with cancer, since the doubling times of most malignancies are considerably longer than 24 h.

Clinical trials testing infusional schedules for paclitaxel have shown mixed results. For example, a 96-h paclitaxel infusion results in a 48% response rate in advanced breast cancer while the same regimen results in a 17% response rate in refractory lymphoma [31, 32]. Similarly, in patients with progressive disease following short-infusion taxane treatment, a 27% response rate has been noted in breast cancer and no response in lymphoma [25, 33]. The present study extends observations of paclitaxel's schedule dependence, and the role of P-glycoprotein in paclitaxel resistance. The data suggest that paclitaxel resistance mediated by P-glycoprotein can be modulated by altering the schedule of administration. The definition of the settings in which infusional therapy is most efficacious requires straightforward clinical study.

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