Anti-Cancer Drugs 2000, **11,** pp. 439–443

Report

Differential impact of Raf-1 kinase activity on tumor cell resistance to paclitaxel and docetaxel

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Docetaxel (Taxotere™) is becoming increasingly important in the treatment of many tumor sites and is unusually active in tumors that are resistant to the structurally similar taxane, paclitaxel. These data suggest that the processes that confer cellular paclitaxel resistance may have a substantially lower impact upon the cytotoxicity induced by docetaxel. We have recently reported that there is a marked Raf-1 kinase dependency of paclitaxel resistance in human cervical and ovarian tumor cell lines. We therefore characterized the impact that inherent and genetically induced variations in Raf-1 kinase activity have on the docetaxel cytotoxicity in human ovarian and cervical cancer cell lines. Our data suggest that docetaxel cytotoxicity is independent of Raf-1 kinase activity in the cell lines studied and that the lack of cross-resistance between these two taxane compounds may be due to the differential impact that Raf-1 kinase activity has on their cytotoxicity. Should these relationships pertain to the clinical situation, these findings could form the basis for a molecular-based triage of patients to receive docetaxel when response to paclitaxel may be unlikely due to high Raf-1 kinase activity. [© 2000 Lippincott Williams & Wilkins.]

Key words: Docetaxel, cervix, molecular determinant, ovarian, paclitaxel, Raf-1 kinase.

Introduction

Docetaxel (Taxotere™) is a semi-synthetic derivative of paclitaxel that is becoming increasingly important in the clinic. Phase I and II trials suggest that docetaxel may be of considerable benefit for patients with recurrent (cisplatin-refractory) ovarian tumors.[1,2] Perhaps one of the most intriguing aspects of docetaxel is that it gives an objective response rate of 18% in paclitaxel-refractory breast tumors.[3] These data suggest that in this sub-set of patients there is no collateral resistance between these structurally related taxanes.

The identification of the processes that differentially affect cellular resistance to paclitaxel compared to docetaxel could create several therapeutic opportunities. For example, provided some form of molecular triage is performed, it could be possible to stratify chemotherapy-naive patients to receive docetaxel rather than paclitaxel if they are likely to be refractory to paclitaxel. In the case of patients who have recurred following front-line treatment with paclitaxel, it may be possible to identify those patients that are likely to respond to second-line docetaxel treatment and spare the remaining patients from docetaxel-related toxicity/morbidity, which can be substantial in some instances.[4] The identification of the molecular determinants of paclitaxel and docetaxel resistance may also provide potential targets for gene therapy (once it has been sufficiently refined to allow the selective targeting of tumor cells).

At present, there is little information of the molecular determinants of docetaxel resistance; with the exception of drug transporters,[5,6] there is virtually no data on the relative importance of paclitaxel resistance mechanisms to cellular docetaxel resistance levels. We have previously reported that Raf-1 kinase activity is a major determinant of paclitaxel resistance in human cervical tumor cells[7] and in ovarian cancer cells that harbor a mutated p53 protein.[8] Consideration of the lack of cross-resistance between paclitaxel and docetaxel in a sub-set of patients in conjunction with our own *in vitro* data raises the possibility that high Raf-1 kinase may not confer cellular resistance to docetaxel as it does to paclitaxel. We thus determined the impact that variations in Raf-1 kinase activity had on the relative paclitaxel and docetaxel sensitivities of $TP53^{mut}$ human tumor cells.

Supported by a grant from the Alberta Cancer Foundation and the Medical Research Council of Canada (MOP-37843).

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Methods and materials

Human tumor cell lines

For these studies, we used four single-cell-derived human cervical tumor cell lines from our previous study on the Raf-1 kinase dependency of paclitaxel cytotoxicity,[7] plus two human ovarian tumor cell lines that have a $TP53^{mut}$: 2780/CP and OAW42/CP. The origins and clonal selection techniques used to isolate the human cervical[9,10] and ovarian[11] tumor cell lines have been previously published. To determine the effect that genetic down-regulation of Raf-1 kinase activity had on docetaxel sensitivity, we used the previously described HT212/9 cell lines that have been transduced with the pcDNA3 vector alone (HT212/9-pc2:1) or with a antisense *RAF1* construct (HT212/9-AS13:5.[7] The Raf-1 kinase activity within these cells and their previously reported paclitaxel sensitivity is presented in Table 1.

The tumor cell lines were maintained as monolayer cultures in DMEM/F12 media, supplemented with 10% fetal calf serum (Life Technologies, Grand Island, NY) and antibiotics. All cell lines were sub-cultured every 4–5 days to ensure exponential growth.

Clonogenic cell survival assays

Two days prior to use, cells from exponentially growing cultures were detached using 0.25% trypsin/1 mM ethylene-diamine-tetraacetic acid (EDTA) at 37°C and placed into fresh 75 cm^2 tissue culture flasks. On the day of the experiment, the tumor cells were detached using 0.25% trypsin/1 mM EDTA at 37°C and washed twice with warm PBS, and resuspended in DMEM/F12 media (supplemented with 10% FCS and antibiotics). The cells were then seeded at densities of between 10^2 and 5×10^4 per 60 mm tissue culture dish and incubated for 3 h at 37°C (5% CO_2/95% air) to allow for cell attachment. The cells were then exposed to graded doses of docetaxel (0–60 nM) or paclitaxel (0–120 nM) for 2 h at 37°C (5% CO_2/95% air), the drug-containing media was then removed and fresh media added. The plates were then incubated for 15 days at 37°C (5% CO_2/95% air), at which time the colonies were fixed in 70% ethanol, stained with 10% methylene blue and those colonies containing greater than 50 cells counted. Each survival curve consisted of a minimum of four drug concentration points. Each dose point was assayed by at least three separate experiments, each assay consisting of three replicate plates. The results presented in this paper represent the pooled data from a minimum of three experiments.

Data handling and presentation

The experimental data were fitted to the linear-quadratic equation:

$$-\ln \mathrm{SF} = \alpha C + \beta C^2$$

where SF is equivalent to the surviving fraction at a given drug concentration, C, and α and β are constants. The data were fitted to a linear-quadratic function using the non-linear regression PRIZM™ software package (Graphpad Software, San Diego, CA). The fraction of cells surviving a clinically relevant concentration of paclitaxel, i.e. 60 nM,[12,13] or docetaxel, i.e. 20 nM,[14] were calculated by substitution of the derived constants and the appropriate drug concentration into the linear quadratic equation.

Table 1. Relative paclitaxel and docetaxel sensitivity of human tumor cells with different Raf-1 kinase activity: kinase determinations were performed at least 3 times on each of at least three Raf-1 immunoprecipitates from a given cell line.

Cell line	Raf-1 kinase activity[a]	Paclitaxel SF_{60}[b]	Paclitaxel IC_{50} (nM)	Docetaxel SF_{20}[c]	Docetaxel IC_{50} (nM)
HT137/5	5.77 (0.54)[d]	0.750	102.9	0.20	8.7
HT180/1	2.91 (0.69)	0.278	33.4	0.04	6.3
HT180/8	5.70 (0.57)	0.849	98.1	0.21	8.7
HT212/9	4.92 (0.41)	0.515	62.0	0.08	6.3
HT212/9-pc2:1	4.52 (0.48)	0.40	49.6	0.07	4.6
HT212/9-AS13:5	0.28 (0.14)	0.20	35.3	0.04	3.5
2780/CP	2.36 (0.27)	0.34	40.7	0.31	15.0
OAW42/CP	2.55 (0.41)	0.58	71.8	0.46	18.3

[a] IU $\times 10^{-3}/10^6$ cells.
[b] Fraction of cells surviving exposure to 60 nM paclitaxel.
[c] Fraction of cells surviving exposure to 20 nM docetaxel.
[d] Standard error of the mean.

Results

The six 'parental' cell lines studied exhibited an 11-fold variation in docetaxel sensitivity, with SF_{20} values (the fraction of cells surviving exposure to 20 nM docetaxel) ranging from 4 to 46%. IC_{50} values for docetaxel ranged from 3.5 to 18.3 nM. These cell lines exhibited a 3.7-fold variation in Raf-1 kinase activity, with Raf-1 kinase activity levels ranging from 2.4 to 5.8×10^{-3} IU/10^6 cells (Table 1). Linear regression analysis revealed that there was no significant relationship between Raf-1 kinase activity and either SF_{20} or docetaxel IC_{50} values for these parameters in these cell lines (Figure 1).

We also determined the impact that translational down-regulation of Raf-1 kinase activity had on docetaxel-induced cytotoxicity. Raf-1 kinase activity was down-regulated by 16-fold in HT212/9 cells that had been stably transfected with the RAF-1 anti-sense construct;[7] however, there was no significant alterations in the docetaxel sensitivity of the 212/9-AS13:5 cells compared to the parental HT212/9 cells (Figure 2). This was in stark contrast to the 4-fold sensitization to paclitaxel that we observe in these cell lines.[7]

A comparison of IC_{50} values for paclitaxel and docetaxel revealed that docetaxel was 8.2 ± 1.3 (range 2.7–11.8) more lethal than paclitaxel (on a molar basis). However, there was no relationship between IC_{50} values for both taxanes (p=0.59) (Figure 3).

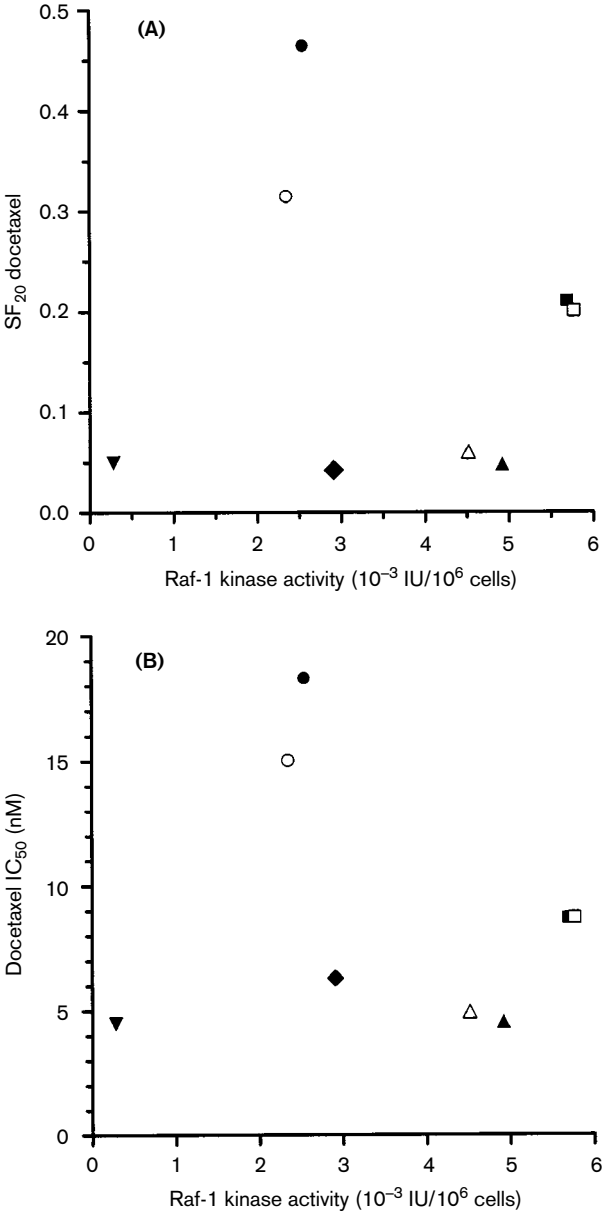

Figure 1. Relationship between Raf-1 kinase activity and cellular docetaxel sensitivity as assessed at (A) SF_{20} or (B) IC_{50} in human HT137/5, □; HT180/1, ◆; HT180/8, ■; HT212/9, ▲; HT212/9-pc2:1, △; HT212/9-AS13:5, ▼; 2780/CP, ○; and OAW42/CP, ●; tumor cell lines.

Discussion

The purpose of these studies was to determine the Raf-1 kinase dependency of docetaxel-induced cytotoxicity in $p53^{mut}$ or $p53^{null}$ human tumor cell lines. The data presented in this study suggest that Raf-1 kinase activity is not a major determinant of *de novo* docetaxel resistance in human cervical and ovarian cancer cells, and that genetic down-regulation of Raf-1

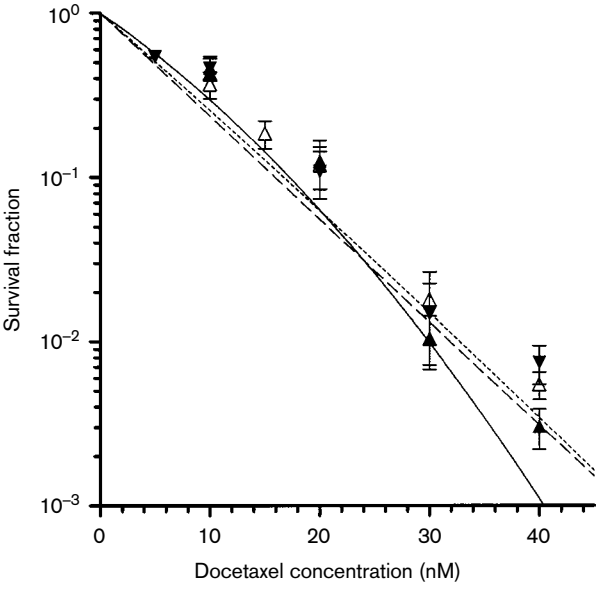

Figure 2. Docetaxel sensitivity of the HT212/9 (▲), HT212/9-pc2:1 (△) and HT212/9-AS13:5 (▼) cell lines.

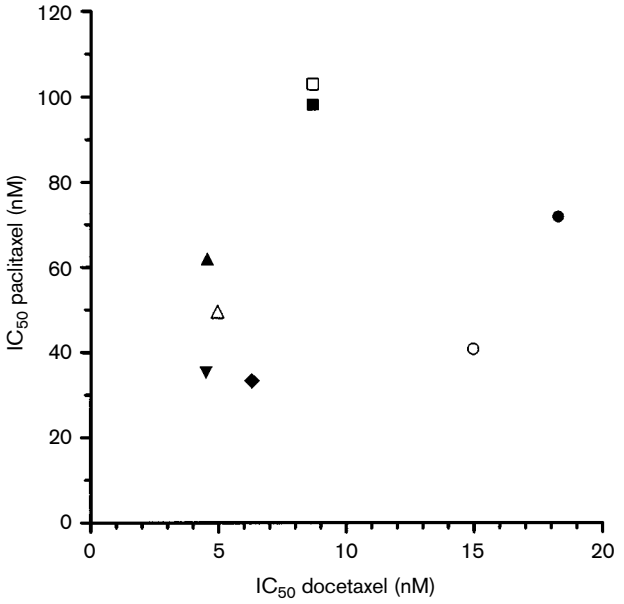

Figure 3. Relative cellular sensitivity to docetaxel and paclitaxel in human tumor cell lines. Symbols are the same as outlined in Figure 1.

kinase activity had no significant impact on the cytotoxicity induced by clinically relevant docetaxel concentrations. The limited impact that Raf-1 kinase activity has on cellular resistance to docetaxel, in comparison to paclitaxel,[7] may partly explain the lack of collateral resistance between these two taxanes in the clinic[3] and *in vitro* (Table 1 and Figure 2).[15]

The primary mechanism of action of docetaxel is through stabilization of microtubules and the subsequent induction of apoptosis. In this regard it is very similar to paclitaxel; however, docetaxel is between 1.3- and 12-fold more potent than paclitaxel on a molar basis (Table 1 and Figure 2).[15] This increased potency of docetaxel has been related to the greater induction of Bcl2 phosphorylation by docetaxel in comparison to paclitaxel. The 100-fold greater level of Bcl2 phosphorylation induced by docetaxel in comparison to paclitaxel[16] would appear to negate, or at least substantially diminish, the anti-apoptotic influence of high Raf-1 kinase activity. A recent study suggests that the antitumor effect of docetaxel in murine tumors was not related to either mitotic arrest nor the induction of apoptosis, but rather to cell lysis.[17] Under such circumstances it is perhaps not surprising that docetaxel-induced cytotoxicity is independent of the anti-apoptotic effect induced by high Raf-1 kinase. However, in these *in vivo* studies docetaxel-induced cell lysis occurs due to the inhibition of neo-angiogenesis by docetaxel,[18] which of course is not a factor in our *in vitro* studies.

Whatever the underlying reasons for the Raf-1 kinase independence of docetaxel cytotoxicity in human cervical and ovarian cancer cells, our observation that high Raf-1 kinase activity is associated with cellular paclitaxel resistance suggests that docetaxel should be prescribed for ovarian cancer patients whose tumors contain high Raf-1 kinase activity. Provided some form of molecular triage is performed, it could be possible to identify patients who are not suitable to receive paclitaxel as first-line treatment (due to high Raf-1 kinase activity levels), who may then receive docetaxel and its associated side effects. Moreover, we have previously suggested[7] that Raf-1 kinase inhibitors such as ISIS 5132 might be a valuable adjuvant for paclitaxel therapy. It seems likely that this treatment protocol may fail in a subset of tumors due to a compensatory up-regulation of Raf-1 kinase activity, under these circumstances docetaxel might be used as salvage therapy.

In summary, we have shown that one explanation for the lack of cross-resistance between paclitaxel and its analog docetaxel is the differential impact that Raf-1 kinase activity has on cellular resistance to these compounds. These findings could form the basis for a molecular based triage of patients to receive docetaxel when response to paclitaxel may be unlikely due to high Raf-1 kinase activity.

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(Received 9 April 2000; accepted 20 April 2000)