Taxotere–5′-deoxy-5-fluorouridine combination on hormone-refractory human prostate cancer cells

Jean-Louis Fischel[a], Jean-Marc Ferrero[a], Patricia Formento[a], Joseph Ciccolini[b], Nicole Renée[a], Jean-Louis Formento[a] and G. Milano[a]

Single-agent docetaxel (Taxotere) treatment has recently demonstrated promising clinical activity in patients with advanced hormone-refractory prostate cancer. Taxanes were recently found to upregulate the tumoral activity of thymidine phosphorylase (TP), a key cellular enzyme [transformation of 5′-deoxy-5-fluorouridine (5′-DFUR) into 5-fluorouracil] in the activation cascade of capecitabine (Xeloda). We tested (cytotoxic effects and molecular mechanisms) the Taxotere–5′-DFUR combination on hormone-refractory prostate cancer cell lines (DU145 and PC3). Cells were exposed to Taxotere and/or 5′-DFUR in three different sequences: Taxotere was given alone for 48 h, then 5′-DFUR was added for 48 h; Taxotere and 5′-DFUR together during 96 h or 5′-DFUR was given alone for 48 h then Taxotere was added for 48 h. The drug sequence Taxotere applied first followed by 5′-DFUR led to synergistic cytotoxic effects on both cell lines; the other sequences resulted in simple additivity. Taxotere did not modify TP activity while it decreased thymidylate synthase activity. There was an increase in CD95 cellular membrane levels following exposure to Taxotere–5′-DFUR, which is in agreement with the supra-additive cytotoxic combination. This observation may serve as a preclinical rationale for a next step testing the Taxotere–capecitabine combination at the clinical level in prostate cancer patients. *Anti-Cancer Drugs* 16:309–316 © 2005 Lippincott Williams & Wilkins.

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Introduction

Capecitabine (N^4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine; Xeloda) is an effective and well-tolerated treatment for head and neck [1], colorectal [2–4], and breast [4–6] cancer. After extensive absorption capecitabine is first transformed to 5′-deoxy-5-fluorocytidine by hepatic carboxylesterase and then to 5′-deoxy-5-fluorouridine (5′-DFUR) by cytidine deaminase, which is found in high concentrations in many human tumor tissues as well as in healthy liver tissue. As a key step, 5′-DFUR is then converted into 5-fluorouracil (5-FU) by thymidine phosphorylase (TP), which is present in higher concentrations in tumors than in healthy tissues [7]. 5-FU is catabolized by the enzyme dihydropyrimidine dehydrogenase (DPD), which is expressed in liver tissue and tumors [8]. Experimental data suggest that the cytotoxic efficacy of capecitabine is improved in cells expressing high levels of TP and low levels of DPD [9]. Clinical data have recently confirmed this experimental observation by demonstrating the role of tumor TP and DPD for predicting the efficacy of 5′-DFUR in the treatment of patients with colorectal cancer [10]. Taxanes were recently found to upregulate the tumoral activity of TP and have shown synergistic cytotoxic activity when combined with capecitabine [11,12]. This synergy has recently been translated into interesting clinical results with the docetaxel–capecitabine combination [13]. A TP-based synergistic, sequence-dependent interaction between an epidermal growth factor receptor inhibitor, ZD1839 (Iressa), and 5′-DFUR has recently been demonstrated by us [14]. It is thus possible that a pharmacologically based induction of TP may be an underlying mechanism sustaining supra-additive cytotoxic interactions with capecitabine.

Single-agent docetaxel (Taxotere) treatment has recently demonstrated promising clinical activity in patients with advanced hormone-refractory prostate cancer [15]. It was thus tempting to apply the Taxotere–capecitabine combination in advanced hormone-refractory prostate cancer in which active treatments are still eagerly awaited [16]. As a preclinical step this two-drug combination was tested on hormone-refractory human prostate tumor cell lines DU145 and PC3. Sequence effects and different molecular mechanisms, including changes in TP activity, were investigated so as to provide a rationale before initiating future clinical investigations.

Materials and methods

Chemicals

DMEM and glutamine were purchased from Whittaker (Verviers, Belgium). FBS was obtained from Dutscher

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(Brumath, France). Penicillin and streptomycin were from Meyrieux (Lyons, France). Taxotere was provided by the hospital pharmacy. Transferrin, 5′-DFUR, BSA, MTT and DMSO were purchased from Sigma (St Quentin Fallavier, France). Deoxy[5-^{3}H]uridine 5′-monophosphate (16 Ci/mmol) was from Amersham Pharmacia Biotech (Little Chalfont UK).

Drug administration schedule

The human prostatic cancer cell lines DU145 and PC3 (androgen resistant) were used in the present study. The PC3 cell line was used to confirm the synergistic cytotoxic interaction for the optimal sequence obtained with the DU145 cell line. Cells were routinely cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 50 000 U/l penicillin and 80 µM streptomycin, in a humidified incubator (Sanyo, Japan) at 37°C with an atmosphere containing 8% CO_2. The first part of the study involved examination of the cytotoxic effects resulting from the combination of Taxotere with 5′-DFUR. Cells were seeded in 96-well microtitration plates (100 µl/well) to obtain exponential growth for the duration of the experiments (initial cell density was 3000 cells/well). After 48 h, cells were exposed to Taxotere and/or 5′-DFUR in three different sequences. Taxotere was given alone for 48 h, then 5′-DFUR was added for 48 h (Schedule A), or Taxotere and 5′-DFUR together during 96 h (Schedule B) or 5′-DFUR was given alone for 48 h then Taxotere was added for 48 h (Schedule C). Eleven concentrations were tested for each drug: the maximum concentrations for Taxotere and 5′-DFUR were 2.5 nM and 10 µM, respectively. At the end of drug exposure, i.e. 96 h after initiation of the first drug, growth inhibition was assessed by the MTT test [17], as follows. After medium renewal, cells were incubated with MTT; after 2 h of exposure, medium was eliminated and DMSO (100 µl) was added. Absorbance at 540 nm was measured using a microplate reader (Labsystems, Helsinki, Finland). Results were expressed as the relative percentage of absorbance compared with controls without drug. Cell sensitivity to Taxotere and 5′-DFUR was expressed as IC_{50}. Experimental conditions were tested in quintuplicate (five wells of the 96-well plate per experimental condition) and in three separate experiments. Dose–effect curves were analyzed using Prism software (GraphPad Software, San Diego, CA).

Drug associations

The cytotoxic effects obtained with the different Taxotere–5′-DFUR combinations were analyzed according to the Chou and Talalay method [18] on Calcusyn software (Biosoft, Cambridge, UK). Interaction between Taxotere and 5′-DFUR was assessed by means of an automatically computed Combination Index (CI). CI was determined at 50 and 75% cell death, and was defined as follows:

$$CI_{A+B} = [(D_{A/A+B})/D_A] + [(D_{B/A+B})/D_B] + [\alpha(D_{A/A+AB} \times D_{B/A+B})/D_A D_B]$$

where $CI_{A+B} = CI$ for a fixed effect (F) for the combination of cytotoxic A and cytotoxic B, $D_{A/A+B}$ = concentration of cytotoxic A in the combination A + B giving an effect F, $D_{B/A+B}$ = concentration of cytotoxic B in the combination A + B giving an effect F, D_A = concentration of cytotoxic A alone giving an effect F, D_B = concentration of cytotoxic B alone giving an effect F, and α = a parameter with value 0 when A and B are mutually exclusive and 1 when A and B are mutually non-exclusive. Synergism is indicated by $CI < 0.8$, additivity by $CI = 0.8$–1.2 and antagonism by $CI > 1.2$; slight synergistic and antagonistic cytotoxic activity are indicated by CI values of 0.8 and 1.2, respectively.

For the application of the Chou and Talalay model cytotoxic agents were used at a fixed dose ratio (e.g. ratio of drug IC_{50}s).

Molecular parameters

The analysis of the different putative molecular mechanisms explaining the observations on cell survival was performed as follows on DU145 cells only. Cells were seeded in 175-cm^2 tissue culture flasks at 1.86×10^6 cells to obtain exponential growth for the duration of the experiments. All investigations were performed in triplicate in separate experiments for all cellular parameters studied (investigations on cell cycle were performed in duplicate).

- *Enzymatic activities and cell cycle analysis*. This was done once the optimal drug sequence was obtained (Schedule A with Taxotere 48 h followed by 5′-DFUR 48 h). Thus, cells were collected following a 48-h exposure to Taxotere (7×10^{-10} M).
- *Apoptosis-related factors*. Levels of Bax and CD95 expression were measured at the end of the optimal sequence (Schedule A). Thus, cells were collected at 96 h in different conditions [drugs together, controls with either medium, or Taxotere alone (7×10^{-10} M) or 5′-DFUR alone (5×10^{-6} M)].

TS activity was measured according to the tritium-release assay described by Spears and Gustavsson [19]. Cytosol (25 µl) was incubated with [^{3}H]dUMP (1 µM final concentration) and 5,10-methylenetetrahydrofolate (0.62 mM final concentration) in a total volume of 55 µl. After 0 (for blank subtraction), 10, 20 and 30 min of incubation at 37°C, the reaction was stopped in ice. Excess [^{3}H]dUMP was removed by adding activated charcoal (300 µl, 15%) containing 4% trichloroacetic acid before 5 min centrifugation at 14 000 g, at room temperature. The ^3H_2O formed during the incubation was then counted in the supernatant by a liquid scintillation

counter (1409 DSA; Wallac, Turku, Finland). Results were expressed as fmol ^3H_2O formed/min/mg protein, based on the linear regression obtained from the incubation times. The sensitivity limit was 10 fmol/min/mg protein. Inter-assay reproducibility was evaluated by repeated analysis of single-use aliquots of a pooled cytosol: $n = 5$, mean = 1, 110 fmol/min/mg protein, SD = 78.59 fmol/min/mg protein, coefficient of variation = 7.08%.

There are two distinct pyrimidine nucleoside phosphorylases present in normal and neoplastic cells: TP, for which the major substrate is thymidine, and uridine phosphorylase (UP), which is responsible for the reversible catalysis of uridine to uracil. As TP is mainly responsible for the catalysis of 5′-DFUR into 5-FU, TP activity was measured in the analyzed samples. Specific inhibitors for TP (TP inhibitor) and UP (PSAU) were applied in order to determine the specific activity of TP in the reaction mixture. PSAU was kindly provided by Dr M. El Kouni (University of Alabama at Birmingham).

The analytical method used for the determination of TP activity was derived from Kubota *et al.* [20]. Cultured cells (10^7) were homogenized in 500 µl lysis buffer [50 mM Tris–HCl (pH 6.8), 1% Triton X-100, 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.02% mercaptoethanol]. The samples were centrifuged at 105 000 *g* for 30 min at 4°C. Protein concentration was determined using the method of Bradford. Supernatants (0.8 mg protein/ml) were incubated for 4 h at 37°C with 10 mM 5′-DFUR and 180 mM potassium phosphate (pH 7.4) ± 100 µM of TP inhibitor or PSAU. The reaction was stopped by the addition of 360 µl of ice-cold methanol to the 120 µl reaction mixture. After removal of the precipitate by centrifugation, an aliquot (dilution of 1/5) of the reaction mixture was applied to the HPLC column (Licrospher 100-RP_{18}). The elution buffer consisted of 50 mM phosphate buffer (pH 6.8) containing 10% methanol. The amount of 5-FU produced was monitored by UV absorbance (262 nm). TP activity was expressed as nmol 5-FU converted/mg protein/h.

Cell cycle was analyzed by FACS according to the Vindelov model [21]. For flow cytometry analysis, 10^6 DU145 cells in exponential growth were treated according to Schedule A and then washed 3 times with citrate buffer. Cell pellets were incubated with 250 µl of trypsin-containing citrate buffer for 10 min at room temperature and then incubated with 200 µl of citrate buffer containing a trypsin inhibitor and RNase (10 min) before adding propidium iodide (200 µl at 125 µg/ml). Samples were analyzed on a Becton Dickinson FACScan flow cytometer using Modfit software, which was also used to determine the percentage of cells in the different phases of the cell cycle. Propidium iodide was excited at 488 nm and fluorescence was analyzed at 620 nm (FL3).

Western blot was performed on total cellular lysates (4×10^6 cells) harvested in 500 µl of lysis buffer Laemmli 1 × (Laemmli 4 ×: 1.6 ml Tris–HCl, 1 M, pH 6.8; 400 mg SDS; 2 ml glycerol; 145 µl β-mercaptoethanol; 12% bromophenol) and heated for 15 min at 95°C. The protein content of cytosolic preparations was determined by the method of Bradford using the Bio-Rad reagent with BSA as a standard. Equal amounts of protein (50 µg/lane) were separated by 12.5% SDS–10% PAGE and transferred onto a nitrocellulose membrane. Prestained molecular weight markers were included in each gel. Membranes were blocked for 60 min in TBS (10 mM Tris–HCl, pH 7.5; 150 mM NaCl) with 5% non-fat milk at room temperature. After blocking, membranes were incubated overnight at 4°C with the appropriate specific anti-human monoclonal antibody (dilution of 1/1000) in TBS with 5% non-fat milk, Bax (mouse anti-human monoclonal anti-Bax antibody; Santa Cruz Biotechnology, Santa Cruz, CA). After washing the membranes 3 times with TBS Tween (5 min each), they were incubated with peroxidase-conjugated secondary antibodies purchased from Dako (dilution of 1/3000; Glostrup, Denmark) at room temperature. A chemiluminescence reaction was performed and the membranes were exposed to ECL hyperfilm according to the manufacturer's instructions (Amersham Pharmacia, Little Chalfont, UK). Quantitative analysis of activities was performed by imaging the autoradiograms and quantitating relative band densities using scan imaging software (Image Master; Pharmacia).

For the detection of the CD95 (APO1-fas) receptor 10^6 cells were trypsinized, washed and exposed to 4 ng/µl of CH11 anti-Fas monoclonal antibody (mAb) for 45 min at 4°C. After two washing steps, cells were resuspended in DMEM containing 1:200 (v/v) goat anti-mouse IgM (Immunotech, Marseilles, France) and incubated for an additional 30 min at 4°C. Cells were then washed twice and cell surface expression of CD95 was assessed by a FACScan. Analysis was carried out on a flow cytometer (FACScan; Becton Dickinson) using CellQuest software. Cells exposed to goat anti-mouse IgM served as negative FITC control. Fas expression was defined as the fluorescence ratio of CH11 Fas mAb:isotype-matched negative control mAb. Relative Fas expression in untreated cells was considered to be 100%.

Statistical analysis

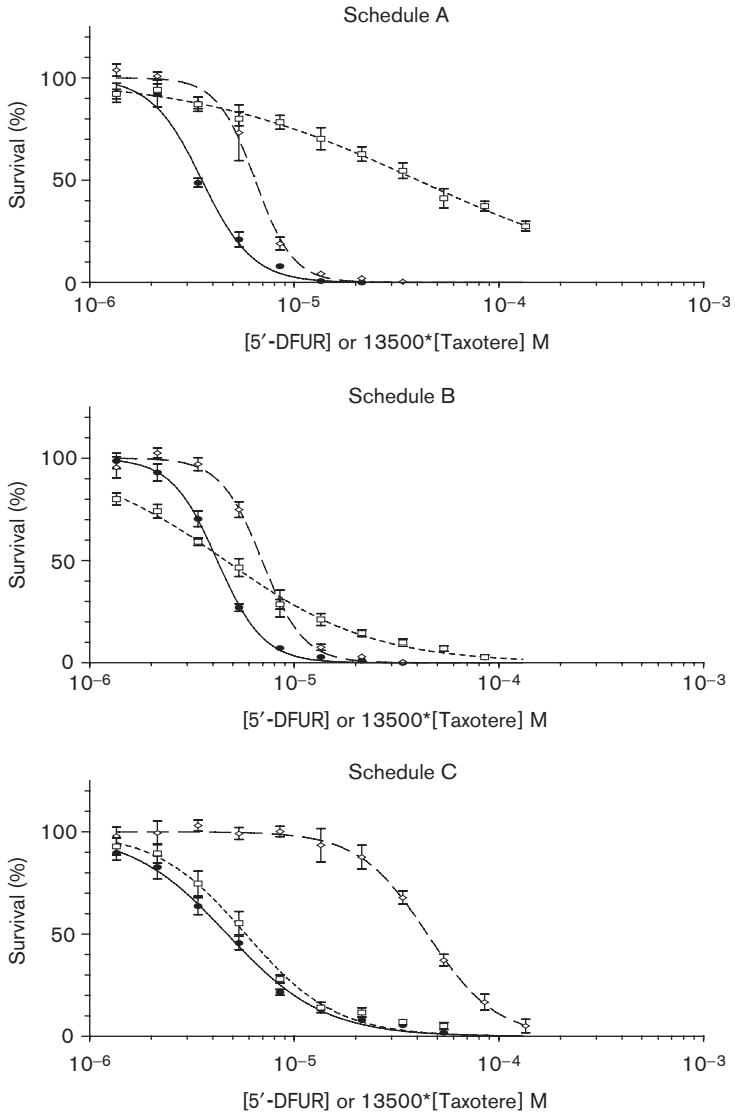
Group comparisons were performed by the non-parametric test of Kruskal–Wallis (more than two groups) or by the Mann–Whitney test (two-group comparison). These tests were performed using Instat software (GraphPad, San Diego, CA).

Results

In the first phase of the study, the three different sequences for drug combination were examined in terms of final cytotoxic effect. Results are shown in Figure 1 and summarized in Table 1 for the CI values. There was a statistical difference in the CI values according to the tested sequence (Table 1). The drug Sequence A, where Taxotere is applied first for 48 h followed by 5′-DFUR 48 h, led to synergistic cytotoxic effects; the other sequences resulted in additivity. A confirmation of the optimal sequence was obtained with PC3 cells (Fig. 2). Combination indexes determined on PC3 cells with the optimal sequence (Taxotere followed by 5′-DFUR) were 0.75 ± 0.05, 0.9 ± 0.1 and 0.6 ± 0.1 (three separate experiments).

Having obtained an optimal sequence allowing a synergistic interaction, we explored different factors which might account for the observed synergistic cytotoxic effects. The impact of Taxotere on cell cycle, and TS activity and TP activity was thus examined. It was found that TP activity is not increased in DU145 cells following pretreatment with Taxotere (Fig. 3); in contrast, Taxotere induces a significant decrease in TS activity (Fig. 3). Regarding the impact of Taxotere on the cell cycle, there was a shift towards an accumulation of cells in the S phase

Fig. 1



Survival curves for DU145 cells for the association Taxotere followed by 5′-DFUR (Schedule A), for the association Taxotere + 5′-DFUR (Schedule B) and for the association 5′-DFUR followed by Taxotere (Schedule C). 5′-DFUR (open squares), Taxotere (open diamonds) and two-drug association (closed circles).

(Fig. 4). Conversely, there were fewer cells in the G_0/G_1 phase after exposure to Taxotere. The proportion of cells in the G_2/M phase was not modified by the treatment with Taxotere.

Molecular factors linked to apoptotic death were then explored following exposure to the optimal combination Schedule A: Taxotere followed by 5′-DFUR. It was noted that the changes in Bax cellular content did not reflect the cytotoxic synergistic effect obtained with the drug combination (Fig. 5). Neither Taxotere nor 5′-DFUR markedly modified the expression of FasR. In contrast there was an increase in CD95 cellular membrane levels following exposure to Taxotere–5′-DFUR, which is in agreement with the supra-additive cytotoxic combination (Fig. 6).

Discussion

The cytotoxic activity of capecitabine is dependent upon the presence of TP which controls, at the target cellular level, the rate of 5-FU delivery. Previous studies by us have underlined the crucial role of TP in sustaining the cytotoxic efficacy of both 5-FU and 5′-DFUR in human tumoral cells transfected with the TP gene and over-expressing TP within a wide range of amplification [22,23]. The study by Sawada *et al.* [11] showed that several cytostatics, such as Taxol, Taxotere, mitomycin C and cyclophosphamide, elevated TP expression in tumor cells. In the same experimental study it was demonstrated that Taxol, in combination with either capecitabine or 5′-DFUR, produced supra-additive antitumoral effects on MX-1 human breast cancer xenografts. An increase in TP activity under the effect of the anti-epidermal growth factor receptor drug Iressa (ZD1839) was also recently reported by us [14]; this upregulation of TP was associated with synergistic cytotoxic effects resulting from the combination of ZD1839 and 5′-DFUR.

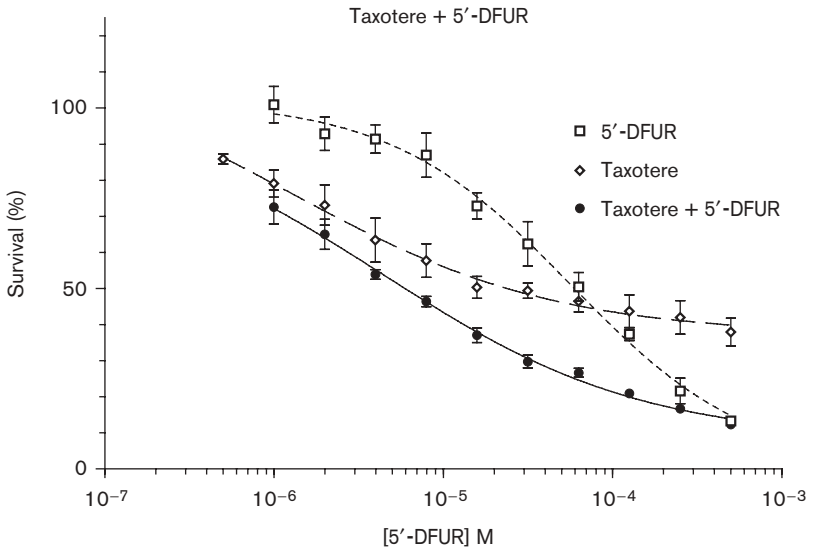
The use of Taxotere brings a real ray of hope to the management of advanced hormone-refractory prostate cancer [15]. On the other hand, an interesting case of a hormone-refractory prostate cancer responding to capecitabine was recently reported [24]. It was thus tempting to check the effects resulting from the Taxotere–5′-DFUR combination on representative human hormone-refractory prostate tumor cell lines, DU145 and PC3. The present preclinical study aimed to examine the impact of the sequence effect between the two drugs and it was shown that synergistic cytotoxic effects were obtained with the combination Taxotere followed by 5′-DFUR. The synergistic combination was observed on the two

Table 1 Drug sequence and cytotoxic effects on the DU145 cell line

Experiment	A	B	C
1	0.71 (S)	1.16 (AD)	0.89 (AD)
2	0.77 (S)	1.3 (AT/AD)	0.87 (AD)
3	0.38 (S)	0.9 (AD)	1.19 (AD)

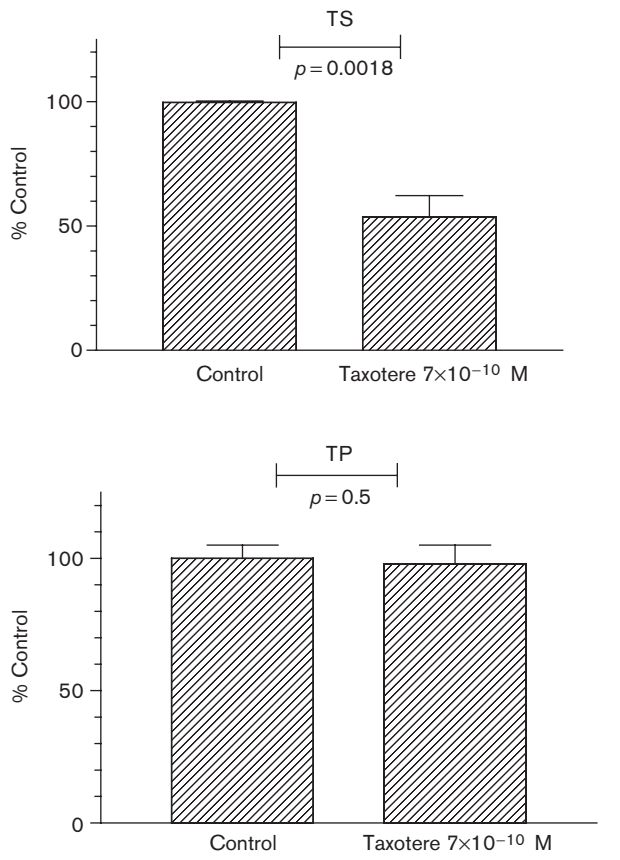
Schedule A: Taxotere alone during 48 h then 5′-DFUR during 48 h. Schedule B: Taxotere + 5′-DFUR during 96 h. Schedule C: 5′-DFUR alone during 48 h then Taxotere during 48 h. Statistical analysis: Kruskal–Wallis test $p = 0.032$. S = synergistic interaction (CI < 0.8); AD = additivity (CI 0.8–1.2) and AT = antagonistic interaction (CI > 1.2). For more details see Materials and methods.

Fig. 2



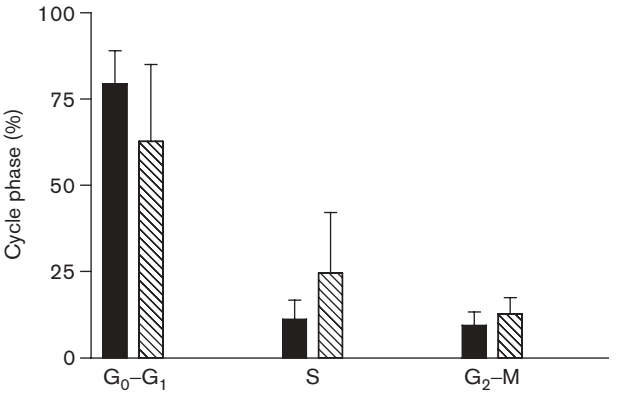
Survival curves for PC3 cells for the association Taxotere followed by 5′-DFUR (Schedule A). 5′-DFUR (open squares), Taxotere (open diamonds) and two-drug association (closed circles).

Fig. 3



Taxotere effects on TS and TP activities after 48 h. Statistical analysis by the Mann–Whitney test.

Fig. 4

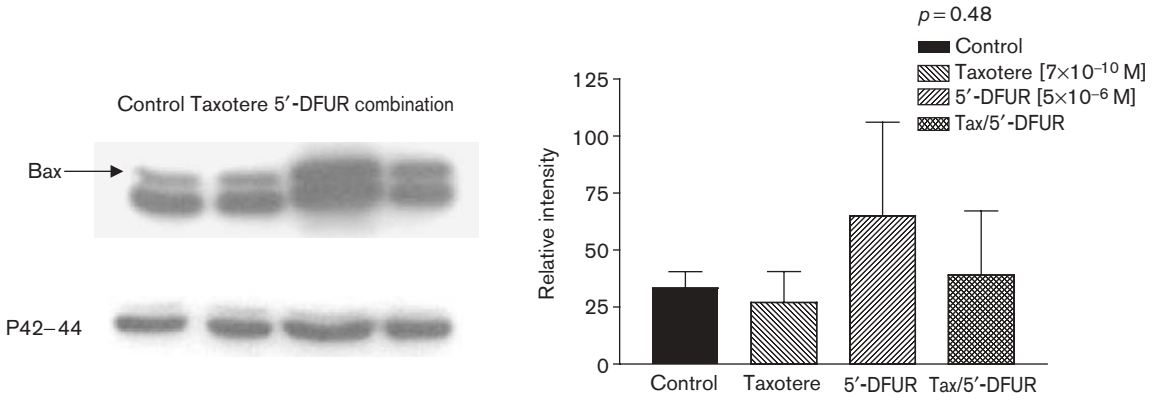


Taxotere effects on cell cycle after 48 h. Control = black and Taxotere = hatched.

hormone-refractory human cancer cell lines DU145 and PC3. It has been previously reported in preclinical and clinical studies that the Fas–FasL system is involved in the antitumoral activity of capecitabine [25,26]. A previous study by Piechocki *et al.* [27] has shown that the induction of apoptosis by Taxotere coincided with an increase in cell FasR expression. Fas–FasL interaction is the extrinsic pathway of apoptotic cell death which is independent of the intrinsic mitochondrial-related pathway, although some cross-talk may occur between these two apoptotic pathways [28]. Taxotere and 5′-DFUR alone slightly trigger CD95–FasR expression on the cell surface. In contrast, FasR levels were found to be increased (+ 30% on average) after combining both drugs. Although this increase was not reaching statistical significance ($p = 0.1$, one-way ANOVA), certainly due to the lack of power of the test regarding the number of samples, this observation suggests that a Fas component was implicated in the apoptosis induction in cells exposed to the double regimen. Bax is one of the key regulators of the mitochondrial-mediated apoptosis required for the release of apoptogens such as cytochrome *c* from mitochondria [29]. The present data did not indicate a significant change of Bax, which might be expected from the supra-additive cytotoxic effect of Taxotere and 5′-DFUR on DU145 cells (Fig. 4). We also examined the expression of Bcl-2, a representative member of mitochondria-related anti-apoptotic factors, but this protein was scarcely measurable in DU145 cells (data not shown).

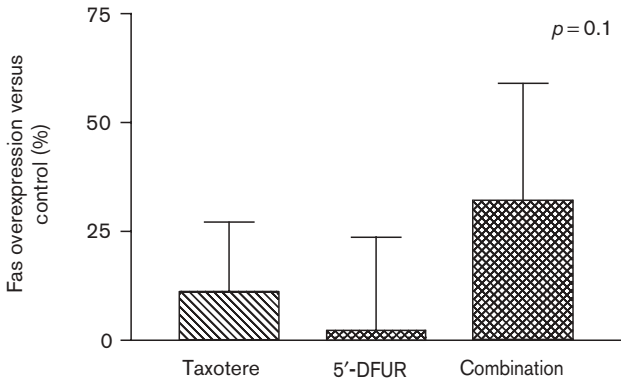
Priority was given to examining Taxotere-induced changes in TP activity in an effort to elucidate the molecular mechanisms underlying the synergistic sequence-dependent combination between this latter drug and 5′-DFUR. DU145 cells showed no change in TP activity following the application of Taxotere. This contrasts with previous findings showing that taxanes can upregulate TP activity [11,12]. The present data thus indicate that a synergistic cytotoxic interaction between Taxotere and 5′-DFUR can occur independently of an upregulation in TP activity. Two Taxotere-induced cellular mechanisms were noted which can explain the supra-additive cytotoxic effects of Taxotere and 5′-DFUR. First, Taxotere treatment led to a marked change in cell cycle profile with a shift towards a higher proportion of cells in the S phase. This cell cycle modification was not strictly related to an increase in cell cycle turnover since there was no change in the G_2/M phase (Fig. 3). On the other hand, it is generally admitted that the activity of antimetabolite drugs, such as 5-FU, is dependent upon cell cycle profile and is more marked on cells being in S phase [30]. This can explain, at least in part, the better activity of 5′-DFUR following Taxotere since this latter drug tended to accumulate cells in S phase. Another cellular impact of Taxotere was the decrease in TS activity observed after application of Taxotere (Fig. 2). This low TS activity is not strictly in contradiction with the concomitant high proportion of cells in the S phase since this elevation in S phase

Fig. 5



Effects of Taxotere, 5′-DFUR and their combination on Bax. Statistical analysis by ANOVA.

Fig. 6



Effects of Taxotere, 5′-DFUR and their combination on FasR. Statistical analysis by ANOVA.

resulted from an accumulation of cells in this phase rather than from an overall increase in cell cycle dynamics. High TS activity has been shown to be a cellular characteristic associated with relative resistance to fluoropyrimidines [31,32]. Consequently, lowering TS activity by administration of Taxotere may be another factor contributing to the supra-additive cytotoxic effect of Taxotere and 5′-DFUR observed in DU145 cells. In total, the present study indicates that the combination Taxotere–5′-DFUR results in a sequence-dependent synergistic cytotoxic interaction on hormone-refractory human prostate cancer cells. Strengthened by molecular analyses, this observation may serve as a preclinical rationale for a next step testing the Taxotere–capecitabine combination at the clinical level.

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