Synergistic cytotoxic interaction in hormone-refractory prostate cancer with the triple combination docetaxel–erlotinib and 5-fluoro-5′-deoxyuridine

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The current reference treatment of hormone-refractory prostate cancer consists mainly of chemotherapy with docetaxel. To improve the management of advanced prostate cancer, one should examine the benefits of adding other agents to docetaxel. We examined the growth inhibitory effects of a triple combination, including the anti-epidermal growth factor receptor drug erlotinib, docetaxel and 5-fluoro-5′-deoxyuridine (the main intermediary metabolite of capecitabine), on the human prostate cancer cell lines PC3 and DU145, which are both devoid of androgen receptors. Marked synergistic cytotoxic effects were observed with the application of the double combination of erlotinib–5-fluoro-5′-deoxyuridine for both cell lines and to a lesser magnitude with the triple combination. For PC3 cells, all conditions resulted in synergistic interactions. The combination between erlotinib and docetaxel resulted in an approximately 50% reduction in thymidylate synthase activity (the molecular target of 5-fluorodeoxyuridine monophosphate, the active capecitabine anabolite) with an higher impact observed with DU145 cells than with PC3 cells. Neither erlotinib nor docetaxel alone displayed marked effects on thymidine phosphorylase activity (the enzyme that governs at the cellular level the final and crucial step in the activation cascade of capecitabine), in contrast to their combination that resulted in a strong increase in thymidine phosphorylase activity in PC3 cells. These data may serve as a rational basis for setting up clinical trials in advanced prostate cancer combining epidermal growth factor receptor-targeting agents like erlotinib together with docetaxel and capecitabine. *Anti-Cancer Drugs* 17:807–813 © 2006 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2006, **17**:807–813

Keywords: anticancer drug combination, 5-fluoro-5′-deoxyuridine, docetaxel, erlotinib, prostate cancer

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Received 22 March 2006 **Accepted** 2 May 2006

Introduction

Numerous studies are underway to assess the role of chemotherapy for patients at various stages of prostate cancer [1]. One of the current treatments of reference in hormone-refractory prostate cancer consists of chemotherapy with docetaxel (Taxotere) combined with prednisone [2]. A recent controlled study by Tannock and coworkers [3] strengthened the importance of the use of docetaxel in prostate cancer by showing that as compared with patients treated by mitoxantrone, those receiving docetaxel had a hazard ratio for death of 0.76 ($P = 0.009$). The following steps of improvement in the management of advanced prostate cancer should examine the benefits of adding other agents to docetaxel. We recently observed [4] that the combination docetaxel–5-fluoro-5′-deoxyuridine (5′DFUR) resulted in a sequence-dependent synergistic cytotoxic interaction on hormone-refractory human prostate cancer, pointing to clinical application of a docetaxel–capecitabine combination (5′DFUR being the main circulating anabolite of capecitabine).

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Numerous molecular mechanisms are linked to the transition to hormone-refractory prostate cancer [5]. The involvement of the human epidermal growth factor receptor (EGFR) signaling family of proteins seems to be the most important [6,7]. We previously observed a synergistic cytotoxic interaction when combining EGFR targeting with 5′DFUR [8]. These supra-additive effects could be explained by an upregulation of thymidine phosphorylase (TP) by the anti-EGFR drug [8]. TP is the enzyme that governs at the cellular level the final and crucial step in the activation cascade of capecitabine [9]. Interestingly, other authors have found that taxanes were able to upregulate the tumor activity of TP and have shown a synergistic cytotoxic activity when combining taxanes with capecitabine [10,11]. This synergy was recently confirmed at the clinical level giving a new standard in the management of advanced breast cancer [12].

On the basis of the above considerations, it was judged as interesting to examine a triple combination including the

clinically relevant anti-EGFR drug erlotinib (Tarceva) together with docetaxel and 5′DFUR on the human prostate cancer cell lines PC3 and DU145, both of which are devoid of androgen receptors. The sequence of drugs tested in the present study was based on previous experiments showing a sequence-dependent synergistic cytotoxic interaction with docetaxel followed by 5′DFUR [4]. For this reason, in the present study, the molecular factors examined so as to explain the observed drug interactions were the key enzymes for 5′DFUR (capecitabine) activity: TP, thymidine synthase (TS) and dihydropyrimidine dehydrogenase (DPD).

Material and methods

Chemicals

Erlotinib (pure product) was kindly provided by Roche (Basel Switzerland). Docetaxel was the clinical formulation and was provided by the pharmacy of our institution. Dulbecco's modified Eagle's medium, penicillin, streptomycin and glutamine were purchased from Whittaker (Verviers, Belgium). Fetal bovine serum was from Dutscher (Brumath, France). 5′DFUR, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethylsulfoxide were purchased from Sigma (St Quentin Fallavier, France) and were of the highest purity available.

Cell lines

Two human prostatic tumor-derived cell lines DU145 (EGFR content 250 000/cell) and PC3 (EGFR content 170 000/cell), both devoid of androgen receptors, were used. Cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 600 μg/l insulin, 500 μg/l transferrin, 50 000 units/penicillin and 80 μmol/l streptomycin in a fully humidified incubator (Sanyo, Osaka, Japan) at 37°C in an atmosphere containing 8% CO_2.

Drug exposure

Cytotoxicity experiments

Cells were seeded in microtitration plates (2000 cells per well). Forty-eight hours after cell seeding, erlotinib (concentration range: 10^{-7} to 10^{-5} mol/l) and/or docetaxel (concentration range: 4×10^{-10} to 4×10^{-9} mol/l) were added for 48 h. At the end of exposure to docetaxel and/or erlotinib, 5′DFUR (concentration range: 10^{-6} to 10^{-3} mol/l) was added for 48 h. The MTT test was performed at the end of exposure to 5′DFUR. This fixed sequence of treatment with erlotinib–docetaxel and 5′DFUR was dictated by our previous experience with two-drug combinations [4,8].

Thymidylate synthase and thymidine phosphorylase measurement

Cells were seeded in Petri dishes (1.23×10^6 cells); 48 h later, erlotinib (final concentration 4×10^{-6} mol/l) and/or docetaxel (final concentration 1.7×10^{-9} mol/l) were added for 48 h. Cells were harvested at the end of exposure to erlotinib and/or docetaxel and cell pellets were frozen.

Cytotoxicity measurement

Growth inhibition was assessed 4 days after incubation with the first drug (erlotinib and/or docetaxel) by the MTT test [13], as described below. Cells were washed with phosphate-buffered saline and incubated with MTT. After 2 h of incubation, MTT was released and coloration was revealed by the addition of 100 μl of dimethylsulfoxide. Absorbance at 450 nm was measured using a microplate reader (Labsystems, Helsinki, Finland). The results were expressed as the relative percentage of absorbance compared with controls without drug. Experimental conditions were tested in sextuplicate (six wells of the 96-well plate per experimental condition) and experiments were performed in quadruplicate at distant intervals. The dose–effect curves were analyzed using Prism software (GraphPad Software, San Diego, California, USA). The antiproliferative activity was expressed by the IC_{50} value (concentration leading to 50% cell survival).

Combination index calculations

The cytotoxic effects obtained with the different drug combinations (erlotinib–docetaxel, erlotinib–5′DFUR, docetaxel–5′DFUR and erlotinib–docetaxel–5′DFUR) were analyzed according to the Chou and Talalay [14] method using the Calcusyn software (Biosoft, Cambridge, UK) by means of an automatically computed combination index (CI). In case of three-drug combination, the Calcusyn program gives the CI for every experimental point, but does not allow us to draw a regression line from the calculated values.

Synergism is indicated by CI below 0.8, additivity by CI values between 0.8 and 1.2, and antagonism by CI above 1.2; slight synergistic and additive cytotoxic activities are indicated by CI values of 0.8 and 1.2, respectively.

Thymidylate synthase activity assay

TS activity was measured according to the tritium-release assay described by Spears and Gustavsson [15]. Cytosol (25 μl) was incubated with [^{3}H]dUMP (1 μmol/l final concentration) and 5,10-methylenetetrahydrofolate (0.62 mmol/l 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 on ice. Excess [^{3}H]dUMP was removed by adding activated charcoal (300 μl, 15%) containing 4% trichloroacetic acid before a 5-min centrifugation at 14 000 *g*, at room temperature. The ^3H_2O formed during the incubation was then counted in the supernatant using a liquid scintillation counter (1409 DSA; Wallac, Turku, Finland).

The results were expressed as femtomoles of ^3H_2O formed per minute per milligram of protein, on the basis of the linear regression obtained from the incubation times. The sensitivity limit was 10 fmol/min/mg protein. Interassay reproducibility was evaluated through repeated analysis of single-use aliquots of a pooled cytosol: $n = 5$, mean = 1110 fmol/min/mg protein, SD = 78.59 fmol/min/mg protein, coefficient of variation = 7.08%.

Thymidine phosphorylase activity assay

Two distinct pyrimidine nucleoside phosphorylases are present in normal and neoplastic cells: TP, for which the major substrate is thymidine, and uridine phosphorylase, which is responsible for the reversible catalysis of uridine to uracil. As TP is mainly responsible for the catalysis of 5′-DFUR into 5-fluorouracil (5FU), TP activity was measured in the analyzed samples. Specific inhibitors for TP (TP inhibitor from Taiho, Japan) and uridine phosphorylase [phenylselenerylacycouridine (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.* [16]. Cultured cells (10^7) were homogenized in 500 µl lysis buffer [50 mmol/l Tris–HCl (pH 6.8), 1% Triton X-100, 2 mmol/l 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.02% mercaptoethanol]. The samples were centrifuged at 105 000 *g* for 30 min at 4°C. Protein concentrations were determined using the method of Bradford. Supernatants (0.8 mg protein/ml) were incubated for 4 h at 37°C with 10 mmol/l 5′-DFUR and 180 mmol/l potassium phosphate (pH 7.4) ± 100 µmol/l 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; Agilent Technologies, Santa Clara, California, USA). The elution buffer consisted of 50 mmol/l phosphate buffer (pH 6.8) containing 10% methanol. The amount of 5FU produced was monitored by ultraviolet absorbance (262 nm). TP activity was expressed as nanomoles of 5FU converted per milligram of protein per hour.

Statistical analysis

Group comparisons were made according to the Mann and Whitney test (Instat; GraphPad).

Results

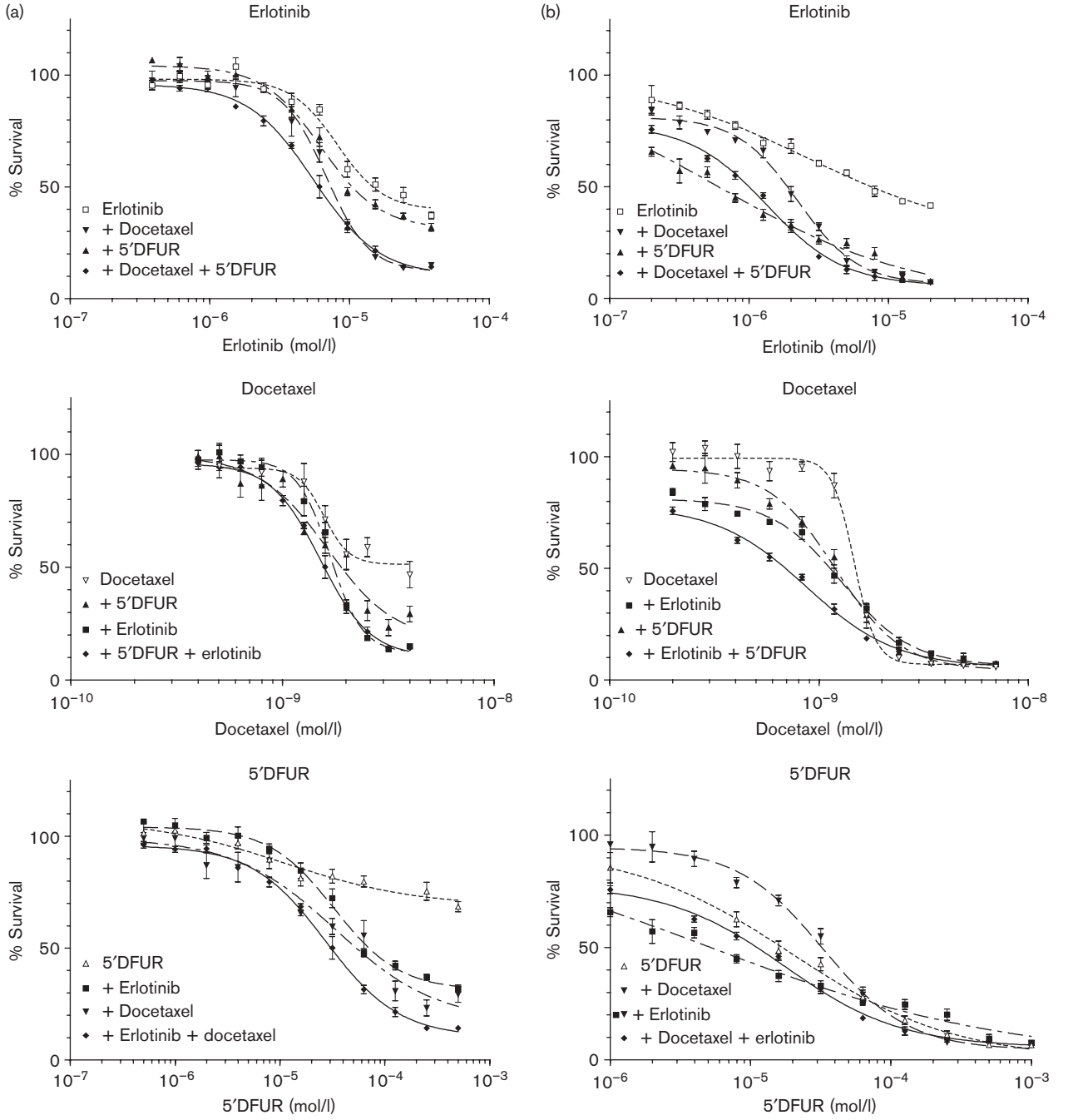
Figure 1 shows the effects on cell growth of drugs given alone or in combination. A specific representation was adopted so as to allow visualization of the relative contribution of each drug to the common effect in the associations. In this way, it appears that for PC3 cells (Fig. 1a) the cytotoxic effect resulting from the triple combination exceeds that observed with each double combination. The advantage of the triple combination was, however, less apparent on DU145 cells, in which docetaxel does not add much more in cytotoxicity to that already obtained with the erlotinib–5′DFUR combination (Fig. 1b). It is noteworthy that AUC_{50} ($IC_{50} \times 96$ h) for docetaxel calculated from our results (1.5×10^{-6} mol × h/l) compares well with published pharmacokinetic data (5×10^{-6} mol × h/l) [17,18] and the erlotinib concentration (4.65×10^{-6} mol/l) matches well with the reported concentration at the steady state (5×10^{-6} mol/l) in treated patients [19]. Two characteristic CI graphs are depicted in Fig. 2(a and b) for the triple combinations on each tested cell line. The whole isobolographic analysis of the combinations according to the Chou and Talalay procedure is summarized in Fig. 3. From these data, it appears that strong synergistic cytotoxic effects were observed with the application of the double-combination erlotinib–5′DFUR for both cell lines and to a lesser degree with the triple combination. For PC3 cells, all conditions resulted in synergistic interactions.

Intracellular molecular factors were examined. The combination between erlotinib and docetaxel resulted in an approximately 50% reduction in TS activity ($P = 0.0189$), with a higher impact observed in DU145 cells than in PC3 cells (Fig. 4). Neither erlotinib nor docetaxel alone displayed marked effects on TP activity, in contrast to their combination resulting in a strong increase in TP activity noted with PC3 cells (Fig. 5, $P = 0.037$).

Discussion

The introduction of docetaxel into the clinic has opened a new era of improvement in the management of advanced hormone-refractory prostate cancer [3,20,21]. Febbo and coworkers [22] have recently reported on neoadjuvant use of docetaxel before medical prostatectomy in prostate cancer patients with high-risk localized lesions. They observed that treatment was well tolerated, and often resulted in a prostate-specific antigen decline of more than 50% and in significant tumor volume diminution [22]. As recently underlined by Engels *et al.* [21], current research into docetaxel-based chemotherapy should include drug combinations so as to improve outcome with an acceptable feasibility. Cases of hormone-refractory prostate cancer responding to capecitabine have been reported [23]. Spicer and coworkers [24] recently published the results of a phase II trial using capecitabine in advanced prostate cancer. The authors concluded that capecitabine as a single drug had limited antitumor activity, but appeared to modulate tumor biology (prostate-specific antigen decrease) and they

Fig. 1

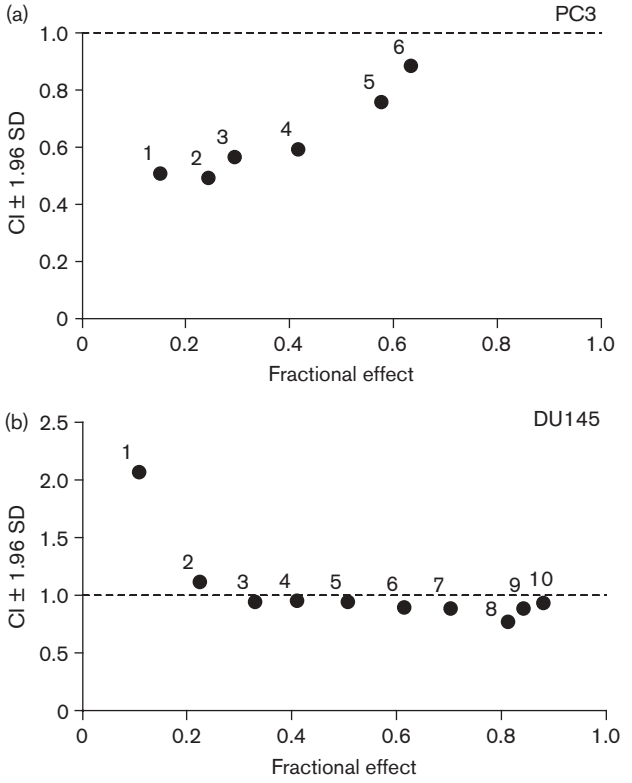


Dose–response curves. Shows a characteristic example of the dose–response curves for PC3 (a) and DU145 cells (b) exposed to erlotinib alone, docetaxel alone, both during 96 h followed by 5-fluoro-5′-deoxyuridine (5′DFUR) alone during 48 h and their combinations. The specific representation is adopted so as to allow visualization of the relative contribution of each drug to the common effect in the associations. Experiments were performed three times for each cell line.

considered that further evaluation of combinations containing capecitabine was justified in hormone-refractory prostate cancer [24].

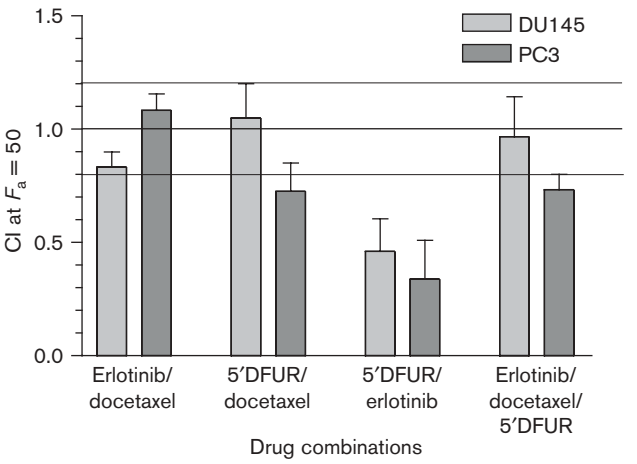
Membrane receptors of the human EGFR family are associated with the hormone-independency of prostate cancer [25]. A recent experimental study by Festuccia and coworkers [26] suggested that blockade of the EGFR pathway can attenuate the invasive potential of prostate cancer cells. Clinical results arising from phase I studies targeting EGFR in advanced prostate cancer were encouraging [27]. A recent phase II study of the EGFR

Fig. 2



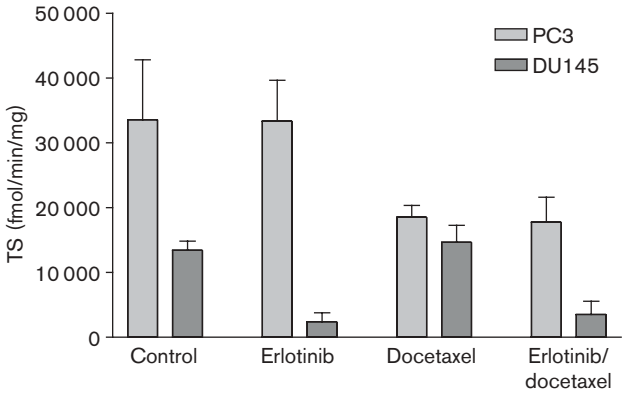
Isobolograms. Shows a typical example of an isobolographic analysis of the triple-drug combination for PC3 (a) and DU145 (b) cells. The curves were automatically constructed by the Compusyn software and the numbers of the combination are indicated above the dots (1 means first combination, 2 means second combination, etc.).

Fig. 3



Combination indexes (CIs). This summarizes the CI values for the different drug combinations and for the 50% growth inhibitory effect ($F_a = 50$). The horizontal solid line for CI = 1 h is the strict limit for additivity and the two other lines at 0.8 and 1.2 represent the envelope of additivity. CI < 0.8 means synergy, 0.8 < CI < 1.2 additivity and CI > 1.2 antagonism. Results are the summary of three experiments for each cell line.

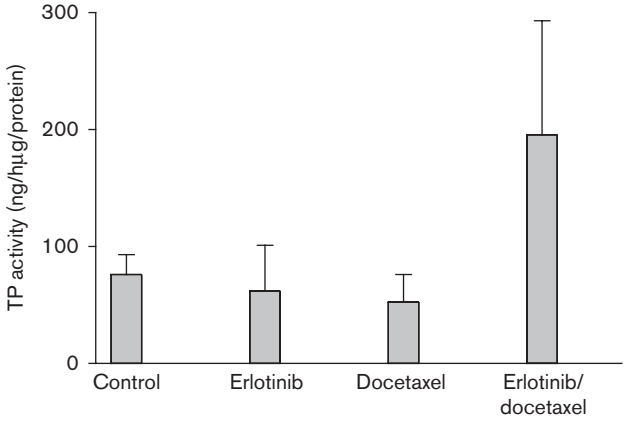
Fig. 4



Thymidilate synthase (TS) activities. This illlustrates the effect of erlotinib, docetaxel and their combination on TS activity in DU145 and PC3 ($P = 0.0189$ between erlotinib–docetaxel and controls, grouped values for DU145 and PC3 cells). Experiments were performed three times for each cell line.

tyrosine kinase inhibitor, gefitinib, however, gave relatively disappointing clinical results as a single therapy [28]. This background stimulated the present experimental study aimed at examining the cytotoxic effect resulting from the triple combination docetaxel–erlotinib and 5′DFUR (key anabolite of capecitabine) on two representative hormone-refractory human prostate cancer cell lines PC3 and DU145. Final cytotoxic effects combined with an isobolographic analysis indicated a supra-additive interaction when associating the three drugs as shown in PC3 cells with the fixed sequence (docetaxel and erlotinib before 5′DFUR). Previous studies, performed by our laboratory [4,8], pointed out that EGFR targeting with a tyrosine kinase inhibitor or with docetaxel was able to develop synergistic cytotoxic effects with 5′DFUR and to impact on key enzymes of the fluoropyrimidine pathway. It was thus observed that the use of an EGFR tyrosine kinase inhibitor induced a marked decrease in TS activity and an increase in TP activity [8]. Docetaxel was not found to notably modify TP activity, but was able to decrease TS activity [4]. The present study indicates that the synergistic cytotoxicity resulting from the triple-drug combinations could be attributed, at least in part, to a favorable combinatory effect on either TS activity or TP activity (Figs 4 and 5). Thus, a more than 50% reduction in TS activity than in the control was observed following the application of tandem erlotinib–docetaxel in DU145 cells (Fig. 4). TS activity has been shown to be a cellular characteristic associated with relative resistance to fluoropyrimidines

Fig. 5



Thymidine phosphorylase (TP) activities. This illustrates the effect of erlotinib, docetaxel and their combination on TP activity in PC3 cells ($P=0.037$, between erlotinib–docetaxel and controls). Experiments were performed three times for each cell line.

including 5′DFUR [29,30]. Consequently, lowering TS activity by administration of both drugs may be a factor contributing to the slight supra-additive cytotoxic effects of the triple-drug combination observed in DU145 cells. Of note, TP activity was increased more than 2-fold in PC3 cells in the presence of the erlotinib–docetaxel combination (Fig. 5). An elevation in TP activity may permit more 5FU to be intracellularly delivered from its prodrug 5′DFUR [9]. This observation is fully consistent with previously published reports showing that higher levels of TP in the target cells are related to an increase in the cytotoxic effect of capecitabine, 5′DFUR and 5FU [31–33]. Previous data have shown that DPD expression can modulate the cytotoxic activity of 5′DFUR [34]. DPD activity was low and at the limit of detection in the tumor cell lines presently investigated (unshown data). The three-drug sequence presently investigated was logically in line with our previous experiments with two-drug combinations [4,8]. To test other sequences with the presently investigated drugs was outside the scope of the study. The present data describe an original combination between docetaxel–erlotinib and 5′DFUR that can result in supra-additive cytotoxic effects on hormone-refractory human prostate cancer cell lines. The impact on cellular determinants of Xeloda activity has been examined and may provide at least a part of the explanation of the mechanistics for the observed effects on cell survival. These data may serve as a rational basis for setting up clinical trials in advanced prostate cancer combining EGFR-targeting agents like erlotinib with docetaxel and capecitabine. This approach has the clinical advantage of potential synergistic antitumor effects and the benefit of the presence of two oral drugs (erlotinib and capecitabine) in this three-drug combination.

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