Anti-Cancer Drugs 2002, **13,** pp. 1011–1016

Preclinical report

Synergistic and antagonistic combinations of drugs in human prostate cancer cell lines *in vitro*

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Microtubulin binding agents such as docetaxel have significant preclinical and clinical activity in the treatment of hormone-refractory prostate cancer. We have previously used median-effect analysis *in vitro* to define both synergistic and antagonistic drug combinations which may be of value in management of human disease. These studies extend our findings in defined prostate cancer cell lines. A semi-automated microtiter culture system was used. Docetaxel was combined with 18 other agents, incubated with DU 145, LnCaP or PC 3 prostate cancer cell lines for 72 h and the cells then incubated with MTT to determine cytotoxic effect. Both doublet and triplet combinations were examined. Synergy and antagonism as measured by the combination index were determined for each combination. The non-mutually exclusive criterion was applied. Docetaxel demonstrated cytotoxic additive effects or synergy with *cis*-retinoic acid, cyclosporin A and vinorelbine in all three cell lines. Docetaxel combined with either epirubicin or doxorubicin displayed cytotoxic synergistic effects in hormone-refractory DU 145 and PC 3 cell lines. In contrast, drugs which have been combined clinically to treat hormone-refractory prostate cancer, i.e. cisplatin, carboplatin or etoposide, were antagonistic when combined with docetaxel. We conclude that combinations of docetaxel with either *cis*-retinoic acid or vinorelbine may offer an enhanced cytotoxic effect in the management of hormone-refractory prostate cancer and need to be evaluated for therapeutic effect. The combination of docetaxel with an anthracycline was also synergistic in the two hormone-refractory cell lines, DU 145 and PC3, thus suggesting a potential role in advanced disease after endocrine failure. Combinations of docetaxel with platinum or etoposide may lead to subadditive effects in treatment. [© 2002 Lippincott Williams & Wilkins.]

Key words: Docetaxel, median-effect analysis, prostate cancer cell lines, synergism.

Supported in part from grants from the Don Monti Foundation and Aventis Pharmaceuticals.

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Introduction

The use of cytotoxic chemotherapeutic agents in the management of hormone-refractory prostate cancer has been believed to be of marginal benefit with concerns that toxicity might outweigh benefits.[1] Mitoxantrone has been demonstrated to have some palliative effect in this disease with no change in survival compared to corticosteroids alone[2,3] and has served as the recognized standard chemotherapy for this disease in the US. However, recent studies have suggested that drugs which interfere with the cancer cell's tubulin functions, e.g. estramustine, taxanes and vinca alkaloids, can exert clinically useful responses with reasonable quality of life.[4–12] Docetaxel is especially attractive as it has a wide antitumor spectrum, is more active than paclitaxel in prostate cancer cell lines *in vitro*[13] and has shown preclinical synergism with a variety of agents.[13] In addition, docetaxel is active both as a single agent and in combination with estramustine.[7,8,14,15] Therefore, a drug regimen incorporating such agents would logically be the basis of treatment of hormone-refractory disease.

The traditional search for new combinations of agents for prostate cancer has been to clinically combine drugs and then test for efficacy in man. However, the number of combinations of agents which can be tested in phase II trials is limited by clinical resources and the numerous potential permutations. This empiric approach also presumes that the new combination would be synergistic or at least additive in the clinical setting. However, it is also possible that the new combination may be antagonistic and that the combination would then be less active clinically than either agent alone or than the sequential application of such drugs. We have previously demonstrated such antagonism exists in

both breast and prostate cell lines *in vitro* by evaluating the combination of etoposide with paclitaxel and the combination of vinorelbine with gemcitabine.[16] In contrast, paclitaxel and vinorelbine demonstrate sequence-dependent synergistic effects in breast cancer cell lines[17] and clinically significant activity when used sequentially.[18] Using three different prostate cancer cell lines, i.e. LnCaP with a mutated androgen receptor,[19] and DU 145 and PC3 which are androgen-independent,[20] we have explored new combinations of agents with docetaxel using the technique of median-effect analysis[21,22] with the intent of identifying new potentially clinically useful treatments. In our previous studies, we have validated this approach against classical isobologram analysis and the technique allows up to three different agents to be studied simultaneously.[13]

Materials and methods

Reagents, tissue culture techniques and median-effect analysis were performed as previously described.[13,16,17,22] In brief, the drugs were obtained in analytic grade from the following sources: cetrorelex (Astra Medica, Frankfurt, Germany), docetaxel (Aventis Pharmaceuticals, Bridgewater, NJ), epirubicin (Calbiochem, San Diego, CA), estramustine (Pharmacia, Peapack, NJ), hydroxyflutamide (Schering Pharmaceuticals, Kenilworth, NJ), PSC 833 (Novartis Pharma Schweiz, Bern, Switzerland) and vinorelbine (Glaxo SmithKline, Research Triangle, NC). The following agents were obtained from Sigma-Aldrich (St Louis, MO): carboplatin, cisplatin, *cis*-retinoic acid, coumadin, cyclosporin A, disulfiram, doxorubicin, etoposide, 5-fluorouracil, mitoxanthrone, *trans*-retinoic acid and vinblastine. Dexrazoxane and procarbazine were obtained from commercial stock.

The three cell lines, LnCaP, DU 145 and PC 3, were obtained from the ATCC (Rockville, MD). Cells used for these experiments had less than 30 passages and were repetitively tested for mycoplasma. The cells were grown to confluence in T 150 tissue culture flasks (Corning Glass Works, Corning, NY) using McCoy's 5A media (Gibco/ BRL, Rockville, MD) for LnCaP, and RPMI 1640 media (Gibco/BRL) for DU 145 and PC 3 with 10% fetal calf serum, supplemented with 2 mM/l glutamine, penicillin and streptomycin at an atmosphere of 5% CO_2. The cells were grown to confluence, treated with 0.05% trypsin and 0.53 mM EDTA (Gibco/BRL) for 10 min, washed, counted, and aliquoted at 5000–8000 cells/well in a total volume of 200 μl/well using 96-well dishes (Falcon 3072; Baxter-Scientific, McGraw Park, IL). The cells were allowed to adhere to the bottom of the plates for 24 h in complete media and then exposed to drug or control in fresh media for 72 h as previously described.[13,16] The cells were harvested, washed and exposed to MTT (Sigma-Aldrich) using a Bio-Rad 3550 microplate reader (Bio-Rad, Hercules, CA) and absorption measured at 595 nm.[16] As previously described, the IC_{50} (the concentration required for 50% inhibition) was determined using the EZ ED_{50} program (Perrella Scientific, Conyers, CA) with all values the means of three to four experiments.

Docetaxel was then combined with the various listed drugs both as doublet combinations and as triplet combinations, incubated, and subjected to the MTT assay as previously described.[13,16] Fixed drug combinations from 0.0156 to 8*N*, where *N* is a value close to the IC_{50}, were evaluated using a minimum of 10 data points which were each repeated in triplicate. The combinations were evaluated for synergism, additive effects or antagonism by median-effect analysis[21] which is based upon the Hill equation, using the computer program of Chou and Chou.[22] In brief, the cytotoxic effects of the drug combination are described by the equation $f_a/f_u=[D/D_m]^m$ where f_a is the fraction of cells affected, f_u is the fraction of cells not affected $(1-f_a)$, D is the dose of drug, D_m is the dose of drug to cause the median effect and m is the slope of the median-effect curve. The combination index (CI) is derived from this relationship with $CI<1$ indicating synergism, $CI=1$ additive effects and $CI>1$ antagonism.[21] We have defined additive effect to be any CI result which is within 1 SD of unity. The model is further modified by an interaction term α which is 0 for drugs with no biologic interaction and is 1 for an interaction (nonexclusive assumption). This interaction is determined by plotting log dose versus log $[f_a/(1-f_a)]$ which gives parallel curves for no biologic interaction and convergence for interaction.[21]

Results

The model is most accurate at the f_{a50}, the point that the drugs affect 50% of the cells. Plots of log dose versus log $[f_a/(1-f_a)]$ indicated that there was interaction between the various agents studied, thus leading to a modification of the CI value by the nonexclusive assumption.[21] This is also a more conservative criterion for assigning synergism. Table 1 lists the IC_{50} for the cell lines with a 72-h

Table 1. IC_{50} (μM/l) values for a 72-h incubation of the listed agent in the three prostate cell lines

Drug	DU 145	PC 3	LnCaP
Aspirin	1210.75 ± 327.22	1587.67 ± 213.28	810.95 ± 106.21
Cisplatin	4.65 ± 1.46	232.08 ± 21.20	5.95 ± 1.68
Carboplatin	92.96 ± 6.57	79.81 ± 9.27	40.91 ± 3.76
Cetrorelix	43.28 ± 4.94	39.79 ± 18.41	10.25 ± 3.03
Coumadin	836.42 ± 70.22	910.65 ± 194.01	481.32 ± 141.34
Cyclosporin A	6.81 ± 0.85	7.36 ± 1.69	4.79 ± 1.11
Disulfiram	83.40 ± 11.25	51.71 ± 8.21	67.61 ± 14.21
Dexrazozane	168.22 ± 26.14	382.21 ± 62.69	213.43 ± 34.77
Doxorubicin	0.0069 ± 0.0012	0.0258 ± 0.0075	0.0202 ± 0.0005
Docetaxel	0.47 ± 0.03	0.25 ± 0.02	0.28 ± 0.02
Epirubicin	0.42 ± 0.13	0.49 ± 0.15	0.28 ± 0.03
Estramustine	20.52 ± 2.07	15.19 ± 0.46	10.97 ± 1.685
Etoposide	0.2782 ± 0.0031	0.1218 ± 0.0195	2.42 ± 0.19
5-Fluorouracil	5.29 ± 1.60	26.97 ± 5.03	6.23 ± 0.26
Hydroxflutamide	164.93 ± 36.40	242.68 ± 58.31	92.11 ± 5.87
Procarbazine	2024.48 ± 267.32	527.15 ± 159.49	432.18 ± 10.42
Cis-retinoic acid	57.68 ± 9.94	51.32 ± 5.48	3.03 ± 8.38
Trans-retinoic acid	40.86 ± 5.06	37.99 ± 1.37	23.05 ± 9.42
Vinblastine	0.0025 ± 0.0009	0.0044 ± 0.0008	0.0072 ± 0.0008
Vinorelbine	0.0052 ± 0.0004	0.0406 ± 0.0112	3.35 ± 0.32

Values (in μM) are mean ± SD of three to eight experiments.

Table 2. Doublet combinations of drugs testing for synergism or antagonism in the three prostate cell lines (CI values shown for f_{a50} and 72-h incubations)

Agents studied	DU 145	PC 3	LnCaP
Docetaxel + cisplatin	1.94 ± 0.04	1.81 ± 0.04	1.03 ± 0.01
Docetaxel + carboplatin	1.35 ± 0.04	1.86 ± 0.04	1.40 ± 0.02
Docetaxel + cetrorelix	0.81 ± 0.09	1.32 ± 0.04	3.47 ± 0.08
Docetaxel + coumadin	2.45 ± 0.05	2.97 ± 0.03	1.28 ± 0.06
Docetaxel + doxorubicin	0.66 ± 0.07	0.83 ± 0.06	3.51 ± 0.07
Docetaxel + etoposide	6.12 ± 0.53	9.94 ± 1.73	6.15 ± 0.69
Docetaxel + vinblastine	2.55 ± 0.02	5.19 ± 0.37	5.95 ± 0.70
Docetaxel + vinorelbine	0.60 ± 0.03	0.96 ± 0.02	0.83 ± 0.06
Docetaxel + aspirin	3.37 ± 0.26	2.34 ± 0.20	1.05 ± 0.13
Docetaxel + *trans*-retinoic acid	2.56 ± 0.65	1.73 ± 0.21	1.37 ± 0.11
Docetaxel + *cis*-retinoic acid	0.77 ± 0.10	0.97 ± 0.12	0.93 ± 0.06
Docetaxel + 5-fluorouracil	0.82 ± 0.31	2.27 ± 0.82	2.25 ± 0.76
Docetaxel + procarbazine	2.74 ± 0.31	0.58 ± 0.19	1.25 ± 0.12
Docetaxel + dexrazoxane	1.33 ± 0.04	1.72 ± 0.05	1.79 ± 0.03
Docetaxel + cyclosporin A	0.54 ± 0.04	0.33 ± 0.04	0.99 ± 0.04
Docetaxel + disulfiram	0.72 ± 0.17	0.75 ± 0.03	2.89 ± 0.14
Docetaxel + epirubicin	0.76 ± 0.06	0.81 ± 0.04	1.18 ± 0.06
Docetaxel + hydroxyflutamide	2.10 ± 0.02	0.27 ± 0.02	1.75 ± 0.05

Mean CI ± SD for two to four experiments, three points per well.

incubation of each drug evaluated. Table 2 displays the CI values obtained in all three prostate cell lines when docetaxel was incubated with the various listed agents for 72 h. Synergism of a couplet with docetaxel in all three cell lines was only seen for vinorelbine with additive effects for *cis*-retinoic acid and for cyclosporin A. However, the cell lines vary in their growth-dependent properties. For the androgen-dependent LnCaP cell line, docetaxel was synergistic with vinorelbine and additive with *cis*-retinoic acid and also with cyclosporin A. A putative reversal agent of drug resistance, disulfiram,[23] led to antagonistic effects in this cell line. In contrast, in the androgen-independent PC 3 and DU 145 lines, doxorubicin, epirubicin, cyclosporin A, disulfiram and vinorelbine, but not vinblastine, demonstrated consistent synergy. Figure 1 illustrates the entire drug interaction in DU 145 prostate cancer cells for

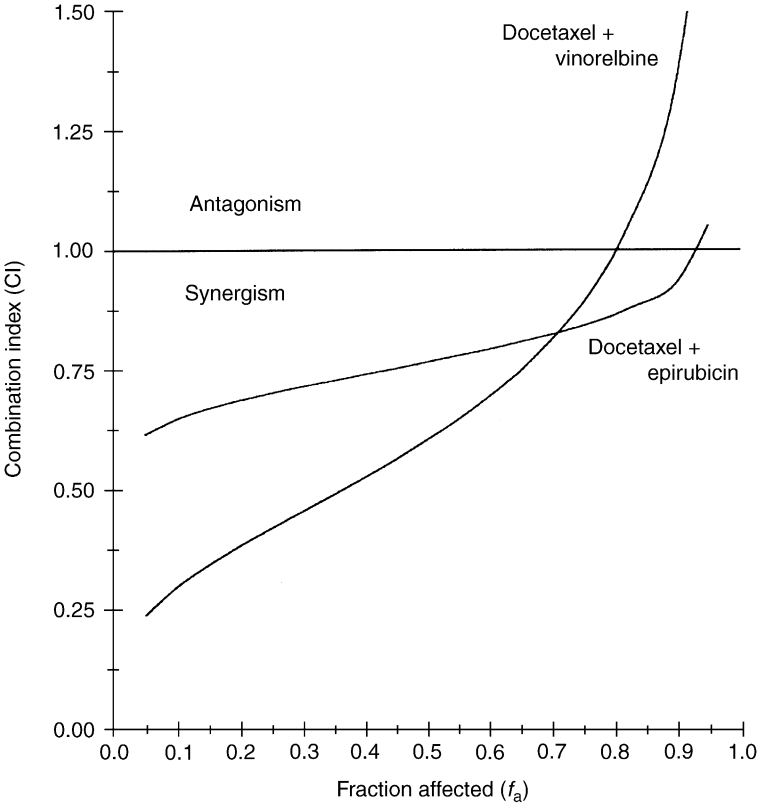

Figure 1. Evidence of synergism for docetaxel and epirubicin over the entire fraction of cell kill (f_a) *in vitro* assayed in DU 145 cells as measured by CI. Docetaxel with vinorelbine is synergistic for f_a values less than 0.75. The model is most accurate at $f_a = 0.5$.

Table 3. Triplet combinations of drugs with the CI ($\pm$ SD) measured at f_{a50} in the three prostate cancer cell lines

	DU 145	PC 3	LnCaP
Docetaxel + EST + retinoic acid-1	1.12 $\pm$ 0.02	2.19 $\pm$ 0.04	1.93 $\pm$ 0.05
Docetaxel + EST + doxorubicin	0.90 $\pm$ 0.02	1.72 $\pm$ 0.04	0.95 $\pm$ 0.04
Docetaxel + EST + coumadin	1.28 $\pm$ 0.03	1.02 $\pm$ 0.03	1.26 $\pm$ 0.06
Docetaxel + EST + dexrazoxane	2.17 $\pm$ 0.27	2.29 $\pm$ 0.45	1.65 $\pm$ 0.05
Docetaxel + EST + PSC 833	0.48 $\pm$ 0.08	0.58 $\pm$ 0.07	0.49 $\pm$ 0.02
Docetaxel + EST + vinorelbine	0.30 $\pm$ 0.01	0.69 $\pm$ 0.06	1.21 $\pm$ 0.26
Docetaxel + EST + epirubicin	0.51 $\pm$ 0.07	0.78 $\pm$ 0.05	1.92 $\pm$ 0.27
Docetaxel + EST + *cis*-retinoic acid	0.79 $\pm$ 0.15	0.93 $\pm$ 0.10	0.51 $\pm$ 0.04
Docetaxel + EST + disulfiram	1.21 $\pm$ 0.19	1.30 $\pm$ 0.17	0.43 $\pm$ 0.06
Docetaxel + EST + cyclosporin A	0.86 $\pm$ 0.06	0.97 $\pm$ 0.03	1.97 $\pm$ 0.28

EST = estramustine.

the combinations of docetaxel with vinorelbine and docetaxel with epirubicin, all three commercially available drugs. Marked synergy is identified. Of note, the platinum drugs, etoposide and dexrazoxane were markedly antagonistic when combined with docetaxel (Table 1).

As this *in vitro* model allows triplet combinations to be evaluated and many such combinations have been brought to clinical trial, we also evaluated a series of triplet combinations in the three prostate cell lines (Table 2). Docetaxel and estramustine were held constant as they have previously demonstrated clinical activity when combined[7,9,14,15] and additional single agents were added to form the triplet. Remarkably, with one exception, the addition of a third agent did not result in additive or synergistic effects across the cell lines. With the exception of the simultaneous application of PSC 833 with estramustine and docetaxel, all the other combinations displayed antagonism (Table 3).

Discussion

We have previously used this approach to identify potentially useful combinations of cytotoxic agents *in vitro* which then could be advanced to the clinic.[13] In further extensions of this initial work, we have identified that the combination of docetaxel with vinorelbine is highly synergistic in both androgen-dependent LnCaP and androgen-independent DU 145 prostate cancer cell lines. Docetaxel with *cis*-retinoic acid is additive in two cell lines while docetaxel with cyclosporin A is synergistic in two cell lines and additive in LnCaP. The combination of docetaxel with vinorelbine has been clinically tested in human breast cancer and human lung cancer with evidence of efficacy,[24–26] but has been under investigation in prostate cancer by only one group with only eight patients studied at the time of the abstract presentation. Early evidence of antitumor effects in hormone-refractory prostate cancer was noted in this study.[27] Docetaxel with *cis*-retinoic acid has not been evaluated in this patient population.

Androgen-insensitive prostate cancer remains a major medical problem despite the use of estramustine–docetaxel combinations. In the androgen-independent cell lines, we have also identified synergy between docetaxel and doxorubicin and docetaxel with epirubicin. These two doublets have been actively investigated in breast cancer with the dosages and toxicities documented.[28,29] However, whether or not this combination has efficacy in hormone-refractory prostate cancer remains unknown. Epirubicin has recently been evaluated both as a single agent and in combination in patients with metastatic prostate cancer with evidence of efficacy and tolerability.[30–34] As docetaxel also has activity clinically in patients with progressive prostate cancer,[5,8] this combination needs to be tested based upon both preclinical and clinical data. Disulfram has been suggested as an agent to reverse drug resistance[35] and was synergistic with docetaxel in the two hormone-independent prostate cell lines. Surprisingly, the platinum drugs demonstrated antagonism in this model system.

In contrast to the doublets, only the triplet combinations of docetaxel, estramustine and PSC 833 and the triplet of docetaxel, estramustine and *cis*-retinoic acid demonstrated synergy in both androgen-dependent and androgen-independent cell lines. As these cell lines have not been selected for resistance, the mechanism of action of PSC 833 (a cyclosporin D analog and MDR-1 inhibitor) may not be through its classical mechanism. We have previously reported that PSC 833 has cytotoxic activity by itself in human prostate cell lines.[36]

An *in vitro* system does not evaluate therapeutic index or the degree of toxicity seen in the use of these synergistic combinations in clinical trials. Another difficulty is that defined cell lines may not reflect the heterogeneity of clinical malignant disease. However, several of the above combinations have been tested in other clinical tumors and determined not to be noxious. Hence, the identified combinations may have a therapeutic role in metastatic prostate cancer and merit further studies.

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(Received 30 July 2002; accepted 27 August 2002)