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ORIGINAL ARTICLE

Schedule treatment design and quantitative in vitro evaluation of chemotherapeutic combinations for metastatic prostate cancer therapy

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Abstract
Purpose Preclinical evaluation is essential for a rational design of combination chemotherapy as some agents, with known mechanisms of action and non-overlapping toxicities may increase the therapeutic index of anticancer drugs, whose clinical success is hindered by side effects and drug resistance. The present study investigated new drug combinations with potential outcome for the treatment of metastatic prostate cancer. This final clinical stage exhibits predominantly hormone-refractory prostate cancer (HRPC) cells but also a minority of hormone responsive cells.
Methods Growth inhibition activity of simultaneous and sequential combinations was evaluated by resazurin assay. In vitro evaluation of synergism, additivity, or antagonism, against prostate cancer cell lines, was performed by the median effect analysis. The importance of dosage, exposure time, drug ratio, and type of treatment were investigated and compared.
Results Most simultaneous combinations of two drugs with different mechanisms of action or of two topoisomerase II inhibitors resulted in mild antagonism of antiproliferative effects, particularly notorious at high cell death. Imatinib–mitoxantrone and ciprofloxacin–etoposide combinations were exceptions, as they yielded additivity and dose reduction index (DRI) values of 2.6 and 3.5-fold for mitoxantrone and etoposide, respectively. Sequential combinations (ciprofloxacin or imatinib pre-treatment) revealed additive growth inhibition effects, translated in much higher DRI values (from 7.0 to 15.3-fold). Moderate synergism was restricted to sequential ciprofloxacin combinations at high cell death.
Conclusions Ciprofloxacin and imatinib significantly improve growth inhibition activity of standard antineoplastic drugs in a schedule-dependent manner and, therefore, may have an important role as adjuvant therapeutic agents in a clinical setting.

Keywords HRPC · Chemosensitization · Imatinib · Dose reduction index · Median effect analysis

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Introduction

Prostate cancer is one of the most life-threatening diseases in western societies. In the USA, it is the most common non-dermatologic cancer diagnosed in men and the second leading cause of male cancer mortality (http://www.cancer.org (2009) official website of American Cancer Society).

Initial therapy against advanced prostate cancer consists of androgen deprivation by surgical or medical castration [1, 2]. In spite of a 18–24-month initial period of regression, the disease may progress and patients virtually become unresponsive to androgen deprivation and have a

median survival of 12 months [1–3]. Standard options for patients with HRPC include second-line hormonal therapy and/or chemotherapy [1, 2]. Usually, chemotherapy is mainly palliative without prolonging life expectancy once the disease becomes refractory [1, 3]. For that reason, new rationally-designed therapeutic approaches are required.

In the current investigation, six chemotherapeutic drugs (standard or innovative in HRPC therapy), representing three classes of antineoplastic mechanisms, were combined in order to determine the most effective two-drug combinations against PC-3 and LNCaP cell lines.

Ciprofloxacin is a fluoroquinolone, widely used in the treatment of urinary tract infections, whose preclinical antitumor activity in bladder and prostate cancer has been reported [4–6].

Doxorubicin represents a first-line treatment for solid and hematological tumors but it exhibits clinical limitations due to dose-related myelosuppression and dose-cumulative cardiotoxicity [7, 8]. Singly, it displays minimal activity in patients with HRPC despite its palliative benefit [1, 7].

Etoposide, a semisynthetic podophyllotoxin-derivate, is used in the treatment of testicular and small cell lung cancers, lymphomas, among others [8, 9]. When administered as monotherapy, orally or by slow IV infusion, it did not show efficacy against HRPC though some combinations with oral etoposide have demonstrated promising results in phase II trials [10].

Imatinib mesylate (Gleevec®) inhibits tyrosine kinase activity of the platelet-derived growth factor receptor (PDGF-R) [11], but its oral administration has shown minimal activity in patients with HRPC with hormone naïve PSA progression after local therapy (phase II trial) [12].

Mitoxantrone is included in clinical chemotherapy for breast cancer, myelocytic leukemia, and lymphomas [8, 13]. Combination with a corticosteroid is the standard palliative treatment for HRPC showing improvement in quality of life, disease progression delay but no survival benefit [2, 14].

Vinblastine, a naturally occurring vinca alkaloid, is used in several therapeutic regimens for the treatment of lymphomas, breast, testicular, bladder, and NSCLC tumors [1, 15, 16] but its single-agent activity is modest in HRPC [1, 3].

The purpose of our study was the in vitro quantitative evaluation of novel two-drug combinations against prostate cancer cell lines in terms of nature of interaction effects, optimal drug ratio, and importance of schedule and sequence of administration. Ciprofloxacin or imatinib combinations with antineoplastic agents were the ones that raised the higher expectations due to non-overlapping toxic effects, possibility of oral administration with high bioavailability, distinct molecular targets which minimize drug resistance and potential to improve tumor drug delivery [6, 11].

Materials and methods

Cell lines and drugs

Doxorubicin hydrochloride (sterile solution) was obtained from Mayne Group Limited (Melbourne, Australia). Ciprofloxacin hydrochloride and imatinib mesylate (Gleevec®) were kindly provided by Bluepharma (Coimbra, Portugal) and by Novartis Pharma (Lisbon, Portugal), respectively. Etoposide, mitoxantrone dihydrochloride and vinblastine sulfate salt were purchased from Sigma (St. Louis, USA). Ciprofloxacin and doxorubicin were diluted in cell culture medium. Etoposide, imatinib, mitoxantrone, and vinblastine stock solutions were prepared in DMSO (Sigma) and diluted to their working concentration with cell culture medium. The highest DMSO concentration used was less than 1% and produced no cytotoxicity in controls (data not shown).

PC-3 and LNCaP cells were obtained from ATCC (Rockville, MD, USA) and DSMZ (Braunschweig, Germany), respectively. Both cell lines, although with different androgen sensitivity, were originally obtained from patients with prostate cancer in a highly metastasized clinical stage which is predominantly hormone-refractory (HRPC). LNCaP and PC-3 cells were isolated from the left supraclavicular lymph node metastasis and from bone metastasis, respectively.

PC-3 cells were grown to confluence in T150 culture flasks (Orange Scientific, Braine-L'Alleud, Belgium), while LNCaP cells were cultured in T150 flasks with CellBIND® surface (Corning®, NY, USA), due to poor attachment properties. Both cell lines were grown to confluence using RPMI 1640 medium (Lonza, Basel, Switzerland) with L-glutamine (2 mM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Lonza) and 100 IU/ml penicillin–100 μg/ml streptomycin (Lonza). Cells were cultured at 37°C in a humidified air incubator (95%) containing 5% CO_2. Before confluence, cells were harvested by trypsinization (500 mg/l (1:250) trypsin-200 mg/l versene (EDTA)) (Lonza).

Single drug-induced cell cytotoxicity studies

PC-3 and LNCaP cells were seeded in 96-well flat bottom plates at 8,000 and 10,000 cells/well, respectively, which allowed linear phase growth throughout the experiment. LNCaP cells required plate coating with 0.001% poly-L-Lysine (Sigma) to improve adherence. Cells were allowed to adhere overnight and then treated with single-drug dilutions (100 μl/well), within the concentration range mentioned in the next section. Control wells consisted of cells incubated with medium.

Cell growth inhibition was evaluated by the resazurin reduction assay as we have previously described in detail

[17], following 48- and 72-h drug incubations at 37°C, without medium change. Absorbance values were measured at 540 and 630 nm using a microplate reader. Cytotoxicity after drug exposure was expressed as cell death relatively to untreated cells (% of control), for each incubation time. Drug concentrations required to inhibit cell growth by 50% (IC_{50}) were determined from dose-response curves created by the GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA) and were compared between the different treatments. All results were reported as mean values ± SD of at least three independent experiments, performed in quadruplicate for ten concentrations tested.

Cytotoxicity studies with simultaneous and sequential drug combinations

After an initial pre-screening assay to evaluate single drug IC_{50} (Table 1), dose range was chosen to cover the concentrations below and above the IC_{50} for each drug against each cell line. Doses were varied such that a constant equipotent (IC_{50}/IC_{50}) ratio was maintained. In practice, ten concentrations from 0.250 to 8 times the IC_{50} were tested in quadruplicate (four wells per each concentration). Several independent experiments (3–15) were performed to evaluate the reproducibility of the obtained IC_{50} values and ultimately calculate an accurate mean value. Agents were studied concurrently (72 h), with both drugs (50 µl each) added to the incubation mixture, or sequentially (48 + 48 h), with the first agent (100 µl) washed out with PBS, before the addition of the second one (200 µl).

Cells incubated with each drug individually and cells incubated with the drug prior to and after medium addition were used as controls for concurrent and sequential treatments, respectively [17]. In controls, drugs were added and removed at the same time as in combination assays. IC_{50} values for all controls and combinations were obtained as described above for single-drug assays.

In another set of preliminary experiments, ciprofloxacin was combined simultaneously with etoposide or mitoxantrone for 72 h at non-equipotent drug ratios, in which the cytotoxic drug was tested at IC_{50} or IC_{70} while ciprofloxacin was added at concentrations corresponding to 1.5, 1, 1/2, or 1/4 times the IC_{50}. Cell death for each combination was determined and compared to the single drug result at the same concentration used in combined treatment. The evaluation of drug combinations at other ratios besides the equipotent one provides an opportunity to estimate the optimal relation that leads to maximal antiproliferative effect.

Median effect analysis of combined effects

As in our previous study [17], the nature of combined interaction effects was analyzed for synergism, additivity, or antagonism by the median effect analysis developed by Chou and Talalay [18–20]. The cytotoxic effects are described by $f_a/f_u = (D/D_m)^m$, where f_a and f_u are the fraction of cells affected and unaffected, respectively, by a dose (D); D_m is the dose inducing the median effect and "m" the coefficient of the sigmoidicity of the dose effect curve. The fractional effect associated with a range of concentrations was determined for each drug alone and for the various drug combinations [17–20] as we have previously described in detail [17].

The nature of drug interaction effects can be determined from the combination index (CI):

$$\mathrm{CI} = \frac{(\mathrm{D})_1}{(\mathrm{D}_x)_1} + \frac{(\mathrm{D})_2}{(\mathrm{D}_x)_2} + \alpha \frac{(\mathrm{D})_1(\mathrm{D})_2}{(\mathrm{D}_x)_1(\mathrm{D}_x)_2} \quad (1)$$

where $\alpha = 0$ and $\alpha = 1$, for drugs with mutually exclusive or non-exclusive mechanisms of action, respectively. $(D_x)_1$ and $(D_x)_2$ denominators are the single-drug doses required

Table 1 Single drug cytotoxicity of tested anticancer agents against PC-3 and LNCaP cells after 48- and 72-h treatments

Therapeutic agents	PC-3 cells				LNCaP cells			
	48 h		72 h		48 h		72 h	
	IC50	SD	IC50	SD	IC50	SD	IC50	SD
Ciprofloxacin (µM)	242.27	22.34	231.38	34.17	244.39	56.47	215.20	40.52
Imatinib (µM)	28.47	3.12	27.64	0.48	24.64	3.86	21.02	2.63
Etoposide (µM)	24.13	7.17	13.00	5.03	1.88	0.67	0.40	0.06
Doxorubicin (nM)	288.67	16.50	253.00	40.99	26.75	4.07	20.94	3.70
Mitoxantrone (nM)	238.46	121.45	89.04	21.92	13.91	3.87	5.57	1.33
Vinblastine (nM)	1.86	0.27	1.24	0.36	ND	ND	ND	ND

Mean IC_{50} values ± SD determined from 3 to 15 independent experiments performed in quadruplicate (four wells per drug concentration) for 48 and 72 h. IC_{50} values for vinblastine against LNCaP were not determined (ND). Cytotoxicity evaluation was performed by the resazurin reduction assay. IC_{50} values were calculated by the GraphPad Prism 5

to achieve a given effect level (f_a). $(D)_1$ and $(D)_2$ numerators are the doses in a given mixture which are isoeffective. CI reflects synergism, additivity, or antagonism when inferior, equal, or superior to one, respectively. We, as others [21], considered additive effect to be any CI result within one SD of unity.

Dose reduction index (DRI) defines the extent of dose reduction that is achieved in a combination, when compared with the dose of each single drug, for a given effect level [20, 22].

Statistical analysis

Data were analyzed using the GraphPad Prism software (version 5.0). Statistical significance of differences between treatment groups was evaluated by one-way ANOVA using the Tukey post-test. A value of $P < 0.05$ was considered significant.

Results

Single drug-induced cytotoxicity study

To evaluate the effect of several therapeutic agents on PC-3 and LNCaP growth, cells were treated with different drug doses for 48- or 72-h incubations. IC_{50} values are summarized in Table 1, and dose–response curves determined for PC-3 cells are depicted in Fig. 1 (not shown for LNCaP cells).

All drugs inhibited cell growth in a time- and dose-dependent manner (Fig. 1, not shown for LNCaP). The most significant differences in individual drug IC_{50} values, obtained for the two cell lines, were verified for etoposide, doxorubicin, and especially mitoxantrone (Table 1). The higher sensitivity of LNCaP cells to those drugs demonstrates that their cytotoxic activity was cell line-dependent.

A preliminary experiment with representative drug combinations (ciprofloxacin–etoposide or –mitoxantrone) demonstrated that only IC_{50} equipotent ratio yielded cell death values higher than the obtained results with single drugs at the concentrations used in combination (data not shown). These results highlighted that IC_{50} equipotent ratio was the optimal combination ratio for further studies.

Median effect analysis of combined effects

Median effect plots were discussed and exemplified in our previous work [17]. In the present study, CI values were calculated by the non-exclusive assumption [19]. This is a more conservative criterion to assess interaction effects since the addition of a third term in Eq. 1 slightly increases the CI value [19]. Median effect plots yielded determination coefficients (R^2) varying between 0.96 and 0.99, which

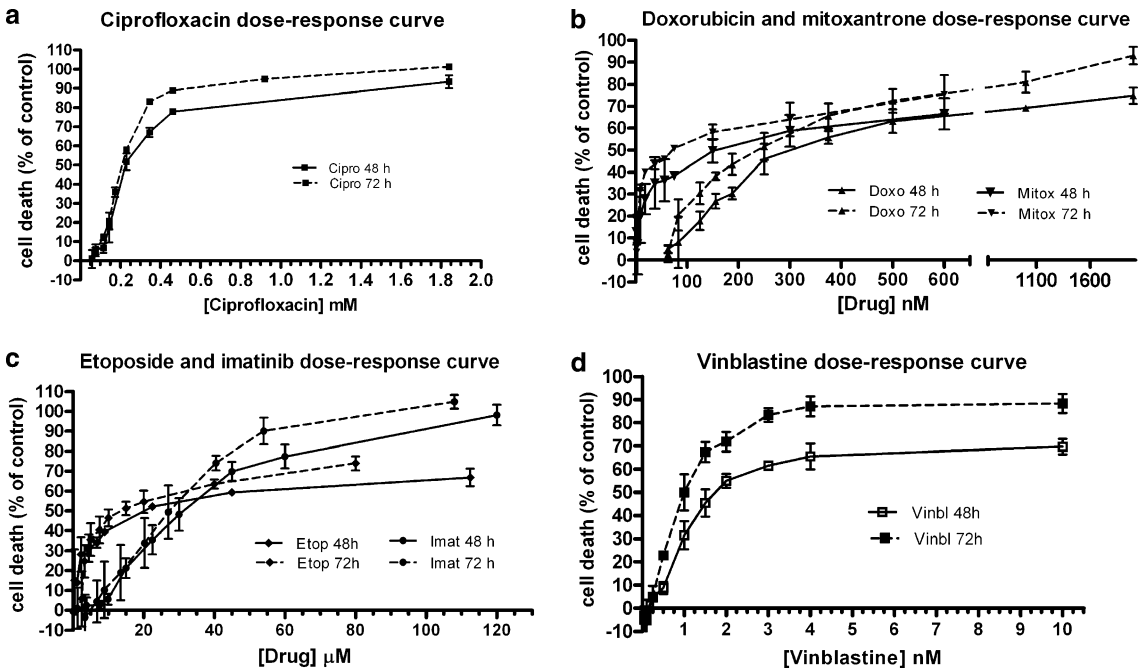

Fig. 1 Dose–response curves obtained for PC-3 cells treated with **a** ciprofloxacin (*filled square*), **b** doxorubicin (*filled triangle*), **b** mitoxantrone (*filled inverted triangle*), **c** etoposide, (*filled losangle*), **c** imatinib (*filled circle*), and **d** vinblastine (*unfilled square*). Cell viability was assessed by the resazurin reduction assay at 37°C for 48-h (*straight line*) and 72-h (*dashed line*) treatment with various drug concentrations. Results are given as a mean cell death (%) relative to untreated controls ± SD of 3–15 independent experiments performed in quadruplicate (four wells per drug concentration)

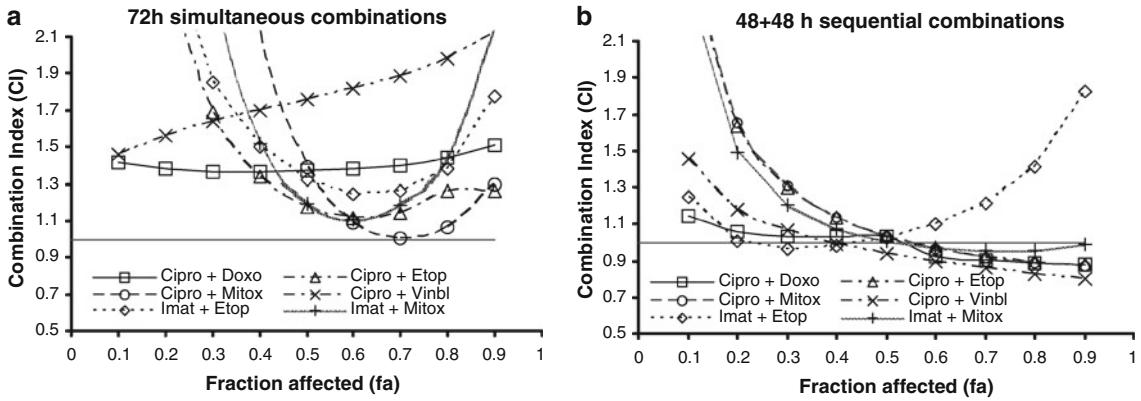

Fig. 2 Graphical representation of CI versus *f*a for **a** 72-h simultaneous and **b** 48 + 48-h sequential drug combinations against PC-3 cells. Mean CI values were compiled from 2 to 4 independent experiments and obtained by median effect analysis. Standard deviations are not presented in this figure to improve clarity but are referred in Table 2 for CI at $f_a = 0.5$. CI values above and below 1.0 indicate antagonism and synergism, respectively, while 1.0 ± SD designates additivity. *Vertical axis* scale was shortened, beginning at CI = 0.5, to better visualize the curves

confirmed the suitability of the method to the data. As before [17], CI value was calculated for $fa_{0.5}$, which is the most reliable and used determination, since the median effect plot may not be accurate at the extremes as it represents a linear estimation of a non-linear function [23].

The CI versus *f*a graph (Fig. 2) illustrates the interaction effects of chosen simultaneous drug combinations (Fig. 2a) that were selected for the following sequential experiments (Fig. 2b) against PC-3 cells (not shown for LNCaP cells). Additionally, Figs. 3 and 4 present mean DRI values (at $fa_{0.5}$ and $fa_{0.9}$), for all tested concurrent and sequential treatments, respectively.

The most promising two-drug combinations and treatment schedules obtained with PC-3 cells on our previous [17] and present studies, in what concerns DRI and CI results, were also evaluated against LNCaP cells in order to compare the combined effects in both cell lines at the same schedule of administration (concurrent 72 h and sequential 48 + 48 h) (Table 2).

For concurrent (Fig. 2a) and sequential (Fig. 2b) schemes, at low *f*a (0.1–0.4), mild to strong antagonism was observed. For a median effect level ($fa_{0.5}$), most concurrent combinations (72 h) were antagonistic in inducing cell growth inhibition (CI > 1) (Fig. 2; Table 2) which translated to low DRI results (close to twofold) (Fig. 3; Table 2). Two exceptions at $fa_{0.5}$ were ciprofloxacin–etoposide (CI = 1.168) and imatinib–mitoxantrone (CI = 1.192) simultaneous combinations that yielded an additive growth inhibition effect (Fig. 2a; Table 2) with higher DRI (3.5 and 2.6-fold) for etoposide (Fig. 3b) and mitoxantrone (Fig. 3d), respectively. For a $fa_{0.9}$ level, simultaneous combinations yielded antagonism, similar or more pronounced than for $fa_{0.5}$ (Fig. 2a), which generally translated in inferior DRI values and with higher SD (Fig. 3). Better CI values (at $fa_{0.5}$ and $fa_{0.9}$), additive or slightly synergistic, were obtained when drug combinations (except imatinib–etoposide) were performed sequentially (Fig. 2b; Table 2). For sequential ciprofloxacin–etoposide and imatinib–mitoxantrone combinations, DRI values were significantly increased for etoposide (7.9-fold) (Fig. 4b) and for mitoxantrone (9.1-fold) (Fig. 4c), comparing to the co-treatment DRI values (Fig. 3b, d). In agreement, for all the other tested sequential combinations, mean DRI values ($fa_{0.5}$) for the second drug were much higher (ranging from 7.0 to 15.3-fold) than those achieved with co-treatment (Fig. 4; Table 2). A central finding of this study is that, in sequential schemes, imatinib appeared to be a more potent sensitizer drug than ciprofloxacin due to higher DRI in combinations comprising the same second drug (Fig. 4; Table 2).

Selected simultaneous combinations were mild antagonistic against LNCaP cells, with DRI values inferior to twofold ($fa_{0.5}$) (Table 2). As observed on PC-3 cells, sequential combinations led to an enhanced cytotoxicity: interaction effects lying in the additivity (pre-treatment with ciprofloxacin) and synergistic (pre-treatment with imatinib) range for *f*a values between 0.5 (Table 2) and 0.9 (data not shown). The DRI values for the second drug ranged from 3.6 to 5.5-fold (Table 2).

Discussion

Since prostate cancer is a known chemoresistant and metastatic disease, the enhancement of chemotherapeutic effects, through new drug combinations and molecular targets, is a valuable therapeutic strategy in order to ultimately improve patient survival.

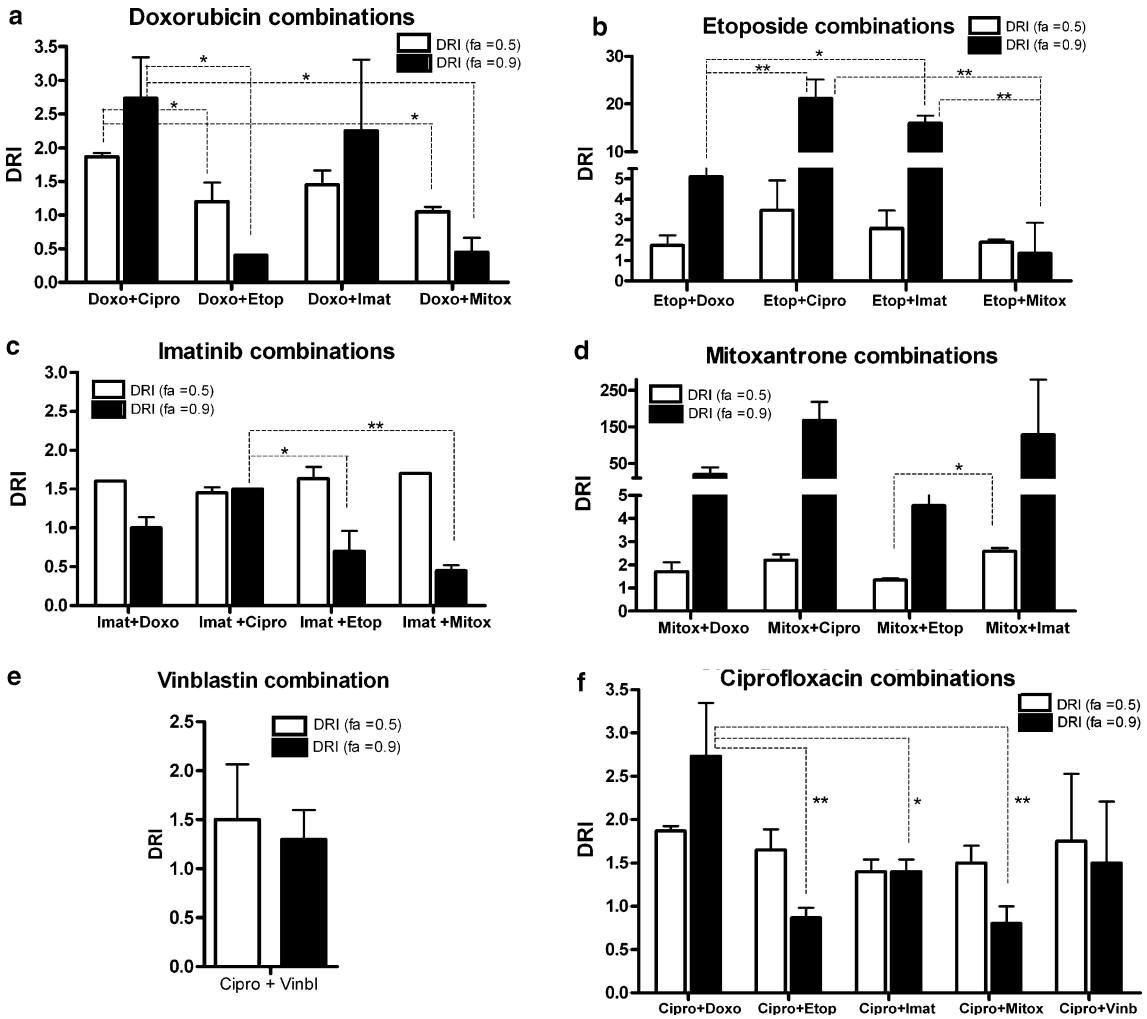

Fig. 3 Graphical representation of DRI values for **a** doxorubicin, **b** etoposide, **c** imatinib, **d** mitoxantrone, **e** vinblastine, and **f** ciprofloxacin in 72-h simultaneous drug combinations against PC-3 cells. For each drug, 10 concentrations, ranging from 0.250 to 8 times the IC_{50} value at 72 h (Table 1), were used for each drug combination, so that the IC_{50} equipotent ratio remained constant. DRI values are presented as mean ± SD of 2–4 independent experiments, for each treatment scheme. DRI results are only expressed for 0.5 (*open columns*) and 0.9 (*shaded columns*) of fractional cell growth inhibition. *Asterisks* indicate drug combinations whose corresponding mean DRI values, for the specified drug at the same effect level ($f_a = 0.5$ or $f_a = 0.9$), are statistically different (*$P < 0.05$, **$P < 0.01$)

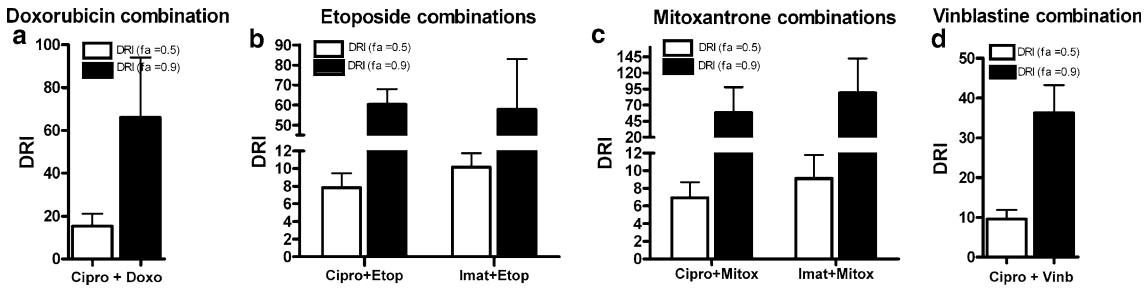

Fig. 4 Graphical representation of DRI values for **a** doxorubicin, **b** etoposide, **c** mitoxantrone, and **d** vinblastine in 48 + 48-h sequential drug combinations against PC-3 cells. For each drug, 10 concentrations, ranging from 0.250 to 8 times the IC_{50} value at 48 h (Table 1), were used for each drug combination, so that the IC_{50} equipotent ratio remained constant. DRI values for cytotoxic agents that sequentially followed ciprofloxacin or imatinib are presented as mean ± SD of 2 or 3 independent experiments, for each treatment scheme. DRI results are only expressed for 0.5 (*open columns*) and 0.9 (*shaded columns*) of fractional cell growth inhibition. No statistical significance between mean DRI values, at the same effect level, was found ($P > 0.05$)

Table 2 Direct comparison of schedule-dependent CI and DRI values against PC-3 and LNCaP cells

Schedule	Drug combinations	PC-3 cells		Interaction effect	LNCaP cells		Interaction effect
		CI	DRI		CI	DRI	
72 h	Cipro/doxo	1.370 ± 0.042	1.9 ± 0.1	Antagonism	1.634 ± 0.231	1.7 ± 0.4	Antagonism
	Cipro/mitox	1.391 ± 0.296	2.2 ± 0.3	Antagonism	ND	ND	ND
	Cipro/etop	1.168 ± 0.243	3.5 ± 1.5	Additivity	1.485 ± 0.155	1.7 ± 0.3	Antagonism
	Cipro/vinb	1.763 ± 0.057	1.5 ± 0.6	Antagonism	ND	ND	ND
	Imat/etop	1.321 ± 0.360	2.6 ± 0.9	Antagonism	ND	ND	ND
	Imat/mitox	1.192 ± 0.019	2.6 ± 0.1	Additivity	2.371 ± 0.348	1.2 ± 0.5	Antagonism
48 + 48 h	Cipro/doxo	1.033 ± 0.178	15.3 ± 5.8	Additivity	1.306 ± 0.429	3.8 ± 0.2	Additivity
	Cipro/mitox	1.030 ± 0.093	7.0 ± 1.8	Additivity	ND	ND	ND
	Cipro/etop	1.033 ± 0.117	7.9 ± 1.6	Additivity	1.051 ± 0.057	3.6 ± 1.9	Additivity
	Cipro/vinb	0.940 ± 0.059	9.6 ± 2.3	Additivity	ND	ND	ND
	Imat/etop	1.025 ± 0.090	10.1 ± 1.6	Additivity	ND	ND	ND
	Imat/mitox	1.007 ± 0.027	9.1 ± 2.7	Additivity	0.746 ± 0.125	5.5 ± 3.4	Synergism

Mean CI or DRI ± SD and nature of interaction effects for 2–4 experiments performed in quadruplicate, for tested drug combinations after simultaneous 72 h and sequential 48 + 48 h. CI and DRI values at $fa_{0.5}$ were determined by the median effect analysis. The presented DRI values refer to the drug combined with ciprofloxacin or imatinib. Several drug combinations were not tested against LNCaP, so CI and DRI values were not determined (ND)

Six agents, representing three categories of antineoplastic mechanisms, were selected because of their minimal clinical effectiveness in patients with HRPC, in spite of the reported in vitro single or combined cytotoxic activity. Combinations were evaluated in order to investigate drug ratio- and schedule-dependent interaction effects between agents with the same or different molecular targets.

The exposure times (72 h and 48 + 48 h) used in the present work were selected as these two-treatment schedules yielded the most promising CI and/or DRI results obtained on PC-3 and LNCaP cells in our previous study [17]. The rationale for the sequential scheme was to pretreat cells with the least potent agent (ciprofloxacin or imatinib) and ultimately decrease the dose and/or exposure time of the following anticancer agent. The reverse schedules were not tested.

Our final results clearly indicate that, for the same tested sequential combinations, similar (cipro/doxo, cipro/etop) or improved (imat/mitox) combined interaction effects may be observed between PC-3 and LNCaP cells (Table 2). However, in all cases, significant lower DRI values were observed for LNCaP cells when compared to PC-3 cells (Table 2). This might be explained by the fact that LNCaP cells were more sensitive to the tested individual drugs and so obtained IC_{50} values are significantly lower (Table 1). That enhanced sensitivity of LNCaP cells to cytotoxic drugs led to similar or improved interaction effects but the decrease on the second drug dose (DRI), which was possible to achieve with combination, was not so accentuated because the IC_{50} of the single drug was already low and so chemosensitization induced by ciprofloxacin or imatinib was not so obvious. Neither imatinib nor mitoxantrone interfere with the androgen signaling pathway and so we hypothesize that the synergistic interaction yielded by the imatinib–mitoxantrone combination on LNCaP was, in fact, a result of the higher sensitivity of these cells to cytotoxic drugs compared to PC-3 cells and was not related to the fact that LNCaP are androgen-responsive.

The irreproducibility of values at $fa_{0.9}$ (high SD) may be explained by the unreliability of the median effect plot at the extremes as it represents a linear estimation of a non-linear function and, for this reason, CI values are generally determined for $fa_{0.5}$ [23]. In spite of the referred caveat, some literature publications state that, for chemotherapeutic purposes, maximal responses are generally required to achieve effective anticancer treatment [22, 24].

Even though ciprofloxacin, doxorubicin, mitoxantrone, and etoposide have topoisomerase II (topo II) as cellular target [8, 13, 25, 26], they have distinct antitumor activities and their combination may result in diverse antiproliferative effects. This can be explained by distinct modes and sites of action on topo II and possible variations in DNA sequences of enzyme action.

Topoisomerase II is an ubiquitous enzyme that catalyses topological interconversions of DNA through double-strand breakage and passage of DNA [8, 27, 28]. Topoisomerase II inhibitors are convenient drugs against prostate cancer since those cells have high levels of enzyme and lack significant *p*-glycoprotein expression, so drug resistance related to this two phenomena is minimal [8, 29]. Doxorubicin, mitoxantrone, and etoposide exert their

antineoplastic effect by stabilizing covalent complexes between topo II and DNA, leading to DNA breaks and cell death [8, 27, 28].

Ciprofloxacin stabilizes irreversibly the cleavable complex and enhances enzyme-mediated DNA breakage, although minimally [30, 31]. It is a non-intercalating drug that acts by stimulating the forward rate of DNA cleavage, without interfering with DNA rejoining [31, 32].

Bromberg et al. [32] proposed a two-drug model suggesting that two molecules must interact independently at the two scissile bonds to increase enzyme-mediated double-stranded DNA breaks. This model is assumed to apply for other drugs that also inhibit enzyme-mediated DNA reunion and share a common interaction domain on topoisomerase II [26]. This competition hypothesis may explain why two-drug combinations including doxorubicin/etoposide/mitoxantrone were found antagonistic after co-administration (Fig. 3). In a potential chemotherapeutic protocol, these drugs should be spaced in time in order to achieve maximal response rate. Schedule-dependent in vitro activity of single etoposide was previously described but against leukemia cell lines [33].

The two-drug model does not apply to drugs, such as ciprofloxacin, that act by stimulating the forward rate of DNA breakage [34]. In the case of quinolones, the presence of one molecule in a single scissile bond is able to distort both strands of DNA and stimulate enzyme-mediated double-strand breakage [34]. Concurrent combinations of ciprofloxacin with another topoisomerase II inhibitor were mild antagonistic or additive (with etoposide) (Table 2). Mild antagonism can be explained by a competition for the site of action on the enzyme and by an opposite action on transcriptional factor NF-κB for apoptosis induction. Anthracyclines, anthracenediones, and fluoroquinolones induce apoptosis by inhibiting the DNA binding activity of NF-κB (hindering a pro-survival mechanism) [5] and through NF-κB activation (leading to pro-apoptotic Fas ligand expression) [35], respectively. Additivity of the simultaneous ciprofloxacin–etoposide combination against PC-3 might be explained by the fact that these drugs may share an overlapping domain on topo II, but not an identical site since the mode of action is different [26]. Etoposide has low affinity for DNA, so it impairs topo II-mediated DNA religation by directly binding to the enzyme without DNA intercalation [13, 25, 36]. Ciprofloxacin and etoposide seem to have also a synergistic action on cell cycle arrest and on apoptosis induction, since both drugs induce late S and G_2/M arrest, increase Bax/bcl-2 ratio, and translocate Bax with concurrent activation of caspase 3 [5, 6, 13].

The lack of competitive inhibition when a topo II inhibitor was administered after ciprofloxacin pre-treatment explains the additivity ($fa_{0.5}$) and synergism ($fa_{0.9}$) of those sequential combinations and the significant enhancement of DRI for the second agent added (Table 2). All these findings are consistent with our previous study [17]. So far, only two studies have demonstrated a chemosensitizer role for ciprofloxacin against PC-3 cells: when combined with etoposide [5] or with doxorubicin [4] against prostate and bladder cancer cells, respectively. However, neither study applied a quantitative method in order to evaluate the antiproliferative effects.

Antagonism of the concurrent ciprofloxacin–vinblastine combination can be justified by their different cellular targets and antagonistic action on cell cycle and on bcl-2. While ciprofloxacin arrests cell cycle at late S/G_2/M phases and down regulates bcl-2 [6], vinblastine prevents microtubule dynamics, with subsequent blockage of mitotic spindle and metaphase arrest, associated with bcl-2 phosphorylation [1, 15, 16]. This might explain why concurrent administration yielded antagonism while sequential treatment resulted in additive ($fa_{0.5}$) or synergistic ($fa_{0.9}$) effects. Similar results were achieved with the ciprofloxacin–docetaxel combination in our previous study [17].

Imatinib prevents platelet-derived growth factor receptor (PDGF-R) activation and subsequent signaling pathway, responsible for cell proliferation, migration, and angiogenesis [11, 37–39]. PDGF-R inhibition is a suitable therapeutic strategy since this receptor is present in 88% of primary prostate cancer and in 80% of metastasis but absent in non-malignant prostatic tissue [38]. In addition, it is known that imatinib decreases interstitial fluid pressure (IFP) in solid tumors, so pre-treatment with imatinib improves tumor delivery of subsequent free or liposomal anticancer drugs in vivo [11, 39]. To date, the molecular mechanisms underlying the cytotoxic interaction between imatinib and other agents are still to be clarified. One possible explanation is that imatinib, by interfering with the mitogenic signal pathway, cannot only directly inhibit cell proliferation but also affect the sensitivity of the cells to a well-established cytotoxic drug. Kübler et al. [38] also described additive to antagonistic effects for imatinib–etoposide concurrent combination (120 h) against PC-3, but additive interactions against DU-145 and LNCaP cells. Other imatinib combinations were also evaluated [38] but none of the herein presented.

Additive and synergistic ciprofloxacin or imatinib–cytotoxic drug combinations might have a therapeutic interest because they conciliate drugs with non-overlapping toxicities and different cellular targets and/or mechanisms of action, so drug resistance is minimized. In addition, the fact that P glycoprotein activity is rare in prostate cancer [29] favors the use of imatinib, whose resistance is known to be P glycoprotein related [40, 41].

Tested concentrations of doxorubicin, etoposide, mitoxantrone, and vinblastine that induced growth inhibition

effects in our in vitro system were in the same range or inferior to the clinically achievable levels [15, 42–44]. However, chemosensitization was produced with ciprofloxacin and imatinib concentrations higher than the ones achievable in human plasma or prostatic tissue [45–48]. The concentrations tested in our study are equivalent to the ones used against prostate cancer cells in similar published studies [5, 6, 38]. In addition, it should be emphasized that the tested dose range had to be used in order to allow the median effect analysis to be performed. Drug concentrations and/or optimized drug:drug ratios that exerted in vitro cytotoxic activity against prostate cancer cells might be translated to the clinic through encapsulation in a drug delivery system. Our research group has successfully developed a liposomal formulation that encapsulates and retains a selected drug combination at the therapeutic drug:drug ratio found in vitro (unpublished results). This formulation, which comprises drug doses that are clinically achievable, is currently undergoing preclinical studies in an animal model of HRPC in order to confirm the previously obtained in vitro results and to better establish the treatment protocol in what concerns the dose and schedule of administration.

In conclusion, the current study provides a rationale for treatment design and quantitative assessment of chemotherapeutic combinations including standard and novel cytotoxic agents for metastatic prostate cancer therapy. This study clarifies, for the first time, the nature of interaction effects and underlines a potential therapeutic outcome for selected combinations including ciprofloxacin or imatinib. Taking into account the systemic and dose-limiting toxicities associated with most antineoplastic drugs, our results suggest that dose reduction, found in some combinations, can improve overall therapeutic index in a future clinical protocol.

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