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ORIGINAL ARTICLE

Ciprofloxacin sensitizes hormone-refractory prostate cancer cell lines to doxorubicin and docetaxel treatment on a schedule-dependent manner

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Abstract
Purpose Combination therapy has generated a significant interest in the clinical setting since certain agents, with known mechanisms of action and non-overlapping toxicities may increase the therapeutic potential of anticancer drugs by decreasing systemic toxicity and overcoming drug resistance. Doxorubicin and docetaxel, two standard antineoplastic agents in hormone-refractory prostate cancer (HRPC) therapy and ciprofloxacin were evaluated singly and in several simultaneous and sequential drug combination schemes, against PC-3 and LNCaP cell lines.
Methods Cellular viability was determined by the resazurin assay and the assessment of synergism, additivity or antagonism was carried out by the median effect analysis. The importance of dose, exposure time and type of administration were investigated and compared.
Results Ciprofloxacin–doxorubicin or docetaxel combinations resulted in prominent additive or synergistic effects in both cell lines, when the cells were pre-treated with ciprofloxacin. These results suggest a rationale for dose reduction of doxorubicin and docetaxel in prostate cancer therapy, since the doses needed to achieve 50% cell death may be decreased by approximately 4- to 15-fold or 3- to 8-fold, respectively, after a pre-treatment with ciprofloxacin. In contrast, the referred combinations yielded moderate antagonistic effects when used concurrently in this in vitro system.
Conclusions Ciprofloxacin sensitized HRPC cells to doxorubicin or docetaxel-induced growth inhibition and, therefore, may play a role as chemosensitizing agent in prostate cancer treatment.

Keywords HRPC · Ciprofloxacin · Chemosensitization · Dose-reduction index · Median effect analysis

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Introduction

Prostate cancer is a significant health problem in most western countries. In the USA, it is the most commonly diagnosed cancer and also one of the leading causes of cancer-related death in men [http://www.cancer.org (2006) Official website of American cancer society].

An effective and well-tolerated first-line treatment for metastatic prostate cancer is androgen withdrawal therapy [19, 34]. In spite of the initial efficacy, hormone-independent sub-populations can emerge and most patients virtually develop hormone-refractory prostate cancer (HRPC) with a median survival of 12 months [12, 19, 34]. Typical options for HRPC include secondary hormonal therapies and/or cytotoxic chemotherapy [19, 34]. Until recently, there had been no chemotherapeutic approach for HRPC able to demonstrate survival benefit; however, docetaxel chemotherapy has shown improvement of

survival in two large randomized trials, reviewed elsewhere [28, 34]. The poor clinical outcome arising from chemotherapy emphasizes the need of a rationally designed therapeutic approach, namely through exploitation of new molecular targets and novel regimens based on drug combination.

In general, a clinical chemotherapeutic regimen consists of combination of drugs in order to achieve a therapeutic efficacy higher than that provided by single drugs, to decrease systemic toxicity and to circumvent drug resistance [9, 16, 38, 39]. Ideally, the combined drugs should have proven single cytotoxic activity, minimal overlapping toxicities and different modes of action. At present, most chemotherapy regimens used in clinic are empiric drug combinations designed in the absence of in vitro experimental data [16, 39].

Ciprofloxacin is an antibiotic, widely used in the treatment of urinary infections and prostatitis, whose preclinical cytotoxic activity in a variety of human tumors, including prostate cancer, has been recently reported [2, 3, 13, 25]. Ciprofloxacin is relatively non-toxic, can be administered orally, presents a high volume of distribution and displays good tissue penetration [2, 13, 25].

Doxorubicin, an anthracycline, has a broad spectrum of activity against solid tumors and hematological malignancies but has significant limitations in cancer treatment due to its severe toxicity, including myelosuppression and cardiotoxicity [21, 23, 38]. Monotherapy with doxorubicin has a relative modest activity in HRPC [19, 20, 23].

Docetaxel is approved for the treatment of HRPC, where it has exhibited an interesting activity either singly or in combination, with improved survival in phase II and III trials [28, 34]. In vitro synergism with several agents has been shown against prostate and other cancer cells [6, 38].

Based on the median effect analysis proposed by Chou and Talalay [8–10], the aim of the present study was the in vitro quantitative evaluation of schedule-dependent cytotoxic effects of ciprofloxacin combinations with doxorubicin or docetaxel against two HRPC cell lines (PC-3 and LNCaP) and determination of the most effective treatment schedules with potential clinical outcome.

Our study demonstrated that pre-treatment with ciprofloxacin, followed by doxorubicin or docetaxel, induced additive or synergistic growth inhibition effects, while concurrent combinations were antagonistic. Our results suggest that the use of lower doses of doxorubicin (and even docetaxel) in a sequential treatment with ciprofloxacin is a promising therapeutic approach in prostate cancer therapy. Therefore, in a potential clinical protocol, those drugs should be optimally scheduled and given sequentially at consistent intervals.

Materials and methods

Cell lines and drugs

Doxorubicin hydrochloride (as sterile solution 2 mg/ml) and docetaxel (analytic grade) were obtained from Mayne Group Limited (Melbourne, Australia) and Fluka Chemie GmbH (Buchs, Switzerland), respectively. Ciprofloxacin hydrochloride was kindly provided by Bluepharma (Coimbra, Portugal). All drug solutions were diluted in cell culture medium. Docetaxel was dissolved in DMSO (Sigma, USA) and diluted to its working concentration with cell culture medium. The final DMSO concentration was less than 0.1% and produced no cytotoxicity in controls (data not shown). Aliquoted stock solutions were stored at −20°C.

PC-3 and LNCaP cells were purchased from ATCC (Rockville, MD, USA) and DSMZ (Braunschweig, Germany), respectively.

PC-3 cells were grown to confluence in T150 tissue 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 cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) with L-glutamine (2 mM), supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) and 100 IU/ml penicillin-100 µg/ml streptomycin (Lonza). Cells were maintained at 37°C in a humidified air incubator (95%) containing 5% CO_2. Before confluence, cells were harvested with trypsin-EDTA [500 mg/L (1:250) trypsin—200 mg/L versene (EDTA)] (Lonza).

Single drug-induced cytotoxicity studies

PC-3 and LNCaP cells were seeded in 96-well flat bottom plates (Orange Scientific) at 8000 and 10000 cells/well, respectively, which allowed linear phase growth throughout the experiment. LNCaP cell line required coating of the plate with 0.001% poly-L-lysine (Sigma) to improve adherence. Cells were allowed to adhere overnight and then treated with dilutions of each drug individually (100 µl/well), within the concentration range further mentioned. Control wells consisted of cells incubated with medium only.

Cell growth inhibition was evaluated by the resazurin reduction assay, following 24, 48, 72 and 96 h drug incubations at 37°C, without any medium change. Resazurin assay is a known indicator of cell viability, allowing continuous monitoring of cell proliferation and/or cytotoxicity, over the incubation period, using a non-toxic reagent that is soluble, stable in culture medium and does not cause cell lysis [31, 33]. Resazurin stock solution (0.1 mg/ml in PBS)

was diluted 10% in culture medium and 200 µl were added after a wash out with PBS. Plates were incubated for 4 h at 37°C. 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. Single-drug concentrations required to inhibit 50% of cell growth (IC_{50}) were determined from dose-response curves plotted 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 three to fifteen independent experiments, performed in quadruplicate for ten concentrations tested.

Cytotoxicity studies with simultaneous and sequential ciprofloxacin combinations

After determination of the average IC_{50} value for every drug, at each incubation time, against both cell lines (further reported in Tables 1 and 2), drugs were combined at the IC_{50} equipotent ratio. For each drug, ten concentrations were combined so that the ratio remained constant. Drug concentration range comprised ten values from 0.250 to 8 times the IC_{50}.

Agents were studied in combination concurrently, with both drugs added to the incubation mixture, or sequentially, with the first agent washed out prior to treatment with the second one. For the concurrent treatment, PC-3 cells were exposed to ciprofloxacin and to the cytotoxic agent simultaneously (50 µl each added to 100 µl of cells in culture medium) for 48, 72 and 96 h. For the 5 sequential administration schedules (24 + 48 or 72 h, 48 + 24 or 48 h and 72 + 24 h) cells were pre-incubated with ciprofloxacin (100 µl) and the second agent (200 µl) was added after wash out with PBS. For the studies with LNCaP cells, only the simultaneous 72 h and sequential 24 + 72/48 + 48 h treatment schemes were selected.

Cells incubated with each drug individually and cells incubated with the drug prior and after medium addition were used as controls for concurrent and sequential treatments, respectively, as performed by Hour et al. [22]. For controls, treatment schedule was the same as in combination assays. IC_{50} values for all treated controls and combinations were obtained as previously described for single drug experiments.

Median effect analysis of combined effects

The combined interaction effects were evaluated for synergism, additivity or antagonism by the median effect analysis developed by Chou and Talalay [8–10]. The cytotoxic effects are described by the median effect equation $fa/fu = (D/D_m)^m$, where fa and fu are the fractions of cells affected and unaffected, respectively, by a dose (D); D_m is the dose causing 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. The median effect plot [log (D) vs. log (fa/fu)] gives parallel lines (for individual drugs and their mixture) if the drugs have the same or similar modes of action, so the effects are mutually exclusive; if the plots for single drugs are parallel but the mixture plot intersects the plot of the more potent drug, the drugs act independently and their effects are mutually non-exclusive. The m and D_m parameters are easily determined from the median effect plot since they correspond to the slope and to the antilog of the x-intercept of the plot, respectively [8–10].

The combination index (CI) evaluates the nature of cytotoxic drugs interactions and is defined as:

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

where $\alpha = 0$ and $\alpha = 1$, for drugs with mutually exclusive or non-exclusive mechanisms of action, respectively. Denominators $(D_x)_1$ and $(D_x)_2$ are drug doses required to achieve a given effect level (fa). Numerators $(D)_1$ and $(D)_2$ are doses of each drug in a given mixture which originates the same fa. For three-drug combinations, the term $(D)_3/(D_x)_3$ is added. CI values reflect synergism, additivity or antagonism when inferior, equal or superior to one,

Table 1 Effect of exposure time on single drug cytotoxicity of three therapeutic agents against PC-3 cells

Therapeutic agents	24 h		48 h		72 h		96 h	
	IC_{50}	SD	IC_{50}	SD	IC_{50}	SD	IC_{50}	SD
Ciprofloxacin (mM)	0.767	0.080	0.238	0.023	0.212	0.023	0.193	0.015
Doxorubicin (µM)	3.937	0.783	0.290	0.017	0.250	0.035	0.173	0.032
Docetaxel (nM)	26.25	2.192	7.655	0.150	3.951	0.148	3.194	0.362

Mean IC_{50} values ± SD for 3–15 experiments in quadruplicate for ciprofloxacin, doxorubicin and docetaxel (mM, µM or nM, respectively) at different incubation periods. Cytotoxicity evaluation was performed by the resazurin assay and IC_{50} values determined by the GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA)

Table 2 Effect of exposure time on single drug cytotoxicity of three therapeutic agents against LNCaP cells

Therapeutic agents	24 h		48 h		72 h	
	IC_{50}	SD	IC_{50}	SD	IC_{50}	SD
Ciprofloxacin (mM)	0.276	0.027	0.244	0.056	0.215	0.041
Doxorubicin (μM)			0.02675	0.004	0.0209	0.004
Docetaxel (nM)			4.139	1.888	4.053	1.884

Mean IC_{50} values ± SD for 3–15 experiments in quadruplicate for ciprofloxacin, doxorubicin and docetaxel (mM, μM or nM, respectively) at different incubation periods. Cytotoxicity evaluation was performed by the resazurin assay and IC_{50} values determined by the GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA)

respectively. We, as others [6], considered additive effect to be any CI result within one SD of unity. CI results were plotted against *fa* for the whole effective range as varied degrees of synergism or antagonism may occur in distinct *fa* levels. This quantitative method takes into account not only the potency (D_m) of each drug and their combinations but also the shape (sigmoidicity) of their dose-effect curves [8–10]. Another advantage of this method is that three agents can be evaluated simultaneously [9].

Dose reduction index (DRI) corresponds to the fold of dose reduction in a combination as compared with the dose of each drug given alone, for a certain effect level [8, 11].

Results

Single-drug cytotoxicity studies

To assess the effect of ciprofloxacin, doxorubicin and docetaxel on cell proliferation, exponentially growing cells were treated with several doses for different incubation periods.

As an example, dose-response curves determined for PC-3 cells are depicted in Fig. 1. IC_{50} values for each drug and time period against PC-3 and LNCaP cells are presented in Tables 1 and 2, respectively. Against both cell lines, all three drugs induced a time and dose-dependent in

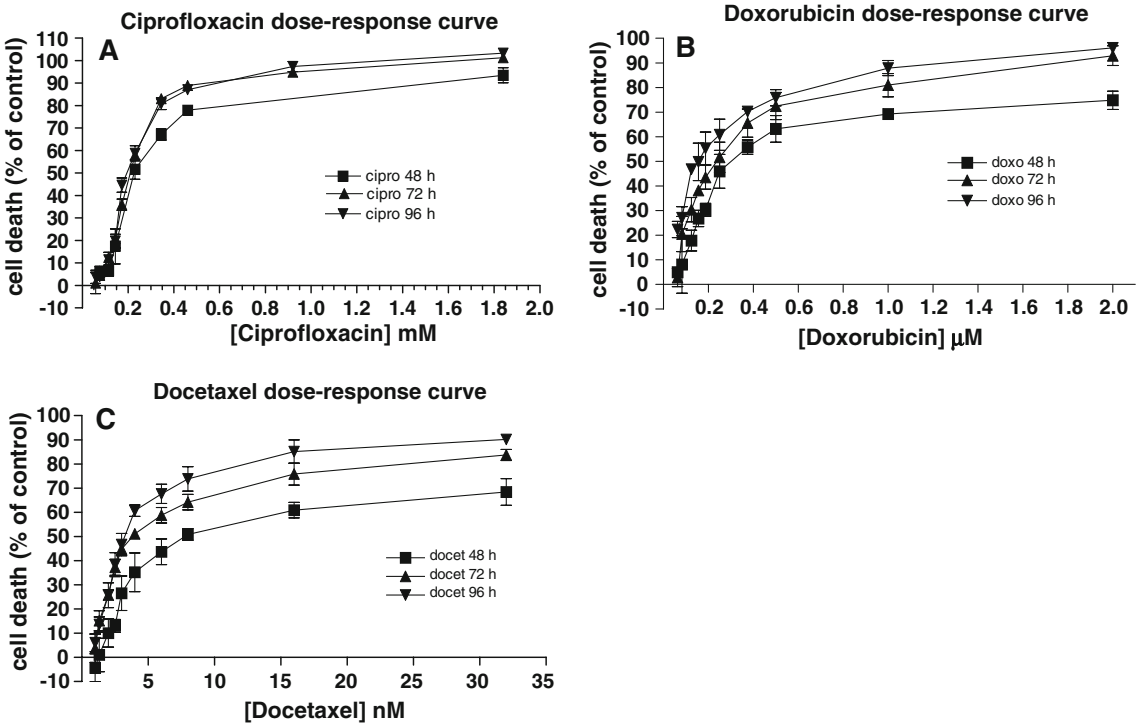


Fig. 1 Dose-response curves obtained for PC-3 cells treated with **a** ciprofloxacin, **b** doxorubicin and **c** docetaxel. Cell viability was assessed by the resazurin reduction assay at 37°C for (*filled square*) 48, (*filled triangle*) 72 and (*filled inverted triangle*) 96 h treatment with ten drug concentrations. Results are given as a mean percentage of non-viable cells relative to untreated controls ± SD of at least three independent experiments performed in quadruplicate

vitro cytotoxic effect (Fig. 1, not shown for LNCaP). Docetaxel was the most potent agent with an IC_{50} in the nanomolar level, whereas ciprofloxacin presented an IC_{50} within the 100-μM range (Tables 1 and 2). Growth inhibition between 0 and almost 100%, relatively to untreated control cells, were easily achieved with the selected concentration range tested (Fig. 1).

The most significant differences in drug IC_{50} values between the two cell lines were verified for doxorubicin and for 24 h exposure of ciprofloxacin (the only drug that would be tested for 24 h on LNCaP). LNCaP cells were approximately 10 times more sensitive to doxorubicin than PC-3 cells for 48 and 72 h treatments. Therefore, the cytotoxic activity of doxorubicin was cell line-dependent (Tables 1, 2).

Median effect analysis of combined effects

A constant IC_{50} ratio (equipotent) was applied so that the contribution of each drug effect would be equal. Representative median effect plots used in the quantitative method are presented in Fig. 2. Since the median-effect plots obtained for the drugs individually were not parallel (different slopes) exclusivity of the drug interaction effects could not be specified [10]. Accordingly, CI values were determined by the mutually exclusive and non-exclusive assumptions [10], but only the ones obtained under the second assumption were considered. This is a more conservative criterion to determine the type of interaction effects since the addition of a third term in Eq. 1 slightly increases the CI value [10]. Median effect plots yielded determination coefficients (R^2) between 0.96 and 0.99, which attests the applicability of the principle. Although the CI value can be expressed for any effect level, the most accurate determination is for $fa_{0.5}$ since the median-effect plot may be unreliable at the extremes as it represents a linear approximation of a non-linear function [27].

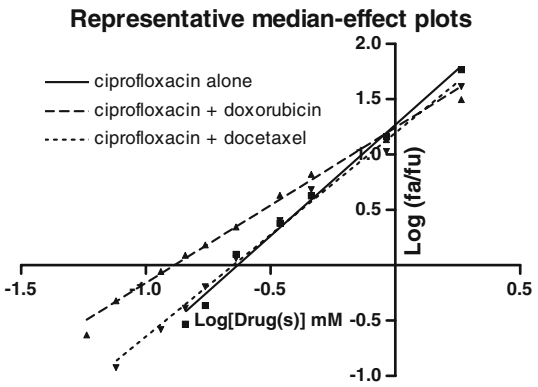


Fig. 2 Example of median-effect plots obtained for ciprofloxacin alone and combinations with doxorubicin and docetaxel. The same plots for doxorubicin and docetaxel were performed in separate graphs due to different concentration range in the *x*-axis

The CI versus *f*a graph (Fig. 3) illustrates ciprofloxacin-doxorubicin (Fig. 3a, b) or -docetaxel (Fig. 3c, d) interactions against PC-3 cells, over the cytotoxic range, for simultaneous and sequential treatment, respectively. Additionally, Figs. 4 and 5 describe the nature of interaction between ciprofloxacin and doxorubicin or docetaxel, against PC-3 cells, by presenting CI and DRI values (at $fa_{0.5}$ and $fa_{0.9}$), for all concurrent and sequential administrations, respectively.

A central finding of the present study is that, for most drug exposure regimens and growth inhibition levels, ciprofloxacin combinations with doxorubicin produced better CI values (lower) and higher DRI values than combinations with docetaxel (Figs. 3, 4 5).

Regardless the drug combination, sequential treatments against PC-3 cells revealed improved CI values (Fig. 3b, d), translated in much better DRI values (Figs. 4b, 5b), as compared to the simultaneous treatments (Figs. 4a, 5a). For a median effect level ($fa_{0.5}$), the concurrent exposure of ciprofloxacin and doxorubicin produced a slight antagonism (CI varying from 1.370 to 1.552) (Fig. 4a), whereas the sequential combinations yielded additivity (CI ranging from 0.997 to 1.169) (Fig. 4b). Doxorubicin doses needed to achieve 50% cell death were reduced by four to approximately 15-fold after a pre-treatment (especially 48 h) with ciprofloxacin (Fig. 4b) but the decrease is only twofold when doxorubicin is given in any concomitant combination (Fig. 4a).

Simultaneous ciprofloxacin–docetaxel combinations against PC-3 cells (Figs. 3c, 5a), yielded CI values, at $fa_{0.5}$, ranging from 1.723 to 2.071 suggesting stronger antagonistic effects comparing to the concurrent ciprofloxacin–doxorubicin combinations (Figs. 3a, 4a). For the sequential treatments ($fa_{0.5}$), only the combinations comprising 48 h or 72 h pre-treatment with ciprofloxacin yielded additive cytotoxic effects whereas those with 24 h ciprofloxacin pre-incubation induced slightly antagonism (Fig. 3d), with inferior DRI values (Fig. 5b). Dose reductions for docetaxel (Fig. 5) were, in most cases, worst than the ones obtained for doxorubicin (Fig. 4), ranging from 4.4 to 7.6 for the sequential schedules and from 1.5 to 1.9 for the concurrent schemes ($fa_{0.5}$).

The most promising schedules of treatment tested on PC-3 cells, in terms of DRI and/or CI results, were also evaluated on LNCaP cells in order to compare the combined effects of the selected combinations. As observed with PC-3 cells, simultaneous ciprofloxacin–doxorubicin or docetaxel combinations were mild antagonistic against LNCaP cells, with DRI values inferior to twofold ($fa_{0.5}$) (Table 3). In contrast, as observed on PC-3 cells, sequential combinations comprising pre-treatment with ciprofloxacin followed by the chemotherapeutic agent led to an enhanced cytotoxicity: interaction effects mostly lying in the

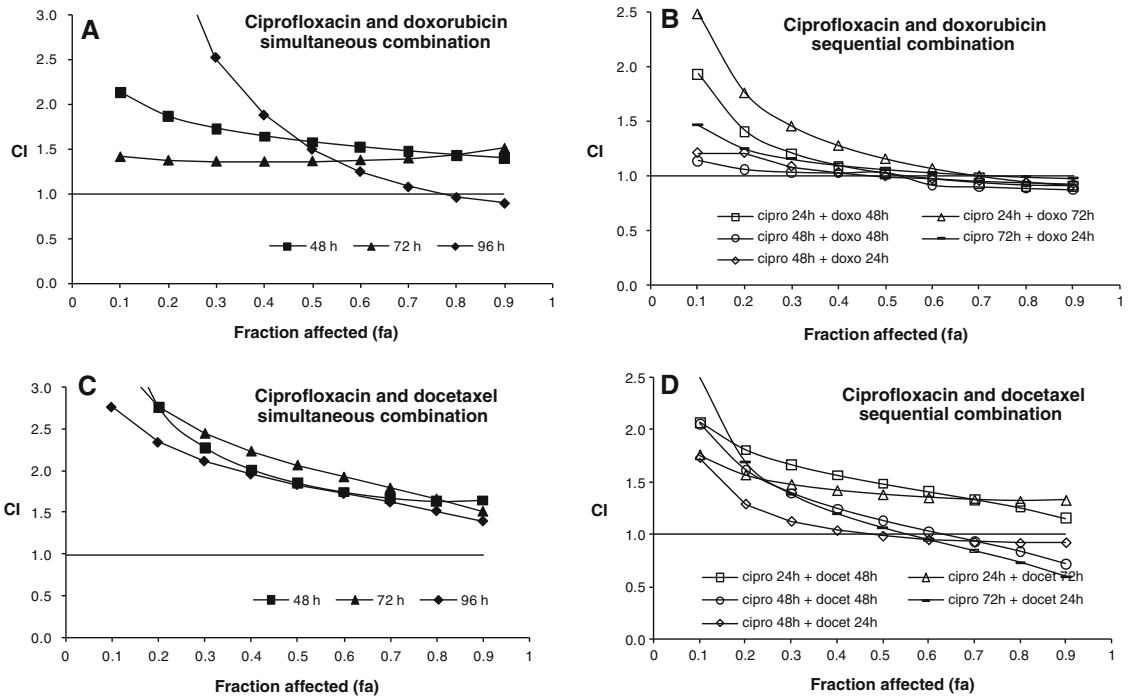
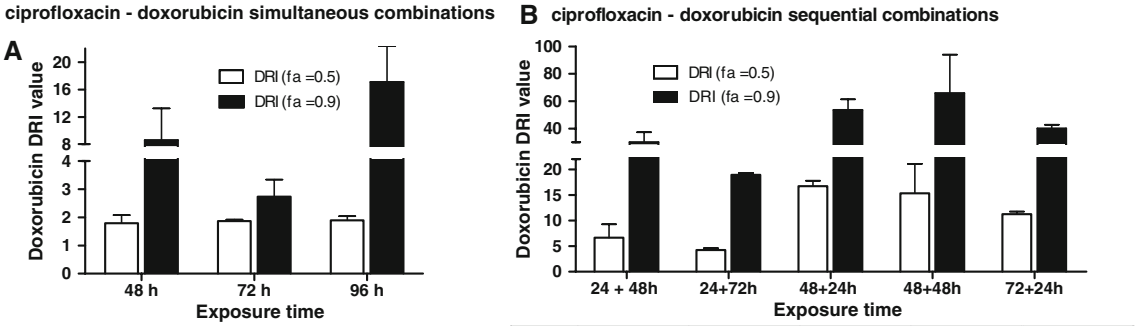


Fig. 3 Graphical representation of CI versus *f*a for ciprofloxacin-doxorubicin (**a**, **b**) or docetaxel (**c**, **d**) simultaneous and sequential combinations, respectively, against PC-3 cells, for different schedules. Mean CI values were compiled from two to four independent experiments and obtained by median effect analysis. CI values above and below 1.0 indicate antagonism and synergism, respectively, while 1.0 ± SD indicate additivity



$CI_{fa0.5}$	1.552 ± 0.122	1.370 ± 0.042	1.499 ± 0.105	1.022 ± 0.182	1.169 ± 0.434	0.997 ± 0.251	1.033 ± 0.178	1.061 ± 0.211
$CI_{fa0.9}$	1.401 ± 0.446	1.514 ± 0.247	0.900 ± 0.172	0.908 ± 0.358	1.063 ± 0.677	0.927 ± 0.403	0.878 ± 0.205	0.980 ± 0.370

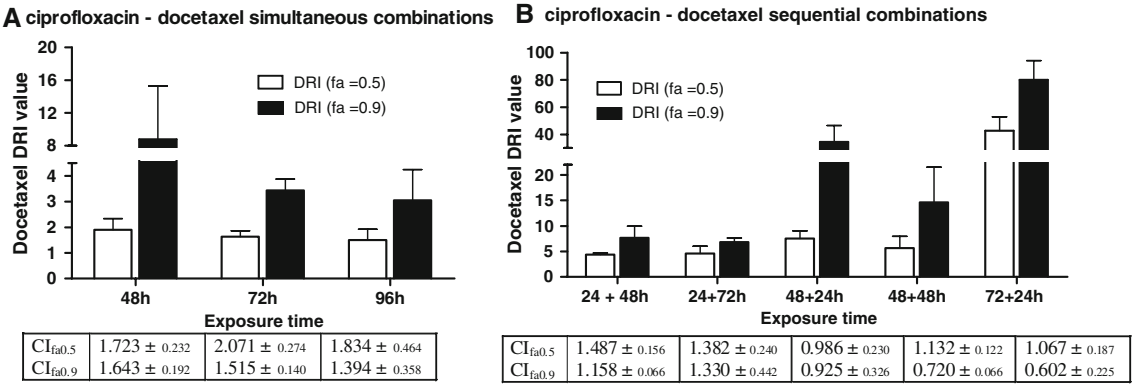
Fig. 4 Graphical representation of DRI and CI values for doxorubicin in **a** concurrent and **b** sequential combinations with ciprofloxacin against PC-3 cells. For each drug, ten concentrations, ranging from 0.250 to 8 times the IC_{50} value (Tables 1, 2), were used for each two-drug combination, so that the IC_{50} equipotent ratio remained constant. DRI values are presented as mean ± SD from 2 or 3 independent experiments for each administration scheme. DRI results are only expressed for 0.5 (*open columns*) and 0.9 (*shaded columns*) of fractional cell growth inhibition. Mean CI ± SD are outlined below the graph

additivity range for *f*a values between 0.5 (shown in Table 3) and 0.9 (not shown) and striking DRI values for the second drug, ranging from 2.6 to 8.1 (Table 3).

The obtained results clearly indicate that, for the same tested combinations, a similar interaction effect is observed for both cell lines. However, a lower DRI value was observed for LNCaP cells (Table 3) as compared to PC-3 cells (Fig. 4 and 5). This might be explained by the fact that LNCaP cells appeared more sensitive to the tested drugs (Table 1 vs. Table 2), so the decrease of IC_{50} (DRI) does not need to be so significant to originate a similar combined effect.

Discussion

The high incidence of recurrence and metastasis, as well as the refraction to chemotherapy make HRPC one of the



$CI_{fa0.5}$	1.723 ± 0.232	2.071 ± 0.274	1.834 ± 0.464
$CI_{fa0.9}$	1.643 ± 0.192	1.515 ± 0.140	1.394 ± 0.358

$CI_{fa0.5}$	1.487 ± 0.156	1.382 ± 0.240	0.986 ± 0.230	1.132 ± 0.122	1.067 ± 0.187
$CI_{fa0.9}$	1.158 ± 0.066	1.330 ± 0.442	0.925 ± 0.326	0.720 ± 0.066	0.602 ± 0.225

Fig. 5 Graphical representation of DRI and CI values for docetaxel in **a** concurrent and **b** sequential combinations with ciprofloxacin against PC-3 cells. For each drug, ten concentrations, ranging from 0.250 to 8 times the IC_{50} value (Tables 1, 2), were used for each two-drug combination, so that the IC_{50} equipotent ratio remained constant. DRI values are presented as mean ± SD from two or three independent experiments for each administration scheme. DRI results are only expressed for 0.5 (*open columns*) and 0.9 (*shaded columns*) of fractional cell growth inhibition. Mean CI ± SD are outlined below the graph

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

Schedule	Drug	PC-3 cells		Interaction effect	LNCaP cells		Interaction effect
	Combinations	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/docet	2.071 ± 0.274	1.6 ± 0.2	Antagonism	2.251 ± 0.375	1.9 ± 0.5	Antagonism
24 + 72 h	Cipro + doxo	1.169 ± 0.434	4.3 ± 0.4	Additivity	0.717 ± 0.046	4.8 ± 1.0	Synergism
	Cipro + docet	1.382 ± 0.240	4.6 ± 1.4	Additivity/antagonism	1.346 ± 0.385	2.6 ± 0.8	Additivity
48 + 48 h	Cipro + doxo	1.033 ± 0.178	15.3 ± 5.8	Additivity	1.306 ± 0.429	3.8 ± 0.2	Additivity
	Cipro + docet	1.132 ± 0.122	5.7 ± 2.3	Additivity/antagonism	1.229 ± 0.042	8.1 ± 1.5	Additivity/antagonism

Mean CI or DRI ± SD and nature of interaction effects for 2 to 4 experiments performed in quadruplicate, for ciprofloxacin–doxorubicin or docetaxel combinations after simultaneous 72 h and sequential 24 + 72 h/48 + 48 h. CI and DRI values at $fa_{0.5}$ were determined by the median effect analysis under the mutually non-exclusive assumption

most challenging cancer malignancies for therapeutic drug combinations studies.

The known preclinical and clinical anti-tumor activities of the drugs tested in this work, and their non-overlapping toxicities, provided the basis for the investigation of a potential therapeutic concurrent and sequential combinatorial effect, against two HRPC cell lines. The rationale of the sequential treatment regimens was to pre-treat cells with ciprofloxacin aiming at sensitizing the cells for the following cytotoxic agent, thus enhancing their anti-tumor effect while decreasing their dose and/or exposure time.

For the two drug combinations (at $fa_{0.5}$ or $fa_{0.9}$) and for both cell lines, we verified that in most administration schedules, small decreases in the CI value were accompanied by significant increases in DRI value (with no relevant proportion being established between these two parameters), with the caveat that at $fa_{0.9}$ the results are much more irreproducible between independent experiments. Even though synergism always results in high DRI values, slight antagonism can also lead to considerable DRI values and may have overall clinical value, as it was pointed out earlier [11, 26]. As therapeutic synergy may result from real synergy, additive effect or even mild antagonism, depending on whether non-overlapping toxicities are observed [26, 36], a better understanding of the mechanisms of action of the drugs is the starting point for a rational drug combination therapy.

Topoisomerase II enzyme regulates DNA topology through sequence-specific cleavage and resealing of DNA strands [4]. Doxorubicin is a topo IIα isotype inhibitor [29, 35] that intercalates with DNA and stabilizes the DNA-drug-enzyme cleavable complex by interfering with the enzyme's ability to religate DNA [4, 7, 29].

Similar to doxorubicin, ciprofloxacin stabilizes irreversibly the ternary cleavable complex, in a concentration dependent manner, but with differences in the mode of action. Unlike doxorubicin, it is a non-intercalating drug that act by stimulating the forward rate of DNA scission, without interfering with the enzyme-mediated religation of DNA [1, 14]. Ciprofloxacin attenuates in vitro topo II-mediated DNA cleavage enhancement by several drugs and inhibits their cytotoxic action in mammalian cells at an

extent similar to the cleavage impairment [15]. It has been established that ciprofloxacin acts as a competitive inhibitor of etoposide because it shares an overlapping domain on topo II [15, 32]. To date, a similar overlapping between ciprofloxacin and doxorubicin has not been described but it is a reasonable presumption because both doxorubicin and etoposide inhibit topo II by hindering enzyme-mediated DNA religation and it is known that those types of drugs share a common interaction domain in the enzyme [15]. Our results are in agreement with this hypothesis as the simultaneous ciprofloxacin-doxorubicin combination was antagonistic against both cell lines. So, these drugs should be sequentially scheduled, otherwise their combinations may be unsuitable for medical application. Furthermore, mild antagonism found in the concomitant exposure may also be explained by a different action on transcriptional factor NF-κB for apoptosis induction [5, 13].

In fact, our results clearly indicate that the sequential exposure of ciprofloxacin followed by doxorubicin led to significant DRI values for doxorubicin and additive effects on PC-3 and LNCaP cells. Sequential treatment may overcome the competitive drug binding on topo-II that occurs when the drugs are co-administered, allowing an inhibitory action on the enzyme spaced in time, which might potentiate the apoptotic mechanism.

Docetaxel suppresses microtubule dynamics with consequent mitotic spindle disruption, leading to late G_2/M arrest and bcl-2 phosphorylation and finally apoptosis [24, 28]. Therefore, the distinct molecular target and interference cell cycle phases might explain the antagonism when ciprofloxacin and docetaxel were co-administered on both cell lines and may justify the less promising results of most sequential combinations when compared with ciprofloxacin-doxorubicin combination.

Particular attention must be devoted to a certain irreproducibility of results at $fa_{0.9}$ due to the referred limitation of the method [27]. Nevertheless, the findings at $fa_{0.9}$ may be crucial since many authors claim that, for chemotherapeutic purposes, an inhibitory effect greater than 90% is generally required to achieve an effective treatment [11, 36].

To date, few studies have evaluated the anti-tumor activity of ciprofloxacin, singly or combined, against prostate [2, 13] or other cancers [3, 25], not to mention a potential timetable-dependence inherent to ciprofloxacin combinations. To our knowledge, only two studies have shown a chemosensitizer role for ciprofloxacin: combination with etoposide against PC-3 cells [13] and with doxorubicin against bladder cancer cells [25]. Both studies were performed in the absence of a quantitative method to assess the nature of combined effects. Herein, it was investigated, for the first time, the in vitro schedule-dependent cytotoxic effects arising from ciprofloxacin combinations with standard doxorubicin and docetaxel, against two HRPC cell lines, using the median effect analysis.

Previous studies have shown that the maximal plasma level for doxorubicin is 5 μM (average 1–2 μM) [17, 20] and the mean peak plasma level for docetaxel is usually 4.6 μM (after i.v. administration of 100 mg/m^2) [http://www.taxotere.com (2006)]. Therefore, doxorubicin and docetaxel concentrations required to achieve biological effects in vitro are in the same range or inferior to the clinical ones.

After oral administration of standard doses or 30-min intravenous infusion of 200 mg of ciprofloxacin, the maximal concentrations reached in plasma are generally 4.2 μg/ml [18] and 3.4 μg/ml [30], respectively. Although urine concentrations are up to 100 times plasma concentrations, the levels in human prostatic tissue are significant lower (2–2.45 times the plasma levels) [30].

Ciprofloxacin concentrations tested in this study are similar to the ones used against prostate cancer cells in previous studies [2, 13] and comprise the dose range required to occur growth inhibition from 0 to 100% and to allow study of interaction effects by the median effect analysis. Chemosensitization was observed with ciprofloxacin concentrations that are not achievable in human plasma or prostatic tissue but only in the bladder epithelium [3, 25]. Nevertheless, concentrations of ciprofloxacin that exert a cytotoxic activity and a chemosensitizer role might be achieved in the prostate through a novel drug delivery strategy (currently being developed) or through intraprostatic chemotherapy. This later type of treatment constitutes a feasible option to translate ciprofloxacin concentrations to clinical practice and has already been studied with doxorubicin [37].

Our study provided clear evidence that the type (concurrent or sequential) and the schedule of administration are important determiners of the cytotoxic patterns when several drugs are combined. Despite the known limitations of in vitro systems, our results clearly support pre-clinical studies in a HRPC animal model (intended to be performed by our group shortly) in order to better establish the therapeutic advantages of our protocol, namely the chosen dose(s) and schedule(s) of administration.

Drug combination studies on tumor cell lines, using a quantitative method to evaluate the nature of drug interactions, allow a more rational design of future chemotherapy protocols. Taking into account the systemic and dose-limiting toxicities associated with doxorubicin and docetaxel, a dose reduction may improve the overall therapeutic index in a potential clinical protocol. In fact, due to its non-toxicity and promising chemosensitizing role, ciprofloxacin might play a role as adjuvant to prostate cancer therapy. Additionally, since it is an antibiotic it may be

important as a potential pre or post-surgical adjuvant to combination chemotherapy.

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