In vitro assessment of cytotoxic agent combinations for hormone-refractory prostate cancer treatment

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We have investigated new drug combinations of potential clinical value for treatment of hormone-refractory prostate cancer. Combinations of paclitaxel, carboplatin and mitoxantrone, and combinations of these three drugs with compounds targeting important pathways for cancer progression, 13-*cis*-retinoic acid and chelerythrine, were assessed. The drugs combinations were incubated for 72 h in steroid-free conditions with two androgen-independent cell lines, DU145 and PC3. Cytotoxicity assay was performed using resazurin and Hoescht 33342. Synergism and antagonism were measured by the combination index, and calculated for each combination by the median-effect method. All six compounds exhibited cytotoxic effects when tested alone. Paclitaxel exhibited the highest and 13-*cis*-retinoic acid the lowest effect on both cell lines. Paclitaxel demonstrated synergism or additivity with 13-*cis*-retinoic acid in both cell lines, whereas antagonistic effects were observed when it was tested in combination with carboplatin. Chelerythrine showed additive effects with mitoxantrone in both cell lines and with paclitaxel in PC3 cells. Our results suggest that combination of paclitaxel and 13-*cis*-retinoic acid, and of chelerythrine with mitoxantrone and paclitaxel, may have clinical value for the treatment of hormone-refractory prostate cancer. *Anti-Cancer Drugs* 16:417–422 © 2005 Lippincott Williams & Wilkins.

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Introduction

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related death of men in industrialized countries [1]. Radical prostatectomy or radiotherapy is curative for patients exhibiting no clinical signs of extra-prostatic involvement. In advanced disease, androgen suppression therapy is the treatment of choice. This consists of medical castration by administration of long-acting luteinizing hormone-releasing hormone analog or surgical castration by orchidectomy with or without anti-androgen treatment. The median duration of response to androgen deprivation is approximately 18–24 months, after which all treated patients develop progressive hormone-insensitive disease.

Treatment options for patients with hormone-refractory prostate cancer (HRPC) remain limited, and include chemotherapy and/or palliation of symptoms. Mitoxantrone is the standard treatment for the palliation of symptoms in patients with HRPC in North America and Europe [2]. However, no survival advantage could be ascribed to mitoxantrone regimens, underscoring the need for new therapies with greater anti-tumor effect to improve outcome for HRPC [3]. Carboplatin and paclitaxel have been identified as a suitable therapeutic option in the setting of HRPC, and are actively studied in clinical trials. However, in monotherapy treatment, they have shown limited efficacy with 22.2 and 17% response in measurable disease for paclitaxel and carboplatin, respectively [4,5]. *In vitro* drug combination studies conducted in well-defined conditions that mimic specific *in vivo* parameters could be helpful in designing and optimizing combination protocols. Indeed, the analysis of *in vitro* drug interactions (synergy, additivity and antagonism) might characterize combinations that would have a greater clinical efficacy as well as combinations to be avoided.

In the present study, we used two androgen-independent prostate cancer cell lines, DU145 and PC3, to identify potential drug combinations for HRPC treatment. The efficacy of two-drug combinations between mitoxantrone, paclitaxel and carboplatin, and then of combinations of these drugs with two compounds targeting pathways playing an important role in cancer proliferation, i.e. 13-*cis*-retinoic acid or chelerythrine, were tested using the median-effect analysis [6–9].

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Materials and methods

Cell lines and culture

The two androgen-independent prostate carcinoma cell lines used here, DU145 and PC3, were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured in androgen deprivation conditions with RPMI 1640 medium (Gibco; Invitrogen, Cergy-Pontoise, France). Phenol red, which has previously been shown to have estrogenic effects, was not added in the culture medium. The latter was supplemented with 10% dextran-coated charcoal (Sigma-Aldrich, Lyon, France)-treated serum (FBS, Bio-West; Sigma) for androgen withdrawal. Testosterone and Δ4-androstenedione concentration were then less than 0.1 ng/ml as assessed by radioimmunoassay (Immunotech immunoassay kits for testosterone and Δ4-androstenedione; Beckman Coulter, Brea, CA). The doubling time of each cell line was determined under androgen deprivation conditions. It was estimated as 14 h for DU145 cells and 43 h for PC3 cells, instead of 16 and 49 h in usual testing (non-filtered serum and RPMI with phenol red). Medium was also supplemented with 0.4 μg/ml gentamicin (Gibco) and 2 mM glutamine (Gibco). Cultures were maintained at 37°C in a 5% CO_2 atmosphere. Medium was changed every 48 h unless specified otherwise and subcultured with 5 × trypsin/EDTA (Gibco).

Cytotoxic compounds and solvents

Mitoxantrone was kindly provided by Wyeth Lederle (Paris, France) and carboplatin by Teva Pharma (Courbevoie, France). Paclitaxel, 13-*cis*-retinoic acid and chelerythrine were purchased from Sigma. Carboplatin, mitoxantrone and chelerythrine were solubilized in sterile water; paclitaxel and 13-*cis*-retinoic acid in dimethylsulfoxide (DMSO).

Cytotoxicity assays

DU145 and PC3 were plated onto 96-well plates at a density of 5000 cells/well. Cells were allowed to attach for 24 h. Drug diluted in culture medium was then added at a constant solvent concentration of 0.5% (v/v) which had no effect on cell growth. Seven drug concentrations were tested in triplicate (three wells per point) versus controls in the presence and absence of solvent. After 72 h, the cytotoxicity was determined using two sequential methods: (i) detection of resorufin, a fluorescent product resulting from reduction of resazurin by cellular metabolism (RRT), and (ii) DNA quantitation by Hoescht 33342 dye (Ho) as previously described [10]. For the RRT assay, medium was removed and 25 μg/ml resazurin solution in RPMI without phenol red was added. After 1 h, plates were read on a microplate fluorometer (Fluoroskan Ascent FL; Thermolabsystem, Helsinki, Finland) with excitation at 530 nm and emission at 590 nm. Then, the resazurin solution was removed and the plates frozen at −80°C. After thawing, a 0.01% (m/v) sodium dodecylsulfate solution was added and incubated for 30 min at room temperature. The plates were then frozen again at −80°C. Finally, a 30 μg/ml Hoescht 33342 solution in 2 × TNE (Tris–HCl 10 mM/EDTA 1 mM/NaCl 2 M) was added and the plates were incubated for 1 h in the dark, at room temperature. Fluorescence was read at excitation 360 nm and emission 460 nm. Dose–response curves were drawn and the concentration giving 50% inhibition (IC_{50}) was calculated using the ALLFIT program [11].

Analysis of the multiple-drug effect by the Chou and Talalay method

Various two-drug combinations were evaluated using the median-effect principle proposed by Chou and Talalay. This mathematical model, previously established for enzyme–substrate interactions, has been extended to multiple drug combinations [12]. The algebraic expression of the median-effect principle is $f_a/f_u = [C/C_m]^m$, where f_a is the fraction of cells affected and f_u is the fraction unaffected by the treatment $(1-f_a)$, C the drug concentration, C_m the concentration required for 50% cell growth inhibition and m the slope of the median-effect curve. When the median-effect plots ($\log = \log[f_a/(1-f_a)]$) of drug 1, drug 2 and their mixture are parallel, the effects of drug 1 and drug 2 are mutually exclusive. If the plots of drugs 1 and 2 are parallel, but the plot of their mixture is concave upward, with a tendency to intersect the plot of the more potent of the drugs, their effects are mutually non-exclusive. From these equations the nature of combinations can be determined from the value of the combination index (CI) that can be calculated using the formula: $CI = (C)_1/(Cx)_1 + (C)_2/(Cx)_2$ for mutually exclusive drugs with the same or similar mechanism of action and $CI = (C)_1/(Cx)_1 + (C)_2/(Cx)_2 + (C)_1(C)_2/(Cx)_1(Cx)_2$ for mutually non-exclusive drugs with independent mechanism of action. $(C)_1$ is the concentration of drug 1, $(C)_2$ is the concentration of drug 2 and (Cx) is the concentration required to produce a median effect, usually defined by the IC_{50}. $CI < 1$ indicates synergism and $CI > 1$ indicates antagonism. An additive effect was defined as Budman *et al.*, i.e. as CI which contains 1 in its confidence interval [13]. Calculation was performed with the computer software CalcuSyn developed by Chou and Hayball [14].

In practice, combinations of drugs were assessed at their equipotent ratio (IC_{50} ratio). A mixture of the two drugs was made at 4-fold their IC_{50} and serially diluted (4 ×, 2 ×, 1.5 ×, 1 ×, 0.75 ×, 0.5 ×, 0.25 ×) to examine drug ratios above and below the IC_{50}, each in triplicate. Controls and assays were performed as described above.

Results

Single-drug cytotoxicity

Figure 1 shows the concentration–effect curve of each compound as measured with the RRT assay. Similar curves were obtained with Ho. All six compounds showed cytotoxic effects with IC_{50} in the range of 3 nM to

125 μM (Table 1). The relative potency against the two cell lines was (highest to lowest): paclitaxel, mitoxantrone, chelerythrine, carboplatin and 13-*cis*-retinoic acid. DU145 cells appeared more sensitive to the drugs than PC3 cells, except to chelerythrine. The two assays gave similar IC_{50} values for all drugs, excepted for carboplatin in PC3 cells in which the IC_{50} measured with RRT was about 2-fold higher than the IC_{50} measured with Ho.

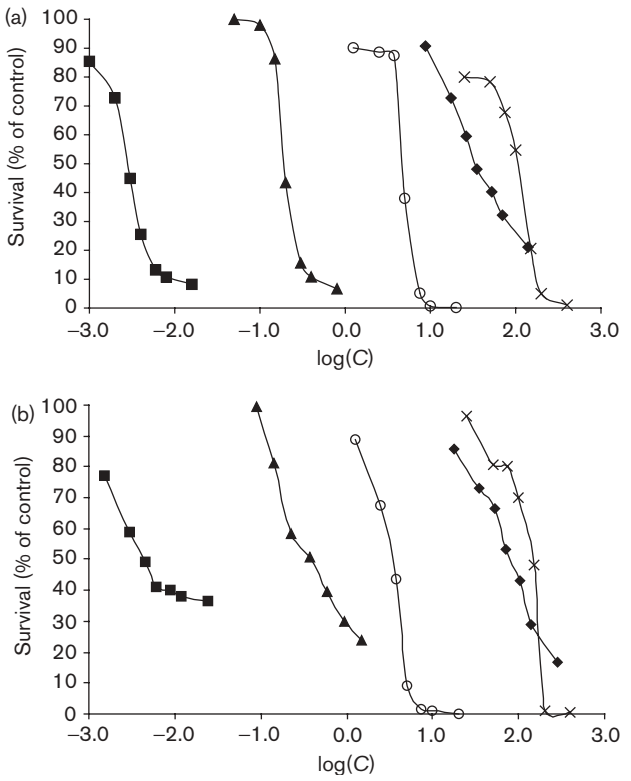
Two-drug combinations

Based on median-effect plot results, the non-mutually exclusive criterion was applied. This analysis is valid when the median-effect plot is linear, as was found in our experiments for each drug alone and in combination (regression values ranged between 0.93 and 0.99). CI values were calculated at f_{a50} because CI results are a linear approximation of a higher-order equation and are not accurate at the extremes [8].

Table 2 shows antagonistic CI values ranging from 1.3 to 2.8 in both cell lines for the three combinations of classical drugs used in HRPC treatment: paclitaxel/carboplatin, paclitaxel/mitoxantrone and carboplatin/mitoxantrone. The plot of the CI versus f_a measured by RRT and Ho illustrates antagonism of paclitaxel/carboplatin over the entire f_a range in both cell lines which decreased with f_a values (Fig. 2a).

To identify new potentially clinically useful treatment for HRPC, paclitaxel, carboplatin and mitoxantrone drugs were then assessed in combination with 13-*cis*-retinoic acid and chelerythrine. Corresponding CIs at f_{a50} are summarized in Table 3. 13-*cis*-Retinoic acid showed synergism when it was tested in combination with paclitaxel in PC3 cells. However, this combination gave only additive results in DU145 cells. Chelerythrine yielded additive effect with mitoxantrone in both cell lines and with paclitaxel only in PC3 cells. The combination of paclitaxel and 13-*cis*-retinoic acid was found synergistic both in PC3 and DU145 cells. However, synergy was observed in PC3 cells at f_a values greater than 0.35 instead of greater than 0.60 in DU145 cells. This may explain the additivity observed in DU145 cells when the combination was used at f_{a50} lower than 0.60 (Fig. 2b). The additive effect of chelerythrine/mitoxantrone combination in both cell lines, with four identical curves except at extreme values, is presented in Fig. 2(c). Figure 2(d) shows the opposite results obtained in DU145 and PC3 cells treated by the paclitaxel/chelerythrine

Fig. 1



Concentration–effect curves in DU145 and PC3 cells. RRT assay after 72 h. (a) DU145; (b) PC3. ■, Paclitaxel; ▲, mitoxantrone; ○, chelerythrine; ◆, carboplatin; ×, 13-*cis*-retinoic acid.

Table 2 **CI of classical drugs used in HRPC treatment in DU145 and PC3 cells**

	DU145		PC3	
	RRT	Ho	RRT	Ho
Paclitaxel + mitoxantrone	2.5 ± 0.7	2.4 ± 0.5	2.7 ± 0.7	1.7 ± 0.5
Paclitaxel + carboplatin	2.8 ± 0.4	2.1 ± 0.4	2.3 ± 0.5	2.6 ± 0.2
Carboplatin + mitoxantrone	1.7 ± 0.2	1.5 ± 0.2	1.7 ± 0.1	1.3 ± 0.2

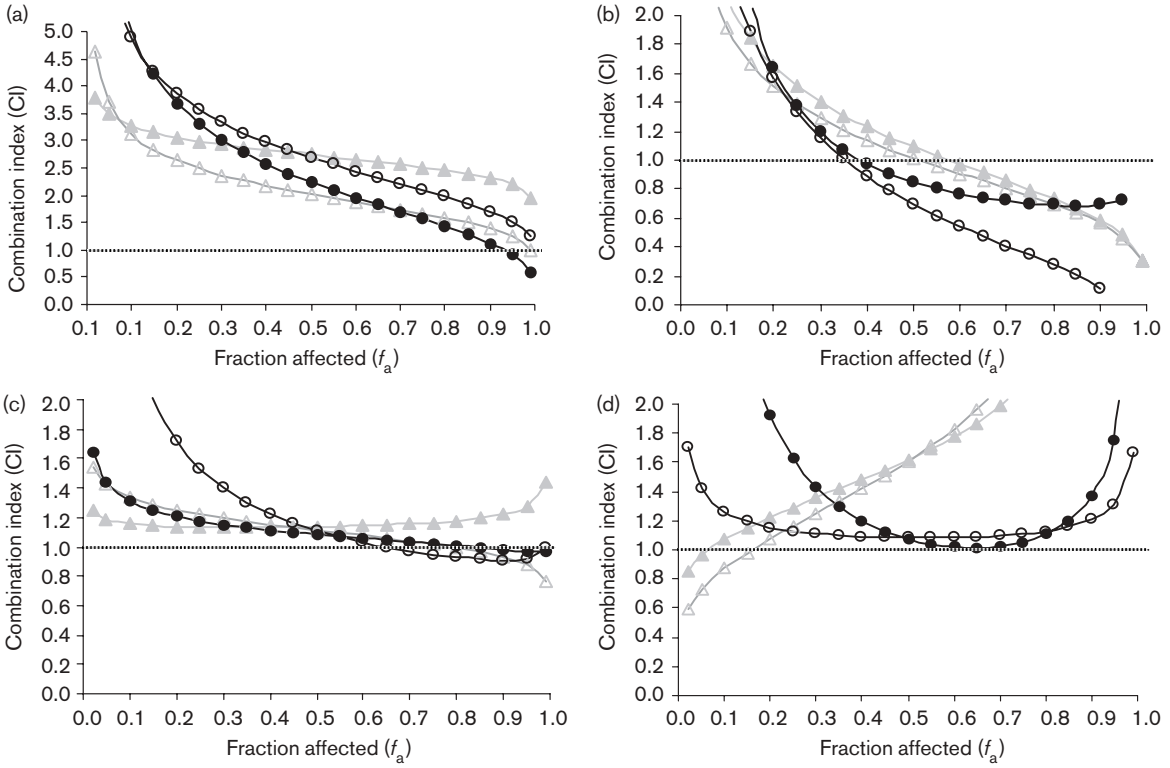
Data represents CI values ± SD (three experiments each in triplicate) at f_{a50} with the RRT and Ho assays after 72 h.

Table 1 **Cytotoxicity of various cytotoxic agents in DU145 and PC3 cells**

	DU145		PC3	
	RRT	Ho	RRT	Ho
Paclitaxel	0.0047 ± 0.0008	0.004 ± 0.009	0.0053 ± 0.0002	0.004 ± 0.001
Carboplatin	36 ± 9	32 ± 12	70 ± 19	40 ± 7
Mitoxantrone	0.3 ± 0.1	0.26 ± 0.08	0.5 ± 0.2	0.4 ± 0.2
13-*cis*-Retinoic acid	95 ± 2	85 ± 11	120 ± 13	125 ± 11
Chelerythrine	4.3 ± 0.9	5.1 ± 0.6	3.9 ± 0.7	4.2 ± 1.1

Data represents IC_{50} (μM) values ± SD (three to five experiments each in triplicate) with the RRT and Ho assay after 72 h.

Fig. 2



CI versus f_a in DU145 and PC3 cells. RRT and Ho assays after 72 h. (a) Paclitaxel + carboplatin; (b) paclitaxel + 13-*cis*-retinoic acid; (c) mitoxantrone + chelerythrine; (d) paclitaxel + chelerythrine. DU145: ▲, RRT; Δ, Ho; PC3: ●, RRT; ○, Ho.

Table 3 CI of 13-*cis*-retinoic acid and chelerythrine with classical drugs used in HRPC in DU145 and PC3 cells

	DU145		PC3	
	RRT	Ho	RRT	Ho
Paclitaxel + 13-*cis*-retinoic acid	1.1 ± 0.1[a]	1.1 ± 0.1[a]	0.84 ± 0.08[b]	0.7 ± 0.2[b]
Carboplatin + 13-*cis*-retinoic acid	2.3 ± 0.7	2.5 ± 0.3	3.2 ± 0.9	2.5 ± 0.6
Mitoxantrone + 13-*cis*-retinoic acid	1.48 ± 0.07	2.3 ± 0.5	4.8 ± 0.2	2.2 ± 0.2
Paclitaxel + chelerythrine	1.6 ± 0.4	1.6 ± 0.4	1.1 ± 0.3[a]	1.0 ± 0.4[a]
Carboplatin + chelerythrine	1.8 ± 0.3	1.5 ± 0.3	1.6 ± 0.1	1.8 ± 0.2
Mitoxantrone + chelerythrine	1.0 ± 0.1[a]	1.1 ± 0.2[a]	1.1 ± 0.2[a]	1.1 ± 0.1[a]
13-*cis*-Retinoic acid + chelerythrine	1.2 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.2 ± 0.1

Data represents CI values ± SD (three experiments each in triplicate) at f_{a50} with the RRT and Ho assays after 72 h. Additivity[a] or synergism[b] are indicated.

combination. A great range of antagonism was observed in DU145, while additivity was observed around f_{a50} values in PC3 cells.

Discussion

In vitro preclinical models have been used with some success on conventional and new combinations of drugs and modulators, and have led to the development of new combined treatment protocols for different tumor types [15].

In the present study, the cytotoxicity of each drug was assessed and presented as IC_{50} measured as either cellular metabolism (RRT) or DNA quantitation (Ho). Used in combination, these two assays validated the results of each test made independently, except for carboplatin in PC3 cells where RRT IC_{50} was 2-fold higher than Ho IC_{50}. A similar observation has been made in human cell lines treated with platinum and other DNA-damaging compounds (Debiton, personal data). However, this difference remains difficult to explain.

Our results demonstrate a clear synergy for the paclitaxel/13-*cis*-retinoic acid combination in PC3 cells and additivity in DU145 cells. Budman *et al.* previously described

synergy between docetaxel and *cis*-retinoic acid by median-effect analysis on both PC3 and DU145 cells [13]. These results underline the clinical potential of taxanes and retinoic acids combinations. Retinoids, which are small lipophilic synthetic derivatives of vitamin A, induce apoptosis *in vitro* and *in vivo* in various prostate cancer cells [16]. The molecular mechanism of this induction remains largely unknown, but appears to be associated with down-regulation of Bcl-2 expression [17]. Since paclitaxel was shown to induce apoptosis via inactivation of Bcl-2 by phosphorylation, it is likely that 13-*cis*-retinoic acid and paclitaxel would have a mutual synergistic action on Bcl-2 [18]. Moreover, using rat kidney cell lines, Zhang *et al.* had demonstrated that mutant p53 cells, which constitutively express Bcl-2, can be given sensitized to paclitaxel by 13-*cis*-retinoic acid and interferon (IFN)-α [19]. Paclitaxel/13-*cis*-retinoic acid combination with IFN-α had been successfully clinically tested. In a recent phase I clinical trial on 13 patients assessed by tumor markers or scans, five had stable disease and two exhibited a 50% decrease of prostate-specific antigen (PSA) [20]. The Eastern Cooperative Oncology Group recently activated a randomized phase II trial which compared weekly administration of paclitaxel plus 13-*cis*-retinoic acid and IFN-α with a combination of estramustine, mitoxantrone and vinorelbine. This trial may help to ascertain the real potency of this combination in the treatment of prostate cancer.

Our data revealed that in combination, paclitaxel and carboplatin were antagonistic. Antagonism was previously described between carboplatin and docetaxel on the same cell lines [13]. In contrast, a synergistic effect was reported for paclitaxel/carboplatin combination in four ovarian cancer cell lines using a clonogenic assay [21]. Based on clinical results on ovarian cancer, the paclitaxel/carboplatin combination has been actively investigated in clinical trials in HRPC and identified as an effective combination with response rates in measurable lesions up to 61.1%. PSA decreased more than 50% in all patients in a recent clinical trial [22,23]. In clinical trials in HRPC, paclitaxel/carboplatin combination was tested in association with estramustine which had demonstrated synergism with paclitaxel during *in vitro* studies [24]. The mechanism of the clinical synergy between paclitaxel/carboplatin is not clear to date. No pharmacokinetic interaction between paclitaxel and carboplatin has been demonstrated [25]. Although an interaction at the cellular level could not be discarded, the anti-angiogenesis effect of taxanes may explain the *in vivo* synergism between paclitaxel with other drugs. Indeed, paclitaxel inhibits human endothelial cell proliferation and tubulogenesis at picomolar concentrations [26,27]. This property is probably the major mechanism involved in the clinical synergy of taxane in combination with carboplatin. This example underlines the limits of the *in vitro* median-effect method for compounds targeting mechanisms other than tumor cell cytotoxicity.

We also assessed combinations of paclitaxel, carboplatin, mitoxantrone and 13-*cis*-retinoic acid with chelerythrine. Chelerythrine competes for the conserved catalytic sites of protein kinase C (PKC), a family of phospholipid-dependent serine/threonine kinases that includes various isoforms [28]. It appears to be a potent and specific inhibitor of the isoforms α and β [6]. As PKCα is involved in cell survival and is over-expressed in several human androgen-independent prostate cancer cell lines, it has been proposed as a possible target for this malignancy [29,30]. A recent study has demonstrated that specific inhibition of PKCα by a hammerhead ribozyme targeting its mRNA enhances the sensitivity of DU145 and PC3 cells to cisplatin and oxaliplatin [30]. However, in single treatment, bryostatin-1, the first PKC inhibitor used in the clinic, has given disappointing results in phase II clinical trials in melanoma, colorectal and renal carcinoma, Hodgkin's lymphoma and chronic leukemia [31]. Recently, Hofmann reviewed the relationship between PKC activity and tumor cell chemosensitivity [32]. He emphasized the great number of conflicting results reported in various *in vitro* and *in vivo* models. These differences may reflect cell-type specificity. The considerable heterogeneity of PKC family members and their complex regulation may also contribute to these discordant results. In contrast to other PKC inhibitors such as bryostatin-1 or flavopiridol, chelerythrine was recently identified as a specific inhibitor of Bcl-x_L, a well-known anti-apoptotic member of the Bcl-2 family [33]. Bcl-x_L mediates a survival mechanism involved in anticancer drug resistance of HRPC cells and down-regulation of this protein was shown to sensitize PC3 cells to apoptotic stimuli [34,35]. Thus, the dual effect of chelerythrine on PKCα and Bcl-x_L makes it very promising for combinations with classical drugs. Although chelerythrine has previously shown additivity with cisplatin in human head and neck squamous cell carcinoma lines using the MTT assay [36], in our model chelerythrine/carboplatin association has shown antagonism in both cell lines with the two assays. Conversely, additivity has been observed in combination with mitoxantrone in both cell lines and with paclitaxel in PC3 cells. Hence, both combinations identified here involving chelerythrine may have a therapeutic interest in HRPC, and justify further investigation in animal models and clinical trials.

The treatment of HRPC remains a therapeutic challenge with a strong need of treatment. Despite the classical limitations of *in vitro* systems, results observed in this study confirm the potential clinical value of 13-*cis*-retinoic acid/paclitaxel combination, and positive results of chelerythrine in combination with mitoxantrone or paclitaxel justify further investigation in animal models and clinical trials to verify the therapeutic interest of these combinations in HRPC.

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