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Low Doses of CPS49 and Flavopiridol Combination as Potential Treatment for Advanced Prostate Cancer

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Abstract: Prostate cancer (PCa) still ranks as the second most frequently diagnosed cancer and metastatic castration-resistant prostate cancer (CRPC) is a foremost cause of men cancer death around the world. The aim of this work was to investigate the selectivity and efficacy of new drug combinations for CRPC. We combined three compounds: paclitaxel (PTX: taxane that inhibits microtubule polymerization); 2-(2,4-Difluoro-phenyl)-4,5,6,7-tetrafluoro-1H-isoindole-1,3(2H)-dione (CPS49; redox-reactive thalidomide analog with anti-angiogenic properties) and flavopiridol (flavo: semi-synthetic flavonoid that inhibits cyclin dependent kinases). We assessed CPS49-flavo or -PTX combinations cytotoxicity in a panel of PCa cell lines and PC3 xenografts. We found that CPS49 enhanced flavo or PTX cytotoxicity in human PCa cell lines while showed resistance in a non-tumor cell line. Furthermore, xenografts generated by inoculation of human prostate carcinoma PC3 cells in *nu/nu* mice showed that CPS49/flavo administration reduced tumor growth both after 2 weeks of co-treatment and after 1 week of pretreatment with a low dose of flavo followed by 2 weeks of co-treatment. PTX and CPS49 combination did not significantly reduce tumor growth in PC3 xenografts. Histological analysis of xenograft PC3 tumor samples from CPS49/flavo combination showed extensive areas of necrosis induced by the treatment. RT-qPCR array containing 23 genes from PC3 cells or PC3 xenografts exposed to CPS49/flavo combination showed that this treatment shut down the expression of several genes involved in adhesion, migration or invasion. In summary, the antitumor activity of CPS49 or flavopiridol was improved by the combination of these compounds and using half dose of that previously reported. Hence, CPS49-flavo combination is a promising new alternative for PCa therapy.

Keywords: CPS49, flavopiridol, paclitaxel, prostate cancer, preclinical study, xenografts.

INTRODUCTION

Prostate cancer (PCa) still ranks as the second most commonly diagnosed cancer in men. Metastatic castration-resistant prostate cancer (CRPC) is a top cause of cancer-related death around the world [1]. Treatment options for patients with PCa include surgery, radiation therapy, hormone therapy, chemotherapy and experimental protocols regulated by approved clinical trials. While there are currently seven agents approved for CRPC and four regimens have shown some survival benefit, those survival prolongations have been shown to be modest and, unfortunately, all patients will eventually progress [2] [www.cancer.gov/cancertopics/treatment/prostate]. Thus, there is a need for the discovery of new agents and regimens for this disease.

The first-line chemotherapy for CRPC includes docetaxel, a taxane that induces apoptosis by blocking microtubule activity during cell division, disruption of the cell cycle, and phosphorylation of bcl-2 [3]. Although treatment with docetaxel shows better survival, it is also associated with toxicity and resistance in many patients. For this reason multiple classes of agents with different mechanisms of action have been studied for additive or synergistic activity with docetaxel [4]. Phase II clinical trials have shown that the combination of different agents with docetaxel increases the efficacy and tolerability, compared with docetaxel alone [3].

One of the agents that have been combined with docetaxel to improve its efficacy is thalidomide, a potent

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angiogenesis inhibitor. In a randomized phase II study of docetaxel plus thalidomide in CRPC, the therapy extended overall survival, and although acceptable safety was reported, some patients presented thromboembolic events [5]. Thalidomide also showed teratogenic effects in pregnant women. Besides these effects had limited its widespread clinical use, this side effect should not be considered for CRPC patient population. Therefore, many structural analogs of thalidomide have been synthesized and tested for their efficacy [6]: 'class I or IMiDs (Immunomodulatory imide Drugs), class II or SelCiDs (Selective Cytokine Inhibitory Drugs)' [7, 8] and class III or N-substituted and tetrafluorinated analogs (CPS49) [6, 9]. CPS49 was reported to selectively kill leukemic cells by increasing intracellular reactive oxygen species (ROS) and further targeting multiple transcriptional pathways [10]. Lenalidomide, another thalidomide derivative with immunomodulatory and anti-angiogenic properties, was combined with docetaxel in a CRPC phase I study. Although the phase II results were promising; phase III results were negative (NCT00988208) [5, 11].

Eventually, therapy needs to be interrupted due to drug toxicity or disease progression due to resistance and treatment should be changed to second-line chemotherapy. Therapy based on PTX (paclitaxel), another class of taxane, had been proposed in docetaxel-pretreated patients. Resistance to one taxol does not imply resistance to another, because these drugs have different binding sites [12]. Hence, combination of PTX and carboplatin in patients with CRPC pretreated with docetaxel had been proposed in a phase II study [13]. This therapy resulted in a well-tolerated regimen with favorable PSA response, although PTX had not been shown to improve overall survival in a large randomized study [14, 15].

'Flavopiridol (flavo) is a semisynthetic flavonoid that inhibits cyclin dependent kinases (CDKs) and shows selective lethality against leukemic cells' [16-19]. Although it's successful performance in several preclinical studies, the advances of flavo to clinical trials have been disappointing. Used as a single agent, flavo produced the stabilization of the disease progression as a result of its cytostatic activity through the inhibition of CDKs. However, not many studies showed any additional effect [17, 20]. Numerous clinical trials using flavo in combination with other agents have yield convincing results. Previously, the co-administration of CPS49 and flavo was tested *in vitro* [21]. These studies showed that this combination was successful targeting transcriptional pathways that enforce selective mitochondrial dysfunction and ROS elevation in leukemia cells [21]. Thus, another rationale for flavo in clinical use could be to minimize the dose in patients while combining with other agents to have lower patient toxicity.

The aim of this work was to test new therapies combining chemotherapeutic drugs to achieve strong tumor size reduction while decreasing toxicity and side effects. We combined low doses of CPS49 with flavo or PTX and investigated these compounds effects *in vitro* and *in vivo* experiments. We found that these combinations inhibit the proliferation of PCa cell lines. In addition, we showed that a half of the effective dose previously reported for flavo and CPS49, significantly decreased prostate tumor growth *in vivo*. Furthermore, the combination of flavo with CPS49 increased tumor cells necrosis and tolerability, targeting adhesion, migration and invasion genes.

MATERIALS AND METHODS

Cell Culture

PCa androgen-sensitive cell line LNCaP (ATCC: CRL-1740) and androgen-insensitive cell lines PC3 (ATCC: CRL-1435), 22Rv1 (ATCC: CRL- 2505) and C4-2 (derived from LNCaP) were grown in RPMI with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified atmosphere at 37ºC. The embryonic human kidney cell line HEK-293 (ATCC**:** CRL-1573) was grown in DMEM with 10% FBS.

Drugs

Flavo (Sanofi) and CPS49 (Celgene Corporation, NJ, USA) were reconstituted in DMSO. PTX (DOSA S.A., Buenos Aires, Argentina) was diluted in ethanol.

Cell Viability (MTS)

Cells were exposed to different concentrations of flavo or CPS49 (0.675; 1.25; 2.5 and 5 μM); or to PTX (6.25; 12.5; 25 and 50 nM) as single treatment or in 1:1 combination for 24 h. Pretreatments were performed with low doses of flavo (0.2 μM) or PTX (5 nM) for 8 h, and then treated with the 1:1 combination (flavo/CPS49 or PTX/CPS49) for 24 h. Cell viability was measured using the MTS assay (Cell Titer 96 wells Aqueous non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA) following the manufacture instructions. Three biological independent experiments were performed for each drug treatment.

RNA Isolation, cDNA Synthesis and Real Time PCR

PC3 cells were treated with flavo and CPS49 and total RNA was isolated using TriReagent (Genbiotech, Buenos Aires, Argentina). cDNA was synthesized from 2 μg of RNA using RevertAid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). Real time PCR (qPCR) was performed as previously described [22] using Taq Polimerase (Fermentas) in a DNA Engine Opticon (MJ Research, Bio-Rad, Hercules, CA, USA) [22]. Average from 3 biological independent experiments was shown. Data were normalized to Actin B (ACTB) expression and vehicle treated control. Primer sequences are indicated in Table **1**.

Cell Cycle Analysis and Apoptosis

PC3 cell line was exposed to flavo and CPS49 treatments for 24 h. Cells were harvested, stained with propidium iodide (PI) or Annexin V-FITC/PI and analyzed by fluorescence-activated cell sorting (FACS) as previously described [22]. Three biological independent experiments were performed.

Clonogenic Assay

Cells were plated at the clonal density of 500 cells per dish (6 cm). After 24 h cells were exposed to flavo, CPS49, PTX or combinations for 3 weeks. Media with drug was replaced every 3 days. Colonies where fixed with 4% paraformaldehyde for 10 min, stained with crystal violet for 10 min,

Table 1. Primer sequences used for RT-qPCR.

Gene Name	RefSeq NM#	Forward Primer	Reverse Primer
SFN	NM_006142	5′CTGTCCAGTTCTCAGCCACA3′	5′CAGGCTACTTCTCCCCTCCT3′
CCNB2	NM_004701	5'TTCTGATGCCTTGCTCTGC3'	5'ATGCGTCCATTTATATCTCTTCC3'
MAD2L1	NM_002358	5'AATACGGACTCACCTTGCTTGTAACTAC3'	5'TGCCATCTTTCCAGGACCTCACC3′
Che1	NM_012138	5'TCAGCCTGTCCCAGAGAGTT3'	5'AGGAGCTGGTCTTCCGTTCT3'
E2F4	NM_001950	5'GGTGTGGGTGCAGCAGAGCA3'	5'CAGGCTGGTGCCTGATGGGG3'
BRCA2	NM_000059	5'AAGCATTGGAGGAATATCGTAGG3'	5'CAGGTTCAGAATTATAGGGTGGAG3'
DDB2	NM_000107	5'TCACTTCCAGCACCTCACAC3'	5'ACGTCGATCGTCCTCAATTC3'
FEN	NM_004111	5'GGCAACCCCGAACCAAGC3'	5'GCTCATAGAGGCATCAATGGC3'
BLM	NM_000057	5'GAATGGTTAAGCAGCGATG3'	5'TCAATACATGGAACTTTCTCAG3'
H3F3B	NM_005324	5'AAAGCCGCCAGGAAAAGC3'	5'CAGACCCACCAGGTACGC3'
BRCA1	NM_007294	5'TGAAATCAGTTTGGATTCTGC3'	5'CATGCAAGTTTGAAACAGAAC3'
SELL	NM_000655	5'GGGCAGTTTAATATGGGTCA3'	5'CATTCTCTGGGTTGGCATTT3'
MMP9	NM_004994	5'AGACCTGGGCAGATTCCAAACC3′	5′GCAAAGGCGTCGTCAATCACC3'
ENG	NM_001114753	5′GCCGTGCTGGGCATCACCTT3′	5′GCTCCCGCTTGCTGGGGGAA3′
VEGF	NM_001025366	5'GCCTTGCCTTGCTGCTCTACC3′	5′GTGATGATTCTGCCCTCCTCCTTC3′
E-Cadherin	NM_004360	5'AAGGTTCACCCAGCACCTTGCA3'	5'GGCAGAGGGACACACCAGTGTAGTAA3'
B-Catenin	NM_001098209	5-CATAACCTTTCCCATCATCGT-3'	5′-TGTGGAGAGTTGTAATGGCA-3′
Vimentin	NM_003380	5'CACTCCCTCTGGTTGATAC3'	5'GTGATGCTGAGAAGTTTCG3'
Fibronectin	NM_002026	5'GCTCATCCGTGGTTGTATCAGG3'	5'TGGTCTGCTTGTCAAAGTGTCC3'
Slug	NM_003068	5'TCGGACCCACACATTACC3'	5'CAGATGAGCCCTCAGATTTG3'
Snail	NM_003068	5'CCTGCGTCTGCGGAACCTG3'	5'GTTGGAGCGGTCAGCGAAGG3'
CCNT1	NM_001240	5′CTTAGCACAGACTTCTTACTTC3'	5'GTGGCGTCAACATACTCC3'
ELL	NM_006532	5′CAGCGTTTCCAGTGTTCC3'	5'CATTGAAGTCGTTCTTGTAGC3'
CtBP1	NM_001012614	5′TACAGCGAGCAGGCATCC3'	5'TGGTCCTTGTTGACACAGTTC3'
Actin B	NM_001101	5'AAGATCATTGCTCCTCCTGAGC3'	5'CATACTCCTGCTTGCTGATCCA3'

and washed with PBS. The colonies were photographed. Each treatment was performed in triplicate.

Xenografts

Six-week old male *nu/nu* mice, weighting 20-25 g, were housed under pathogen-free condition following the University of Buenos Aires animal care guidelines. Animal protocols were approved by the Animal Ethics Committee from the University of Buenos Aires. PC3 cells ($5x10^6$ cells) were inoculated *s.c.* into the right flank of each mice. When tumors reached between 50 and 100 mm^3, mice were randomly distributed into different groups (6 mice per group) and drugs were administrated intraperitoneally (i.p.). To evaluate the effects of each drug alone, we injected: *i)* 4 weekly injections of flavo (6 mg/kg/day) for two weeks; *ii)* 4 weekly injections of CPS49 (6 mg/kg/day) for two weeks; *iii)* 3 weekly injections of PTX (12 mg/kg/day) for two weeks; or *iv)* 4 weekly injections of DMSO (control) for 2 weeks (Fig **3A**). To evaluate the effects of drug combinations we injected: *i)* Co-treatment flavo/CPS49: 4 injections of DMSO for one week and 4 weekly injections of flavo (6 mg/kg/day) plus CPS49 (6 mg/kg/day) for the following two weeks; *ii)* Pre-treatment: 4 injections of flavo (2 mg/Kg/day) for one week, and 4 weekly injections of flavo (6 mg/kg/day) plus CPS49 (6 mg/kg/day) for the following two weeks; *iii)* Co-treatment PTX/CPS49: 3 injections of PTX (12 mg/kg/day) plus CPS49 (6 mg/kg/day) for two weeks; *iv)* 4 weekly injections of DMSO (control) for 3 weeks (Fig **3C**). All drug doses were half of the previously reported concentrations. Mice were weighed once a week. Mice were sacrificed 24 h after last injection. Tumors were extirpated and sectioned for histology and total RNA isolation as described [22]. The length and width of the tumor were meas-

ured three times per week using a digital caliper. Tumor volume was calculated as follows: 0.523 x length x width^2 [22]. In addition, lungs, livers and kidneys were resected and histological analyses were performed.

Histology

Tumors were fixed in a PBS-10% formaldehyde solution and paraffin embedded (FFPE). Microscopic sections were stained with hematoxylin-eosin (H&E) and Masson´s trichrome technique for fibrous and reticular fibers, and examined by light microscopy as previously described [23].

Statistical Analysis

Results are presented as mean ± standard deviation (SD) of *"n"* separate independent experiments. To ascertain statistical significance one-way ANOVA followed by Tukey´s multiple comparison test and two-way ANOVA followed by Bonferroni test was performed using GraphPad Prism version 4.00 with a threshold of $P< 0.05$. For *in vivo* experiments, two independent experiments were performed using 6 mice per group of drug treatment. Comparison for *in vivo* experiments was made with one-way ANOVA followed by Dunnett´s test, with $P< 0.05$ as the criterion for statistic significance.

RESULTS

Flavopiridol or Paclitaxel are Cytotoxic Compounds for Human PCa Cell Lines

To assess drug cytotoxicity, we exposed human androgen-sensitive (LNCaP), androgen-insensitive (PC3, C4-2 and 22Rv1) prostate tumor cell lines and the non-tumor HEK293 cell line to increasing concentrations of CPS49, flavo or PTX. As shown in (Fig. **1A**), all the PCa cells tested were highly sensitive to PTX (50 nM). Interestingly, only androgen insensitive cells were high to moderate sensitive to flavo (5 μM), showing 22Rv1 cells the highest cytotoxic response (Fig. **1A**). All the cell lines tested were resistant to the CPS49 concentrations assayed (0.675 to 5 μM). Furthermore, it was previously reported that PBMCs normal cells were resistant to CPS49 or flavo [21], interestingly, the non-tumor HEK293 cell line was insensitive to all the tested compounds.

In summary, PTX and flavo are cytotoxic agents against PCa cell lines, showing certain selectivity considering that the non-tumor cells were resistant.

Flavopiridol and Paclitaxel Alone or in Combination with CPS49 Decrease PCa Viability

Based on our previous findings that flavo and CPS49 combination was more efficient for killing leukemia cells than single-drug treatments [21], we first treated PCa and HEK293 cell lines with different 1:1 drug combinations for 24 h (*co-treatment*). We observed that LNCaP and HEK293 non-tumor cells were resistant to this combination (Fig. **1B**). A two-way ANOVA analysis revealed that all androgen insensitive cells were sensitive to flavo agent. Also, non-additive effect or interaction effect was observed when using combined treatment with CPS49 (Fig. **1B**).

We previously reported that the initial treatment with low doses of flavo sensitizes cells to a subsequent co-treatment with flavo and CPS49 [21]. In this paper, we investigated the effect of flavo pretreatment (0.2 μM for 8 h) plus 24 h CPS49/flavo co-treatment on this panel of PCa cell lines (*pre-treatment*). As shown for co-treatment, 22Rv1, C4-2 and PC3 cells showed moderate sensitivity to this type of treatment while LNCaP and HEK293 cells were resistant (Fig. **1C**). Interestingly, by performing a two-way ANOVA analysis, we found that *pre-treatment* significantly synergized the *co-treatment* in PC3 cells, showing that this alternative is the most cytotoxic combination tested in PC3 cells.

In order to investigate new drug combinations for advanced PCa treatment, we exposed cell lines to CPS49 plus PTX for 24 h (co-treatment), or pre-treated with 0.5 nM PTX for 8 h and then 24 h of PTX plus CPS49 (Fig. **1B-C**). All PCa cell lines were highly sensitive to both combinations. Statistical analysis from co-treatment revealed that all PCa cell lines resulted sensitive to PTX exposure. However, the non-tumor cell line showed moderate sensitivity to these combinations. Interestingly, although no significant interaction was found for PTX and CPS49, LNCaP cells turned less sensitive to PTX when combined with CPS49 treatment as two-way ANOVA and Bonferroni post hoc analysis revealed. However, this effect was abolished by PTX pre-treatment (Fig. **1B-C**).

Flavopiridol and Paclitaxel Alone or in Combination with CPS49 Inhibited PC3 Colony Formation

To determine whether flavo, CPS49, PTX or their combinations influence colony proliferation, we exposed PC3 cells to these agents during 21 days and assessed colony formation. Flavo, PTX or their combinations with CPS49 dramatically decreased the number of PC3 colonies (Fig. **2A**). No effect was produced by CPS49 on the number of colonies compared to control (Fig. **2A**); therefore, CPS49 was not altering cell proliferation. Since all the doses of flavo and PTX tested completely abolished colony formation, we could not detect any drug synergism given by the co-treatment with CPS49.

Flavopiridol Alone or in Combination with CPS49 Induces Cell Cycle Arrest and Apoptosis in PC3 Cells

It was previously demonstrated that Flavo inhibited CDKs [16] and induced cell cycle arrest in different cell lines [24, 25]. To investigate whether low doses of flavo or its combination with CPS49 induced PC3 cell cycle arrest, we determined DNA content by PI staining and FACS in PC3 cells exposed to flavo or CPS49 plus flavo combinations. As shown in (Fig. **2B**), flavo alone or in combination with CPS49 (either co-treatment or pre-treatment) induced cell accumulation in G2/M phase decreasing the percentage of cells in G1-phase. These results demonstrated that CPS49 did not interfere with the ability of flavo to arrest cell cycle when PC3 cell lines were exposed to the drugs combination. Therefore co-treatment and pre-treatment caused a slight increase in S-phase cells, which could indicate an S-phase blockage.

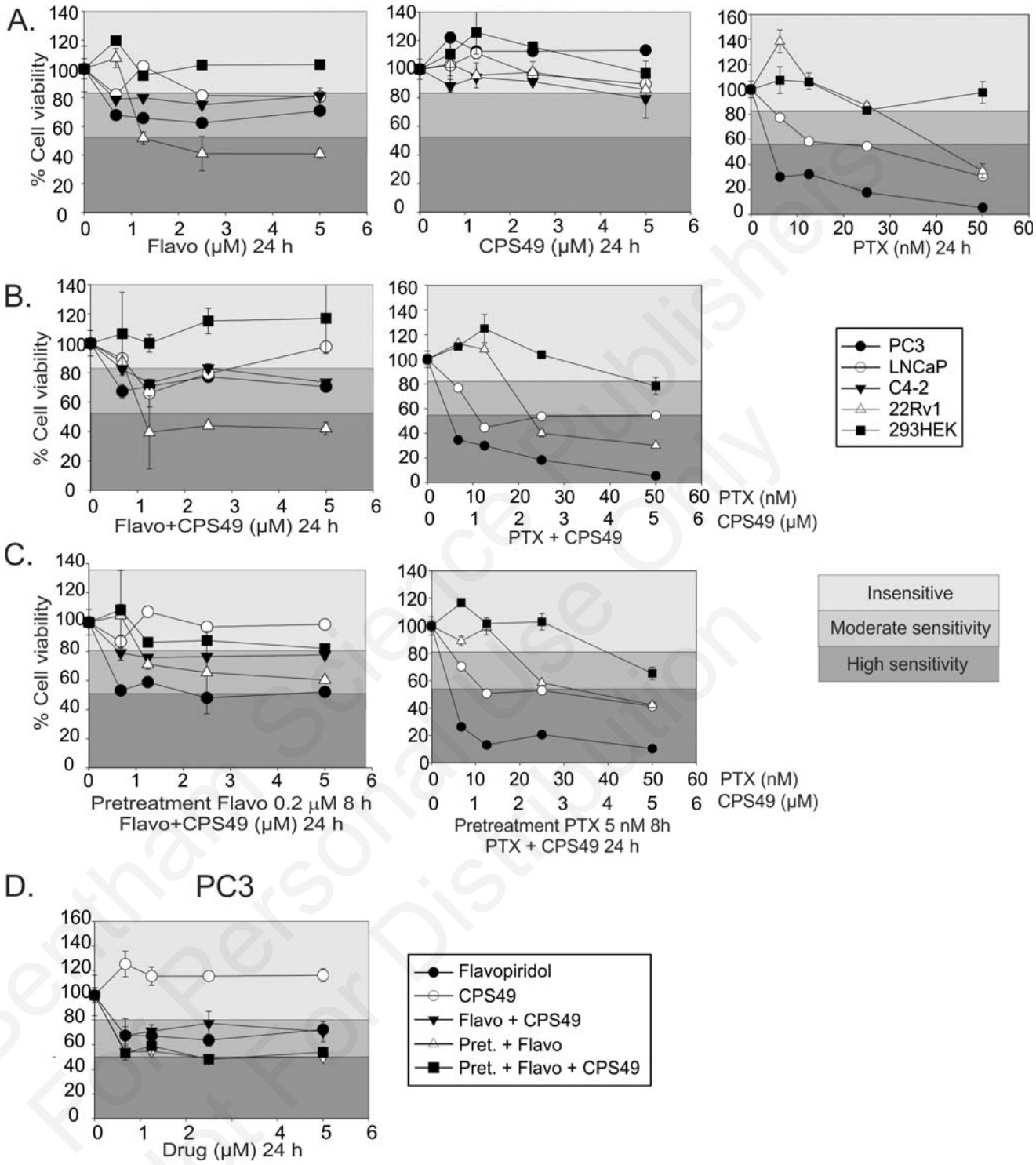

Fig. (1). CPS49 and flavopiridol or paclitaxel combinations are cytotoxic treatments for PCa cell lines. Viability was determined by MTS assay in PCa cells lines (PC3, LNCaP, C4-2, 22RV1) and non-tumor cell line HEK293 after exposure to: *A*. different concentrations of flavo, CPS49 or PTX for 24 h; ***B***. increasing concentrations of 1:1 combination of CPS49 with flavo or PTX for 24 h; or ***C***. flavo (0.2 μM) or PTX (5 nM) for 8 h and then 1:1 combination of CPS49 with flavo or PTX. Data were normalized to vehicle treated cells (DMSO). Points indicate average and standard deviation from 3 independent experiments. ***D***. Viability in PC3 cells after all treatments. PC3: Flavo or Flavo + CPS49 *versus* pretreatment flavo = $p<0.05$ (student´s t-tests). High sensitive = 0 to 50% of cell viability. Moderate sensitive = 50 to 80% of cell viability. Insensitive = 80 to 100% of viability.

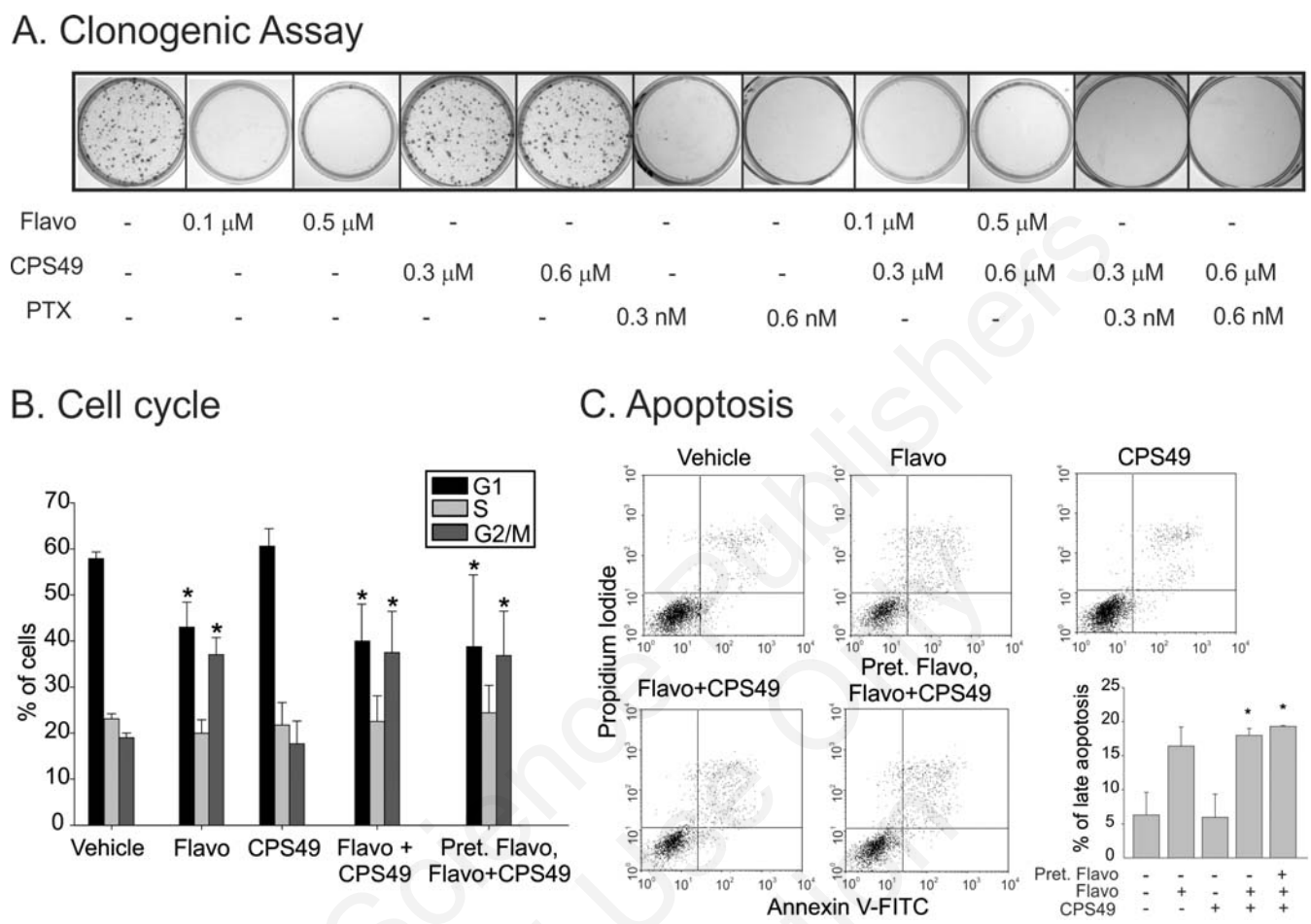

Fig. (2). CPS49 and flavopiridol combination induces cell cycle arrest and apoptosis in PC3 cells. ***A.*** Cells were plated in 6 cm Petri dishes and exposed to different concentrations of flavo, CPS49, PTX or combinations for 3 weeks. Colonies were stained with crystal violet and photographed**.** Triplicates were performed for each treatment. One representative plate for each treatment is shown. ***B***. Cells were exposed to flavo (2.5 μM), CPS49 (2.5 μM), combination of flavo and CPS49 (2.5 μM), or 0.2 μM pretreatment of flavo, and then co-treatment of flavo and CPS49 (2.5 μM) for 24 h. Cells were stained with propidium iodide and analyzed by FACS. Histogram shows the percentage of cells in G1, S and G2/M phases. Average from 3 biological independent experiments is shown. G1: Flavo, Flavo+CPS49, Pret. Flavo *versus* vehicle = $p<0.05$; G2/M: Flavo, Flavo+CPS49, Pret. Flavo *versus* vehicle = $p<0.01$. ***C.*** PC3 cells were treated with flavo (2.5 μM), CPS49 (2.5 μM) or 1:1 combination with or without flavo pretreatment for 8 h. After 24 h, cells were counted and stained with Annexin V-FITC and PI and analyzed by FACS. Bars represent the average and standard deviation of late apoptosis from 3 biological independent experiments. Significance was analyzed performing a t-Test. Asterisk means a significant difference from control ($p < 0.05$).

In addition, flavo has been associated with selective induction of apoptosis in hematopoietic cell lines [26] through the activation of pro-apoptotic genes or down regulation of anti-apoptotic genes [27]. In this work we assessed apoptosis using Annexin V/PI double staining and FACS analysis in PC3 cells treated with low doses of flavo or in combination with CPS49 (co-treatment or pre-treatment). Figure **2C** shows that all treatments significantly increased the percentage of late apoptotic cells compared to control.

In order to determine whether these drugs induced senescence *in vitro*, we exposed PC3 cell lines to low doses of flavo or in combination with CPS49 (co-treatment or pre-treatment). We found that these drugs did not induce senescence (Supplemental Fig. **1**).

Altogether these results demonstrate that a low dose of flavo as single agent or combined with CPS49 induces cell cycle arrest and apoptosis in PC3 cells.

Flavopiridol as Single Agent or in Combination with CPS49 Decrease Prostate Tumor Growth

It was previously reported that high doses of flavo (10 μM) [28] or CPS49 (12 mg/kg/day) [9] were needed to inhibit PC3 tumor growth using murine xenografts. It was also reported that 20 mg/kg/day of PTX inhibited tumor growth in LNCaP xenografts [29].

With the aim of reducing drug side effects in patients while controlling drug effect over tumor growth, in this work we injected PC3 cells *s.c.* into nude mice. Three weeks after injection, mice were exposed to half of the reported dose of flavo, CPS49 or PTX as indicated in (Fig. **3A**). PC3 cells were selected for the *in vivo* studies because these cells showed the highest response *in vitro*. Tumor volume was measured every 2 days during 37 days. Using these reduced drug concentrations, PTX was not effective to diminish tu-

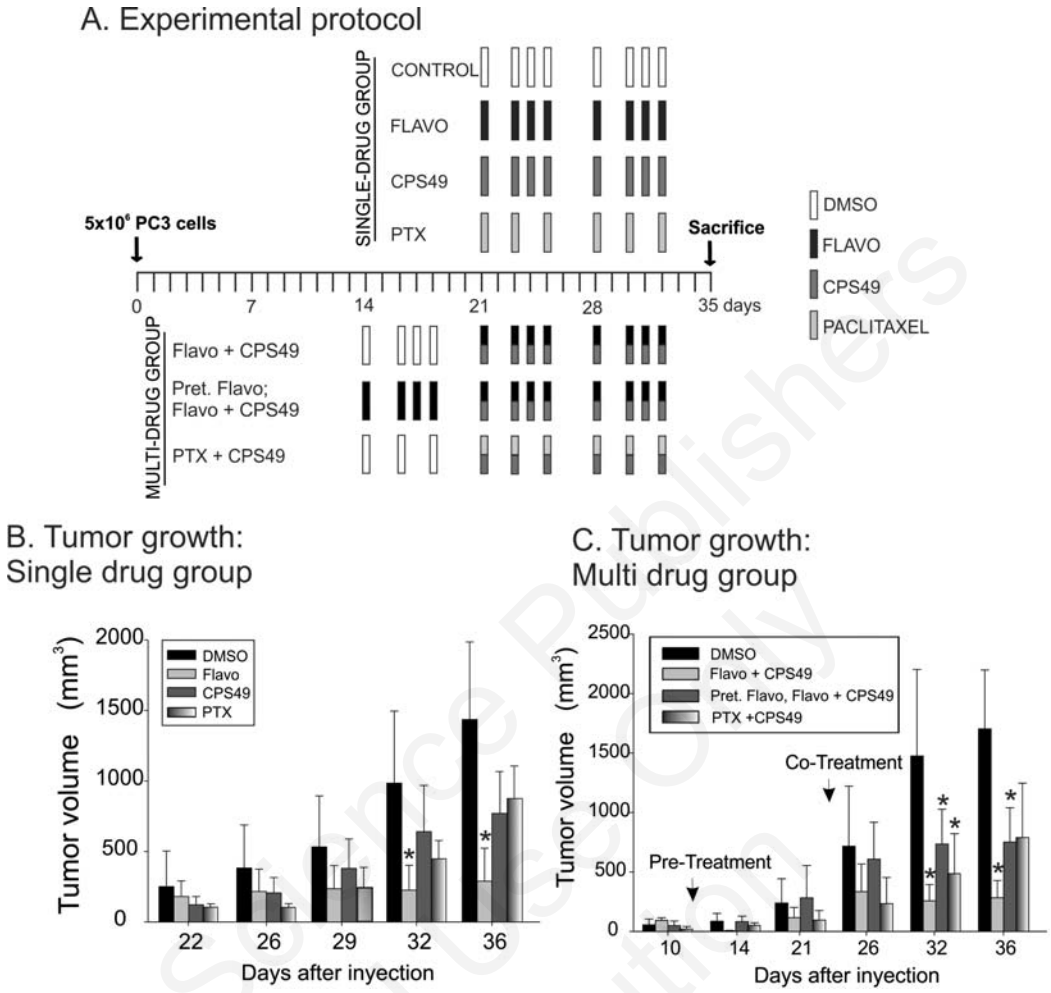

Fig. (3). Flavopiridol or its combination with CPS49 significantly decreases prostate tumor growth. ***A.*** *Schematic representation of mice protocol for single drugs (upper panel): Nu/nu* mice were inoculated *s.c.* with PC3 cells. At day 21, when tumor reached 100 mm^3, mice were randomly distributed into 4 groups (6 mice per group), and were administrated drugs i.p. Control group received injections of DMSO four times per week; flavo or CPS49 groups received four injections (6 mg/Kg/day) per week; PTX group received three injections (12 mg/kg/day) per week. *Schematic representation of mice protocol for drug combinations (lower panel): Nu/nu* mice were inoculated *s.c.* with PC3 cells. At day 15, mice were randomly distributed into 4 groups and were administrated drugs i.p. Control group received injections of DMSO four times per week; Flavo + CPS49 group received four injections (6 mg/Kg/day each drug) per week during two weeks; Pret. Flavo, Flavo + CPS49 group received 2 mg/Kg/day of flavo during one week, and then four injections of flavo and CPS49 (6 mg/Kg/day) per week during two weeks; PTX + CPS49 group received PTX (12mg/kg/day) and CPS49 (6 mg/kg/day) 3 injections per week during 2 weeks. *B-C.* Tumor size was measured 3 times per week with a digital caliper and tumor volume was calculated. Graph indicates average and standard deviations of tumor volumes from six mice. *$p < 0.05$. Two independent experiments were performed using 6 mice per drug treatment.

mor growth when compared with non-treated control mice, but flavo significantly decreased tumor growth (Fig. **3B**). However, mortality was higher in flavo-treated mice (50%) compared to other groups: control, CPS49- and PTX-treated animals (<5%).

In order to assess drug toxicity in mice, we measured animals' body weight every week along the complete experiment and analyzed the organ histology (liver, lung and kidney) at the end point. Non-significant differences were observed in mice body weight and all animal organs showed conserved histology when compared with the control group (Table **2**).

Histological analysis showed poorly differentiated adenocarcinomas with fibro-reticular epithelial type frame preserved in control mice group. Flavo or PTX treatments induced microscopic foci of tumor cells necrosis and fibroreticular frame loss (Fig. **4**). CPS49 administration induced numerous foci of tumor cells with different degrees of necrosis, reactive leukocytes infiltration and moderate fibroreticular frame loss (Fig. **4**).

To increase drug response efficacy, we assessed the effect of low doses of CPS49 in combination with flavo or PTX *in vivo*. We injected PC3 cells into nude mice and after two weeks we administrated three different drug combinations: flavo plus CPS49 (1:1) for 2 weeks (co-treatment group); pre-treatment with low doses of flavo for one week and flavo plus CPS49 (1:1) for 2 weeks (pre-treatment group); and the combination of PTX and CPS49 for 2 weeks (Fig. **3A**).

Table 2. Mortality and drug toxicity in mice.

	Mortality (%)	Mice body weight	Liver, lung and kidney histology
Control	2	No changes	Conserved
Flavo	50	No changes	Conserved
CPS49	2	No changes	Conserved
PTX	2	No changes	Conserved
Flavo + CPS49	2	No changes	Conserved
PTX + CPS49	2	No changes	Conserved

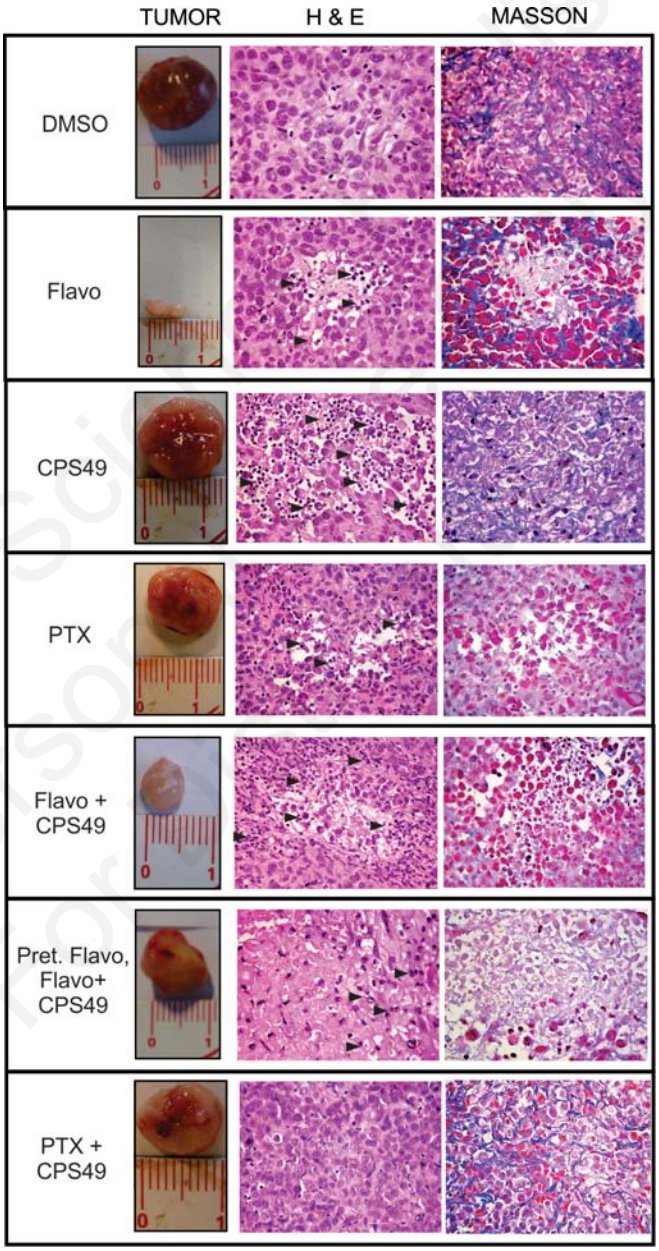

Fig. (4). Flavopiridol and CPS49 combination induces extensive necrosis in PC3 xenograft tumor samples. Nude mice were exposed as described in Figure 3. After animals were sacrificed, tumors were sectioned for histology. Microscopic sections were stained with H&E and Masson`s trichrome and examined by light microscopy. One representative photograph of tumor size, H&E and Masson´s trichrome staining of each group is shown (400x).

As shown in Fig **3C**, flavo and CPS49 combination significantly reduced tumor growth (83% compared to control mice group) after 2 weeks of co-treatment. Furthermore, one week of pre-treatment and 2 weeks of co-treatment also reduced tumor growth (54% compared to control mice group) (Fig. **3C**). Histological analysis of the xenograft samples showed extensive necrosis areas induced by the treatments with moderate to complete fibro-reticular frame loss (Fig. **4**). The mortality rate for these treatments was lower than the one observed in the flavo mice group (less of 5% of mice died with these combinations) (Table **2**).

Interestingly, low doses of PTX in combination with CPS49 were unable to reduce tumor growth using PC3 xenografts (Fig. **3C**). Furthermore, histological analysis of these tumors showed relative well-preserved tumor cells and fibro-reticular frame (Fig. **4**).

Altogether, these results indicate that CPS49 enhanced flavo antitumor effects and induced tumor necrosis while diminishing mice mortality rate. PTX in combination with CPS49 did not show significant differences compared to PTX alone.

CPS49 and Flavopiridol Combination Modulates Several Molecular Targets

To identify the drug molecular targets, we isolated RNA from PC3 cells exposed to CPS49, flavo or their combinations. Furthermore, we also extracted total RNA from PC3 xenograft growing in nude mice as described above. We performed RT-qPCR using specific primers for the following genes set: (i) DNA damage and cell cycle regulators: SFN, CCNB2, MAD2L1, CHE, E2F4, BRCA2, DDB2, FEN, BLM, H3F3B; (ii) Adhesion, migration and invasion: SELL, MMP9, ENG, VEGF; (iii) Epithelial-Mesenchymal transition (EMT) markers: B-CATENIN, E-CADHERIN, VIMENTIN, SLUG, SNAIL, FIBRONECTIN; iv) Transcription regulators: CCNT1, ELL, CtBP1.

As shown in Fig. **5**, the expression of most of the analyzed targets significantly decreased after both drug combinations in PC3 cell lines. Furthermore, the expression of the set of genes involved in adhesion, migration and invasion (SELL, MMP9, ENG, VEGF) were also significantly diminished in tumor xenografts from mice treated with flavo/ CPS49 combinations (co-treatment or pretreatment), (Fig. **5**).

DISCUSSION

The common treatment for patients with CRPC is chemotherapy based on docetaxel. Currently, there are seven agents approved for CRPC and four regimens have shown to increase survival. However, the survival benefits have been modest and, unfortunately, all patients will eventually progress. Thus, there is a need for new drugs and regimens for PCa treatment. The advance of targeted therapeutics against cancer, with higher specificity for tumor cells is one of the key goals of anti-cancer research.

The purpose of this work was to improve the therapies against advanced PCa reducing drug doses and side effects. In this work, we found that a reduction of flavo dose to a half of the previously reported was effective to reduce tumor growth but still the use of this single drug shows very high mortality (50%). Furthermore, CPS49 used as single agent was also effective to reduce tumor growth and did not induce mortality, but this treatment resulted less efficient compared to flavo alone. Moreover, the combination of flavo plus CPS49 increased both tumor cells necrosis and efficacy/tolerability.

Why CPS49 and flavo combination show higher tolerability than flavo alone *in vivo* is unclear. It was previously reported that CPS49 seemed to antagonize the capability of flavo to stabilize E2F-1 activity in leukemia cells [21]. Thus, one explanation could be that this antagonist effect might differentially affect the response *in vivo.*

We further investigate the molecular mechanism involved in this therapy. We found that low doses of flavo reduced cell proliferation inducing cell cycle arrest and apoptosis. Although CPS49 single treatment did not modulate these processes, the co-administration of flavo plus CPS49 reduced viability compared to the single treatment turning advanced PCa cells highly sensitive to these combinations.

Tumor angiogenesis has been considered as a key target for cancer therapy. Angiogenesis inhibitors only have a small effect on cancer patient survival. In addition, several anti-angiogenic agents have severe side effects. Therefore, the identification of the mechanisms underlying these toxicities might facilitate the optimization of the therapeutic strategies designed.

In this work, we tested the toxicity and the capacity to reduce tumor growth of the anti-angiogenic agent CPS49 in PCa pre-clinical models. In fact, the CPS49 anti-angiogenic properties could explain the extensive necrosis foci observed in the combined treatment with flavo and the diminished expression of VEGF detected in those tumors.

It was previously reported that this tetrafluorinated thalidomide analogue was potent and efficacious to block microvessel outgrowth in the rat aorta as well as HUVEC proliferation and tube formation at 12.5 μM [9]. Furthermore, it was reported in leukemic cells that CPS49 in combination with the cell cycle inhibitor flavo, provoked selective cytotoxicity related to mitochondrial dysfunction and ROS elevation [21]. The assessment of the transcriptional targeting of CPS49 and flavo combinations revealed that these drugs initiated a cell specific transcriptional program that modulated nuclear factor-kB (NF-kB), E2F-1, and p73 activity to promote mitochondrial instability, stimulating the expression of the proapoptotic factors p73, BAD, BAX and PUMA and simultaneously reducing the antiapoptotic genes expression: MDM2, MCL1, BCL-xL, XIAP and SURVIVIN [21]. Based on these findings, the co-administration of CPS49 and flavo emerges as a promising new therapeutic combination for PCa.

The rationale of this study was to investigate the mechanism of action and efficacy of the combinatorial use of the CPS49 with other agents as a therapeutic strategy for PCa.

In contrast, CPS49 combination with PTX was highly cytotoxic *in vitro*, but did not significantly decrease the tumor growth *in vivo*. These results suggest that CPS49/PTX

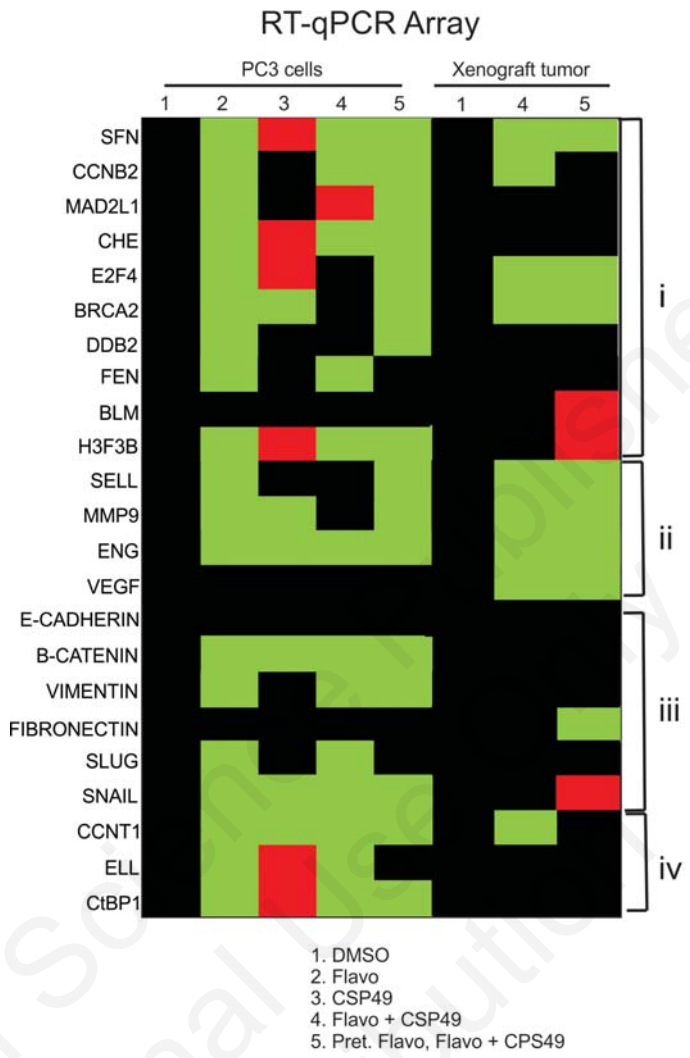

Fig. (5). CPS49 and flavopiridol combination modulates several molecular targets. RT-qPCR array was carried out from PC3 cells and PC3 xenografts, as described in Materials and Methods. Figure represents the average of three biological independent experiments in PC3 cells or the average of three animals from the same group for *in vivo* experiments. Significantly induced genes were colored in red, significantly repressed genes were plotted in green and black represents no change. Data were normalized to Actin B and control ($p<0.05$). i: DNA damage and cell cycle regulator genes; ii: Adhesion, migration and invasion genes; iii: EMT marker genes; iv: Transcription regulator genes.

combination should be tested in a different dose/schedule regimen or combined with other taxanes such as docetaxel.

One important observation from our results emerges from the tumors histological analysis. All the tested drugs administered as single agents induced microscopic foci of tumor cells necrosis and fibro-reticular frame loss. Nevertheless, the histological analysis of PC3 xenografts from CPS49 plus flavo treated mice showed significantly induced necrosis. Interestingly, the administration of low doses of PTX plus CPS49 showed relative well preserved tumor cells, highlighting again that this combination was not effective for PCa xenografts using this particular therapeutic strategy.

As mentioned above, high doses of flavo or CPS49 were reported to be necessary to reduce tumor volume in preclinical models. Here we describe a new strategy that reduces tumor volume using lower doses of these drugs. Differential adjustment of doses and sequential addition of flavo and CPS49 are practical approaches that should be tested for better therapeutic benefit against PCa.

The expression of most of the targets analyzed was significantly decreased after both drug combinations in PC3 cell lines. In the xenograft tumors from mice exposed to flavo plus CPS49 combinations (co-treatment or pretreatment), we found that the set of genes involved in adhesion, migration or invasion (SELL, MMP9, ENG,VEGF) were significantly down regulated. However, a longer list of genes should be analyzed to specifically unveil the molecular mechanism for these drugs and combinations.

CONCLUSION

In summary, we demonstrated that low doses of CPS49 combined with flavo is a good strategy to reduce tumor growth in PCa preclinical models which warranty further studies for the clinical use.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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