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Article

Anti-Cancer *In Vivo* and *In Vitro* Evaluations of Combinations of Cisplatin and Masticadienonic Acid Isolated from *Amphypterygium Adstringens*.

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Abstract:
Cisplatin (CDDP) is widely used to treat several types of cancer. However, CDDP induces nephrotoxicity. This toxicity could be avoided, applying a lower cisplatin dose; however, it could induce a lesser therapeutic activity. In this paper, we present the cytotoxic activity against prostate human cancer cell line (PC-3) of the combination of CDDP with masticadienonic acid (MDA), a triterpene isolated from *Amphypterygium adstringens.*
The combinations **A** (half of the IC_{50}), **B** (IC_{50}) and **C** (twice IC_{50} of the compounds) in a radio 1:1 were evaluated. Our results showed that the **B** and **C** combinations presented synergism effect. However, **B** combination showed almost 100% inhibition of cell proliferative activity and increased apoptosis compared with those presented by each compound apart. A pretreatment of MDA 24 h to cells before the CDDP, AMD or B combination administration result in a resistance to the treatments. A xenograft study using PC-3 cells showed that the combination of 47.5 mM/kg (AMD) plus 4 mM/kg (CDDP) administered weekly for 3 weeks reducing the tumor volume in approximately 47 %. However, the combination of 47.5 mg/kg (AMD) plus 2 mg/kg (CDDP) administered every third day for 21 days reduce, approximately 82% of tumor compared with mice no treated.

Keywords: cisplatin; masticadienonic acid; *Amphypterygium adstringens*; antitumor; xenograft.

1. Introduction

Globally, more than 1.4 million new prostate cancer cases were diagnosed in 2020. The crude incidence rate was 36.0 per 100,000 males and the ASIR was 30.7 per 100,000 males [1]. Although surgical intervention is the primary therapy used in the initial stages of prostate cancer, chemotherapy remains an essential strategy to fight these cancerous processes. It can be used as a neoadjuvant or adjuvant treatment. Also, chemotherapy could be used against recurrent and metastatic cancers. Platinum-based drugs are widely used as chemotherapy agents [2, 3]. However, although cisplatin (CDDP) is not frequent used as anti-cancer drug due to its high nephrotoxicity, yet it is utilized to treat patients with recurrent tumors [4]. One way to avoid cisplatin's toxicity is to use lower doses administered in conjunction with other drugs. In cancer, drug combination therapy is essential. This type of therapy has recently been a superior treatment strategy [5, 6, 7]. For example, a randomized clinical trial on the effects of low-dose cisplatin on 89Sr therapy for painful bone metastases from prostate cancer has been reported [8]. In another study, CDDP's antitumoral effect against colon cancer was enhanced by combination with aspirin [9]. Therefore, there is in need to search for chemosensitizers that can increase the

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therapeutic effect of cisplatin and, at the same time, overcome the multi-drug resistance and side effects.

Our systematic studies about antitumoral triterpenes investigated the *Amphipterygium adstringens* masticadienonic (AMD) and 3a-OH masticadienonic acids (3OH-AMD) tirucallane-type triterpenoids showed that these triterpenic acids inhibited tumor growth, *in vivo* and induced cell death by apoptosis [10]. In addition, AMD is the most potent Pol β inhibitor found so far. DNA polymerase β (Pol β) is an error-prone enzyme whose up-regulation is a genetic instability enhancer as well as a contributor to cisplatin resistance in tumor cells [11].

Considering the above, we decided to evaluate the cytotoxicity of AMD and CDDP combinations against prostate human cancer cells (PC3) and anticancer agents against tumors induced by PC-3 cells in a xerographic study. To cover a wide range of equivalent concentrations, we decided to evaluate different doses based on the IC_{50} of each compound. *In vitro* experiments, the combinations were **A** (half of the IC_{50}), **B** (IC_{50}), and **C** (2 X IC_{50}) of the compounds in a radio 1:1. Although combinations **B** and **C** showed synergism; however, the **B** combination was the best treatment increasing both inhibitions of anti-proliferative activity and apoptosis compared with those presented by AMP and CDDP in individual treatment. It is worth noting that a pre-treatment of AMD 24 h before CDDP administration induced a lower cytotoxic activity. The best antitumor combination *in vivo* was 47.2 mg/kg (AMD) plus 2 mg/kg (CDDP) administered every third day, reaching approximately 85% of tumor inhibition. However, the same activity was achieved by AMD at 47.2 mg/kg doses.

2. Results

Cytotoxicity in vitro of MDA and CDDP combinations on PC3 cells

Dose and effect data were obtained from the Sulforhodamine B assay. The AMD and CPPD IC_{50} were 47.5±04 and 15.87±0.8 μM respectively, The MDA and CDDP combinations were evaluated as are indicated in the experimental section. Our results showed that almost 100 % of anti-proliferative activity was achieved by combination **B** (MDA IC_{50} 47.5 μM and CDDP IC_{50} 15.87 μM) against PC3 cells. It is worth to note that **A** (0.5xIC_{50} 23.75 μM MDA and 0.5xIC_{50} 7.93 μM CDDP) combination achieve a 50 % of the anti-proliferative effect (Figure 1).

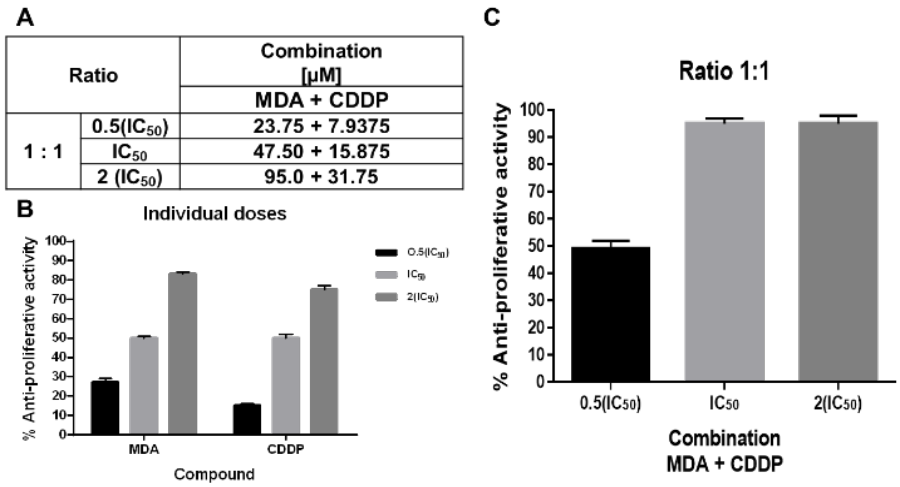

Figure 1. *Cytotoxic evaluation of AMD y CDDP combinations on PC-3 cells*

Synergistic Effect

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Dose and cytotoxic effect were subjected to CompuSyn program [12]. The parameters, M, Dm and r were calculated from the median-effect equation and the median-effect plot [13]. M is slope (sigmodicity). Dm indicate potency and r is linear correlation coefficient. Combination index (CI) was calculated from the CI equation algorithms using CompuSyn software. CI=1, <1 and >1 indicates additive effect, synergism and antagonism, respectively [13]. Dose-reduction index (DRI) was calculated from the DRI equation using CompuSyn software. DRI=1 indicates no dose reduction, whereas DRI>1 and <1 indicate favorable and unfavorable dose-reduction, respectively [13].

Compound [μM]		Fractional	Parameters[a]				DRI[c]
MDA	CDDP	Inhibition (*fa*)	m	Dm	r	CI[b]	[MDA;CDDP]
$(D)_1$							
23.75		0.27					
47.5		0.5					
95		0.83	1.91363	42.3148	0.98973		
	$(D)_2$						
	7.9375	0.15					
	15.875	0.50					
	31.75	0.75	2.04373	16.6102	0.99171		
$(D)_1$+$(D)_2$							
23.75	7.9375	0.49				1.02634	[1.76087;2.18132]
47.5	15.875	0.95				0.44139	[4.37593;4.69771]
95	31.75	0.95	2.15282	25.6052	0.86602	0.88279	[2.18796;2.34885]

Table 1. Combination synergism test of masticadienonic acid with cisplatin against prostate human cancer cells (PC3).

Cell apoptosis analysis

Our results showed that the **B** combination in the PC3 cell line induced 17.4 % and 67.1 % of late and early apoptosis (Figure 2). While the MDA and CDDP at IC_{50} doses caused 12.6 and 14.1 % of late apoptosis and 56.7% and 57.7% of early apoptosis, respectively. (Figure 2).

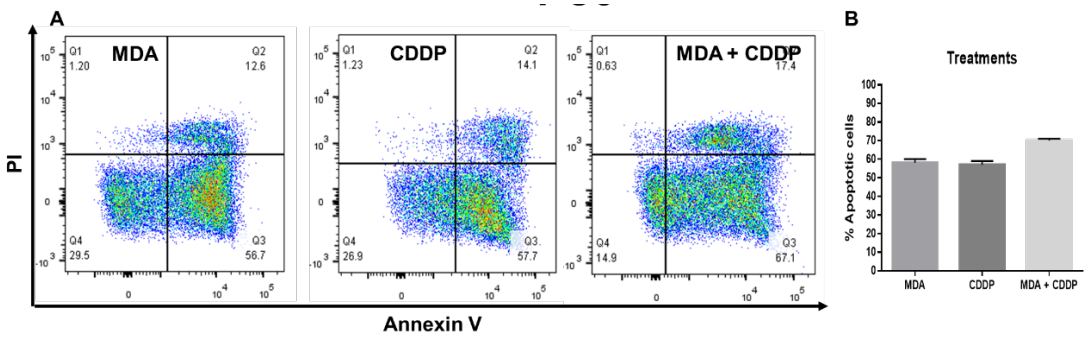

Figure 2. Plot diagrams of the apoptosis and percentage of apoptotic cells, induce by MDA, CDDP at IC_{50} doses and **B** combination, in prostate human cancer cell line (PC3).

Pre-treatment of PC-3 cells with AMD.

When the PC-3 cells were previously treated with AMD at different doses for 24 h, they presented resistance to the treatments with CDDP combinations (Figure 3)

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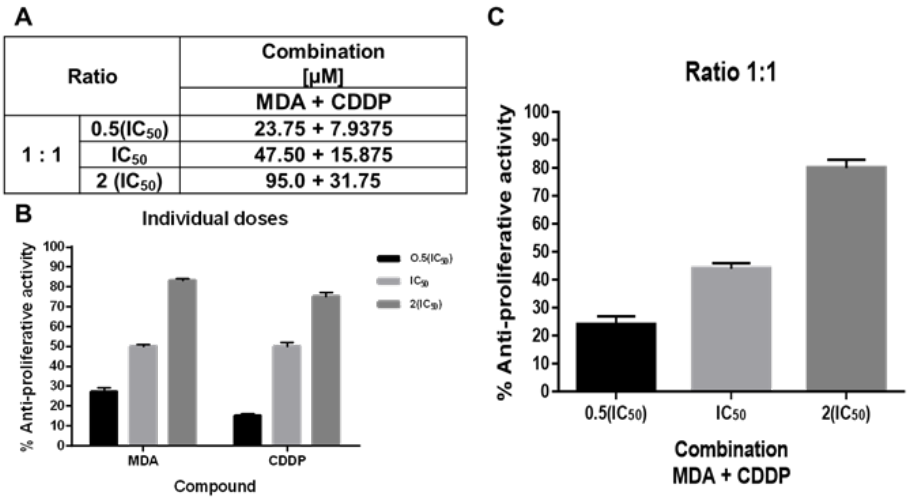

Figure 3. Pre-treatment of PC-3 cells with AMD.

The figure 3A shows the compounds concentrations used. The figure 3B shows the MDA and CDDP percentage of anti-proliferative activity in PC-3 cells. The figure 3C shows the anti-proliferative activity of **A**, **B** and **C** combinations in PC-3 cells previously treated with AMD. Assays were performed in triplicate. ± SD.

*Treatment of non-cancerous MCF10A cell with **A**, **B** and **C** combinations*

MCF10A cells were treated with the **A**, **B** or **C** combinations in analogous way like PC3 cells. The results showed that the **B** and **C** combinations induced approximately 60 % of cytotoxicity in not cancerous cell MCF10A (Figure 4). While **A** combination induced less 10% of MCF10A cell death.

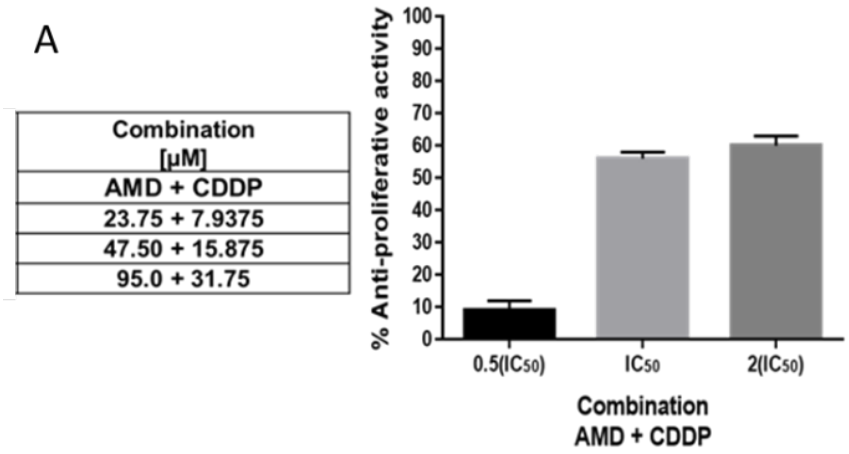

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Figure 4. *Anti-proliferative activity of masticadienonic acid and cisplatin combinations in MCF10A cell line.* The MCF10 cells were treated with AMD and CDDP for 48 h and the cell viability was determined by Sulforhodamine B assay. Assays were performed in triplicate. ± SD.

AMD and CDDP combination inhibited tumor growth

To assess the antitumor activity of AMD and CDDP, these compounds were evaluated in a xenograft model inducing a tumor with PC-3 cells in *nu/nu* mice. The results indicated that AMD at doses of 47.5 and 95 mg/kg reduced tumor volume by approximately 85 and 80%, respectively, while CDDP evaluated at doses of 2 and 4 mg/kg reduced tumour by approximately 66 and 82%, respectively. (Figure 5). Considering the above results, we decided to evaluate the combinations of AMD 47.5 mg/kg plus 4 mg/kg CDDP given every week for three weeks, and AMD 47.5 mg/kg plus 2 mg/kg provided every third day for 21 days.

The administration of AMD at 47.5 mg/kg doses plus CDDP at 4 mg/ kg doses weekly for three weeks reduced tumor growth by 59.2% (Figure 5). However, the administration of AMD at 47.5 mg/kg plus CDDP at 2 mg/ kg three times a week for three weeks showed a reduction of 85.33% compared to those untreated mice. Nonetheless, the same percentage of antitumor activity was shown by AMD at 47.5 mg/kg doses (Figure 5).

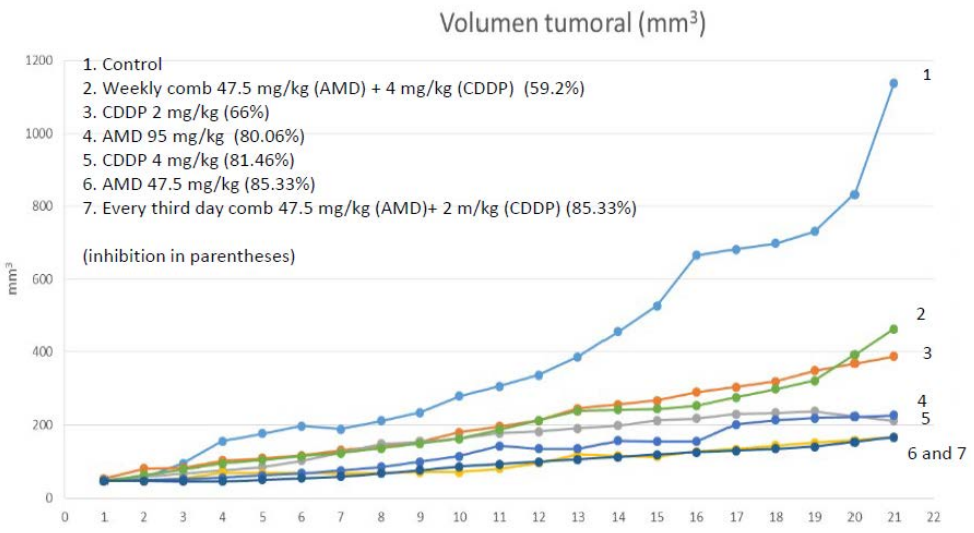

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Figure 5. Antitumor activity of CDDP, AMD, and some of their combinations

The variation of the weight of the animals was calculated, considering the initial and final weight. The only treatment where a slight loss of weight observed was the combination of the 47.5 mg/kg dose of AMD and 2 mg/kg of CDDP given every other day for three weeks (Figure 6).

3.

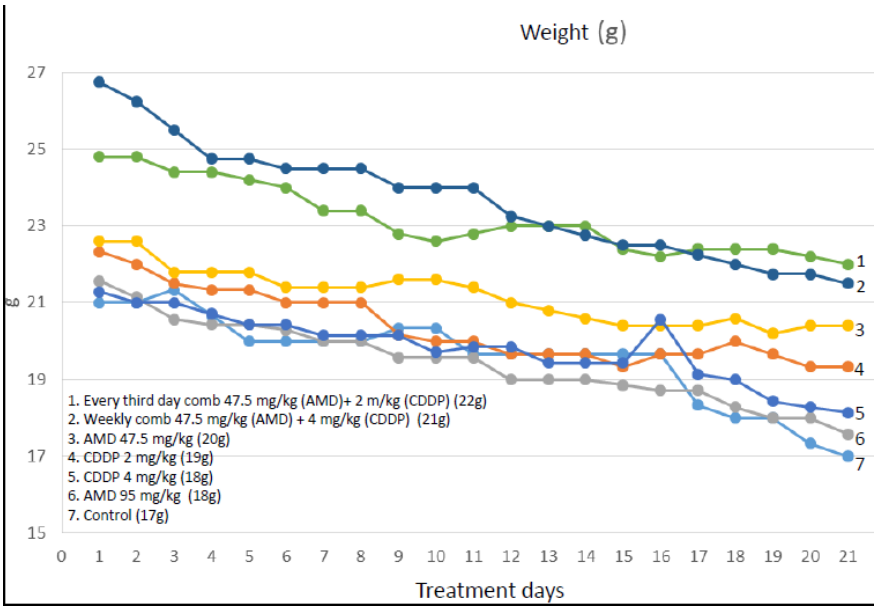

Figure 6. Weight variation of the animals in the different treatments compared to the control

Discussion

Our results showed that combinations **A**, **B**, and **C** presented higher citoxic activity against PC-3 cells than AMD and CDDP separately. The combinations were tested simultaneously (Figure 1).

The combination index (CI) was used to determine the types of drug interactions where CI < 1 indicates synergistic effect, CI = 1 indicates additive effect, and CI > 1 represents antagonistic effect. Our findings showed that the **A** combination induce an additive effect, while **B** and **C** administration result to be synergic combinations (Table 1).

In synergistic drug combinations, such as **B** the dose of each drug (15.87 μM CDDP and 47. 50 μM MDA) used in the combination to achieve a specific measurable level of effect (in this case 100% inhibition) will be reduced compared to the dose needed to achieve the same level of effect when the drugs are administered individually (31.75 μM CDDP and 95.0 μM MDA). This parameter is known as the dose reduction index (DRI). The reduced dose which will reduce toxicity at the increased effect would lead to beneficial clinical consequences. Our findings showed that the **B** combination has the best DRI results (Table 1). Furthermore, **B** combination showed slightly higher apoptotic activity than AMD and CDDP at the IC_{50} dose (Figure 2).

It is known that AMD inhibit Poly β an error-prone enzyme which contribute to cisplatin resistance in tumor, so we hypothesized that if the pretreatment of PC-cells with AMD, 24 h before treatment with CDDP a more significant cytotoxic activity could be

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obtained. Unexpectedly, the pretreatment of PC-3 cells with AMD made that these cells were less susceptible to the cytotoxicity induced by CDDP (Figure 3). Further studies are required to find out the possible mechanism exerted by PC-3 cells when treated with non-lethal doses of AMD to reduce the cytotoxic activity exerted by CDDP. It is remarkable that this resistance develops in just 24 hours.

In a xenograft model inducing a tumor with PC-3 cells in nu/nu mice. The results indicated that AMD at doses of 47.5 and 95 mg/kg reduced tumor volume by approximately 85 and 80%, respectively, while CDDP evaluated at doses of 2 and 4 mg/kg reduced tumor by approximately 66 and 82%, respectively. (Figure 5). Considering the above results, we decided to evaluate the combinations of AMD 47.5 mg/kg plus 4 mg/kg CDDP given every week for three weeks, and AMD 47.5 mg/kg plus 2 mg/kg provided every third day for 21 days.

The administration of AMD at 47.5 mg/kg doses plus CDDP at 4 mg/ kg doses weekly for three weeks reduced tumor growth by 59.2% (Figure 5). However, the ad-ministration of AMD at 47.5 mg/kg plus CDDP at 2 mg/ kg three times a week for three weeks showed a reduction of 85.33% compared to those untreated mice. Nonetheless, the same percentage of antitumor activity was shown by AMD at 47.5 mg/kg doses (Figure 5). These results showed that AMD and CDDP combination did not showed additivity or synergy in the anti-tumor activity.

4. Materials and Methods

Cell culture and reagents

Roswell Park Memorial Institute medium (RPMI-1640, BIO-L500-500) and FBS (fetal bovine serum, BIO-S1650-500) and Trypsin (BIO-L0931-100) were obtained from Biowest company (Riverside, MO, USA). Dimethyl sulfoxide (D4540-100) and Cisplatin (cis-Diammineplatinum (II) dichloride, 479306-1G) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Prostate human cancer (PC3), human colon cancer (HCT116) and normal human mama epithelial (MCF10A) cell lines were obtained from the American Type Culture Collection (ATCC). The cells were routinely cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (SFB), 250 µg/mL streptomycin sulfate, 250 U/mL penicillin, 0.625 µg/mL amphotericin B and 2 mmol L-glutamine and incubated in a humidified cell incubator with an atmosphere of 5% CO2 at 37 °C until 860-70% confluent. FITC Annexin V apoptosis Detection Kit and Propidium Iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of secondary metabolites.

Masticadienonic and 3α-OH masticadienonic acids were isolated from *Amphipterygium adstringens*, as previously reported (Martínez et al 1990)

MDA and CDDP IC_{50} determination

PC3 cells were seeded in a 96-well plate (150 µL of medium RPMI-1640 which contained 10,000 cells) and allowed to grow overnight. Cisplatin and masticadienonic acid were dissolved in DMSO and diluted with RPMI-1640 medium to final concentrations. Control groups contained only an equivalent volume of each cell line and RPMI-1640 + 10% FBS. The sulforhodamine B assay evaluated the MDA and CDDP cytotoxicity in PC3 cells. The cancer cells were exposed to MDA concentrations ranges between 12.5 to 100 µM for 48 h. Similarly, the cancer cells were administrated with CDDP concentrations ranges between 2.5 to 20 µM during 48 h. The bioactivity of MDA and CDDP was determined from 50% growth inhibition (IC_{50}) in the treated cells. The results are shown in Table 1.

MDA and CDDP combinations

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The cytotoxicity of CDDP to PC3 cells was evaluated at 7.93 µM (0.5xIC_{50}), 15.87 µM (IC_{50}) and 31.75 µM (2xIC_{50}) doses. While the doses of MDA administrated were 23.75 µM (0.5 x IC_{50}), 47.50 µM (IC_{50}) and 95.0 µM (2x IC_{50}). The cells were seeded in a 96-well plate and grew overnight (150 µL of the medium, which contained 10,000 cells). The plate was then incubated for 24 h at 37 ° C in 5% CO2. Subsequently, they were washed with PBS, and 150 µL of the following MDA and CDDP 1:1 combination: **A** (0.5x IC_{50}), **B** (IC_{50}) or **C** (2x IC_{50}) were added. The plate was incubated for 48 h at 37 ° C in a 5% CO2 atmosphere. Control groups contained only an equivalent volume of each cell line and RPMI-1640 + 10% FBS. The results are shown in figures 2C and 2F.

Cell apoptosis analysis

PC3 cells were seeded on 60-mm dishes for 24 h, later were treated with MDA or CDDP alone or with the combinations for 48 h. Cells were harvested, washed twice with PBS, and stained with Annexin V-FITC and propidium iodide (PI). Mortality of the cells was determined using a FACSCalibur flow cytometer (BD Biosciences, CA).

Pre-treatment of PC-3 cells with AMD.

The cells were seeded in a 96-well plate and allowed to grow overnight (150 µL of the medium, which contained 10,000 cells). Subsequently, they were washed with PBS, and 150 µL of medium RPMI-1640 with different AMD concentrations were added. The plate was incubated for 24 h (as a pre-treatment) at 37 ° C in a 5% CO_2 atmosphere. After 24 h, they were washed with PBS, and 150 µL of a new medium the CDDP was added at different concentrations and incubated for another 24 h, completing a total of 48 h of treatment. Control groups contained only an equivalent volume of each cell line and RPMI-1640 + 10% FBS.

MCF10A treatment

MCF10A cells were seeded in a 96-well plate and grew overnight (150 µL of the medium, which contained 10,000 cells). The plate was then incubated for 24 h at 37 ° C in 5% CO2. Subsequently, they were washed with PBS, and 150 µL of the different MDA or CDDP combinations were added for each case. The MDA and CDDP combinations 1:1 at 0.5x IC_{50}, IC_{50} and 2x IC_{50} doses were evaluated. The plate was incubated for 48 h at 37 ° C in a 5% CO2 atmosphere. Control groups contained only an equivalent volume of each cell line and RPMI-1640 + 10% FBS. The results are shown in figures 2A treated like PC3 and 2B treated like HCT116.

Statistical analysis.

All experiments were assayed in triplicate (n= 3)

Data are expressed as means ± SEM. All statistical analyses were performed using GraphPad Pro. Prism 5.0 (GraphPad, SanDiego, CA). Student's t-test and two-way ANOVA were employed to analyze the differences between sets of data. A p value < 0.05 was considered statistically significant.

Analysis of combination results by median-effect equation

The tables of median-effect equation are constructed from the contents generated by CompuSyn Report. Dose data were obtained from the Sulforhodamine B assay (Figure 2) -average value of triplicate- and were subjected to CompuSyn analysis. Parameters were calculated from the median-effect equation and the median-effect plot. M is slope, signifies shape; Dm signifies potency; and r is linear correlation coefficient, (**a**)**.** Combination index (CI) was calculated from the CI equation algorithms using CompuSyn software. CI=1, <1 and >1 indicates additive effect, synergism, and antagonism, respectively (**b**). Dose-reduction index (DRI) was calculated from the DRI equation and algorithm using CompuSyn software. DRI=1, >1, and <1 indicates no dose-reduction,

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favorable dose-reduction, and not favorable dose-reduction, respectively, for each compound in the combination (**c**).

Xenotransplant Assay

The mice were acquired in the Bioterium of the National Institute of Medical Sciences and Nutrition Salvador Zubiran (INCMNSZ) in Mexico City. The mice were kept in Micro-Isolator® cages in a pathogen-free environment, fed ad libitum and kept at room temperature of 22 C, relative humidity of 55% and dark light cycles of 12/12 h. Approval by the Animal Research Committee was granted before performing the experimental procedures, which were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of INCMNSZ (the institutional registration code BQO-1488-15/17-1). Scheme 1: once weekly for three weeks. 4–6 week-old male nu/nu mice were distributed in 3 groups of 6 mice each. The mice were implanted with $1.5X10^6$ PC-3 cells. The cells were re-suspended in 0.1 mL of PBS for subcutaneous inoculation in the right flank of the animal's back.

The treatments initiated once the tumors reached a volume of approximately 50 mm^3 (Day 0). The mice received i.p. treatment once weekly for 3 weeks (days of administration: 0, 7, and 14).

The treatments groups were as follows:

47.5 mg/kg AMD
95 mg/kg AMD
4 mg/kg of CDDP
2 mg/kg of CDDP
47.5 mg/kg (AMD) + 2 mg/kg (CDDP)
Negative control: 10% DMSO and sesame oil extra virgin Inés®

Scheme 2: three times a week for three weeks. 4–6 week-old male nu/nu mice were distributed in 3 groups of 6 mice each. Mice care was performed as in Scheme 1. The mice were implanted with 1.5 X 10^6 PC-3 cells. The cells were inoculated as previously described. The treatments initiated once the tumors reached a volume of approximately 50 mm^3 (Day 0).

The mice received i.p. treatment three times a week for three weeks (days of administration: 0, 2, 4, 7, 9, 11, 14, 16, 18). The study groups were as follows:

47.5 mg/kg (AMD) + 2mg (CDDP)
Positive control: 2 mg/kg of Cisplatin
Negative control: 10% DMSO and sesame oil extra virgin Inés®

The mice in both schemes were weighed daily. The tumor was measured with a calibrator (Vernier digital). The tumor volume was calculated using the formula $V = \pi/6$ x (larger diameter x [smaller diameter]^2). The experiment lasted 21 days, after which the animals were humanely sacrificed and the tumor was removed, preserved in 10% formaldehyde, and embedded in paraffin for future immunohistochemical test.

5. Conclusions

The combinations of AMD and CDDP named **A**, **B** and **C** showed good cytotoxic activity against the PC-3 human prostate cancer cell line. These effects can be explained by the different mechanisms of action of CDDP and AMD. Combinations **B** and **C** showed synergy, while A was only additive. Notwithstanding the foregoing, blend **A** (0.5 IC_{50} of AMD and CDDP) showed approximately 50% cytotoxic activity. Indicating that this combination is a suitable candidate for further study since the toxicity of CDDP is lower than in the other combinations. For example, **A** combination induces only 10% cell death in the noncancerous line MCF10 while **B** and **C** combinations induce approximately 50 % of MCF10 cell death. A flow cytometric analysis showed that combination **B** induces cell death by apoptosis. However, the results of synergy or additivity were not reflected in the

in vivo tests, where the best combination of AMD (47.5 mg/kg) and CDDP (17 mg/Kg) showed the same tumor´s inhibition of 85% than AMD at 47.5 mg/kg doses alone.

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Conflicts of Interest: The authors declare no conflict of interest.

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