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# Cytotoxic effect of cryptopleurine and its interaction with cisplatin in A549 lung cancer cells

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**Abstract:** In this study, the effect of the plant alkaloid cryptopleurine (CRP) on the cytotoxic activity of cisplatin (CDDP) in non-small cell lung carcinoma A549 cells was investigated. Cells were treated with various concentrations of CRP and CDDP, either individually or in combination. Cell viability was assessed using the MTT assay, and drug interactions were evaluated employing the Chou-Talalay method. The results did not demonstrate a synergistic interaction between CDDP and CRP, instead, the combination exhibited an antagonistic effect in vitro. Further analyses using flow cytometry and fluorescence microscopy revealed no significant induction of apoptosis following combined treatment. Most cells remained viable and only a minimal increase in necrosis was observed. While morphological alterations and reduced cell proliferation were detected, these effects did not correspond to classical apoptotic features, suggesting the involvement of alternative non-apoptotic mechanisms.
These findings indicate that CRP does not enhance CDDP cytotoxicity and should not be considered a chemosensitizer of CDDP. Rather, CRP may be more appropriately evaluated as an independent or alternative approach under the tested conditions.

**Keywords**: human lung adenocarcinoma A549; cisplatin; cryptopleurine; synergistic effect.

## Introduction

Lung cancer remains one of the most common and most aggressive types of cancer with a high mortality rate worldwide, with incomplete efficacy of conventional chemotherapy and the development of resistance remaining key clinical problems. CDDP, one of the most widely used cytostatic drugs in the treatment of non-small cell lung cancer (NSCLC), often leads to the development of resistance and toxic effects that limit its therapeutic potential (Liao et al., 2020; Oncu et al., 2024). It is a well-known chemotherapeutic drug in the treatment of solid tumors, including NSCLC. After entering the cell, it undergoes aquation (removal of chloride ions and their replacement with water molecules) becoming a highly reactive complex capable of covalently binding to DNA. The resulting adducts, primarily 1,2-nitro- and interchain cross-links between purine bases, disrupt the structure of DNA, preventing replication and transcription thereby activating the cellular response to DNA damage and subsequent apoptosis of tumor cells (Dasari and Bernard Tchounwou, 2014; Dasari et al., 2022). In addition to directly damaging nuclear DNA, CDDP can also lead to production of reactive oxygen species (ROS) and damage mitochondrial DNA, which increases stress in the cell and activates the intrinsic apoptotic pathway. These mechanisms contribute to its cytotoxic effect and suppress the proliferation of rapidly dividing cancer cells (Kiss et al., 2021; Mohiuddin and Kasahara, 2021). Under conditions of oxidative stress, excessive ROS can damage cellular proteins, lipids, and DNA, leading to lethal lesions in cells that contribute to carcinogenesis (Dasari and Bernard Tchounwou, 2014). CDDP induces ROS triggering cell death besides DNA damage. Formation of ROS depends on CDDP concentration and exposure time. Despite its efficiency, clinical use of CDDP is often limited by the development of resistance (e.g., increased activity of DNA repair mechanisms, efflux pumps, or changes in apoptosis pathways) and undesirable side effects such as nephrotoxicity, hepatotoxicity, cardiotoxicity, and ototoxicity, which are the subject of intensive research and efforts to optimize therapies (Dasari and Bernard Tchounwou, 2014; Liao et al., 2020). Combination therapy is a common strategy for cancer treatment that allows dose reduction of individual drugs and simultaneous treatment of multiple targets (Wang et al., 2021).
In recent years, research focus has shifted to natural compounds with anti-tumor potential that can either independently inhibit tumor cell proliferation or synergistically enhance the effect of existing chemotherapeutic agents. Such combined approaches have also been experimentally demonstrated for various natural compounds in A549 cell lines; certain flavonoids and plant alkaloids exhibit increased cytotoxicity when administered concomitantly with CDDP

(Boța et al., 2024; Hu et al., 2016). Phenanthroquinolizidine alkaloids, represented by cryptopleurine and similar compounds, exhibit significant in vitro antitumor activity, suppressing cancer cell growth at low nanomolar concentrations through inhibition of cell synthesis and other mechanisms (Chemler, 2009). These derivatives can induce cytotoxic effects characterized by apoptosis, with $IC_{50}$ in the micromolar range while avoiding P-glycoprotein resistance (Kubíčková et al., 2019). CRP can be found in various plants of the families *Asclepiadaceae, Moraceae*, and *Urticaceae* (e.g., *Boehmeria cylindrica, Boehmeria pannosa*) (Chemler, 2009). Studies investigating biological activity of CRP have shown that the lone electron pair on the nitrogen atom plays a crucial role as an electron donor in reactions with electrophilic molecules, and the hexacyclic structure around nitrogen is also critical for its cytotoxic activity (Choi et al., 2015). CRP has a wide range of applications and biological effects in medicine, including antiamoebicidal, antiviral, antifungal, and anti-inflammatory activities (Chen et al., 2012). In recent years, its antitumor potential has been investigated. However, its overall mechanism of action has not yet been fully elucidated (Chemler, 2009). Several biological effects on eukaryotic cells have been identified, such as inhibition of protein synthesis and nucleic acid synthesis, as well as interaction with the NF-κB signaling pathway through its blockade, ultimately leading to growth inhibition and induction of apoptosis in cells (Chen et al., 2012).

These findings suggest that CRP does not act as a chemosensitizer of CDDP but may exert an antagonistic effect; therefore, it should be considered as an independent or alternative therapeutic approach.

## Material and Methods

***Biological model***

Human lung adenocarcinoma cell line A549 was cultured in HAM'S F-12 Kaighn's modified medium supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were passaged twice a week after reaching 80 % confluence. The cells were cultured in a humidity-controlled atmosphere with 5 % $CO_2$ at 37 °C.

***Reagents***

CDDP was purchased from Hospira (USA). Hoechst 33342, DMSO, Annexin V, Fetal bovine serum (FBS), propidium iodide (PI), and trypsin (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) were purchased from Sigma Aldrich (Saint-Louis, MO, USA)/Merck (Darmstadt, Germany). Cell culture HAM'S F-12 Kaighn's modification was purchased from Cytiva (Utah, USA). Cryptopleurine was purchased from Cayman Chemical Company (Michigan, USA).

***Determination of cell viability and concentration***

A key step before each experiment was to determine cell concentration and viability to prevent variability in results and ensure consistency across experiments. Cell viability was determined using trypan blue staining, and cell concentration was determined using a Countess II Automated Cell Counter.

***Subculturing A549 cells***

A549 cells were passaged at approximately 80 % confluence. The cells were washed in PBS, treated with trypsin, and incubated at 37 °C and 5 % $CO_2$ for 5 minutes. Trypsinization was stopped by adding culture medium, and the cells were transferred to a new Petri dish with fresh medium. The cells were then cultured under standard conditions.

***Evaluation of lethal concentration ($LC_{50}$) of CRP and CDDP***

To determine $LC_{50}$, cell viability assay (MTT) was used. Mean cell viability values obtained after treatment with CRP and CDDP were used to calculate $LC_{50}$ values, fitting the data to a modified Hill equation using the Origin 2018 software:

$$Viability\,(\%) = 100 \times \left(1 - \frac{c_{CDDP(CRP)^n}}{LC_{50}^n + c_{CDDP(CRP)^n}}\right) \qquad (1)$$

Where $n$ is the Hill coefficient, and $c$ is the concentration of CRP (nmol/L) or CDDP (μmol/L) that caused 50 % cell death in the tested cell population. The cells were seeded onto 96-well microtiter plates at the density of $4 \times 10^5$ cells/ml in the cell culture. As adherent cells, they were treated with CRP (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 nmol/L) or CDDP (2,5, 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560 μmol/L) for 24 h. After incubation, the plate was centrifuged at 600× g for 10 min at 20 °C. The culture medium was then removed and replaced with serum-free medium containing 0,25 mg/ml MTT. The cells were incubated for 4 h at 37 °C under shaking at 250 rpm. Subsequently, the plate was centrifuged at 900× g for 10 min at 20 °C, the supernatant was discarded, and 150 μL DMSO were added to each well to dissolve the formazan crystals. Absorbance was measured at 540 nm using a Synergy H4 Hybrid Microplate Reader.

***In vitro evaluation of CDDP and CRP interaction in A549 cells***

To determine whether the tested combinations of the chemotherapeutic agent (CDDP) and the plant

alkaloid (CRP) induced synergistic, additive, or antagonistic effects. The interaction between CRP and CDDP was analyzed using the Combination Index (*CI*) based on the Chou-Talalay method (Chou, 2010; Chou and Talalay, 1984). This index quantifies whether the combination of two drugs acts synergically ($CI < 1$), additively ($CI = 1$), or antagonistically ($CI > 1$). Results obtained from the MTT assay were used to calculate *CI* values using Microsoft Excel according to the following Eq. 2:

$$CI = \frac{(D)_1}{(D_X)_1} + \frac{(D)_2}{(D_X)_2} \quad (2)$$

Where $D$ represents combined doses of substances 1 and 2, and $D_x$ represents single-substance doses for the same effect.

***Flow cytometry analysis***

Cells were seeded into small Petri dishes to achieve 40—50 % confluence within 24 hours. Then they were treated with CDDP and CRP at selected concentrations based on preliminary analysis. After reaching 80 % confluence (24 hours), the cells were trypsinized and centrifuged at 400× g for 5 min. Then, the supernatant was removed, and the cells were washed with culture medium without serum and phenol red, followed by centrifugation under the same conditions. The supernatant was discarded, and the cells were resuspended in $Ca^{2+}$ containing binding buffer supplemented with 0,2 µL AV per 100 µL of buffer. The cells were incubated for 15 min in the dark at room temperature. Immediately before measurement, 1 µL of propidium iodide (PI) was added to the cell suspension to a final concentration of 10 µg/mL and mixed thoroughly. Cell death modalities were analyzed using a BD Accuri C6 flow cytometer.

***Fluorescence microscopy***

Cells were seeded at low density (approximately $3 \times 10^4$ cells) onto poly-L-lysine-coated coverslips and cultured in small Petri dishes. After 24 h, they were treated with CDDP and CRP at a concentration at which a synergistic effect was observed. Following 24 h of incubation, the coverslips with adherent cells were removed from the Petri dishes and incubated with a staining mixture containing AV (0,2 µL/100 µL HEPES), Hoechst 33342 (5 µg/mL), and PI (10 µg/mL) in HEPES buffer. After 15—20 min of incubation at room temperature in the dark, the cells were washed with HEPES, mounted onto microscope slides with immersion oil, and immediately observed using a Zeiss Axio imager A1 fluorescence microscope equipped with a camera at 100× magnification.

***ROS production***

Cells were seeded onto 24-well microplates at the density of $1 \times 10^5$ cells per well. After 24 h of incubation, CDDP and CRP were added and the cells were incubated for another 24 h. After 20 h, 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) was added to the treated cell to the final concentration of 10 µmol/L per well, while 30 % $H_2O_2$ was added to the positive control to the final concentration of 100 µmol/L per well. The cells were then returned to the incubator for additional 4 h. Subsequently, the medium was aspirated from the wells and replaced with PBS (pH 7.4) containing 5 µg/mL Hoechst 33342. The cells were incubated for 10 min in a shaking thermoblock at 250 rpm and 37 °C. Fluorescence intensity of individual samples was measured using a Synergy H4 Hybrid Microplate Reader at the following excitation/emission wavelengths: DCF, 500/530 nm; Hoechst 33342, 361/461 nm.

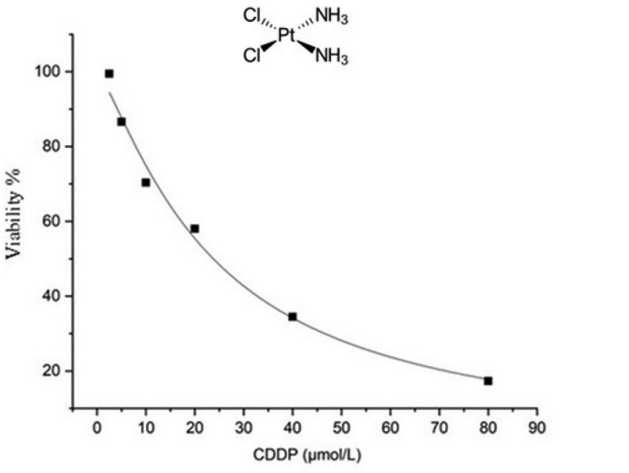
## Result and discussion

***Determination of $LC_{50}$ for CDDP and CRP in A549 cells***

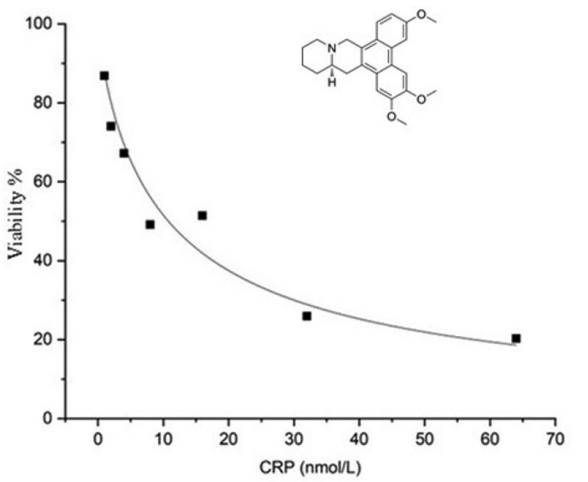
A549 cells were exposed to increasing concentrations of CDDP and CRP for 24 h resulting in a concentration-dependent decrease in cell viability. Based on the data obtained, dose-response curves were constructed and $LC_{50}$ values were calculated (Fig. 1).

***Evaluation of combined cytotoxic effects of CDDP and CRP***

This study aimed to determine the potential synergistic effect of CRP in combination with CDDP on the viability of A549 tumor cells. We hypothesized that the addition of CRP enhances the cytotoxic effect of CDDP, resulting in reduced cell viability at the same CDDP concentration. The combination of both compounds was intended to achieve a higher cytotoxic effect than CDDP alone, thereby confirming their synergistic interaction. To investigate the synergistic effects of CRP and CDDP, three concentrations of CRP (5.4, 10.8, and 21.6 nmol/L) were tested in combination with various concentrations of CDDP (11.89, 23.78, and 47.56 µmol/L). The results demonstrate that CRP does not enhance the cytotoxic effect of CDDP in A549 cells (Fig. 2). Combination index (*CI*) analysis revealed *CI* values were predominantly above 1, indicating an antagonistic interaction between CRP and CDDP (Fig. 3). At the CDDP concentration of 23.77 µmol/L combined with 21.6 nmol CRP, A549 cell viability decreased




| A 549 | LC50 (μmol/L) | $R^2$ | n | START (%) | END (%) |
|---|---|---|---|---|---|
| CDDP | 23,77 ± 1,17 | 0,999 | 1,26 ± 0,08 | 100 ± 0 | 0 ± 0 |




| A 549 | LC50 (nmol/L) | $R^2$ | n | START (%) | END (%) |
|---|---|---|---|---|---|
| CRP | 10,8 ± 1,95 | 0,995 | 1,26 ± 0,13 | 100 ± 0 | 0 ± 0 |

**Fig. 1.** Determination of cell viability concentration dependence. Concentration-dependent effect of CDDP (1A) and CRP (1B) on A549 cell viability is expressed as percentage. Cells were exposed to the indicated concentrations of CDDP or CRP for 24 h. Calculated parameters of the modified Hill equation describe the concentration-dependent effects of CRP and CDDP on A549 cell viability.
Data shown in Fig. 1 represent the mean values obtained from three independent experiments.

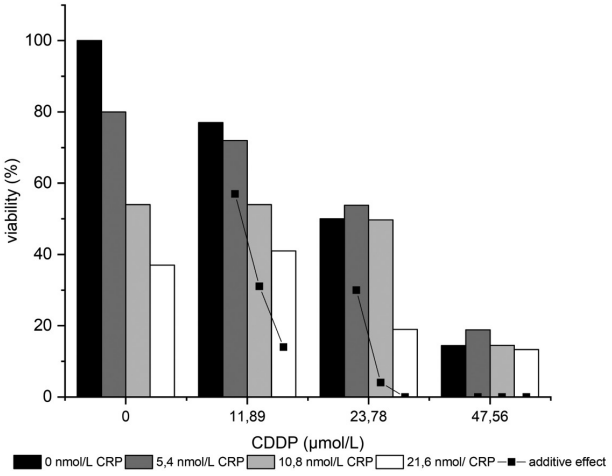



**Fig. 2.** Dependence of A549 cell viability (%) on different concentrations of CDDP and CRP administered alone or in combination. Black lines represent the additive effect of CDDP and CRP. If there is synergy between the drugs, the bars are below the black line. In case of a non-synergistic effect, the bars appear either above (antagonism) or close (additive effect) to the black line. Cell viability values represent the average of three independent experiments. Cells were treated with the indicated concentrations of the substances for 24 hours.

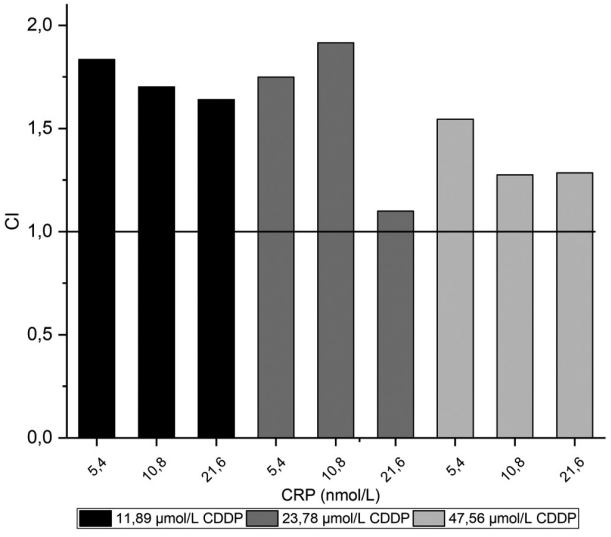



**Fig. 3.** Determination of *CI*. Relationship between the combination index (*CI*) and various combined concentrations of cisplatin (CDDP) and cryptopleurine (CRP). *CI* values < 1 indicate synergistic effect of the drug combination, *CI* = 1 corresponds to additive effect, and *CI* > 1 suggests antagonism.

to 19 ±0.9 % (Fig. 2). Similarly, treatment with 11.89 μmol/L CDDP in combination with the same CRP concentration (21.6 nmol/L) resulted in cell viability reduction to 39 ±2 % (Fig. 2), while *CI* values again induced antagonism between the two agents. In contrast, at the highest CDDP concentration tested (47.56 μmol/L), the addition of CRP did not markedly alter the A549 cell viability compared to CDDP alone, which is consistent with *CI* values remaining at or above 1, further con-

firming the antagonistic interaction. Synergistic effect can be expected when responding to treatment combining two drugs (Fig. 2, bars), which is higher than the summed effects of the drugs when used individually (Fig. 2, black line) (Šimoničová et al., 2023). However, in the A549 cell line, no synergistic effect was observed indicating that the CRP may be used only as an alternative to CDDP rather than in combination therapy in this case. To quantify the interaction between CDDP and CRP, the CI method was applied (Chou, 2010). Figure 3 (Fig. 3) shows *CI* values for individual combinations of the tested compounds proving the antagonistic effect of CDDP and CRP combination.

***Changes in intracellular ROS level***

Intracellular reactive oxygen species (ROS) production in A549 cells was evaluated following treatment with CDDP and CRP, either alone or in combination. Treatment with CDDP and CRP led to an increase in relative fluorescence intensity compared to the control group (C). However, this increase did not reach statistical significance ($p > 0.05$). No significant differences in ROS levels in treated and control cells were observed (Fig. 4).

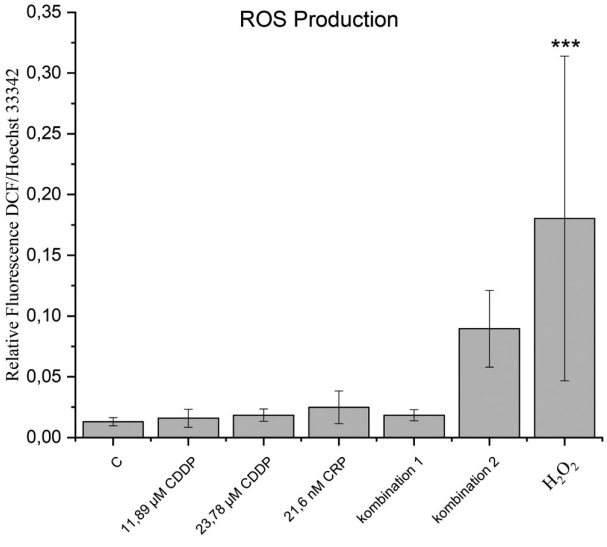



**Fig. 4.** Reactive oxygen species (ROS) production in A549 cells treated with CDDP and CRP after 24 hours. Relative fluorescence detection after exposure of A549 to 100 µmol/L $H_2O_2$ using $H_2$DCFDA as a fluorescent cell-permeable ROS indicator. Each bar represents MEAN ± standard deviation (n = 6). Results were analyzed by one-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001 versus negative control (C, nil concentration CDDP or CRP).

***Identification of cell death type***

To assess whether cell death was induced by combination treatment, A549 cells were treated for 24 h with combinations of CDDP (11.89 µmol/L or 23.78 µmol/L) and CRP (21.6 nmol/L). Based on the combination index analysis, these combinations exhibited antagonistic rather than synergistic effect. The mode of cell death induced by the combination of CDDP and CRP was evaluated by flow cytometry using Annexin V (AV) and propidium iodide (PI) double staining. In the control group (C; A549 + AV + PI), the proportion of viable cells ($AV^-/PI^-$) was 85.71 %, apoptotic cells accounted for 0.98 %, and necrotic cells ($AV^-/PI^+$) represented 7.44 % (Tab. 1). Following treatment with 11.89 µmol/L CDDP in combination with 21.6 nmol/L CRP (sample 1), showed a slight decrease in viable cells (83.40 %), accompanied by a low proportion of apoptotic cells (0.70 %). Treatment with 23.78 µmol/L CDDP combined with 21.6 nmol/L CRP (sample 2) resulted in a further reduction in viable cells, while the proportion of apoptotic cells remained minimal (0.14 %). In both treated samples, an increased proportion of necrotic cells was detected compared with the control indicating predominance of necrotic cell death. Overall, apoptosis was not confirmed in any of the tested samples. The results did not demonstrate a distinct apoptotic profile following combined treatment with CDDP and CRP. (Fig. 5).

To visualize nuclear morphological changes and assess apoptosis, A549 cells were stained with Hoechst 33342, AV, and PI after 24 h of treatment with combinations of CDDP and CRP. The results are shown in Fig. 6. In the control group, adherent A549 cells exhibited typical fibroblast-like morphology. Hoechst 33342 nuclear staining revealed regular, homogeneous nuclei without signs of chromatin condensation or fragmentation, indicating the absence of apoptosis. No AV or PI fluorescence was observed in control cells. Similar observations were made in cells treated with the combinations of 11.89 µmol/L CDDP + 21.6 nmol/L CRP and 23.78 µmol/L + 21.6 nmol/L CRP. Although treated cells partially lost their adherent morphology and exhibited changes, such as cell rounding and cytoplasmic contraction, Hoechst 33342 staining showed only mildly increased nuclear fluorescence without characteristic apoptotic morphology. Consistently, no AV and PI fluorescence was observed in these samples, which is in agreement with the flow cytometry results. Overall, these findings indicate that while combinations of CDDP and CRP induce morphological alterations in A549 cells, they do not trigger pronounced apoptotic features detectable by fluorescence microscopy.

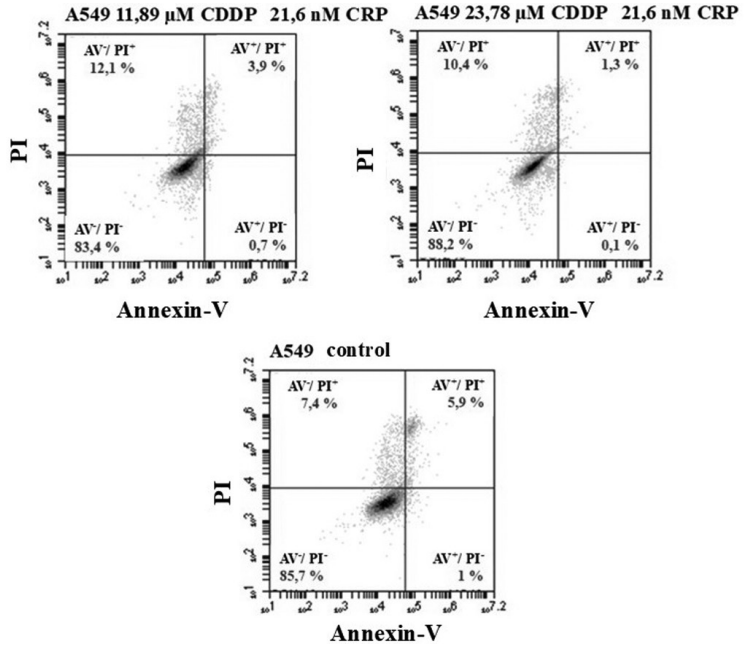



**Fig. 5.** Representative flow cytometry plot showing the distribution of A549 cells according to the type of cell death following treatment with CDDP and CRP, as determined by double staining with AV and PI.

**Tab. 1.** Measured percentage distribution of individual types of cell death in A549 cells determined by flow cytometry.

| Control/sample | $AV^-PI^-$ | $AV^+PI^-$ | $AV^+PI^+$ | $AV^-PI^+$ |
|---|---|---|---|---|
| **C + AV + PI** | 85.71 % | 0.98 % | 7.44 % | 5.87 % |
| **Sample 1 AV + PI** | 83.40 % | 0.65 % | 12.07 % | 3.88 % |
| **Sample 2 + AV + PI** | 88.16 % | 0.14 % | 10.43 % | 1.27 % |

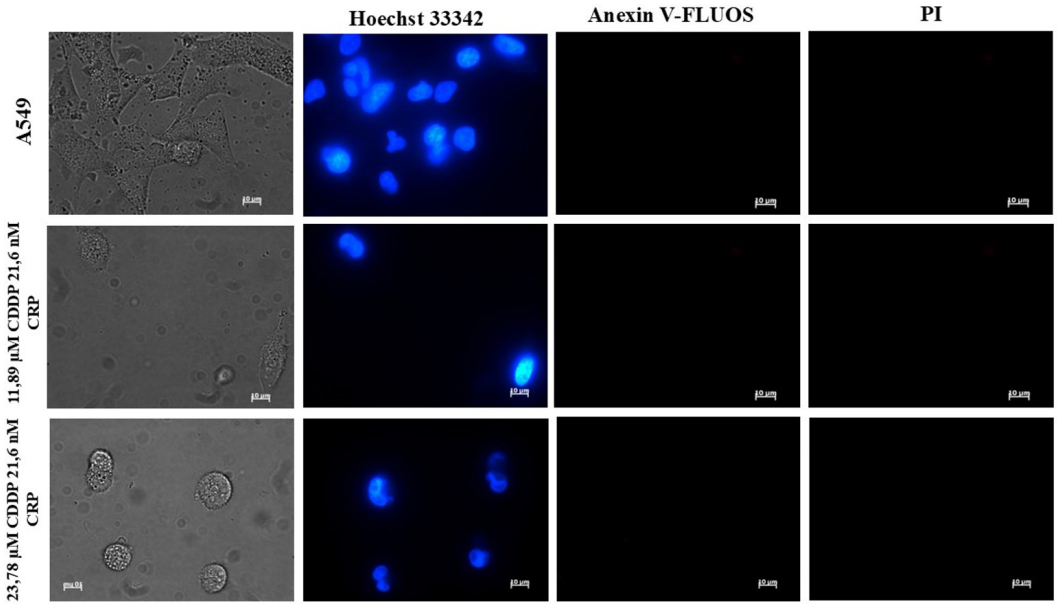



**Fig. 6.** Representative fluorescence microscopy images showing the type of cell death in A549 cells following treatment with CDDP and CRP, stained with AV, Hoechst 33342, and PI.

## Conclusion

Combination of CRP with CDDP did not lead to enhanced cytotoxic effect of CDDP in A549 cells. CI analyses indicated values above 1, demonstrating antagonistic rather than synergistic interaction between CRP and CDDP. Although treatment with the combination resulted in reduced cell viability compared with untreated controls, this effect did not exceed the expected additive response. The lack of synergistic interaction suggests that CRP does not potentiate the anticancer activity of CDDP nor does it support the use of this combination as an effective adjunct to conventional chemotherapy aimed at dose reduction. Overall, the results indicate that reduced A549 cell viability after combined treatment with CRP and CDDP is not mediated by synergistic cytotoxicity or apoptosis but may instead be associated with antagonistic interactions, altered cellular stress responses, or inhibition of cell proliferation. In conclusion, the combination of CRP with CDDP does not enhance the anticancer efficacy of CDDP in A549 cells and exhibits antagonistic interaction under the tested conditions. These findings suggest that CRP should not be considered a chemosensitizer of CDDP but rather an independent or alternative approach.

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