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Elucidation of Anticancer Mode of action of Betulinic acid-Cisplatin Conjugates on Lung cancer A549 cells In vitro

Kranthi Vanchanagiri[1], Thomas Müller[2], Reinhard Paschke[1]

[1]Biozentrum, Martin Luther University Halle–Wittenberg, Weinbergweg 22, 06120, Halle, Germany
[2] Department of Hematology/ Oncology, Martin-Luther-University of Halle-Wittenberg, Halle, Germany
Corresponding Author: Reinhard Paschke, Biozentrum, Martin Luther University Halle–Wittenberg, Weinbergweg 22, 06120, Halle, Germany.

Abstract: Betulinic acid (1) and Cisplatin (CP) are apoptosis causing antitumor agents. In our previous report we demonst a-ted the synthesis, characterization and in vitro anticancer activity of novel conjugates containing Betulinic acid (1) at one end and Cisplatin (CP) similar ligand at other end [Emmerich et al., 2014]. The property of presence of two cytotoxic groups is called "Double loading". Preliminary results showed that all novel conjugates exerted a dose dependent antiproliferative action at micromolar concentrations towards target tumor cell lines. In order to complement the previous results, in this report we looked for the anticancer mechanism of action of novel conjugates with their precursor molecules on A549 lung cancer cell line, which contributes to the understanding of the cytotoxic potential and their structure-activity relationship. A series of investigations had been carried out successfully which includes Microscopic studies, DNA Fragmentation assay, Cell cycle analysis, Annexin V/Propidium Iodide (PI) assay and Caspase assays. Interestingly, the results revealed that novel conjugates retain cytotoxicty and they induce apoptotic cell death which is identical to that of Betulinic acid (1).

Keywords: Betulinic acid derivatives; Cisplatin; A549 cells; Cytotoxicity; Double loading; Flow cytometry; Apoptosis; Caspases

I. Introduction

Currently, the development of new anticancer agents focused on discovering diverse compounds with either novel structures or a new mechanism(s) of action. Betulinic acid ((3β)-3-Hydroxy-lup-20(29)-en-28-oic acid, **1**) is a known bioactive pentacyclic triterpene, which has gained a lot of attention in the recent years since it exhibits a variety of biological and medicinal properties [1-4]. Platinum complexes are clinically used as adjuvant therapy of cancers aiming to induce tumor cell death. Cisplatin (*cis*-Diammineplatinum(II) dichloride, **CP**) is one of the most potent chemotherapy drug widely used for cancer treatment. Betulinic acid (**1**) and **CP** both are promising antitumor agents, both induce the apoptotic cell death of cancer cells [5, 6]. Both has broad spectrum anticancer activity and shown to be effective against a variety of carcinoma cell lines derived from lung, ovarian, cervical, head and neck carcinomas, as well as from lymphoma, neuroblastoma, medulloblastoma, gliob-lastoma, and other types of tumours [7-9]. Additionally Betulinic acid (1) has potential clinical value as anti-HIV, anti-bacterial and anti-malarial agent [10-12]. Discovery of novel plant derived natural products as potential new lead compounds for anticancer agents as well as the modi fication of the new lead compounds is continuing goals o four laboratory [13-17]. Combinational therapy is common in the field of chemotherapy. The efficiency of this therapy depends strongly on the nature of the single components: how they can be delivered, how they are metabolized, and how and to which extent they can enter the cell [18-21]. Therefore it could be advantageous when the components are covalently linked to each other. Based on this theory we have developed a concept to combine two bioactive fragments into one molecule. The aim was to find out if a combination of two different apoptosis causing structures would lead to a significant in fluence on the overall cytotoxicity of the conjugates. In our previous studies, a series of Betulinic a cid **(1)**and its derivatives containing Cisplatin **(CP)** simil ar ligands were designed and synthesized. The characteri zation, preliminary *in vitro* antitumor cytotoxicity (IC_{50}) and selectivity of these compounds were studied and

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reported in the previous paper [22]. With the goal of evaluating the anticancer mode of cell death, based on the promising cytotoxicity (Table1), the compounds **3, 3($PtCl_2$), 5, 5($PtCl_2$)** along with **1** and **CP** (Figure 1) were further selected for the more extensive investigations *i.e.*, cell cycle analysis, Annexin V and Caspase activation assays along with microscopic investigation and DNA fragmentation assay.

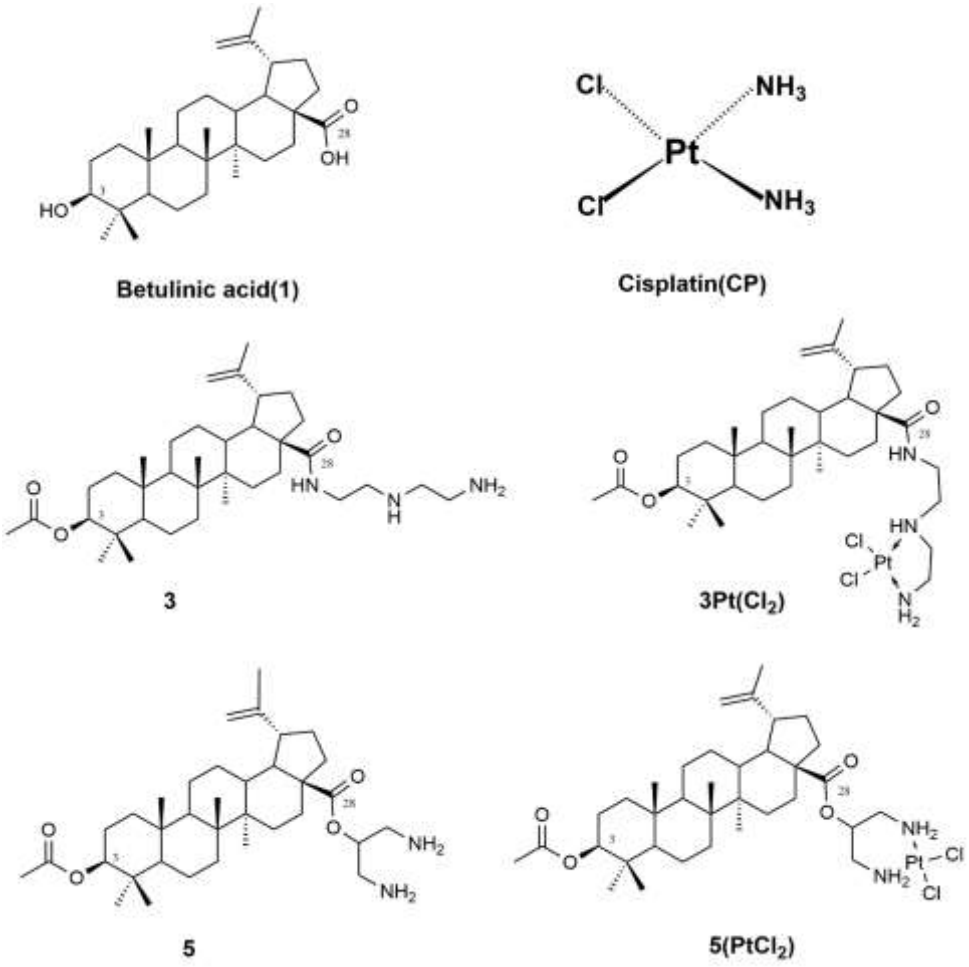

Figure 1. Structures of Betulinic acid (**1**) and its derivatives **3**, **5,** Cisplatin **(CP)**, and the novel conjugates **3($PtCl_2$), 5($PtCl_2$)**.

Apoptosis is an energy dependent controlled process and generally involves the activation of caspases, a group of cysteine proteases, which trigger a complex cascade of events that carry the cell to death [23, 24]. There are two types of apoptotic caspases: initiator (apical) caspases and effector (executioner) caspases. Initiator caspases (e.g. caspase 2, 8, 9, and 10) cleave inactive pro-forms of effector caspases, there by activating them. Effector caspases (e.g. caspase 3, 6 and 7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process. Apoptotic pathways are potential targets for therapeutic modulation. Apoptosis is either triggered by internal cellular stress (TYPE II-Intrinsic pathway) or extracellular signals (TYPE I-Extrinsic pathway). Extrinsic pathways directly activate executioner caspases (caspase 3) through initiator caspases (caspase 8 and 9) ultimately leading to cell death. In intrinsic pathways, death signals are conducted through mitochondria, increasing permeability that leads to the release of Cytochrome C. Cytosolic Cytochrome C binds Apaf-1 to activate the apoptosome and caspase 9 which ultimately leads to downstream activation of executioner caspase 3 [23, 25-27].

There exists a great deal of interest in probing the structural features responsible for the pharmacological effects of Betulinic acid **(1)** and its Cisplatin **(CP)** similar conjugates and to further optimize its activity profile. The evaluation of the whole set of results would lead to new insights in the field of drug development especially when two cytotoxic groups are combined within one molecule. The two main objectives of this project are: 1)to scrutinize the anticancer mode of cell death induced by these novel conjugates and to establish the structure

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activity relationship, further that could contribute to the understanding of the cytotoxic profile of this class of compounds, 2) to answer weather this "double loading" lead to a significant influence on the overall cytotoxicity.

II. Materials and methods

A. Preparation of solutions of compounds

Test compounds (Figure 1) were provided by Prof. Dr. Reinhard Paschke, "Laboratory of Medicinal Pharmaceut ical chemistry" at "Martin-Luther University Halle-Wittenberg. Stock solutions (20 mM) of Betulinic acid **(1), 3, 5** were prepared in DMSO and **CP, 3($PtCl_2$), 5($PtCl_2$)** were prepared in Dimethylformamide (DMF). The IC_{80} concentrations of compounds further diluted in RPMI 1640 nutrient medium (PAA Laboratories, Pasching, Austria) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich, Steinheim, Germany) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria).

B. Cell line and culture conditions

The lung cancer A549 cell line (kindly provided by BioSolutions Halle GmbH, Halle, Germany) was maintained as monolayers in RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich, Steinheim, Germany) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria) at 37 °C in a humidified atmosphere with 5% CO_2. Subconfluent monolayers of cells were used in all experiments.

C. Apoptosis tests

C.1. Microscopic Investigation

The microscopic investigation was performed to identify the morphological behaviour of treated cancer cells compare to untreated controls. 5.0 x 10^4 A549 cells were seeded in µ-Slide (chambered coverslip) with 8 wells (Sigma Aldrich, Germany) with 500 µL nutrient medium and kept at 37 ^oC and 5% CO_2. After 24 h medium was replaced with compounds **1, 3, 3($PtCl_2$), 5, 5($PtCl_2$), CP** at their respective IC_{80} concentration. After 48 h drug treatment the medium was discarded and the chamber slide was air dried for 2 min. The microwells of the chamber slide washed with 500 µL of PBS (with Ca^{2+} and Mg^{2+}) and rinsed thoroughly. The PBS was discarded and the chamber slide was air dried for 5 min. 20 µL of PBS (with Ca^{2+} and Mg^{2+}) was added to each well, covered with coverslip and visualized using Bright field microscope (390-700 nm, Carl Ziess GmbH, Germany) .

C.2. DNA fragmentation assay

2.0 x 10^5 A549 cells were seeded in 25 cm^2 flasks (10 mL RPMI medium), then flasks kept in incubator at 37 ^oC, 5% CO2. After 24 h medium replaced with IC_{80} concentration of the compounds **1, 3, 3($PtCl_2$), 5, 5($PtCl_2$), CP.** After 48 h drug treatment floating cells induced by drug exposure were collected, washed with PBS and lysed with DNA lysis buffer (100 mM Tris HCl pH 8.0; 20 mM EDTA; 0.8% SDS; all from Sigma Aldrich). Then cells were treated with RNAse A at 37 ^oC for 2 h. 10 µL proteinase K (20 mg/mL) was added to the sample and incubated at 50ºC overnight. 2% agarose gel was prepared (6 g agarose in 300 mL TAE-Buffer + 15 µL Ethidium bromide). 10 µL DNA loading buffer (6X) was added to samples and loaded on to the gel. The DNA samples were electrophoresed on a 2% agarose gel for 2 h at 40 V. The gel was examined and photographed by an UV Transilluminator (Biometra GmbH, Germany).

C.3. Cell Cycle analysis

Cell cycle analysis assessed by flow cytometry (Attune Acoustic Focusing Cytometer, Applied Biosystems, USA). 2 × 10^5 A549 cells were seeded in 25 cm^2 cell culture flasks with 10 mL of nutrient medium and kept at 37 ^oC, 5% CO_2. After 24 h of incubation, the medium was replaced with compounds **1, 3, 3($PtCl_2$), 5, 5($PtCl_2$), CP** at their respective IC_{80} concentration. Following 24 h and 48 h of incubation, the adherent cells and the supern atant were harvested, centrifuged (1500 rpm, 5 min, 4 ^oC) and the pellet was washed with PBS. Cells (1 × 10^6 cells/mL) were fixed with ethanol (70%, -20°C, for 2 h). Fixed cells were centrifuged and the pellet resuspended in 1 mL staining buffer (PBS + 2% FCS + 0.01% NaN_3) and centrifuged. Further the cell pellet was resuspended in 100 µL of RNase A (1 mg/mL) and incubated at 37 °C, for 30 min. 1 mL propidium iodide (PI) (20 mg/mL of staining buffer) was added and the samples were kept in dark at room temperature for atleast 30 min before the analysis. Doublet cells were excluded from the measurements by plotting BL-2A against BL-2H. For each cell cycle distribution 20,000 events were collected. Each sample was measured in triplicates and the results were compared with untreated controls. Cell Cycle distribution was calculated using Attune software (Life technologies, Darmstadt, Germany).

C.4. Annexin V-staining

Apoptotic cells were detected using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, BD Biosciences, Heidelberg, Germany). 4 × 10^5 A549 cells were seeded in 25 cm^2 cell culture flasks. After 24 h of incubation, the medium was replaced with IC_{80} concentration of the compounds **1, 3, 3($PtCl_2$), 5, 5($PtCl_2$), CP.** Following 24 h and 48 h of incubation, adherent and floating cells were harvested, centrifuged (1500 rpm, 5 min, 4 ^oC) and washed with 1 mL PBS

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(with Ca^{2+}/Mg^{2+}). The cell pellet was resuspended in AnnexinV binding buffer (BioLegend®, San Diego, US) to a concentration of 1×10^6/ml. 100 µL of each sample was stained with PI solution (3 µL, 1 mg/ml) and FITC Annexin V solution (5 µL, BioLegend®, San Diego, US) for 15 min in the dark at room temperature. After adding Annexin V binding buffer (400 µL) the suspension was analysed using the Attune® FACS machine (life technologies, Darmstadt, Germany). For each sample 20,000 events were collected. Each sample was measured in triplicates and the results were compared with untreated controls.

C.5. Caspase 3, 8 and 9 activation

8.0 x 10^5 A549 cells were seeded in 25 cm^2 cell culture flasks. After 24 h of incubation, the medium was replaced with IC_{80} concentration of the compounds **1, 3, 3($PtCl_2$), 5, 5($PtCl_2$), CP.** Following 24 h, 48 h and 72 h of incubation, cells were harvested by mild trypsinization and washed twice with PBS buffer (with Mg^{2+} and Ca^{2+}). The cell pellet was resuspended in PBS buffer (with Mg^{2+} and Ca^{2+}) to a concentration of 1×10^6/mL. 300,000 cells were treated with Caspase 3, 8 and 9 staining kit solution (1 µl, PromoKine, Germany) for one hour at 37 °C and 5 % CO_2. Following the incubation the cell samples were washed twice in caspase washing solution (PromoKine, Germany), resuspended in 300 µl caspase washing solution (PromoKine, Germany) and analysed using Attune® FACS machine (Life technologies, Darmstadt, Germany). For each sample 20,000 events were collected and duplicates were measured.

III. Results and Discussion

A. Cytotoxicity on A549 cell line

Previously the test compounds evaluated for their cytotxicity against broad spectrum of human cancer cell lines from different hisotgenic origin [22]. The IC_{50} and IC_{80} are defined as the concentrations of the compound at which 50% and 80% cell inhibition relative to untreated control cells. It was known that those with IC_{50}<20 µM are considered highly active, values between 20-75 µM are moderately active, and those between 75-165 µM are slightly active or inactive [28]. The compounds showed dose-dependent antitumoral activity.

The IC_{50} and IC_{80} values of the compounds on A549 cells were estimated from the semi-logarithmic dose response curves (Figure 2.) calculated by GraphPad Prism6 and summarized in Table 1.

Table 1. IC_{50} ± S.E and IC_{80}±S.E values of the investigated compounds against A549 cell line.

Compound	A549 [IC_{50}±S.E]	A549 [IC_{80}±S.E]
1	13.3±0.82	23.3±1.26
3	2.05±0.34	2.59±0.49
3($PtCl_2$)	17.32±0.00	24.5±0.54
5	12.58±0.24	23.0±0.91
5($PtCl_2$)	17.85±1.87	51.3±2.09
CP	1.15±0.05	2.0±0.58

Values are derived from dose-response curves obtained by measuring the percentage of viable cells relative to untreated controls after 96 h exposure of the test compounds against A549 (lung carcinoma) cell line .Data shown as the average from the three independent experiments.

The lead compound **1** (IC_{50} = **1**3.3 µM) shared similar range of IC_{50} value with compound **5** (IC_{50} = 12.58 µM).The introduction of platinum ligands into Betulinic acid **(1)** led to no significant loss of activity, however the presence of platinum groups [compound **3($PtCl_2$),** IC_{50} = 17.32 µM and compound **5($PtCl_2$),** IC_{50} = 17.85 µM] did not have any influence on the overall cytotoxicity of the compounds.

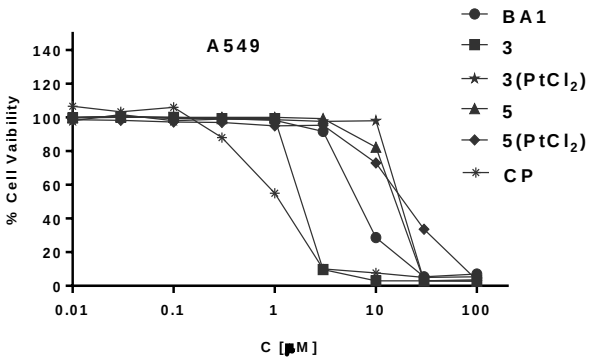

Figure 2. Cytotoxicity dose-response curves of **1, 3, 5, CP, 3($PtCl_2$), 5($PtCl_2$)** thereof on A549 cell line determined by SRB Assay (one representative of three independent experiments).

B. Apoptosis tests All Compounds were investigated regarding their potential to induce apoptosis.

B.1. Microscopic examination

Apoptotic cells share morphological features of cell shortening, loss of intercellular adhesion, membrane blebbings, apoptotic bodies, and DNA laddering. However, it is well known that morphological changes

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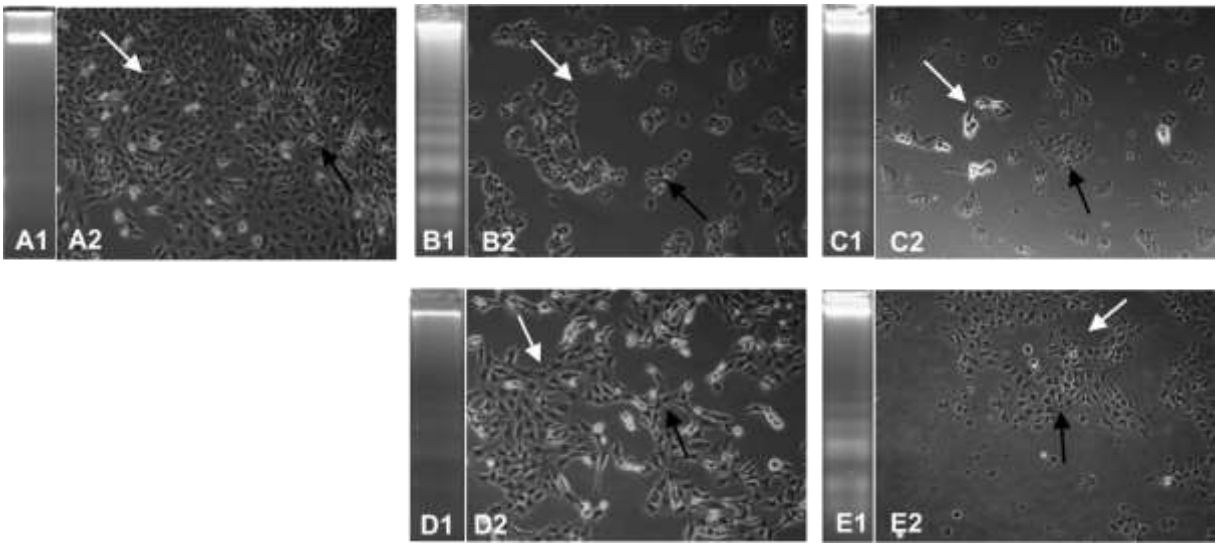

Figure 3. DNA laddering assay [A1-E1] and Bright field microscopy images (A2-E2) of A549 cell treated with IC_{80} concentration of **3**[B1, B2], **3(PtCl$_2$)** [C1, C2], **5**[D1,D2], **5(PtCl$_2$)** [E1,E2] for 48 h compared to untreated control [A1,A2].

are the basis of functional changes; in turn, functional changes would affect the morphological structure [24, 29]. Untreated A549 cells showed typical epithelial morphology with tight cell-cell contacts marked by white arrows and cuboid or triangle--shape to spindle-shape cells marked by black arrows (Figure 3.A2). Treatment of cells with test compounds for 48 h induced marked changes in cell morphology (Figure 3 and Figure S1). Treated cells showed loosened cell-cell contacts marked by white arrows and prolonged cell extrusions marked by black arrows (Figure 3[B2-E2] and Figure S1[B2, C2]). Compared to untreated control many cells detached from the monolayer of treated cells. The detached cells undergone programed cell death which is further characterized by DNA fragmentation assay (Figure 3 [A1-E1] and Figure S1[A1-C1]). From the results it was confirmed that the compounds induce morphological changes and there is no distingusible diffrences observed for A549 cells treated with different test compounds.

B.2. DNA fragmentation

DNA fragmentation is a consequence of apoptosis, which is a type of programmed cell death. Apoptosis is characterized by fragmentation of DNA, under stress and when the compounds treated with antitumor agents apoptotic signal endogenous endonucleases are activated with subsequent cleavage of chromatin DNA into internucleosomal fragments of 180 base pairs or its multiples [30]. DNA laddering assay performed for floating cells collected from untreated and treated A549 compound **5** typical DNA ladder patterns were observed for compounds **1, 3, 3(PtCl$_2$)**, **5(PtCl$_2$)** and **CP**. Weak DNA ladder pattern was seen in case of compound **5**. However the occurrence of DNA ladders could indicate that all compounds inducing programmed cell death i.e., apoptosis. Further investigations performed to characterize the compound induced apoptotic cell death.

B.3. Cell cycle perturbations

Cell cycle analysis by flow cytometry performed to test whether the compound induced apoptotic cell death associated with arrest of any of cell cycle phases. DNA histogram analysis revealed that the relative amount of cells distributed in different phases of cell cycle. In general cells that suffer apoptosis can be detected as a subdiploid peak (SubG1) by flow cytometry. SubG1 peak corresponds to cells with fragmented DNA, a feature of the apoptotic cell death. When compared to control, except **CP** after 24 h all test compounds (Figure 4, Figure 6 and Figure S2) caused increase in the number of apoptotic cells (SubG1-peak). After 24 h the amount of cells in the different cell cycle phases decrease in the same ratios for compounds **1** and **3**. After 48 h, (except **CP**) further exposure to the compounds didn't induce significant cell cycle arrest in any cell cycle phase but rather directly provoked an increase of number of apoptotic cells (SubG1) with concomitant decline of all other cell cycle phases (Figure 5, Figure 6 and Figure S2). After 24 h **CP** was found to show "S" phase arrest and after 48 h it caused "G2/M" phase arrest which was associated with slight increase in the number of cells in SubG1 (Figure 6 and Figure S2).

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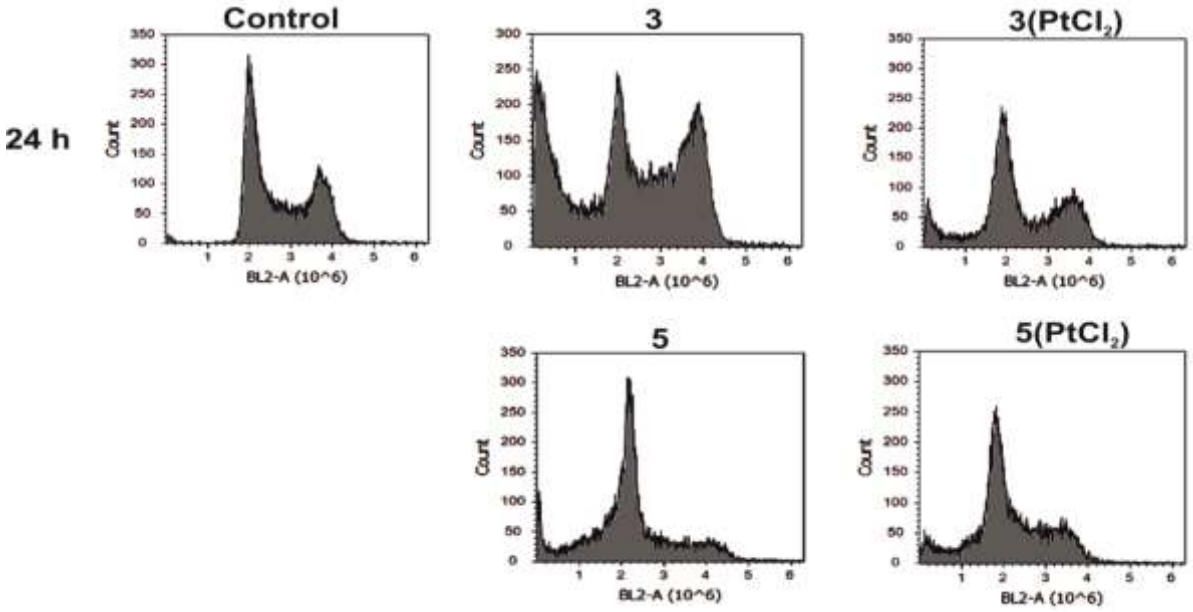

Figure 4. Distribution of A549 cells in each cell cycle phase after treatment with IC_{80} concentration of **3, 3($PtCl_2$), 5, 5($PtCl_2$),** for 24. The results were obtained by the analysis of Propidium iodide stained DNA amount by flow cytometry. The histograms are representative of three independent experiments.

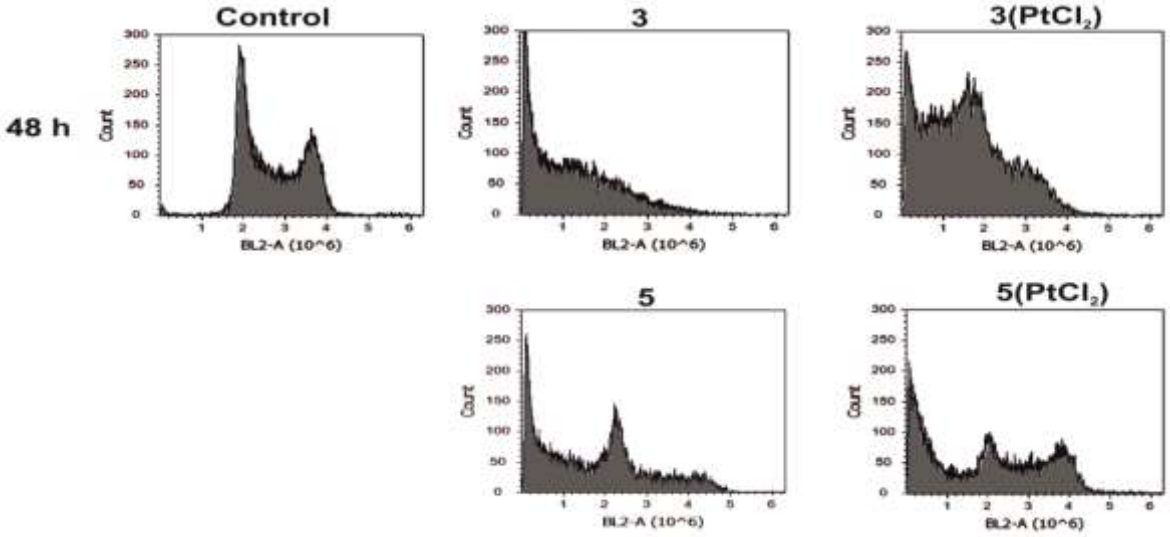

Figure 5. Distribution of A549 cells in each cell cycle phase after treatment with IC_{80} concentration of **3, 3($PtCl_2$), 5, 5($PtCl_2$),** for 48 h. The results were obtained by the analysis of Propidium iodide stained DNA amount by flow cytometry. The histograms are representative of three independent experiments

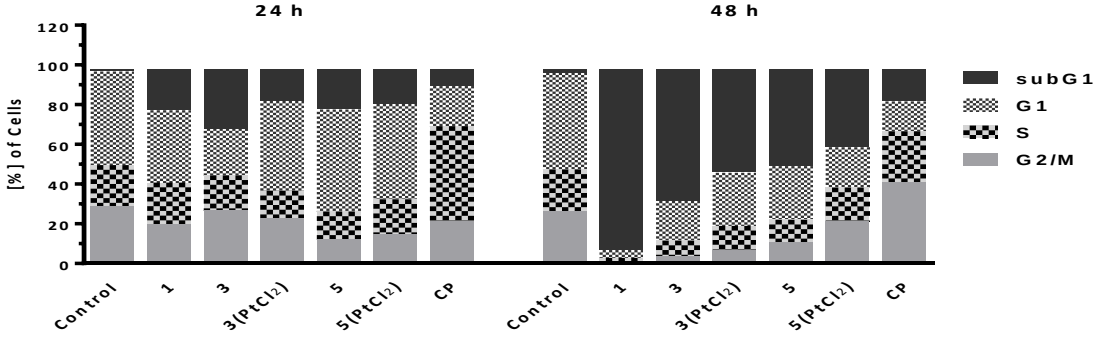

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Figure 6. Percentage of cells in each cell cycle distribution phase (mean of three independent experiments) of A549 cells treated for 24 h and 48 h with compounds **1, 3, 3($PtCl_2$), 5, 5($PtCl_2$), CP** at their IC_{80} concentrations compared to untreated control.

B.4. Externalization of Phosphotidylcerine - Annexin V assay

Early events in apoptotic process are loss of plasma membrane asymmetry accompanied by translocation of phosphatidylserine (PS) from the inner to the outer membrane leaflet there by exposing PS to the external environment [29]. The conjugate phospholipid binding protein AnnexinV-FITC binds to PS and used to detect early apoptotic cells. PI (Propidium Iodide) is an intercalating agent and a fluorescent molecule used to stain cells that had lost membrane integrity (Late apoptotic/Necrotic). Quantitative assessment of apoptosis in A549 cells treated with IC_{80} concentration of the compounds for 24 h and 48 h was carried out by FACS analysis using Annexin V-FITC/PI-staining (Figure 7, Figure 8 and Figure S3). When compared to control after

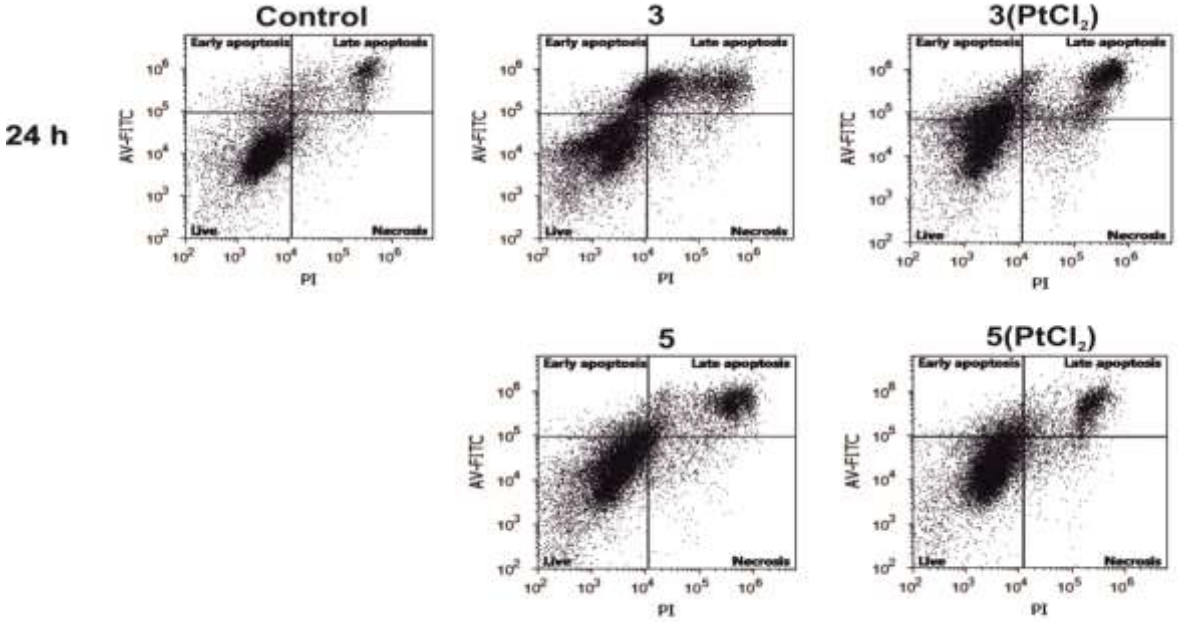

Figure 7. Annexin V assay of A549 cells untreated and treated with compounds **3, 3($PtCl_2$), 5, 5($PtCl_2$), CP** at their IC_{80} concentrations for 24 h (as indicated), the floating and attached cells harvested further stained and analysed by flow cytometry.

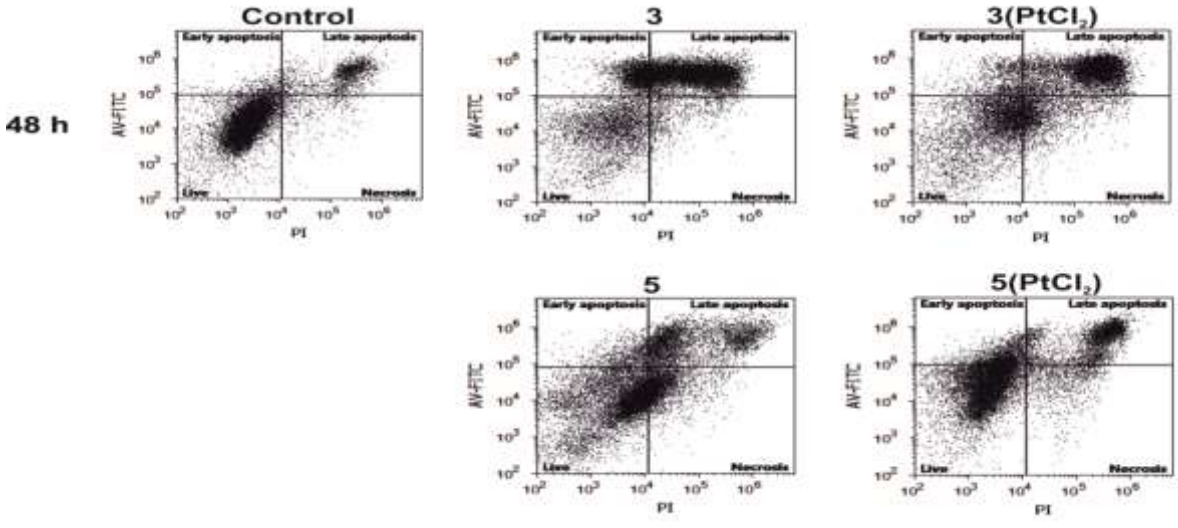

Figure 8. Annexin V assay of A549 cells untreated and treated with compounds **3, 3($PtCl_2$), 5, 5($PtCl_2$), C**P at their IC_{80} concentrations for 48 h (as indicated), the floating and attached cells harvested further stained and analysed by flow cytometry.

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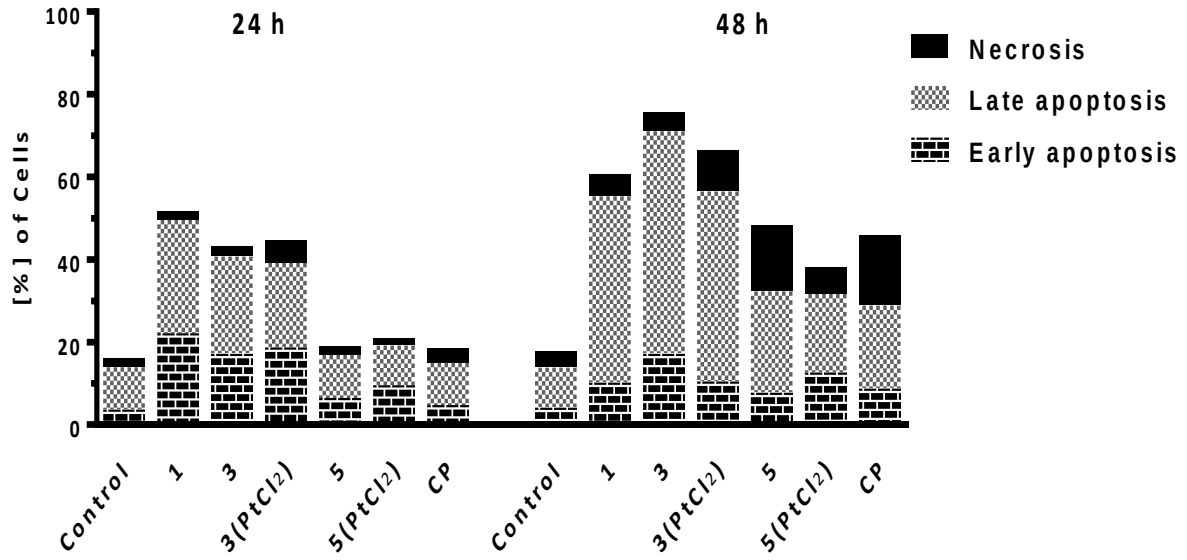

Figure 9. Annexin V/PI staining of A549 cells untreated and treated with compounds **1, 3, 3($PtCl_2$), 5, 5($PtCl_2$),** CP at their IC_{80} concentrations for 24 & 48 h (as indicated). Data (percentages of early and late apoptotic cells) shown as the mean of three independent experiments.

24 h an increase of the number of apoptotic cells was observed for A549 cells treated with compounds **1**, **3** and **3($PtCl_2$)**, while no significant increase of amount of apoptotic cells was observed for cells treated with **5, 5($PtCl_2$)** and **CP** (Figure 7, Figure 9 and Figure S3). After 24 h an increase of around 25%-36% apoptosis

(Early apoptosis + Late apoptosis) noticed for cells treated with **1**, **3** and **3($PtCl_2$),** and in case of **5, 5($PtCl_2$)** and **CP** only 1%-5.5% apoptosis was verified. After 48 h around half of the (42%) cells treated with **1**, **3($PtCl_2$)** were apoptotic and more than 50% (66.23%) of the cells treated with **3** were apoptotic, in addition a slight increase (around 15%-18.5%) of apoptotic cells was seen for cells treated with **5, 5($PtCl_2$)** and **CP** (Figure 8, Figure 9 and Figure S3). In contrast considerable amount of necrotic cells was exhibited for cells treated with **5** and **CP** was neglected in rest of compounds.

B. 5. Activation of initiator and effector caspases- sign of apoptotic cell death

A hallmark of apoptosis is the activation of caspases [31-33]. To gain insight into the molecular changes of the new derivatives induced apoptosis, we analyzed whether caspases are involved as effectors in the apoptotic induced cell death. The activation of the the upstream caspases 8, 9 and the downstream caspase 3 were tested by using fluorescent labelled inhibitors of caspases (FLICA) by flow cytometry (Figure 10).

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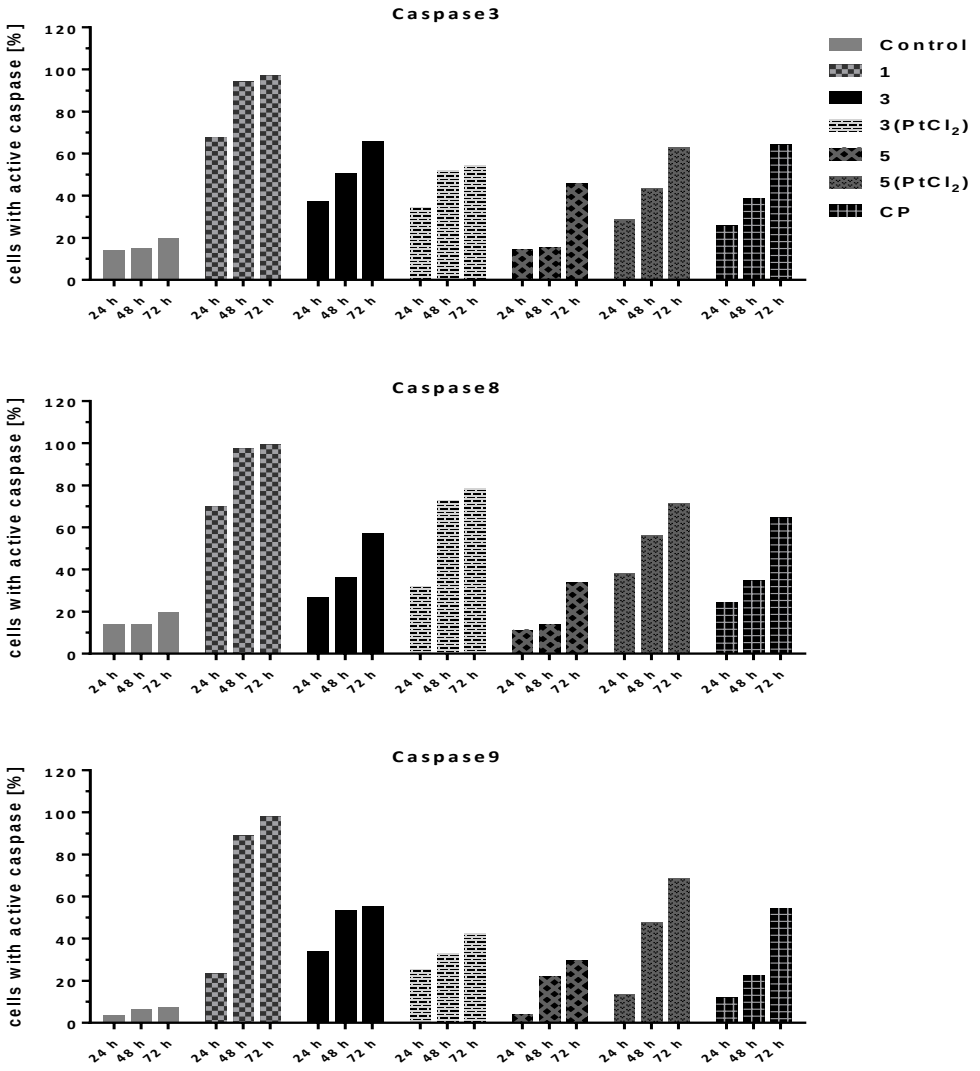

Figure 10. Caspase assays of A549 cells untreated and treated with compounds **1, 3, 3($PtCl_2$), 5, 5($PtCl_2$), CP** at their IC_{80} concentrations for 24 h, 48 h and 72 h (as indicated). Data shown as the mean of two independent experiments.

During 24 h treatment Betulinic acid **(1)** caused the activation of caspase 3 and 8, whereas caspase 9 was less activated than other caspases (Figure 10). However the up regulation of both of three caspases by **1** was increased to maximum towards the end of the treatment (48 h and 72 h) which evidence that apoptosis induced by Betulinic acid **(1)** traverse the mitochondrial or intrinsic pathway. All the three caspases were equally upregulated by **3** and **3($PtCl_2$)** through out the treatment period. No significant up regulation of any of caspases was observed for compound **5** during initial treatment (24 h). At the end of treatment (72 h) the up regulation of caspase 3 is slightly higher than that of caspase 8 and 9. Caspase 3 and 8 were well upregulated by **5($PtCl_2$)** and **CP**, while activation of caspase 9 was not observed during 24 h treatment but it was upregulated upon further exposure (48 h and 72 h) to the compounds.

IV. Conclusions

The search for new candidates for anticancer agents is a very competitive field in research and many different approaches have been made. This investigation not only tries to find new derivatives that are more potent than the existing ones but also intends to make a contribution to scrutinize the mechanism of action. Among the panel of Betulinic acid **(1)** derivatives, **3** was found to be highly cytotoxic and it induced apoptosis mediated by caspase cascade pathways similar to Betulinic acid **(1)**. The presence of alkyl amide (polyamine) at C-28 position leads to DNA damage followed by apoptotic cell death and finally it contributes to the high toxicity of **3** [34, 35]. Besides the classical caspase dependent apoptosis, also caspase independent cell death may exist maintaining key characteristics of apoptosis [36, 37]. Notably compound **5** bypassed the caspase dependent mechanism evidenced by occurrence of floating cells after treatment, showing

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weak DNA fragmentation and morphological changes despite no significant caspase activity. When compared to precursors the compounds with two cytotoxic groups **3($PtCl_2$), 5($PtCl_2$)** retained the cytotoxicity. Both compounds did not induce cell cycle arrest in any cell cycle phase but rather directly provoked an increase of SubG1-peak with concomitant decline of all other cell cycle phases and, concurrently induced apoptosis mediated by activation of downstream (Caspase 3) and upstream caspases (Caspase 8,9) more or less similar to that of lead compound Betulinic acid (**1**). Cisplatin **(CP**) is a DNA damaging agent (apoptosis-intrinsic pathway) [38-42]. To induce apoptosis it has to enter the nucleus followed by DNA damage takes place [38, 39]. In case of Betulinic acid-Cisplatin conjugates the Cisplatin **(CP**) ligand not directly linked to Betulinic acid **(1)** skeleton instead, it was covalently linked to C-28 alkyl amide group (a polyamine spacer). When A549 cells treated with these conjugates inside the cells the ligands either may not be cleaved or apoptosis caused by Betulinic acid fragment is much quicker than by interaction of the platinum with DNA.

To summarize, the apoptosis induction by Betulinic acid-Cisplatin conjugates was documented and the cytotoxic activity was retained for the novel conjugates with two cytotoxic groups, and they found to show similar apoptotic induction to that of Betulinic acid **(1)**. The structure of novel conjugates just influenced the ability to enter the membrane and to alter the mitochondrial membrane potential, this could also be one of the reason for their different activities [44]. In conclusion, the desi-gn of a more effective and more systematic structural variations of Betulinic acid-Cisplatin conjugates must be carried out in order to get more clarity and more reliable structure activity relationship. Nevertheless, the results open up the possibility of double loading with two cytotoxic groups and the double loaded conjugates deserved to be proceed for further developmental strategies.

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Author Contributions

Kranthi Vanchanagiri designed the study, performed all experimental procedures, analyzed the data and drafted the manuscript. Reinhard Paschke, and Thomas Müller aided in study design, analyzed the data and reviewed the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary data:

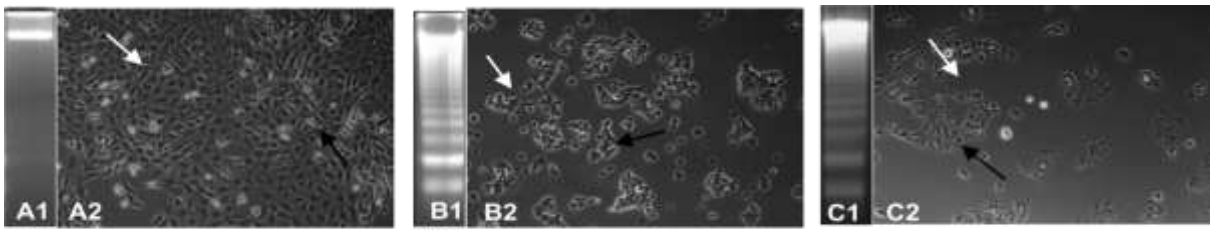

Figure S1: DNA laddering assay [A1-E1] and light microscopic images (A2-E2) of A549 cell treated with IC_{80} concentration of **1**[B1, B2], **CP**[C1, C2] for 48 h compared to untreated control [A1,A2].

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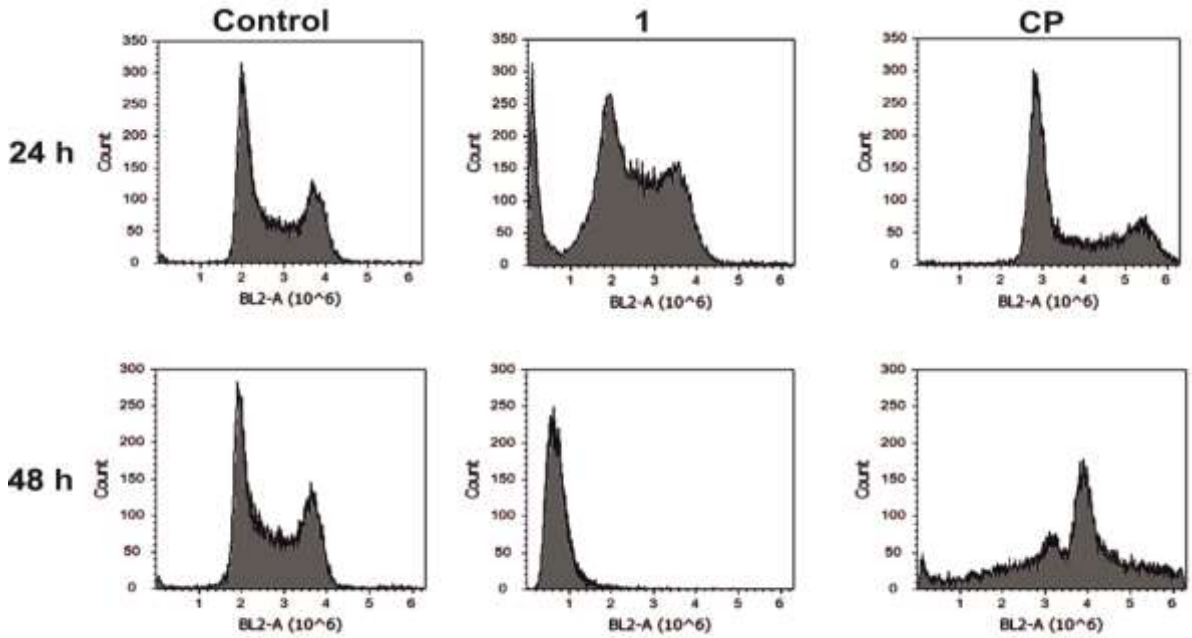

Figure S2: Cell Cycle distributions of A549 cells untreated and treated with compounds **1** and **CP** at their IC_{80} concentrations for 24 & 48 h (as indicated). The DNA was stained with PI and the cells were analyzed by flow cytometry.

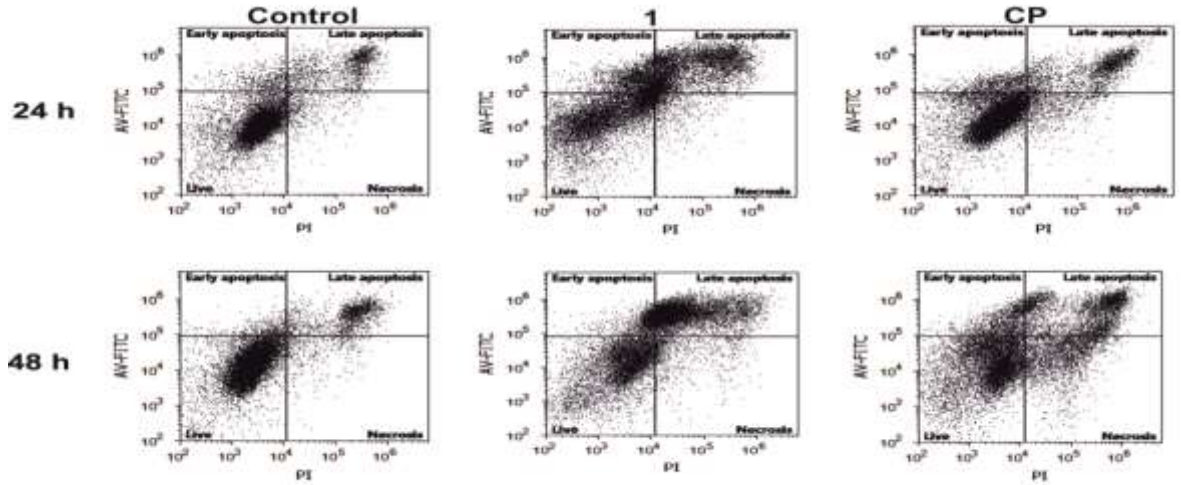

Figure S3: AnnexinV staining of A549 cells untreated and treated with compounds **1** and **CP** at their IC_{80} concentrations for 24 & 48 h (as indicated). After harvesting the cells were stained and flow cytometry analysis was performed.

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