



Original Article

Apoptotic Effects of Sanguinarine on the Organ of Corti 1 Cells: Comparison with Cisplatin

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OBJECTIVE: Sanguinarine is an alkaloid obtained from the root of *Sanguinaria canadensis* and other plants from the Papaveraceae family and is well known to possess a broad range of biological functions, such as antimicrobial, antifungal, anti-inflammatory, and antineoplastic activities. We aimed to specify the *in vitro* effect of sanguinarine on the House Ear Institute-Organ of Corti 1 (HEI-OC1) cells and to compare this effect with the ototoxic effect of cisplatin (CDDP).

MATERIALS and METHODS: We performed cell proliferation assay for determining the *in vitro* effect of sanguinarine alone and compared it with the effect of cisplatin. Flow cytometry annexin-V apoptosis detection was performed.

RESULTS: We found that sanguinarine and CDDP inhibited the cell growth in a dose-dependant manner in HEI-OC1 cells after 24 h of incubation. In sanguinarine-treated group, apoptosis was 6.6%, necrosis was 26.7%, and the cell viability was 66.7%. Further, in CDDP-treated group, apoptosis was 5.6%, necrosis was 45.4%, and the cell viability was 48.7%. According to the annexin-V apoptosis detection results, we found that sanguinarine caused 3.9% apoptosis and 1.3% necrosis, while CDDP caused 2.9% apoptosis and 20% necrosis on HEI-OC1 cells.

CONCLUSION: Our findings suggested that lower doses of sanguinarine are promising antineoplastic agents, which did not indicate any toxic effect on HEI-OC1 cells. Application of these data to clinical practice requires further support by *in vivo* studies.

KEYWORDS: Cisplatin, sanguinarine, ototoxicity, HEI-OC1 cells

INTRODUCTION

Sanguinarine (13-methyl-benzodioxolo-[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium) is an alkaloid obtained from the root of *Sanguinaria canadensis* and other plants from the Papaveraceae family^[1]. The mechanism of action of benzophenanthrid alkaloids is associated with the cell death signaling pathway, and apoptosis induction is shown in various cancer cell lines. Apoptosis caused by sanguinarine has been shown through different ways, such as mitochondrial damage, nuclear factor kappa-light-chain enhancer of activated B cells activation, and cell cycle arrest^[2]. Sanguinarine is shown to inhibit microtubule polymerization and can intercalate double-stranded DNA^[3,4]. The caspase activation, depletion of cellular glutathione, downregulation of extracellular signal-regulated kinases, modulation of B-cell lymphoma 2 family, and upregulation of DR-5 are the mechanisms of antitumoral action of sanguinarine^[4-7]. It is shown that the cytotoxic activity of benzophenanthridine is proportional to the DNA-binding feature and induction of DNA fragmentation. According to recent studies, the cytotoxic and DNA damaging effect of sanguinarine is more specific to cancer cells than to normal cells^[4,8]. We have already demonstrated that sanguinarine causes apoptosis in human neuroblastoma cells. We determined the cell death mechanism of sanguinarine was particularly via cytotoxic and apoptotic effects occurring by changing the apoptotic gene expressions in human SH-SY5Y and Kelly neuroblastoma cell lines^[9].

Cisplatin (cisdiammine-dichloro-platinum, CDDP) is one of the most important chemotherapeutic agents used in the treatment of both adult and childhood cancers. However, its dose-limiting adverse effects, such as ototoxicity, nephrotoxicity, myelosuppression, gastrointestinal toxicity, neurotoxicity, and cardiovascular damage, have been shown to be an important obstacle for its utility and therapeutic profile^[10]. It is found that 25% patients undergo sensorineural hearing loss because of cisplatin ototoxicity. The incidence of ototoxicity can be affected by factors, such as the route of administration, age, dietary factors, genetic factors, serum protein levels, and a history of cranial irradiation exposure. CDDP can produce both superoxide ions and active oxygen species,

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such as hydroxyl radicals, and may inhibit antioxidant enzymes in normal tissues. This ototoxicity is not limited to hairy cells [11-14]. Because of side effects that limit the cisplatin use, recent studies have focused on finding a novel agent as effective as CDDP with less or no adverse effects. Clinical studies regarding herbal drugs for protection and/or therapy of tumors have recently become extensive in cancer treatment [13, 15, 16].

The House Ear Institute-Organ of Corti 1 (HEI-OC1) is a suitable cell line for *in vitro* studies on the ototoxic effect of chemotherapeutic agents, such as CDDP. As HEI-OC1 cells carry the molecular characteristics of the organ of Corti sensory cells, the underlying molecular mechanism of ototoxicity and candidate protective agents against hearing loss caused by ototoxic drugs can be well clarified [17]. Therefore, we aimed to specify the *in vitro* effect of sanguinarine on HEI-OC1 cells and to compare this effect with the ototoxic agent, CDDP. To our knowledge, this study was the first to assess the effect of sanguinarine in *in vitro* ototoxicity model.

MATERIALS and METHODS

This study was approved by the local ethics committee of Dokuz Eylül University Medical School.

HEI-OC1 Cell Culture

The HEI-OC1 cell line was provided by Professor F. Kalinec. It is an immortalized cell line obtained from cochlear cultures of the immortal mouse. Kalinec et al. [17] have proposed that the HEI-OC1 cell line was an excellent *in vitro* system with 10% fetal bovine serum (FBS) at 33°C under 10% CO₂ in air. The cells were grown in 75-cm² culture flasks with no antibiotics and were passaged to 1:2 two times in a week. The cells were cultured in 96-well plates as 20000 cells/well. Further, after attaching to the plate after 24 h of incubation, we performed dose-dependant cell proliferation analysis of sanguinarine and CDDP.

WST-1 Cell Proliferation Assay

CDDP (Hexal; Sandoz, Novartis, Bangladesh), Sanguinarine (Sigma-Aldrich Co; St. Louis, MO, USA), and WST1 (Roche Applied Science; Mannheim, Germany) were utilized in this study. They were newly prepared before all experiments. Cells were exposed to different doses of sanguinarine (0, 2.5, 5, 10, and 20 µM) and CDDP (20 and 40 µM) for 24 h, and the cell viability was analyzed using Cell Proliferation Reagent WST-1 assay kit. WST-1 is a colorimetric assay that measures the cell viability based on the cleavage of tetrazolium salts to formazan by mitochondrial dehydrogenases in viable cells. After treatment, cells were incubated with 10-µl WST-1 solution/well for 2 h at 37°C. The 96-well plates were read at 440 nm with a reference wavelength at 630 nm using a multidetection microplate reader (Thermo Instruments Inc; Shanghai, China).

Flow Cytometry Analysis of Apoptosis

The rate of apoptosis among HEI-OC1 auditory cells treated with 40-µM CDDP alone and 5-µM sanguinarine alone for 24 h as well as the untreated control group was determined using annexin V-FITC (BD Annexin V-FITC Apoptosis Detection Kit; Roche Diagnostics GmbH, Germany). The flow cytometric analysis was performed using BD Accuri C6 flow cytometry. The gating strategies were performed based on the distribution of the unstained and one-stained (FITC- Annexin V or propidium iodide only) control cells on the scatterplot.

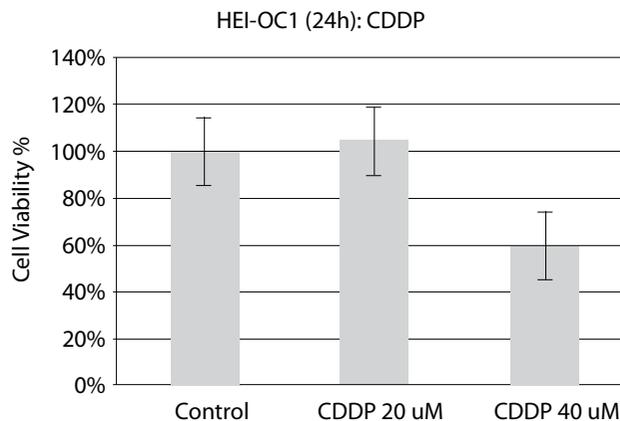


Figure 1. Cell proliferation assay results of CDDP for HEI-OC1 cells (24 h)

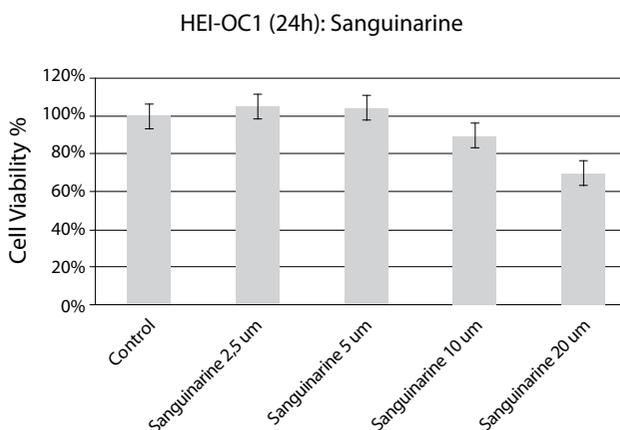


Figure 2. Cell proliferation assay results of sanguinarine for HEI-OC1 cells (24 h)

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences 15.0 for Windows (SPSS; SPSS Inc., Chicago, IL, USA), and p values less than 0.05 were considered as statistically significant. Non-parametric Mann-Whitney U test with Bonferroni correction were also used. All treatment experiments were repeated at least three times/six condition to generate statistically relevant data.

RESULTS

Cell Culture and Survival Analysis According to Dose Range of CDDP and/or Sanguinarine

We cultivated HEI-OC1 cells with 20- and 40-µM CDDP for 24 h, and CDDP inhibited cell growth in a dose-dependant manner in HEI-OC1 cells. CDDP inhibited 50% cell growth at 40-µM dose when incubated for 24 h in the cells (Figure 1). We cultivated HEI-OC1 cells with sanguinarine (2.5, 5, 10, and 20 µM) for 24 h. Sanguinarine inhibited cell growth in a dose-dependant manner after 24 h of incubation. These cell proliferation assay results of sanguinarine for HEI-OC1 cells are shown as in Figure 2. The cell viability was 105% in a low sanguinarine dose (2.5 µM) and decreased to 70% in the highest dose of sanguinarine (20 µM). Sanguinarine did not inhibit cell growth at 5-µM dose (104%; Figure 2).

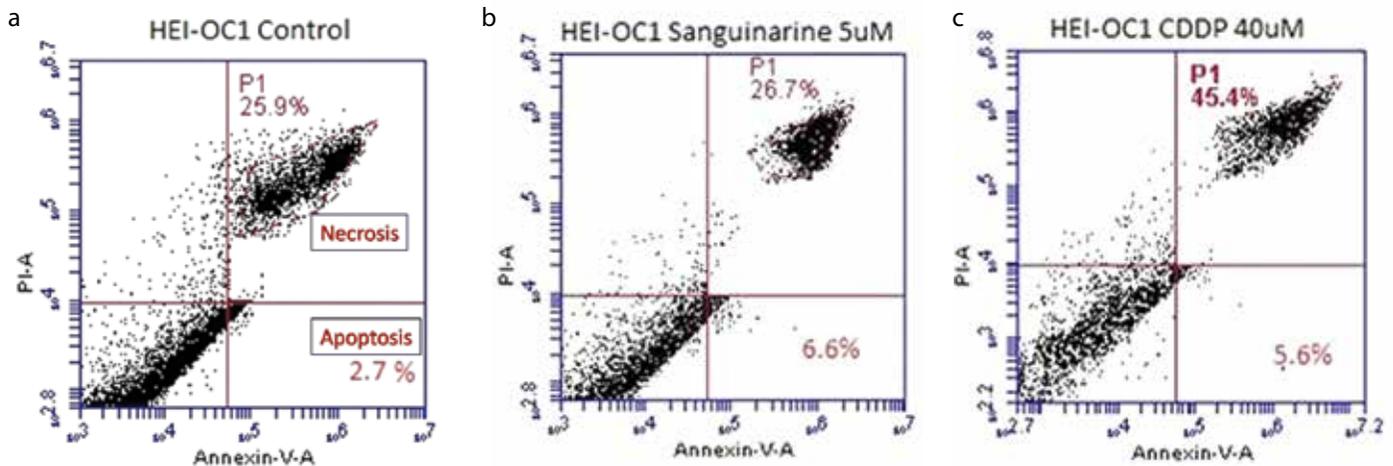


Figure 3. a-c. The rate of apoptosis and necrosis among HEI-OC1 auditory cells in the control group (a), treated with 40- μ M CDDP alone (b), and 5- μ M sanguinarine alone for 24 h (c)

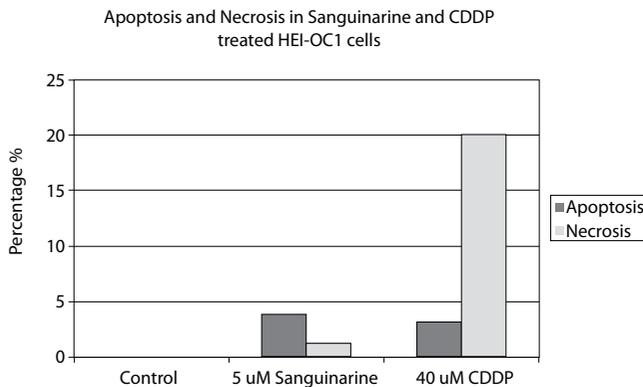


Figure 4. Apoptotic and necrotic percentages of sanguinarine (5 μ M)- and CDDP (40 μ M)-treated HEI-OC1 cells determined by flow cytometry annexin-V and PI analysis

Apoptosis in Number

When we evaluated apoptotic and necrotic cell death in the control group, apoptosis was 2.7%, necrosis was 25.9%, and the cell viability was 71.9% (Figure 3a). In the sanguinarine (5 μ M)-treated group, apoptosis was 6.6%, necrosis was 26.7%, and cell viability was 66.7% (Figure 3b). In the CDDP-treated group, apoptosis was 5.6%, necrosis was 45.4%, and cell viability was 48.75% (Figure 3c). According to these findings, when we re-calculated the percentages in association with the control group, we found that sanguinarine caused 3.9% apoptotic cell death and 1.3% necrosis, while CDDP caused 2.9% apoptotic cell death and 20% necrotic cell death, as shown in Figure 4.

DISCUSSION

CDDP is a highly effective chemotherapeutic agent widely used for treating various adult and pediatric cancers; however, it has dose-limiting serious adverse effects, including nephrotoxicity, neurotoxicity, and ototoxicity. As a result of this obstacle, recent studies have focused on finding a novel agent as effective as CDDP with less or no adverse effects. An ideal chemotherapeutic agent should be effective on cancer cells, and it should not affect normal cells. Adverse effects of cisplatin on auditory function resulting with progressive sensorineural hearing loss have been documented in various studies, and although the histopathology of cisplatin ototoxicity has been

described well, the molecular mechanisms underlying this hearing loss are not completely understood [13, 18]. When we incubated HEI-OC1 cells with 20- and 40- μ M CDDP, which carry the molecular characteristics of the organ of Corti sensory cells and can be used as an ototoxicity model *in vitro*, CDDP revealed cytotoxic effects in a dose-dependant manner. Furthermore, CDDP inhibited 50% cell growth at 40- μ M dose after 24-h incubation. This finding supports the literature that CDDP shows cytotoxic effect on cochlear cells.

Sanguinarine is well known to possess a broad range of biological functions, such as antimicrobial, antifungal, anti-inflammatory, and antineoplastic activities [19-21].

Sanguinarine-induced apoptosis has been detected in human epidermoid carcinoma (A431)^[8], human prostate cancer (LNCaP, DU-145, PC-3)^[22, 23], and breast cancer (MCF-7) cell lines^[7], human endocervical (HeLa)^[24], human melanoma (M4Beu), human colon adenocarcinoma (DLD-1), lung non-small cell carcinoma (A549)^[7], human uveal melanoma (OCM-1)^[25], histiocytic lymphoma (U937), myeloid leukemia (ML-1a)^[6], human erythroleukemia (K562, JM1) cell lines^[6, 26-28], immortalized human keratinocytes (HaCaT)^[26] and human primary fibroblasts^[7, 26]. In addition to this information, we found that sanguinarine did not cause ototoxic effect on cochlear cells, while cisplatin caused 20% necrosis on cochlear cells. Moreover, when we evaluated the effect of sanguinarine on HEI-OC1 cell proliferation, we found that sanguinarine did not change the cell viability in lower doses (2.5 and 5 μ M); however, it inhibited the cell growth in higher doses, i.e., 10 and 20 μ M in approximately 11% and 20%, respectively. These findings revealed that lower doses of sanguinarine could be promising reagents not affecting normal HEI-OC1 cells. Although sanguinarine was found to be inhibiting cell growth in higher doses, the percentage of this suppression (20%) was less than that caused by CDDP (50%) in the dose optimized for *in vitro* application. Our data supported previous findings that sanguinarine was found to inhibit diverse human tumor cells at micromolar concentrations, without affecting normal cells^[29]. Moreover, in our previous study, we had found that sanguinarine revealed cytotoxic and apoptotic properties in Kelly (nmyc amplification-positive, bad prognosis) and SHSY5Y (nmyc amplification-negative, good prognosis) human neuroblastoma cells^[9].

In conclusion, an ideal chemotherapeutic agent must be selective to cancer cells and nontoxic to normal cells. CDDP, which is the most important chemotherapeutic agent used in the treatment of both adult and childhood cancers, has ototoxic effect on hairy cells, leading to hearing loss. Therefore, recent studies have focused on finding novel agents as effective as CDDP with no ototoxicity. In our unpublished data, we have found that sanguinarine revealed cytotoxic and apoptotic properties in human neuroblastoma cells, which led us to question whether it has cytotoxicity on normal cells. When we questioned the effect of sanguinarine on normal cochlear cells, we found that sanguinarine did not cause necrosis on HEI-OC1 cells, while CDDP caused 20% necrosis. Hence, our data suggested that lower doses of sanguinarine are promising agents that did not indicate any toxic effect on HEI-OC1 cells. Application of these data to clinical practice requires further support by *in vivo* studies.

Ethics Committee Approval: This study was approved by the local ethics committee of Dokuz Eylul University Medical School.

Informed Consent: Due to the research related to the cell lines, informed consent was not used in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - E.Ç., G.K.; Design - S.A., G.K., E.D.; Supervision - S.A., N.O.; Materials - N.O.; Data Collection and/or Processing - A.P., Z.A.; Analysis and/or Interpretation - P.E.; Literature Review - E.Ç.; Writing - E.Ç.; Critical Review - S.A., N.O.

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Conflict of Interest: No conflict of interest was declared by the authors.

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