



# Tannic acid enhances cisplatin effect on cell proliferation and apoptosis of human osteosarcoma cell line (U2OS)

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## Abstract

**Background** The increase in cases of chemoresistance of cisplatin for osteosarcoma treatment has called for the need to establish a new treatment regime. Tannic acid (TA) possesses a potent antiproliferative effect against various cancers. Therefore, this study investigated the effect of TA combined with cisplatin on human osteosarcoma cell lines (U2OS).

**Methods** MTT assay was used to determine the half-maximal inhibitory concentration ( $IC_{50}$ ), while the combination index (CI) value was utilized to analyze the interaction within each combination. The antiproliferative effect of the treatment was evaluated by trypan blue exclusion assay. The morphological changes of cells were observed under a phase-contrast inverted microscope. The nuclear morphology and percentage of apoptosis cells were evaluated by using the Hoechst 33258 staining and annexin V/PI assay, respectively.

**Results** The U2OS cells showed cytotoxic effect when treated with TA and cisplatin, with  $IC_{50}$  at 4.47  $\mu\text{g}/\text{mL}$  and 16.25  $\mu\text{g}/\text{mL}$ , respectively. The TA demonstrated no significant inhibition effect on the normal human fetal osteoblast cells (hFOB 1.19); yet, interestingly, a potent proliferative effect was indicated. Synergistic interaction was triggered when TA was combined with cisplatin at percentage ratios of 90:10 and 85:15. Meanwhile, antagonistic interaction was induced in the combination at percentage ratios of 75:25 and 50:50. On the other hand, a significant antiproliferative effect with prominent morphological alteration was detected in the cells treated with a combination of TA and cisplatin at the percentage ratio of 90:10. Additionally, combination-treated cells demonstrated the highest percentage of apoptosis cells, with distinct chromosomal condensation, nuclear fragmentation, reduction of nuclear volume, and notable apoptotic body.

**Conclusion** Therefore, there is a high potential for the inclusion of TA in the cisplatin-based chemotherapeutic regimen of osteosarcoma.

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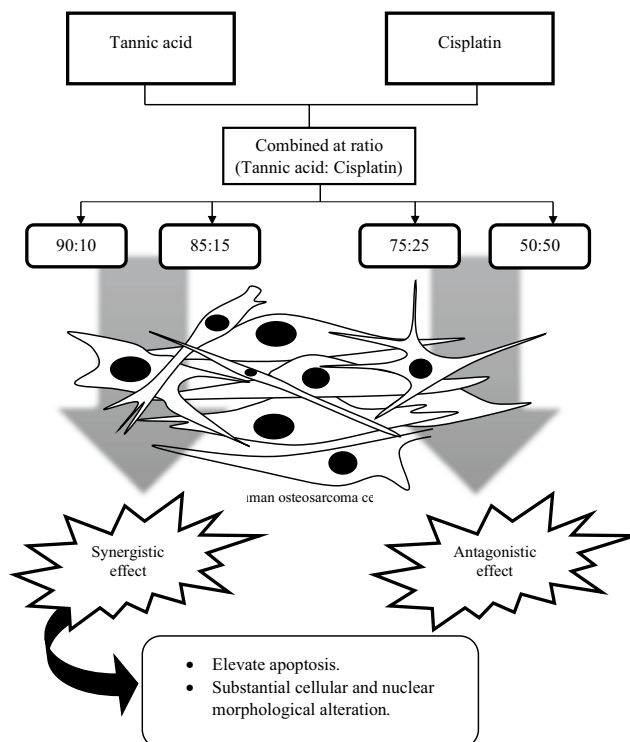
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## Graphic abstract



**Keywords** Tannic acid · Cisplatin · Combination treatment · Antiproliferative activity · Apoptosis · Osteosarcoma

### Abbreviations

CI	Combination index
CO <sub>2</sub>	Carbon dioxide
Cells/mL	Cells per milliliter
FITC	Fluorescein isothiocyanate
FACS	Fluorescence-activated cell sorting
hFOB 1.19	Human fetal osteoblast cells
IC <sub>50</sub>	Half-maximal inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mg/mL	Milligram per milliliter
µg/mL	Microgram per milliliter
OD	Optical density
PI assay	Propidium iodide assay
ROS	Reactive oxygen species
Saos-2	Sarcoma osteogenic-2
TA	Tannic acid
U2OS	Human osteosarcoma cells
UV	Ultraviolet

### Introduction

Osteosarcoma is a primary malignant bone tumor, which originates from mesenchymal stem cells. It is developed due to the uncontrolled production of osteoid or immature bone and commonly occurs in the metaphyseal region of the long bone. Osteosarcoma accounts for 60% of all bone sarcomas [1]. Osteosarcoma cases are detected in 3–4.5 million of the population annually, and among these cases children are most affected [2]. Rapid bone development among children and teenagers has been highlighted as the cause of osteosarcoma development in that age group [3].

Osteosarcoma can be treated with chemotherapy, surgery, and radiation therapy. The standard treatment component of osteosarcoma is chemotherapy [4]. Cisplatin (cis-diamminedichloroplatinum) is one of the potent chemotherapeutic drugs that is frequently applied for the treatment of osteosarcoma. Cisplatin is a platinum-based drug and it is predominantly used in combination with other chemotherapeutic drugs [5]. According to Mohanty et al. [6] a combination treatment of cisplatin with other drugs has substantiated the chemotherapeutic response of osteosarcoma. Due to the higher treatment efficacy, the

treatment regimen which consisted of cisplatin is considered as the primary treatment for osteosarcoma.

The emergence of chemotherapy has improved the disease remission of osteosarcoma, especially among patients with localized tumor [7]. To date, the 5-year survival rate of patients with localized osteosarcoma is between 65% and 70% [8]. However, despite the advancement of osteosarcoma treatment, the 5-year survival rate among patients with metastasis tumor is still low, which is approximately 10%–30% [9]. These survival rates have remained stagnant over the past three decades, especially with metastatic osteosarcoma. Presently, the highest concern regarding osteosarcoma is that it is also recognized to be resistant to the currently used chemotherapeutic drugs [10]. Long-term exposure of the tumor to a chemotherapeutic agent has induced deoxyribonucleic acid (DNA) mutation and consequently increased the endurance of cells [11]. This chemoresistance problem has limited the effectiveness of chemotherapy on osteosarcoma. Besides that, adverse side effects induced by the chemotherapeutic drugs are also among the disputations that needed to be solved. Therefore, the search for a novel agent is urgently required to circumvent these problems.

Combination treatment is widely applied in cancer treatment due to its ability to enhance treatment efficacy. Several new combinations are currently being studied to increase the survival rate of osteosarcoma. Due to the chemoresistance problem toward the currently available drugs, more attention is given to the natural occurrence of chemopreventive agents. The bioactive substances extracted from the natural plant have been acknowledged to have considerably lower toxicity and can be easily obtained [12]. Moreover, natural substances are described as capable of ameliorating the total effect and reduce the toxicity of conventional drugs [13]. Polyphenol is one of the largest categories of phytochemicals. It represents the major secondary metabolites in the plant and is ubiquitously present in most medicinal plants [14]. Tannic acid (TA) is one of the polyphenolic compounds under the group of nonflavonoids-hydrolyzable tannins. Previous studies have reported that TA is an effective natural antioxidant and a significant radical scavenging agent [15, 16]. Besides that, TA also actively induces the production of reactive oxygen species [17]. Furthermore, it has been reported that TA possesses a potent antiproliferative effect against multiple types of cancers [17, 18]. Pro-oxidative and antioxidative effects of an agent have simultaneously worked to inhibit the proliferation of cancer cells [19] to concurrently improve the therapeutic effect [20]. Pro-oxidative effect induces the related signaling pathway to effectively execute apoptosis in cancer cells [19]. Other than that, antioxidative property is also beneficial in reducing the adverse side effect of medication by selectively inducing cancer cell inhibition, sparing the surrounding normal cells [20].

Nagesh et al. [17] reported that treatment of prostate cancer cells with TA resulted in an upregulation of the associated apoptotic markers. In a study conducted by Liu et al. [21], the highest growth inhibition of the human osteosarcoma cell line (Saos-2) was found when it was treated with condensed tannin extracted from *Caulis spatholobi* (Chinese medicinal plant). Several studies have also reported therapeutic enhancement when polyphenol was combined with cisplatin for osteosarcoma treatment [22, 23]. To date, the effect of TA and its combination with cisplatin on human osteosarcoma cells remains unknown. Therefore, this study was conducted to investigate the effect of TA and combination of TA and cisplatin against human osteosarcoma cell line (U2OS) by determining the effect on cell viability, synergistic interaction, antiproliferation, apoptosis event as well as the morphological changes of U2OS.

## Materials and methods

### Cell culture

#### Cell revival and subculture

The human osteosarcoma cells, U2OS (HTB-96<sup>TM</sup>) and normal human fetal osteoblast cell line, hFOB 1.19 (CRL-11372<sup>TM</sup>) were used in this study. Both cell lines were purchased from the American Type Culture Collection (ATCC<sup>®</sup>)(Manassas, VA 20,110). U2OS was maintained in McCoy's 5A modified medium (Invitrogen, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Massachusetts, USA) and 1% (v/v) penicillin–streptomycin (Gibco, Gaithersburg, USA). Meanwhile, hFOB 1.19 was maintained in Dulbecco's modified Eagle medium F12 nutrient mixture (DMEM/F12<sup>TM</sup>, 1:1) (Invitrogen, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Massachusetts, USA), and 1% (v/v) penicillin–streptomycin (Gibco, Gaithersburg, USA). The cells were incubated in a humidified atmosphere of 5% CO<sub>2</sub> incubator at 37 °C and closely monitored every 24 h. All cell culture-related works were conducted in Biosafety Cabinet (BSC) Class II to maintain sterile conditions.

#### Preparation for cell treatment

TA used is a naturally extracted compound from fruit and seeds of *Phyllanthus emblica* (Indian gooseberry). The purified TA was purchased from Chemfaces (Daejeon, South Korea) and cisplatin was purchased from Targetmol (Boston, USA). Both compounds were prepared by diluting in dimethyl sulfoxide (DMSO) (Nacalai Tesque, Japan). In this experiment, the cisplatin acted as a positive controlled drug, while the untreated cells served as a negative control. The

treatment groups comprised cells treated with TA and a combination between TA and cisplatin. Stock solution of TA and cisplatin were prepared at 10 mg/mL. For combination treatment, TA and cisplatin were combined at a concentration of 10 mg/mL and several percentage ratios (TA:cisplatin) (v:v) of 90:10, 85:15, 75:25, and 50:50 to produce the final stock concentration of 10 mg/mL. The stock solutions of each agent were serially diluted to produce working solution at several ranges of concentration (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.02, and 0.01 mg/mL).

The cells were initially seeded at  $5 \times 10^4$  cells/mL in 96-well plates and incubated overnight at 37 °C in 5% CO<sub>2</sub> incubator for cell adhesion. Then, the cells were treated with serially diluted agents that were prepared previously. The treated cells were incubated in a 5% CO<sub>2</sub> incubator at 37 °C for 72 h. The tests were conducted in three independent experiments in triplicate to ensure the reliability and acceptance of the results obtained.

### MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Nacalai Tesque, Japan) was performed to obtain the value of half-maximal inhibitory concentration (IC<sub>50</sub>) of U2OS and hFOB 1.19. The method used was modified from Li et al. [24]. The MTT working solution was prepared at 5 mg/mL in phosphate buffer saline (PBS) solution. Then, the MTT solution was pipetted in each well of the control and treatment groups. The 96-well plate was then wrapped with aluminum foil and incubated in 5% CO<sub>2</sub> incubator at 37 °C for 4 h. After the incubation, the supernatant was aspirated from the wells and it was replaced with DMSO to solubilize the purple formazan crystal. The 96-well plate was then shaken for 30 min to ensure complete solubilization. The absorbance (OD) was measured at 570 nm by using the ELISA microplate reader (Tecan, Switzerland). The DMSO was used as blank. The percentage of the viable cell was calculated by using the following formula:

$$\text{Percentage of viable cells (\%)} = \frac{(\text{OD value of treated cells} - \text{OD value of blank})}{(\text{OD value of untreated cells} - \text{OD value of blank})} \times 100$$

The dose–response curve of cell viability (%) against the final concentration was plotted, and the IC<sub>50</sub> values were identified by using the GraphPad Prism software, version 9.

### Combination analysis

TA and cisplatin were combined at several percentage ratios (TA: cisplatin) (v:v) with decreasing order of cisplatin dose, which were 90:10, 85:15, 75:25, and 50:50. The chosen

combination ratios were modified from Tsakalozou et al. [25]. TA and cisplatin were combined at a concentration of 10 mg/mL according to the selected ratios and serially diluted to produce concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.02, and 0.01 mg/mL. The percentages of cell viability of each serially diluted combined agent of every selected ratio were used for combination analysis. The combination analysis was conducted according to a method described by Chou [26] using the combination index (CI). CI allowed the quantitation of multiple drug interactions based on the calculation by using the CI equation. The CI value was measured automatically with the CompuSyn software. The obtained CI value specified the degree of drug interactions in which CI < 1 indicated synergistic effects between the two drugs used, CI = 1 indicated additive effects, and CI > 1 indicated antagonistic effects [27].

### Trypan blue exclusion assay

Antiproliferative activity was evaluated through the utilization of trypan blue exclusion assay. Cells at  $5 \times 10^4$  cells/mL were initially seeded and incubated overnight at 37 °C in 5% CO<sub>2</sub> incubator. The cells were then treated with cisplatin, TA, and the combination of TA and cisplatin at IC<sub>50</sub>. After 24, 48, and 72 h, the cells were trypsinized with 0.25% trypsin/EDTA (Invitrogen, Massachusetts, USA). The cells were then stained with trypan blue exclusion dye solution for cell counting. Countess™ Automated Cell Counter (Invitrogen, Massachusetts, USA) was utilized to count the number of cells in each treatment and control group. Graph number of viable cells versus time of each treatment and control group was plotted and analyzed.

### Cell morphology observation by phase-contrast inverted microscope

The morphological changes of U2OS cells were observed and compared after treatment with cisplatin, TA, and the combination of TA and cisplatin. Cells at concentration

$5 \times 10^4$  cells/mL were seeded and incubated overnight at 37 °C in 5% CO<sub>2</sub> incubator. After that, the cells were treated with cisplatin, TA, and the combination of cisplatin and TA at IC<sub>50</sub> for 24, 48, and 72 h at 37 °C in 5% CO<sub>2</sub> incubator. Morphological changes like cellular shrinkage, blebbing, and chromatin condensation were determined using a phase-contrast inverted microscope (Carl Zeiss, Germany). Three images were captured for each treatment and control group at 20X magnification.

## Hoechst 33258 staining

Nuclear morphological changes of U2OS cells in response to the treatment with cisplatin, TA, and the combination of TA with cisplatin were identified with Hoechst 33258 staining. Initially, the cells were seeded at  $5 \times 10^4$  cells/mL in six-well plate and incubated overnight at 37 °C in 5% CO<sub>2</sub> incubator. The cells were then treated with cisplatin, TA, and the combination of TA with cisplatin at IC<sub>50</sub>. After 24, 48, and 72 h post-treatment, the cells were washed with phosphate buffer saline (PBS) and soaked in ice-cold methanol for 15 min. Following that, the cells were fixed with 4% paraformaldehyde in PBS at 4 °C for 20 min and rinsed with PBS before being immersed in 10 µg/mL Hoechst 33258 dye solution in the dark for 10 min. The stained cells were observed using Olympus BX41 Fluorescence microscope (Olympus, Japan) for the presence of condensed chromatin, nuclear fragmentation, and apoptotic body. Three images were captured for each treatment and control group at 40× magnification under ultraviolet (UV) excitation light at 350 nm.

## Flow cytometry analysis

Flow cytometry analysis was utilized to count the percentage of apoptotic cells. U2OS cells at  $5 \times 10^4$  cells/mL were initially seeded in six-well plates and incubated overnight at 37 °C in 5% CO<sub>2</sub> incubator. Following that, the cells were treated with cisplatin, TA, and the combination of TA and cisplatin at IC<sub>50</sub> and incubated at 37 °C in 5% CO<sub>2</sub> incubator. After 24, 48, and 72 h of incubation, the cells were trypsinized with 0.25% of trypsin/EDTA (Invitrogen, Massachusetts, USA) and washed with pre-chilled PBS (4 °C) twice. Annexin V–FITC Apoptosis detection kit (BD Biosciences,

USA) was utilized according to the manufacturer's protocol. The cells were re-suspended in a binding buffer and stained with the annexin-V and propidium iodide solution at an equal ratio. The stained cells were incubated for 15 min at room temperature in the dark afterward. Following incubation, the binding buffer was added again, and the cells were analyzed immediately within 1 h with Becton Fluorescence-activated cell sorting (FACS) flow cytometer (BD Corporation, USA). The obtained data were analyzed by using the FlowJo software (BD Biosciences, USA).

## Statistical analysis

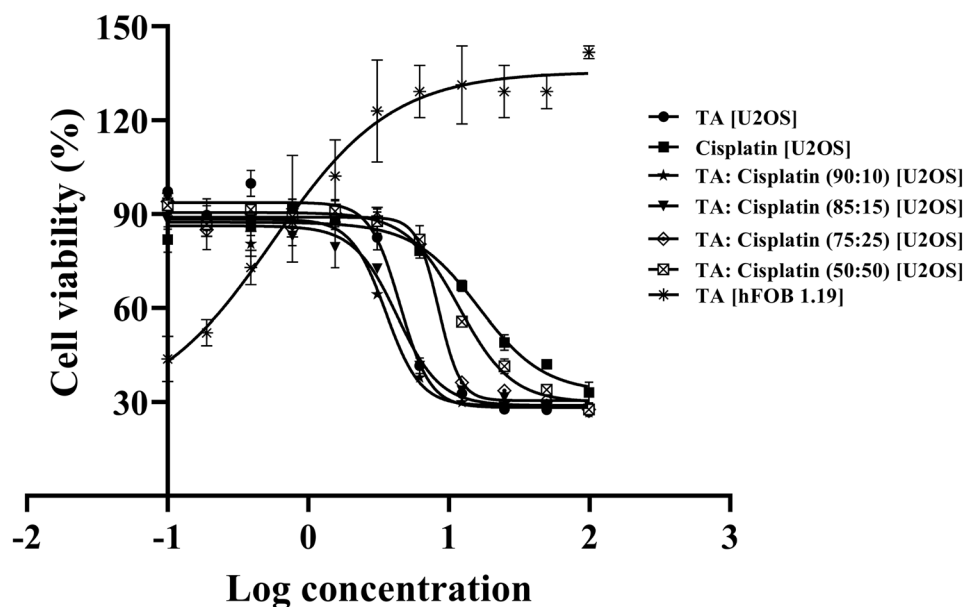
Data obtained were expressed as mean  $\pm$  SEM (standard error mean) from three independent experiments ( $n = 3$ ). The obtained data were initially tested for the normality and homogeneity of variance through the Shapiro–Wilk test. Then, a statistical comparison was performed through the utilization of two-way repeated measure ANOVA, followed by Tukey's honest significant difference (HSD) post hoc test. The result was considered statistically significant if  $p < 0.05$ . Each analysis was conducted using the IBM SPSS Statistics version 26.

## Results

### The combination of TA with cisplatin promoted cytotoxicity on U2OS but not to normal hFOB 1.19

After 72 h of treatment, cisplatin, TA and the combination of TA with cisplatin showed different cytotoxicity effects on U2OS cells. Figure 1 shows the percentage viability of

**Fig. 1** Percentage of cell viability (%) against log concentration of TA, cisplatin, and the combination of TA and cisplatin at percentage ratios (TA: cisplatin) (v:v) of 90:10, 85:15, 75:25, and 50:50. The viability of U2OS cells was determined by MTT assay. The values were expressed as mean  $\pm$  SEM from three independent ( $n = 3$ ) experiments. U2OS is the human osteosarcoma cell line, hFOB 1.19 is the normal osteoblast cell line for normal cell control



U2OS cells against log concentration of the treatment used. IC<sub>50</sub> of TA was lower (4.47 µg/mL) than the controlled drug of cisplatin (16.25 µg/mL). In the combination treatment group, the combination of TA and cisplatin at the percentage ratios of 90:10, 85:15, 75:25, and 50:50 showed to have IC<sub>50</sub> of 3.56 µg/mL, 4.28 µg/mL, 8.46 µg/mL, and 11.81 µg/mL, respectively (Table 1). The combination at the percentage ratios of 90:10 and 85:15 exhibited lower IC<sub>50</sub> than the TA treatment group. The IC<sub>50</sub> of cisplatin, TA, and the combination of TA and cisplatin at the ratio of 90:10 were then used to treat the cells in subsequent experiments.

Notably, no significant inhibition effect was observed when TA was treated on normal human fetal osteoblast cells (hFOB 1.19). Moreover, TA was indicated to induce hFOB 1.19 proliferation (Fig. 1). The IC<sub>50</sub> value of TA against hFOB 1.19 cells was at 0.56 µg/mL.

### The combination of TA with cisplatin exerted a synergistic antitumor effect on U2OS

The combination effect of TA and cisplatin was determined against the U2OS cells. Table 2 shows the CI value for the combination of TA and cisplatin at each percentage ratio. Synergistic activity was determined when TA and cisplatin were combined at the 90:10 and 85:15 percentage ratios. The obtained CI values were below 1 (0.699 and 0.769, respectively). The combination of TA and cisplatin at the percentage ratios of 75:25 and 50:50 showed a CI value of 1.357 and 1.596. CI value that was higher than 1 indicated an antagonistic interaction between TA and cisplatin.

### The combination of TA with cisplatin enhanced antiproliferative activity on U2OS

As depicted in Fig. 2, the number of viable cells was reduced at each time point in all treatment groups, especially in the group receiving the combined treatment. A two-way repeated measure ANOVA indicated a significant effect for the treatment agents [ $F_{3, 8} = 127.39$ ,  $p = 0.001$ ], time [ $F_{3, 24} = 5.32$ ,  $p = 0.006$ ] and treatment agents  $\times$  time interaction [ $F_{9, 24} = 29.13$ ,  $p = 0.001$ ]. Post hoc analysis by using Tukey's test revealed that treatment with cisplatin, TA, and the combination of TA and cisplatin significantly lowered the number of viable cells compared to the untreated ( $p = 0.001$ ) after 24, 48, and 72 h of

**Table 2** Combination index (CI) value for the combination of TA and cisplatin

Combination ratio (TA: Cisplatin)	90:10	85:15	75:25	50:50
CI value	0.699	0.796	1.357	1.596
Indication	Synergism	Synergism	Antagonism	Antagonism

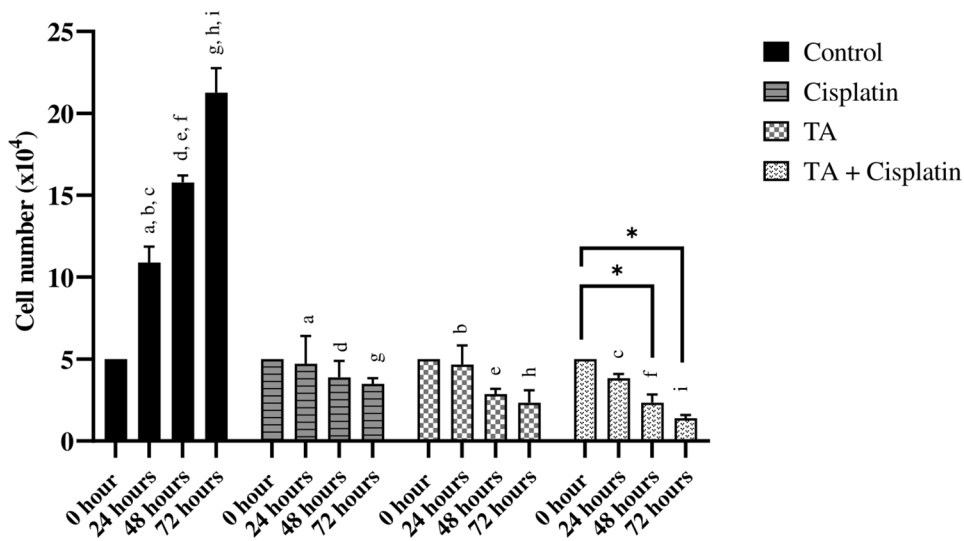
treatment. Further, the combined treatment significantly lowered the number of viable cells after 48 ( $p = 0.016$ ) and 72 h ( $p = 0.018$ ).

### The combination of TA with cisplatin increased morphological changes on U2OS

The morphological changes of the U2OS cells in each group were analyzed after 24, 48, and 72 h in the four groups [control group (untreated), cisplatin treatment, TA treatment, and the combination of TA and cisplatin treatment groups]. After 24 h of treatment, some of the TA- and combination-treated cells began to change into a rounded shape with denser nuclear chromatin (Fig. 3C, D). The majority of the cisplatin-treated cells appeared unchanged with the normal spindle-like look (Fig. 3B). After 48 h of treatment, the TA- and combination-treated cells were sparsely distributed and only a few cells showed normal spindle shape. However, a large group of cells appeared to be oval and round shaped with denser nuclear chromatin. In addition, the cells also appeared to shrink and bleb with notable chromatin condensation (Fig. 3G, H). Meanwhile, only a few cisplatin-treated cells were shown to have chromatin condensation (Fig. 3F). After 72 h of treatment, the cells appeared sparsely distributed with deteriorated shapes in all treatment groups (Fig. 3J, K, L). Treatment with TA changed the morphology of the cells into oval and round shaped, with a blebbing- and shrinkage-like appearance. Chromatin condensation was spotted in most of the cells (Fig. 3K). The morphological alteration was more prominent in the combination treatment group. Most of the combination-treated cells were observed to be dead, marked by the high deterioration of cell shape and significant chromatin condensation (Fig. 3L).

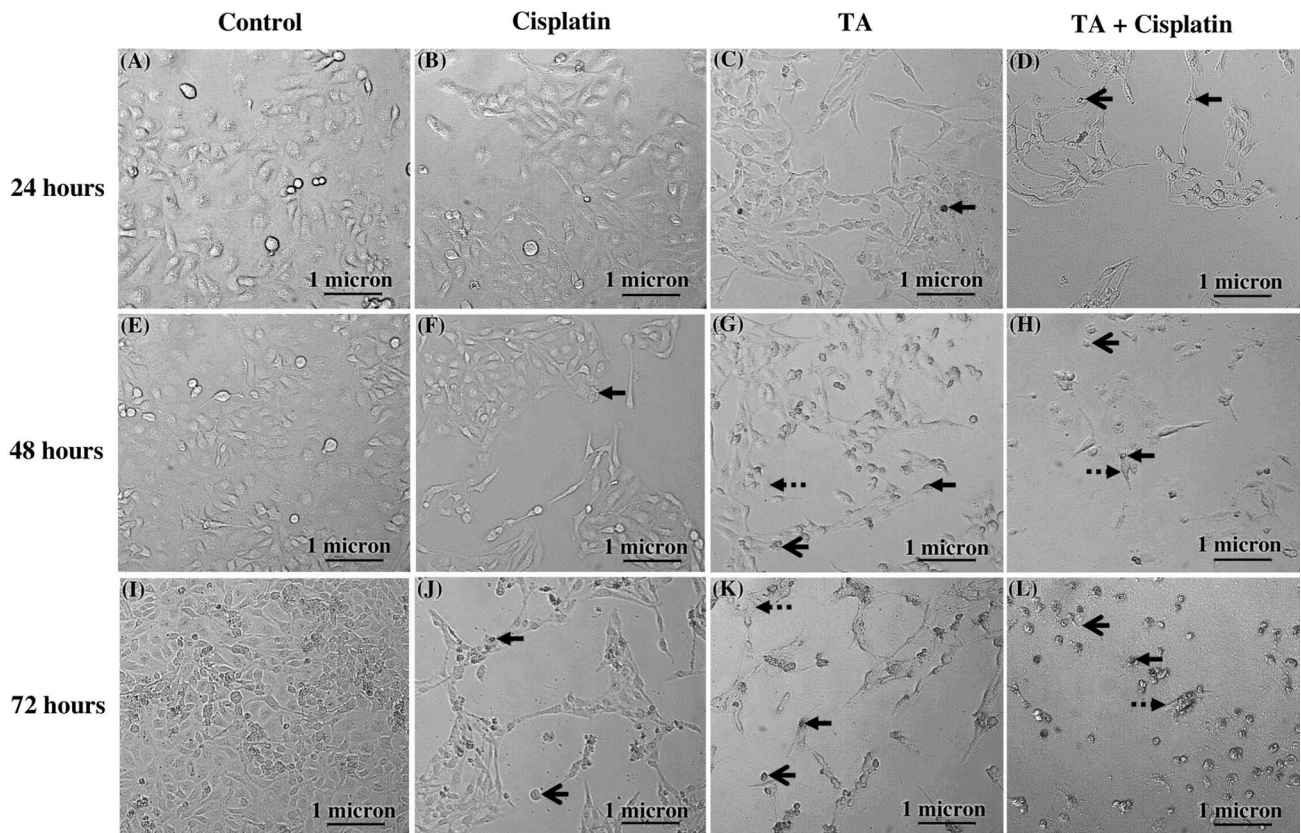
**Table 1** Value of half-maximal inhibitory concentration (IC<sub>50</sub>) of U2OS and hFOB 1.19 cells

Treatment	TA	Cisplatin	90:10	85:15	75:25	50:50
IC <sub>50</sub> in U2OS (µg/ml)	4.47	16.25	3.56	4.28	8.45	11.81
Log <sub>10</sub> IC <sub>50</sub>	0.65	1.21	0.55	0.63	0.93	1.07
IC <sub>50</sub> in hFOB 1.19 (µg/ml)	0.56	–	–	–	–	–
Log <sub>10</sub> IC <sub>50</sub>	0.25	–	–	–	–	–



**Fig. 2** U2OS cell number ( $\times 10^4$ ) in response to treatment for 0, 24, 48, and 72 h in four groups [control, cisplatin treatment, TA treatment, and the combination of TA and cisplatin treatment groups] determined by trypan blue exclusion assay. The bar represents mean  $\pm$  standard error mean (SEM). The asterisk (\*) indicates a sig-

nificant difference ( $p < 0.05$ ) within a similar group in different treatment hours. Groups that shared the same letter showed significant differences among the different groups in the similar treatment hour. The statistical analysis was conducted using Two-way repeated measure ANOVA followed by Tukey's post hoc test



**Fig. 3** Morphological changes of control and treated U2OS cells with cisplatin, TA, and the combination of TA and cisplatin. (A, B, C, D) Morphology of U2OS after 24 h of treatment. (E, F, G, H) Morphology of U2OS after 48 h of treatment. (I, J, K, L) Morphology of

U2OS after 72 h of treatment. Cell morphologies were observed and captured by a phase-contrast inverted microscope (Carl Zeiss, Germany) at 20 $\times$  magnification. [  $\blackleftarrow$  represent chromatin condensation,  $\blackleftarrow$  represent cells shrinkage,  $\blackleftarrow$  represent cells blebbing]

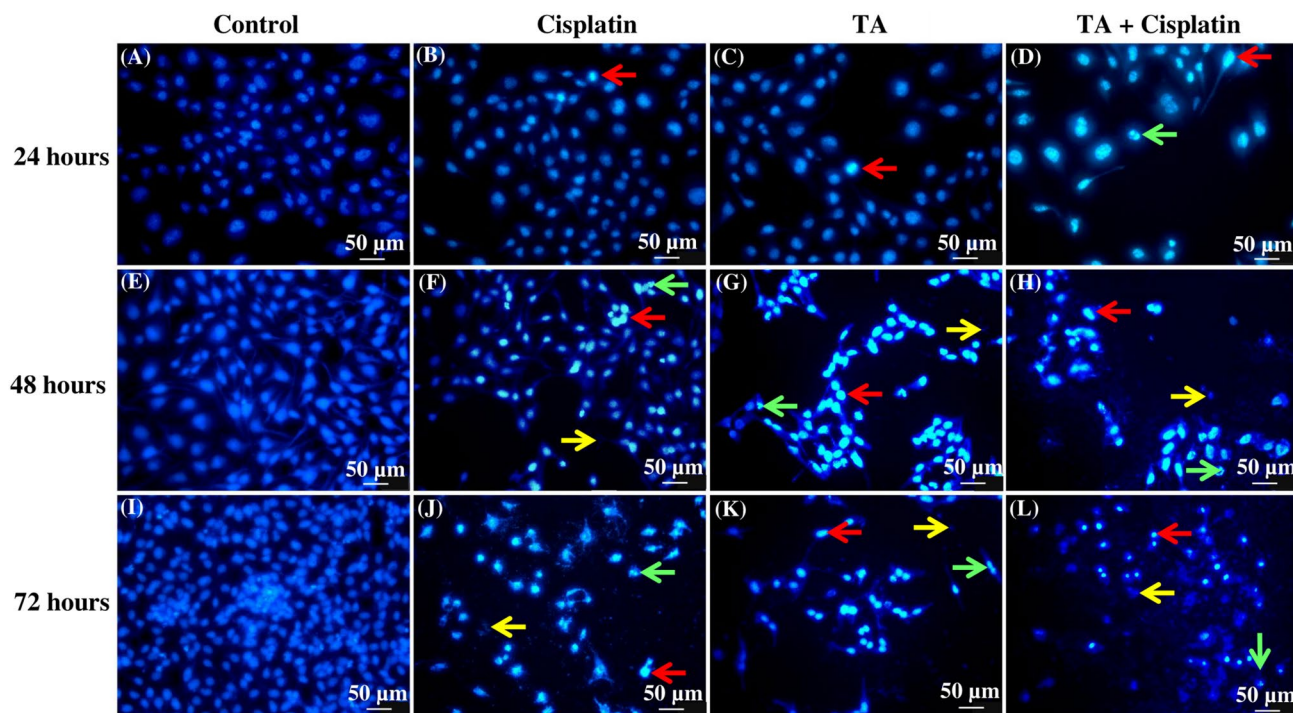
## The combination of TA with cisplatin elevated nuclear morphological alteration on U2OS

Combination-treated cells showed obvious morphological changes compared to the control (untreated), cisplatin-, and TA-treated cells. Typically, the nuclei of the untreated cells demonstrate homogenous stain and even distribution with less bright blue fluorescence being emitted. Meanwhile, the treated cells have brighter emissions compared to the untreated cells. In general, changes in the nuclear morphology of treated cells were observed in a time-dependent manner and the nuclear morphology of the combination-treated cells was prominently altered compared to the cisplatin- and TA-treated cells. Treatment with cisplatin and TA for 24 h induced chromatin condensation in several cells, indicated by the brighter emission of blue fluorescence light (Fig. 4B, C). Nevertheless, chromatin condensation was not observed in the untreated cells (Fig. 4A). Meanwhile, most of the combination-treated cells showed condensed chromatin and nuclear fragmentation (Fig. 4D). After 48 h of treatment, several cisplatin-, TA-, and combination-treated cells were observed with distinguished nuclear alteration such as nuclear fragmentation, chromatin condensation, and reduction of nuclear volume. Besides that, the apoptotic body also

appeared (Fig. 4F, G, H). After 72 h of treatment, most of the cells in all treatment groups had significant nuclear alteration. Most of the cisplatin-, TA-, and combination-treated cells (Fig. 4J, K, L) had the symptoms of chromatin condensation, nuclear fragmentation, and reduction of nuclear volume. The apoptotic body was prominently observed in the combination-treated cells (Fig. 4L) compared to cisplatin-, and TA-treated cells (Fig. 4J, K).

## The combination of TA with cisplatin boosted apoptosis on U2OS

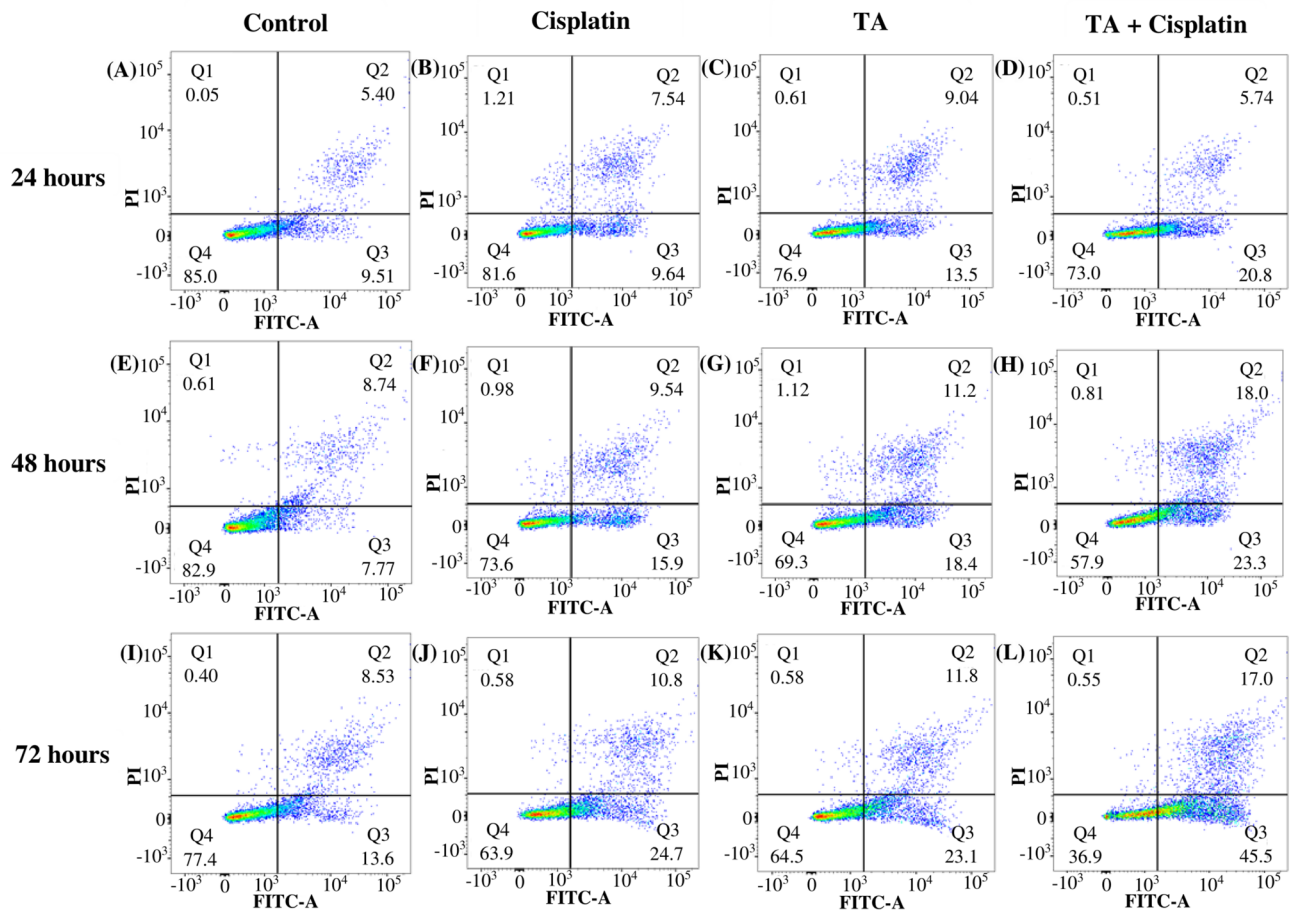
Treatment with cisplatin, TA, and the combination of TA and cisplatin for 24, 48, and 72 h resulted in changes in the percentage of viable cells, early apoptotic, late apoptotic, and necrotic cells. Figure 5 illustrates the four divided quadrants representing the percentage of the viable cells (Q4), early apoptosis (Q3), late apoptosis (Q2), and necrosis (Q1) of control (untreated) and treated cells after staining with annexin V-FITC and propidium iodide (PI). Treatment with cisplatin, TA, and the combination of TA and cisplatin reduced the percentage of viable cells compared to untreated cells at 24, 48, and 72 h of treatment (Fig. 6A). A two-way repeated measure ANOVA showed a significant effect of



**Fig. 4** Nuclear morphological changes of control and treated U2OS cells with cisplatin, TA, and the combination of TA with cisplatin. (A, B, C, D) Nuclear morphology of U2OS after 24 h of treatment. (E, F, G, H) Nuclear morphology of U2OS after 48 h of treatment. (I, J, K, L) Nuclear morphology of U2OS after 72 h of treatment. Hoechst 33258 nuclear staining was utilized, and the images were

visualized and captured by Olympus BX41 fluorescence microscope (Olympus, Japan) under UV excitation light at 350 nm at 40× magnification. The scale bar is labeled at 50 µm. Red arrows represent nuclear chromatin condensation, green arrows represent nuclear fragmentation, yellow arrows represent the apoptotic body





**Fig. 5** Apoptosis dot plot of control and treated U2OS cells with cisplatin, TA, and the combination of TA with cisplatin. (A, B, C, D) Dot plot of U2OS after 24 h of treatment. (E, F, G, H) Dot plot of U2OS after 48 h of treatment. (I, J, K, L) Dot plot of U2OS after

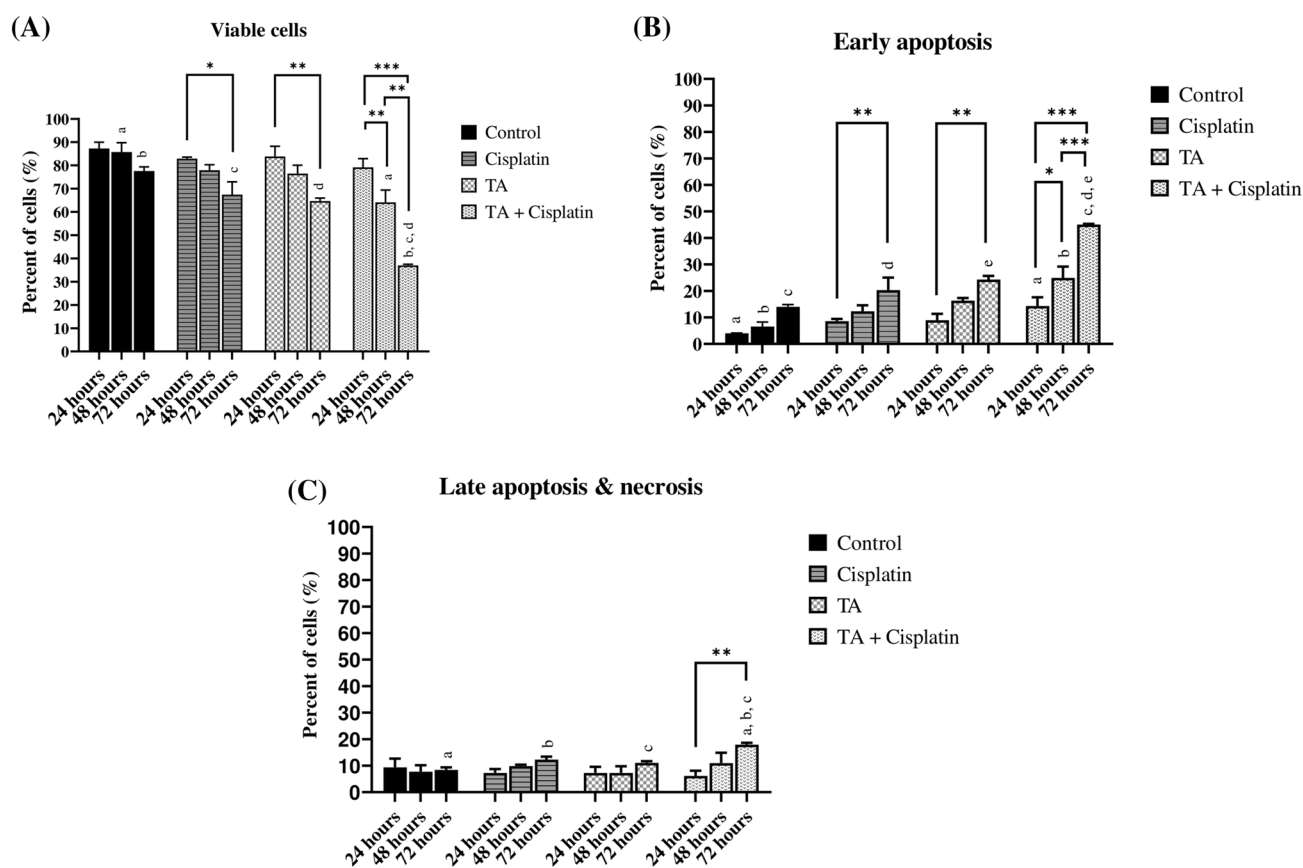
72 h of treatment. Cells were analyzed by using flow cytometry with Annexin V–FITC/PI double staining assay and sorted into four quadrants which are viable cells (Q4), early apoptotic cells (Q3), late apoptotic cells (Q2), and necrotic cells (Q1)

treatments [ $F_{3, 8} = 14.87$ ,  $p = 0.001$ ], time [ $F_{2, 16} = 63.87$ ,  $p = 0.001$ ] and treatments  $\times$  time interaction [ $F_{6, 16} = 6.83$ ,  $p = 0.001$ ]. Post hoc analysis with Tukey's test indicated that the percentage of viable cells dropped significantly in the combination treatment group after 72 h compared to the 48 h ( $p = 0.001$ ) and 24 h ( $p = 0.001$ ). Treatment with TA alone also indicated a significant reduction of the viable cells after 72 h of treatment compared to 24 h ( $p = 0.007$ ). After 48 h of treatment, the percentage of viable cells in the combination treatment group was significantly reduced compared to the untreated group ( $p = 0.029$ ). Besides, the combination treatment significantly reduced the percentage of viable cells compared to the untreated ( $p = 0.001$ ), cisplatin ( $p = 0.001$ ) and TA treatment ( $p = 0.001$ ) after 72 h of treatment.

As demonstrated in Fig. 2B, the percentage of early apoptotic cells increased consecutively at each time in every treatment group. Analysis with two-way repeated measure ANOVA indicated a significant effect of treatments [ $F_{3, 8} = 26.01$ ,  $p = 0.001$ ], time [ $F_{2, 16} = 65.01$ ,  $p = 0.001$ ]

and treatments  $\times$  time interaction [ $F_{6, 16} = 5.18$ ,  $p = 0.004$ ] on U2OS cells. Post hoc analysis with Tukey's test revealed a significant increase in the percentage of early apoptotic cells after 48 h ( $p = 0.024$ ) and 72 h ( $p = 0.001$ ) of treatment with the combination of TA and cisplatin. After 24 and 48 h, the combination treatment had elevated percentage of early apoptotic cells compared to the untreated group ( $p = 0.047$  and  $p = 0.006$ , respectively). Treatment for 72 h with the combination of TA and cisplatin also resulted in a significant surge of the early apoptotic cells compared to the untreated ( $p = 0.002$ ), cisplatin ( $p = 0.001$ ), and TA ( $p = 0.002$ ) treatment. On the other hand, TA treatment also significantly augmented the percentage of early apoptotic cells ( $p = 0.001$ ) after 72 h.

The proportion of late apoptotic and necrotic cells were increased after treatment at a longer time. A two-way repeated measure ANOVA identified a significant time of treatment [ $F_{2, 16} = 7.05$ ,  $p = 0.006$ ] to the induction of late apoptosis and necrosis. However, there was no significant



**Fig. 6** Apoptosis analysis of control and treated U2OS cells with cisplatin, TA, and the combination of TA with cisplatin. Percentage of (A) viable, (B) early apoptosis, (C) late apoptosis and necrosis of U2OS cells after 24, 48, and 72 h of treatment. Cells were stained with Annexin V–FITC/PI double staining assay and analyzed using a flow cytometer. The bar represents mean  $\pm$  standard error mean

(SEM). The asterisk (\*) indicates a significant difference ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ) within a similar group in different treatment hours. Groups that shared the same letter showed significant differences among the different groups in the similar treatment hour. The statistical analysis was conducted using Two-way repeated measure ANOVA followed by Tukey's post hoc test

influence of the treatments used [ $F_{3,8} = 1.10$ ,  $p = 0.405$ ] as well as treatments  $\times$  time interaction [ $F_{6,16} = 1.97$ ,  $p = 0.130$ ] to the occurrence of the late apoptosis and necrosis. Post hoc analysis with Tukey's test revealed that treatment with the combination of TA and cisplatin for 72 h significantly induced the percentage of late apoptotic and necrotic cells ( $p = 0.002$ ). After 72 h of treatment, the combination treatment significantly elevated the percentage of late apoptotic and necrotic cells compared to the untreated ( $p = 0.001$ ), cisplatin ( $p = 0.011$ ) and TA ( $p = 0.003$ ) treatment.

## Discussion

In recent years, natural polyphenols have been given considerable attention in oncotherapy research due to their antitumor activity. Tannic acid (TA) is a natural polyphenol compound that can be extracted from numerous medicinal plants [18] and it is known for its capability as an anticancer

agent [28]. In this study, we have found that TA effectively induced cytotoxic effects on U2OS cells. The  $IC_{50}$  value of TA on U2OS cells was lower than that of cisplatin, whereby it was considered to exhibit better potency. The inhibition of U2OS cells by TA was determined in a dose-dependent manner. A study by Nam et al. [29] found that TA effectively inhibited the activity of the tumor cell proteasome. Proteasome inhibition directly disturbs the regulatory network of the tumor cells, which leads to a profound effect on cell growth [30]. Besides that, several polyphenolic compounds were also reported to inhibit the proliferation of human osteosarcoma cells [31, 32]. According to Valavanidis et al. [33], the number and location of the hydroxyl groups in the polyphenolic structure played an important role in its anti-cancer property. Thus, the inhibition of TA on U2OS could be due to the influence of the same factor and mechanism.

Nevertheless, TA reacted differently on normal human fetal osteoblasts cells (hFOB 1.19). The proliferation of hFOB 1.19 was induced with no significant cell inhibition

or toxicity, similar to a previous report [34]. Unlike TA, cisplatin is known to be invasive on the normal osteoblast cells. It interferes with the regulation of protein synthesis and DNA replication of osteoblasts to consequently disrupt the mechanism of bone formation as well as induce deleterious side effects in the patients [35]. Meanwhile, TA induces a potent cytotoxicity effect on the osteosarcoma without destroying the surrounding healthy osteoblasts.

In an effort to increase the treatment efficacy and reduce the adverse side effect of cisplatin, we tested the combined effect of TA and cisplatin at several percentage ratios, 90:10, 85:15, 75:25, and 50:50. The pharmacological interactions between the combined drugs were evaluated by calculating the combination index (CI) value using the CompuSyn software program. The CI value obtained allowed characterizing the specific interaction between TA and cisplatin through the cutoff value of CI, in which if the CI value was lower than 1, it indicated a synergistic effect; if the CI value equaled 1, it indicated an additive effect; and if the CI value was higher than 1, it indicated an antagonistic effect. Synergistic effect is defined as drug–drug interaction that induces an overall effect more than the sum of individual effects of each drug. Meanwhile, the additive effect is defined as a drug–drug interaction that gives an overall effect equal to the sum of individual effects of each drug, whereas antagonistic effect is defined as drug–drug interaction that promotes an overall effect lower than the sum of individual effects of each drug [36]. The synergistic effect is the most desired drug–drug interaction in the application of the combination treatment [37].

The drug-to-drug interaction was mainly influenced by the composition ratio and concentration of each drug used [38]. Wide ranges of combination ratios were used in this study to obtain a broader insight into the drug–drug combination effect at different doses and compositions. The aim is to establish a regimen with a lower dose of cisplatin as it is known to have undesirable side effect and chemotherapeutic resistance. Based on the result obtained, the combination at percentage ratios of 90:10 and 85:15 showed a favorable synergism. The combination with the lowest dose of cisplatin (90:10) had the lowest CI value to indicate the most favorable synergism. Thus, dose reduction of cisplatin could successfully be done. Mokhtari et al. [39] reported that the approach of the chemotherapeutic combination potentially reduced the occurrence of drug resistance and adverse side effects while simultaneously enhanced the treatment efficacy. For example, usage of curcuminoid in combination with chemotherapeutic drugs reduced the chemotherapeutic adverse side effects with better treatment efficacy on solid tumors [40]. Therefore, apart from demonstrating better potency and efficacy, the use of TA with cisplatin would hopefully reduce the risk of toxicity and development of drug resistance.

The combination of TA and cisplatin also showed a better growth-inhibitory effect on U2OS cells compared to the individual treatment with TA or cisplatin. It might indicate that TA enhanced the therapeutic effect of cisplatin on osteosarcoma. The synergistic interaction between the combined agents induced the destructive effect on U2OS cells, which directly inhibited its proliferation. The synergistic effect might also reflect the complement interaction between the mechanisms of action of each agent to consequently elevate the antiproliferative effect in the combined treatment compared to the single-agent treatment. As previously reported, the inhibition of cancer cells requires a sophisticated mechanism of action [41]. Thus, the complement interaction induced by synergism could contribute to the effective inhibition of cancer cells. Meanwhile, an antagonistic interaction is a less favorable interaction, as it reduces the therapeutic efficacy. Molecules that interact antagonistically tend to mask and impede one another which would hinder the effective action of each drug on the cells [42]. As observed in this study, the combination that induced antagonistic interaction (75:25 and 50:50) had lower potency than the combination that induced synergistic interaction (90:10 and 85:15) (Fig. 1).

U2OS is a pleomorphic cell, in which the majority of the cells were spindle, elongated shape [43], rhombus-like or angularly structured and adhered to the plate [44]. In this study, the morphology of the combination-treated cells was prominently distorted compared to the cisplatin- and TA-treated cells. The synergistic interaction between TA and cisplatin proved to induce severe defects to the cell's structure. More cells were observed with oval, shrunk shape, bleb-like, and with chromatin condensation. The cellular shrinkage occurred due to the loss of cell volume [45]. Meanwhile, the bleb-like appearance resulted from the protrusion of the plasma which made the cells appear bulky [46]. Furthermore, condensed chromatin was also detected. Chromatin condensation might indicate that the cells were in a vulnerable condition after treatment. Nuclear chromatin compaction is one of the distinguished phenomena of apoptotic execution [47]. Normally, these morphological features are described as the morphological hallmark of the dead cells due to the apoptosis program cell death [48].

Treatment agents are considered toxic when distinct changes are observed in the cell's nucleus in response to the treatment [49]. Changes in the nuclear morphology of the neoplastic cells contribute to a significant role in the assessment of tumor malignancy [50]. The types of alteration of the nuclear morphology might leave a clue that reflects the ways of cell death: apoptosis, necrosis, or autophagy [51]. In this study, Hoechst 33258 staining was used to stain the untreated and treated cells to evaluate its nuclear morphology. It was found that the nuclear morphological structure of U2OS cells was explicitly altered after treatment, especially

after 72 h. Overall, the treated cells showed chromatin condensation, nuclear fragmentation, nuclear volume reduction, and the appearance of the apoptotic body. It was reported that these types of morphologies are the common morphological features of apoptosis [52].

Apoptosis is described as one of the mechanisms of cell death. It is controlled by a series of signaling pathways that is responsible for the activation of cell destruction [53]. Meanwhile, the cancer cell is characterized by possessing the malfunction of the death machinery with uncontrolled cell proliferation [54]. It is necessary to prevent the cancer cells from proliferating by continuously inducing apoptosis. In this study, flow cytometry analysis was conducted to quantitatively determine the percentage of apoptotic cells. The cells treated with the combination of TA and cisplatin showed the highest percentage of apoptosis compared to the cells treated with TA or cisplatin alone. As determined earlier, the nuclear and cellular morphology of the combination-treated cells were prominently altered each time compared to the other treatment groups. The findings confirmed that the combination of TA and cisplatin effectively induced apoptosis with dreadful cellular and nuclear morphological alterations to promote the highest cell obstruction.

The synergistic effect of the combination of TA and cisplatin might play an important role in promoting the highest cell inhibition and apoptosis. Cell inhibition would occur when the cells stop from proliferating and there is an increment in the number of cell death. However, apoptosis resistance has become a problem in the current cancer therapy, as it reduces the therapeutic efficacy and limits treatment options. Thus, one of the major goals in developing new cancer treatments is to induce a higher number of apoptotic cell death and enhance therapeutic efficacy. Combination treatment with TA and cisplatin appears to be an ideal candidate for a new therapeutic regimen for osteosarcoma, as it efficiently promotes apoptosis synergistically. Polyphenol was widely reported as a phytochemical compound that directly influenced apoptosis in numerous cancer cells [55]. The combination of the polyphenolic compound with the currently used chemotherapeutic drugs has been widely studied and could possibly provide a safer treatment and higher treatment efficacy. In a previous study, the synergistic interaction between cisplatin and polyphenol oleandrin was also found to enhance the killing effect against human osteosarcoma cells [23]. Nevertheless, usage of TA was said to possibly induce stomach irritation, nausea, vomiting, and migraine [56]. In any study of new drug discovery, there will always be a concern regarding the potential side effects and chemoresistance problems that might arise over time. Due to that, details in vivo and clinical trial evaluations are vital to be conducted to establish a regimen with a permissible limit of usage. Thus, issues of excessive side effects and chemoresistance can be curbed and avoided.

Although the combination of TA and cisplatin has synergistically augmented apoptosis on U2OS cells, the underlying mechanism remains unclear. Thus, several limitations in this study should be addressed. First, the pro-oxidative and anti-oxidative profiles of TA and its combination with cisplatin were not evaluated. TA was reported to have antioxidant properties [16]. The intracellular activity of the antioxidative enzymes might reflect the antioxidative effect of TA. Antioxidative enzyme profiling in osteosarcoma cells is required to determine the antioxidative level of TA, especially when it is used in combination with cisplatin. On the other hand, TA was also reported to be a potent pro-oxidative agent on cancer cells. It is known that apoptosis can be stimulated by ROS [57], and the combination of TA and cisplatin might be capable to boost ROS production and consequently elevate apoptosis. As apoptotic cells were increased in response to treatment with a combination of TA and cisplatin, it is necessary to evaluate whether the observed effect is due to the elevation of intracellular ROS induced by both agents. Second, the expression of the related apoptotic marker has not been evaluated. Apoptosis is a tightly regulated process that yields both morphological and biochemical alteration. Even though the numbers of apoptotic cells were found to increase, biochemical alteration still represents the main key in the apoptotic event [58]. Thus, it is imperative to further investigate the expression of the related apoptotic markers that play a vital role in the apoptosis regulatory process to validate the findings.

In conclusion, the combination of TA and cisplatin induced a prominent cytotoxicity effect with a profound nuclear morphological alteration and highest apoptosis on U2OS cells compared to individual treatment. The overall morphology of the combination-treated cells was remarkably altered compared to the cells in other treatment groups. The synergistic interaction between TA and cisplatin could influence the observed effects and consequently enhance the drug's potency and therapeutic efficacy. Other than that, TA did not induce cytotoxicity effect to the normal human fetal osteoblast cells (hFOB 1.19). Nevertheless, proliferation was indicated. Hence, the combination of TA and cisplatin has high potential to be developed as a chemotherapeutic regimen for osteosarcoma treatment.

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**Author contributions** Study conception or design: HH and HA; data processing, collection, perform experiment: MZK and NMH; analysis

and interpretation of results: MZK and NMH; draft manuscript preparation, visualization: MZK; critical revision of the paper: HH, HA, AA and SS; supervision, funding acquisition: HH; final approval of the version to be published: MZK, HH, HA, NMH, AA, and SS.

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## Declarations

**Conflict of interest** The authors declare no conflict of interest.

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