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MOLECULAR-BIOLOGICAL PROBLEMS OF DRUG DESIGN AND MECHANISM OF DRUG ACTION

PROTECTIVE EFFECTS OF LIGANDROL AGAINST CISPLATIN-INDUCED MOUSE MUSCLE CELL LINE TOXICITY

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Cisplatin is an effective chemotherapeutic agent, but skeletal muscle dysfunction and fatigue are serious side effects after its administration. Ligandrol is a new nonsteroidal oral selective androgen receptor modulator used for muscle strengthening. The present study aimed to demonstrate the protective effects of ligandrol against cisplatin-induced muscle toxicity. Cytotoxic effects were determined by cell viability (MTT) and lactate dehydrogenase (LDH) assays in mouse muscle cell lines (C2C12). The oxidant/antioxidant status was demonstrated by total antioxidant capacity (TAC) and total oxidative status (TOS). Cell cycle analysis, DNA fragmentation, caspase 3/7 activities, lipid peroxidation, and intracellular calcium concentration levels were evaluated. Irritant effects were determined by the egg chorioallantoic membrane method. Our results showed that combination treatment with cisplatin of ligandrol significantly ($p < 0.05$) increased cell viability, TAC levels, and caspase 3/7 gene expression, while significantly ($p < 0.05$) reducing TOS, LDH release, DNA fragmentation, lipid peroxidation, and intracellular calcium concentration levels compared to the cisplatin alone. Ligandrol significantly ameliorated cisplatin-induced irritant effects. This result suggested that ligandrol had a significant protective effect against cisplatin-induced muscle toxicity.

Keywords: Cisplatin; ligandrol; mouse muscle cell line; oxidative stress; toxicity.

INTRODUCTION

Cisplatin was one of the first platinum-based compounds that was approved by the U.S. Food and Drug Administration for cancer treatment. Cisplatin is an effective chemotherapeutic anticancer agent used in the treatment of various types of malignancies such as leukemia, lymphomas, sarcomas, breast, testicular, ovarian [1]. Its cytotoxic activity in cancer cells occurs by damaging DNA, inhibiting DNA replication and transcription, and inducing apoptotic cell death [2]. However, cisplatin is associated with serious side effects involved in neurotoxic, hepatotoxic, cardiotoxic, nephrotoxic, and hematotoxic damage in chemotherapic treatments [3]. Another serious side effect of cisplatin is muscle dysfunction characterized by loss of skeletal muscle mass, muscle fatigue, loss of body fat, and inflammation [4]. Despite the toxic effects of cisplatin on healthy tissues and cells, it is still one of the most attractive anticancer drugs used in the treatment of various solid tumors. Thus, potent pharmacological applications minimizing or counteracting cisplatin-induced muscle dysfunctions are urgently needed. The combination of cisplatin with different compounds is one of the effective methods to help its effectiveness and reduce its toxicity [5, 6].

Selective androgen receptor modulators (SARMs) are a new class of androgen receptor ligands and have a selective mimicking ability similar to androgen drugs [7]. SARMs have been studied as potential treatments for breast and prostate cancer, osteoporosis, sexual dysfunction, multiple sclerosis, Alzheimer's disease, benign prostatic hyperplasia, muscular dystrophy, and muscle wasting associated with a variety of diseases [8]. In particular, SARMs have shown promising results against cachexia resulting from weight loss, muscle wasting, and decreased appetite in cancer studies [9, 10]. Ostarine, which is a very popular anabolic

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SARM, improved lean body mass and physical performance in healthy elderly men and elderly women without androgenic side effects in cancer cachexia [11]. Enobosarm has been demonstrated to be a potential protective agent for the prevention and treatment of cancer-related muscle wasting by increasing muscle mass without undesirable side effects [12, 13]. Taylor, et al. (2012) showed that enobosarm may play an important role in enhancing the physical function of older cancer patients. Ligandrol (LGD-4033) is a SARM androgen receptor with high selectivity and affinity and is used as a popular supplement by bodybuilders as they are safer alternatives to steroids because of their muscle and bone strengthening, fat-burning, endurance-enhancing properties [15]. A human clinical study on 76 healthy men showed that ligandrol significantly increased lean body mass [16], whereas Roch, et al. [15] revealed that ligandrol caused a significant improvement in muscle structure in a rat model study

Despite these promising potential effects of ligandrol on muscle structure, there has not been any study on the ameliorating effect of ligandrol on the muscle system in chemotherapeutic treatment. To this end, this study aims to reveal the protective effects of ligandrol against cisplatin-induced muscular dysfunction, by observing parameters associated with cell viability, lactate dehydrogenase activity (LDH), cell cycle analysis, caspase 3/7 activity, DNA fragmentation, oxidant/antioxidant status, lipid peroxidation, intracellular calcium concentrations, and irritant effects.

EXPERIMENTAL

Cell viability

Cytotoxicity was determined on mouse muscle cell line (C2C12) (ATCC- CRL-1772) by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay, which measures the reducing activity of viable cell calorimetrically [17]. Cell lines with an initial concentration of 1×10^5 cells/mL were seeded as 100 µL into a 96-well microplate. Then, cell lines were cultivated for 24 h at 37°C in an 85% humidified atmosphere of 5% CO_2. After 24 h of incubation, the supernatant was removed. Cell lines were diluted and treated with cisplatin (Sigma-Aldrich, CAS 15663-27-1; 10 µM) and different concentrations (0.5, 5 and 50 mg/L) of ligandrol (CAS 1165910-22-4) and their combinations. At 24 h of the incubation, the MTT solution with a concentration of 5 g/L (30 µL/well) was added to the cell lines, and the solution was incubated for 4 h at 37°C. The formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the optical density was measured at 570 nm (Tecan Infinite 200 PRO, Switzerland). Each sample was tested in triplicate.

Lactate dehydrogenase (LDH) assay

LDH leakage assay was determined using the LDH Assay Kit (Cat no. ab102526, Abcam, Cambridge, UK) on the culture medium of a new set of cells exposed to cisplatin (10 µM), ligandrol (0.5, 5, and 50 mg/L) and their combinations for 24 h. 100 µL of culture medium was transferred to a new 96-well microplate, and 100 µL of LDH reaction solution was added to each well, after which the absorbance was measured at 490 nm using an ELISA plate reader (BMG Labtech, Ortenberg, Germany) after 30 min. Mitomycin-C (Mitc) (10^{-7} M) was used as a positive control. Each sample was tested in triplicate.

Total antioxidant capacity (TAC) and total oxidative stress (TOS) activities

TAC and TOS levels were measured in the cellular media using a commercial kit (Rel Assay Diagnostics®, Gaziantep, Turkey) according to the manufacturer's instructions. Cells for these experiments were treated with cisplatin (10 µM), ligandrol (0.5, 5, and 50 mg/L), and their combination, then incubated at 37°C in 85% humidified 5% CO_2 for 2 h. In the TAC assay, potential antioxidants in the culture medium caused a reduction of the ABTS (2,2'-azino-bis-3-ethyl benzothiazoline-6-sulfuric acid) radical. Briefly, 500 µL of the Reagent 1 solution from the kit was added to the quartz cuvette containing 30 µL of cell culture medium treated with ligandrol, cisplatin, and their combination, and the initial absorbance was measured at 660 nm after 30 sec. Then 75 µL of Reagent 2 solution was added to the same cuvette and the absorbance was measured at 660 nm after 5 min incubation. The assay was calibrated with Trolox and the results were expressed in terms of mM Trolox equivalent per liter (mmoL Trolox Equiv/L). TOS assay was based on the conversion of the ferrous ion–chelator complex to ferric ion via oxidants present in the culture medium. To determine the TOS level, 500 µL of Reagent 1 was mixed with 75 µL of the cell culture medium treated with ligandrol, cisplatin, and their combination, then the absorbance of each sample was measured at 530 nm after 30 sec. Subsequently, 15 µL of Reagent 2 was added to the mixture, and the absorbance was again read at 530 nm. The treatments were calibrated with hydrogen peroxide (H_2O_2), and the data were expressed as µM H_2O_2 equivalent per liter (µmoL H_2O_2 Equiv/L). H_2O_2 (25 µmoL/L) and ascorbic acid (10 µmoL/L) were used as the positive controls for the TAC and TOS analysis, respectively. Each sample was tested in triplicate.

Measurement of DNA fragmentation

DNA fragmentation was determined by the Cell Death Detection ELISA Plus Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the cells (1×10^5 cells/well) were seeded in a 96-well plate, and then the cells were exposed to cisplatin (10 µM), ligandrol (0.5, 5, and 50 mg/L) and their combina-

tion for 24 h. 20 mL of the cytoplasmic fractions were transferred into a streptavidin-coated 96-well with anti-DNA antibodies and incubated for 2 h at room temperature. After the washing period, 2,2-azino-di-(3-ethylbenzthiazoline sulphonate) diammonium salt was added, and the absorbances were measured at 405 nm using an ELISA reader (BMG Labtech, Ortenberg, Germany). Each sample was tested in triplicate.

Cell cycle analysis

C2C12 cells were seeded in 6-well plates at 1×10^5 cells/ml for 24 h and treated with cisplatin (10 μM), ligandrol (0.5, 5, and 50 mg/L) and their combination for 24 h. The cell cycle phase was determined using a Muse™ Cell Cycle Assay Kit (Merck Millipore, Germany) according to the manufacturer's instructions. Briefly, cells were trypsinized with PBS and fixed with 70% cold-ethanol. Muse™ Cell Cycle reagent was added to the obtained cell pellet, and the solution was incubated for 30 min. Cell percentages in G0/G1, S, and G2/M phases were calculated by the Muse™ Cell Cycle analyzer (Merck Millipore, Germany). Each sample was tested in triplicate.

Caspase 3/7 expression levels

Caspase 3/7 expression levels were determined with the Caspase-Glo® 3/7 Assay (Promega). Cells were seeded in 96-well plates at a concentration of 1×10^5 mL and exposed to cisplatin (10 μM), ligandrol (0.5, 5, and 50 mg/L) and their combination for 24 h. After incubation, caspase-Glo® 3/7 reagent (100 mL) was added to each well, and the luminescence was measured after a 10-minute incubation. Each sample was tested in triplicate.

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid (TBA) test, which determines the levels of malondialdehyde (MDA), the final product of lipid peroxidation. 2 mL of TBA mixture (0.375% TBA, 15% trichloroacetic acid, 2 N hydrochloric acids) was added to 1 mL of cell lysate, then the solution was heated in a water bath for 20 minutes and centrifuged at 1000 g for 10 min. The absorbance of the color formed in the *n*-butanol phase was measured at 532 nm using an ELISA reader (BMG Labtech). Each sample was tested in triplicate.

Intracellular calcium concentrations

Intracellular calcium concentration analysis was performed with a slight modification of the method of [18]. C2C12 cells were treated with cisplatin (10 μM), ligandrol (0.5, 5, and 50 mg/L) and their combination at 37°C. After 24 h, the cells were washed with phosphate-buffered saline (PBS). 3 mL of ultra-high purity nitric acid was added to the solution. The solution was evaporated on a heating plate until the solution was clear and colorless. The cells were then resuspended in 0.2% nitric acid, and the calcium content was calculated by inductively coupled plasma-optical emission spectrometry (ICP-OES). Intracellular calcium levels are expressed as ng/10^6 cells. Each sample was tested in triplicate.

Hen's egg test chorioallantoic membrane (HET-CAM) irritation test

Irritation effects of cisplatin (10 μM), ligandrol (0.5, 5, and 50 mg/L), and their combination were determined by a chorioallantoic membrane model on fertilized hen's eggs with a slight change of the procedure of Güner and İlhan [18]. Fertile Leghorn chicken eggs weighing 50-60 g were purchased from commercial sources (Giresun, Turkey). Fertilized hen's eggs were placed into an incubator with a conveyor rotation system at 37 ± 1°C and 80 ± 2% humidity for 7 days. On day 7, the eggs were opened on the snub side, sucked off through a hole in the pointed side, and then a round piece of shell (3-4 cm diameter) was removed carefully with forceps. Then, the inner membrane was carefully removed with forceps, without injury to the blood vessels. After this stage, the eggs were divided into six groups; Group I: DMSO (as a negative control), Group II: Sodium hydroxide (NaOH) (0.1 mol/L) (positive control), Groups III-V: 0.5, 5, and 50 mg/L ligandrol, Group VI: 10 μM cisplatin concentration, Group VII-IX: 0.5 mg/L ligandrol + cisplatin, 5 mg/L ligandrol + cisplatin, 500 mg/L ligandrol + cisplatin. The samples were loaded onto a Whatman filter paper, CAM applied, and the samples were incubated. At the end of the 24-hour incubation period, the irritation effect was examined under the stereomicroscope, following the scoring table shown below [19]:

$$IS = [(301 - h) \times 5]/300 + [(301 - l) \times 7]/300 + \\ + [(301 - c) \times 9]/300,$$

where h is the time of vascular hemorrhaging, l is the time the first vascular lysis occurred, and c is the time the first vascular coagulation occurred.

Irritation classification was determined based on IS: 0.0–0.9 non-irritation, 1.0–4.9 slight irritation, 5.0–8.9 moderate irritation, and 9.0–21.0 severe irritation. For every test compound, five eggs were utilized. All samples were tested in triplicate at different times.

Statistical analysis

Statistical analysis was done via SPSS 21 (SPSS, Chicago, IL, USA). The experimental data were analyzed by one-way analysis of variance (ANOVA) and differences between the treatment and control groups were determined by Duncan's test. Pearson's r coefficient was used to determine correlations between data. Obtained data were expressed as means ± SD in the three independent experiments and $p < 0.05$ was accepted as significant. All assays were run in triplicate. Controls represent untreated samples.

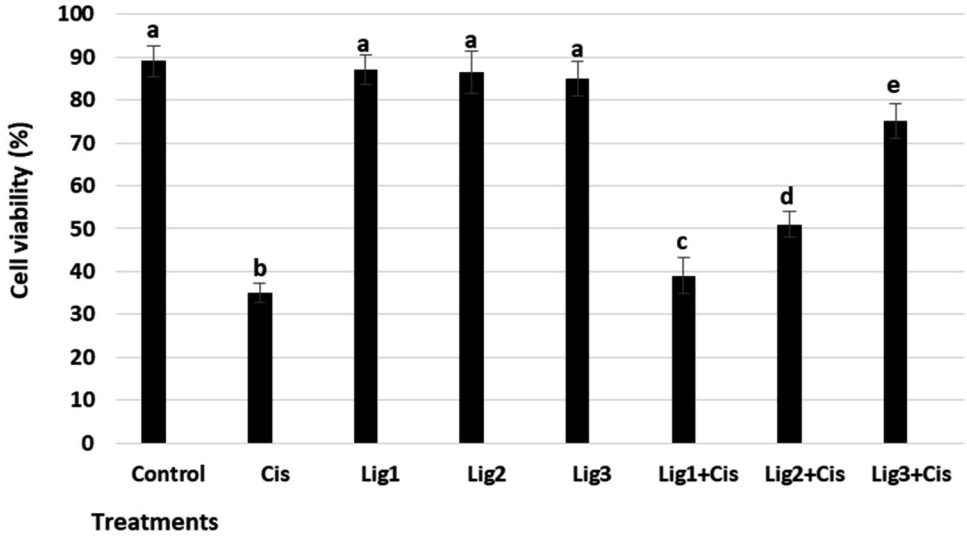

Fig. 1. Cell viability rates of C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

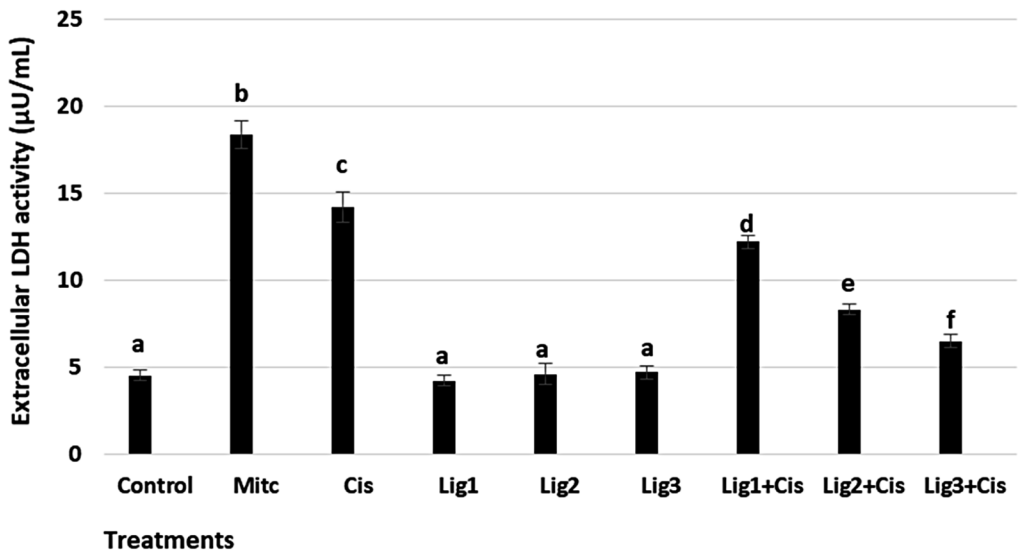

Fig. 2. Extracellular LDH activity of C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin. Mitc (10^{-7} M) was used as the positive control. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

RESULTS

Cell viability

As seen in Fig. 1, the viability level of the C2C12 cells treated with cisplatin significantly decreased ($p < 0.05$) by a 2.54-fold change compared to the control. Ligandrol did not cause a significant change in viability levels, even at the highest concentration. However, decreases in cell viability caused by cisplatin significantly compensated ($p < 0.05$) with an increase of approximately 1.1, 1.45, and 2.14-fold increase with the addition of 0.5, 5, and 50 mg/L concentrations of ligandrol, respectively.

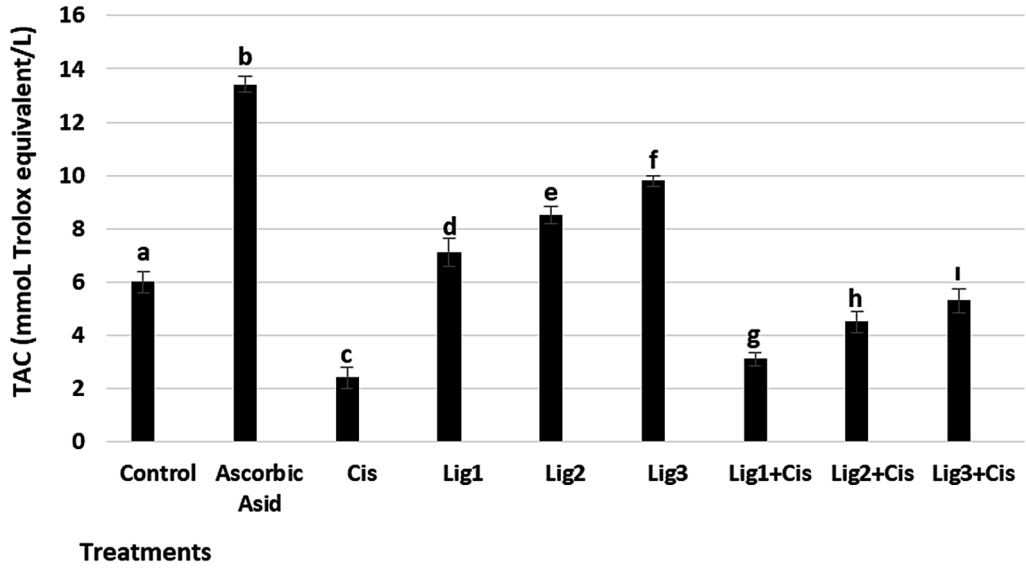

Fig. 3. Total antioxidant capacity of C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin. H_2O_2 (25 μmoL/L) and ascorbic acid (10 μmoL/L) were used as the positive controls for TAC activity. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

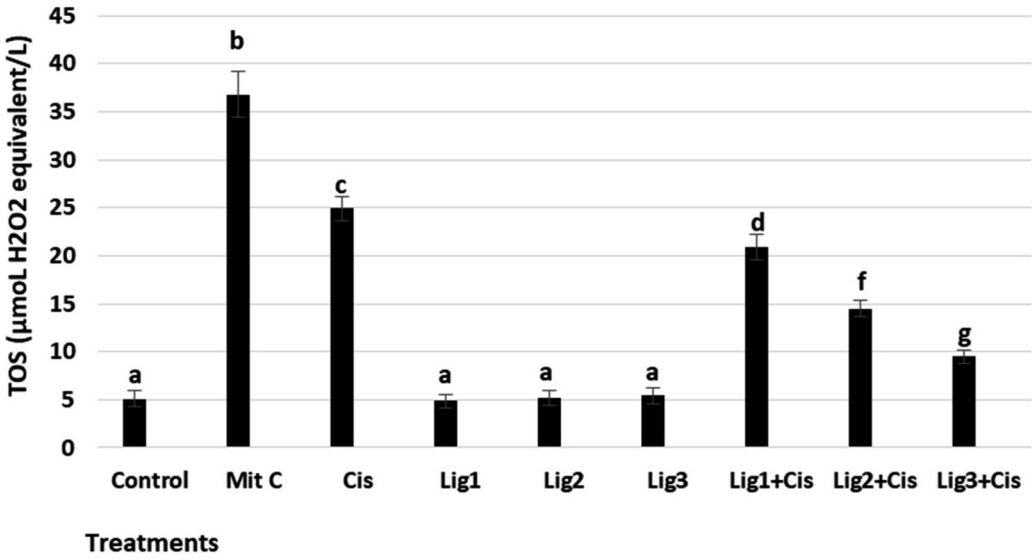

Fig. 4. Total oxidative stress levels in C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin. H_2O_2 (25 μmoL/L) and ascorbic acid (10 μmoL/L) were used as the positive controls for TOS activity. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

Lactate dehydrogenase (LDH) assay

Cytotoxic effects after treatments of cisplatin and ligandrol were revealed by LDH assay (Fig. 2). MitC (positive control) and cisplatin-only treatments caused a significant increase ($p < 0.05$) of LDH release by 4.1 and 3.15-fold change compared to the untreated control. Ligandrol treatment did not cause a significant change in the LDH levels. Combination treatments of 0.5, 5, and 50 mg/L concentrations of ligandrol with cisplatin significantly decreased by a 1.16, 1.71 and 2.18-fold change ($p < 0.05$) in the LDH release treatment, respectively.

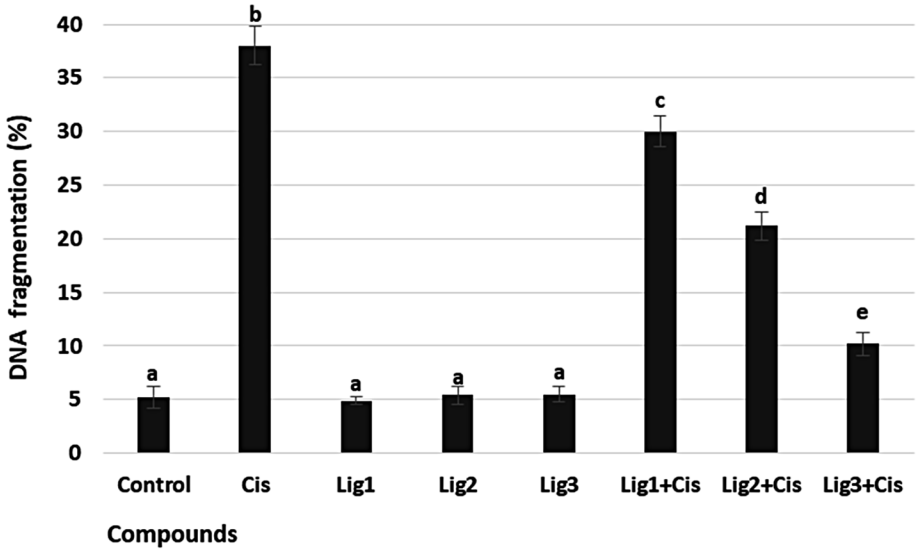

Fig. 5. DNA fragmentation rate in C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

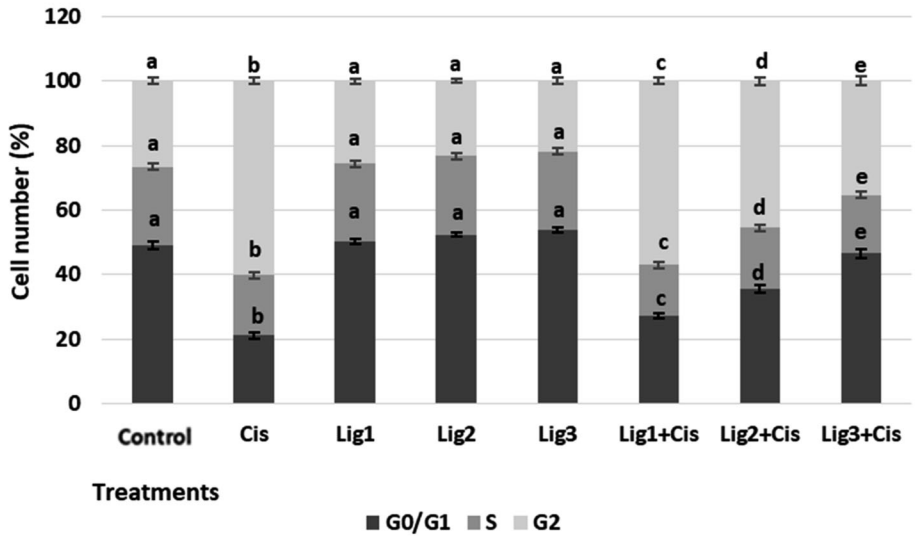

Fig. 6. Determination of cell number during cell cycles G0/G1, S and G2 in C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

Total antioxidant capacity (TAC) and total oxidative stress (TOS) activities

Oxidant/antioxidant status on C2C12 cells exposed to cisplatin and ligandrol were determined by TAC and TOS assay. 0.5, 5, and 50 mg/L concentrations of ligandrol significantly increased ($p < 0.05$) the TAC levels by 1.2, 1.4, and 1.6-fold changes compared to the untreated control, respectively, while the cisplatin-only treatment significantly decreased ($p < 0.05$) by a 2.5-fold change. Combination treatments of 0.5, 5, and 50 mg/L concentrations of ligandrol with cisplatin significantly decreased by a 1.3, 1.87, and 2.2-fold

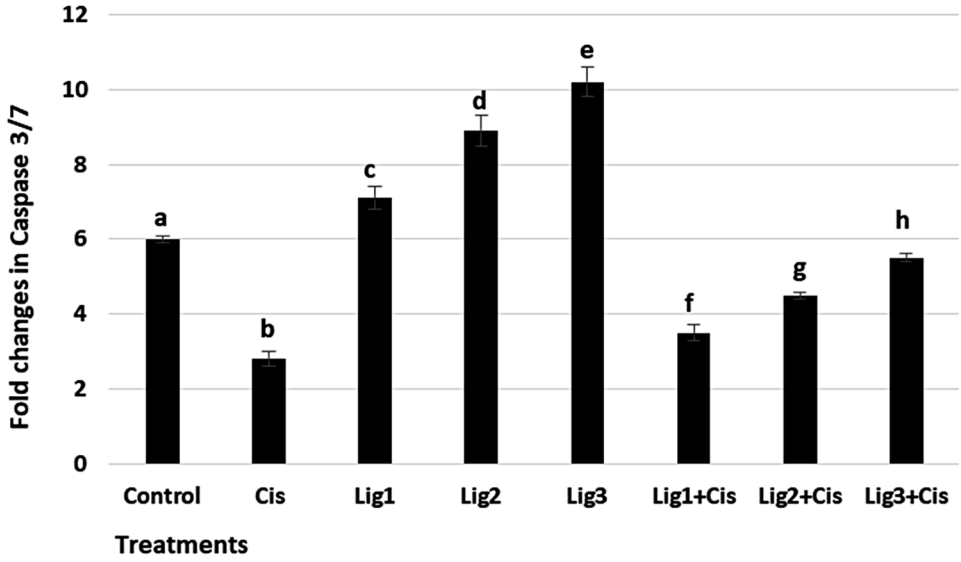

Fig. 7. Fold changes of Caspase 3/7 in C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

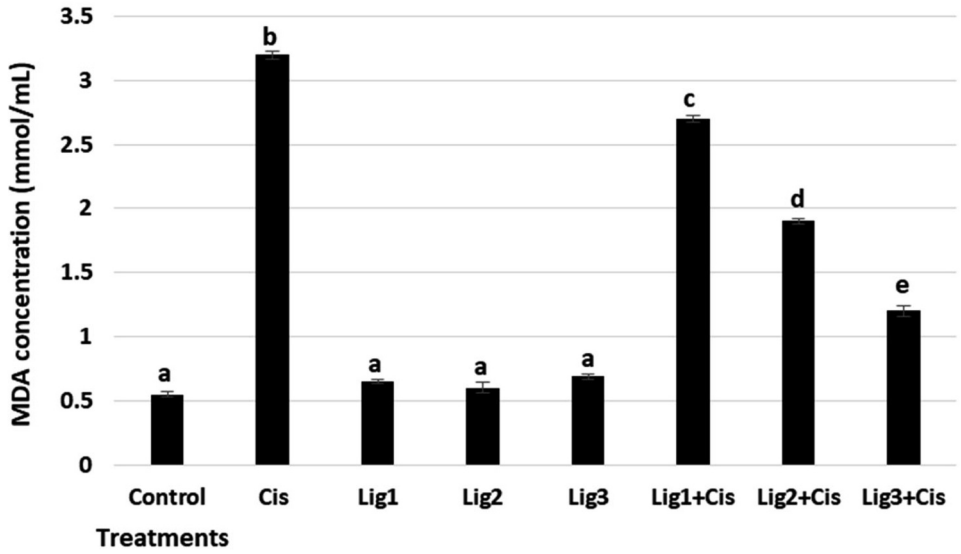

Fig. 8. TBA test for measuring MDA concentrations of C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin to determine lipid peroxidation levels. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

change ($p < 0.05$) in the LDH release treatment compared to the cisplatin-only treatment, respectively (Fig. 3).

When the TOS levels were investigated, Mitc and cisplatin significantly increased ($p < 0.05$) by a 7.1 and 4.9-fold change compared to the untreated control, respectively. Increasing concentrations of ligandrol did not cause a significant change in the TOS levels. Ligandrol at 0.5, 5, and 50 mg/L concentrations significantly decreased ($p < 0.05$) cisplatin-induced TOS levels by a 1.19, 1.71, and 2.62-fold change compared to cisplatin alone, respectively (Fig. 4).

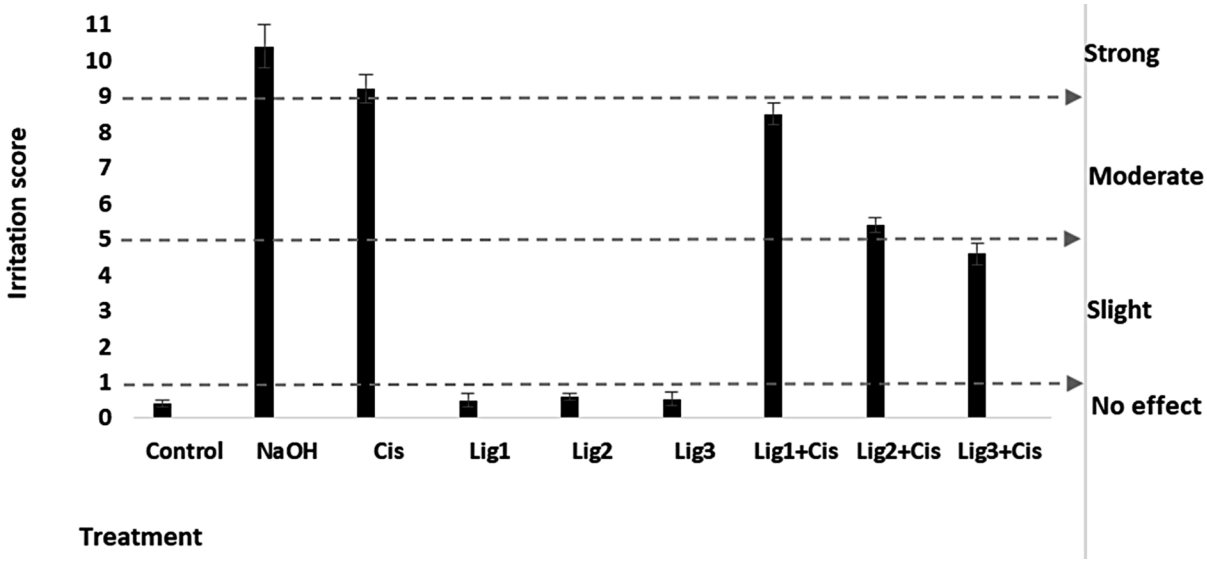

Fig. 9. Irritation scores after treatments using cisplatin (10 μM), ligandrol (0.5, 5, and 50 mg/L) and their combinations on membrane surfaces up to 5 min. Values represent means ± SD of at least three experiments. Bars indicated by the different letters show significant differences at the $p < 0.05$ level. Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

Measurement of DNA fragmentation

As seen in Fig. 5, DNA fragmentation levels on C2C12 cells after treatment of cisplatin significantly increased ($p < 0.05$) by a 7.3-fold change compared to the control, while ligandrol alone did not cause a change in the DNA fragmentation rate. Combination treatments of 0.5, 5, and 50 mg/L concentrations of ligandrol with cisplatin significantly decreased ($p < 0.05$) the DNA fragmentation levels by changes of 1.26, 1.79, and 3.72 fold, respectively.

TABLE 1. Intracellular calcium concentrations of C2C12 cells that are treated with cisplatin (10 μM), ligandrol (0.5, 5 and 50 mg/L) alone or in the presence of cisplatin as a result of calcium release

Treatments	Intracellular calcium concentration ($ng/10^6$ cell)
Control	8.78 ± 0.78^a
Cis	13.14 ± 1.21^b
Lig1	8.75 ± 0.8^a
Lig2	8.81 ± 0.85^a
Lig3	8.83 ± 0.93^a
Lig1+Cis	12.54 ± 1.2
Lig2+Cis	11.64 ± 1.05^d
Lig3+Cis	10.26 ± 1.02^e

Values represent means ± SD of at least three experiments. Vertically, different letters (a, b, c, d and e) show significant differences in intracellular Ca concentration at the $p < 0.05$ level.

Cis: Cisplatin, Lig1: 0.5 mg/L concentration of ligandrol, Lig2: 5 mg/L concentration of ligandrol, Lig3: 50 mg/L concentration of ligandrol.

Cell cycle analysis

Cell population changes in G0/G1, S, and G2/M phases after treatment of cisplatin and ligandrol were evaluated by cell cycle analysis (Fig. 6). Cisplatin treatment significantly decreased ($p < 0.05$) the number of cells in the G0/G1 phase by 2.32-fold change compared to the control, while significantly increasing ($p < 0.05$) the number of cells in the G2/M phase 2.5-fold change. Combination treatments of 0.5, 5, and 50 mg/L concentrations of ligandrol with cisplatin significantly increased ($p < 0.05$) cell population in the G0/G1 phase by 1.28, 1.68, and 2.2 compared to the cisplatin treatment, while significantly decreasing ($p < 0.05$) it in the G2/M phase by 1.05, 1.32, and 1.7, respectively.

Caspase 3/7 expression levels

Apoptotic activation on C2C12 cells exposed to cisplatin and ligandrol was revealed by changes in caspase 3/7 gene expression (Fig. 7). Cisplatin caused significant downregulation ($p < 0.05$) caspase 3/7 gene expression by a 2.14-fold change compared to the untreated control, while increasing concentrations (0.5, 5, and 50 mg/L) of ligandrol significantly upregulated ($p < 0.05$) in caspase 3/7 expression levels by 1.18, 1.48, and 1.7, respectively. Decrease in cisplatin-induced caspase 3/7 gene expression significantly upregulated ($p < 0.05$) by the addition of ligandrol at 0.5, 5, and 50 mg/L concentrations by 1.25, 1.6, and 1.96-fold increase, respectively.

Lipid peroxidation

Lipid peroxidation levels on C2C12 cells after treatments of cisplatin and ligandrol were determined by measurement of MDA concentration levels (Fig. 8). Cisplatin significantly increased ($p < 0.05$) the MDA level by a 5.8-fold change compared to the control. Increasing concentrations of ligandrol did not cause significant changes in MDA concentration. Simultaneous treatment of 0.5, 5, and 50 mg/L concentrations of ligandrol with cisplatin significantly decreased ($p < 0.05$) the MDA concentration by a 1.18, 1.68, and 2.6-fold change compared to the cisplatin treatment, respectively.

Intracellular calcium concentrations

Intracellular calcium release after treatments of cisplatin, ligandrol and their combination are depicted in Table 1. Cisplatin treatment significantly increased ($p < 0.05$) intracellular calcium levels with a value of 13.14 ± 1.21 on C2C12 cells compared to the untreated control, while a combination treatment of ligandrol with cisplatin significantly compensated ($p < 0.05$) the calcium levels between 12.54 ± 1.2 to 10.26 ± 1.02 compared to the cisplatin only treatment, in a concentration-dependent manner.

Irritation effect

Irritant effects on the CAM surface of ligandrol and cisplatin are shown in Fig. 9. Cisplatin treatment caused a strong irritant effect (9.23 ± 0.6), while ligandrol had no irritant effect. Ligandrol at 0.5, 5 and 50 mg/L concentrations significantly compensated by reducing the cisplatin-induced strong irritant effects to moderate and slight with values of 8.52 ± 0.4, 5.41 ± 0.3, and 4.75 ± 0.2, respectively.

DISCUSSION

Skeletal muscle disorders (muscle weakness and fatigue) can be frequently encountered in cancer patients treated with cisplatin. The molecular mechanisms of cisplatin-induced muscle dysregulation are often associated with oxidative stress, impaired mitochondrial function, lipid metabolism disorders, ubiquitin-dependent proteolysis, autophagy, and alteration in Ca^{2+} homeostasis [20]. Oxidative stress is a mechanism underlying cisplatin-induced toxicity and cisplatin can contribute directly or indirectly to oxidative stress by interacting with molecular oxygen and undergoing redox cycling or by reducing antioxidant levels [21–23]. In this study, the first devastating effect of cisplatin on muscle cells was evidenced by a reduction in cell viability via both elevated oxidative stress levels and decreased total antioxidant status. A recent report declared that cisplatin-treated C2C12 muscle cells showed a decrease in cell viability through abnormal ROS accumulation following increased malondialdehyde levels, which the cell viability could compensate for via a combination treatment that balanced intracellular oxidative radical levels [24]. Leciejewska, et al. [25] reported an increase in proliferation and cell viability of muscle cells after treatment with ostarine. Similarly, this study revealed that ligandrol protected against cisplatin-induced muscle damage through both increased plasma TAC levels and decreased TOS levels.

Cisplatin-induced oxidative stress severely affects the mitochondrial process through a cascade of events such as LDH leakage, inhibition of calcium uptake, lipid peroxidation, and ultimately disruption of mitochondrial membrane integrity [26]. The LDH enzyme is present in many cells and leakage of LDH is an important sign of the cell membrane damage and oxidative effects caused by the toxic agent [27]. Previous studies have revealed that the increase in LDH release after loss of membrane integrity is one of the most important mechanisms of cisplatin-mediated toxicity [28, 29]. Several studies showed that cisplatin-induced muscle wasting might be associated with overcumulating intracellular Ca^{2+} levels followed by increased protein degradation. Under low intracellular chloride ion concentrations, cisplatin hydrolyzes to reactive species. These hydrolyzed forms inhibit oxidative phosphorylation and mitochondrial respiration, leading to a significant increase in calcium levels, disrupting calcium homeostasis and initiating primary events such as lipid peroxidation and enzyme inhibition [30]. A study conducted on the relationship between intracellular calcium and oxygen radicals in cisplatin-induced kidney cell damage has shown that intracellular production of ROS increased with the increase of calcium, which, in turn, caused an increase in the release of LDH from cells [31]. Combinational strategies significantly enhanced mitochondrial function by rectifying calcium imbalances, regulation of enzymatic activation and reduction of oxidative stress in cisplatin-induced cytotoxicity [30, 32]. Roch, et al. [15] revealed that elevation of cellular LDH enzyme levels after administration of ligandrol might indicate enhanced muscle structure. Similarly, combinational treatment with cisplatins of ligandrol significantly inhibited the increased intracellular Ca^{2+} level, MDA concentration and LDH release. This phenomenon suggested that ligandrol could protect membrane integrity by supporting primary antioxidant mechanisms that scavenge superoxide produced in mitochondria and maintaining Ca^{2+} homeostasis [20].

Cisplatin becomes activated when it enters the cell, and chlorides on cisplatin are replaced with water molecules in the cytoplasm. The newly formed electrophile binds to the N7 reactive center on the purine residues, disrupting the DNA structure and cell cycle, and leading to cell apoptosis [33]. Cisplatin-induced weight loss may be characterized by changes in protein synthesis as a result of a change in the apoptotic pathway [20]. Cisplatin-based chemotherapy treatment induces NFêB activity, which is associated with muscle wasting, following an increase in DNA damage [20, 34]. This cisplatin-induced DNA damage strongly induces cell death if they are not properly repaired or processed. In this context, the cell population ratio in cell cycle periods is one of the most important methods used in the interpretation of

DNA damage caused by cytotoxic effects [35]. In a study on cisplatin-induced gene changes, cell cycle, and apoptosis of promyelocytic leukemia, the cell cycle analysis indicated that, at lower doses of cisplatin treatment, the cells were arrested at sub-G1, S, or G2 checkpoints, and as cisplatin dose and treatment time increases, the cells started accumulating more in sub-G1 phase [36]. In a study indicating similar results, Swift and Golsteyn [37] declared that HT-29 cells treated with cisplatin were arrested mostly at the G2/M phases. Our results showed that the increased population in the G2/M cell cycle in muscle cells exposed to cisplatin might be due to the defects in the repair mechanisms and possible DNA damage in the cells. However, increased DNA fragmentation levels were significantly compensated by an increase in the G0/G1 cell population and caspase 3/7 gene expressions after ligandrol treatment. This phenomenon was related to the entrainment of damaged cells into apoptosis following upregulation of proapoptotic genes through transcriptional changes in mitochondrial and cellular receptors. In a study conducted by Nyquist, et al. [38], it was found that SARMs (GTX-024, GTX-027, SARM-2F, and T8039) repressed the growth of prostate cells and induced concordant transcriptional changes and the expression of luminal differentiation genes, but blocked entry into the S phase. These results indicated that different ligand characterizations of SARMs impacted the different cell cycle checkpoints to arrest the cells at a certain cell cycle phase.

Many clinical studies have revealed that some patients receiving cisplatin-based chemotherapy have a significantly elevated risk of vascular compliations, such as cardiovascular, thrombosis, and coronary syndromes [39, 40], which raises concerns about the effects of cisplatin therapy on the vascular system. Unfortunately, the mechanism underlying the toxic effects of chemotherapeutic agents on skeletal muscle circulation has not been fully elucidated. Some experimental and clinical studies have explained chemotherapeutic platin drug-induced muscle wasting with changes in muscle mitochondrial function, signaling pathways and muscle endothelium [41, 42]. Several studies reported an increase in the vascular risk of patients, depending on endothelial damage and vascular endothelial dysfunction in cisplatin-based chemotherapy [43–45]. In this study, cisplatin induced a strong irritant effect with vascular hemorrhage and lysis in a chorioallantoic membrane model, mimicing vascular toxicity, and this vascular irritant effect significantly decreased, in a ñoncentration-dependent manner, in the presence of ligandrol. Vascular endothelial cells are important cellular components of blood vessels that play important roles in vascular growth, death, and physiological function; additionally, androgen receptors may contribute to the modulation of endothelial and fibroblast cells through the induction of apoptotic signal pathways or through mechanisms regulating oxidative stress [46–49]. This phenomenon suggests that ligandrol might enhance endothelial cell function by regulating apoptotic gene expression and/or inhibiting vascular oxidative stress against cisplatin-induced vascular toxicity.

CONCLUSION

This study provides the first evidence for the protective effects of ligandrol against cisplatin-induced toxic effects on muscle cells and the vascular system. Briefly, besides its vascular remodeling ability, ligandrol significantly improved cell proliferation, DNA damage, biochemical changes, membrane integrity and irritant effects through the inhibition of increased oxidative stress or enhancing the antioxidant system in cisplatin-exposed muscle cells. Therefore, ligandrol supplementation, considering that it had no toxic effects on muscle cells, can be used as a potential agent against cisplatin-induced muscle toxicity in chemotherapeutic treatment.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Authors' contribution

The study was designed by Z?, TA and AG. The experimental studies were performed by Z?. Statistical analysis was performed by TA and AG. All authors contributed to the writing of the manuscript. TA and AG are responsible for supervising the manuscript.

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