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The Synergistic Effect of Theranekron and Cisplatin on the Neuroblastoma (SH-SY5Y) Cell Line

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ABSTRACT

AIM: To assess the combined antitumor potential of theranekron (Trn) and cisplatin in neuroblastoma (NB), a pediatric tumor characterized by high prevalence and mortality rates. The investigation involved examining the interaction between theranekron (10-100 μM) and cisplatin (40 μM) within this cell line.

MATERIAL and METHODS: The cytotoxicity of Trn was evaluated using MTT assays, cell volume analyses, apoptosis assessments, and measurements of mitochondrial membrane potential. These evaluations were conducted over a 24-hour incubation period.

RESULTS: The coadministration of 100 μM Trn with cisplatin exhibited a cytotoxic effect of approximately 60%. Furthermore, it led to a reduction of up to 38% in cell volume. Notably, SH-SY5Y cells demonstrated an early apoptosis rate of 34.4%, accompanied by an eightfold decrease in mitochondrial membrane potential compared to the control group.

CONCLUSION: Trn demonstrated synergism and significantly enhanced the efficacy of cisplatin ($p<0.001$). Nevertheless, further in-depth investigations are necessary to elucidate the intricacies of these synergistic effects.

KEYWORDS: Cisplatin, SH-SY5Y, Synergism, Theranekron

INTRODUCTION

Neuroblastoma (NB) is a pediatric tumor originating from neural crest (NC)-derived cells, which often exhibit defective sympathetic neuronal differentiation due to genomic and epigenetic abnormalities (23). While primarily observed in children, it can occasionally manifest in adolescents and adults (20). Despite intensive therapeutic interventions such as chemotherapy, radiotherapy, and immunotherapy, over half of the patients have a survival rate below 50%, and metastatic conditions are frequently detected upon diagnosis (2). The absence of a standardized therapeutic approach for relapsed disease remains a challenge (14).

Cisplatin (Cis) is a well-established chemotherapy drug employed in the treatment of various human cancers (18,33). It elicits complex defense mechanisms that simultaneously activate or inhibit multiple genes, leading to DNA damage response, repair pathways (12), cell cycle arrest, and apoptosis (27,30). However, the response to Cis treatment varies, and drug resistance is a significant impediment to its efficacy (17). Cisplatin resistance often becomes apparent when high doses are administered as part of intensive treatment protocols, necessitating cytotoxic concentrations up to 50-100 times greater than those effective in sensitive cells (11). To enhance drug effectiveness, combination therapies involving other drugs or substances are utilized to potentiate the drug's action. While such combinations can amplify the therapeutic effect, they may also increase the risk of additional toxic effects.

Theranekron® (Trn), a homeopathic remedy extracted from the venom of the spider *Tarantula cubensis* and available as an injectable solution, is widely used by veterinarians to treat various ailments, such as caries, arthritis, abscesses, injuries, panaritium, and laminitis (6). This preparation is believed to stimulate the immune system, enhance vitality, and naturally suppress inflammation in proliferative lesions (25). Theranekron is associated with four key pharmacological prop-

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erties: demarcation (separation of two tissues), regeneration, resorption, and anti-inflammatory action (26).

The potential anticancer properties of Trn have been explored in canine mammary tumors (8,9) and various human cancer cell lines (breast, prostate, lung osteosarcoma) (6,13). While clinical veterinary studies have demonstrated the anticancer effects of Theranekron, especially in combination with other applications, its impact on different cancer cell lines remains relatively unexplored. Limited studies have hinted at its substantial potential (4,6), yet extensive *in vivo* and *in vitro* investigations are crucial, particularly concerning human cancer cell lines. This study aimed to assess the cytotoxic effects of theranekron alone and in conjunction with cisplatin on the neuroblastoma cell line SH-SY5Y.

MATERIAL and METHODS

Trn, commercially available in 50 mL bottles, is an alcoholic extract of Tarantula cubensis used as a solution (Richter Pharma, Wels, Austria). Human neuroblastoma SH-SY5Y cells were acquired from ATCC (USA) and cultured according to the manual provided by the manufacturer. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma-Aldrich Inc. (Louis, Missouri, USA).

Cell Culture Model

SH-SY5Y neuroblastoma cells obtained from flasks were seeded into 96-well plates with eight replicates per group at an approximate density of 5000 cells per well. The plates were then placed in a 37°C incubator with 5% CO_2. The cell culture medium comprised 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and Dulbecco's modified Eagle medium (DMEM). The experiment commenced once the cells had reached approximately 80% confluence on the well surfaces. For the experiment, Trn was administered alone at concentrations of 10, 25, 50, 75, and 100 µM. Additionally, Trn was coadministered with cisplatin (Cis) at 40 µM, which also served as a control drug. The choice of 40 µM cisplatin was based on its IC_{50} value reported in the literature (15). The drug solutions were prepared by homogenizing the specified volumes in the culture medium and applying 100 µL per well. Wells designated as controls received the same volume (100 µL) of the culture medium. As a positive control, cisplatin was adjusted to a concentration of 40 µM and added in an equivalent volume (100 µL).

MTT Test

The MTT assay is a widely used standard method for colorimetrically assessing cell viability. In this assay, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] undergoes reduction by mitochondrial reductase activity, leading to the formation of purple-colored formazan crystals. At the end of the experiment, the culture medium in the 96-well plate was removed 24 hours after drug administration. A total of ten microliters of prepared MTT solution was added to each well, followed by the addition of the prepared culture medium to complete the volume. Subsequently, the cells were incubated under appropriate conditions for 4 hours to allow the formazan crystals to form. After this incubation period, the medium was aspirated, and dimethyl sulfoxide (DMSO) was employed to dissolve the formazan crystals (21). Once the formazan crystals were fully dissolved within the wells, readings were taken using a microplate reader set at a wavelength of 450 nm.

Cell Volume Analysis

Cell volume analysis was used to confirm the cytotoxic effect of Trn. A rapid and sensitive method for cytotoxicity measurement was established using the flow cytometry analysis system. Trn volumes (10, 25, 50, 75, and 100 µM) were used to determine the final experimental concentration (31). Finally, the final experimental Trn concentrations were specified as 75 and 100 µM, as the IC_{50} of Trn was above 90 µM. The Cis concentration was added to all Trn combinations at a constant 40 µM. Trn and Trn+Cis stock solutions were diluted in RPMI medium containing 10% FBS. The final volume was adjusted to 1 mL with RPMI medium per well of a 24-well microplate. A total of 3×10^5 cells were exposed to Trn and Trn+Cis in the dark for 1 h at 37°C. Histograms and dot plots were generated, with FSC-H representing cell volume. A total of 10,000 events were obtained in the region corresponding to SH-SY5 neuroblastoma cells.

Mitochondrial Membrane Potential (ΔΨm) Test

Mitochondrial membrane potential loss was quantitatively determined by flow cytometry using a JC-1 labeling kit (BD MitoScreen Mitochondrial Membrane Potential Detection Kit, BD, USA) according to the manufacturer's protocol. At least 10,000 events were analyzed by flow cytometry (BD Accuri C6 Plus, San Jose, CA, USA).

Apoptosis Detection Using Annexin V Staining

Cells ($1x10^6$ cells/sample) were stained with Annexin V-FITC (BD Biosciences, San Jose, CA, USA). Apoptosis induced by Trn and/or Cis in SH-SY5 cells was quantified by flow cytometry and software (BD Accuri C6 Plus, San Jose, CA, USA). The apoptosis rate was quantified.

Statistical Analysis

Data obtained in the study were analyzed statistically with SPSS for Windows, Version 17.0. (SPSS Inc. Chicago, USA) software. All tests were conducted by one-way analysis of variance (ANOVA) with Tukey's post hoc test. Data were expressed as the mean ± standard deviation (SD). A p-value of <0.001 was considered significant.

RESULTS

MTT and Cell Volume Results

Through MTT analysis, we systematically evaluated the potential neurotoxic effects of Trn on the SH-SY5Y neuroblastoma cell line. In the initial phase of our investigation, diverse concentrations of Trn (ranging from 1 µM to 100 µM) were introduced to the SH-SY5Y cell line. However, it was evident that Trn, when administered in isolation, did not exhibit any discernible cytotoxicity toward SH-SY5Y cells. While not at-

taining statistical significance, a marginal increase in the cell population was observed at lower concentrations. Subsequently, we subjected the SH-SY5Y cell line to a fixed dose of 40 μM cisplatin in combination with varying concentrations of Trn (10, 25, 50, 75, and 100 μM). The outcome of this combined application of Trn and Cis was the cumulative and interactive effect of the two agents. During this second experimental phase, we observed a lack of cytotoxicity when SH-SY5Y cells were exclusively treated with Trn. However, a notable cytotoxic impact was apparent at different Trn concentrations when coadministered with Cis. Remarkably, the extent of cytotoxicity exhibited a direct correlation with increasing Trn concentrations. Most notably, the most profound toxic effect was witnessed at a concentration of 100 μM Trn combined with 40 μM Cis, resulting in a reduction of cell viability to approximately 38% relative to the control group (Figure 1). This phenomenon of decreasing cell viability was consistently mirrored by cell volume measurements, a supplementary analytical approach that we undertook using Trn doses of 75 μM and 100 μM in accordance with the IC_{50} value. Intriguingly, while there were no variations in cell volumes observed when Trn was administered independently compared to the control, a conspicuous reduction in cell volume was noted in response to increasing Trn concentrations in the presence of Cis (Figure 1B). The findings from both MTT analysis and cell volume assessments were in alignment, reflecting a congruent pattern: a decrease in viable cell count corresponded directly with a reduction in cell volume. These results underscored the interplay between Trn and Cis and emphasized the potential for cumulative and synergistic cytotoxic effects, particularly at higher Trn concentrations.

Apoptosis Detection

As a result of MTT analysis, the Trn+Cis IC50 value was determined to be 92.32 μM, and 75 and 100 μM Trn were used for apoptosis detection. Apoptosis induction was measured after 24 hours of treatment. The early apoptosis percentages were 0.28% in the control group, 3.14% in the Cis group, 6% in the 100 μM Trn group, and 34.4% in the 100 μM Trn + Cis group. The percentages of late apoptotic cells were 0.97%, 8.74%, 13.5%, and 18.8%, respectively (Figure 2B). The percentages of viable cells were measured as 85%, 80%, 70%, and 42%, respectively. The percentage of viable cells was similar to other MTT and cell volume measurements. This further increased the reliability of the results we obtained using different tests.

Mitochondrial Membrane Potential Results

Mitochondrial membrane potential (MMP) results were determined quantitatively by flow cytometry as the ratio compared to the control group by taking P1/P2 values. No difference was detected between the administration of cisplatin to SH-SY5Y cells and the control group in which no treatment was applied. Values were similar in both groups. There was a significant difference in Trn treatment applied at different concentrations ($p<0.001$). The values were determined to be 0.76 and 0.7 for 75 and 100 μM Trn applications, respectively, and at these values, the MMP was 25-30% lower than that of the control group for both doses (Figure 3B). In the Trn SH-SY5Y cell line, it decreased MMP more than it affected cell viability. In Trn (75, 100 μM)+Cis treatment, MMP values were determined to be 0.47 and 0.12. There was a difference of 0.58 between the 100 μM single application dose of Trn and the dose applied with Cis. When we compared both results with the control group, it was seen that there was a 30% reduction

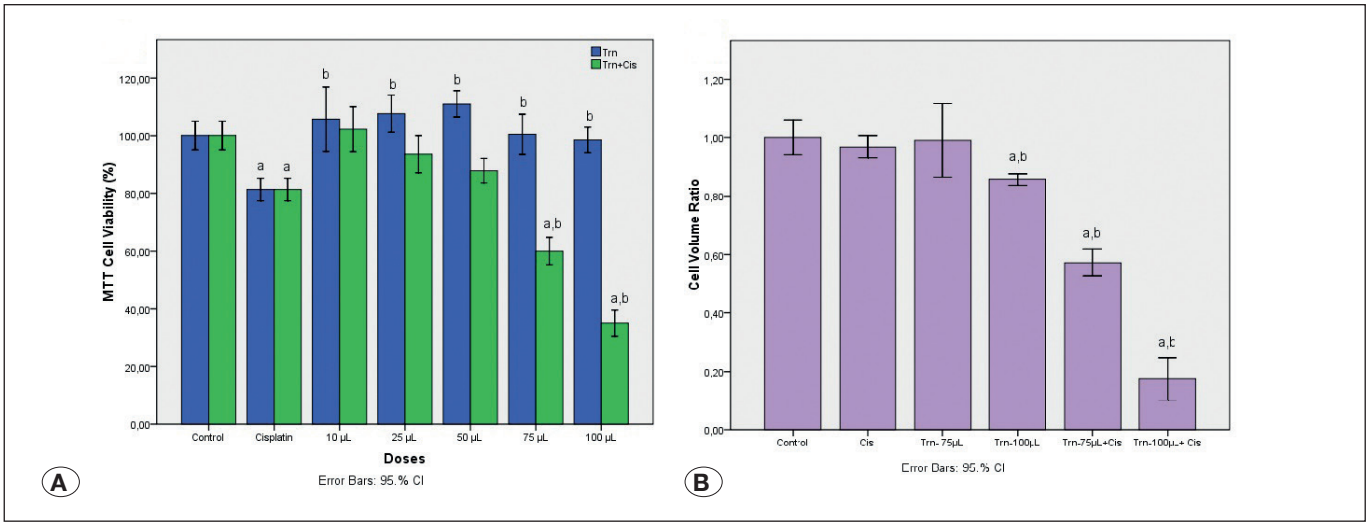

Figure 1: MTT cell viability and cell volume ratios. **A)** Cell viability. After MTT analyses were performed, the values of the control group were compared with those of the other groups, and the values are given as percentages. **B)** Cell volume values were taken and compared with the control group. Values were expressed proportionally. Cis was applied at 40 μM at all Trn concentrations. For both analyses, the substance was administered for 24 h. Percentages are given as the mean ± standard deviation and n: 8 for each group. Control; untreated SH-SY5Y cells, Cis; 40 μM cisplatin, a; compared to the control group, $p<0.001$, b; represents $p<0.001$ according to the Cis group.

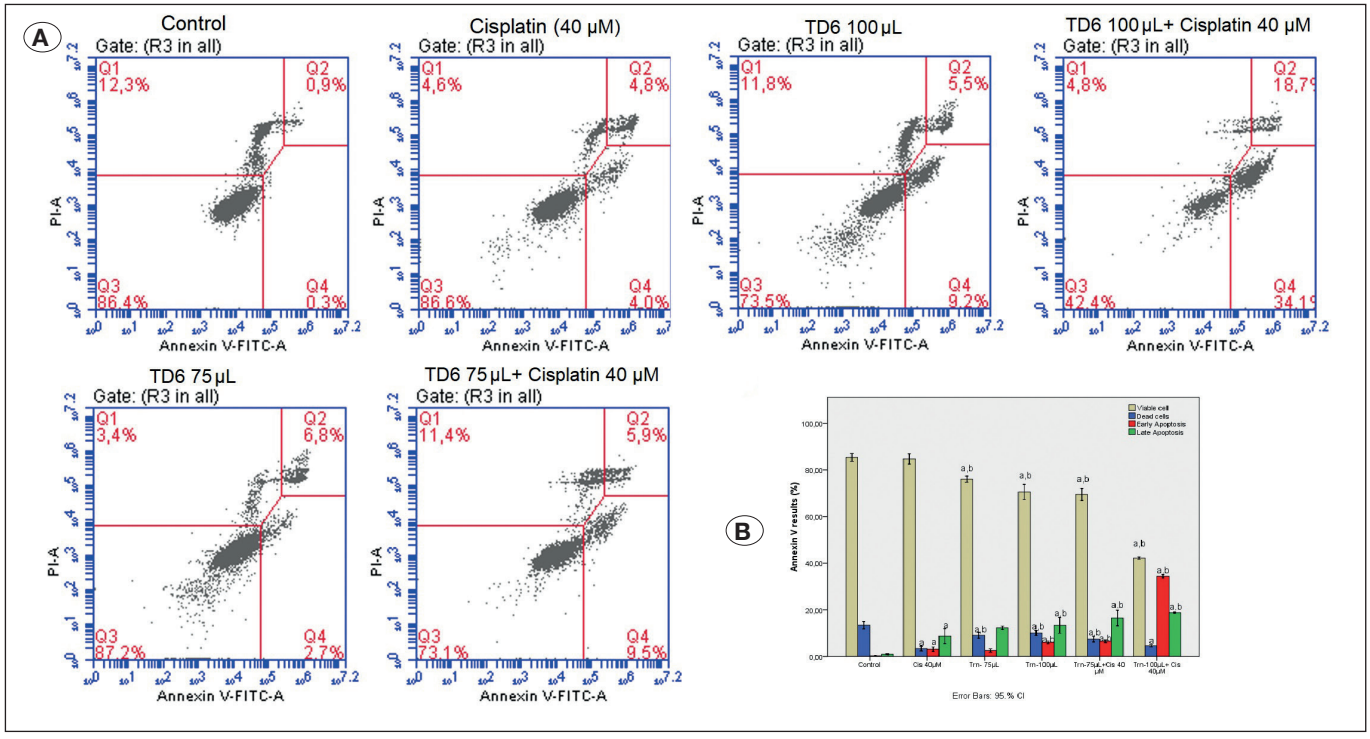

Figure 2: Apoptosis findings. **A)** Sample Annexin V results of all the groups analyzed are given. Here, we describe necrotic cells with Q1 Annexin-FITC$^-$ and PI + staining index, late apoptotic cells with Q2 Annexin-FITC + and PI +, healthy cells with Q3 Annexin-FITC$^-$ and PI$^-$ staining index, and Q4 Annexin-FITC + and PI$^-$ represent early apoptotic cells with a staining index. **B)** Graphs of early apoptosis, late apoptosis, necrosis, and viable cell percentages. It represents the mean ± standard deviation values of all Annexin V analyses on the graph, and it is n:8 for each group. a: control, b represents p<0.001 according to the Cis group.

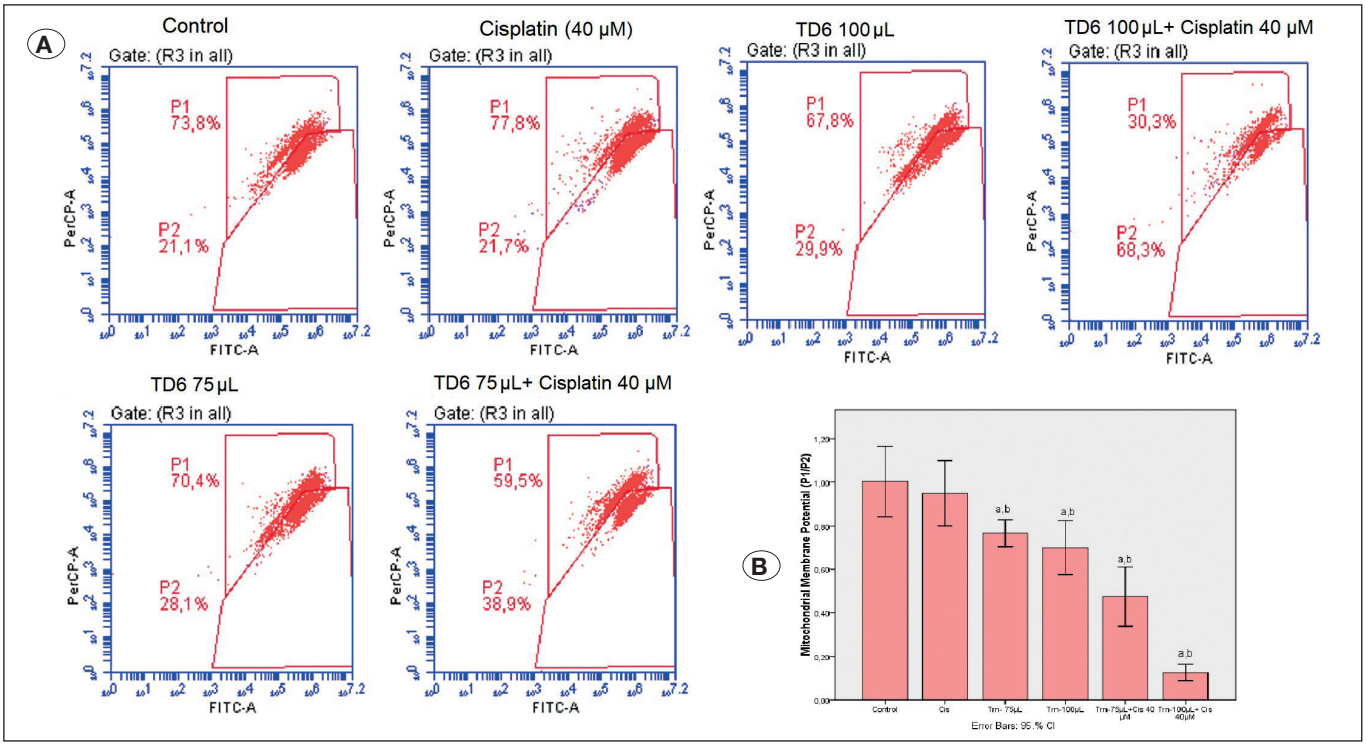

Figure 3: Mitochondrial membrane potential results. **A)** Sample images of JC-1 analysis by flow cytometry of all groups. **B)** Graph of P1/P2 ratios. Values are given as the mean ± standard deviation and n: 8 for each group. a; according to control, b; according to Cis, p<0.001 was considered significant.

in the dose where only Trn was applied and 88% when it was applied together with Cis. The MMP of the control group was approximately 8 times higher than that of the Trn 100 µM + Cis MMP group, and MMP was decreased at this rate.

DISCUSSION

Trn has been investigated under both *in vivo* and *in vitro* experimental conditions. Prior to presenting our own findings, it is valuable to highlight some of the animal studies conducted on Trn. In a rat femur fracture model, Trn demonstrated the potential to expedite fracture healing during the early stage of bone union. This was achieved through the reduction of acute inflammatory processes and the enhancement of fracture strength, angiogenesis, osteoblast proliferation, and bone formation (10). Additionally, in rabbits with experimentally induced rupture of the superficial digital flexor tendon (SDFT), Trn treatment effectively mitigated inflammation, improved clinical symptoms, and positively influenced the structural organization and biomechanical properties of the injured tendon (22). Trn also exhibited a notable anti-inflammatory effect on acetic acid-induced colonic inflammation through the NF-kB signaling pathway, while its anti-apoptotic effects were found to be independent of this pathway (24). In a comparison study involving rats with surgically induced endometriosis, Trn displayed more significant regression of endometriotic foci than medroxyprogesterone acetate (MPA) or leuprolide acetate (LA). Furthermore, the recurrence rate after discontinuing Trn therapy was lower than that observed in the other groups (3). Moreover, Trn was found to reduce axonal and myelin damage following sciatic nerve injury in rats with compression-type peripheral nerve damage, suggesting a neuroprotective effect attributed to its anti-inflammatory impact on proinflammatory cytokine levels (16). In another rat interstitial cystitis model, intravesical Trn (Theranekron) instillation demonstrated an anti-inflammatory effect by reducing inflammatory parameters such as IL-6, TNF-alpha, and MPO in the bladder tissue. Additionally, Trn increased hydroxyproline and fibroblast proliferation, thus accelerating tissue healing (1). Furthermore, in rats with experimentally induced wounds on the back skin, Trn expedited epithelialization, resulting in accelerated wound healing (7). In the context of burn injuries, Trn has been shown to enhance the viability of the stasis zone and support wound healing (28). In response to a foot-and-mouth disease outbreak in cattle in Iran, a comparative study was conducted between normal treatment (consisting of lunixin meglumine and oxytetracycline injections with daily dressing of lesions using 4% sodium carbonate) and Trn treatment. The results indicated that Trn was significantly more effective ($p<0.05$) in addressing both systemic and local symptoms of foot-and-mouth disease in infected cattle (19). These animal studies offer valuable insights into the diverse therapeutic potential of Trn across various medical conditions and underscore its relevance in different fields of research.

Our study investigated the effects of Trn and cisplatin (Cis) on cell viability and cell death with different parameters. Initially, only Trn was applied, but no cytotoxic effect was observed at the concentrations (1-100 µM) applied to the SH-SY5Y cell line compared to the control group ($p>0.05$). After these findings were obtained, cisplatin (in the IC50 (40 µM) inhibitory concentration specified in the literature for SH-SY5Y), which was not specific to the period and was also indicated in the treatment of neuroblastoma, was added to different doses of Trn (10-100 µM). In this study, no significant difference was observed in the doses where Trn was used alone. However, at the doses used with Cis, the two substances caused cell death. In particular, it was determined that the viability decreased to 37% when Trn (100 µM) + Cis (40 µM) was applied (Figure 1A). The findings show that the ethanol contained in Trn has toxic effects on different cell lines (metastatic AGS and nonmetastatic human gastric cell lines (MKN-45) and human embryonic kidney cell line (HEK-293) noncancer cells as controls) (32). However, in our study, no toxic effects were detected at any of the doses of Trn administered (Figure 1A). That is, there was no difference ($p>0.05$) with all concentrations (10-100 µM) treated with only Trn and the control group (Trn 0 µM). No toxic effect was observed in MTT analyses performed on SH-SY5Y cells. According to these findings, it is not possible to state that ethanol in Trn also causes toxicity that reduces cell viability (Figure 1A).

Then, apoptosis tests were performed to understand the cause of this cell death more clearly. As a result of these tests, it was determined that early apoptosis was 60 times higher in the Trn (100 µM) + Cis (40 µM) application compared to the control. In addition, compared to the control group, the total apoptosis rate was approximately 30 times higher, and the late apoptosis rate was approximately 15 times higher. The percentages of early and late apoptosis in the Cis group were 3% and 9%, respectively. These apoptosis rates were 0.6% and 1% in the control group, respectively. The apoptosis percentage values are given in Figure 2. According to these data, when the treatment groups with Cis were evaluated among themselves, no significant difference was observed in the percentages of late apoptosis. However, when the early apoptosis rates were examined, the percentage of early apoptotic cells after Trn (100 µM) + Cis (40 µM) treatment increased significantly. This finding showed that Trn (100 µM) + Cis (40 µM) application was more effective in the early apoptosis of Cis and Trn SH-SY5Y cells. Another point is that while cell death is not observed in Trn application alone, cell death is observed in doses administered together with Cis, suggesting the possibility of a synergistic effect between Trn and Cis. If an evaluation was made based on MTT results alone, the viability was 85% in the group using Cis alone. No cytotoxicity was detected at the concentrations where only Trn was applied. However, at the concentrations applied as Cis (40 µM) and Trn 75 and 100 µM, the viability was determined to be 61% and 34%, respectively. This finding shows that Trn and Cis interact. There is an interesting point here. The cytotoxic effect of Trn and Cis at the dose applied alone was tolerated by Trn, up to a certain concentration (10, 25 and 50 µm) of Trn at the doses they were applied together. While the cell viability was 80% at the dose where cis was applied, these viability rates were determined to be 101%, 93% and 87%, respectively, at the concentrations applied with 10, 25 and 50 µM Trn. These results are interesting. The cytotoxic effect of Trn and

Cis was suppressed at certain rates in Trn+Cis application up to 50 μM Trn. However, the cytotoxic effect of Cis increased at Trn concentrations of 75 μM and above. Unfortunately, it was not possible to conduct curative tests to elucidate the mechanism of this interaction in this study. According to the MTT results alone, the suspicion of such an interaction is high. In addition, when we evaluate other analysis results, we see similar results. These results strengthen the suspicion that there is a synergistic interaction. In this synergistic interaction, one of the issues that arouses curiosity and needs to be investigated is how Cis and Trn interact between their molecular structures and whether they form ligands. While there was no significant difference in Trn doses applied alone compared to the control, both MTT and Annexin analysis results were statistically significant with all groups of Trn (100 μM) + Cis (40 μM) application (p=0.0000). Trn may be an excellent alternative therapeutic drug for treating malignant neoplasms as an apoptosis-inducing agent (29). Studies on many different cancer cell lines (MCF7 (human mammary gland cancer cell line) and HN5 (human head and neck cancer cell line) (6), MCF-7, Saos-2 (human osteosarcoma), and A549 (human non-small cell lung cancer) (29) have shown that Trn alone is toxic to cancer cells. However, we did not observe any toxic effects of Trn doses that we applied to SH-SY5Y cells alone. Toxic effects may be seen at higher Trn doses. However, Trn doses applied with these findings did not reveal any toxic effects.

Trn significantly induces caspase-3 activation in cancer cells (MCF7 and HN5). It causes DNA fragmentation in cells, but this effect is low in healthy cells (HEK293), and there is insufficient evidence about the effect of its structures on other apoptosis pathways (6). The Bax/Bcl-2 ratio was increased in cells obtained from bone (Saos-2) and breast (MCF-7) cancers. In addition, the apoptotic effect of Trn with different molecular structures has been explained in androgen-sensitive prostate cancer cells (LNCaP), epithelial metastatic prostate cells (DU145) and normal prostate cells (PNT1A) (5). It has been stated that the colonial growth of prostate cancer cells is significantly limited by Trn treatment and that androgen-sensitive prostate adenocarcinoma cells are more sensitive to Trn than normal prostatic cell lines (5). Similarly, in our study, we observed that apoptosis was induced in the neuroblastoma SH-SY5 cell line. This was particularly evident with the proportional excess of early apoptosis rather than late apoptosis. However, these findings could not be supported by more detailed molecular targets. The effects of Trn on the molecular pathway of apoptosis need to be investigated further in future studies. Another issue is that the combination of Trn and Cis caused cytotoxicity in the SH-SY5Y cell line, but one of the shortcomings of our study is the question of what effect it would have on healthy neuron cells. Future studies on this subject should also be investigated.

CONCLUSION

In this investigation, we explored a novel territory by assessing the impact of Trn on SH-SY5Y cells. When employed in isolation, Trn exhibited no discernible influence on SH-SY5Y cells. However, when administered in conjunction with cisplatin at the prescribed doses, Trn demonstrated a twofold effect. It not only prompted early apoptosis within the cells but also induced a noteworthy reduction in cell viability, concurrent with a substantial decline in mitochondrial membrane potential. Furthermore, a significant diminution in the volume of SH-SY5Y cells was observed. Notably, this study did not delve into the molecular and immunohistopathological mechanisms that govern the initiation of apoptosis in these cells. These findings, however, do suggest the potential for synergistic interactions between Trn and cisplatin. Nevertheless, a more comprehensive exploration of these synergistic dynamics is warranted. Additionally, it is imperative to extend the investigation to ascertain the effects of Trn on healthy cells.

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ETHICS DECLARATION

The authors assert that, in accordance with prevailing ethical guidelines and regulations governing research involving cell lines, no Institutional Review Board (IRB) approval was deemed necessary for this study. The research exclusively employed cell lines as the primary material, therefore the study do not necessitate formal ethical approval.

AUTHORSHIP CONTRIBUTION

The author (NT) confirm responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

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