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Exploring apoptotic pathways in SH-SY5Y neuroblastoma cells: combined effects of napabucasin and doxorubicin

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ABSTRACT

Background: Neuroblastoma often begins in infancy and one of the most common types of cancer among children is someone. Napabucasin (NP) (BBI608), a natural naphthoquinone emerging as a novel inhibitor of STAT3, has been found to effectively kill cancer stem-like tumor cells. On the other hand, the effect of Napabucasin on SH-SY5Y cells is currently unclear. The effects and mechanisms of NP and doxorubicin (DX) on human metastatic neuroblastoma cells were investigated.

Materials and methods: In this study, human neuroblastoma cells line (SHSY-5Y) were used. Apoptotic activation of NP and DX via the *Bcl-2/Bax* signaling pathway was evaluated by qRT-PCR, western blot and Tali cytometry. It was also detected by MTT, a cell viability test.

Results: NP and DX antiproliferative and invasive effected to SH-SY5Y cells. Additionally, NP induced apoptosis by pausing the cell cycle. Moreover, NP treatment inhibited the expression of Bcl-2, which is associated with apoptosis, while it clearly inhibited the expression of *Bax* and *CASP3* genes.

Conclusions: As a results showed that NP and DX suppressed the proliferation of neuroblastoma cells and could do this through apoptotic pathways. NP can be used to

suppress metastasis of SHSY-5Y cells as an inhibitor of the apoptosis pathway *Bcl-2*. It is thought that NP, which provides tumor suppression through an apoptotic mechanism, may be an alternative treatment agent in neurological cancers such as neuroblastoma.
Keywords: napabucasin, apoptosis, SH-SY5Y, neuroblastoma, doxorubicin

INTRODUCTION

Neuroblastoma is one of the most common and deadly pediatric cancers, characterized by its aggressive nature and poor prognosis in advanced stages. Despite significant advancements in conventional therapies, including chemotherapy, radiation, and surgery, the survival rates for high-risk neuroblastoma patients remain dismally low. As a result, there is a novel therapeutic approaches that can enhance the efficacy of existing treatments. Napabucasin, a STAT3 inhibitor, has emerged as a promising candidate due to its ability to target cancer stem cells and disrupt key signaling pathways involved in tumor progression and resistance. When used in conjunction with Doxorubicin, a cornerstone chemotherapeutic agent, the potential for both synergistic and antagonistic interactions warrants thorough investigation.

Among the deadly diseases in the world, cancer is one of the diseases with the highest annual mortality rate. Cancer, which is the leading cause of death in China, is the second leading cause of death in the United States [19, 24]. Although surgery, chemotherapy and radiotherapy are the most common traditional methods for many types of treatment, recently immunotherapy, targeted chemotherapy and new therapeutics such as hormone drugs, cytotoxic compounds, biological response modifiers, etc. have become the main clinical treatment options in many developing countries due to promising results in certain patients. It has been included in the treatment [22]. Despite various anticancer drugs, their use is limited and endangered due to their side effects and the drug resistance they create. Therefore, the discovery of anticancer drugs with high effectiveness and minimal toxicity is important.

Neuroblastoma is one of the most common types of cancer in children and infants, and onset is common in infancy [15]. Treatments include surgery, chemotherapy, immunotherapy and radiotherapy. Despite the use of numerous anticancer drugs, it is very difficult to prevent the proliferation and invasion of neuroblastoma cancers [1, 25]. Targeting and downregulating signal transducer and transcription activator 3 (STAT3), which contains a wide range of genes, has been reported in all types of cancer (50–90%) [27]. However, the number of studies revealing the basic roles of STAT3 in neuroblastoma and proving its effectiveness is limited [7, 18]. There are studies showing that napabucasin (BBI608) acts by disrupting the self-

renewal mechanism of cancer stem cells via apoptosis in many types of cancer, especially in colon and pancreatic cancer [14, 16]. NP, which is newly discovered and is being developed and transformed into a more effective molecule every day, is an agent that can be administered orally as a STAT3 inhibitor. Combinations with many chemotherapy drugs used in current treatments are being tried [17, 24, 28].

The aim of this study is to reveal the combined effects of Napabucasin and Doxorubicin on apoptosis induction in neuroblastoma cells. The aim of the combination of these agents used was to reveal their synergistic and antagonistic interactions. Based on these interactions, it is aimed to provide new therapeutic strategies for neuroblastoma treatment and ultimately to improve patients.

MATERIALS AND METHODS

Culture and passage of cells

SH-SY5Y (CRL-2266™, American Type Culture Collection (ATCC) (human metastatic neuroblastoma cell line) was used in the study. SH-SY5Y cells line were cultured in F12 and EMEM (1:1 ratio) medium in containing 1% penicillin/streptomycin, 10% FBS and 2 mM L-glutamine. The cells were grown in sterile incubators at 37°C and 5% CO_2. The cells used in the study were used for analysis after the 5th passage and the study was terminated at most at the 15^{th} passage.

Mycoplasma control in cell lines

Mycoplasma controls of the studied cell line were performed. Mycoplasma contamination controls were performed by DNA fluorescence labeling with bisbenzimide (Hoechst 33258, Sigma). With the direct method; cell lines produced in an antibiotic-free medium for 3 passages were seeded in two 48-well cell culture plates with an initial cell count of 30,000. Cells produced in the medium containing antibiotics were used as the control group. Then, fixation was performed with 4% paraformaldehyde for 15 minutes. In accordance with the conditions specified by the company for bacterial and fixed cell staining, Bisbenzimide (Hoechst 33258) was incubated for 15 minutes at concentrations of 1 μg/mL and 5 μg/mL and the cells were visualized with a fluorescent microscope.

Determination of antagonistic-synergistic effect and formation of experimental groups

Stock solutions of Doxorubicin and Napabucasin were prepared using 1:1 absolute ethanol (Merck, Kenilworth, NJ, USA) and distilled water. 1 mM stock solutions were prepared for

NP and DX. The final concentration of the carrier in the wells was reduced to 0.1%. After the SH-SY5Y cells were multiplied, they were seeded in 96-well plates with a digital pipette at a density of 5 × 103 cells. After incubation for one night, single and combined applications of NP and DX were performed. In order to reveal the antagonist-synergist interaction between NP and DX, 8 different doses were applied starting from 10 μM concentration, serial dilution and dosing squares, and 64 different doses were applied. After 48 hours of incubation, cell viability was determined by MTT assay. After 48 hours of application of the agents, spectrophotometric readings were performed at 492 and 650 nm wavelengths with the microplate reader Multiskan GO (ThermoScientific, USA). The value obtained from the carrier-loaded control group was determined as 100% viability and the comparative viability rate was determined accordingly.

- **Group 1: Control group:** Only vehicle application was performed.
- **Group 2: Doxorobucin (DX) group:** 0.77 μM was applied.
- **Group 3: Napabucasin (NP) group:** 2.1 μM was applied.
- **Group 4: Doxorobucin + Napabucasin (DX + NP1):** 0.7 μM + 0.3 μM were applied.
- **Group 5: Doxorobucin + Napabucasin(DX + NP2):** 0.7 μM + 1 μM were applied.

TALI image based cytometer analysis

Apoptosis determination studies with Tali cytometer were performed according to the kit procedure using Tali® Apoptosis Kit–Annexin V AlexaFluor® 488 and PropidiumIodide (Life Technologies, Carlsbad, CA, USA). For the apoptosis determination study with the TALI cytometer, cells were planted in a 24-well plate with 1 mL of medium at approximately 5 × 104 cells/well. Depending on the proliferation status of the cells, they were incubated in a CO_2 for 1–2 days. Application was made with the application doses determined in the previous study. At the end of the 48-hour incubation period, the medium in the 24-well plates was removed. 1 mL trypsin EDTA was added to these wells, incubated for 10 minutes, and the cells were collected with an automatic pipette and placed in Eppendorf tubes. They were centrifuged at 700 rpm for 2 minutes. At the end of the centrifugation process, the upper trypsin and supernatant were completely removed and the cells that settled at the bottom of the tube were used for analysis. After incubation, 25 μL of the mixture in the tubes was poured onto special slides prepared for Tali and read with the Tali apoptosis analysis program.

RNA isolation and cDNA synthesis

SH-SY5Y cells used in the study were incubated in culture dishes until the logarithmic phase occurred after they were seeded. Then, IC50 doses of control, DX, NP and DX + NP1 and DX + NP2 doses were applied. RNAs were obtained by RNA isolation after 48 hours. Mini Purelink RNA kit (Thermo, Waltham, MA, USA) was used for RNA isolation and the kit protocol was applied. It was equalized to 1000 ng/10 μL with ultrapure water using Optizen NanoQ micro volume spectrophotometer (Mecasys, Suwon, South Korea). After synchronization, complementary DNA synthesis was performed to amplify RNAs. For this, High-Capacityc DNA Reverse Transcription Kit (Life Technologies) was used. After cDNAs were obtained, they were stored at –20°C to be used in the subsequent stages of the study.

Gene expressions

In the study, *BCL-2*, *BAX* and *CASP3* in control, NP and DX groups of SH-SY5Y cells; Expression levels of *BCL-2*, *BAX*, *CASP3* mitochondrial apoptosis pathway genes were analyzed by qRT-PCR method. The primers are given below, in 5'-3' order.

***Bcl-2*: F: ATGTGTGTGGAGAGCGTCAA, R: ACAGTTCCACAAAGGCATCC;**

***Bax*: F: TTCATCCAGGATCGAGCAGA, R: GCAAAGTAGAAGGCAACG;**

***Casp3*: F: GGTATTGAGACAGACAGTGG, R: CATGGGATCTGTTTCTTTGC;**

***β-Actin*: F: CCTCTGAACCCTAAGGCCAAC, R: TGCCACAGGATTCCATACCC;**

GAPDH: F:CGGAGTCAACGGATTTGGTCGTAT,R:GCCTTCTCCATGGTGGTGAAGAC.

cDNAs obtained from RNAs were used for gene expression. cDNAs were amplified in qRT-PCR according to the protocol of Pathway Scanner by Micromolecules qPCR Master Mix (Thermo).

Western blot

Cells were seeded into T75 flasks in 3 replicates for each of the control, NP and DX groups and drug applications were performed after clicking on the cells that adhered to the bottom of the flask. The incubated cells were lysed for protein isolation and the proteins that settled to the bottom were collected from the centrifuged tubes. In the study, *Bcl-2* (sc-492); *Bax* (B-9) (sc-7480); active *Caspase-3* (31A1067) (sc-56053) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin [ACTN05 (C4)] primary antibodies were obtained and then protein levels were determined by Western blot. Following SDS-PAGE electrophoresis, specific primary antibodies and secondary antibodies were applied and visualized by

chemiluminescence method (ChemiDoc MP System) and the results were analyzed by the system program.

Statistical analysis

Gene expressions, apoptotic cell rates and differences between means of the control, NP and DX groups were determined using one-way ANOVA, Tukey HSD test and SPSS 20 program, and significance was taken as $p \leq 0.05$.

RESULTS

MTT assay findings

The viability of SH-SY5Y cells was evaluated by various assays after treatment with different concentrations of NP and DX (0.1 and 10 μM) and vehicle control (0.1% DMSO) for 48 h. NP and DX exhibited a significant dose-dependent reduction effect on the viability of neuroblastoma cells (Fig. 1). In addition, half-maximum inhibitory concentrations were determined depending on the doses of NP and DX applied at different concentrations on SH-SY5Y cells. Accordingly, IC50 was determined as 2.10 μM for NP and IC50 as 0.769 μM for DX (Fig. 1). NP1 + DX and NP2 + DX groups were formed according to the IC50 values obtained. In order to determine the antagonist and synergist interactions, 8 different DX and NP doses prepared by serial dilution were combined and a total of 64 different dose applications were performed with the dose x response square model (Fig. 2). After the applications, the death rates on the SH-SY5Y cell line were determined with combenefit software when NP and DX were used together (Fig. 2).

Mycoplasma controls

Mycoplasma controls were performed on the SH-SY5Y cells studied. Mycoplasma contamination controls were performed by DNA fluorescence labeling with bisbenzimide (Hoechst 33258). No mycoplasma contamination was detected in the SH-SY5Y cell line (Fig. 3).

LOEWE scoring (antagonism–synergism)

NP and DX applications caused an increasing lethal effect in SH-SY5Y cell line depending on the dose. In NP + DX combinations, it was shown that NP added to DX application caused lethal effect on cancer cells while at the same time it could significantly reduce DX dose. This effect was evident in all combinations of NP + DX. It was determined that NP + DX

combinations had higher effects than the IC50 doses applied alone. It was seen that NP and DX created a synergistic effect with LOEWE scoring (Fig. 4). This effect was also statistically significant.

Apoptosis findings

In the study, TaLi image-based cytometry results after Annexin V:PI staining performed with the TaLi apoptosis kit are given in Figure 4. In the apoptotic staining performed with the Annexin V staining kit, it was observed that the cells undergoing apoptosis increased in NP + DX combination (Fig. 5). It was determined that the number of viable cells showed a statistically significant decrease compared to the control group. The effectiveness of NP in accelerating the apoptotic process was revealed.

Bcl-2, Bax, Caspase-3 gene expression and protein levels findings

Then, neuroblastoma cells were treated with Napabucasin and doxorubicin for 48 hours, and apoptotic protein expressions were analyzed by qRT-PCR. We found that *Bcl-2* expression was significantly reduced, *Bax* expression was increased, and *Casp3* expression was increased compared to the control group (Fig. 5). Additionally, apoptotic cells were counted by TALI cytometric analysis (Fig. 6). Compared to the control group, the number of apoptotic cells and dead cells increased significantly in the NP and DX applied groups. Synthesis was evaluated with the Click-iT Edu Imaging Kit. Collectively, Napabucasin upstream and downstream affected cell proliferation and in vitro apoptosis protein levels. According to the results of Western blot analysis, the analysis results showed parallelism. While there was a decrease in *Bcl-2* protein level compared to the control group, there was a significant increase in *Bax* and *Casp3* protein levels (Fig. 7).

DISCUSSION

The study were investigated the antagonistic and synergistic effects of NP and DX on the induction of apoptosis in neuroblastoma cells. These findings demonstrate a complex interaction between these two agents, highlighting both their potential therapeutic benefits and limitations. Napabucasin, a novel STAT3 inhibitor, has shown promise in targeting cancer stem cells and overcoming resistance mechanisms. When combined with doxorubicin, a well-established chemotherapeutic agent, we observed that the apoptotic response in neuroblastoma cells was significantly enhanced at certain concentrations, suggesting a synergistic effect. However, at other concentrations, the combination appeared to reduce

efficacy, indicating an antagonistic interaction. These results underscore the importance of optimizing dosage and treatment schedules to maximize the therapeutic potential of Napabucasin and Doxorubicin in neuroblastoma treatment.

Neuroblastoma is one of the most common types of cancer among children, which begins to appear in infancy [20, 24]. Radiotherapy, chemotherapy, immunotherapy and surgical methods are used for its treatment in the clinic [8]. Despite the diversity in its treatment and the testing of many anti-cancer drugs, neuroblastoma is a type of cancer whose proliferation and migration are difficult to prevent [2, 6]. It has been reported that drugs used in current treatment cause cancer cell death, but are insufficient to stop the invasion of cancer cells. Therefore, innovative treatments are needed to prevent or stop the invasion. In this context, innovative therapeutic approaches that target cancer cells and aim to stop their invasion are among the greatest expectations, especially reducing drug toxicity and improving patient survival. Studies have shown that phenolic compounds have effective results in many cancer studies and that new targets can be determined for innovative approaches in the light of these studies [9, 21].

The anti-oxidant effects of natural products have been determined by many studies. With their radical scavenging effects, phenolic compounds also reduce the risk of cancer formation [26]. In one of the in vitro studies, the anticancer activity of NP was reported [23]. These compounds were reported to exhibit a wide range of biological effects on carcinogenesis, in addition to their primary antioxidant effects. It was reported that the anti-proliferative and anticarcinogenic effects of these compounds were due to their aromatic rings and hydroxyl groups, which are similar to other phenolic compounds [10]. In this study, it was determined that NP exhibited an anti-proliferative effect on the SHSY-5Y cell line and that this effect was further increased by the DX combination. The number of studies showing the use of the NP and DX combination in cancer treatments is limited. Therefore, the proliferation suppression feature and apoptosis inducing ability of the obtained NP on SHSY-5Y cells should be investigated in more detail.

Induction of apoptosis in cancer cells constitutes one of the anticancer bases of the newly discovered Napabucasin, and many studies have reported that NP can activate both intrinsic and extrinsic apoptosis pathways to initiate the apoptotic process. In nonhodgkin lymphoma, cell viability was reduced by application of different concentrations of NP ranging from 0.001 to 2.0 μM for 48 and 72 hours, and again in diffuse large B-cell lymphoma, cells were found to be cleaved by *caspase-3* and Poly (ADP-Ribose) Polymerase-1 (PARP). It has been reported to be effective by significantly increasing its expression [12, 29]. In addition, it has

been reported that NP application is effective in drug-resistant lung cancer cells by driving the cells to apoptosis [17]. In cisplatin-resistant small cell lung cancer, NP also induced apoptosis by degrading PARP and suppressing the antiapoptotic proteins Mcl-1 and survivin [11]. In our study, it was observed that apoptosis was induced via *Bcl-2*/Bax/Casp3. Apoptosis was observed to increase with the combination of NP and DX.

There are few in vivo animal model studies on the anticancer effects of NP. It has been reported that NP, especially when applied as monotherapy, causes significant tumor shrinkage in xenograft mouse models of human acute myeloid leukemia, osteosarcoma and hepatocellular carcinoma [3, 5, 10, 29]. Another study reported that life span was extended in mice treated with glioma and melanoma [4, 5]. In addition, it has been reported that NP treatment inhibits bone osteolysis and bone resorption in BABL/c mice [10]. It also suppresses metastasis in mice with pancreatic cancer model [11]. Supporting the results of these studies, the main mechanisms of NP's anticancer effects in animal models include inhibiting cancer stem cells and suppressing their proliferation. NP treatment decreased Ki67-positive cells while also increasing the number of apoptotic cells; this suggests that NP triggers cancer cell apoptosis [12]. In our study, N accelerated cell death by triggering apoptosis through *Blc2*, *Bax* and *Casp3*, which are responsible for the mitochondrial apoptosis pathway.

It has been shown that anticancer effects are obtained from combination therapy of NP with other drugs through synergistic interaction. Napabucasin has been reported to interact synergistically with doxorubicin, docetaxel, and paclitaxel in a diffuse large B-cell lymphoma [13], prostate cancer [28], and ovarian cancer tumor model [10], respectively. The mechanism that reveals its synergistic potential may be the inhibition of cancer cells or the ability to induce apoptosis. In our study, it was observed that the apoptotic effect increased and death in cancer cells accelerated as a result of the combination of NP and DX.

CONCLUSIONS

We showed that Napabucasin has anti-proliferative and apoptotic effects on the human neuroblastoma cells. Also NP showed a synergistic effect with cell DX and induced apoptosis. NP treatment, taken together with DX, may be a new approach to suppress the progression and improve the prognosis of SH-SY5Y cells. We have shown that NP in combination with DX increases the levels of *Bcl-2*, *Bax* and *Casp3*, which regulate the proliferation of neuroblastoma cells, but further studies are needed to determine the intracellular mechanisms that produce this effect.

ARTICLE INFORMATION AND DECLARATIONS

Data availability statement

All data can be obtained from the corresponding author.

Ethics statement

This study does not require ethical approval.

Authors' contribution

İÜ: conceptualization, data curation, formal analysis, Investigation, methodology, software, supervision, validation, visualization, writing — original draft, writing — review & editing.

MCT: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing — original draft, writing — review & editing.

İÖ: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing — original draft, writing — review & editing.

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Conflict of interest

The authors declare that they have no competing interests.

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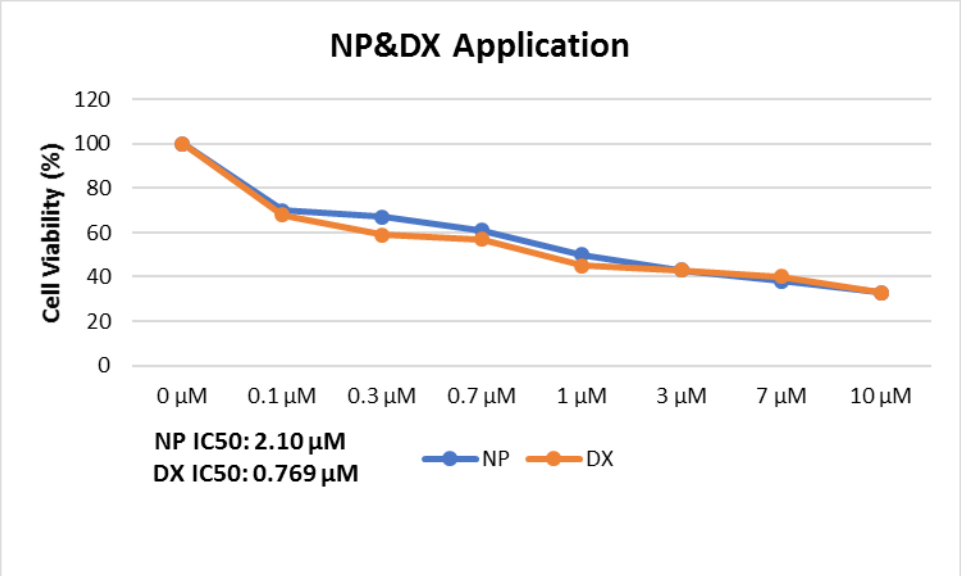

Figure 1. % Cell viability determined by MTT assay in SH-SY5Y cell line at different concentrations of NP and DX. DX — doxorubicin; MTT assay — 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; NP — nanoparticles.

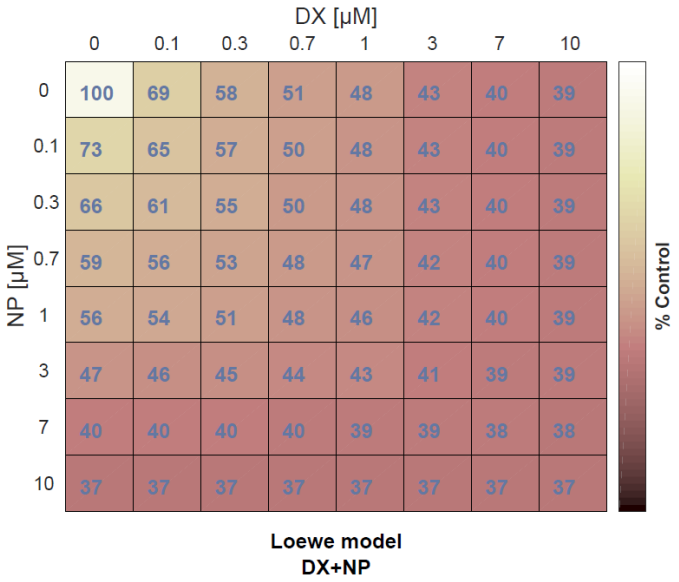

Figure 2. 64 Different doses of NP and DX in SH-SY5Y cell lines for 48 hours. DX — doxorubicin; NP — nanoparticles.

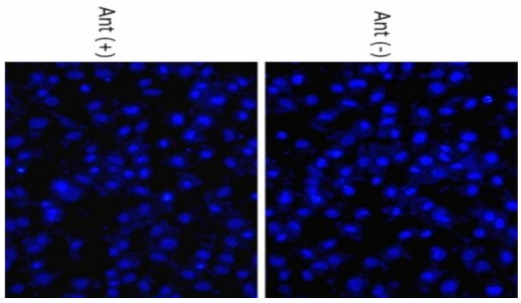

Figure 3. Staining of passages of SH-SY5Y cells produced in media containing antibiotics (Ant+) and not containing antibiotics (Ant–) with different concentrations of Bisbenzimide (Hoechst 33258). Magnification: ×20.

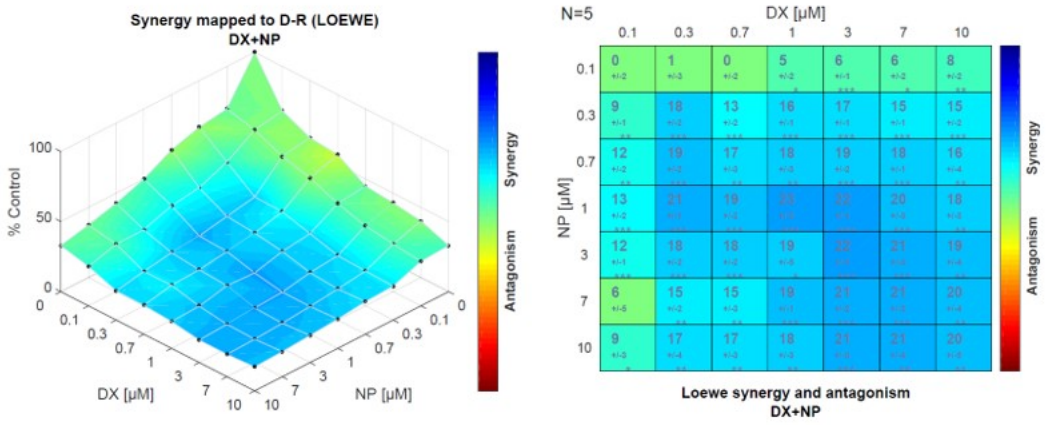

Figure 4. Antagonist and synergist effects of 64 different doses of DX and NP administration for 48 hours (LOEWE scoring). DX — doxorubicin; NP — nanoparticles.

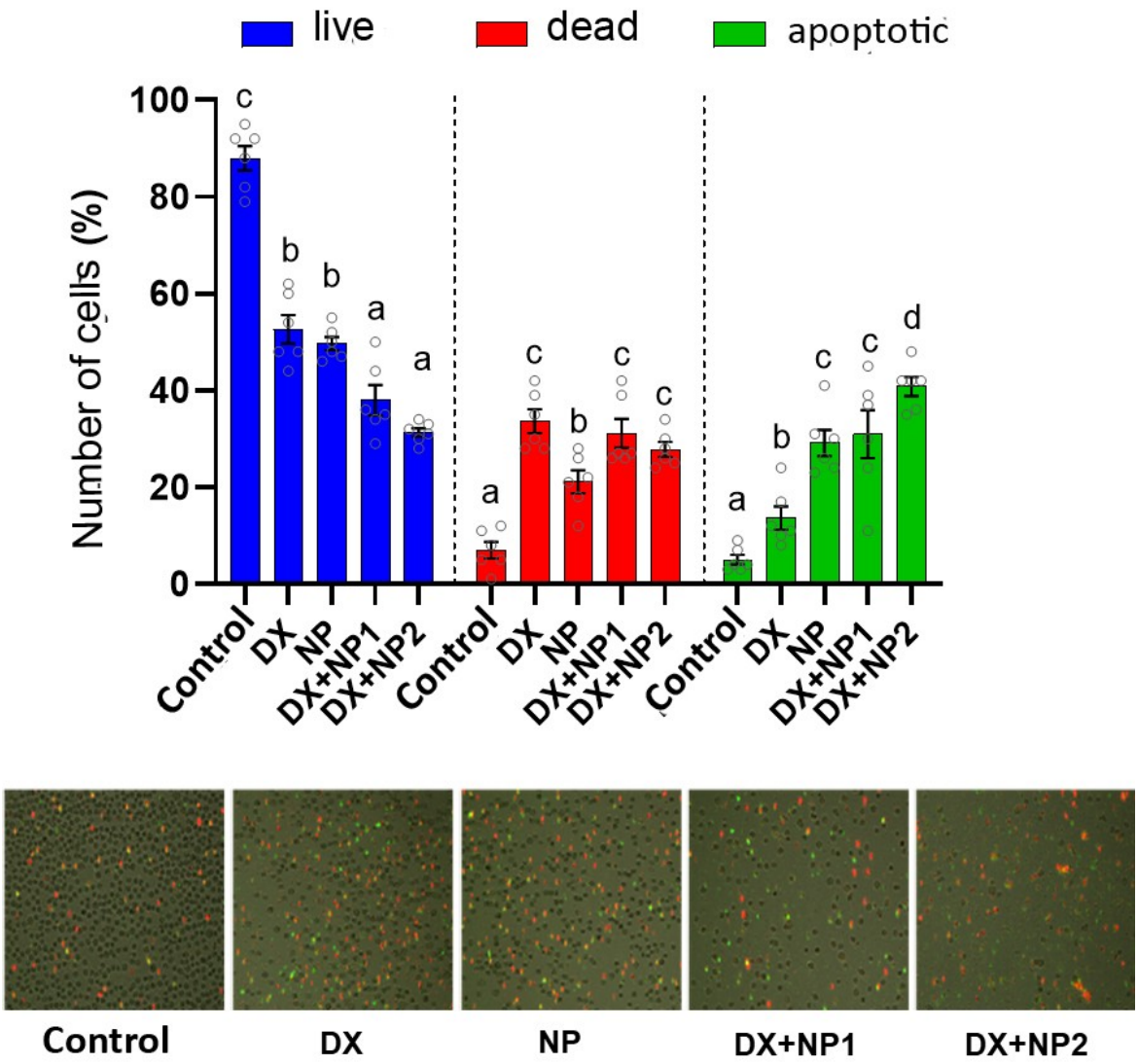

Figure 5. Control, DX IC50, NP, DX + NP1 and DX + NP2 for 48 hours proportions of live, dead, and apoptotic cells in cell populations determined by TALI image-based cytometry analysis. Data are mean±standard error. The averages marked with different letters within each group were statistically different. One-way ANOVA, Tukey HSD test, $p \leq 0.05$. DX — doxorubicin; NP — nanoparticles.

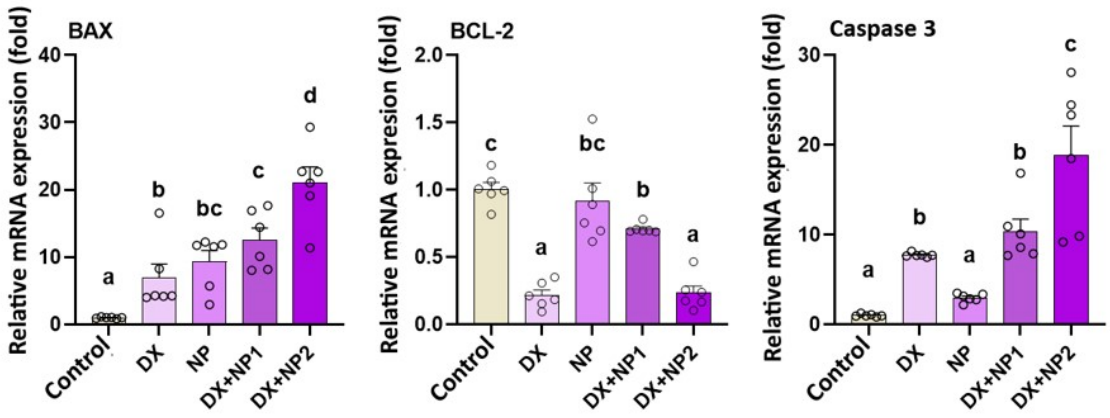

Figure 6. Relative fold values of *Bcl-2*, *Bax* and *Casp3* gene expressions 48 hours after single and combined application of NP and DX in SH-SY5Y cell line. [a, b, c, d, bc] means marked with different letters are statistically different, one-way ANOVA, Tukey HSD test, $p \leq 0.05$. DX — doxorubicin; NP — nanoparticles.

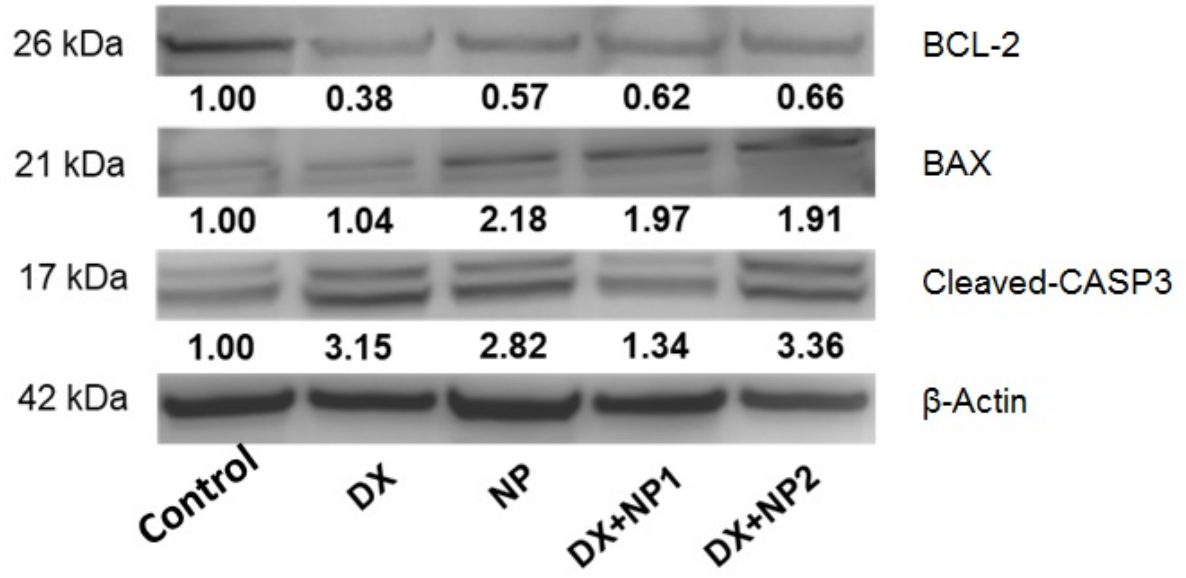

Figure 7. Control, NP and DX were applied to SH-SY5Y cell line. *Bcl-2*, *Bax* and active *Casp3* protein levels at the end of 48 hours. (All data were obtained from SH-SY5Y cell line treated with control, target protein/actin = 1. (given as relative fold change). DX — doxorubicin; NP — nanoparticles.