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Neuropeptide Y improves cisplatin-induced bone marrow dysfunction without blocking chemotherapeutic efficacy in a cancer mouse model

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Running title: Protective role of NPY in bone marrow dysfunction during cancer therapy

Keywords: Bone marrow dysfunction, Cancer, Chemotherapy-induced side effects, Neuropeptide Y, Hematopoietic stem cell

ABSTRACT

Cisplatin is the most effective and widely used chemotherapeutic agent for many types of cancer. Unfortunately, its clinical use is limited by its adverse effects, notably bone marrow suppression leading to abnormal hematopoiesis. We previously revealed that neuropeptide Y (NPY) is responsible for the maintenance of hematopoietic stem cell (HSC) function by protecting the sympathetic nervous system (SNS) fibers survival from chemotherapy-induced bone marrow impairment. Here, we show the NPY-mediated protective effect against bone marrow dysfunction due to cisplatin in an ovarian cancer mouse model. During chemotherapy, NPY mitigates reduction in HSC abundance and destruction of SNS fibers in the bone marrow without blocking the anticancer efficacy of cisplatin, and it results in the restoration of blood cells and amelioration of sensory neuropathy. Therefore, these results suggest that NPY can be used as a potentially effective agent to improve bone marrow dysfunction during cisplatin-based cancer therapy.

INTRODUCTION

The majority of cancer therapies are based on chemotherapeutic agents with cytotoxic effects, which cause cancer cell death by directly damaging DNA or by inhibiting cell division. Unfortunately, these agents are non-specific, thus, their administration often induces extended toxic effects in normal tissue as well (1). Cisplatin, which is one of the most widely used chemotherapeutic drugs (2–5), has been employed for the treatment of solid cancers such as ovarian, testicular, uterine, breast, stomach, brain, head-neck, and lung cancer (6–9). Although cisplatin has potent anticancer effects, its use is limited by various side effects such as neurotoxicity, nephrotoxicity, ototoxicity, and particularly bone marrow suppression (4, 10–13). Cisplatin-induced bone marrow damage is accompanied by acute nerve injury in the bone marrow (BM), resulting in sensory and autonomic neuropathy. Patients that have previously received cisplatin show irreversible chronic bone marrow failure, leading to the impairment of hematopoietic stem cells (HSCs) and bone marrow regeneration (13–15). Therefore, it is important to prevent bone marrow dysfunction during conventional chemotherapy using cisplatin without diminishing its anticancer efficacy.

Neuropeptide Y (NPY) is secreted from the brain or sympathetic nerves in the autonomic system and its involvement in a variety of physiological processes, including

food intake, energy storage, anxiety, stress, and pain perception, is well known (16–18). Several studies have reported that NPY is implicated in the regulation of cell death processes (19). Particularly, our recent study demonstrated that NPY can prevent sensory neuropathy and reduction in HSC abundance by protecting sympathetic nervous system (SNS) fibers in cisplatin-treated mice, suggesting the therapeutic potential of NPY to improve chemotherapy-induced bone marrow suppression (20, 21).

In this study, we have indeed demonstrated the protective effect of NPY against cisplatin-induced bone marrow dysfunction in a mouse model of ovarian cancer. Moreover, we found that NPY did not influence the chemotherapeutic effects of cisplatin, while protecting against reduction in HSC abundance and nerve injury from bone marrow impairment, suggesting its potential clinical utility as a protective agent for patients treated with chemotherapy.

RESULTS

NPY does not affect the anticancer efficacy of cisplatin in an ovarian cancer mouse model

Prior to determining the mitigating effect of NPY on cisplatin-induced bone marrow damage in a mouse model of cancer, we first tested whether NPY has influence on the anticancer efficacy of cisplatin. To establish a cancer xenograft mouse model, A2780 human ovarian cancer cells were transplanted subcutaneously in female athymic nude mice. After cancer establishment, the mice were randomized into 3 groups. One group was subjected to intraperitoneal injection (i.p.) with 10 mg/kg cisplatin, the second group was treated with 10 mg/kg cisplatin plus 50 μg/kg NPY, and the control group was treated with phosphate-buffered saline (PBS); the treatment duration was 7 weeks (Fig. 1A). During the observation period of 7 weeks, the PBS-treated group showed continuous increase in body weight and tumor growth, whereas the cisplatin-alone or cisplatin plus NPY-treated groups did not show any gain in weight and tumor volume (Fig. 1B, C). At the end of 7 weeks, the average tumor weights were 9.38 g in the PBS group, 3.34 g in the cisplatin group, and 1.12 g in the cisplatin-plus-NPY group (Fig, 1D, E). Interestingly, the cisplatin plus NPY-treated group showed a tendency for further decrease in tumor weight. Therefore, these results suggest that NPY did not affect the

anticancer efficacy of cisplatin therapy in an ovarian cancer mouse model.

NPY prevents cisplatin-induced reduction of HSC abundance in BM

Decrease in the number of HSCs, which are responsible for the regeneration and repopulation of all blood cell lineages, is a main pathogenesis mechanism in cisplatin-induced bone marrow dysfunction (13, 15, 22). To examine whether NPY could prevent reduction in HSC abundance upon cisplatin treatment in our ovarian cancer model, we analyzed phenotypic HSCs in BM by flow cytometry. Although no significant differences in the numbers of bone marrow nucleated cells (BMNCs) were observed between the groups (Fig. 2A), the PBS- and cisplatin-treated groups in the cancer-induced groups showed a marked decrease in the number of $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ (LSK) and $LSKCD48^{-}CD150^{+}$ (LT-HSCs) cells compared to the PBS-treated sham group. However, cisplatin plus NPY-treated group in the cancer-induced groups showed increase of BM HSCs as normal condition (PBS-treated sham group) (Fig. 2B–D). These results implied that NPY treatment recovered reduction of BM HSCs, and promoted its survival. Moreover, NPY prevented the reduction of blood cell lineages observed in the cisplatin-treated group (Table 1). Taken together, these results suggest that NPY could ameliorate cisplatin-induced HSC impairment in an ovarian cancer mouse model.

NPY counteracts cisplatin-induced reduction of SNS fiber and EC abundance in the BM

Recent studies reported that cisplatin-induced SNS injury in BM induces sensory neuropathy, and impairs HSC function by reducing the number of endothelial cells (ECs), which are cells of the BM microenvironment cell related to HSC survival (15, 23). In addition, our previous study demonstrated that NPY is required for the maintenance of HSC function by protecting SNS fibers and EC survival within the BM (20, 21). Here, we confirmed these protective roles of NPY during chemotherapy in an ovarian cancer model, where cisplatin-induced sensory neuropathy was ameliorated by NPY treatment (Fig. 3A). The decrease in the number of SNS fibers, which were stained with an antibody against the catecholaminergic enzyme tyrosine hydroxylase (Th), and of ECs was also prevented in the NPY-treated group compared to that by cisplatin alone (Fig. 3B–C). The cytotoxic effects of cisplatin induce direct cell death in cancers and even in normal tissues, and NPY can protect against cell death by regulating apoptosis signaling (19–21). We observed increased apoptosis levels in the BM of PBS- or cisplatin-treated groups, whereas the cisplatin plus NPY-treated group showed significant reduction in apoptosis (Fig. 3D). Taken together, these results strongly

suggest that NPY can prevent sensory neuropathy and bone marrow damage during cisplatin therapy in an ovarian cancer model.

DISCUSSION

Chemotherapy-induced BM suppression is a severe side effect of cancer therapy. Moreover, in cancer patients, chronic BM damage due to chemotherapy is accompanied by impaired HSC function and mobilization, and leads to hematopoiesis abnormalities (13, 14, 24, 25). Neurotoxicity due to chemotherapy drugs such as cisplatin can damage autonomic nerves in the BM and compromise hematopoietic regeneration, suggesting that neuroprotection may preserve hematopoietic reserves and regulate HSC mobility after chemotherapy (15). Recently, we demonstrated new roles of NPY as a regulator of HSC survival and mobilization (20, 21, 26). In particular, we revealed that NPY treatment prevented cisplatin-induced HSC impairment and reduced cisplatin-induced apoptosis of SNS fibers and cells of the BM microenvironment, showing that the neuroprotective effects of NPY could mitigate HSC dysfunction due to chemotherapy (20, 21). However, the protective effects of NPY in BM has not been fully explored during cisplatin therapy in cancer mouse models.

In this study, we first tested whether NPY diminished the chemotherapeutic effects of cisplatin in any ovarian cancer mouse model. The results show that NPY did not inhibit the anticancer efficacy of cisplatin; instead, the decrease in the tumor weight

upon cisplatin treatment was more pronounced in the presence of NPY, suggesting that NPY might enhance the anticancer efficacy of cisplatin (Fig. 1). In addition, we observed that NPY treatment prevented the cisplatin-induced HSC suppression, resulting in the recovery of the complete blood count (Fig. 2). This is important because NPY can be administered in patients who either have undergone chemotherapy or suffer from disorders with abnormal hematopoiesis. The sympathetic nerve fibers regulate HSC survival and trafficking by acting on cells of the BM niche, and destruction of nerve fibers in the BM causes critical peripheral neuropathies (13–15). Our results show that decrease in Th fiber abundance by cisplatin in an ovarian cancer model was prevented by NPY treatment, leading to the mitigation of sensory neuropathy. Moreover, NPY significantly counteracted the cisplatin-induced reduction in EC abundance and increased apoptosis levels in the BM (Fig. 3). Taken together, these observations indicate that in an ovarian cancer mouse model, NPY could protect against sensory neuropathy and BM damage without blocking the chemotherapeutic efficacy of cisplatin.

Nephrotoxicity, which is another side effect of chemotherapy, also limits the use of cisplatin in cancer therapy. We recently confirmed that NPY treatment could attenuate cisplatin-induced nephrotoxicity by regulating the pro-apoptotic pathway, resulting in protection against renal dysfunction (27). This our previous study suggests

that NPY can also be used as an effective agent for renoprotection, as well as neuroprotection. During the last few years, several researchers have demonstrated that a broad range of natural compounds may mitigate the side effects of chemotherapy. Importantly, NPY is a stable peptide that is naturally synthesized in the human body. Therefore, therapeutic applications using NPY may provide clinical benefits and reduce side effects in patients previously treated with chemotherapy.

MATERIALS AND METHODS

Ovarian cancer xenograft mouse model

Six- to 8-week-old, female, athymic nude mice (BALB/c Slc-nu/nu) were purchased from SLC (Japan). The A2780 cells were obtained from ECACC (93112519, Salisbury, Wiltshire/UK) and cultivated in RPMI-1640 medium supplemented with 10% FBS, and 1% streptomycin and penicillin (all from Gibco). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Female athymic nude mice were inoculated with 5×10^6 A2780 cells in 100 μl saline by a subcutaneous injection at the flank. After inoculation, tumor growth was monitored for approximately 2 weeks, using a vernier caliper. When the tumor size reached approximately 200 mm^3, the animals were randomly divided into 3 groups to receive a 7-week treatment with cisplatin, cisplatin plus NPY, or PBS as a control. During the treatment, body weight and tumor size were monitored once a week. A block randomization method was used to divide the animals into different experimental groups. To eliminate bias, investigators were blinded during data collection and analysis. Mice were housed under a 12-hour day-night cycle with free access to tap water and food pellets. All mouse studies were approved by the Kyungpook National University Institutional Animal Care and Use

Committee.

Drug treatment

Cisplatin (Enzo; 10 mg per kg of body weight, once per week) was used for chemotherapy, and the mice received i.p. injections of cisplatin for 7 weeks. To investigate the protective effect of NPY against cisplatin-induced BM dysfunction, the mice received i.p. injections of NPY (Bachem; 50 μg per kg body weight, H-6375) daily during the 7-week cisplatin-treatment period. After 1 h from the last injection, the BM and blood were collected and analyzed.

Flow cytometry

The BM was flushed from the tibia and femur of each mouse. Red blood cells (RBCs) were lysed once for 5 min at 4 °C in 0.15 M NH_4Cl (StemCell Technologies), washed once with PBS (Gibco), and counted using a cell counter (LUNA™ Automated Cell Counter). For the detection of LSK cells and LT-HSCs, $Lineage^+$ cells were removed by magnetic depletion using biotinylated lineage-specific antibodies (CD5, CD45R, CD11b, Gr-1, and Ter-119), followed by depletion with MACs beads conjugated to a monoclonal anti-biotin (Miltenyi Biotec). $Lineage^-$ cells were stained with

phycoerythrin PE-Cy7-conjugated antibodies to Sca1 (558162), APC-conjugated antibodies to c-kit (553356), FITC-conjugated antibodies to CD48 (557484), and PE-conjugated antibodies to CD150 (561540), all from BD Biosciences. Cells were further stained with streptavidin-pacific blue (PB) (Invitrogen, S11222). Data were collected on a BD AriaIII (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

Quantification of sensory neuropathy by the heated-pad assay

To evaluate the effects of different treatments on the sensory response, we performed the hot-plate test, as previously described (28). We used a flattering hot plate (Panlab, Harvard Apparatus) maintained at 50 °C. Mice were individually placed on top of the heated surface, and the time to the first episode of nociception (jumping or paw licking) was measured. The cutoff time was 60s. Between measurements, the heated surface was thoroughly cleaned with detergent and ethanol, and the temperature was allowed to stabilize to 50 °C.

Immunofluorescence staining of BM sections

Frozen BM sections were prepared and immunostained according to a previously published method (29). The BM sections were fixed in dry ice/hexane, and incubated

first with the primary antibody and then with a secondary antibody conjugated with Alexa488 (Life Technologies). Immunofluorescence data were obtained and analyzed using a laser scanning confocal microscope equipped with the Fluoview SV1000 imaging software (Olympus FV1000; Japan). The MetaMorph software (Molecular Devices) was used to calculate the average intensity and cell number. Antibodies used were as follows: Th (Millipore, AB152 or AB318, 1:250 dilution), and CD31 (BD Biosciences, 550300, 1:50 dilution). TUNEL assays were performed using the In Situ Cell Detection Kit, Fluorescein (Roche Diagnostic) according to the manufacturer's instructions.

Statistical analysis

One way analysis of variance (ANOVA) was conducted, followed by Tukey's HSD test for comparisons between more than two groups. All statistical analyses were performed using the SPSS statistical software. A value of $P < 0.05$ was considered to denote statistical significance.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests**.**

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FIGURE LEGENDS

Fig. 1. Influence of NPY on the therapeutic efficacy of cisplatin in a mouse ovarian cancer model.

(A) Experimental design to investigate the effect of NPY in cisplatin-induced bone marrow dysfunction. A cancer mouse model was established in athymic nude mice by inoculation of A2780 ovarian cancer cells. After the tumors had grown to approximately 200 mm^3, the animals were randomly divided into 3 groups, which were treated with PBS (daily), 10 mg/kg cisplatin (once a week), or 10 mg/kg cisplatin (once a week) plus 50 μg/kg NPY (daily). (B) Body weight and (C) tumor volume of each group during treatment (left) or after 7 weeks (right) (n = 5 mice per group). (D) Tumor weight after 7 weeks. (n = 5 mice per group). (E) Representative mice and dissected tumors. *P < 0.05. All error bars indicate the standard errors of the mean (S.E.M.).

Fig. 2. Mitigation of cisplatin-induced reduction in HSC abundance by NPY

treatment.

(A) Number of BMNCs of each group after 7 weeks of treatment (n = 5 mice per group). (B) Representative flow cytometry plot from the BM of each group (n = 3–4 mice per group). The BM was gated first based on Lineage$^-$ cells, then based on Sca-1$^+$ c-kit$^+$ to detect LSK cells, and finally based on CD48$^-$ CD150$^+$ cells to detect LT-HSCs. (C and D) Percentage of LSK (Lineage$^-$ Sca-1$^+$ c-kit$^+$) cells and LT-HSCs (Lineage$^-$ Sca-1$^+$ c-kit$^+$ CD48$^-$ CD150$^+$) in the BM of each group (n = 3~4 mice per group). *P < 0.05. All error bars indicate S.E.M.

Fig. 3. Protective effect of NPY against cisplatin-induced sensory neuropathy and cell death in the BM microenvironment.

(A) Quantification of sensory neuropathy in each group (n = 5 mice per group). (B) Left, representative immunofluorescence images to detect the presence of Th$^+$ fibers. Scale bar, 50 μm. Right, quantification of Th$^+$ fibers in the BM of each group (n = 5 mice per group). (C) Left, representative immunofluorescence BM images of CD31$^+$ ECs. Scale bar, 40 μm. Right, number of CD31$^+$ ECs per femur in each group (n = 5 mice per group). (D) Left, representative immunofluorescence images of the BM showing apoptosis by TUNEL staining. Scale bar, 50 μm. Right, percentage of apoptotic cells in

BM of each group (n = 5 mice per group). *P < 0.05. All error bars indicate S.E.M.

Table 1. Increased blood cell recovery in NPY-treated mice models of cancer with cisplatin treatment. Complete blood counts of each group (n = 5 mice per group).

	Sham	Cancer		
	PBS	PBS	Cisplatin	Cisplatin/NPY
WBC (K/ml)	0.9±0.1	0.3±0.03*	0.5±0.1*	0.8±0.1 #
RBC (M/ml)	8.7±0.2	5.5±0.2*	6.8±0.7*	8.3±0.2 #
Hgb (g/dl)	14.7±0.2	10.8±0.5	14.5±0.2	14.2±0.1
HCT (%)	75.0±0.8	58.0±3.1*	69.7±3.5	71.5±0.6
MCV (fl)	86.1±1.1	113.6±3.0	85.9±0.8	89.5±4.0
MCH (pg)	16.8±0.2	21.2±0.8	17.9±0.9	17.4±0.5
MCHC (g/dl)	19.6±0.1	18.6±0.2	19.7±0.2	19.5±0.3
RDW-CV (%)	19.0±0.4	26.4±2.8	20.3±1.4	18.7±0.6
PLT (K/ml)	1002.8±18.2	895.5±63.1	856.5±79.8	946.6±36.0 #

WBC: white blood cells; RBC: red blood cells; Hgb: hemoglobin; HCT: hematocrit;

MCV: mean cell volume; MCH: mean corpuscular hemoglobin content; MCHC: mean corpuscular hemoglobin concentration; RDW-CV: red cell distribution width-coefficient of variation; PLT: platelets; MPV: mean platelet volume. *P < 0.05 versus Sham/PBS group. #P < 0.05 versus Cancer/Cisplatin group.

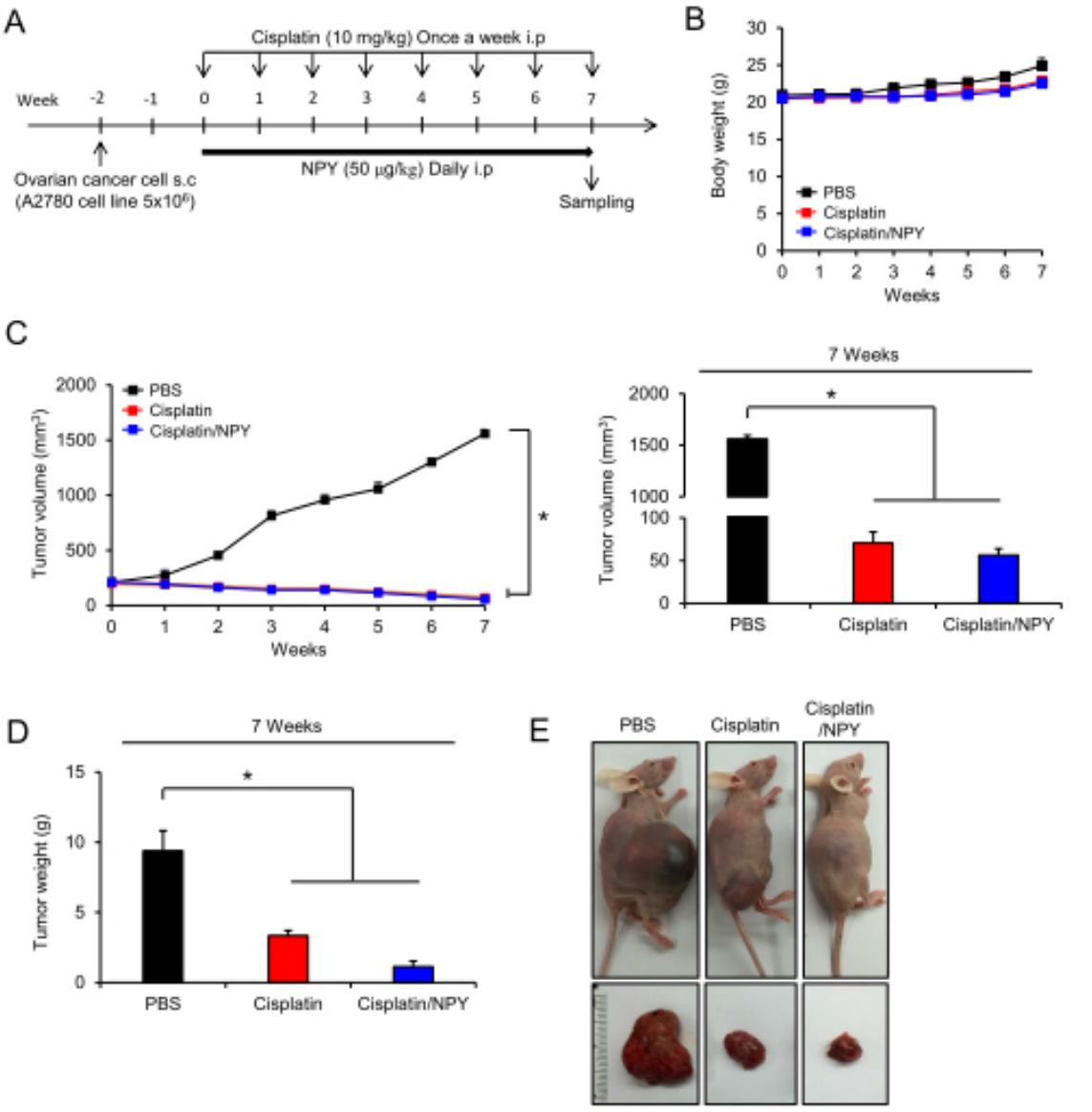

Figure 1

Fig. 1 Figure1

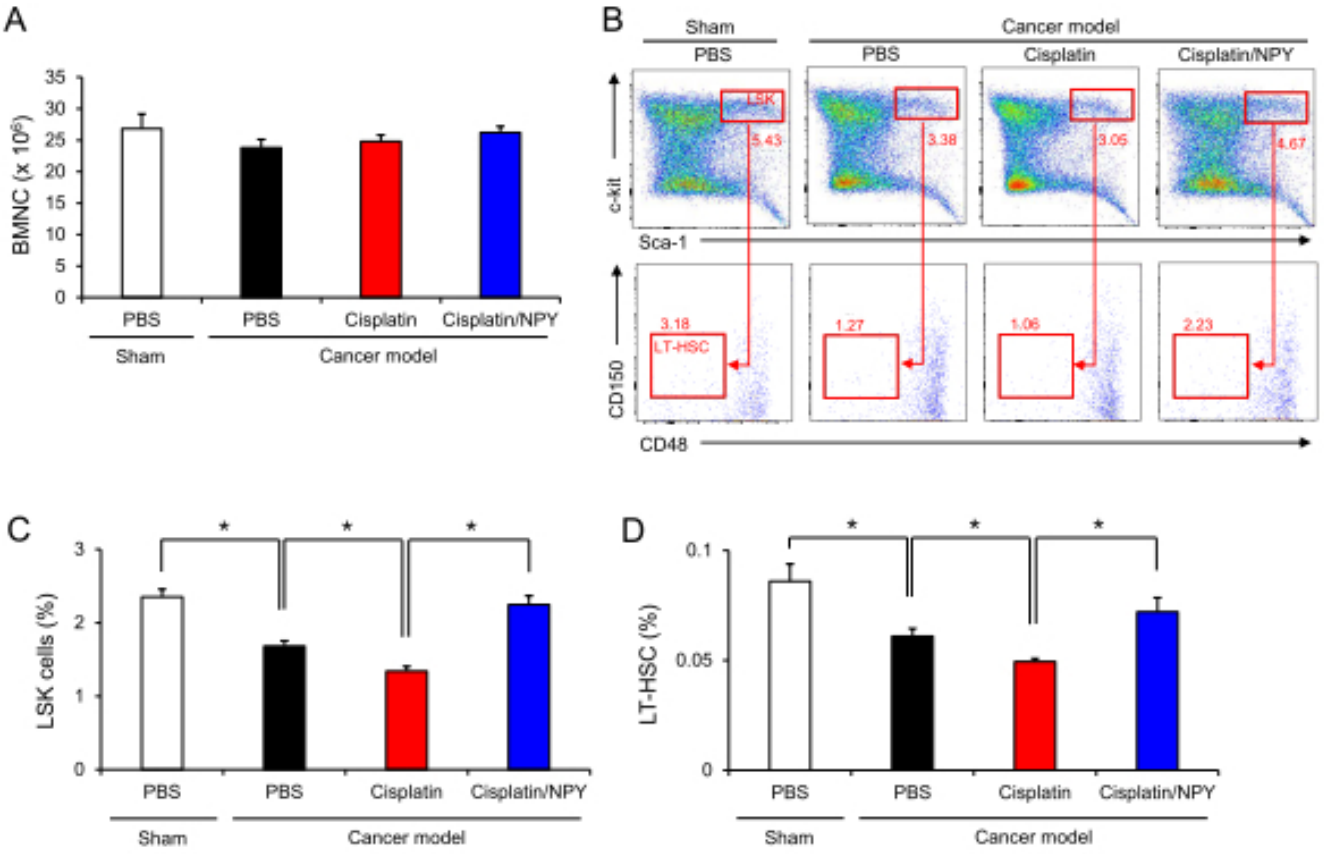

Figure 2

Fig. 2 Figure2

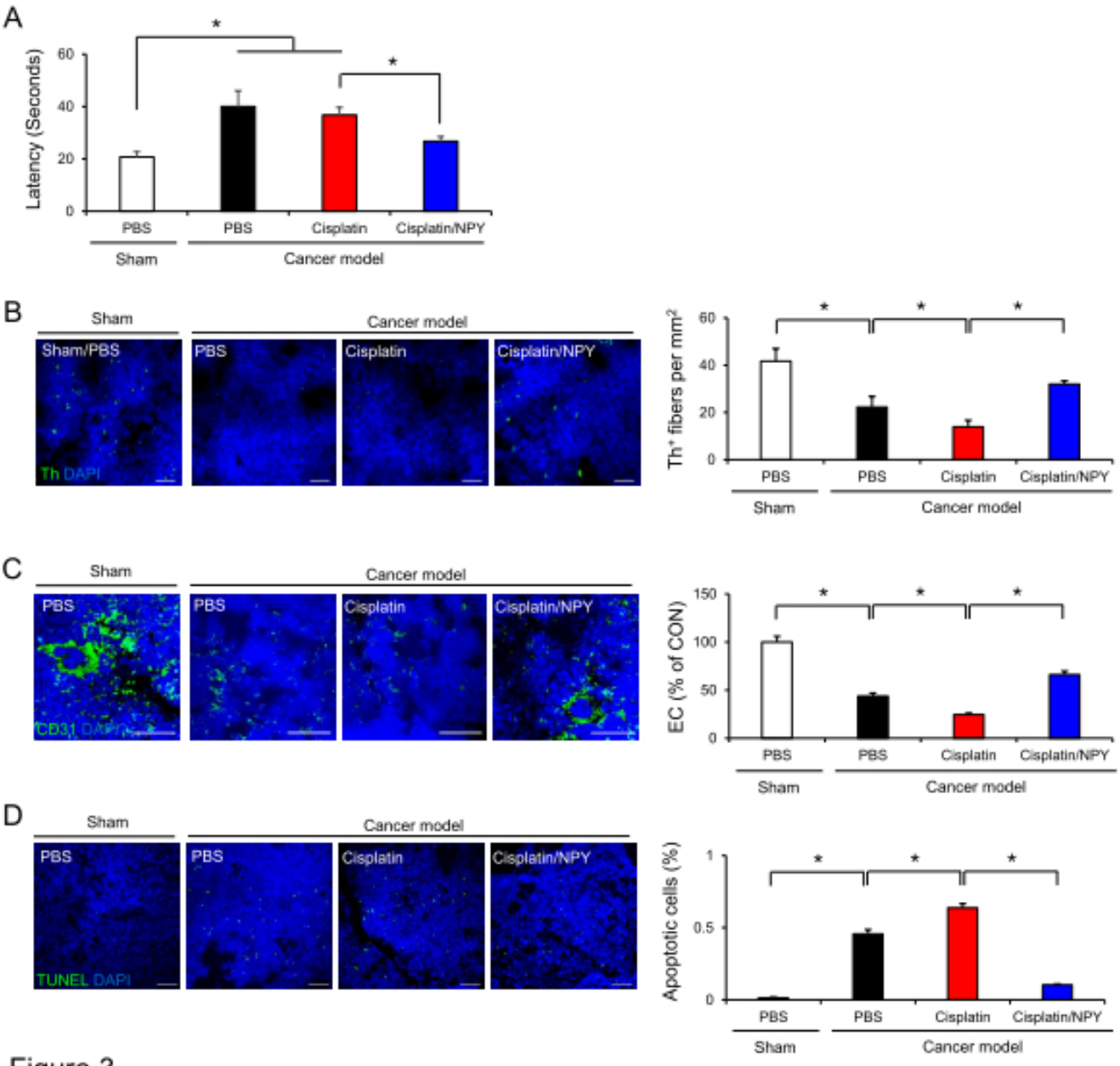

Figure 3

Fig. 3 Figure3