

Silencing of survivin using YM155 induces apoptosis and chemosensitization in neuroblastomas cells

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Abstract. – OBJECTIVES: Aggressive cell growth and chemoresistance are notorious obstacles in neuroblastoma therapy. Accumulating evidence suggests that survivin is preferentially expressed in cancer cells and plays a crucial role in cell division and apoptosis dysfunction. Thus, in the present study, we investigated whether silencing of survivin, using a novel small-molecule survivin suppressant, YM155 could suppress the proliferation and induce chemosensitization of neuroblastoma cells.

MATERIALS AND METHODS: SH-SY5Y human neuroblastomas cells were treated with YM155 (10 to 500 μ M) and/or chemotherapeutic agent cisplatin for 72 hours, and cell viability, apoptosis, mRNA and protein expression level were then evaluated. Furthermore, the efficacy of YM155 combined with cisplatin was further examined in established xenograft models.

RESULTS: YM155 suppressed expression of survivin, inhibited the proliferation and induced apoptosis in SH-SY5Y cells in a concentration-dependent manner. Reduced levels of survivin sensitized SH-SY5Y to the chemotherapeutic agent cisplatin. YM155 showed antiproliferative effects and induced tumor regression and apoptosis in established SH-SY5Y xenograft models. Cisplatin showed antitumor activity against SH-SY5Y cells, it did not induce survivin upregulation. Combination treatment of YM155 and cisplatin induced a greater rate of apoptosis than the sum of the single-treatment rates and promoted tumor regression without enhanced body weight loss in the SH-SY5Y xenograft models.

CONCLUSIONS: The concomitant combination of YM155 with cisplatin induced more intense apoptosis compared with each single treatment *in vivo* and *in vitro*. YM155 in combination with cisplatin is well tolerated and shows greater efficacy than either agent alone in mouse xenograft models.

Key words:

Neuroblastoma, Survivin, YM155, Apoptosis, Chemotherapy.

Abbreviations

MYC: myelocytomatosis gene; IAP: inhibitor of apoptosis; SAO: survivin antisense oligonucleotides; PBS: phosphate buffered saline; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; RT-PCR: reverse transcriptase-polymerase chain reaction; TBE buffer: Tris/Borate EDTA buffer; TDT buffer: terminal deoxynucleotidyl transferase buffer; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; DMSO = dimethyl sulfoxide.

Introduction

Neuroblastomas (NB) are pediatric tumors that originate from the embryonal precursor cells of the sympathetic nervous system. MYCN amplification, gain of 17q, and deletion of 1p are frequently occurring genetic abnormalities in neuroblastoma and all correlate with a bad prognosis. Risk stratification is based on tumor stage according to the 'International Neuroblastoma Staging System' (INSS), age of the patient, and genetic risk factors as MYCN amplification and deletion of 1p. Patients with low-risk tumors can be treated by surgery alone and have a very good prognosis. However, patients with high-risk disease are treated with intensive chemotherapy, surgery, and high-dose myeloablative therapy to eradicate minimal residual disease. Despite extensive treatment, children with high-stage neuroblastoma have a poor prognosis with 20-35% overall survival¹⁻². Advanced NB patients generally have a poor prognosis with currently approved therapeutic agents, underscoring the need for more effective treatment options.

Survivin is a member of the inhibitor of apoptosis (IAP) protein family and has been implicated in both cell survival and regulation of mitosis in cancer³⁻⁵. Survivin is highly expressed in all primary tumor types⁴ but is undetectable in most normal differentiated tissues, with the exception of placenta, testes, and rapidly dividing cells such as CD34+ bone marrow stem cells⁶. Because a correlation ex-

ists between high expression of survivin in tumors and poor survival among patients with various cancers including NB⁷⁻¹¹, survivin is considered a putative novel target in various cancer therapies.

Many studies found survivin inhibition by survivin antisense oligonucleotides (SAO) or siRNA (shRNA) could induce apoptosis and decrease the viability of human neuroblastoma cells¹²⁻¹⁴. Studies have shown that chemoresistance in neuroblastoma cell line SK-N-MC can be correlated to an overexpression of the inhibitor of apoptosis surviving¹⁵.

YM155(1-(2-Methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho[2,-d]imidazolium bromide) is a small-molecule inhibitor of the antiapoptosis protein survivin. YM155 was selected as a specific inhibitor of survivin expression from high throughput screening using a survivin promoter luciferase assay.

In preclinical experiments, YM155 inhibited survivin mRNA and protein expression in a dose- and time-dependent manner, resulting in activation of caspases and apoptosis induction in a broad array of human tumor cell lines¹⁶. YM155 induced tumor regressions in mice-bearing established human tumor xenografts¹⁶. Furthermore, YM155 enhanced the antitumor activity of cytotoxic agents in several cell lines¹⁷.

Here, we investigated whether silencing of survivin using YM155 shows distinct antitumor activity and inducing of chemosensitization in preclinical NB models.

Materials and Methods

Cell culture

SH-SY5Y cells were obtained from the American Type Culture Collection. The SH-SY5Y cells are aggressive and poorly differentiated. Cells were cultured in 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 2 mM *L*-glutamine and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ atmosphere.

Reagents

YM155 were obtained from Xin Xing Tang Biotechnology Co., Ltd. Beijing, China. Cisplatin was from Deyao, Co., Ltd., Dezhou, China. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture

medium [DMSO final concentration, 0.1% (v/v)]. Drugs were dissolved and diluted in saline immediately prior to administration.

Cell growth inhibition Assay

The SH-SY5Y cell lines were used to determine the inhibitory effect of YM155 on cell growth using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (8 × 10³/well) were plated in 96-well plates and then cultured in medium with or without various concentrations of YM155 or/and cisplatin alone or in combination. Control cultures received 0.1% DMSO. Three days after the treatment, the percentage of viable cells in each well was examined by MTT assay (Sigma Chemical Co, St. Louis, MO, USA), using the SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Determination of apoptosis

Cells were harvested by trypsinization and washed with PBS. They were fixed in ice-cold ethanol, washed, resuspended in PBS and treated with RNase A. Finally, cells were stained with propidium iodide (Sigma-Aldrich, Bornem, Belgium). The stained cells were analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, Erembodegem, Belgium) and DNA content was quantified using CellQuest Pro software (BD Biosciences, Erembodegem, Belgium). Apoptosis was analyzed by assessing sub-G0 events in the euploid cell fraction.

Western blot analysis

Protein was extracted using western blot assay. Extracted protein (20 mg) samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking at room temperature with TBS-T buffer containing 10% Blocking One (Nakalai Tesque, Kyoto, Japan), each membrane was incubated overnight at 4°C with the primary antibody anti-survivin (Cell Signaling, Beverly, MA, USA), mouse anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed with TBS-T and incubated with horseradish peroxidaseconjugated secondary antibody for 1 hour at room temperature. Proteins of interest were visualized by enhanced chemiluminescence using ECL (Amersham, Buckinghamshire, UK).

RT-PCR

SH-SY5Y were cultured in medium with or without various concentrations of YM155 or/and

cisplatin alone or in combination. Control cultures received 0.1% dimethyl sulfoxide. For RNA extraction, Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's protocol and the RNA concentration was determined using the NanoDrop ND-1000. cDNA was made from 1 mg of the extracted RNA with 12.5 pM t12 primer in mQ at 70°C for 10 min. A mix was added with the final concentrations of 2 mM MgCl₂, 0.5 mM dNTP, 1×Fs-buffer, and superscript III (Invitrogen, 100 U) in mQ. The reaction was performed at 50°C for 60 min and 70°C for 15 min. The primers (Biolegio, Nijmegen, The Netherlands) used for PCR were as follows: forward: 5'-GCATGGGTGCCCC-GACGTTG-3', reverse: 5'-GCTCCGGCCA-GAGGCCTCAA-3'. RT-PCR were performed in a final concentration of 312.5× diluted cDNA, 1 ng/ml forward primer and reverse primer, and 2× diluted reddymix in mQ. After activation of Taq at 94°C, PCR followed with 28 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min with a final extension at 72°C for 5 min. Equal volumes of PCR products were electrophoresed through a 1% agarose gel in TBE buffer.

In vivo antitumor activities against SH-SY5Y s.c. xenograft model

Four-old male nude mice (BALB/c nu/nu) were purchased from the Lab Center of Shandong University. SH-SY5Y cells (2×10^6) were injected into the flanks of the mice and allowed to reach a tumor volume of $> 100 \text{ mm}^3$ in tumor volume ($\text{length} \times \text{width}^2/2$). YM155 was i.v. administered five times a week for 3 weeks. Cisplatin was administered i.v. 5 times (once every 4 days). Maximum tolerated dose (MTD) was determined in a separate study. Body weight and tumor diameter were measured twice a week, and tumor volume was determined ($\text{length} \times \text{width}^2/2$). Observation continued until the day 21. The survivin expression levels of frozen tumors was analyzed by immunocytochemistry and the apoptotic index by TUNEL.

Immunocytochemistry

Immunohistochemical studies were done on 4-μm-thick sections derived from zinc-fixed, paraffin wax-embedded tumor tissue blocks. These tumors were harvested at the end of the experiments (after 21 days). Sections were subsequently dewaxed, rehydrated, and had endogenous peroxidase activity quenched before survivin specific

immunohistochemical staining. After specific staining or hematoxylin and eosin staining, sections were dehydrated in alcohol and xylene and subsequently mounted.

TUNEL assay

Briefly, frozen tumor samples were cut on a cryostat and got 4 μm-thick sections. Slices were fixed with acetone and soaked in 3% hydrogen peroxide-methanol solution for 20 min, then rinsed in PBS for 3 times. Fixed sections were incubated with proteinase K (20 μg/ml) for 20 min at 37°C and washed with TBS for 3 times. Then they were incubated with TdT buffer containing digoxigenin labelled desoxyuridinetriphosphate (DIG-UTP) and dATP for 2 h at 37°C, and immersed in stop solution for 30 min at room temperature. After washing, the sections were incubated with strept avidin-biotin-peroxidase complex (SABC) antibody solution for 60 min at 37°C, followed by incubation with DAB for 20 min. Slices were counterstained with hematoxylin and examined with a fluorescence microscope.

Statistical analysis

Results are expressed as relative change compared with DMSO controls and are given as the mean±SEM. The statistical differences between groups were tested using a two-tailed Student's *t*-test. Statistical significance was assumed for *p*-values < 0.05 .

Results

YM155 inhibits endogenous survivin expression in SH-SY5Y cells in vitro

We confirmed the *in vitro* effect of YM155 on endogenous survivin expression in SH-SY5Y human NB cells (Figure 1). YM155 administered from 10 to 500 nM significantly suppressed survivin expression in a dose-dependent manner (Figure 1A) and time-dependent manner, as observed at 12 h after the drug addition (Figure 1B). YM155 also suppresses survivin at the mRNA levels in a dose-dependent manner (Figure 1C) and time-dependent manner (Figure 1D), which suggests that the suppression of survivin by YM155 is through transcriptional inhibition of the survivin gene promoter. In this research, survivin was completely inhibited by 100 nM treatment, 100 nM was used for further study.

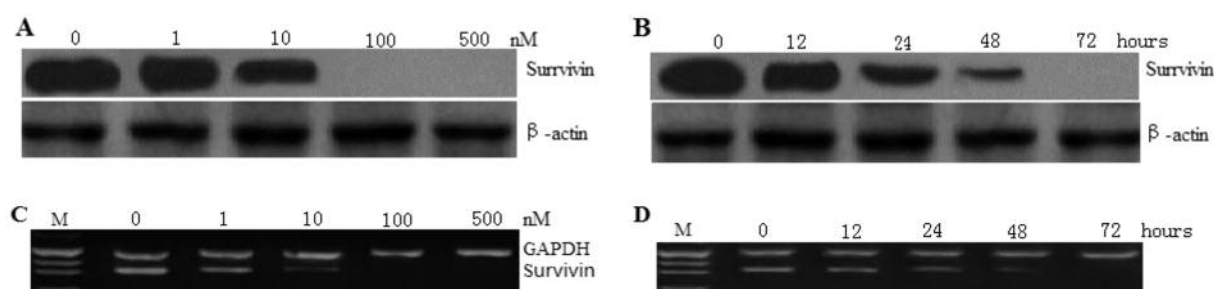


Figure 1. YM155 suppresses survivin expression in human SH-SY5Y cells. **A**, SH-SY5Y cells were treated with YM155 (1, 10, 100, and 500 nM) for 72 h (control: 0.1% DMSO). Cells were analyzed to detect survivin protein by Western blot analysis. **B**, SH-SY5Y cells were treated with YM155 (100 nM) for the indicated periods of time. Cells were analyzed to detect survivin protein by western blot analysis. **C**, SH-SY5Y cells were treated with YM155 (1, 10, 100, and 500 nM) for 72 h (control: 0.1% DMSO). Cells were analyzed to detect survivin mRNA by RT-PCR analysis. **D**, SH-SY5Y cells were treated with YM155 (100 nM) for the indicated periods of time. Cells were analyzed to detect survivin mRNA by RT-PCR analysis. Expression levels of survivin mRNA were normalized to that of GAPDH.

YM155 induces cancer cell apoptosis *in vitro*

To determine induction of cell death by YM155 in SH-SY5Y cells, SH-SY5Y cells were treated with YM155 for 72 h, after which cell viability was assessed with MTT method and the triggering of apoptosis by flow cytometry assay. At concentrations from 1 to 100 μ M, significantly decreased the viability of cells in a dose-dependent manner (Figure 2A). When exposed to YM155, SH-SY5Y showed a concomitant increase in the apoptotic cells (Figure 2B). These results suggest that YM155 induces apoptosis in SH-SY5Y cells.

Cisplatin treatment did not affect survivin levels in SH-SY5Y cells

SH-SY5Y cells were stimulated with various concentrations (0.05–2 μ M) of cisplatin for 72 hours *in vitro* and survivin levels were measured using western blot assay. Cisplatin treatment for 24–72 hours had no significant effect on survivin levels in the SH-SY5Y cells (Figure 3).

YM155 enhances cisplatin-induced apoptosis

SH-SY5Y cells treated with either YM155, cisplatin, or both agents were analyzed by flow cytometry assay to assess the *in vitro* effects on apoptosis induction (Figure 4). Apoptotic cells analysis revealed that exposure to 10 μ M YM155 increased the apoptotic cells by 21.7% (Figure 2B), and exposure to 0.05, 1 and 2 μ M cisplatin increased the apoptotic cells by 2.14%, 10.6 and 15.4%, respectively. In contrast, combined with cisplatin and YM155, the apoptotic cells were increased by 46.2%, 58.8% and 63.5%, respectively, which was greater than the sum in either treatment alone (Figure 4).

Antitumor effects of YM155 combined with cisplatin in SH-SY5Y cells xenograft models

We evaluated the *in vivo* antitumor activity of YM155 with IV injection in SH-SY5Y s.c. xenografted mice. Mice receiving daily i.v. bolus injections of YM155 at 2.5 mg/kg showed a maximum tumor growth inhibition of 40% compared with the control (Figure 5A). YM155 shows time-dependent antitumor activity *in vivo*. Mice receiving daily i.v. of cisplatin, 5 times (once every 4 days), showed a maximum tumor growth inhibition of 14% compared with the control (Figure 5A). YM155 in concomitant combination with cisplatin significantly inhibited tumor growth compared with each single-

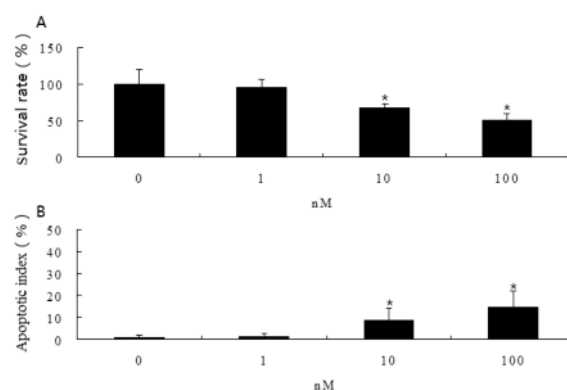


Figure 2. YM155 induces apoptosis in human SH-SY5Y cells *in vitro*. SH-SY5Y cells were treated with YM155 (1, 10 and 100 μ M) for 72 h (control, 0.1% DMSO). Columns, mean ($n = 3$); bars, SE. *, $p < 0.05$, versus DMSO. Cell viability was determined by MTT (**A**). Apoptotic cells were detected by flow cytometry assay (**B**).

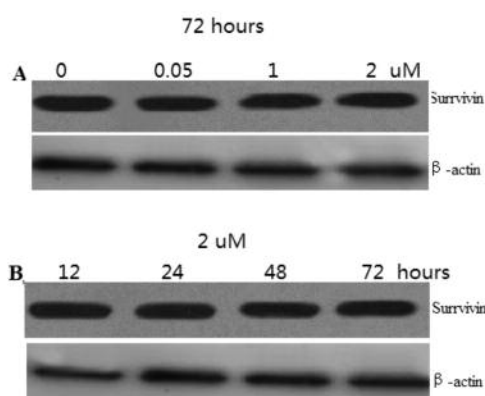


Figure 3. Cisplatin treatment does not influence survivin levels *in vitro*. SH-SY5Y cells were stimulated with 0.05-2 μ M of cisplatin for 72 hours (A), or 2 μ M cisplatin for 12-72 hours (B). No significant effect on survivin levels in the SH-SY5Y cells was shown.

compound group ($p < 0.01$) in SH-SY5Y established tumors. However, each monotherapy treatment induced tumor regression, which was followed by successive tumor regrowth during the observation period. No significant decrease in body weight was observed in the combination group as compared with the cisplatin group. These results indicated that YM155 in combination with cisplatin was tolerated in mice and enhanced the *in vivo* tumor response to cisplatin.

YM155 suppresses survivin *in vivo*

To confirm whether YM155 alters survivin expression in tumors during drug treatment, we evaluated the effect of YM155 on intratumoral survivin expression. The control animals showed rapid tumor growth from day 0 to 21 days, no change in intratumoral survivin was found. In contrast, animals treated with YM155 showed tumor regression from day 0 to day 21 days, and a clear decrease in intratumoral survivin levels was observed. These results show that the rapid tumor regression induced by YM155 suppresses intratumoral survivin protein levels, and that YM155 shows potent antitumor activity in human SH-SY5Y models by suppressing intratumoral survivin levels. However, cisplatin treatment does not influence survivin levels *in vivo* (data not shown).

YM155 combined with cisplatin induces apoptosis in SH-SY5Y cells xenograft models

The apoptotic index was 8.47% and 3.46% in YM155 and cisplatin groups, respectively. How-

ever, YM155 in concomitant combination with cisplatin significantly induces apoptosis compared with each single-compound group ($p < 0.01$) in SH-SY5Y cells xenograft models (Figure 5C). These results indicated that YM155 in combination with cisplatin enhanced the *in vivo* tumor inhibition by inducing apoptosis.

Discussion

More than half of the neuroblastoma patients over 1 year old have advanced metastatic disease at the time of diagnosis¹⁸. For these patients, the overall survival rate remains less than 50%. Therefore, a new therapeutic strategy is critically needed. Here, we examined the therapeutic potential of YM155 alone and in combination with cisplatin using SH-SY5Y cells *in vitro* and preclinical NB SH-SY5Y cell models. We found that YM155 exhibited significantly antiproliferative activity and showed tumor regression in SH-SY5Y cells *in vitro* and established xenograft models. In an *in vitro* combination study, YM155 decreased cisplatin-induced survivin accumulation at G2/M cell-cycle arrest and synergistically enhanced antitumor activity of cisplatin in SH-SY5Y cells. In addition, YM155 concomitantly combined with cisplatin resulted in greater tumor reduction than each single treatment in established xenograft models. Taken together, these findings suggest that YM155 may be a promising candidate for NB therapy as a novel apoptosis inducer with survivin-suppressive activity.

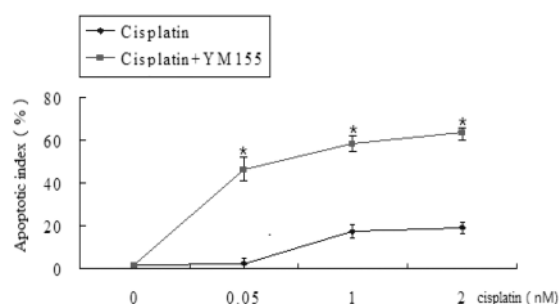


Figure 4. *In vitro* effects of combined YM155 and Cisplatin treatment on cell apoptosis. SH-SY5Y cells treated with either YM155 (10 μ M), Cisplatin (0.05, 1 and 2 μ M), or both agents for 72 hours were analyzed using flow cytometry assay to assess the *in vitro* effects on apoptosis induction. Columns, mean ($n = 3$); bars, SE. *, $p < 0.01$.

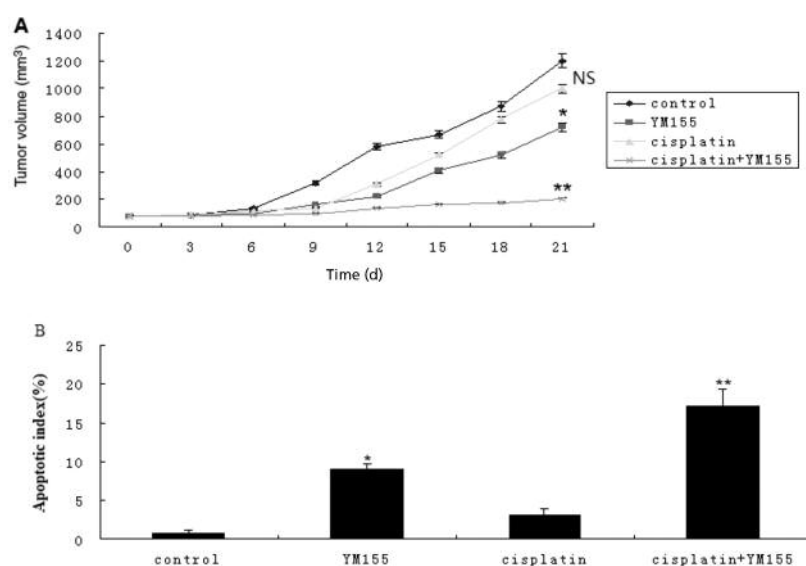


Figure 5. Tumor regression and surviving suppression induced by YM155 combined with cisplatin in s.c. xenografted SH-SY5Y tumors in mice.

A, YM155 was i.v. administered five times a week for 3 weeks or/and Cisplatin was administered i.v. 5 times (once every 4 days). Efficacy in each treated group was statistically evaluated by t test at day 21. *, $p < 0.05$; **, $p < 0.01$, versus control. Points, mean ($n = 6$); bars, SE. **B**, Effect of YM155 and cisplatin on apoptotic index in each treated group by TUNEL assay. Columns, mean ($n = 3$); bars, SE. *, $p < 0.05$; **, $p < 0.01$.

As cancer cells develop heterogeneity through a series of accumulated genetic changes, it was unclear whether the cytoprotective potential of survivin and therapeutic potential of YM155 would be differentiated by tumor type, specific somatic mutation, or cell-cycle progression. Our present findings suggest that NB cells may acquire a cytoprotective phenotype by mitigating apoptosis induction through overexpression of survivin. In addition, NB cells became sensitive to apoptosis induction when survivin was down-regulated using YM155. Further extensive research for survivin-mediated apoptosis defects may allow the rational stratification of NB patients who would most likely respond to YM155.

Combinational chemotherapy is typically employed to achieve a better response rate than that of monotherapy and is generally designed empirically using drugs that act through different cytotoxic mechanisms with less overlapping toxicity. Here, we found when YM155 was concomitantly combined with cisplatin, more intense apoptosis was observed compared with each single treatment. In addition, YM155 potentiated antitumor activity of cisplatin without an enhancement of body weight loss in established xenograft models.

In a toxicologic study, short-term exposure at high blood concentrations caused cardiotoxicity in the form of atrioventricular block and myocardial degeneration/necrosis, as well as nephrotoxicity, mainly displayed as proximal tubular necrosis and increased serum creatinine. In contrast,

long-term exposure at low blood concentrations by 168-hour continuous infusion did not cause cardiotoxicity¹⁹. In this study, low concentration of YM155 displayed strong antitumor effect. We, therefore, suggested that YM155 may be as a safety and effect agent for NB treatment.

Conclusions

In clinical settings, cisplatin treatment is effective against several types of human cancers. Despite its clinical benefits, the long-term treatment of cisplatin is often limited due to development of drug resistance and cumulative side effects. Therefore, drugs that circumvent cisplatin resistance without overlapping side effects represent ideal candidates for developing novel combinatorial therapeutic regimen for treating NB. Although cancer cells can acquire resistance to cisplatin through various mechanisms, our findings suggest that survivin may be involved in the mitotic survival pathway and counteract cisplatin induced apoptosis in NB cells. Further clinical investigation of YM155 as an apoptosis inducer, either alone or in combination with cisplatin, for the treatment of malignant NB is warranted.

Conflict of interest

The Authors declare that they have no conflict of interests.

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