Targeting of Survivin Inhibits Neuroblastoma Cell Proliferation

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INTRODUCTION

High-risk neuroblastoma (NB) is an aggressive pediatric tumor which develops from the extracranial sympathetic nervous system and accounts for almost 15% of all childhood cancer-related deaths. Survivin is known to be involved in controlling cell division and apoptosis and belongs to the inhibitor of apoptosis protein family. Oncogenic activation of survivin has been reported in different cancers including NB. In the present study, we analyzed genomic datasets of 1135 NB patients and found that high expression of survivin coding gene BIRC5 strongly correlates with poor overall and event-free survival of NB patients, and more aggressive tumors have significantly higher BIRC5 levels. To understand and evaluate the biological effects of survivin inhibition on NB growth, five experiments were performed. The cell viability assay (MTT) results from six cell lines- 3 MYCN-non-amplified cell lines (SH-SY5Y, SK-N-AS, CHLA-255), and 3 MYCN-amplified cell lines (NGP, LAN-5, IMR-32) demonstrated the inversely proportional relationship between increased dose, and tumor growth. Clonogenic and Spheroidal experiments further confirmed a noteworthy reduction of colony formation and reduced tumor size respectively, with increased dose. Utilizing Annexin V apoptosis assays and Click-iT EdU cell proliferation assays, the effect on Cell Cycle and Apoptosis were assessed, which revealed that in comparison to control, increased dose promoted apoptosis and prevented the advancement of the cell cycle.

OBJECTIVES

- To evaluate the effects of a small molecule inhibitor of survivin.
- To determine in vitro effects of survivin inhibition on NB cell proliferation, apoptosis, and cell cycle.
- To identify a novel therapeutic approach to treat high-risk neuroblastoma.

RESULTS

Figure 1. BIRC5 promotes NB progression.

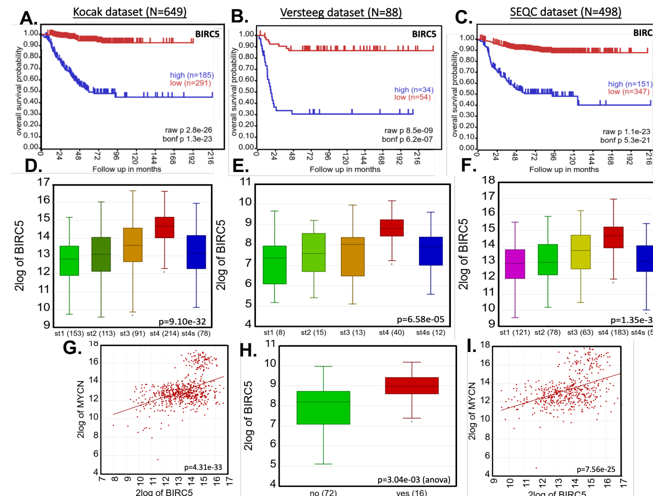
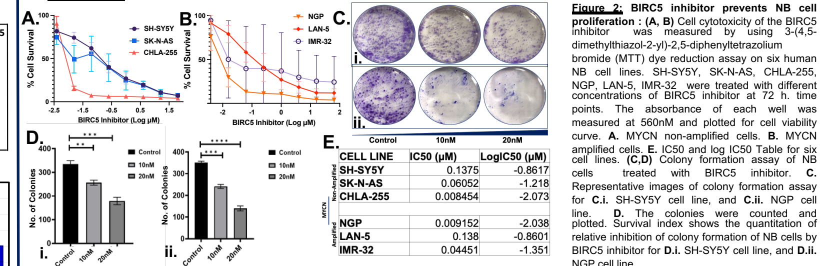


Figure 1: BIRC5 promotes NB progression. **(A, B, C)** Kaplan Meier curves shows the overall probability of NB survival in correlation with BIRC5 expression. **A.** 649 patients in Kocak dataset. **B.** 498 patients in SEQC dataset. **C.** 88 patients in the Versteeg dataset. **(D, E, F)** R2-analysis showing correlation of BIRC5 expression levels to neuroblastoma disease stages. **D.** Kocak. **E.** Versteeg. **F.** SEQC. **(G. , I.)** R2 Kocak., SEQC datasets, shows the correlation of BIRC5 with MYCN. **H.** R2-versteeg dataset. Analysis, shows higher BIRC5 expression in MYCN-amplified cases in comparison to MYCN-non-amplified cases.

Figure 2. BIRC5 inhibitor prevents NB cell proliferation.



	CELL LINE	IC50 (µM)	LogIC50 (µM)
MYCN Non-Amplified	SH-SY5Y	0.1375	-0.8617
	SK-N-AS	0.06052	-1.218
	CHLA-255	0.008454	-2.073
MYCN Amplified	NGP	0.009152	-2.038
	LAN-5	0.138	-0.8601
	IMR-32	0.04451	-1.351

Figure 2: BIRC5 inhibitor prevents NB cell proliferation : (A, B) Cell cytotoxicity of the BIRC5 inhibitor was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay on six human NB cell lines. SH-SY5Y, SK-N-AS, CHLA-255, NGP, LAN-5, IMR-32 were treated with different concentrations of BIRC5 inhibitor at 72 h. time points. The absorbance of each well was measured at 560nM and plotted for cell viability curve. **A.** MYCN non-amplified cells. **B.** MYCN amplified cells. **E.** IC50 and log IC50 Table for six cell lines. **(C,D)** Colony formation assay of NB cells treated with BIRC5 inhibitor. **C.** Representative images of colony formation assay for **C.i.** SH-SY5Y cell line, and **C.ii.** NGP cell line. **D.** The colonies were counted and plotted. Survival index shows the quantitation of relative inhibition of colony formation of NB cells by BIRC5 inhibitor for **D.i.** SH-SY5Y cell line, and **D.ii.** NGP cell line.

Figure 3. BIRC5 inhibitor prevents NB 3D spheroidal growth.

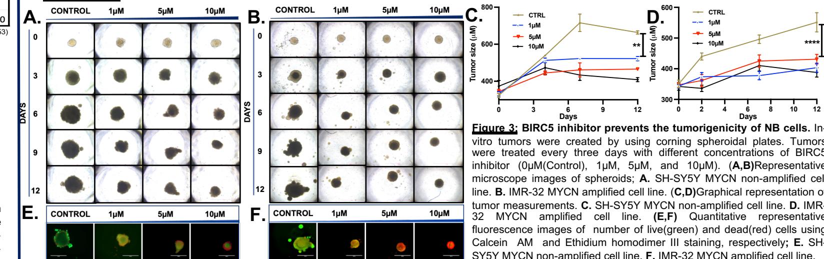


Figure 3: BIRC5 inhibitor prevents the tumorigenicity of NB cells. In-vitro tumors were created by using corning spheroidal plates. Tumors were treated every three days with different concentrations of BIRC5 inhibitor (0µM(Control), 1µM, 5µM, and 10µM). **(A,B)**Representative microscope images of spheroids; **A.** SH-SY5Y MYCN non-amplified cell line. **B.** IMR-32 MYCN amplified cell line. (**C,D)**Graphical representation of tumor measurements. **C.** SH-SY5Y MYCN non-amplified cell line. **D.** IMR-32 MYCN amplified cell line. **(E,F)** Quantitative representative fluorescence images of number of live(green) and dead(red) cells using Calcein AM and Ethidium homodimer III staining, respectively**; E.** SH-SY5Y MYCN non-amplified cell line. **F.** IMR-32 MYCN amplified cell line.

Figure 4. BIRC5 inhibitor induces apoptosis and blocks cell cycle progression in NB.

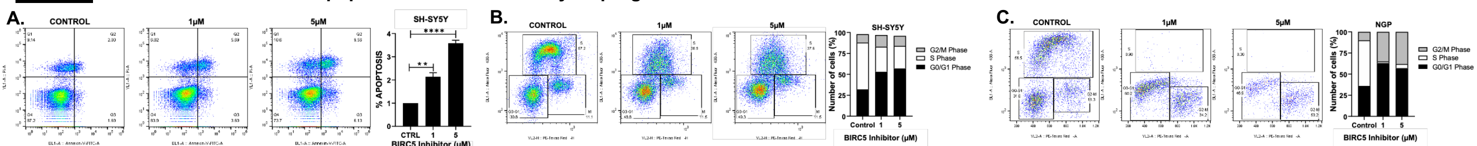


Figure 3: BIRC5 inhibitor induces Apoptosis and blocks Cell Cycle progression in NB Cells. (A,B,C) : BIRC inhibitor induces apoptosis in NB cells and blocks cell cycle. Both MYCN amplified and MYCN non-amplified cells were treated with different concentrations of BIRC inhibitor (Control, 1µm, 5µm) for 16hrs. **A.** Representative Flow cytometer images and graphical representation of % Apoptosis in SH-SY5Y. **(B.,C.)** Representative Flow cytometer images and graphical representation of cell cycle analysis performed using Attune cell cycle assay kit in: **B.** SH-SY5Y, and **C.** NGP cell lines.

METHODS

R2 Genomic Analysis: Neuroblastoma patient datasets of Kocak (N-649), Versteeg (N=88), and SEQC (N=498) were analyzed using R2 Genomic Analysis and Visualization Platform. These datasets were analyzed for overall survival correlation and gene expression.

Cell Culture: The human neuroblastoma MYCN-non-amplified(SH-SY5Y, SK-N-AS, CHLA255), and MYCN-amplified(NGP, LAN-5, IMR-32) cell lines were cultured in a 5% CO_2 atmosphere with 95% humidity at 37°C in RPMI-1640 supplemented media.

Viability Assay: Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after 72 hours incubation.

Spheroidal Assay: Spheroidal assay was performed using corning 3D-sheroidal plates, and Biotium live/dead kit..

Clonogenic Assay: Colony formation was evaluated using 0.5 % crystal violet staining.

Apoptosis and Cell Cycle Assay: Apoptosis and cell cycle assays were performed using Annexin V apoptosis assay kit and Click-iT EdU cell proliferation assay in the Attune NXT flow cytometer.

Statistical Analysis: All experimental data was obtained using three biological replicates for each of the two technical replicates done.

CONCLUSION/ FUTURE WORK

- Survivin inhibition significantly inhibits NB cell proliferation, colony growth, blocks cell cycle progression, induce apoptosis, and inhibit 3D spheroid tumor formation and growth in a dose-dependent manner.
- Overall, our data highlights the importance of survivin as a target in NB, and the potential of survivin inhibition as a novel therapeutic approach for NB.
- In future efforts, we will combine the survivin inhibitor with chemotherapy drugs to develop a less-toxic and more-effective therapeutic approach for NB treatment.

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