RESEARCH ARTICLE

Promoting Effects of Sanguinarine on Apoptotic Gene Expression in Human Neuroblastoma Cells

Emre Cecen[1]*, Zekiye Altun[2], Pinar Ercetin[2], Safiye Aktas[2], Nur Olgun[3]

Abstract

Neuroblastoma is the most common extracranial solid tumor in children. Approximately half of the affected patients are diagnosed with high-risk poor prognosis disease, and novel therapies are needed. Sanguinarine is a benzophenanthridine alkaloid which has anti-microbial, anti-oxidant and anti-inflammatory properties. The aim of this study is whether sanguinarine has *in vitro* apoptotic effects and which apoptotic genes might be affected in the human neuroblastoma cell lines SH-SY5Y (N-myc negative), Kelly (N-myc positive, ALK positive), and SK-N-BE(2). Cell viability was analysed with WST-1 and apoptotic cell death rates were determined using TUNEL. After RNA isolation and cDNA conversion, expression of 84 custom array genes of apoptosis was determined. Sanguinarine caused cell death in a dose dependent manner in all neuroblastoma cell lines except SK-N-BE(2) with rates of 18% in SH-SY5Y and 21% in Kelly human neuroblastoma cells. Cisplatin caused similar apoptotic cell death rates of 16% in SH-SY5Y and 23% in Kelly cells and sanguinarine-cisplatin combinations caused the same rates (18% and 20%). Sanguinarine treatment did not affect apoptototic gene expression but decreased levels of anti-apoptotic genes NOL3 and BCL2L2 in SH-SY5Y cells. Caspase and TNF related gene expression was affected by the sanguinarine-cisplatin combination in SH-SY5Y cells. The expression of regulation of apoptotic genes were increased with sanguinarine treatment in Kelly cells. From these results, we conclude that sanguinarine is a candidate agent against neuroblastoma.

Keywords: Sanguinarine - neuroblastoma - apoptosis - gene expression

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Introduction

Neuroblastoma is the most common extracranial solid tumors of childhood and the most frequently diagnosed neoplasm during infancy (Missaoui et al., 2011; National cancer institute, 2014). It accounts for more than 7% of malignancies in patients younger than 15 years and around 15% of all paediatric oncology deaths. Children younger than 18 month of age fare better than older children with the same disease stage. Despite agressive conventional treatment or bone marrow transplantation, long-term survival rates for older children with advanced stage disease rarely exceed 40%. N-myc amplification predicts a poor outcome in all age/stage groups. Approximately half of all patients with neuroblastoma are diagnosed with high-risk poor prognosis disease, and novel therapies are needed (Brodeur et al., 2011; Mehdiabadi et al., 2013; Wiangnon et al., 2011; Gao et al., 2014; Zhang et al., 2014).

Sanguinarine (13-methyl (1, 3) benzodioxolo (5, 6-c)-1, 3-dioxolo (4, 5-i) phenanthridinium), which is derived from the root of Sanguinaria canadendid and other poppy fumaria species, is a benzophenanthridine alkaloid and a structural homologue of chelerythrine. Its principle medicinal use to date is in dental products based on its anti-bacterial, anti-fungal, and anti-inflammatory activities, which reduce both gingival inflammation and supragingival plaque formation (Godowski, 1989; Kuftinec et al., 1990; Laster and Lobene, 1990). Several recent studies have showed that sanguinarine, at micromolar concentration, inhibits the growth of cancer cells, and this inhibition of growth is associated with cell cycle arrest and the stimulation of apoptosis (Ahsan et al., 2007; Choi et al., 2008; Han et al., 2013a; Jang et al., 2009). The aim of this study is to determine in vitro effect of sanguinarine against neuroblastoma and to question which apoptotic genes contributes its apoptotic effect.

Materials and Methods

Chemicals

Sanguinarine chloride hydrate, ≥598 (HPLC) (S5890-

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5MG SIGMA), cisplatin, and DMSO and were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines and culture conditions

Human neuroblastoma cell lines SH-SY5Y (N-myc negative), Kelly (N-myc positive, ALK positive) SK-N-BE(2) (N-myc positive, chemoresistant) were used in the experiments. SH-SY5Y and SK-N-BE(2) cell lines were grown in DMEM medium (Dulbeccós Modification of Eagle's Medium, PAA Laboratories, Austria) and Kelly cell line was grown in RPMI 1640 medium. Each medium was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin (PAA Laboratories, Pasching, Austria). All cell lines were cultivated in a humidified incubator at 37ºC in a 5% CO_2 atmosphere.

Application of agents

For dose optimisation of sanguinarine and cisplatin, cells were cultivated in 96 well plates in incubator. Each agent and dose were given into six wells. Each dose were applied for 24, 48, 72 hours. Each experiment was repeated three times. Doses of sanguinarine were between 0-10uM (0,1 uM, 0.25 uM, 0.5 uM, 1 uM, 2 uM, 2.5 uM, 4 uM, 5 uM, 8 uM, 10 uM) and doses of cisplatin were between 5-200 uM. After end of time MTT cell viability assay were applied. 50% Lethal dose and optimal time were selected for each cell line and agent for further analysis (24 hours Sanguinarine 5 uM for SH-SY5Y and Kelly; Cisplatin 8 microuM for SH-SY5Y and 20 uM for Kelly cell line; SK-BE(2) cell line was resistant to both cisplatin and sanguinarine at all doses so that further analysis was not performed).

For RT PCR analysis, cells were grown in 25 cm^2 flasks (three flasks for each cell line), then they were treated with pre-optimized doses of sanguinarine, cisplatin and sanguinarine -cisplatin combination for 24 hours. After washing with PBS cells were collected with cell scrabber for RNA isolation.For apoptosis detection cells were exposed to preoptimised doses of cisplatin, sanguinarine and combinations for 24 hours incubation period at 96 well plate to Kelly and SH-SY5Y cells.

Cell viability assay

MTT ((3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide), Sigma–Aldrich Corp.), cells were seeded at approximately $1x10^4$/well in a final volume of 200 uL in 96-well microtiter culture plates. Cells were seeded with at least 6 replicates for each group. After 24 hours of plating, incubation was continued for another 24 hours in absence (control) or presence of agents. At the end of the incubation period, colorimetric WST-1 assay was performed with the Cell Proliferation Reagent WST-1 assay kit (Roche Applied Science, Mannheim, Germany), according to manufacturer's instructions. The reaction was allowed to proceed for 4 hours at 37°C. The intensity of the resulting color developed, which is the reflection of number of live cells, was measured at a wavelength of 450 nm against a reference wavelength at 630 nm by ELISA reader (Thermo Multiscan Ascent, Instruments Inc, USA). All values were compared to those corresponding controls. The mean of triplicate experiments for each dose was used to calculate the 50% cell growth inhibitor doses of chemicals and combinations.

TUNEL (TdT-mediated dUTP nick end labeling)

Cells were exposed to preoptimised doses of cisplatin, sanguinarine and combinations for 24 hours incubation period at 96 well plate and apoptotic cell death was monitored with TUNEL assay that can detect fragmented DNA in the nucleus during apoptosis (GenScript TUNEL Apoptosis Detection Kit Cat. No.L00299, for Adherent Cells, FITC-labeled POD). The kit was applied on wells according to manufacturer's instructions. After fixation and washing, tunel reaction mix containing Equilibration Buffer, FITC-12-dUTP and TdT was applied for 60 minutes at 37°C. Assay was done with Olympus fluorescence microscope using excitation wave 450-500 nm and emission wave 515-565 nm (green). Six wells per condition were used and 5000 cells per well were evaluated and scored as % of apoptosis per all cells.

RNA isolation and apoptosis gene expression analysis by real time PCR

Most of the important apoptotic and anti-apoptotic genes were designed as a standard apoptotic gene array from SABiosciences and 84 apoptosis related gene expressions were evaluated in this study. After each cell line was cultured at 25 cm^2 flasks and agents and combinations were applied for 24 hours, cells were collected by cell scaber; RNA isolation and complementary DNA (cDNA) converting and expression of 84 standard array genes of human apoptosis (SABiosciences, PAHS-012A) was determined by Real-Time PCR for each condition. PCR array (84 genes, 5 housekeeping genes, 1 genomic DNA control, 3 reverse transcriptase control, 3 positive PCR control) were studied on 96 well PCR plate. Total RNA extraction was done according to manufacturer's instructions (Macharey Nagel RNA Isolation Kit). cDNA synthesis was done by RT2 First Strand Kit (QIAGEN Cat No. 330401). For gene expression analysis, real time PCR on ABI PRISM 7000 Sequence Detection System was applied on standard arrayed plates. SABiosciences's PAHS-012A array included master mix, primary for each gene or housekeeping genes matched on array code. Only cDNA and SYBR Green was loaded on PCR 96 well plates. The protocol was loaded as one cycle of10 minutes at 95°C, followed by 45 cycles of 15 seconds 95°C and 1 minute 65°C each. SYBR Green Fluorescence was the detection method.

Cp values on excel file listed according to A1-H12 array codes were uploaded on http://www.sabiosciences.com/pcr/arrayanalysis.php, online free array analysis system. Fold changes of each condition compared with control cells of each cell line were calculated. Genes that showed increase or decrease more than 5 folds were taken into consideration for expression changes. The list of genes in this PCR array was shown in table 1. A list of the genes analyzed in this profile is also available online (http://www. sabiosciences.com/rt_pcr_product/HTML/

PAHS-012A.html).

Statistical analysis

Statistical analyses were performed using the SPSS 15.0 software program. Mann-Whitney U test was used for cell viability and apoptosis number comparison. P< 0.05 was considered statistically significant. This study was approved by Local Ethics Committee.

Results

Cell viability

Sanguinarine caused cell death in a dose dependent manner in neuroblastoma cell lines except SK-N-BE(2) cells. The effective 50% LD at 24 hours of Sanguinarine was 5 uM for SH-SY5Y and Kelly. The effective 50% LD at 24 hours of Cisplatin was 8 uM for SH-SY5Y and 20 uM for Kelly cell line; SK-BE(2) cell line was resistant to both cisplatin and sanguinarine at all doses (Figure 1 and 2). Sanguinarine caused cell death equivalant to cisplatin on both neuroblastoma cell line. Combination of sanguinarine and cisplatin did not gain synergistic effect.

Apoptosis results

Apoptotic cell death ratios were determined in 18% in SH-SY5Y (control 1%) and 21% in Kelly (control 3%) human neuroblastoma cells with sanguinarine at 5 uM dose.

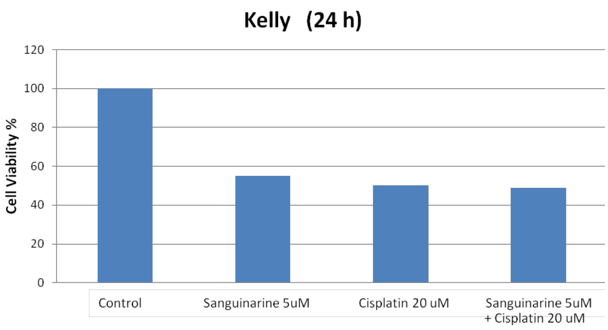


Figure 1. The Cell Viability Values of Kelly Cells after The Application of 20 uM Cisplatin, 5 uM Sanguinarine and Combination of 5 uM Sanguinarine –20 uM Cisplatin

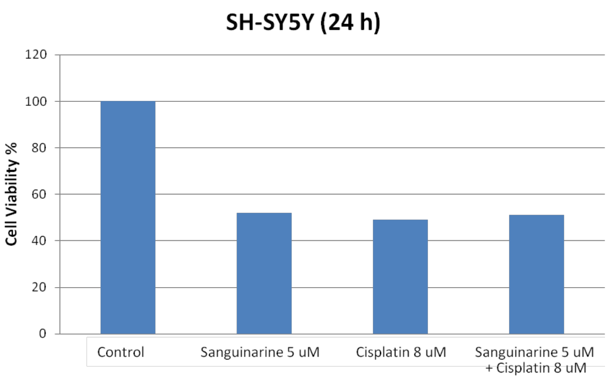


Figure 2. The Cell Viability Values of SH-SY5Y Cells after the Application of 8 uM Cisplatin, 5 uM Sanguinarine and Combination of 5 uM Sanguinarine –8 uM Cisplatin

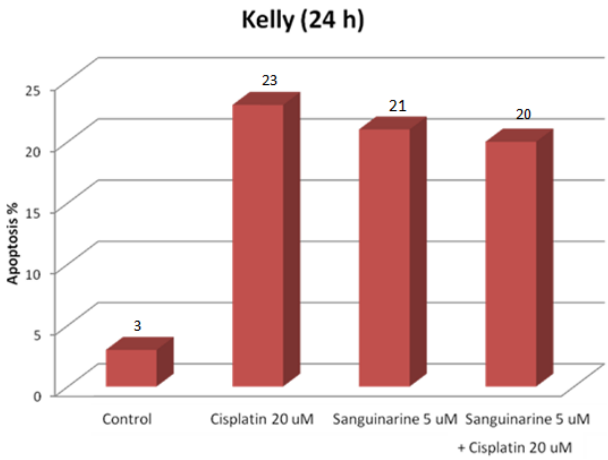


Figure 3. Results of Apoptosis % Detected by TUNEL for Kelly Cells after The Application of 20 uM Cisplatin, 5 uM Sanguinarine and Combination of 5 uM Sanguinarine –20 uM Cisplatin

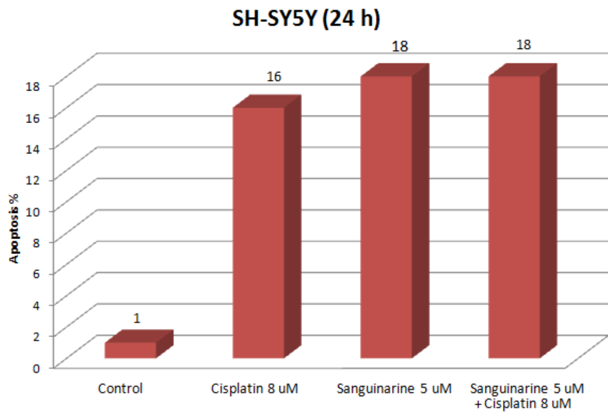


Figure 4. Results of Apoptosis % Detected by TUNEL for Kelly Cells after The Application of 20 uM Cisplatin, 5 uM Sanguinarine and Combination of 5 uM Sanguinarine –20 uM Cisplatin

Cisplatin caused apoptotic cell death ratios were 16% in SH-SY5Y and 23% in Kelly cells. Sanguinarine-cisplatin combinations caused cell death as same ratios (Figure 3 and 4).

Mann Whitney U test confirmed statistically significant difference for cell viability and apoptosis number of % of sanguinarine and cisplatin comparing to control groups for each cell line (p<0.05).

Gene expression results

Sanguinarine treatment did not affect apoptototic gene expressions but showed especially by decreasing the expression of anti-apoptotic genes NOL3 and BCL2L2 in SH-SY5Y cells. Caspase gene expressions and TNF related gene expressions were affected by sanguinarine-cisplatin combinations in SH-SY5Y cells. The expression of apoptotic genes were increased by sanguinarine in Kelly cells.

Gene expression results for kelly cells

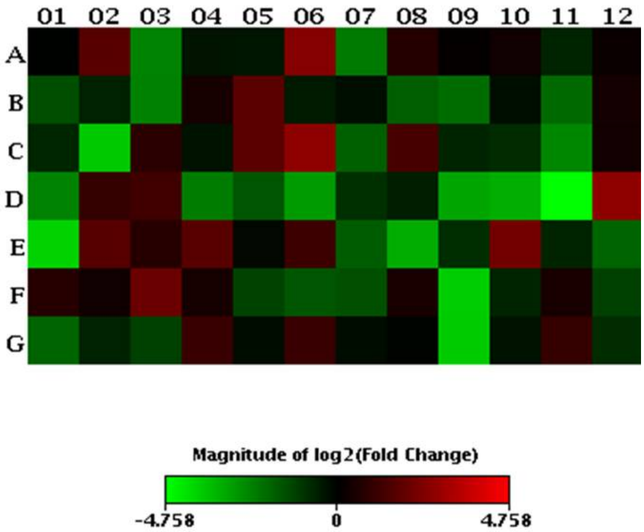
Cisplatin increased apoptotic genes expression such as BCLAF1, CASP9, CASP3, DFFA, CIDEB, FADD,

BID, CFLAR, LTBR, DAPK1, CASP2, BAD, NOD1, TNFSR25 besides also increased some anti-apoptotic gene expression. Sanguinarine increased apoptotic genes expression such as CFLAR, BCLAF1, DAPK1, CIDEP, NOD1, CASP3, FADD. But it also increased some anti-apoptotic gene expression. Sanguinarine caused decrease expression of anti-apoptotic genes such as BIRC8, CD40LG, CD40. Sanguinarine and cisplatin combination increased apoptotic more genes expression such as BCLAF1,CFLAR, BID, CASP3, FADD, CASP2, LTBR, BNIP3, BCL10, CARD8, TRAF3. Besides also increased some anti-apoptotic gene expression. Combination caused decrease in expression of anti-apoptotic genes such as BIRC8, BIRC3, CD40LG (Figure 5).

Gene expression results for SH-SY5Y cells

Sanguinarine did not cause any increase in gene expression of apoptotic genes but caused decrease in anti-apoptotic gene expression (especially NOL3, BCL2L2, HRK genes).

Cisplatin increased apoptotic genes expression such as CIDEA, TNF, CD70 ve TNFRSF1A, and decreased expression of anti-apoptotic gene BNIP2. Sanguinarine and cisplatin combination increased apoptotic more genes expression such as CASP1, FASLG, CASP10, CASP4, CASP5, LTA, TRADD, TNF, CASP7, CIDEA, TNFRSF9, PYCARD, CD70, CASP8. Combination caused decrease in expression of anti-apoptotic genes more such as IGF1R, BAG3, BRAF, BCL2L2, BIRC2, BNIP1, MCL1, AKT1,



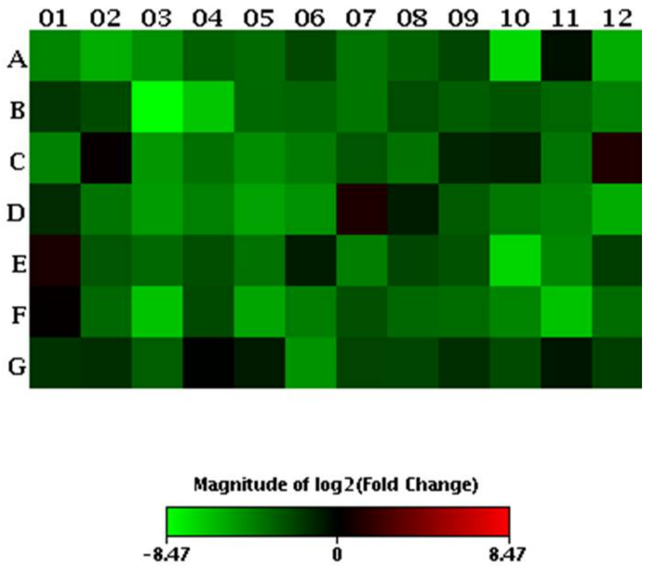
Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABL1 **-1.03** B	AKT1 **3.08** B	APAF1 **-5.45** B	BAD **-1.28** B	BAG1 **-1.34** B	BAG3 **5.67** A	BAG4 **-4.88** B	BAK1 **1.60** B	BAX **1.06** A	BCL10 **1.24** B	BCL2 **-1.58** OKAY	BCL2A1 **1.13** B
B	BCL2L1 **-2.75** B	BCL2L10 **-1.57** B	BCL2L11 **-5.34** B	BCL2L2 **1.33** B	BCLAF1 **3.17** A	BFAR **-1.41** B	BID **-1.20** B	BIK **-3.33** B	NAIP **-4.08** A	BIRC2 **-1.20** B	BIRC3 **-3.91** B	XIAP **1.29** B
C	BIRC6 **-1.63** B	BIRC8 **-13.52** B	BNIP1 **1.73** B	BNIP2 **-1.25** A	BNIP3 **3.23** A	BNIP3L **6.38** A	BRAF **-3.50** B	NOD1 **2.45** B	CARD6 **-1.60** B	CARD8 **-1.77** B	CASP1 **-5.85** B	CASP10 **1.27** B
D	CASP14 **-5.45** B	CASP2 **1.94** A	CASP3 **2.32** B	CASP4 **-5.02** B	CASP5 **-3.07** B	CASP6 **-7.56** B	CASP7 **-1.84** B	CASP8 **-1.48** B	CASP9 **-8.62** B	CD40 **-9.83** B	CD40LG **-27.05** B	CFLAR **6.38** A
E	CIDEA **-15.32** B	CIDEB **3.08** B	CRADD **1.63** B	DAPK1 **3.10** B	DFFA **-1.10** B	FADD **2.16** B	FAS **-3.27** B	FASLG **-9.43** A	GADD45A **-1.81** B	HRK **4.42** B	IGF1R **-1.60** B	LTA **-3.67** B
F	LTBR **1.65** A	MCL1 **1.22** OKAY	NOL3 **3.95** B	PYCARD **1.34** B	RIPK2 **-2.34** B	TNF **-3.05** B	TNFRSF10A **-2.78** B	TNFRSF10B **1.35** B	TNFRSF11B **-14.30** B	TNFRSF1A **-1.61** B	TNFRSF21 **1.35** B	TNFRSF25 **-2.29** B
G	CD27 **-3.62** B	TNFRSF9 **-1.54** B	TNFSF10 **-2.28** B	CD70 **2.05** B	TNFSF8 **-1.16** B	TP53 **2.05** B	TP53BP2 **-1.16** A	TP73 **-1.06** B	TRADD **-14.00** B	TRAF2 **-1.25** B	TRAF3 **1.94** A	TRAF4 **-1.79** B

Figure 5. Heatmap of 84 Evaluated Apoptosis Related Genes Showing Effect of Sanguinarine at Gene Expression Degrees Comparing to Control for Kelly Cells; Green color indicates lower expression of genes and red color indicates higher expressions of genes

XIAP, BNIP3, BNIP3L, BFAR, HRK, NOL3, DFFA BAD, DAPK1, CASP9, TNFRSF21, BCL10, BCLAF1 in a sinergistic manner (Figure 6 and 7).

Discussion

The antiproliferative and/or pro-apoptotic activities of sanguinarine have been demonstrated in cells derived from several human cancers including epidermal (Ahmad et al., 2000), keratinocytes (Adhami et al., 2003; Reagan-Shaw et al., 2006), prostate (Malikova et al., 2006b; Adhami et al., 2004; Huh et al., 2006), cervical (Ding et al., 2002), breast (Choi et al., 2008; Debiton et al., 2003; Holy et al., 2006), leukemia (Han et al., 2008; Weerasinghe et al., 2001c; Weerasinghe et al., 2001b; Weerasinghe et al., 2001a), lymphoma (Hussain et al., 2007), melanoma (Burgeiro et al., 2013; Hammerova et al., 2011; Serafim et al., 2008), colon (Lee et al., 2012; Matkar et al., 2008), colorectal (Han et al., 2013a; Lee et al., 2012; Pica et al., 2012), gastric (Choi et al., 2009), pancreatic (Ahsan et al., 2007), lung (Jang et al., 2009), neuroendocrine (Larsson et al., 2010), osteosarcoma (Park et al., 2010), and in rat glioblastoma cells (Han et al., 2007), bladde (Han et al., 2013b). However, the impact of sanguinarine on



Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABL1 **-22.32** A	AKT1 **-54.19** B	APAF1 **-27.10** B	BAD **-9.13** B	BAG1 **-11.24** B	BAG3 **-5.21** A	BAG4 **-14.22** A	BAK1 **-9.06** B	BAX **-4.96** A	BCL10 **-150.12** A	BCL2 **-1.45** A	BCL2A1 **-55.72** B
B	BCL2L1 **-3.48** B	BCL2L10 **-5.39** B	BCL2L11 **-354.59** B	BCL2L2 **-98.36** A	BCLAF1 **-10.78** OKAY	BFAR **-10.13** A	BID **-14.83** A	BIK **-5.82** B	NAIP **-8.22** A	BIRC2 **-6.92** A	BIRC3 **-10.27** B	XIAP **-18.77** A
C	BIRC6 **-19.70** A	BIRC8 **1.14** B	BNIP1 **-33.36** B	BNIP2 **-13.27** A	BNIP3 **-25.99** A	BNIP3L **-16.91** A	BRAF **-7.36** B	NOD1 **-13.93** A	CARD6 **-2.33** B	CARD8 **-2.07** B	CASP1 **-14.32** B	CASP10 **1.99** B
D	CASP14 **-2.68** B	CASP2 **-14.42** A	CASP3 **-35.51** A	CASP4 **-19.29** B	CASP5 **-42.52** B	CASP6 **-29.24** B	CASP7 **1.89** B	CASP8 **-1.95** B	CASP9 **-7.78** A	CD40 **-15.67** B	CD40LG **-20.39** B	CFLAR **-54.19** A
E	CIDEA **1.85** B	CIDEB **-7.31** B	CRADD **-11.00** A	DAPK1 **-6.06** B	DFFA **-13.55** A	FADD **-1.95** B	FAS **-18.51** B	FASLG **-5.10** B	GADD45A **-6.73** B	HRK **-141.04** A	IGF1R **-23.10** A	LTA **-4.20** B
F	LTBR **1.07** OKAY	MCL1 **-10.93** OKAY	NOL3 **-89.26** A	PYCARD **-5.58** B	RIPK2 **-46.21** B	TNF **-18.38** B	TNFRSF10A **-6.45** B	TNFRSF10B **-10.93** A	TNFRSF11B **-12.30** B	TNFRSF1A **-22.01** A	TNFRSF21 **-92.41** A	TNFRSF25 **-12.30** B
G	CD27 **-3.12** B	TNFRSF9 **-2.89** B	TNFSF10 **-8.69** B	CD70 **-1.04** B	TNFSF8 **-1.80** B	TP53 **-29.65** A	TP53BP2 **-4.82** A	TP73 **-5.06** B	TRADD **-2.89** B	TRAF2 **-5.58** A	TRAF3 **-1.69** OKAY	TRAF4 **-4.14** A

Figure 6. Heatmap of 84 Evaluated Apoptosis Related Genes Showing Effect of Sanguinarine at Gene Expression Degrees Comparing to Control for SHSY5Y Cells; Green color indicates lower expression of genes and red color indicates higher expressions of genes

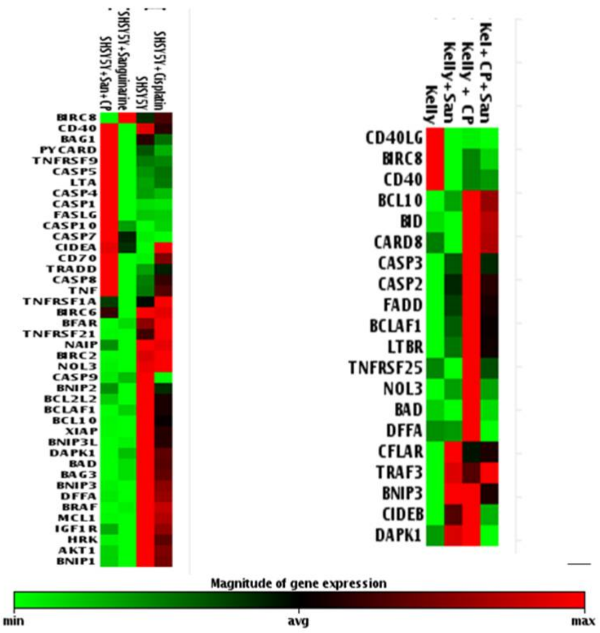


Figure 7. Clustergram of Effects of Agents and Combination on given Genes in SHSY5Y and Kelly Cells; Green color indicates lower expression of genes and red color indicates higher expressions of genes

neuroblastoma cells has not been shown. In the present study, we demonstrated that induced anti-proliferative effects observed in human neuroblastoma cells were related to induction of apoptosis. Sanguinarine treatment resulted in a dose-dependent decrease in the viability of both neuroblastoma cell line, as shown by data in Figure 1 and 2. We found that the addition of sanguinarine to the cultures induced cell death by apoptosis (Figure 3 and 4).

Sanguinarine-mediated apoptosis has been shown occurring through multiple pathways, including activation of nuclear factor-ϰB (NF-ϰB) (Chaturvedi et al., 1997), cell cycle arrest (Adhami et al., 2004), and mitochondrial damage (Adhami et al., 2003). Sanguinarine has been reported to arrest the cell cycle providing an increase in cyclin-dependent protein kinase inhibitor (CKI) expression and a decrease in cyclin D1, D2 and E, and CDK2, 4 and 6. Apoptosis induced by sanguinarine may be mediated by both caspase-9-dependent mitochondrial pathways, or the death receptor pathway, in which caspase-8 is activated. The activation of caspase-3 and cleavage of PARP and the downregulation of Bcl-2 and c-FLIP may mediate sanguinarine-induced apoptosis (Malikova et al., 2006a; Malikova et al., 2006b; Kim et al., 2008).

Various genes have been determined as either inducers or repressors of apoptosis. In our study, sanguinarine did not cause any increase in apoptotic gene expressiongenes but caused decrease in anti-apoptotic gene expression, especially NOL3, BCL2L2, HRK genes on the *SH-SY5Y* Cells. The activation of caspases can be regulated by molecules in the Bcl-2 family. In the cytosol, cytochrome c activates caspase-9, which in turn activates effector caspases such as caspase-3. We also demonstrated that, for Kelly cells, sanguinarine increased expression of apoptotic genes such as CFLAR, BCLAF1, DAPK1, CIDEP, NOD1, CASP3, FADD. We have investigated the expression of several apoptotic or anti-apoptotic genes, but did not examine the protein products of these genes. About the effect of chelerythrine which is a benzophenanthridine alkaloid and a known protein kinase C inhibitor, as an inhibitor of BclXL-Bak BH3 peptide binding, on neuroblastoma cells only one study has been published (Chan et al., 2003). This study showed that SH-SY5Y human neuroblastoma cells were treated with chelerythrine underwent apoptosis via the mitochondrial pathway through a mechanism that involves direct targeting of Bcl-2 family proteins. Chelerythrine, triggers cytocrome C release from isolated mitochondria but had no effect on NF-ϰB activation like sanguinarine.

In conclusion, as far as we know, our study is the first report regarding effects of sanguinarine on neuroblastoma. We determined that, sanguinarine was shown to cause cell death against human neuroblastoma SH-SY5Y and Kelly cells via cytotoxic and apoptotic mechanisms. Sanguinarine is a candidate agent against neuroblastoma and its effect should be explored by further in vivo animal models.

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