Betulinic Acid and Cisplatin Inhibit Metastasis Related Genes in Neuroblastoma Cells Due to Their N-MYC Status

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ABSTRACT

Betulinic acid-(BA) has apoptotic effects on neuroblastoma. This study explored the effects of cisplatin-(CP), BA and combination of BA and CP (BA-CP) on cell proliferation, apoptotic cell death and expressions of metastasis related genes in neuroblastoma. In this study, N-MYC positive and negative neuroblastoma cell lines, KELLY and SHSY5Y, were treated with BA, CP and BA-CP combinations. BA-CP combinations showed synergistic activity on cell growth inhibition and apoptotic cell death in both neuroblastoma cell lines. In SHSY5Y cells BA, CP or BA-CP treatments induced downregulation most of the metastatic genes' expressions. In KELLY cells, while CP treatment decreased some metastatic gene expressions, BA or BA-CP treatment reduced only DENR and RB1. BA and BA-CP showed prominent effect on metastasis related gene expressions in predominantly KELLY cells. The results of this study suggest that BA may be a promising anti-cancer agent for neuroblastoma treatment, whereas CP is still the most suitable chemotherapeutic drug for aggressive neuroblastoma.

Keywords: Betulinic acid, N-MYC, Metastases genes, Neuroblastoma, Cisplatin

ÖZET

Betulinik Asit ve Sisplatin Nöroblastom Hücrelerinde N-MYC Durumlarına Göre Metastaz İlişkili Genleri İnhibe Eder

Betulinik asid-(BA), nöroblastom üzerinde apoptotik etkiye sahiptir. Bu çalışma sisplatin-(CP), BA ve BA ile CP kombinasyonunun (BA-CP) nöroblastomda hücre çoğalması, apoptotik hücre ölümü ve metastaz ilişkli gen ekspresyonları üzerindeki etkilerini araştırmıştır. Bu çalışmada, N-MYC pozitif ve negatif, KELLY ve SHSY5Y, nöroblastom hücrelerine BA, CP ve kombinasyonları uygulanmıştır. BA-CP kombinasyonu her iki nöroblastom hücre hattında hücre gelişimi inhibisyonu ve apoptotik hücre ölümü üzerine sinerjik aktivite göstermiştir. SHSY5Y hücrelerinde BA, CP veya BA-CP uygulamaları, metastatik gen ekspresyonlarının çoğunun downregülasyonunu indüklemiştir. KELLY hücrelerinde CP uygulaması bazı metastatik gen ekspresyonlarını azaltırken, BA ve ya BA-CP kombinasyonları metastaza bağlı gen ekspresyonları üzerinde belirgin bir etki göstermiştir. Bu çalışma sonuçları, BA'nın neuroblastom tedavisi için umut verici bir anti-kanser ajan olabileceğini önermekle birlikte, CP halen özellikle agresif nöroblastom için en uygun kemoterapötik ilaçtır.

Anahtar Kelimeler: Betulinik asit, N-MYC, Metastaz genleri, Nöroblastom, Sisplatin

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INTRODUCTION

Neuroblastoma (NB) is the third common cancer (6%) among all childhood cancers and related with 15% of all pediatric cancer deaths.[1] Approximately 50% patients have in metastatic disease and those have very poor outcome and in spite of intensive therapeutic regimens including high-dose chemotherapy and myeloablative regimens.[2-4] N-MYC status of tumor is one of the significant factors in determining clinical risk groups.[4,5] N-MYC overexpression is related to poor prognosis, advanced disease stage and rapid tumor progression.[4] Furthermore, the biological mechanism of NB has not been fully elucidated and genetic changes that may define prognosis are still being investigated. Though a minority of N-MYC target genes involved in cell proliferation and apoptosis have been identified, N-MYC target genes responsible from cell migration and metastasis remain elusive.

Cisplatin (CP) is one of the commonly used anti-apoptotic chemotherapeutics in solid tumours, especially in childhood neuroblastoma. However, CP has serious side effects in children including nephrotoxicity, neurotoxicity and ototoxicity.[6-8] New or alternative therapeutic agents are demanded for patients with neuroblastoma.

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid, BA), a pentacyclic triterpene, is found in many plant species derived from white birch trees. BA has several biological effects, including anti-viral, anti-bacterial, anti-inflammatory and anti-tumor effects.[9] While it induces cell death on tumour cells, it is not found as cytotoxic on normal cells.[10] BA has been shown to induce apoptosis in several tumors such as melanoma, neuroectodermal tumors, head and neck squamous carcinomas, prostate and breast carcinomas.[11-15] In combinationatorial therapies, BA and anti-cancer drugs, ionizing radiation or TRAIL increases the anti-cancer activity on tumor cells.[16,17] Furthermore, Cisplatin and BA showed synergistic cytotoxic effect and the induction of apoptosis in SCC25 head and neck cancers cells under combination treatment.

CP is a main chemotherapeutic agent is still used in neuroblastoma treatment but the metastasis related genes expressions have not been studied especially with using CP and BA combinations. The aim of this study was to determine the possible anti-proliferative, apoptotic effects and find the differences between metastasis-associated gene expressions with BA, CP, and BA-CP treatments in both N-MYC negative and positive neuroblastoma cells.

MATERIAL AND METHODS

Cell Culture

The human neuroblastoma cell lines SH-SY5Y (N-MYC negative) and KELLY (N-MYC positive) were obtained from DSMZ (Germany). SH-SY5Y cells were grown in DMEM and KELLY cells were grown in RPMI-1640 with 10% fetal bovine serum, 1% L-glutamine and penicillin/streptomycin respectively in a 5% CO_2 incubator at 37ºC. Seeding density was $5x10^4$cells/cm^2 in both cell lines.

BA and CP were purchased from Sigma–Aldrich (USA). CP was freshly dissolved in isotonic solution (0.9% NaCl) prior to all experiments and immediately diluted into mediums. Stock solution of BA (100 mM) was prepared in DMSO. CP was used in 7.5 and 20 μmol doses in the cell growth inhibition assays with 24 h incubations as previously described.[7] In BA-CP combination group, BA was used at 1 μmoll doses with CP. Twenty-four hours after seeding (time 0) medium of cells were removed and exchanged with serum free medium (control) and CP or BA plus medium. In BA-CP combination group, cells were incubated with BA 1 hour and then CP was added and incubated additional 24 h.

Inhibition of cell growth in response to CP was assessed by 3-(4,5-dimethylthiazol-2yl)-tetrazolium 2,5-diphenyltetrazoliumbromide (MTT) (Sigma–Aldrich, USA) assay and lactate dehydrogenase (LDH) Cytotoxicity Kit (Roche Diagnostics Mannheim, Germany) according to manufacturer's instructions.

Briefly, cells were detached by tyripsin-EDTA and $5x10^4$ cells/ml resuspended in mediums and seed into 96-well culture plates with six replicates. After 24 h of plating, incubation was continued for another 24 h in absence (control) or presence of BA, CP and BA-CP. At the end of the incubation period, the reaction was terminated by adding 10 ul MTT reagent to each well. The reaction was allowed

Table 1. Genes in Metastasis Array

Cell Adhesion Genes: Cell to Cell Adhesion: apc, cd44, cdh1 (cadherin-1 / e-cadherin), cdh11, cdh6, fat, fxyd5, itga7, pnn, syk, vegf. Transmembrane Receptors: cd44, itga7, itgb3, rpsa (lamr1). Other Genes Related to Adhesion: ctnna1, fn1, mcam, mgat5 (acetylglucosaminyltransferase v), mtss1. **Extracellular Matrix Proteins:** Matrix Metalloproteinases: mmp10, mmp11, mmp13, mmp2, mmp3, mmp7, mmp9. MMP Inhibitors: timp2, timp3, timp4. Other ECM Proteins: col4a2 (collagen α2(iv)), hpse (heparanase). **Cell Cycle Genes:** Regulation of the Cell Cycle: hras, il1b, kras, tgfb1 (tgf-β1), vegfa. Negative Regulation of the Cell Cycle: apc, brms1 (brms1), cdkn2a, mtss1, nf2, nme1, nme2, pten, rb1, tp53. Cell Cycle Arrest and Checkpoint: cdkn2a, myc (c-myc), rb1, tp53. **Other Genes Related to Metastasis:** cst7, ctsk, ctsl1 (cathepsin l), cd82 (kai1), kiss1 (kiss-1), metap2, nme4.	**Cell Growth and Proliferation Genes:** Negative Regulation of Cell Proliferation: cdkn2a, ctbp1, gnrh1, il1b, mdm2, nf2, nme1, nme2, sstr2. Positive Regulation of Cell Proliferation: igf1, il18, tshr, vegfa. Growth Factors and Hormones: gnrh1, hgf (scatter factor), igf1, tgfb1 (tgf-β1), vegfa. Cytokines and Chemokines: ccl7, cxcl12, il18, il1b, tnfsf10. Receptors: cxcr4, ephb2, fgfr4, flt4, kiss1r, il8rb, met, nr4a3, plaur (upar), rorb, sstr2, tshr. Other Genes Related to Growth: denr, ewsr1, hras (c-hras), myc (c-myc), set, src (c-src), syk, trpm1. **Apoptosis genes:** Induction of Apoptosis: htatip2, il18, timp3, tnfsf10, tp53. Anti-apoptosis: htatip2, tgfb1. Other Genes Related to Apoptosis: cxcr4, il1b. **Transcription Factors and Regulators:** Transcription Factors: etv4, htatip2, mta1, myc (c-myc), mycl1, nme2, nr4a3, rb1, rorb, smad4, tcf20, tp53. Regulators: chd4, ewsr1, smad2.

to proceed for 4 h at 37^{0}C. The formazan crystals were dissolved by adding solubilization solution. The intensity of the color developed, which is the reflection of number of live cells, was measured at a wavelength of 450 nm by ELISA reader (Thermo, Instruments Inc, USA). All values were compared to the corresponding controls. All assays were performed with 6 replicates.

LDH cytotoxicity assay was done using picked cell culture supernatants at the end of the 24 h incubation periods. Supernatants were added into reaction mixture of catalyst and dye solution and incubated about 30 minutes at room temperature. The stop solution was added on the wells at the end of the incubation period. The changes of absorbance's due to color changes was measured at 490/590 nm.

Assessment of Apoptosis

Human neuroblastoma cells were exposed to CP, BA and BA-CP combinations at 24 h incubation period and apoptotic cell death was monitored with using TUNEL (TdT-mediated dUTP Nick End Labeling) assay that can detect fragmented DNA in the nucleus during apoptosis (GenScript Apoptosis Detection Kit). The kit was applied on slides according to manufacturer's instructions at the end of the experiments. 5000 cells per condition were assessed and recorded as percentage of apoptosis per all cells.

Analysis of Metastatic Gene Expressions

After experimental procedure, RNA isolation (Qiagen) was done from neuroblastoma cell cultures. Complementary DNA (cDNA) synthesis was obtained by thermal cycling. Specific primers for mRNA were analyzed by RT-PCR array (SABiosciences).

Changes in expressions of mRNA of human tumor metastasis related genes in metastasis array

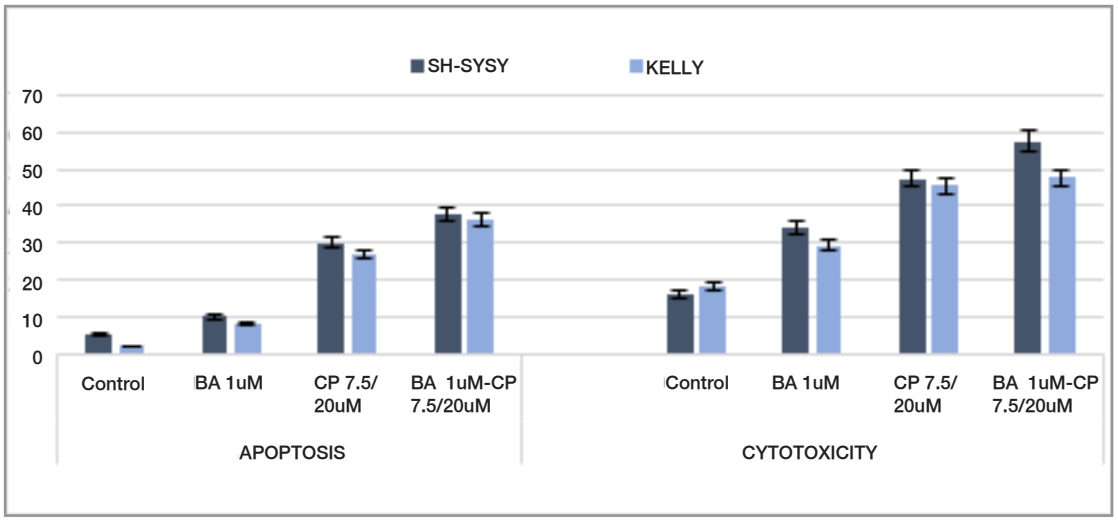

Figure 1. Betulinic acid (BA), Cisplatin (CP) and BA-CP combinations significantly induced apoptosis and cytotoxicity in both SH-SY5Y (N-MYC negative) and KELLY (N-MYC positive) neuroblastoma cells at 24 h. Values represent the mean ± SEM of 6 observations (Mann-Whitney-U, *p< 0.05 versus control, # p< 0.05 versus CP).

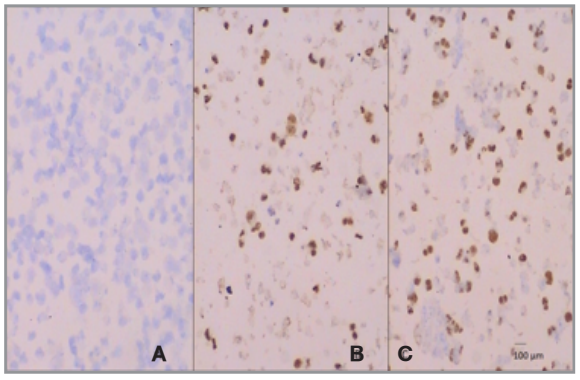

Figure 2. Apoptotic cell death (TUNEL) photos of control, Cisplatin (CP) and BA-CP combinations groups in KELLY (N-MYC positive) neuroblastoma cells at 24 h.

A: Kelly neuroblastoma cells: Control; B: Kelly, cisplatin 28% apoptosis; C: Kelly, betulinic acid+Cisplatin 35% apoptosis

and also neuronal migration related some human genes like as ADAM13, FRZLD4, TRIO, DDX1, NDRG1 and INTGA6 were evaluated by quantitatively.

After RNA isolation and cDNA converting, expression of 84 custom array gene panel of tumor metastatic genes (SABiosciences, PATS028A) was determined by RT-PCR for each condition. Custom genes of primers were supplied by the SABiosicences as standardized primers and used in this study. These genes are listed below in Table 1. Fold changes of gene expressions according to control of each condition were calculated at manufacturer's online free data PCR expression analysis page (www.sabiosciences.com). Housekeeping genes of B2M, HPRT1, RPL13A, GAPDH and ACTB genes were used in this study. Fold changes of each condition were calculated according to $2^{-\Delta\Delta C(t)}$ and compared with their controls. Genes that showed increase or decrease more than three folds were taken into consideration for expression changes.

Statistical Analysis

All statistical analyses were performed using the SPSS 15.0 software program. All results were expressed as means± SEM. Continuous variables were compared with the Mann-Whitney-U rank sum test. All treatment experiments were repeated at least three times to generate statistically relevant data. P< 0.05 was considered statistically significant.

RESULTS

BA and CP Decreased Neuroblastoma Cell Viability and Induced Apoptosis

BA increased cell cytotoxicity in a dose dependent manner from 1-100 μM in both neuroblastoma cells. CP inhibited 50% of cell growth at 7.5 and 20 μM doses for 24 h incubations in SH-SY5Y and KELLY cells (Figure 1). CP, BA and CP-BA com-

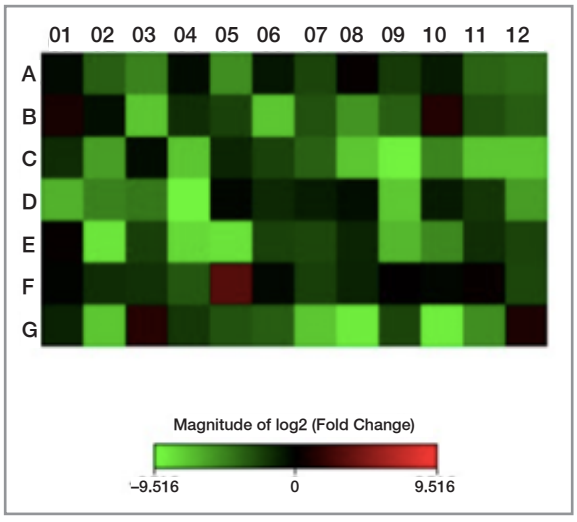

Figure 3. Heatmap representation of BA-CP versus control in SHSY5Y cells

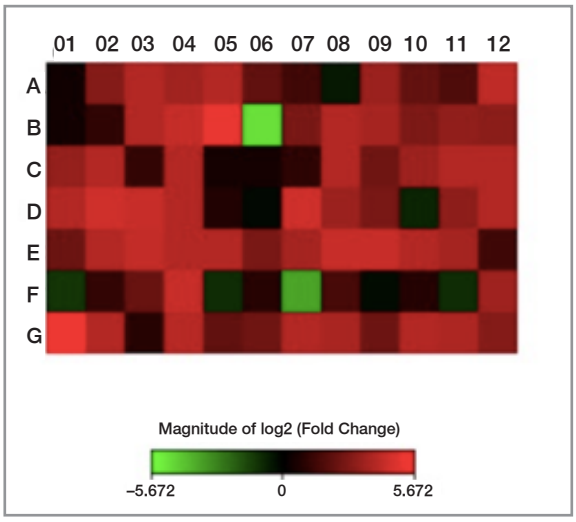

Figure 4. Heatmap representation of BA-CP versus control in KELLY cells

bination treatments caused a statistically significant induction of apoptosis in SH-SY5Y and KELLY cells as shown in Figure 1 and Figure 2 ($p< 0.05$). According to these results, CP-BA combinations showed synergistic activity on cell growth inhibition and apoptotic cell death. BA (1 μM) and CP (7.5 μM and 20 μM respectively) incubated for 24 h in the cells were used in gene expressions experiments (Figure 1).

Metastatic Gene Expressions in N-MYC Negative SH-SY5Y Cells

CP increased DENR, METAP2, NME2, PNN, SET, SMAD2 and SMAD4 gene expressions in N-MYC negative SH-SY5Y cells. IGFR1, MET, MMP7, MMP13, TNSF10 and TRPM1) were downregulated with CP treatment in SH-SY5Y cells (data not shown). Only PNN gene expression increased with BA treatment SH-SY5Y cells (data not shown).

BA-CP treatment reversed the effects CP treatment and decreased the expression of DENR, METAP2, NME2, PNN, SET, SMAD2 and SMAD4 genes in SH-SY5Y cells (Figure 3, Table 1).

Moreover, BRMS1, CST7, CTBP1, CSTL1, ETV4, KISS1R, HRAS, HTATIP2, IL18, IL1B, IL8RB, ITGA7, KISS1, MMP10, MMP13, MMP3, MMP7, MYC, SYK, TIMP4, TNFSF10 and TRPM1 gene expressions were decreased with BA-CP treatment in comparison with CP treatment alone (data not shown). Interestingly, N-MYC was the most down-regulated gene among all genes in response to BA-CP combinatorial treatment (Figure 3).

In addition, BA alone treatment was compared with BA-CP combinatorial treatment. Interestingly, CDH1, CST7, CTBP1, CSTL1, CXCL12, CXCR4, ETV4, FGFR4, FN1, KISS1R, HGF, HTATIP2, IL8RB, ITGA7, ITGB3, CD82, KISS1, MCAM, MDM2, MET, MGAT5, MMP10, MMP13, MMP2, MMP7, MMP9, MTA1, MYC, MYCL1, NME4, PLAUR, SSTR2, TIMP4 and TNFSF10 genes expressions were much more downregulated in BA treatment alone in comparison with BA-CP combinatorial treatment (Figure 3). In addition, BA treatment alone has shown to decrease the metastatic gene expressions in a higher rate than CP alone treatment.

Metastatic Gene Expressions in N-MYC Positive KELLY Cells

CP treatment increased CDH1, CXCR4, FXYD5, MCAM and SSTR2 gene expression in KELLY cells. CDH11, CDH6, COL4A2, EPHB2, IL18, MGAT5, RORB, and TIMP3 genes were decreased in response to CP treatment (data not shown).

BA treatment alone increased the expression of most of the metastatic genes in bad prognostic Kelly cells, whereas DENR and RB1 gene expressions were downregulated (data not shown).

BA-CP treatment increased the gene expressions of BRMS1, CCL7, CD44, CDH11, and CHD4 in comparison to CP treatment alone in KELLY cells (Figure 4, Table 1).

CP treatment alone decreased the expressions of CDH11, CDH6, COL4A2, EPHB2, IL18, MCAT5, RORB and TIMP3 genes in KELLY cells however the effect was reversed with BA-CP combinatorial treatment (Figure 4). Moreover, CP-BA treatment in KELLY cells increased the CDH1, CXCR4, FXYD5, MCAM and SSTR2 and decreased DENR and RB1 gene expressions when compared with CP treatment alone.

Comparison of Gene Expressions of N-MYC Negative Versus N-MYC Positive Neuroblastoma Cells

All metastatic genes in the gene array panel except DENR and PNN, were expressed higher in SH-SY5Y cells when compared to KELLY cells. In CP alone treated groups, CDH1, CXCR4, FXYD5, MCAM and SSTR2 gene expressions were found to be increased in KELLY cells and decreased in SH-SY5Y cells (data not shown). CP treatment reduced the number of metastatic gene expression N-MYC negative SH-SY5Y cells when compared with N-MYC positive KELLY neuroblastoma cells.

BA alone treatment has shown adverse effect in metastatic gene expression levels in N-MYC negative SH-SY5Y cells and N-MYC positive KELLY cells (data not shown). While BA alone treatment upregulated metastatic gene expressions in KELLY, downregulated in SH-SY5Y cells. In addition, when compared to CP alone with BA alone treatments on especially bad prognostic KELLY cells, our results have been showed more metastatic genes were in higher expression levels affected in negatively manner with CP treatment (data not shown).

BA-CP treatment inversely affected the expression of metastatic genes in both N-MYC negative and N-MYC positive human neuroblastoma cells (Figure 3, Figure 4). BA-CP treatments showed the increased the metastatic gene expressions in especially bad prognostic KELLY cells but when compared to other treatments it did not as good as CP treatments on these cells (Figure 3, Figure 4).

Migration related genes such as ADAM13, FRZLD4, TRIO, NDRG1 and INTGA6 were not expressed in both cell lines. Following BA or BA-CP treatments only DDX1 gene expression were increased in SH-SY5Y cells while decreased in KELLY cells.

DISCUSSION

It is still problematic to find optimal treatment modalities in NB and other childhood tumours. Oncogene N-MYC expression level is one of the most important clinical prognostic risk factor in NB.[2,4] Therefore, SH-SY5Y (N-MYC negative) and KELLY (N-MYC positive) cell lines, an appropriate model for determination of N-MYC associated prognosis. To date, there had been few studies about metastatic gene expressions comparing N-MYC negative and N-MYC positive neuroblastoma cells in the literature.

This study had demonstrated that BA could be used as an anti-proliferative and anti-apoptotic agent in human neuroblastoma cells. Similar to our results, BA treatment had previously shown to lead apoptosis via both caspase 8 and caspase 3 activation in SHEP neuroblastoma cells.[18] Moreover, in metastatic melanoma cells, BA induced p53 expression, which is observed in poor prognostic KELLY cells in our study.[19] Also, BA delayed the formation and also inhibited proliferation, invasion and angiogenesis of breast tumors in nude mices.[20] Metastasis or hypoxia related therapy resistances are important factors for malign progression of cancers cells such as glioblastoma. Glycosylated derivative of BA was shown to be effective in killing of hypoxic glioblastoma cells by down-regulating TNF-α, iNOS and NF-κB mRNA expressions.[21] Abnormal expression of chemokines and their receptors, such as CXC chemokine receptor-4 (CXCR4), positively correlated with the development, progression and metastasis of tumour cells.[22] Treatments of cyclophosphamide and pirarubicin reduced the ex-

pressions of CXCR4 in especially CXCR4 highly expressed neuroblastoma cells. In agreement with this, we have shown that CXCR4 expressions was decreased with BA alone and BA-CP combination treatment in SH-SY5Y cells while upregulated in KELLY cells.

CDK4 which participates in cell cycle regulation, is inhibited by Cyclin Dependent Kinase Inhibitor 2A (CDKN2A).[23] Inhibition of CDK4 was shown to induce apoptosis as result of PARP cleavage in melanoma cells. In our study, CP stimulated downregulation of CDKN2A might be related to apoptosis observed in SH-SY5Y cells while not in KELLY cells. In another study, it was demonstrated that BA decrease Bcl-2, Cyclin D1 expressions while increase Bax expressions in SK-NA-S neuroblastoma cells.[24] Furthermore BA was shown to inhibit the migration of tumor cells such as glioma, lung carcinoma and medulloblastoma cells. In our study, we showed that growth factor IGF-1 expression was increased with BA treatment in N-MYC negative SH-SY5Y cells while decreased in N-MYC positive KELLY cells. In parallel with our results, BA treatment was shown to downregulated IGF-1 expression in human endometrial adenocarcinoma cells.[25] BA treatment also inhibited collagen biosynthesis associated to IGF-1 receptor signalling in those cells. In this present study, BA-CP treatment was shown to upregulate cell adhesion and angiogenesis related gene VEGF expression in SH-SY5Y cells whereas downregulate in KELLY cells. Interestingly, in a recent study similar to our findings indicated that BA and mithramycin combination up-regulated VEGF expression in human pancreatic cancer cells.[26]

Matrix metalloproteinase-2 (MMP-2) and MMP-9, were well-known genes involved in invasion of cancer cells and also had been associated with invasion of the SH-SH5Y cells.[27] N-MYC increased the activity of MMPs. In one study, it was indicated that expression of BCL2 led to an increase in expression and secretion of MMP2, further co-expression of N-MYC and BCL2 suppressed the expression MMP2 antagonist, tissue inhibitor of metalloproteinase (TIMP) TIMP-2 in SHEP NB cells.[28] TIMP-2 was also assumed as metastasis suppressor. In our study CP alone, BA alone and CP-BA treatments declined MMP-2, MMP-9, TIMP-2 gene expression levels in SH-SY5Y cells. In contrast, BA alone and BA-CP treatments elevated the expression levels of MMP-2, MMP-9 and TIMP2.

In our study, we analysed further metastatic gene expressions levels between our conditions. We also shown that MMP-3, MMP-7, MMP-10, MMP-11, MMP-13 and their antagonist TIMP-3 and TIMP-4 gene expressions were diminished with CP alone, BA alone and CP-BA treatments in SH-SY5Y cells, whereas in KELLY cells, expressions of these genes only decreased with BA alone and BA-CP.

Furthermore, it was suggested that BA might have preventive effect on bone loss in patients with bone metastasis of breast cancer through inhibiting MMP-2, MMP-9 and cathepsin K secretion.[15] N-MYCN and N-MYC-associated activation of a microRNA-9 (miR-9), targeted and suppressed E-cadherin expression and contributed to epithelial mesenchymal transition.[29] In the present study, we demonstrated that E-cadherin expression decreased in a higher rate with BA>BA-CP>CP in N-MYC negative neuroblastoma cells while increased with CP>BA-CP>BA treatments in N-MYC positive and poor prognostic cells. Our findings are supported by another study which showed that CDH1 mRNA-gene and protein expressions decreased in metastatic neuroblastoma tissues when compared to primary tumour tissues.[30] In this regard, BA has promising agent that CDH1 inactivation is important for metastasis of neuroblastoma.

Furthermore, epithelial mesenchymal transition related transforming growth factor-β1 and CD44 mRNA expressions decreased with CP, BA, CP-BA treatments in N-MYC negative neuroblastoma cells while increased with BA-CP and BA treatments in N-MYC positive neuroblastoma cells. BA-CP treatments showed the increased the metastatic gene expressions in especially bad prognostic KELLY cells but when compared to other treatments it did not as good as CP treatments on these cells. Furthermore, in KELLY cells, only BA treatment induced the metastatic gene expressions when compared to CP and BA-CP treatments with the levels of gene expressions.

Only DENR and a tumor suppressor gene of RB1, expressions were decreased with BA treatment

in both cell lines. In a recent study showed that DENR is related to initiation of eukaryotic translation and might be involve in proliferation and tissue growth.[31] In this regard, BA could be evaluated as a tissue growth inhibiting agent by DENR gene expression in neuroblastoma.

BA is a potential chemotherapeutic agent because of it is not cytotoxic on normal cells but toxic on tumor cells.[10] Moreover, it shows low toxicity in fibroblasts, lack of toxicity to neurons.[32] BA has remarkable anti-proliferative activity in a wide range of cancer cells including breast, prostate, colon, melanoma, glioma and neuroblastoma, and it might be accepted as a candidate agent for adjuvant treatment of human cancers in a short time.[11, 21-22]

In conclusion, neuroblastoma treatment is a still problematic due to disease heterogeneity and adverse effects of the chemotherapeutics. Cisplatin is the main chemotherapeutic drug especially in N-MYC positive bad prognostic patients but it has some dose limiting serious side effects like as nephrotoxicity, ototoxicity and neurotoxicity in children. Therefore, new chemicals and drugs should be developed for the treatment of neuroblastoma. In this study, BA alone, BA-CP combinations and CP treatments showed prominent effect on tumor metastasis related gene expressions in particularly in good prognostic N-MYC negative SH-SY5Y neuroblastoma cells. Moreover, BA and BA-CP combinations decreased the metastatic gene expressions like as CP treatment in poor prognostic N-MYC positive KELLY neuroblastoma cells. The results of this study suggest that BA may be a promising anti-cancer agent for neuroblastoma treatment, whereas CP is still the most suitable chemotherapeutic drug for aggressive neuroblastoma. The effects of BA on metastasis and related gene expressions in neuroblastoma animal tumor models *in vivo* should be investigated.

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