Biological Activity of a Thiobarbituric Acid Compound in Neuroblastomas

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Abstract. *Background/Aim:* We have previously reported the identification of the cytotoxic chemotype compound-I (CC-I) from a chemical library screening against glioblastoma. *Materials and Methods:* The biological activity of CC-I on drug-resistant neuroblastomas [e.g., HFE gene variant C282Y stably transfected human neuroblastoma SH-SY5Y cells (C282Y HFE/SH-SY5Y), SK-N-AS] was characterized using cell culture models and in vivo mouse tumor models. *Results:* CC-I had potent cytotoxicity on therapy-resistant neuroblastoma cells and limited cytotoxicity on human primary dermal fibroblast cells. In addition, CC-I showed a robust anti-tumor effect on therapy-resistant human neuroblastoma C282Y HFE/SH-SY5Y cells but not on SK-N-AS cells in a subcutaneous tumor model. CC-I induced phosphorylation of heat shock protein 27 (HSP27), protein kinase B (Akt), and c-Jun N-terminal kinase (JNK) in C282Y HFE/SH-SY5Y neuroblastoma cells. *Conclusion:* CC-I may be an effective therapeutic option for therapy-resistant neuroblastomas, especially if they express the C282Y HFE gene variant. Its anti-tumor effects are possibly through HSP27-Akt-JNK activation.

Neuroblastoma is the second most common solid tumor discovered in children (1). It begins in the nerve cells outside of the brain, most often in the adrenal glands located on top of both kidneys. Nearly 90% of neuroblastomas are diagnosed by age 10 (2). In the United States, neuroblastoma occurs in about 800 children (age up to 14) each year, which accounts for 6% of all childhood cancers (3). The

International Neuroblastoma Risk Group Staging System was developed by the International Neuroblastoma Risk Group for the standardization of neuroblastoma risk classification and for clinical trials (4). Risk factors for neuroblastoma include age at diagnosis, disease stage, tumor histology, and N-myc proto-oncogene protein (MYCN) amplification status (5). Although the 5-year survival rate for low-risk neuroblastoma is 95%, the 5-year survival rate for high-risk neuroblastoma is only 40-50% (3).

About half of the neuroblastomas are metastatic at diagnosis, and advanced disease remains difficult to treat successfully despite the aggressive multimodality therapy. Treatment for neuroblastoma usually involves a variety of approaches such as chemotherapy, radiation, surgery, stem cell transplantation, biological agents, and immunotherapy (6, 7). The majority of patients with high-risk neuroblastoma will relapse despite receiving aggressive multimodal therapy, while an additional 10% to 20% will be refractory to induction therapy (8, 9). Because of disease heterogeneity among high-risk cases, subsequent management with salvage chemotherapy can be very challenging. Therefore, developing new treatment agents against neuroblastoma, especially therapy-resistant neuroblastoma, is urgently needed.

We previously reported the identification of compound CC-I, a thiobarbituric acid derivative, from the screening of the ChemBridge small molecule library compounds, as an effective agent against drug-resistant glioblastomas (GBMs) (10). We showed that CC-I had a dose-dependent cytotoxic effect in Temozolomide resistant astrocytoma cells, caused apoptotic cell death and cell cycle arrest at S and G₂/M phases, inhibited tumor growth without toxicity, and inhibited topoisomerase II α . We also demonstrated that CC-I and its analogs had cytotoxicity and anti-tumor effect on human melanoma, breast cancer, colon cancer, pancreatic cancer, and lung cancer cells (11, 12). In melanoma cells (CHL-1, UACC903), the two most potent compounds induced PARP cleavage and inhibited anti-apoptotic Bcl-2, Bcl-xL and Survivin in a dose-dependent manner (11).

In this study, we characterized the cytotoxicity and began to evaluate the underlying mechanisms of action which

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could, at least in part, be responsible for the anti-cancer activity of CC-I in therapy-resistant human neuroblastoma cell lines, such as SK-N-AS and C282Y HFE/SH-SY5Y cells, and subcutaneous neuroblastoma tumor models.

Materials and Methods

Materials. Cell culture reagents, including DMEM/F12 medium, L-glutamine, and Trypsin-EDTA were purchased from Life Technologies (Grand Island, NY, USA). Fibroblast basal medium and fibroblast growth kit-serum free were ordered from American Type Cell Culture (ATCC). Fetal bovine serum (FBS) was obtained from Gemini Bio-Products (West Sacramento, CA, USA). The compound CC-I was synthesized as described before (11). The final compound was purified by silica gel column chromatography and characterized by nuclear magnetic resonance (NMR) and mass spectra. The purity level of CC-I was $\geq 99\%$.

Cell culture, cell proliferation, treatment, and cytotoxicity assay. To determine the efficacy of CC-I against therapy-resistant human neuroblastomas, we used three human neuroblastoma cell lines (SK-N-AS, CHLA-171, and C282Y HFE variant stably transfected SH-SY5Y cells (C282Y HFE/SH-SY5Y)). The SH-SY5Y cell line was generated from the bone marrow of a 4-year-old female, whereas the SK-N-AS cell line was derived from the metastatic site of a 6-year-old female. The CHLA-171 cell line was established from the progressive disease of a 101-month-old male neuroblastoma patient. The three cell lines had non-amplified MYCN; however, the p53 status was different. While the SH-SY5Y and CHLA-171 cells had wild type p53, SK-N-AS cells expressed the p53 β isoform (13). The CHLA-171 cells had non-functional p53 (14). Drug-resistant human neuroblastoma SK-N-AS cells were purchased from ATCC (CRL-2137) and maintained in DMEM supplemented with 10% FBS and 1X Penicillin-Streptomycin (10,000 U/ml). Acquired drug-resistant human neuroblastoma CHLA-171 cell line was obtained from the Children's Oncology Group and cultured in IMDM supplemented with 10% FBS, 4 mM L-glutamine, 1X ITS (5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenous acid). The human neuroblastoma SH-SY5Y cell lines, purchased from ATCC (CRL2266), stably transfected with wild type (WT) or C282Y HFE mutant were maintained in DMEM/F12 media supplemented with 10% FBS, 1% antibiotics (Penicillin-Streptomycin), 1X non-essential amino acid, and geneticin (200 μ g/ml) (15). Authentication of the cell line was performed by Genetica (Burlington, NC, USA) *via* STR DNA profiling. Adult human normal primary dermal fibroblasts (nHDF) (ATCC PCS-201-012) were maintained in fibroblast basal medium with a growth supplement. All the cell lines were cultured in a CO₂ incubator at 37°C.

For cell proliferation assays, WT or C282Y HFE/SH-SY5Y cells were plated at a density of 10,000 cells per well in 24-well plates and then, live cell number was counted using 0.2% Trypan blue staining at different days (n=3). Also, the same cells were plated at a density of 4,000 cells per well in 96-well plates and then, cell growth was measured with the MTS cell proliferation assay for ten days.

For the cytotoxicity assays, the overnight cultured cells (*e.g.*, 8,000 cells/well for C282Y HFE/SH-SY5Y; 20,000 cells/well for WT HFE/SH-SY5Y) were exposed to compounds or DMSO vehicle control for 48 h and then, cytotoxicity was evaluated by the MTT assay at the end of the cell culture period. In another cytotoxicity assay, the overnight cultured cells (*e.g.*, 4,000 cells/well for SK-N-AS, CHLA-171, nHDF) were exposed to compounds or DMSO for

72 h and then, cytotoxicity was evaluated by the MTT assay at the end of the cell culture period. For molecular mechanism studies, the CC-I concentration depended on exposure time and LC₅₀ (50% lethal concentration). For example, if the exposure time to the compound was short (2-24 h), the concentration of compound was high (5-20 fold higher than LC₅₀ which is determined for 2-3 days treatment). CC-I was dissolved in DMSO to make a stock solution before dilution for treatment. The LC₅₀ of CC-I was determined using statistical software (GraphPad Prism version 7) as a general indicator of a chemical's toxicity.

Apoptosis and necrosis analysis. Apoptosis and necrosis assays were performed using the Annexin V-FITC Apoptosis kit (V13242, Invitrogen Molecular Probes, Eugene, OR, USA) or Apoptosis/Necrosis detection kit (ab176749, Abcam, Cambridge, MA, USA). In brief, cells (2 $\times$ 10⁶ per cell culture dish) were cultured up to 24 or 48 h with CC-I (10-36 μ M) or actinomycin D (40-80 nM, for apoptosis) or hydrogen peroxide (500 μ M for necrosis) as positive controls (10, 16, 17). After collecting and washing in cold HANK's buffer, the cells in 100 μ l assay buffer were incubated with 2 μ l of Apopxin Green Indicator (100 $\times$), 1 μ l of 7-Aminoactinomycin D (7-AAD) (200 $\times$), and 1 μ l of CytoCalcein Violet 450 for 30 min at room temperature in the dark. The stained cells were analyzed by flow cytometry with the green color reporting apoptotic cells and red color reporting the late-stage apoptotic/necrotic cells.

Cell cycle analysis. C282Y HFE/SH-SY5Y cells were cultured for 1 or 2 days with or without CC-I (18, 36 μ M) for cell cycle analysis. After washing with cold HANK's buffer, the cells were fixed in ice-cold 70% ethanol overnight at -20°C. Then, the cells were incubated with propidium iodide (100 μ g/ml) and RNase A (20 μ g/ml) for 15 min at 4°C (protected from light) and cell cycle was analyzed using BD FACS Calibur Flow Cytometry Analyzer (BD Biosciences, San Jose, CA, USA).

Subcutaneous tumor model for neuroblastoma. The anti-tumor effect of CC-I was evaluated using neuroblastoma (C282Y HFE/SH-SY5Y, SK-N-AS) subcutaneous tumor nude mouse xenograft models. In brief, 6-8 weeks old female Severe Combined Immunodeficiency (SCID) mice (strain #236, total n=18) or athymic nude mouse (strain #490, total n=13) (Charles River Laboratories, Wilmington, MA, USA) were implanted at the flank with ~200 μ l (5 $\times$ 10⁶ cells per mouse) medium of neuroblastoma cells (18, 19). When the subcutaneous tumors reached approximately 80-100 mm³ in size (2-3 weeks after cell implant), the mice were randomly divided into control and treatment groups keeping similar tumor volume range in each group. For the CC-I- treated groups, the stock compound was dissolved in 100% DMSO and then diluted with ethanol to prepare a working solution (5% DMSO and 12% ethanol, or DMSO/PEG400/Solutol) on the injection day. CC-I was injected intraperitoneally once a week for 3-7 weeks using 70% of the maximum tolerated dose (MTD) (10). The vehicle-treated control group was injected intraperitoneally with the same regime. The health and survival of the mice were monitored daily. The body weight and tumor size of the mice were measured once a week. Tumor volume (V) was measured with a Vernier caliper. It was calculated according to the formula $V=(a^2/2) \times b$, where *a* and *b* are the minor and major axes of the tumor foci, respectively. In the subcutaneous tumor model, we terminated the experiment after the final injection or when the tumors began to interfere with normal

functions such as eating, drinking, moving or Body Condition Scoring (a non-invasive and effective assessment of an animal's physical wellbeing) was less than 2. If the tumor was ulcerated or necrotic, it was treated with a triple antibiotic daily using a sterile Q-tip and was entirely covered. To test the toxicity of compounds, we euthanized the mice with ketamine/xylazine (100/10 mg/kg body weight, intraperitoneally) and a toe pinch was performed to make sure the animals were completely under anesthesia. Once there was no response, a cardiac heart puncture was performed to obtain blood from the animal. After that, the mice were subjected to cervical dislocation to assure humane animal destruction. The blood toxicity (liver and kidney toxicity) was evaluated using an automated chemistry analyzer machine (Roche Cobase MIRA, Bellport, NY, USA) and kits manufactured by Thermo Electron (Louisville, CO, USA). During the *in vivo* study, mice were housed in a virus-free barrier facility under a 12-h light-dark cycle, with access to food and water *ad libitum*. All animal living conditions were consistent with standards required by the Association for Assessment and Accreditation of Laboratory Care International (AAALAC International). All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC # 47185, #47464) of Penn State College of Medicine.

Western blotting. Human neuroblastoma cells were treated with various concentrations of CC-I for 24 h to determine protein expression. After the cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA), protein concentration was determined using the Pierce BCA protein assay kit (Pierce, Rockford, IL, USA). The proteins were separated by electrophoresis, transferred onto a PVDF membrane and probed with the following primary antibodies: pan-Akt (#4691), phospho-Akt (p-Akt) (Ser473) (#4060), SAPK/JNK (#9252), p-SAPK/JNK (Thr183/Tyr185) (#4668), c-Jun (#9165), p-c-Jun (Ser73) (#3270), HSP27 (#2402), p-HSP27 (Ser78) (#2405) (all from Cell Signaling, Danvers, MA, USA), and β -actin (A5441, Sigma-Aldrich). The antibodies for JNK and p-JNK were diluted to 1:500. All the other primary antibodies were incubated at 1:1,000 dilution. The primary antibody reaction was performed at 4°C overnight. The Amersham ECL anti-mouse or anti-rabbit secondary HRP antibodies (#NA931, #NA934, GE Healthcare, Piscataway, NJ, USA) were used at 1:5,000 dilution at room temperature for 1 h. The protein signals were detected using Western Lightning Plus ECL (PerkinElmer, Waltham, MA, USA) and Amersham Imager 600 system (GE Healthcare). The signal intensity of the protein bands was analyzed by ImageQuant TL software (GE Healthcare), and protein intensity was normalized to β -actin levels. The protein expression in the CC-I-treated cells was compared with the protein expression in vehicle-treated control cells. The western blotting experiment was performed using three different biological samples.

Statistical analysis. All of the data were subjected to statistical analysis using the student *t*-test when comparing two groups. We used one-way ANOVA followed by the Tukey-Kramer test for more than two group comparisons, to determine if the differences are significant. For comparisons of time course or concentration data, we performed repeated measures two-way ANOVA followed by the Tukey-Kramer test. The LC_{50} (50% lethal concentration) of CC-I was determined using simple logistic regression analysis of GraphPad Prism software (version 7) as a general indicator of

toxicity. In the *in vivo* subcutaneous tumor model study, we used two-way ANOVA models followed by Tukey's multiple comparison test for tumor volume comparison. For the *in vitro* and *in vivo* study, data are displayed as mean $\pm$ standard error of the mean (SEM). Differences among means were considered statistically significant when the *p*-value was less than 0.05.

Results

Cytotoxicity of CC-I to HFE expressing human neuroblastoma SH-SY5Y cells. We have previously reported that C282Y HFE/SH-SY5Y cells are drug- and radiation-resistant (20). We confirmed our previous finding (21) that cells expressing the C282Y HFE proliferated at a much higher rate than the WT HFE/SH-SY5Y cell lines based on cell number counting (Figure 1A) and MTS cell proliferation assay (Figure 1B). Compound CC-I was more cytotoxic to the therapy-resistant and rapidly growing human neuroblastoma C282Y HFE/SH-SY5Y cells compared to the therapy-sensitive WT HFE/SH-SY5Y cells (Figure 1C). The LC_{50} of CC-I on WT HFE/SH-SY5Y and C282Y HFE/SH-SY5Y cells was $3.1 \pm 0.3 \mu M$ and $1.8 \pm 0.3 \mu M$, respectively.

Cytotoxicity of CC-I to human neuroblastoma and fibroblast cells. CC-I was also cytotoxic to the other drug-resistant neuroblastoma cells, such as SK-N-AS and CHLA-171, in a dose-dependent manner, but was relatively less toxic to normal human dermal fibroblasts (nHDF) (Figure 1D). The LC_{50} of CC-I on fibroblast cells was $9.3 \pm 3.9 \mu M$, whereas on SK-N-AS and CHLA-171 cells the LC_{50} of CC-I was $3.1 \pm 0.9 \mu M$ and $2.1 \pm 0.1 \mu M$, respectively.

Cell death (late apoptotic/necrotic vs. early apoptotic) and cell cycle analysis in CC-I treated human neuroblastoma cells. Compound CC-I induced a higher rate of late apoptosis/necrotic cell death than early apoptotic cell death, in a dose-dependent manner in C282Y HFE/SH-SY5Y cells (Figure 2A and B). Moreover, CC-I-treated C282Y HFE/SH-SY5Y cells showed a significant decrease in the G0/G1 phase and an increase in the S and G2/M phases compared to untreated cells (Figure 2C and D).

CC-I (10 μM) induced a higher rate of late apoptosis (top right area) compared to early apoptosis (bottom right area) or necrosis (top left area) after 3 h of treatment of SK-N-AS cells (Figure 3A and B). Following 24 h of treatment, CC-I induced both late apoptosis and necrosis, almost 6-fold higher compared to controls (14.3% vs. 2.3% for late apoptosis; 3.0% vs. 0.5% for necrosis) (Figure 3A and C).

Anti-tumor effect of CC-I on human neuroblastoma subcutaneous mouse tumor model. To assess the anti-tumor effect of CC-I on the *in vivo* neuroblastoma tumor model, we used C282Y HFE/SH-SY5Y and SK-N-AS subcutaneously injected mouse tumor models. As shown in Figure 4A, weekly

Figure 1. Effect of C282Y HFE and CC-I on the survival of neuroblastoma cells. (A-B). Effect of C282Y HFE on cell proliferation. A. Cells were plated at a density of 10,000 cells per well in 24 well plates, and then live cell number was counted following 0.2% Trypan blue staining at 2, 5, and 8 days (n=3). #p<0.001 - compared to WT HFE/SH-SY5Y. B. Cells were plated at a density of 4,000 cells per well in 96 well plates and then cell growth was measured by the MTS cell proliferation assay for ten days (n=3). Data are displayed as mean±SEM. Some of the error bars on the graph are smaller than the indicated symbol in the figure. #p<0.001 - compared to WT HFE/SH-SY5Y. C. Cytotoxicity of CC-I on SH-SY5Y cells stably transfected with wild type (WT) or C282Y HFE mutant. Overnight cultured cells (2×10^4 cells/well for WT HFE/SH-SY5Y cells and 8×10^3 cells/well for C282Y HFE/SH-SY5Y cells, due to different cell proliferation rate) were treated for 48 h and then the MTT cytotoxicity assay was performed. Data are displayed as mean±SEM (n=3). *p<0.05 - compared to vehicle control. D. Cytotoxicity of CC-I on drug-resistant human neuroblastoma cells (SK-N-AS, CHLA-171) and normal human dermal fibroblasts (nHDF). The cells (4×10^3 cells/well) were cultured overnight and exposed to different concentrations of CC-I for 72 h and then the MTT assay was performed. Experiments were performed in triplicate and repeated at least three times. Data are displayed as mean±SEM (n=3). **p<0.01 between nHDF and SK-N-AS.

intraperitoneal injections of CC-I completely inhibited tumor growth in SCID mice compared to control mice that received vehicle injections (p<0.001). Neither liver nor kidney toxicity was observed based on the levels of liver and kidney enzymes in the serum (Figure 4B). However, CC-I showed no anti-tumor effect on the SK-N-AS subcutaneous mouse tumor model compared to the vehicle-treated control mice.

HSP27-Akt-JNK expression in CC-I-treated human neuroblastoma cells. The expression of several proteins involved in signal transduction was determined by measuring the phosphorylation levels of proteins. We examined whether CC-I affects the expression of Akt and p-Akt in C282Y

HFE/SH-SY5Y cells, because p-Akt expression was increased in CC-I-treated human glioblastoma (unpublished observation) and lung cancer cells (12) as was shown in a human phospho-kinase array at an early treatment time. Besides, Akt is a crucial protein involved in cell death (22), and the activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling is a poor prognostic indicator of neuroblastoma (23). While the expression of p-Akt was not increased after 24 h exposure of C282Y HFE/SH-SY5Y cells to CC-I (36 μ M) compared to the vehicle control group, Akt expression was decreased (Figure 5A). The ratio of phosphorylated Akt expression levels to Akt levels was three-fold enhanced by 16 μ M of CC-I after 24 h exposure

Figure 2. Analysis of apoptosis/necrosis and cell cycle in CC-I-treated C282Y HFE/SH-SY5Y cells. (A, B) Cell death was monitored with early apoptotic and late apoptotic/necrotic cell markers following 24 h exposure of CC-I in C282Y HFE/SH-SY5Y cells. Cell death was determined using recombinant annexin V conjugated to fluorescein, followed by flow cytometric analysis (Molecular Probes). Early apoptotic cell death is shown in panel A. Panel B shows late apoptotic/necrotic cell death. Actinomycin D was used as a positive control to induce apoptotic cell death. There was a pronounced dose-dependent increase in late apoptotic/necrotic cell death in the CC-I-treated C282Y HFE/SH-SY5Y cells. Data are displayed as mean±SEM (n=3). The symbols indicate a significant difference compared to the control. (* $p<0.05$; ** $p<0.01$; # $p<0.001$) (C, D) Cell cycle analysis of C282Y HFE/SH-SY5Y cells after CC-I treatment (18 or 36 μ M) for 24 h (C) or 48 h (D). The cells were stained with propidium iodide and then analyzed for cell cycle distribution using a FACScan analyzer. CC-I treatment significantly increased the S and G₂/M cell population but decreased that of the G₀/G₁ phase. Data are displayed as mean±SEM (n=3). Some error bars are too small to be visible. The symbols indicate a significant difference compared to the control. (* $p<0.05$; ** $p<0.01$; # $p<0.001$).

(Figure 5B). CC-I significantly increased the expression of the two isoforms of p-JNK (Thr183/Tyr185) (46, 54 KDa) (Figure 5A and B). Phosphorylated c-Jun levels were also elevated in the CC-I-treated cells compared to control cells; however, c-Jun levels were also increased by CC-I (Figure 5A and B). An increase in the levels of the phosphorylated form of HSP27 (Ser78) was also observed in CC-I-treated C282Y HFE/SH-SY5Y cells (Figure 5A and B).

Discussion

We demonstrated the cytotoxicity, apoptosis/necrosis, cell cycle arrest, and anti-tumor effect of compound CC-I on

neuroblastomas. The cell death mechanisms activated by CC-I may be mediated by HSP27-Akt-JNK activation (*i.e.* increased expression of the phosphorylated form of the proteins).

We found that p-JNK expression (*i.e.* JNK activation) was significantly increased in CC-I-treated neuroblastoma cells. This finding is consistent with our previous results showing activation of JNK in CC-I-treated lung cancer cells (12). JNKs have critical roles in stress signaling pathways and many cellular functions such as cell proliferation, differentiation, cellular senescence, and cell death (24, 25). Several compounds have shown an antineoplastic effect by activating JNK in cancers such as ovarian cancer and lung cancer (26-28). The activation of JNKs may be pro- or anti-apoptotic

Figure 3. Apoptotic and necrotic cell death in CC-I-treated SK-N-AS cells. (A) Representative FACS analysis showing apoptotic and necrotic death of SK-N-AS cells treated with CC-I. (B, C) The average percentage for apoptosis and necrosis of SK-N-AS cells treated with CC-I or other chemicals was assayed by FACS. The SK-N-AS cells were exposed to CC-I (10 μ M), H₂O₂ (500 μ M), or actinomycin D (AcD, 40 nM) for 3 h (B) or 24 h (C). H₂O₂ (500 μ M) and actinomycin D (40 nM) were used as necrosis and apoptosis-inducing agents, respectively. Apoptosis and necrosis assay were analyzed by FACS using Apoptosis/Necrosis Detection Kit (ab176749, Abcam). The cell number in the compound-treated group was compared with the vehicle-treated control group by t-test. (* p <0.05; ** p <0.01).

depending on the downstream signaling factors that are activated and/or the duration of the activation of JNKs (29). Also, the sustained activation of JNK is associated with apoptosis, whereas acute and transient JNK activation is involved in cell proliferation (30, 31). Moreover, it is known that JNKs play a central role in both death receptor-mediated apoptosis, and mitochondria-mediated apoptosis. In the present study, we demonstrated Akt-JNK activation in CC-I treated neuroblastomas. This is consistent with the current knowledge that the JNK signaling pathway is one of the downstream pathways of PI3K/Akt signaling (32).

Moreover, we found increased p-HSP27 expression but decreased HSP27 expression in CC-I-treated neuroblastomas.

HSP27 is a small heat shock protein that has multiple functions. The expression of HSP27 was increased in lung cancer tissues and the serum of patients with lung cancer (33). Lung cancer patients with increased HSP27 levels were associated with poorly differentiated cancer (33). Therefore, our present result supports that CC-I induced neuroblastoma cell death is associated with decreased HSP27 and increased p-HSP27 levels. Moreover, HSP27 can directly bind to Akt and increase its phosphorylation (34), and also inhibit apoptosis by the direct interaction and activation of Akt (35). Combined, these observations suggest that the mechanism of CC-I-induced cell death in susceptible neuroblastoma cells is *via* activation of the HSP27-Akt-JNK pathway. Although C282Y HFE can

Figure 4. Anti-tumor effect of CC-I in therapy-resistant C282Y HFE/SH-SY5Y subcutaneous mouse tumor model. (A) Mice were implanted with five million human neuroblastoma SH-SY5Y cells stably-transfected with the C282Y HFE variant. When the tumor size ranged from 80-100 mm³, CC-I in 5% DMSO and 12% ethanol was injected intraperitoneally at a concentration of 25 mg/kg body weight once a week for 7 weeks (n=10). The vehicle in the control group was administered in the same volume and regimen (n=8). The data are displayed as mean±SEM. The error bars are small to be visible in the graph. (B) Liver and kidney toxicity of CC-I (25 mg/kg body weight) to the subcutaneous neuroblastoma (C282Y HFE/SH-SY5Y) tumor SCID mice. The data are displayed as mean±SEM (n=8 for control, n=10 for CC-I). CC-I inhibited tumor growth and was not lethal in any of the treatment groups. SGOT: Serum glutamic-oxaloacetic transaminase; AST: aspartate aminotransferase; SGPT: serum glutamic pyruvic transaminase; ALT: alanine aminotransferase; BUN: blood urea nitrogen.

induce both an unfolded protein response and an endoplasmic reticulum overload response in human embryonic kidney HEK293 cell lines (36), it is unknown whether there is a link between C282Y HFE and HSP27-Akt-JNK pathway.

In the neuroblastoma cells, CC-I induced more late apoptotic/necrotic cell death than early apoptotic cell death. This was in sharp contrast to the data from astrocytoma cells treated with the same concentration of CC-I, where it induced more apoptosis than necrosis (10). This difference can be explained by the differences in cell lines and cancer mechanisms. However, in both astrocytomas and neuroblastomas, CC-I induced S and G₂/M phase cell cycle arrest (10). Several anti-tumor agents, including topoisomerase II poisons (*e.g.*, doxorubicin, etoposide), cause S and G₂/M arrest in cancer cells (37, 38). These results suggest that CC-I may exert toxicity by inhibiting topoisomerase of neuroblastomas, as we observed in astrocytomas (10).

In the neuroblastoma *in vivo* tumor models, CC-I-treated mice showed no indication of liver or kidney toxicity. This result is consistent with our previous blood toxicity data in CC-I treated intracranial xenograft mice (10). Even though CC-I showed a robust anti-tumor effect on C282Y HFE/SH-SY5Y subcutaneous mouse tumor model, it did not have an anti-tumor effect in the SK-N-AS cancer model. CC-I was effective in the cell culture model for both of these cell lines although the SH-SY5Y cells were more sensitive. Thus, the discrepancy in the *in vivo* study may reflect this difference. There are also genetic background differences between the mouse strains (SCID mouse strain #236 *vs.* athymic nude mouse strain #490). While the immunodeficient athymic nude mouse that we used for the SK-N-AS tumor model is only T-cell deficient, the SCID mouse used for C282Y HFE/SH-SY5Y cells *in vivo* tumor model is both T-cell and B-cell deficient. We used the two different mouse strains because C282Y HFE/SH-SY5Y cells do not form subcutaneous tumors in the athymic nude mouse strain #490 but form tumors in the SCID mouse strain #236. Another potential reason could be the difference in tumor growth rate of the two cell lines (C282Y HFE/SH-SY5Y *vs.* SK-N-AS) in the subcutaneously implanted mouse tumor models. The tumor development in C282Y/SH-SY5Y cells injected mice showed much slower growth rate than the SK-N-AS cells injected mice. Thus, CC-I had anti-tumor effect in slow growing tumors. Therefore, the *in vivo* differences may be due to the rapid growth of SK-N-AS subcutaneous tumors and the decreased sensitivity to CC-I indicating a higher dose or increased dosing frequency of CC-I may be required to observe the anti-tumor effect.

In the *in vitro* cytotoxicity study, CC-I showed a more potent cytotoxic effect on C282Y HFE/SH-SY5Y cells compared to WT HFE/SH-SY5Y cells. The C282Y HFE/SH-SY5Y cells grow faster than WT HFE/SH-SY5Y cells. This suggests that C282Y HFE/SH-SY5Y cells have shorter doubling times and

Figure 5. Western blotting and signal intensity analysis in C282Y-HFE/SH-SY5Y cells treated with CC-I (24 h). (A) Representative images of western blotting. β -actin was used as an internal control. Western blotting was performed using three different biological samples. (B) Band intensities were compared between compound-treated and vehicle-treated controls. For example, (pAkt vs. Akt)/ β -actin refers to the pAkt to total Akt ratio. Signal intensity is displayed as fold mean $\pm$ SEM to vehicle controls. (n=3) * p <0.05; ** p <0.01.

more cancer characteristics compared to WT HFE/SH-SY5Y cells. Thus, CC-I is more effective in C282Y HFE/SH-SY5Y cells compared to WT HFE/SH-SY5Y cells, like most other anticancer agents/drugs targeting fast-growing cells. Larsson *et al.* reported that treatment with a compound is crucial during the first 24 h, because there is similar LC₅₀ between the different populations of cells with different doubling times compared to longer exposure times, like 48 h (39). These results suggest that there is another target or cell death mechanism for CC-I in the C282Y HFE/SH-SY5Y cells. The efficacy of CC-I on other types of cancer cells expressing C282Y HFE at different levels and different dose/administration plan of CC-I should be further studied in the future, using various background mouse strains to evaluate the specificity of CC-I.

The present and previous results (21) indicate that C282Y HFE impacts neuroblastoma characteristics such as therapy resistance and cell proliferation. Several studies reported that single nucleotide polymorphisms (SNPs) in genes [e.g., ATP binding cassette subfamily C member 1 (ABCC1); caspase 8 (CASP8); ERCC excision repair 1, endonuclease non-catalytic subunit/ERCC excision repair 4, endonuclease non-catalytic subunit (ERCC1/XPF or ERCC1/ERCC4); poly(ADP-ribose) polymerase 1 (PARP1); base excision repair (BER); methyltransferase like 14 (METTL14)] are risk factors for neuroblastoma development and patient survival (40-45). Our data indicate that the frequency of the C282Y HFE SNP in neuroblastoma patients should also be determined in future clinical studies.

The present study has several limitations. First, we determined CC-I-mediated apoptotic/necrotic cell death by DNA-binding dye propidium iodide and fluorescent-tagged annexin V staining using flow cytometry at a single time point. It does not include other parameters such as morphological, biochemical, and molecular events. Second, we did not study whether the altered levels of HSP27-Akt-JNK by siRNA or inhibitors still leads to CC-I-mediated cell death. Future studies should focus on in-depth understanding of the mechanism of action of CC-I.

In conclusion, we demonstrated an anti-tumor effect of CC-I on C282Y HFE stably expressing neuroblastomas using *in vitro* cell culture and *in vivo* mouse tumor models. The cytotoxicity of CC-I was mediated by late apoptosis/necrosis, cell cycle arrest in the S and G2/M phases, and activation of HSP27-Akt-JNK pathway. The present results suggest that CC-I can be an effective therapy for C282Y HFE expressing neuroblastomas or slowly developing tumors through HSP27-Akt-JNK activation.

Conflicts of Interest

Dr. Connor is founder of NuHope LLC that holds the patent for the compound tested in this study. Dr. Lee has a financial interest in NuHope and is a co-inventor on the patent covering the compound in this study along with Dr. Connor.

Authors' Contributions

Conceptualization [Sang Y Lee]; Literature search and analysis [Sang Y Lee]; Data generation [Sang Y Lee, Becky Slagle-Webb]; Writing – original draft preparation [Sang Y Lee]; Writing – review and editing [Sang Y Lee, Becky Slagle-Webb, Arun K Sharma, James R Connor]

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