Association Between Iron and Cholesterol in Neuroblastomas

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Abstract. *Background/Aim: Neuroblastoma is the most common childhood extracranial solid malignancy. Although cancer cells need iron and lipids for active cell division, possible links between iron and lipid metabolism in neuroblastomas have not been studied. Materials and Methods: We evaluated the levels and association between iron and cholesterol on in vitro neuroblastoma cancer models. Results: We found that the levels of iron and cholesterol are diverse among neuroblastoma cell lines. There is a bi-directional association between iron and cholesterol in drug-resistant neuroblastoma SK-N-AS cells. In drug-resistant neuroblastoma cells, low concentration of an iron chelator did not have an impact on iron levels, but on cellular cholesterol levels. Furthermore, a cholesterol decreasing agent, simvastatin, influenced both iron and cholesterol levels in drug-resistant neuroblastoma cells. Conclusion: Cholesterol decreasing agents may be more effective than iron chelators for drug-resistant neuroblastoma treatment.*

Neuroblastoma, the most common extracranial solid tumor in children, accounts for about 7.5% of all childhood cancers and ~15% of all pediatric oncology-related deaths (1, 2). Approximately 50% of neuroblastoma patients have metastatic disease and adverse tumor-specific biological features at the time of diagnosis (3, 4). The variation seen in neuroblastoma is thought to arise from arrest and deregulation of normal development of the neural crest (5). The two most common

risk factors for neuroblastomas are age and genetic factors. Both the International Staging System (6) and the International Neuroblastoma Risk Group Staging System (7) note that children less than 18 months of age have the best prognosis while those whose tumors have metastasized to distant parts of the body have the poorest outcome when over 18 months of age. Presently, genetic mutations are also used in the staging of neuroblastomas. These can include germline mutations (8, 9) (*e.g.*, *ALK, MYCN*), or somatic events (10-13) (*e.g.*, somatic mutations, copy number variation, somatic DNA-amplification, somatic chromosomal gains and losses). Most children with neuroblastoma are treated with chemotherapy. Those with high-risk neuroblastomas often receive a combination of drugs as the primary treatment, either prior to and/or after surgery.

Iron is an essential trace metal for normal as well as malignant cancer cells. In general, cancer cells exhibit increased iron dependence, hence increased stores of body iron are thought to be linked to increased cancer risk (14, 15), and the cellular iron level is closely associated with cancer characteristics. The proliferation of cancer cells requires large amounts of lipids, the building blocks for the cell's biological membranes. Seven of the enzymes needed for cholesterol synthesis are iron-dependent (16). Interestingly cholesterol accumulation in either blood or the tumor has been documented in patients with prostate, oral, and endometrial cancer (17-20). Studies interrogating the association between iron *per se* and cholesterol metabolism are limited. There is an excellent review paper for the link between iron and lipids in human neurodegenerative disease (21). Data from a study carried out over an 18-year time span indicated that iron-induced oxidation of serum lipids is important in cancer pathogenesis (22). Mainous 3rd *et al*. also reported that high iron levels raise the risk of cancer in people with high cholesterol (23). However, there appear to be no reports describing a relationship between iron and cholesterol metabolism in childhood cancers. To address this question, we investigated the relationship between iron and cholesterol in neuroblastoma cells using an *in vitro* cell culture model. The

overreaching hypothesis behind this work is that the identification of a unique relationship between iron and cholesterol in treatment-resistant neuroblastomas could provide the basis for the development of new treatment options.

Materials and Methods

Materials. All cell culture reagents including Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12, cat #11330-057, lot #1750072) and Iscove's Modified Dulbecco's Medium (IMDM, cat #12440-079, lot #1663817) were purchased from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS, cat #100-106, lot #A27D00D) was ordered from Gemini Bio-Products (West Sacramento, CA, USA) and lipoprotein-deficient serum was ordered from Sigma-Aldrich (cat #S5394, lot #SLBL5464V) (St. Louis, MO, USA). Deferoxamine (DFO) (cat #D9533), di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) (cat #SML0483, lot #092M4615V), Di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT) (cat #SML0186), U18666A (cat #U3633, lot #023M4603V), and simvastatin (cat #S6196, lot #053M4728V) were purchased from Sigma-Aldrich. Ezetimibe (cat #S1655) and Triapine (cat #S7470) were obtained from Selleckchem (Houston, TX, USA). Stock concentrations of chemicals were prepared in sterile ddH$_2$O or DMSO (Sigma, cat #D2650, lot #RNBD6333) and appropriately diluted as needed.

Cell culture. Human neuroblastoma cell lines (SH-SY5Y, SK-N-AS) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Drug-resistant human neuroblastoma cell lines (CHLA-136, CHLA-171), established from tumors of patients who were at various stages of therapy (24, 25), were obtained from the Children's Oncology Group. Authentication of human neuroblastoma cell lines was performed by Genetica (Burlington, NC, USA) *via* STR DNA profiling. Drug-resistant neuroblastomas are the cell lines with LC90 values (drug concentration lethal for 90% of treated cells) greater than the clinically achievable drug levels for at least two of the four agents commonly used in neuroblastoma therapy (melphalan, carboplatin, etoposide, vincristine) (25). Cells were maintained at low passage when possible and new cultures started after either 2-3 months or when cell passage number exceeded ten. Neuroblastoma cells were cultured in DMEM/F12 with 10% FBS, 1% antibiotics (Penn-Strep, Life Technologies, cat #15140-122, lot #1751324), and 1% non-essential amino acids (Life Technologies, cat #M7145, lot #RNBD2211). Acquired drug-resistant neuroblastoma cells (CHLA-136, CHLA-171) were cultured in Iscove's Modified Dulbecco's Medium containing 10% or 20% FBS (for experimental studies and cell maintenance, respectively), 4 mM L-glutamine (Life Technologies, cat #25030081), 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenous acid (Life Technologies, cat #41400045 – 100X Insulin-Transferrin-Selenium).

Measurement of iron and cholesterol content. Human neuroblastoma cells were cultured for 1-3 days in 6 well plates with complete cell culture medium or lipoprotein-deficient serum-containing medium for iron measurements. Intracellular iron concentration was determined on cell extracts prepared from 1×10^5 cells using the QuantiChrom Iron Assay Kit (BioAssay Systems, cat #DIFF-250, lot #BE11A11) (Hayward, CA, USA) following the manufacturer's instructions (26). To determine cholesterol content, neuroblastoma

cells were cultured for 1-3 days in 6 well plates in medium containing lipoprotein deficient serum. Biovision's cholesterol assay kit (cat #K603-100, lot #1A280603) (Milpitas, CA, USA) was used to determine the cholesterol content of homogenates of 1×10^6 cells, as previously described (27).

Association between iron and cholesterol. To determine the association between iron and cholesterol, SK-N-AS and CHLA-171 cells cultured in lipoprotein deficient medium were treated with the iron chelator DFO, as performed previously (28), before monitoring cholesterol concentration as well as iron level. To determine the effect of a reduction in cholesterol concentration on the iron level in SK-N-AS and CHLA-171 cells, cells cultured in lipoprotein deficient medium were treated with simvastatin, a 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase inhibitor (29), and then both iron level and cholesterol concentration were determined.

Cytotoxic effects of iron chelators and cholesterol decreasing agents on neuroblastoma cells. Human neuroblastoma cells ($1-2\times10^4$/well) were treated with either different concentrations of an iron chelator (DFO, Dp44mT, triapine, DpC) (30), a HMG-CoA reductase inhibitor (simvastatin) (29), the desmosterol Δ24-reductase inhibitor (U18666A) (31), or a cholesterol absorption inhibitor (ezetimibe) (32) for 48 h in cell culture medium (for iron chelators) or medium containing lipoprotein deficient serum (for cholesterol decreasing agents). Reduced FBS was used because the flux of cell cholesterol is linked to the type and concentration of lipoproteins in serum as well as transport proteins expressed by the cells (33, 34). Cell cytotoxicity was determined using the alamarBlue assay (InVitrogen, Cat #DAL700, Lot #559787) (Grand Island, NY, USA,). The EC50s for each chemical treatment were ascertained using GraphPad Prism software (version 7).

Statistical analysis. Treatment data are shown as the percentage of vehicle-treated control cells. Each experiment was repeated at least three times with 3-6 replicates. For each treatment concentration, the level of iron and cholesterol in the two groups was compared using the two-sample Student's *t*-test or two-way ANOVA for comparison of multiple samples. Data were displayed as mean±standard error of the mean (SEM). Two-way ANOVA models were used to analyze data obtained from *in vitro* cytotoxicity experiments. Group differences in cell viability were examined at each concentration by slicing the group-concentration interactions. All analyses were performed using statistical software SAS version 9.4 (SAS Institute, Cary, NC, USA) or GraphPad Prism 7. Differences among means are considered statistically significant when the *p*-value is less than 0.05.

Results

Iron and cholesterol levels of neuroblastoma cells. Iron and cholesterol levels were determined for representative samples of human neuroblastoma cells. At day 2, iron levels in the drug-sensitive SH-SY5Y cells and drug-resistant SK-N-AS cells were higher compared to drug-resistant CHLA-136 and CHLA-171 neuroblastoma cells (Figure 1A). The cholesterol levels in the CHLA-171 cells were significantly higher than other tested neuroblastoma cells (Figure 1A). During the cell culture times, iron levels increased, but cholesterol levels decreased (Figure 1B-D).
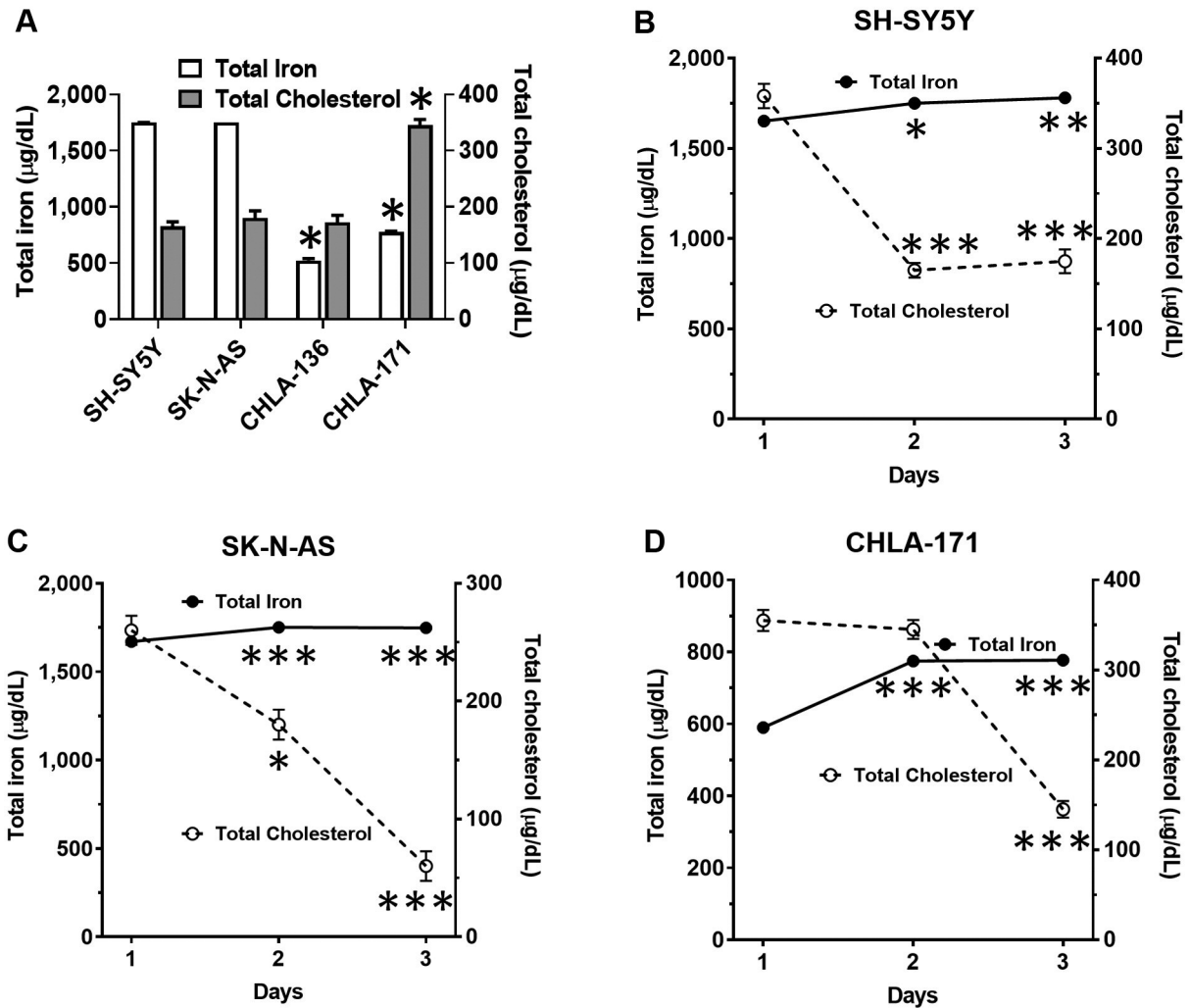
Figure 1. *Iron and cholesterol content of human neuroblastoma cells. (A) Human neuroblastoma cells were cultured in complete cell culture medium for 2 days for iron measurements. Intracellular iron concentrations were determined using the QuantiChrom Iron Assay Kit. To determine cholesterol content, neuroblastoma cells were cultured for 2 days in medium containing lipoprotein-deficient serum. Intracellular cholesterol content was determined for 1×10^6 cells using the Biovision cholesterol assay kit. Thirty μg of cell protein extract were used for the iron assay, and 50 μl from a total of 200 μl of the extracted sample were used for the cholesterol assay. Concentrations of total iron or cholesterol are given in μg/dl. Results represent the mean of three independent experiments with error bars corresponding to standard error. In the case of small errors, bars may not be visible. Asterisks indicate a significant difference compared to the iron or cholesterol level of SH-SY5Y cells (*p<0.05). (B-D) The cells were cultured for 3 days and then the iron and cholesterol content were determined as described above. Asterisks indicate a significant difference compared to the iron or cholesterol level on day 1. *p<0.05, **p<0.01, ***p<0.001.*

Association between iron and cholesterol in neuroblastoma cells. To determine the possible association between cellular iron and cholesterol concentrations, drug-resistant SK-N-AS and CHLA-171 cells were treated with either the iron chelator DFO or simvastatin in lipoprotein-deficient serum-containing medium prior to monitoring their iron or cholesterol concentration. In both cells (SK-N-AS, CHLA-171), DFO decreased cholesterol levels, although an alteration in iron level was not seen (Figure 2A-B, E-F).

Simvastatin treatment affected both cholesterol and iron levels in the SK-N-AS cells (Figure 2C-D). Unexpectedly, treatment of CHLA-171 cells with 2.5 μM simvastatin resulted in increase of both cholesterol and iron levels, while at 5 μM it had no effect (Figure 2G-H).

Effect of iron chelators and cholesterol decreasing agents on neuroblastoma cells. To investigate whether iron chelators and/or inhibitors of cholesterol uptake or synthesis would
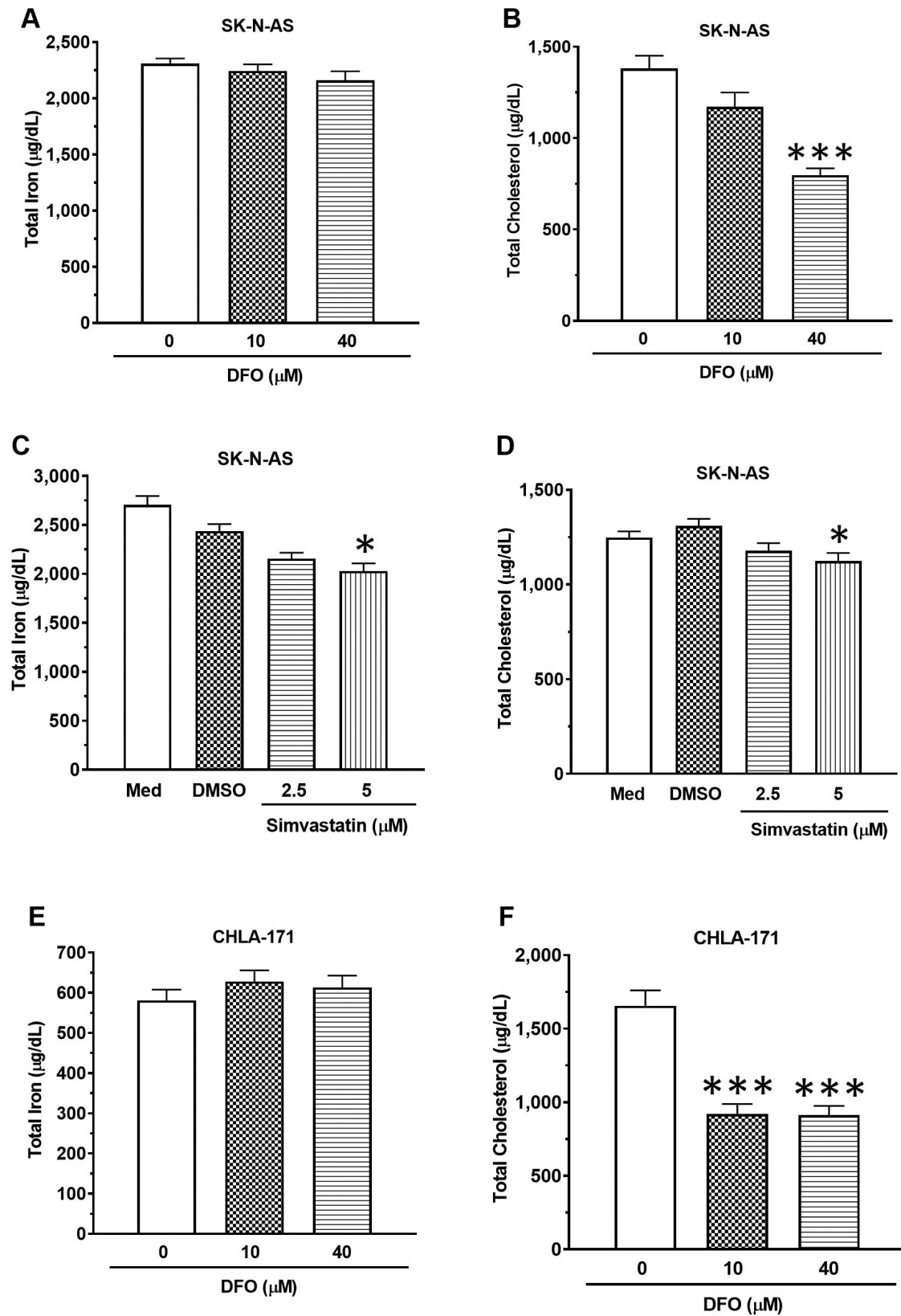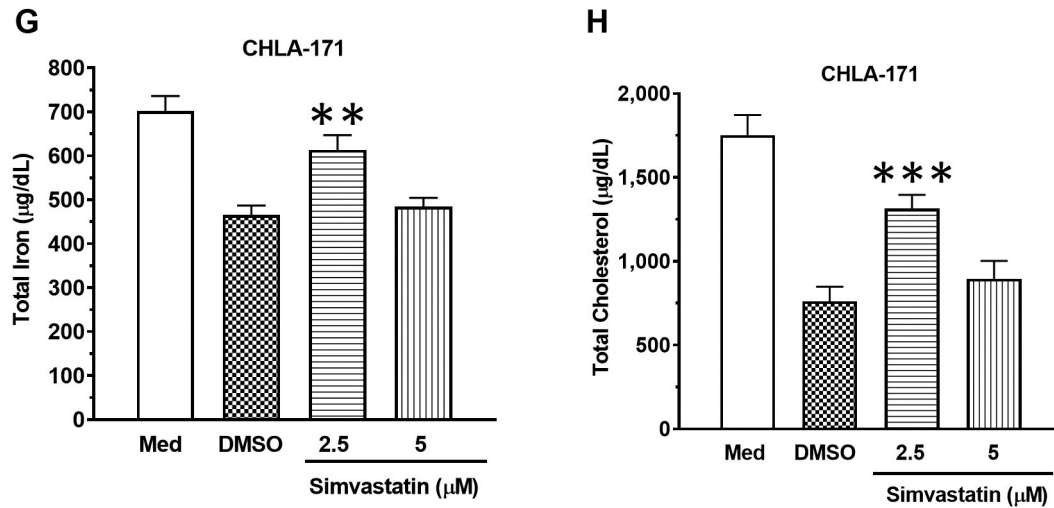
Figure 2. *Continued*

Figure 2. *Effect of DFO or simvastatin on iron and cholesterol levels in SK-N-AS and CHLA-171 cells. SK-N-AS and CHLA-171 cells cultured in lipoprotein-deficient serum-containing medium were treated with vehicle or different doses of DFO (A-B, E-F) or simvastatin (C-D, G-H) for two days prior to determination of iron or cholesterol. Intracellular iron and cholesterol contents were determined using the QuantiChrom Iron Assay Kit and Biovision cholesterol assay kit, respectively. Concentrations of total iron or cholesterol are given in μg/dl, as described in the legend of Figure 1. Results shown indicate the mean±SEM of three independent experiments. The asterisks indicate a significant difference compared to the control group. *p<0.05, **p<0.01, ***p<0.001.*

inhibit the growth of neuroblastoma cells *in vitro*, we examined their effect on cell viability. When determining the effect of cholesterol decreasing agents, cells were maintained in medium containing 1% FBS in order to minimize the effect of serum cholesterol. SK-N-AS cells were found to be less susceptible to the iron chelator DFO (Figure 3A) but more sensitive to simvastatin, an inhibitor of HMGCoA reductase, than SH-SY5Y cells (Figure 3B). The response to ezetimibe, an inhibitor of cholesterol uptake by cells, was similar for both the SH-SY5Y and SK-N-AS cells (Figure 3C). Meanwhile, CHLA-171 cells were more sensitive to simvastatin than other drugs (Figure 3D). A comparison of the cytotoxicity of DFO with that of other iron chelators developed for cancer treatment indicated that it was less toxic to SK-N-AS and CHLA-171 cells (Figure 3E and F) than Dp44mT, triapine, and DpC. Of the four iron chelators tested, DpC was most cytotoxic.

Discussion

The present study evaluated the relationship between iron and cholesterol in drug-resistant human neuroblastoma cells to determine whether altering either or both might provide a potential treatment strategy. Using known drug-resistant neuroblastoma cells, we found a partial association between cellular iron and cholesterol concentrations.

To study the relationship between iron and cholesterol metabolism, we focused on the drug-resistant SK-N-AS and

CHLA-171 cells. As far as we know, analyses of the levels of iron and cholesterol in neuroblastoma cells, especially those derived from tumors of drug-resistant patients, have not been previously reported. Although we hypothesized that drug-resistant SK-N-AS and CHLA-171 cells would have increased iron and cholesterol, there was no apparent association between the level of iron/cholesterol and drug resistance in the cells studied. Instead, we observed that as iron levels increased, cholesterol levels decreased during their time in culture. These data indicate that neuroblastoma cells may take up iron for use during cell proliferation, while cholesterol accumulation may depend on its rate of usage during cell proliferation, availability in the culture medium, and rate of synthesis by the cells. Although there are several publications discussing either the role of iron or of cholesterol in cancer, those describing the effects of iron and cholesterol in cancer is limited. One study of the effects of iron described the interaction between ferroptosis (accumulation of iron-dependent lethal lipid peroxides) and lipid metabolism in cancer (35). The postulate drawn from their observations is that because cancer cells require higher levels of iron and lipid metabolism for rapid growth, they are more susceptible to ferroptosis. Excess iron is associated with cancer cell proliferation, migration, regulation of DNA replication and the cell cycle, as well as the tumor microenvironment (36, 37). Studies of the effect of iron on neuroblastoma and Ewing's sarcoma indicated that iron functioned in formation of N-glycolyl neuraminic acid, a sugar found in sialylated
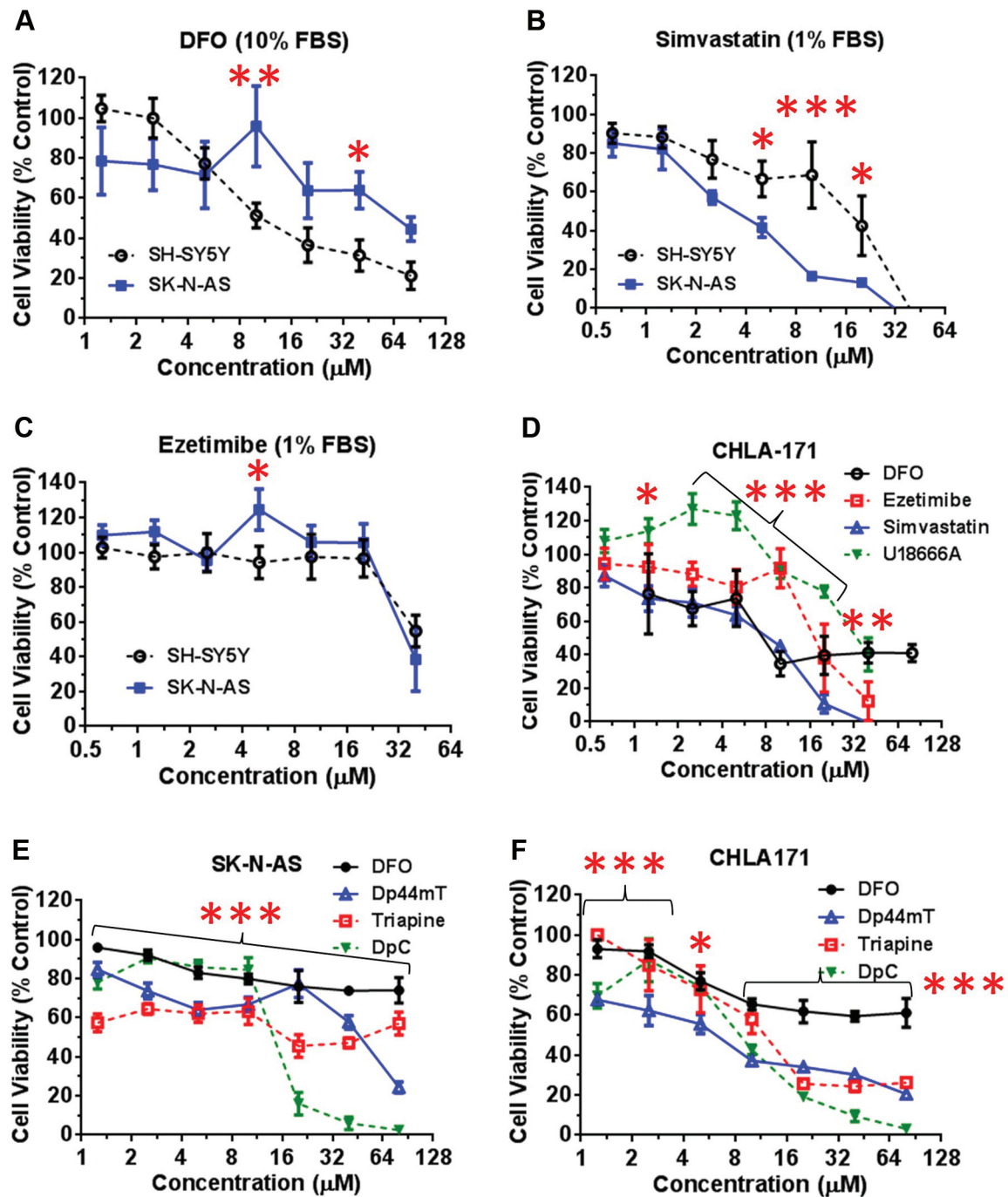
Figure 3. *Cytotoxic effects of iron chelators and cholesterol decreasing agents on neuroblastoma cells. (A) Human neuroblastoma cells (SH-SY5Y, SK-N-AS) were cultured overnight in complete cell culture medium prior to a 2-day exposure to the iron chelator DFO. (B-C) Human neuroblastoma cells (SH-SY5Y, SK-N-AS) were cultured overnight in complete cell culture medium prior to exposure to either simvastatin or ezetimibe, cholesterol decreasing agents, in medium containing 1% FBS for 2 days. (D) Human neuroblastoma CHLA-171 cells were cultured overnight in complete cell culture medium prior to a 2-day exposure to either an iron chelator (DFO) or cholesterol decreasing agent (simvastatin, ezetimibe, or U18666A) in a medium containing 1% FBS. (E, F) Human neuroblastoma cells (SK-N-AS, CHLA-171) were cultured overnight in complete cell culture medium prior to a 2-day exposure to an iron chelator, either DFO, Dp44mT, triapine, or DpC. (A-F) In each instance after two days of exposure, cytotoxicity was ascertained using the alamarBlue assay. Results represent the means of three independent experiments with error bars corresponding to standard errors. For each plot, symbols indicate statistically significant differences between treatments at the indicated concentrations. *p<0.05, **p<0.01, ***p<0.001.*

glycosphingolipids in cancer, but not in healthy human tissue (38). In a cohort of the Framingham study it was found that high iron coupled with either a high level of very low-density lipoprotein cholesterol or a low level of high density lipoprotein cholesterol appeared to increase cancer risk (23). Therefore, our results and previous reports suggest that iron in conjunction with its effects on lipid metabolism plays a role in cancer development. Examples of how cholesterol and derivatives thereof might function in cancer cell progression include binding to sterol response element binding proteins, nuclear sterol receptors, lipid particle receptors (39, 40), and the formation of lipid rafts (glycolipid enriched microdomains) (41, 42), that can function in signal transduction and affect membrane fluidity. The finding that cholesterol and gangliosides are present in lipid rafts and the composition of the sialic acid present on gangliosides can be modified by the presence of iron, further supports the need for interrogation of how iron and cholesterol affect neuroblastoma behavior.

In this study, the iron chelator, DFO, did not cause a decrease in iron in the drug-resistant SK-N-AS and CHLA-171 cells. This observation agrees with an earlier report that nonheme iron was not altered in iron treated hepatocytes cultured in media containing DFO (43). In contrast, Liu *et al*. found that DFO depleted intracellular iron in non-aggressive human breast cancer MCF-7 cells. At the same time, it significantly increased the intracellular iron level in aggressive breast cancer MDA-MB-231 cells (44). Therefore, failure of DFO treatment to alter iron levels in drug-resistant neuroblastoma cells may be due to i) activation of transferrin receptor-mediated or non-transferrin receptor-mediated iron uptake for the iron complement, ii) the difference between drug-sensitive cells and drug-resistant cells, iii) cell type (*e.g*., neuroblastomas vs. breast cancer). The current findings are based on two drug-resistant neuroblastomas (SK-N-AS and CHLA-171). To address this possibility, the effect of DFO treatment on iron levels of neuroblastoma cells should be assessed relative to its effect on other types of cancer cells. While DFO did not affect iron content, it did result in a decreased cholesterol level in both SK-N-AS and CHLA-171 cells. This observation, coupled with the finding that iron level was decreased in SK-N-AS cells by the HMGCoA inhibitor simvastatin, indicates that a bi-directional association between iron and cholesterol may exist in human neuroblastoma SK-N-AS cells. The bi-directional association between iron and cholesterol appears to be cell dependent since it was not seen in CHLA-171 cells.

Previously, we reported that C282Y HFE/SH-SY5Y neuroblastoma cells showed resistance to chemotherapy and radiation treatment (45), but were significantly more sensitive to the iron chelator DFO than WT HFE/SH-SY5Y cells (28). These observations coupled with the observation that C282Y HFE/SH-SY5Y cells were more sensitive to U18666A than WT HFE/SH-SY5Y cells (27) supported

interrogation of whether iron chelation therapy and cholesterol decreasing agents could be effective against drug-resistant neuroblastomas. The finding that DFO had a mild cytotoxic effect, but did not completely kill drug-resistant neuroblastoma SK-N-AS cells agrees with the observation that DFO did not have anti-tumor effects in either the IMR-32 or JBN-1 neuroblastoma xenograft nude mouse model (46). Iron chelators have antioxidant and free radical scavenging activity (47). Both functions are essential for cancer treatment. As expected from the effect of iron chelators, a potent antioxidant N-acetyl-L-cysteine (NAC) protected SH-SY5Y cells from oxidative stress and cell cytotoxicity (48).

Among the tested iron chelators, DpC had the strongest cytotoxic effect on the SK-N-AS and CHLA-171 cells, while simvastatin was the most cytotoxic of the cholesterol-decreasing agents tested. The later result suggests that the rate-limiting enzyme (HMG-CoA reductase) of cholesterol synthesis is critical for survival of drug-resistant neuroblastoma cells. The anti-tumor effect of DpC on a neuroblastoma animal model (orthotopic SK-N-LP/Luciferase xenograft) has been reported (49). Although *MYCN* amplification is observed in about 30% of high-risk neuroblastomas (4), other high-risk neuroblastomas lacking *MYCN* amplification such as those caused by SK-N-AS cells also acquire multidrug resistance. The SK-N-AS cells express higher levels of multidrug resistance-related protein 1 and sphingolipid than SK-N-DZ cells (50). The SK-N-AS cells also maintain drug resistance through overexpression of neuronal apoptosis inhibitory protein (51). While both CHLA-136 and CHLA-171 cells are multidrug resistant, p53 is functional in CHLA-136 cells, but not in CHLA-171 cells (24). These data support the possibility that alterations in iron and cholesterol metabolism are part of the cell's drug resistance mechanism, similar to other factors (*MYCN* amplification, multidrug resistance-related protein 1, functional p53 expression) identified in neuroblastomas.

The results obtained by studying several established neuroblastoma cell lines to ascertain the association between iron and cholesterol underscore the variability seen in the response of neuroblastomas to different therapeutic agents as well as the need for further studies. For example, in addition to testing the efficacy of other statins and iron chelators, and combinations of iron chelators and cholesterol decreasing agents, studies should be done to ascertain the most effective approach to treat neuroblastomas. The finding that the cell lines have different doubling times and optimum cell culture conditions as well as more subtle differences related to the tumors from which they were derived may have influenced our results. Therefore, to determine whether an association exists between iron and cholesterol and whether it is found in the majority of human neuroblastomas an extended study of multiple cell lines derived from different human neuroblastomas is needed. Such studies could monitor the

efficacy of other iron chelators (*e.g.*, Dp44mT) and other cholesterol decreasing agents (*e.g.*, lovastatin) alone and together on cells in culture. Those that seem promising would then be tested on multiple *in vivo* neuroblastoma tumor models. While possible molecular mechanisms for the effects of both iron and cholesterol on cancer development are discussed above, assays to determine which one(s) cause the significant effect(s) found in multiple neuroblastoma cell lines should be done. For example, the effect of iron and cholesterol alteration on cell signaling pathways, and expression of proteins needed for lipid synthesis could be assessed.

In summary, our results indicate that drug-resistant neuroblastoma cells may have a diverse pattern of iron and cholesterol levels. There is a bi-directional association between iron and cholesterol in human neuroblastoma SK-N-AS cells. The results of *in vitro* studies of human neuroblastoma cells indicated that they were susceptible to iron chelators and cholesterol inhibitors, however, further investigation is needed to ascertain whether alteration of the tumors' iron and/or cholesterol metabolism can be used to enhance treatment of neuroblastomas refractory to approaches currently in use.

Conflicts of Interest

Authors' Contributions

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