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# Synthetic high-density lipoprotein nanoconjugate targets neuroblastoma stem cells, blocking migration and self-renewal☆

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ABSTRACT

*Background:* Pathways critical for neuroblastoma cancer stem cell function are targeted by 4,19,27-triacetyl withalongolide A (WGA-TA). Because neuroblastoma cells and their cancer stem cells highly overexpress the scavenger receptor class B type 1 receptor that binds to synthetic high-density lipoprotein, we hypothesized that a novel mimetic synthetic high-density lipoprotein nanoparticle would be an ideal carrier for the delivery of 4,19,27-triacetyl withalongolide to neuroblastoma and neuroblastoma cancer stem cells.

*Methods:* Expression of scavenger receptor class B type 1 in validated human neuroblastoma cells was evaluated by quantitative polymerase chain reaction (qPCR) and Western blot. In vitro cellular uptake of synthetic high-density lipoprotein nanoparticles was observed with a fluorescence microscope. In vivo biodistribution of synthetic high-density lipoprotein nanoparticles was investigated with IVIS imaging. Self-renewal and migration/invasion were assessed by sphere formation and Boyden chamber assays, respectively. Viability was analyzed by CellTiter-Glo assay. Cancer stem cell markers were evaluated by flow cytometry.

*Results:* qPCR and Western blot analysis revealed a higher level of scavenger receptor class B type 1 expression and drug uptake in N-myc amplified neuroblastoma cells. In vitro uptake of synthetic high-density lipoprotein was almost completely blocked by excess synthetic high-density lipoprotein. The synthetic high-density lipoprotein nanoparticles mainly accumulated in the tumor and liver, but not in other organs. Synthetic HDL-4,19,27-triacetyl withalongolide showed a 1,000-fold higher potency than the carrier (synthetic high-density lipoprotein) alone ($P < .01$) to kill neuroblastoma cells. Additionally, a dose-dependent decrease in sphere formation, invasion, migration, and cancer stem cell markers was observed after treatment of neuroblastoma cells with synthetic high-density lipoprotein–4,19,27-triacetyl withalongolide A.

*Conclusion:* Synthetic high-density lipoprotein is a promising platform to improve the delivery of anticancer drug 4,19,27-triacetyl withalongolide A to neuroblastomas and neuroblastoma cancer stem cells through SR-B1 targeting in vitro and in vivo.



Neuroblastoma (NB) accounts for approximately 15% of cancer-related pediatric deaths and is the most common extracranial solid tumor affecting children.[1] Despite aggressive combination therapy options, over 50% of children with high-risk disease will relapse within the first 5 years after diagnosis.[2,3] In patients whose disease progresses or relapses, median survival drops to less than 1 year, and the 10-year overall survival (OS) is abysmal (6.8% for patients who progress and 14.4% for patients who relapse).[4] In addition to a poor OS, surviving patients with high-risk NBs are often left with

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considerable long-term deficits, including hearing loss, cardiac dysfunction, infertility, secondary malignancies, learning disabilities, and vision and skeletal problems, along with many others. Collectively, this underscores the critical and urgent need for the development of more effective and less toxic therapies for treating NB.[5]

Currently, targeted therapies using antiangiogenic drugs, anaplastic lymphoma kinase antagonists, and PI3K/Akt/mTOR inhibitors are in phase I/II clinical trials either alone or in combination with conventional chemotherapy for refractory or recurrent NB.[6] A major limitation to which each of these treatments is susceptible is that cancer cells have an innate ability to develop resistance to single-target drugs through several mechanisms, including recruitment or up-regulation of alternative survival pathways. It has been postulated that this drug resistance may in part be due to the presence of a small population of progenitor cells called cancer stem cells (CSCs) that can escape resistance and recapitulate the tumor and form spheres. For the last 6 years, our group has been studying the multitargeted anticancer benefits of natural withanolides isolated from the *Physalis* plant.[7–14] Not only are these natural products extremely safe in vivo with a large therapeutic index, but they also function through a unique mechanism of action, leading to the simultaneous and selective inhibition of multiple key regulatory pathways in cancers. In NB cells, withanolides target the specific pathways involved in their rapid proliferation, invasion, metastatic spread, poor survival, and escape resistance. These natural compounds utilize a multitargeted approach through their unique mechanism of action, inducing an oxidative stress response coupled with a novel means of inhibiting heat shock protein (HSP90) chaperone function through blockade of CDC37 docking, thereby preventing kinase activation by HSP90.[10] In NBs, these mechanisms target key tumorigenic proteins that are implicated in NB-CSC functions, such as invasion, drug resistance, and poor survival.[8,15–17] Because withanolides are safe and selective and carry potent multitargeted anticancer effects in NB, they represent a novel yet suitable drug strategy to overcome many of the barriers identified for the successful treatment of NBs.

Despite being effective anticancer therapeutics both in vitro and in vivo, withanolides have low solubility in plasma and a short circulation half-life of only 1 hour,[18] limiting their translational and clinical suitability. To overcome these limitations, we have developed the unique approach of encapsulating withanolides in synthetic high-density lipoprotein (sHDL) nanoparticles. These nanoparticles offer several advantages over other synthetic nanocarriers, including liposomes, micelles, and inorganic and polymeric nanoparticles. Most notably, they have an ultrasmall size (8–12 nM in diameter), are already in numerous clinical trials with excellent safety and tolerability in humans, and have a long circulating half-life.[11] In addition to improved circulation and stability, the sHDL nanoparticles target tumor cells via their highly overexpressed surface receptor, scavenger receptor class B type 1 (SR-B1), for which HDL is a ligand.[19] Hence, in the present study we have developed a novel synthetic HDL mimetic nanoparticle (sHDL) to encapsulate 4,19,27-triacetyl withalongolide A (WGA-TA) and evaluated particle and drug uptake as well as the efficacy of SR-B1-mediated targeting of NB cancer stem cell functions such as migration, invasion, and sphere formation.

## Methods

### *Cell lines*

Human NB cell lines SH-EP, SH-SY5Y, IMR-32, and SK-N-AS were validated as authentic by standard DNA fingerprinting and were grown in 2D culture. The cells were grown in Minimum Essential Medium (MEM) (Thermo Fisher, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Co, St. Louis, MO) and 1% penicillin and streptomycin (Thermo Fisher). For the growth of IMR-32 cells, the media was additionally supplemented with 1% MEM Non-Essential Amino Acids (100X; Sigma Chemical Co), 2 mmol/L of L-Glutamine (200 mmol/L; Sigma Chemical Co), 1 mmol/L sodium pyruvate (100 mm/L; Sigma Chemical Co), and 1,500 mg/L of sodium bicarbonate (7.5%; Sigma Chemical Co). The cell lines were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ in air.

### *Preparation of sHDL WGA-TA nanoparticles*

sHDL nanoparticles were loaded with either the dye [DiO (benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolyliden e)-1-propenyl]-, perchlorate), or DiR (DilC$_{18}$(7) (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide)] or the drug withanolide (WGA-TA), using the lyophilization method as described in our previous reports.[20]

### *Cell proliferation assay*

Approximately 5,000 NB cells per well were seeded in a 96-well plate in triplicate. As a control, WGA-TA alone, sHDL alone, and untreated cells were used. Following 24 hours' incubation time, cells were treated with serial dilutions of either sHDL or sHDL-WGA-TA or WGA-TA starting at 20 μM. Viability after 72 hours was evaluated by CellTiter-Glo luminescent assay as per the manufacturer's protocol (Promega Corp, Madison, WI). The luminescence was quantified via a BioTek Synergy Neo plate reader (Bio-Tek, Winooski, VT). Cell viability ratios were calculated using GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA).

### *Real-time polymerase chain reaction and Western blot analysis for SR-B1*

RNA from NB cell lines SK-N-BE(2), IMR-32, SH-EP, SH-SY5Y, SK-N-AS, the positive control NCI-H295R, and the negative control (Jurkat and fibroblast cells) were extracted using Qiagen RNA isolation kit (Qiagen Sciences, Valencia, CA). The 500 ng of RNA was reverse transcribed using superscript RT kit from Thermo Fisher. Quantitative polymerase chain reaction (PCR) was performed in a step-1 real-time PCR machine using SR-B1 and actin specific primer sets. Relative gene expression levels were calculated after normalization with internal controls. The expression of SR-B1 was further confirmed at the protein level by Western blot (WB) analysis as previously described, and actin was used as a loading control.[12]

### *Uptake of DiO-labeled sHDL nanoparticles by NB cells in vitro*

SR-B1-mediated uptake of dye by the NB cell lines as well as the positive control NCI-H295R and the negative control Jurkat was evaluated after incubating the cells for 4 hours with the long-chain dialkylcarbocyanines lipophilic tracer DiO (Invitrogen, Carlsbad, CA) labeled sHDL nanoparticle. Additionally, SR-B1-mediated uptake was further confirmed after small interfering (si)RNA-mediated SR-B1 knockdown by standard protocols. Briefly, SK-N-BE(2) cells grown to 80% confluency were transfected with validated silencer select non-targeting as well as SR-B1 targeting siRNA using Lipofectamine RNAiMAX transfection reagent as per the manufacturer protocol (Thermo Fisher Scientific, Carlsbad, CA). After 48 hours, cells were treated with DiO-labeled sHDL, and part of the cells were collected for WB analysis to verify knockdown of SR-B1. For blocking, the cells were treated with 10-fold excess of blank sHDL for 1 hour prior to the addition of the nanoparticles. The fluorescent images were taken using a Nikon fluorescent microscope after fixing the cells with paraformaldehyde. The nuclei

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