Synthetic HDL Nanoconjugate Targets Neuroblastoma Stem Cells, Blocking Migration and Self-Renewal

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Abstract

Background—Pathways critical for neuroblastoma (NB) cancer stem cell (CSC) function are targeted by 4,19,27-triacetyl withanolide A (WGA-TA). Since NB cells and their CSCs highly overexpress the SR-B1 receptor that binds to sHDL, we hypothesized that a novel mimetic sHDL nanoparticle will be an ideal carrier for the delivery of WGA-TA to NB and NB-CSCs.

Methods—Expression of SR-B1 in validated human NB cells was evaluated by qPCR and Western blot (WB). *In vitro* cellular uptake of sHDL nanoparticles was observed with a fluorescence microscope. *In vivo* bio distribution of sHDL 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 Cell-Titer Glow (CTG) assay. CSC markers were evaluated by flow cytometry.

Results—qPCR and WB analysis revealed a higher level of SR-B1 expression and drug uptake in N-myc amplified NB cells. *In vitro* uptake of sHDL was almost completely blocked by excess sHDL. The sHDL nanoparticles mainly accumulated in the tumor and liver, but not in other organs. sHDL-WGA-TA showed a 1000-fold higher potency than the carrier (sHDL) alone

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($p < 0.01$) to kill NB cells. Additionally, a dose-dependent decrease in sphere formation, invasion, migration and CSC markers was observed after treatment of NB cells with sHDL-WGA-TA.

Conclusion—sHDL is a promising platform to improve the delivery of anticancer drug WGA-TA to NBs and NB-CSCs through SR-B1 targeting *in vitro* and *in vivo*.

INTRODUCTION

Neuroblastoma (NB) accounting for approximately 15% of cancer-related pediatric deaths is the most common extra-cranial 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 progress or relapse, median survival drop 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 considerable long-term deficits, including hearing loss, cardiac dysfunction, infertility, secondary malignancies, learning disabilities, 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 anti-angiogenic drugs, anaplastic lymphoma kinase (ALK) 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 that each of these treatments is susceptible to is that cancer cells have an innate ability to develop resistance to single-target drugs through several mechanisms including recruitment or upregulation 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 re-capitulate the tumor and form spheres. Our group has been studying for the last 6 years the multi targeted 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 multi-targeted 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 function such as invasion, drug resistance, and poor survival (8, 15-17). Since withanolides are safe, selective, and carry potent multi-targeted 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 1h (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 nanoparticles (sHDL). These nanoparticles offer several advantages over other synthetic nanocarriers including liposomes, micelles, and inorganic and polymeric nanoparticles. Most notably they have an ultra-small size (8–12 nm in diameter), they are already in numerous clinical trials with excellent safety and tolerability in humans, and they 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 I (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, IMR32 and SK-N-As were validated authentic by standard DNA fingerprinting and were grown in 2D-culture. The cells were grown in minimum essential medium (MEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO) and 1% penicillin and streptomycin (Life Technologies, Grand Island, NY). For the growth of IMR32 cells the media was additionally supplemented with 1% MEM non-essential amino acids (100X; Sigma-Aldrich), 2mM of L-glutamine (200mM; Sigma-Aldrich), 1 mM sodium pyruvate (100mM; Sigma-Aldrich), and 1500mg/L of sodium bicarbonate (7.5%; Sigma-Aldrich). The cell lines were incubated at 37°C in a humidified atmosphere of 5% CO₂ 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)-benzoxazolydene)-1-propenyl]-, perchlorate), or DIR (DiIC18(7) (1,1'-Dioctadecyl-3,3',3'-Tetramethylindotricarbocyanine Iodide)] or the drug The withanolide (WGA-TA), using the lyophilization method as described in our previous reports (20).

Cell Proliferation assay

Approximately 5000 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 h 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 h was evaluated by CellTiter-Glo (CTG) luminescent assay as per the manufacturer's protocol (Promega, Madison, WI). The luminescence was quantified via a BioTek Synergy Neo plate reader (BioTek, Winooski, VT). Cell viability ratios were calculated using GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA).

Real time polymerase chain reaction (RT-PCR) and western blot analysis for SR-B1

RNA from NB cell lines SK-N-Be(2), IMR32, 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). 500 ng of RNA was reverse transcribed using superscript RT kit from Life Technologies. Quantitative polymerase chain reaction (PCR) was performed in a step-1 real-time PCR (RT-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 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 h with the long-chain dialkylcarbocyanines lipophilic tracer DIO (Invitrogen, NY) labeled sHDL nanoparticle. Additionally, SR-B1 mediated uptake was further confirmed after siRNA 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 h, cells were treated with DiO labeled sHDL and part of the cells were collected for western blot analysis to verify knockdown of SR-B1. For blocking, the cells were treated with 10-fold excess of blank sHDL for 1 h 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 were stained with 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent stain binding to DNA.

Sphere formation assay

NB cell lines IMR32, SK-N-Be(2) and SK-N-As were plated at a density of approximately 250 cells per well in a 96 well ultralow attachment plate (Corning, Corning, NY) in mammoCult medium (STEMCELL technologies, Vancouver, BC, Canada) with varying concentrations of sHDL, WGA-TA, and sHDL-WGA-TA starting at 7.7 nM and the ability of cells to form spheres was observed for one week. The number of spheres formed (>25 cells) was counted using a light microscope (LM).

Boyden chamber assay for migration and invasion

NB cell lines IMR32, SK-N-Be(2) and SK-N-As cells were suspended in serum free MEM-medium supplemented with penicillin/streptomycin and 0.5 or 1 μ M drugs (WGA-TA, sHDL-WGA-TA, or sHDL). An equal number of cells with drugs were placed on the upper well of either Matrigel coated 8-micron polycarbonate membranes (Corning, Corning, NY) or standard membranes and 10% FBS at the lower well served as a chemoattractant. After 48 h incubation, membranes were fixed in 2% paraformaldehyde and stained with 1% crystal violet in 20% methanol for 20 min. The membranes were washed with distilled water, non-

migratory cells from the insert interior were removed using cotton-tipped swab. The migration and invasion were quantified as the number of cells per field using LM.

Evaluation of markers of NB CSCs by flow cytometry

IMR 32 and SK-N-Be(2) cells were treated with 0.5 μ M drugs (WGA-TA, sHDL-WGA-TA, or sHDL) for 24h. The cells were collected, washed with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and then incubated with phycoerythrin (PE)-conjugated CD133 or CD114 (Miltenyi Biotech, Germany) antibodies for 30 min. Following incubation, the cells were washed with PBS containing 0.5% BSA and analyzed by CyAn ADP analyzer (Beckman Coulter, Brea, CA). DAPI staining at 1 μ g/mL was used to exclude dead cells from analysis.

In vivo distribution of DiR sHDL nanoparticle by IMR32 xenograft

To characterize the distribution of the nanoparticle, whole body imaging was conducted on IMR32 tumor-bearing mice using IVIS imaging at the University of Michigan imaging core. DiR-dye labeled sHDL (0.6% DiR) was injected by i.p. (Intraperitoneal) at 1mg/Kg dose and the whole body imaging was performed after 24 h. At the end of 24 h, animals were sacrificed and major organs including the spleen, liver, heart, lung, kidneys, and tumor were harvested, weighed and imaged to characterize the bio distribution of DiR-sHDL.

Statistical Analysis

All the experiments were repeated in triplicate and the values are presented as average plus or minus standard deviation. The p-values were calculated by one way analysis of variance using Dunnett's multiple comparison test using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA) and the p-values of <0.05 were considered statistically significant.

RESULTS

The HDL receptor SR-B1 is highly expressed in N-myc amplified neuroblastoma cells

For sHDL-WGA-TA to specifically target malignant NB cells, high levels of the HDL receptor SR-B1 expression are necessary. Therefore, we examined both mRNA and protein levels of SR-B1 in NB cells as well as in SR-B1 negative and positive control cell lines by real time PCR (Fig.1A) and Western blot analysis (Fig.1B). Compared to the negative control (fibroblast and Jurkat) cell lines, mRNA level expression of SR-B1 for N-myc amplified (IMR32 and SK-N-Be (2)) and non-amplified cell lines (SH-EP, SK-N-As and SH-SY5Y) were 6-7 fold and 1-3 fold higher respectively, ($p < 0.001$ vs controls). Results were similar for the protein expressions of SR-B1 as N-myc amplified cells expressed 18-fold higher levels of SR-B1 compared to negative control cells ($p < 0.001$), whereas non-amplified cells expressed only 6-8 fold higher levels of SR-B1 protein compared to the negative controls ($p < 0.05$). As expected the positive control adrenocortical carcinoma cell line, NCI-H295R had very high mRNA and protein expression levels of SR-B1 vs. the negative control or the NB cell lines ($p < 0.0001$).

NB cells uptake DIO sHDL nanoparticles in vitro through its receptor SR-B1

The ability of NB cancer cells with various levels of SR-B1 to take up the sHDL nanoparticles through SR-B1 receptor and the effect of sHDL composition on internalization of sHDL's cargo by SR-B1 was examined after a 4-h incubation of cells with DIO (Invitrogen, NY)-labeled sHDL. The cellular uptake of the nanoparticle showed increased uptake for SR-B1 high expressers (IMR32, SK-N-BE (2) and the positive control) compared to the low expressers (SH-EP and SK-N-As) and the negative control. To further confirm that the uptake was mediated through SR-B1, the cells were either pre-treated with 10-fold excess of sHDL or SR-B1 was knocked down using siRNA and then DIO labeled nanoparticles was added. As expected, the uptake was completely blocked by either excess sHDL or by knocking down SR-B1 (Fig.2A and B), indicating receptor-mediated uptake of the drug by NB cells.

WGA-TA encapsulated sHDL nanoparticle reduces the viability of NB cells

The viability of sHDL nanoparticles either alone or after encapsulation with WGA-TA was determined by cell Titer-Glo assay (Fig.3). Treatment of cells with sHDL nanoparticle alone had minimal effect on the viability of cells ($IC_{50} > 10 \mu M$) whereas treatment with either WGA-TA or sHDL-WGA-TA was highly effective in reducing the viability of NB cells at nM concentrations ($IC_{50} = 25-55 nM$), which was 4-10 fold greater than that of normal fibroblast cells (Table 1).

WGA-TA encapsulated sHDL nanoparticle targets NB cancer stem cells

To evaluate whether the novel WGA-TA encapsulated sHDL nanoparticle targets NB cancer stem cells, sphere formation assay (indicator of stem like cells) as well as analysis of CSC markers (CD114 and CD133), Boyden chamber migration and invasion assays were performed. Starting at a very low concentration of 7.8 nM the drugs prevented sphere formation in a dose dependent manner and at about 62.5-125 nM sphere formation was completely prevented compared to either the control or sHDL nanoparticle treated cells ($p < 0.001$). This indicated that sHDL-WGA-TA particles likely target NB CSCs (Fig.4). Analysis of markers of CSCs revealed 40-50% and 10-20% decrease in the levels of markers CD133 and CD114 respectively after treatment with either WGA-TA or sHDL-WGA-TA compared to controls ($p < 0.01$), whereas sHDL alone showed no significant changes in levels compared to control for both IMR32 and SK-N-Be (2) cells (Fig.5). Another important property of cancer cells is migration leading to metastasis. Therefore, we analyzed migration and invasion of NB cells through Boyden chamber (Fig.6). Treatment of NB cells with 1 μM sHDL WGA-TA or WGA-TA for 48 h was highly effective in blocking the migration and invasion of cells whereas sHDL alone did not have any effect.

IMR32 xenografts uptake DiR sHDL nanoparticle in vivo

To assess the bio distribution of the sHDL nanoparticles, whole-animal imaging and *ex vivo* imaging of major organs of tumor-bearing mice were performed 24 h following i.p injection of either DiR-sHDL or DiR-liposomes. The results showed efficient accumulation of the nanoparticles in the tumor (Fig.7A) only in mice injected with sHDL nanoparticle but not in mice injected with liposomes (Supplemental Fig.1). In addition, IVIS imaging also indicated

high uptake of the sHDL nanoparticles in the liver, which plays a critical role in regulating cholesterol, whereas other organs showed minimal uptake (Fig.7B).

DISCUSSION

Despite recent advances in chemotherapy and surgery, the long term survival rate of patients with advanced metastatic NB is a dismal 18-30% (21). Systemic toxicity associated with chemotherapeutic agents is a serious problem, especially in pediatric cancer patients. Hence, novel anticancer compounds from natural products represent a largely untapped resource for development of new treatment options for pediatric tumors such as NB. This area recently has surged given the need for identification of novel compounds with anticancer benefits and low toxicity (22).

Withanolides are the key bioactive components present in the Solanaceae family of plants; including the Ashwagandha (*Withania somnifera*) plant, whose medicinal properties have been known for decades in Indian Ayurveda medicine. Withanolides are a group of naturally occurring 28 carbon steroidal lactones with an ergostane skeleton in which C-22 and C-26 are oxidized to form a six membered δ -lactone ring. Over the last several years our group has identified and purified more than 40 natural and semi-synthetic withanolide compounds with potent antitumor activity from the native *Physalis* plant (23, 24). We have explored the *in vitro* efficacy and the mechanism of action of novel natural withanolides that have shown potent anticancer properties both *in vitro* and *in vivo* in various cancer models with potential for targeting several cancers including NB.

Previous studies with our lead compound WGA-TA, exhibited high potency in inducing apoptosis, modulating the cell cycle, and targeting key oncogenic pathway proteins implicated in the poor survival of NB (Oncotarget, 2018). However, the poor water solubility and short half-life of WGA-TA *in vivo* may limit its use in the clinic. We sought to address these issues by using a nanocarrier that can solubilize the drug and improve the pharmacokinetic profile.

Recently we have reported that synthetic HDL (sHDL) composed of phospholipids and ApoA-1 mimetic peptides (25) can mimic the function of endogenous HDL to transport cholesterol from peripheral tissues to the liver for metabolism. These sHDL nanoparticles have also been shown to efficiently transport its cargo to SR-B1 expressing tumor cells (9, 26-28). Due to the presence of a hydrophobic space in the lipid bilayer, sHDL nanoparticles can significantly improve the solubility and transportability of poorly soluble anticancer drugs such as WGA-TA.

Furthermore, synthetic HDLs (sHDL) have been developed and clinically tested for the treatment of atherosclerosis. Clinical trials confirmed that sHDL therapeutics are safe at high doses of 20-40 mg/kg infusions and have a prolonged plasma circulation time of 8-14 h (29, 30). These features of sHDL may overcome the limitations of WGA-TA and facilitate its use in the clinic.

Our results indicate that HDL receptor SR-B1 is highly expressed in NB cells, especially N-myc amplified cells compared to normal cells. Consistent with previous results with retinoid

encapsulated sHDL that were non-toxic to normal cells, our sHDL-WGA-TA nanoparticles were effective in targeting NB cancer cells without significant effect on normal fibroblast cells (30). Similar to other malignant cancers (31), NB cells over express SR-B1 and these cells take up the sHDL nanoparticles in an SR-B1 dependent manner. Our novel sHDL delivery system for WGA-TA will be highly suitable for targeting high risk NBs that are known to have amplified N-myc. NB tumors are heterogeneous and CSC model suggests that treatment failure is due to the presence of self-renewal of a small population of tumor initiating cells (32). Hence, we examined the self-renewal and migratory properties of NB cells after treatment with sHDL-WGA-TA nanoparticles. Consistent with our previous report in which WGA-TA can target thyroid CSCs (33) and prevent self-renewal and migration, the sHDL-WGA-TA nanoparticles were able to block both self-renewal and migration in NB-CSCs whereas sHDL nanoparticles alone had no effect on these CSC functions.

Overall, our results demonstrate that novel withanolide-encapsulated sHDL nanoparticles target NB tumors both *in vitro* and *in vivo* through the HDL receptor SR-B1. Additionally, our results suggest that these novel nanoparticles decrease the viability of NB cells as well as potentially target NB CSC functions such as self-renewal, migration, and invasion. Given these encouraging findings, future *in vivo* translational studies are warranted to further evaluate this drug's potential as a novel therapy for NB, especially related to its safety and efficacy on CSC inhibition, tumor growth, invasion, and metastatic spread.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. A and B: Expression levels of SR-B1 in NB cell lines

mRNA and protein levels of SR-B1 in both N-myc amplified and non-amplified cell lines by RT-PCR and western blot analysis. As a positive control, NCI-H295R was used. Jurkat and fibroblast cell lines were used as negative control. mRNA level expression was measured by standard curve method using actin as an internal control. As a loading control for western blot, the same blot was stripped and probed for actin.

Figure 2. A and B: *In vitro* uptake of DIO labeled s-HDL nanoparticle by NB cells
 SR-B1^{high} and low expressing NB cells, positive control (NCI-H295R) and negative control (Jurkat and fibroblast) cell lines were treated with DIO labeled sHDL nanoparticle (green) for 4 h and the accumulation of the nanoparticle was examined using a fluorescent microscope. The nuclei of the cells were labeled with DAPI (blue). The cells were either pretreated with sHDL for 1 h or SR-B1 was knocked down to block the HDL receptor and uptake was imaged using a fluorescent microscope. The knockdown efficiency was verified by western blot analysis.

Figure 3. *In vitro* viability of NB cells after drug treatment

The viability of NB cells; NCI-H295R and fibroblast were determined after treating the cells with varying concentrations of either sHDL or WGA-TA or sHDL WGA-TA nanoparticle. Percentage viability was determined by MTS assay and the IC₅₀ values were calculated using GraphPad Prism software (TABLE-1).

Figure 4. Concentration dependent prevention of self-renewal of NB cells by WGA-TA loaded sHDL and WGA-TA

Sphere formation after treatment of NB cells (IMR32, SK-N-Be (2) and SK-N-As) with varying concentrations of either sHDL or WGA-TA or sHDL WGA-TA nanoparticles for a week was examined by counting the number of spheres (greater than 50 cells per sphere) formed under a light microscope. All experiments were repeated thrice and the values are represented as mean $\pm$ standard deviation. Representative spheres from IMR32 cells are shown.

Figure 5. Markers of NB CSCs are down regulated by WGA-TA loaded sHDL and WGA-TA Cell surface markers CD133 and CD114 were analyzed after treatment of human NB cells IMR 32 and SK-N-Be (2) with drugs (sHDL, WGA-TA, sHDL WGA TA). Representative flow cytometry figures are shown for SK-N-Be (2) and the results for both the cells were shown below.

Figure 6. Dose dependent blocking of migration and invasion of NB cells by WGA-TA loaded sHDL and WGA-TA

Boyden chambers coated with matrigel and control (uncoated) were used for estimating invasion and migration of NB cells (IMR32, SK-N-Be (2) and SK-N-As) after treating them with varying concentrations of either sHDL or WGA-TA or sHDL WGA-TA nanoparticles for 48 h. MEM supplemented with 10% FBS was used as a chemo attractant. The number of cells that have passed through the membrane was examined by counting the number of cells under a light microscope. Percentage of migration and invasion were calculated after normalization to control untreated cells. Each experiment was repeated thrice and the values are represented as mean $\pm$ standard deviation. Representative figures from IMR32 cells are shown.

Figure 7. A and B: *In vivo* uptake of DIR labeled s-HDL nanoparticles by IMR32 Xenografts
A. Approximately 5 million IMR32 cells were injected into nude mice for developing IMR32 xenografts. DIR labeled sHDL nanoparticles were injected i.p. and images were taken at time 0 and 24 h post injection using IVIS imaging system. **B.** *Ex vivo* images of organs and tumor at 24 h. A Representative image from n=3 is shown.

Table 1

IC50 values of NB cells and fibroblast cells after treatment with varying concentrations of drugs (sHDL or WGA-TA or sHDL WGA-TA)

IC50 in nM	sHDL	WGA-TA	sHDL-WGA-TA
IMR32	9982±5	24.46±1.43	26.44±0.94
SK-N-BE(2)	>10000	58.88±3.13	59.26±4.2
SH-EP	>10000	55.61±1.1	56.21±2.79
SK-N-As	>10000	21.62±1.1	33.55±3.32
Fibroblast	>10000	192.5±26.8	283.8±34.9