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Isatin Inhibits SH-SY5Y Neuroblastoma Cell Invasion and Metastasis through LSD1 Activity Inhibition

Shaobo Cong[#1], Haoyue Luo[#2], Yanan Hua[1], Xue Li[1], Fangling Wang[1], Li Zhang[1], Zheng Zhang[1], Ning Li[1], Lin Hou[1]

[1]Department of Biochemistry and Molecular Biology, Medical College of Qingdao University, Qingdao, Shandong 266021, China
[2]Medical College of Qingdao University, Qingdao, Shandong 266071
[#]Co first author, these authors contributed equally to this work

Abstract: Isatin has received much attention in recent years due to its anti-cancer properties[1], which offer important medical benefits. Isatin is an endogenous oxidized indole with a wide spectrum of behavioral and metabolic effects[2] and is commonly found in mammalian tissues and fluids[3]. It has many possible uses on the biomedical field[4] [5]and has also been investigated as a potential anti-cancer drug. However, its effects on neuroblastoma (NB) cells is still a mystery. This research aimed to elucidate the effects of Isatin on neuroblastoma cells metastasis and invasion and the underlying mechanism. Neuroblastoma cells viability was tested by CCK8. NB cells invasion and migration ability were tested by transwell and wound healing experiment. The mRNA relative expression of related molecules are detected by Rt-PCR and q-PCR. The protein relative expression of related molecules are detected by Simple western blotting. Our results demonstrated that isatin could inhibit neuroblastoma cell proliferation, invasion, and migration in a dose-dependent manner. Moreover, isatin increases the expression level of H3K4m1, PTEN. All results support the potential anti-metastatic effect of isatin in neuroblastoma cells.

Keywords: Isatin, Neuroblastoma, PTEN, LSD1, Invasion

Highlights: Isatin inhibits SH-SY5Y neuroblastoma cell invasion and metastasis.
Isatin decrease the expression of PTEN by inhabiting the activity of LSD1.

Introduction: As one of the most common solid extracranial neoplasm found in children, neuroblastoma (NB) accounts for more than 7% of malignancies in patients younger than 15 years old and around 15% of all pediatric oncology deaths[6, 7] .Although substantial improvement in outcome of certain well-defined subsets of patients has been observed during the past few decades, the outcome for children with a high-risk clinical phenotype has not improved much[8] . Neuroblastoma is a disease of the Sympatico adrenal lineage of the neural crest, and therefore tumors can develop anywhere in the sympathetic nervous system. Around half of all cases are currently classified as high-risk for disease relapse, with overall survival rates less than 40% despite intensive multimodal therapy[9]. The leading cause of death in NB patients is metastasis which for the most of time goes to the bone marrow in an unexplained way. Therefore it is important to learn more about the specific mechanism underlying NB invasion and metastasis and [10]find safer and better compounds which inhibit NB[4] invasion and metastasis.

Localized in the outer membrane of mitochondria，Monoamine oxidase（MAO）catalyzes the oxidative deamination of neuroactive cells. Increased monoamine oxidase (MAO) activity was recently shown to accompany apoptotic cell death of various neuronal cells following growth factor deprivation[11]. Monoamine oxidase A (MAOA) was reported to induce epithelial to mesenchymal transition thus mediating the growth, invasiveness, and metastasis of tumor cells.

As a member of MAO[12], lysine specific demethylase 1 (LSD1) which specifically removes the dimethyl and monomethyl modifications of H3K4 under the participation of FAD[13] is strongly associated with development ,invasiveness and metastasis of tumor cells[14]. For instance,LSD1 can be recruited by SNAG domain to E-cadherin promoter for transcription suppression and EMT [15]. As an epigenetic marker, LSD1 overexpression is one of the characteristics of malignant tumors [16] and has been correlated with malignant progression of multiple cancers, including primary neuroblastic tumors, ER-negative breast cancer

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Lin Hou (Correspondence)
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[17] and poorly differentiated neuroblastomas[18].LSD1 can also form co-inhibitory complex with Snail's SNAG domain which helps recruit LSD1 to PTEN promoter for H3K4 demethylation [19] and repress the transcription of PTEN [20].

PTEN, on the one hand, exert its role as a tumor suppressor by downregulating the PI3K/ AKT signaling pathway which is highly related to invasion and metastasis of cancer cells [21, 22] .For instance, PTEN inhibits the migration and invasion of HepG2 cells by coordinately decreasing MMP expression via the PI3K/AKT pathway[23]. On the other hand, PTEN interacts with focal adhesion kinase (FAK) by reducing its tyrosine phosphorylation and negatively regulates cell interactions with the extracellular matrix[24] to inhibits cell migration, spreading, and focal adhesion formation[25].

Previous researches have showed that Isatin is an endogenous indole which inhibits monoamine oxidase (MAO) B and may induce SH-SY5Y cells death in dose and time dependent manner [26]. In recent time, isatin was proved to inhibit SH-SY5Y cell migration and invasion in various pathways. For instance, isatin may inhabit SH-SY5Y cell migration and invasion by downregulating MMP-2/MMP-9 expression[27] or through MAO/HIF-1 alpha/CXCR4 signaling[28]. However, the precise mechanisms involved in the anti-metastasis activity of isatin are still not well elucidated.

In this study, we assume that through inhabiting LSD1 activity which may cause H3K4 demethylation, isatin may upregulate the expression of PTEN which then negatively regulates SH-SY5Y cell invasion and metastasis through PI3K-AKT signaling pathway and PTEN/ p-SHC /p-FAK signaling pathway. To testify our assumption, we apply Rt-PCR, Western blotting, Trans well experiment to test these molecules involved. The results were in line with expectations.

1.Materials and methods

1.1 Cell culture

SH-SY5Y cells (Chinese Academy of Sciences) were cultured in DMEM/high glucose (Hyclone, USA) supplemented with 10% fetal bovine serum (BI, California, USA) at 37°C with 5% CO_2 and 98% relative humidity in a culture incubator. Cells were incubated with different concentrations (0, 50, 100, 200μmol/l) of isatin (Sigma, California, USA, 99.0%) for 24, 48, or 72h when 80 percent of the plate had been covered. Thenceforth, the cells (about9×10^5cells/well) were harvested and used for proliferation, migration, and protein analysis.

1.2 Cell viability assay

The viability of cells was measured by CCK-8 assay. The cells were seeded independently in a 96-well plate with a final volume of 100μl of complete culture medium containing 1×10^4 cells/well then exposed to different concentrations (0, 25, 50, 100, 200, 300, 400, 500,600， 700μmol/l) of isatin (six wells for each concentration) and incubated for48h /72h at 37°C. After treatment, 10μl CCK-8 solution was added to each well and incubated at 37°C for 3h. The absorbance of the samples was measured at 450nm with a Microplate Reader (SynergyH1; Bio Tek, Vermont, USA). Each independent experiment was run for three times.

1.3 Cell migration and invasion assay.

SH-SY5Y Cells were seeded with serum-free medium in a six-well plate and exposed to isatin (0, 50,100, and 200μmol/l) for 48h. Thereafter, use a micropipette tip to scratch a wound in per well. Cells were monitored during regrowth, and images were captured at different time points (0, 12, 24, and 48h). The transwell invasion was assayed in Boyden chambers (Millipore, California, USA). 2×10^5 cells/ well in 200μl serum-free medium were pretreated with isatin for 24h and added to the upper chamber coated with matrigel, while medium with 10% FBS was added to the lower chamber. At the end of incubation, the cells on the upper surface were completely removed and the filter was fixed in methanol and stained with crystal violet staining. Cells invading the Matrigel of the filter were counted under an inverted microscope. Data were expressed as the average cell number in five fields and repeated for three times.

1.4 Simple Western Blotting

SH-SY5Y cells were lysed in RIPA buffer (Solabio, Beijing, China) with protease inhibitor cocktail (Sigma，Germany) on ice for 1h and then centrifuged for 20min at 10000rpm. The supernatant was collected and protein concentration was measured with BCA assay (Beyotime, Jiangsu,China).Prepare standard pack reagents ： Open the standard pack. There are four different tubes contains four different reagents with four different colors. Pierce foil of the clear tube which contains DTT with pipette tip and add 40μL deionized water in it and gently mix it by pipette to make a 400mM solution. Pierce foil of the pink tube which contains fluorescent 5× master mix with pipette tip then add 20μL 10×Sample buffer and 20μL prepared DTT solution in it and gently mix it by pipette. Pierce foil of the white tube which contains biotinylated ladder with pipette tip then add 16μL deionized water ,2μL10×Sample buffer and 2μL prepared DTT solution and gently mix it by pipette. Transfer entire volume of ladder to the 0.6mL tube(blue). Dilute 10×sample buffer 1：100 with water to make 0.1× sample buffer. Dilute protein lysate with 0.1×sample buffer and adjust the final protein concentration to 0.2 mg/ml. Denature samples and biotinylated ladder at 95℃ for 5mins then store them on ice. Dilute primary antibody with Antibody Diluent2 (1:50). The secondary HRP Conjugate is provided in the detection module

and ready to use. Combine 200µL Lumino-S and 200µLPeroxide supplied in the detection module in a microcentrifuge tube. Gently pipette up and down to mix and store it on ice. Add all these reagents into the wells of the plate (as shown in Fig.1A below) for immunoassay. Load the desired assay in Compass software. Open Wes' door. Insert a capillary cartridge into the cartridge holder. The interior light will change from orange to blue. Remove the assay plate lid and hold plate firmly on bench and carefully peel off evaporation seal. Pop any bubbles observed in the Separation Matrix wells with a pipette tip. Place the assay plate on the plate holder. Close Wes' door. Click the Start button in Compass. When the run is complete, discard the plate and cartridge. Data are collected and analyzed by Compass software.

1.5 Quantitative real-time RT-PCR assay

Total RNA was extracted from cultured SH-SY5Y cells with Trizol reagent (Solabio, Beijing, China). A reverse transcription kit (Transgene, Beijing, China) was used to construct the template cDNA for real-time PCR with Trans Start Probe Rt-PCR Super Mix (Transgene, Beijing, China). The data were gathered on a Bio-Rad (California, USA) One-Step Plus system. Primer sequences were show in table1.

Table1

Gene	Forward	Reverse
LSD1	5'TGGTGGTAACAGGTCTTGGAGG 3'	5'GGCTTCATAAAGTGGGCATTTTTG3'
PTEN	5′AGTTCCCTCAGCCGTTACCT3′	5'ATTTGACGGCTCCTCAACTG3′
GAPDH	5'GGAGCCAAAAGGGTCATCATCT3'	5'AGGGGCCATCCACAGTCTTCT-3'

1.6 Statistical analysis

The data shown in this study were obtained from three independent experiments and represented as mean ± SD. The corresponding data were compared by one-way ANOVA analysis of variance using GraphPad prism 6 statistical software (GraphPad, La Jolla, CA, USA), and significance was set at P value less than 0.05.

2. Results:

2.1 Cell viability is detected by CCK8

Isatin inhibited the invasion and metastasis of NB cells in vitro. The growth inhibition effect of isatin on SH-SY5Y was tested by CCK-8 assay. As shown in （Fig. 1B）, isatin at various concentrations influenced the survival rate of SH-SY5Y cells after treatment for 48 h/72h. The noncytotoxic concentration of isatin that resulted in more than 80% cell survival is below 200µmol/l. This concentration range was used in subsequent experiments.

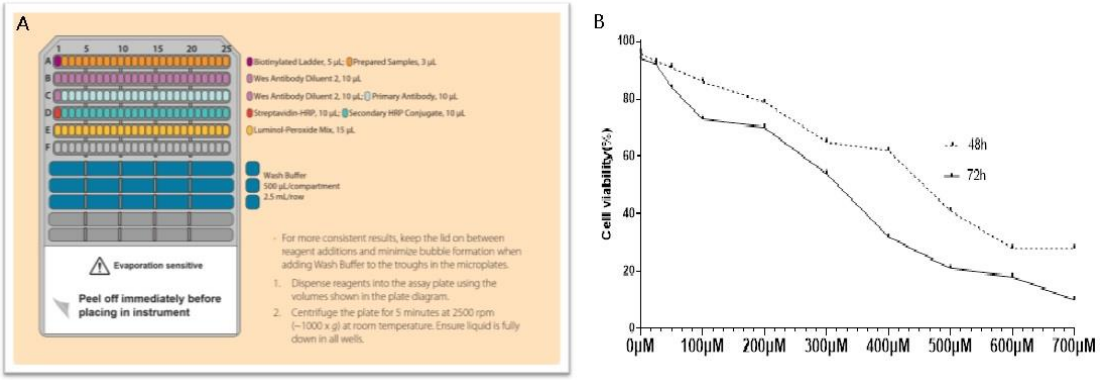

Fig.1 All simple western reagents are added into the wells of the plate as Fig.1A shows

Effect of isatin on the proliferation of neuroblastoma cells. Cells were incubated with 0, 25, 50, 100, 200, 300, 400, 500, 600,700µmol/l of isatin for 48 or 72h in CCK-8 assay(Fig.1B). *P<0.05

2.2 NB cells invasion and migration ability are decreased compared with control group.

The wound healing rates of SH-SY5Y cells treated with isatin were significantly lower than those of untreated cells. At 48h, the wound in control cells was almost closed while cells treated with isatin still showed a noticeable wound (Fig. 2A). In addition, isatin at 200µmol/l concentration decreased the cell invasion to 20%, as compared with control cells (Fig. 2B ,Fig.2C). Meanwhile, isatin had an anti-proliferation effect on SH-SY5Y cells (Fig.1B). All these results suggested that isatin had a potentially inhibitory effect on NB cell metastasis.

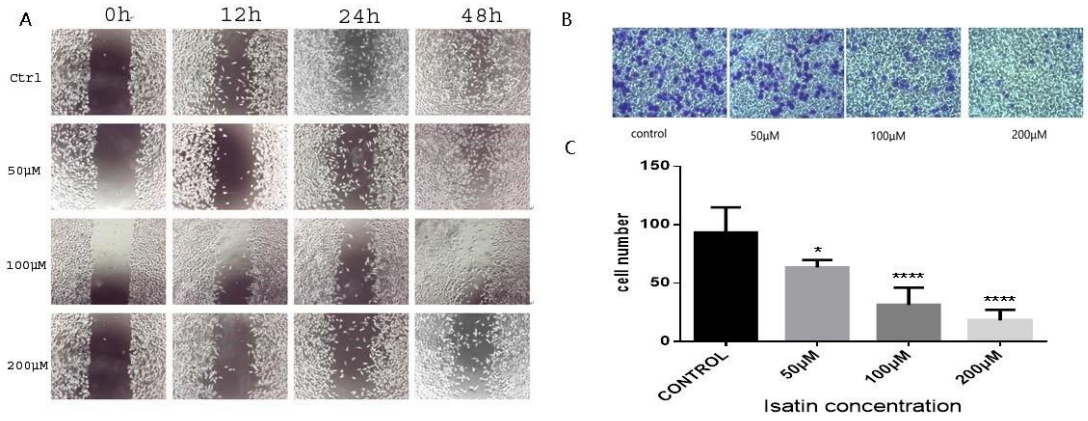

Fig.2 Isatin inhibits SH-SY5Y cell migration and invasion. (Fig.2A) Effect of isatin on neuroblastoma cell migration under a ×100 light scope using an inverted microscope. (Fig.2B) Statistical analysis of SH-SY5Y cell matrigel invasion counted in five random views(Fig.2C). *p<0.05, **P<0.01 compared with control

2.3 The expressions of H3K4 m1 and PTEN are increased

As reported in previous studies, isatin inhibits MAO activity[29]. Here we investigated whether isatin modulates LSD1 activity by testing the expression of its target protein H3K4 m1[13]. When incubated with 200μmol/l isatin, the protein expression of H3K4m1 was significantly increased compared with that of control cells (Fig.3A, Fig.3B) indicating isatin may influence SH-SY5Y cell invasion and metastasis through inhibiting LSD1 activity. It's been reported that LSD1 may sustains carcinoma cell proliferation through the PI3K/AKT pathway[30]. But whether LSD1 decrease the expression of PTEN ,a typical inhibitor of PI3K/AKT pathway[31, 32] , remains unclear in NB cells. To settle this argument, we tested related molecules through Simple Western Blotting and Rt-PCR. We found that the protein expressions of PTEN (Fig.3C, Fig3D) in SH-SY5Y cells exposed to isatin are increased in a dose-dependent manner and so are the mRNA expression (Fig.3E).

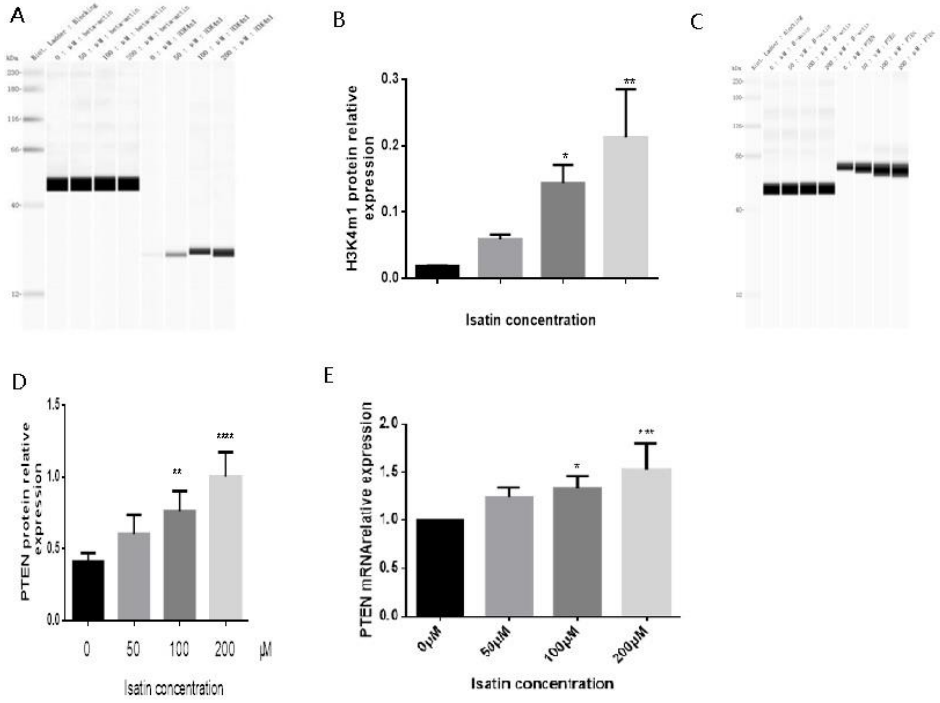

Fig3 Isatin increases the protein expression of H3K4m1(Fig.3A, Fig.3B) and PTEN (Fig.3C, Fig.3D) in SH-SY5Y cells after 48h of treatment with isatin as detected by simple western blot analysis. Statistical analysis of the Rt-PCR expression of PTEN(Fig.3E). Values are expressed as mean ± SD. *P<0.05, **P<0.01 compared with control.

2.4 The expression of LSD1 hasn't change

Intriguingly, Neither the expression protein (Fig.6A, Fig. 6B) nor mRNA relative expression (Fig.6C) of LSD1 has been fund changed in this study suggesting that isatin may not inhibit cell invasion by decreasing the expression of LSD1 but by inhibiting the enzyme activity of it.

All these results suggested that isatin might inhibit LSD1 activity thus increase PTEN expression which inhibit SH-SY5Y cell invasion and metastasis through PI3K/AKT signaling and PTEN/p-SHC/p-FAK signaling.

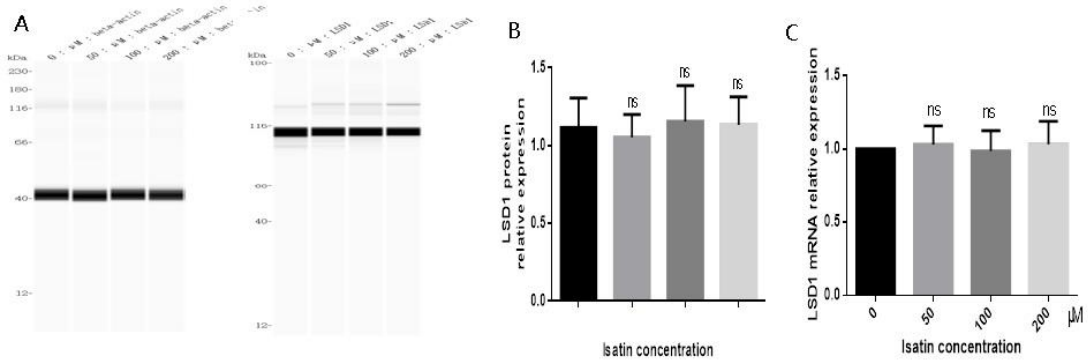

Fig.4 The protein (Fig.6A ,Fig.6B) and mRNA(Fig.6C) relative expressions of LSD1 in SH-SY5Y cells hadn't change after 48h of treatment with isatin as detected by Simple western blot analysis. Values are expressed as mean±SD. *P<0.05, **P<0.01 compared with control.

Discussion

Prior work has documented the effectiveness of isatin in preventing cancer cells proliferation and progression. Havrylyuk[33] ,for instance, reports that isatin possess remarkable antiproliferative influence on cancer. However, these studies have either just focus on isatin's antiproliferation property or have not clarified the anti-invasive mechanism of isatin in NB cells. In this study we investigated isatin's anti-invasive influence on NB cells and tried to reveal its mechanism beneath through techniques like Western blotting, Rt-PCR. We found that as isatin was added in SH-SY5Y cells, the expression of H3K4m1 which is the substrate of LSD1 was increased, while the expression of LSD1 hasn't change. Indicating that instead of decrease the expression of LSD1，isatin is much more likely to downregulate H3K4m1 expression by inhibiting the activity of LSD1. As the expression of H3K4m1 was upregulated, the expression of PTEN was increased as well. What's more, the expressions of downstream molecules of PTEN signaling such as p-SHC, p-FAK,p-PI3K,p-AKT,p-mTOR were decreased. These findings suggest that the mechanism of isatin's anti-invasion and anti-metastasis effect on SH-SY5Y cells is that isatin increases the expression level of H3K4m1 which then active PTEN signaling by inhabiting the activity of LSD1. Most notably, this is the first study to our knowledge to systematically investigate the influence of isatin on PTEN signaling related molecules in NB cells. However, some limitations are worth noting. Although our hypotheses were supported statistically by the results of our biochemical experiments in cells but whether the mechanism suits in animals or human remains unknown. Further study should therefore focus on isatin's effect on the tumors of animals.

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