Isatin inhibits the proliferation and invasion of SH-SY5Y neuroblastoma cells

PINGPING XU1, LIN HOU1, CHUANXIA JU2, ZHENG ZHANG1, WENYAN SUN1, LI ZHANG2, JINLIAN SONG3, YUQIANG LV1, LU LIU1, ZHIXIANG CHEN1 and YANHUI WANG1

Correspondence to: Professor Lin Hou, Department of Biochemistry, Medical College, Qingdao University, Qingdao, Shandong 266021; zhangyiwei@qdu.edu.cn

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Abstract. Isatin has been shown to initiate apoptotic processes in SH-SY5Y neuroblastoma cells. The aim of the current study was to investigate whether isatin is also capable to alter the proliferation and migratory ability of SH-SY5Y cells. The results demonstrated that the proportion of SH-SY5Y cells in G0 phase was significantly increased following treatment with isatin for 48 h with simultaneous downregulation of cyclin D1 expression. In addition, isatin significantly inhibited cell migration and invasion, along with decreases in matrix metalloproteinase (MMP)2 and MMP9 expression. In addition, isatin reduced the levels of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) in a concentration-dependent manner. These results demonstrated that isatin induces G0-phase arrest in SH-SY5Y cells, possibly by decreasing cyclin D1 expression as well as inhibiting their migration and invasiveness, probably by reducing MMP2 and MMP9. These effects may be exerted by isatin via a downregulating the levels of pSTAT3.

Introduction

Neuroblastoma, which are derived from the sympathetic nervous system, represent the forth most common type of extracranial malignant solid tumor in children (1). A variety of treatment approaches, including surgery, immunotherapy, apoptosis-inducing therapy, myeloablative chemotherapy and radionuclide therapy, are used in the clinic for inhibiting the rapid growth of neuroblastoma (2,3). In the spite of the development of numerous anti-cancer drugs and therapies, the five-year survival rate remains <75% owing to the high proliferation and migratory ability of neuroblastoma (4,5). Innovative therapeutic approaches using migratory inhibitors are expected to improve patient survival due to enhanced efficacy as well as reduced drug-associated toxicity.

1H-indole-2,3-dione (isatin) is a promising heterocyclic drug with numerous beneficial biological activities, including anti-bacterial, anti-fungal and anti-tumor properties (6). Derivatives of isatin have been demonstrated to exert inhibitory effects on tyrosine kinases and cyclin-dependent kinases (CDKs) as well as anti-angiogenic effects in tumor cells (7-11). Previous studies by our group suggested that isatin has marked pro-apoptotic effects on the SH-SY5Y neuroblastoma cell line in vitro and in vivo (12,13). The present study investigated the anti-proliferative and anti-invasive effects of isatin on SH-SY5Y cells as well as the underlying molecular mechanisms.

Materials and methods

Cells and cell culture. The SH-SY5Y human neuroblastoma cell line was purchased from Peking Union Medical College (Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 µg/ml streptomycin and 100 U/ml penicillin (both Solarbio Science & Technology Co., Ltd., Beijing, China). The cells were cultured at 37°C in an humidified atmosphere of 95% air and 5% CO2. Upon 70% confluency, isatin [in a 5 mM stock solution in 0.1% dimethyl sulfoxide (DMSO); Sigma-Aldrich, St. Louis, MO, USA] was added with final concentrations of 100, 200 or 400 µM. Following incubation for 48 h, the cells were harvested and subjected to analysis.

Flow-cytometric analysis. The treated cells were harvested by centrifugation and washed three times with phosphate-buffered saline. The cells were fixed with ice-cold 75% ethanol for 18 h, stained with propidium iodide (Sigma-Aldrich) and then analyzed by flow cytometry (FACSCanto; BD Biosciences, Franklin Lakes, NJ, USA) to detect the cell cycle. A minimum of 10,000 events were analyzed in each experiment, and the results were analyzed using ModFit LT software, version 3.2 (Verity Software House, Inc., Topsham, ME, USA).

Key words: isatin, human neuroblastoma cell, proliferation, invasion, matrix metalloproteinase, cyclin D1, signal transducer and activator of transcription 3
Table I. Cell cycle distribution of SH-SY5Y cells treated with isatin for 48 h as determined by flow cytometry.

<table>
<thead>
<tr>
<th>Group</th>
<th>G_1 phase</th>
<th>S phase</th>
<th>G_2/M phase</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>54.18±0.47</td>
<td>36.16±0.64</td>
<td>9.66±1.12</td>
</tr>
<tr>
<td>100 µM isatin</td>
<td>66.23±0.51</td>
<td>28.76±0.91</td>
<td>5.01±1.04</td>
</tr>
<tr>
<td>200 µM isatin</td>
<td>67.40±0.17</td>
<td>28.98±0.68</td>
<td>3.62±0.51</td>
</tr>
<tr>
<td>400 µM isatin</td>
<td>73.39±2.12</td>
<td>17.68±0.78</td>
<td>8.92±1.71</td>
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</table>

Values are expressed as the mean ± standard deviation. *P<0.01 compared with control; *P<0.01 compared with 100 µM isatin; **P<0.01 compared with 200 µM isatin.

**Invasion assay.** The invasive potential of SH-SY5Y cells was examined using Transwell inserts (Corning Inc., Corning, NY, USA). The membranes were coated with Matrigel (BD Biosciences) for 30 min. SH-SY5Y cells were trypsinized (Thermo Fisher Scientific, Inc.), re-suspended in serum-free medium and counted following serum starvation for 12 h. The bottom wells of the Transwell inserts were filled with DMEM containing 10% FBS. Cells (2×10^5 in 200 µl serum-free medium) were added to the upper compartment of each Transwell insert and incubated for 24 h in the absence or presence of isatin (100 or 200 µM). Cells that failed to migrate through the filter following incubation were removed using a sterile cotton swab, while invaded cells on the lower side of the filter were fixed with methanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and stained with 0.1% crystal violet (Solarbio Science & Technology Co., Ltd.). The number of invaded cells in five random fields of the Transwell membrane was counted under a microscope (CKX41; Olympus Corporation, Tokyo, Japan).

**Cell survival assay.** An MTT assay was conducted in order to assess the survival of SH-SY5Y cells. Cells (10^5 cells/well) were seeded into 96-well plates and isatin was added to a final concentration of 100 µM-400 µM 24 h later. Following incubation for 48 h, the cells were incubated with MTT (1 mg/ml; Sigma-Aldrich) for 3 h at 37°C, after which formazan crystals were dissolved in 100 µl DMSO. The absorbance was measured at 490 nm using a microplate reader (Synergy H1; BioTek Instruments, Inc., Winooski, VT, USA). The suppression rate was calculated using the following formula: Suppression rate = (1-A/C) x 100%, where A and C represent the number of cells treated with or without isatin, respectively. The MTT assay was performed six times.

**Monolayer wound healing assay.** Cells were seeded into individual wells of a six-well culture plate and grown to confluence. In order to suppress the contribution of cell proliferation, cells were grown in serum-free medium for 12 h; furthermore, cells were treated with mitomycin (10 µg/ml; Bio Basic Canada, Inc., Markham, ON, Canada) for 3 h prior to wounding. A sterile 10-µl pipette tip was then used to perform a longitudinal scratch in the confluent monolayer. The cell debris and medium were removed by aspiration and substituted with 2 ml fresh serum-free medium. Images were captured at 0, 12, 24, 36 and 48 h after wounding (corresponding to 12, 24, 36, 48 and 60 h post-treatment) using an inverted microscope (CKX41; Olympus Corporation). Ten randomly selected points along each wound, which were used to mark the horizontal distance between the initial wound and the migrated cells, was measured. All images were processed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

**Reverse-transcription quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from SH-SY5Y cells cultured in the presence or absence of isatin using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. Total RNA was converted into cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland), under the following conditions: 50°C for 1 h followed by 85°C for 5 min. cDNA was subjected to qPCR amplification in triplicate experiments using SYBR Green qPCR Master Mix (Takara Biotechnology Co., Ltd.) in a Real-Time PCR System (LightCycler® 96; Roche Diagnostics). The qPCR amplification conditions were as follows: 95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 30 sec. Quantification relative to GAPDH was performed using the 2^-ΔΔCq method (14).

Real-time PCR was performed using the following primers (Shanghai Sunny Biotech Co., Ltd., Shanghai, China): GAPDH forward, 5'-AACAGGCCCTCAAGATCATCAGCAA-3' and reverse, 5'-GACTGTGGTCATGAGTCCCTCCA-3'; MMP2 forward, 5'-TTCCCTGGCAAGCCAGAATG-3' and reverse, 5'-CTCCAGCGGGCCAAGTGG-3'; MMP9 forward, 5'-GTCACTGCAGGTATGCG-3' and reverse, 5'-GCCCCACTTCTTGTGCGT-3'.

**Protein extraction and western blot analysis.** SH-SY5Y cells were harvested and treated with isatin for 48 h. The cells were lysed in radioimmunoprecipitation assay buffer (Solarbio Science & Technology Co., Ltd.) for 20 min on ice. The homogenate was centrifuged for 5 min at 13,200 x g and the protein concentration in the supernatant was quantified using the bicinchoninic acid protein assay (Beyotime Institute of Biotechnology, Inc., Shanghai, China). Following storage at -80°C, 30 µg protein from each sample was separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sigma-Aldrich) and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were individually blocked with 5%...
bovine serum albumin (Amresco, LLC, Solon, OH, USA) and then incubated with mouse anti-β-actin monoclonal antibody (1:1,000; TA-09; Zhongshan Golden Bridge Biotechnology, Beijing, China), rabbit anti-cyclin D1 monoclonal antibody (1:1,000; 2978; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-matrix metalloproteinase (MMP)2 polyclonal antibody (1:1,000; 4022; Cell Signaling Technology), rabbit anti-MMP9 monoclonal antibody (1:3,000; ab76003; Abcam, Cambridge, UK) and rabbit anti-phosphorylated signal transducer and activator of transcription 3 (pSTAT3) monoclonal antibody (1:2,000; Tyr705; Cell Signaling Technology) for 2 h at room temperature, followed by further incubation at 4˚C overnight. The blots were washed three times for 10 min each in Tris-buffered saline (Sangon Biotech Co., Ltd., Shanghai, China) containing 0.1% Tween 20 (TBST; Bio Basic Canada, Inc.) and then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit (1:2,000; BA1054; Boster Biological Technology, Ltd., Wuhan, China) and goat anti-mouse (1:5,000; ZB-2305; Zhongshan Golden Bridge Biotechnology) monoclonal antibodies for 1 h at room temperature. Following three washes in TBST for 10 min each, proteins were detected using an Enhanced Chemiluminescence Plus kit (Cyanagen, Bologna, Italy) by a chemiluminescence system (Fusion FX7; Vilber Lourmat, Collégien, France). Densitometric analysis was performed with Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Each experiment was performed at least three times. Values are expressed as the mean ± standard deviation. Statistical analysis included one-way analysis of variance, which was performed using SPSS software, version 20.0 (IBM SPSS, Armonk, NY, USA). When the differences between average levels among several groups were statistically significant, the Bonferroni multiple-comparisons test was performed. *P<0.05 was considered to indicate a statistically significant difference.

Results

Isatin enhances the G1-phase population of SH-SY5Y cells. Following administration of isatin for 48 h, cell cycle analysis was performed by flow cytometry. The results revealed that isatin significantly increased the proportion of cells in G1 phase.
XU et al: ISATIN INHIBITS NEUROBLASTOMA CELL PROLIFERATION AND INVASION

Figure 3. Isatin treatment decreased the protein and mRNA expression of MMPs in SH-SY5Y cells. Relative mRNA expression of (A) MMP2 and (B) MMP9 normalized to GAPDH. (C) Representative western blot of MMP2 and MMP9 protein in SH-SY5Y cells incubated with isatin for 48 h. Expression of (D) MMP2 and (E) MMP9 protein was quantified by densitometric analysis. β-actin was used as a loading control. Lanes/groups: 1, control cells; 2-4: SH-SY5Y cells treated with 100, 200 or 400 µmol/l isatin, respectively. Values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 compared with control; #P<0.01 compared with 100 µM isatin. MMP, matrix metalloproteinase.

Table II. Inhibitory effect of isatin on human SHSY-5Y cells, as measured by an MTT assay.

<table>
<thead>
<tr>
<th>Group</th>
<th>OD&lt;sub&gt;490&lt;/sub&gt;</th>
<th>Suppression rate (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.2401±0.0280</td>
<td>--</td>
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<tr>
<td>Isatin</td>
<td></td>
<td></td>
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<tr>
<td>100 µM</td>
<td>0.1976±0.0218&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.8±1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>200 µM</td>
<td>0.1832±0.0301&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.5±3.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>400 µM</td>
<td>0.1796±0.0201&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>22.1±2.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard deviation. *P<0.05 vs. the control group; <sup>a</sup>P<0.05 vs. the 100 µM isatin group; <sup>b</sup>P<0.01 vs. control.

(P<0.01) and significantly decreased the proportion of cells in S phase (P<0.01), while the proportion of cells in the G<sub>2</sub>/M phase was not altered (Table I). Compared with the 100 and 200 µM groups, treatment with 400 µM isatin resulted in a significantly higher increase in G<sub>1</sub>-phase and decrease in S-phase populations (P<0.01). These results suggested that isatin significantly caused G<sub>1</sub>-phase arrest in SH-SY5Y cells.

Isatin impedes the expression of cyclin D1. As cyclin D1 activation regulates the transcription of genes associated with cell proliferation (15), the present study investigated the impact of isatin on the expression of cyclin D1. As shown in Fig. 1, isatin significantly reduced the protein expression of cyclin D1 compared with that in the control group (P<0.01). Furthermore, cyclin D1 expression in the 400 µM isatin group was significantly decreased compared with that in the 100 and 200 µM groups (P<0.01).

Isatin inhibits the invasive and migratory capacity of SH-SY5Y cells. The impact of isatin on the invasion and migration of neuroblastoma cells was assessed using in vitro Transwell and wound-healing assays. As illustrated in Fig. 2A and B, 200 µM isatin significantly restrained the invasiveness of SH-SY5Y cells (P<0.01). Furthermore 200 µM isatin reduced the migratory ability of these cells after 36 h (P=0.046) and 48 h of incubation (P=0.035) (Fig. 2C and D). These results suggested that isatin reduces the invasion and migration of the SH-SY5Y human neuroblastoma cell line.

Isatin inhibits the proliferation of SH-SY5Y cells. The effect of isatin on the proliferation of SH-SY5Y cells was investi-
gated using an MTT assay. As is shown in Table II, isatin significantly inhibited the proliferation of SH-SY5Y cells (P<0.01), as compared with the control. In addition, the OD_{570} and suppression rate of SH-SY5Y cells were significantly decreased (P<0.05). Notably, the 400 µM isatin group resulted in a more significant decrease, as compared with the 100 µM group (P<0.05).

Isatin reduces the expression of MMP2 and MMP9. As it is known that MMP-2 and MMP-9 expression is relevant to metastasis and progression of neuroblastoma (16), the present study assessed the impact of isatin on MMP2 and MMP9 mRNA and protein expression in SH-SY5Y cells. As shown in Fig. 3A and B, 100 µM isatin significantly reduced the mRNA expression of MMP2 (P=0.010) and MMP9 (P=0.040); however, at this concentration, isatin did not significantly affect the protein expression of these MMPs (P=0.520 and P=0.661) (Fig. 3C-E). Of note, at 200 and 400 µM, isatin significantly inhibited the mRNA and protein expression of MMP2 and MMP9 compared with that in the control (P<0.01).

Isatin restrains the phosphorylation of STAT3. As pSTAT3 has a major role in tumor formation, as it is the point of convergence of multiple signaling pathways triggered by growth factors, cytokines and oncogenes. Considerable evidence has implicated STAT3 in the regulation of cellular apoptosis, tumor proliferation, invasion/metastasis and angiogenesis (31-33). Target genes of STAT3 include several members of the MMP family, D-type cyclins, vascular endothelial growth factor (VEGF) and B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) (34-37). The results of the present study showed that isatin inhibited the expression of MMP family and D-type cyclins. Previous studies by our group indicated that isatin regulates Bcl-2/Bax and VEGF expression (12,13). Hence, isatin may downregulate these genes by restraining the phosphorylation of STAT3.

The present study confirmed that isatin is an effective inhibitor of neuroblastoma-cell proliferation and metastasis. It inhibits the proliferation of SH-SY5Y cells by reducing the expression of cyclin D1 and impedes cell migration and invasion by decreasing MMP2 and MMP9. The observed effects of isatin on tumor-cell migration and proliferation are likely to be associated with pSTAT3. Isatin is a promising candidate for the clinical treatment of human neuroblastoma, which should be evaluated in in vivo studies.

Acknowledgements

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References

Xu et al: ISATIN INHIBITS NEUROBLASTOMA CELL PROLIFERATION AND INVASION


