The endogenous oxindole isatin induces apoptosis of MCF-7 breast cancer cells through a mitochondrial pathway

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Abstract. Isatin is an endogenous indole in mammalian tissues and fluids that is expected to have antitumor effects in human breast cancer cells. Human breast cancer cells (MCF-7) were exposed to isatin at various concentrations (0, 50, 100, 200 µmol/l) for 48 h. Apoptotic features were demonstrated by nuclei staining with Hoechst 33258 and flow cytometry. Bcl-2 and Bax mRNA were analyzed via reverse transcription-polymerase chain reaction. Bcl-2, Bax, the inhibitor of caspase-activated DNase (ICAD), and cytochrome c protein were analyzed by western blot analysis. Apoptosis, caspase-9 and -3 activation and mitochondrial depolarization were assayed by flow cytometry. The results showed that isatin induced apoptosis of MCF-7 cells. Furthermore, Bcl-2 expression was decreased and the ratio of Bcl-2 to Bax was significantly decreased by isatin. The mitochondrial transmembrane potential was markedly decreased and the release of cytochrome c into the cytosol was elevated following treatment with isatin. At the same time, caspase-9 and -3 were stimulated, followed by the degradation of ICAD, a caspase-3 substrate.

Introduction

As a result of the increasing number of breast carcinomas, breast cancer has become the most common malignancy and is currently one of the leading causes of mortality in women (1-2). Although systematic chemotherapy remains a frequently used clinical strategy, due to the development of multidrug resistance and severe side-effects (3,4), there is still a need to identify novel agents to enhance the effectiveness of breast cancer treatment.

In the past years, synthesis and characterization of novel antitumor compounds with known biological activity have represented a field of research that has created expectations for more specific and less toxic therapies (5). Isatin (an alternative name for 1H-indole-2,3-dione; formula, C8H5O2N), an endogenous indole in mammalian tissues and fluids, possesses a wide range of biological activities, such as anxiogenic, sedative and anticonvulsant activities, and is a potent antagonist of atrial natriuretic peptide receptors in vitro (6-8). However, previous studies have focused on its potent anticaner properties (9,10). In addition, many indole-based compounds appear to act as inhibitors of various protein kinase families, particularly receptor tyrosine kinases (RTKs) and serine/threonine-specific protein kinases such as the cyclin-dependent kinases (CDKs) (11,12). Oxindole sunitinib malate, as a kinase inhibitor, was recently approved by US FDA for the treatment of advanced renal carcinoma and gastrointestinal stromal tumors, which underscores the increasing interest in isatins as a new class of antineoplastic agents. It was also shown that isatin and its analogs inhibited the proliferation of some cancer cells, including colon HT29, breast MCF-7, lung A549 and melanoma UACC903 cells and is a dual inhibitor of tubulin polymerization and the Akt pathway (13). Therefore the aim of this study was to further clarify the anticancer mechanism of isatin itself, which may offer an opportunity to design effective safe drugs with minimal toxicity for the treatment of breast carcinoma.

It is widely believed that the mitochondrial pathway plays a critical role in the apoptotic process (14), particularly the mitochondrial Bcl-2 protein family. This family consists of a large group of apoptosis-regulating proteins that modulate the apoptotic mitochondrial pathway (15) and includes both anti-apoptotic proteins, such as Bcl-2, Bcl-xl and Mcl-1, and pro-apoptotic proteins, including Bax, Bad and Bak (16). These proteins regulate mitochondrial membrane permeability by either promoting or suppressing the release of apoptogenic proteins from organelles. Therefore, we sought to assess the in vitro effects of isatin in MCF-7 cells and to further confirm the anticancer effect of isatin through a mitochondrial pathway.

Materials and methods

Cell line and cell culture. The human MCF-7 cell line was obtained from Peking Union Medical College. The cells

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were maintained in DMEM medium supplemented with 15% heat-inactivated fetal calf serum at 37°C in a tissue culture incubator with 5% CO₂ and 98% relative humidity. When 70% of the plate was covered by cells, isatin (dissolved in 200 µl DMSO to obtain a 5 mmol/l solution) was added to the final concentration of between 50 and 200 µmol/l. Following a 48 h incubation period, the cells were harvested and used for apoptosis, mRNA and protein analysis.

**Nuclear staining.** The cells used for nuclear staining were seeded in 6-well plates (10⁷ cells/well). After 48 h of treatment with isatin, the cells were fixed with 4% paraformaldehyde for 1 h at room temperature, washed with PBS, stained with 10 µg/ml Hoechst 33258 (Sigma) for 10 min at 37°C in the dark and then washed with PBS. The apoptotic features of cell death were established by staining cell nuclei with the DNA-binding fluorochrome Hoechst 33258 and assessing chromatin condensation by fluorescence microscopy (BX-50, Olympus, Tokyo, Japan).

**Flow cytometric analysis.** The treated cells were harvested by centrifugation and washed three times with PBS. The cells were fixed with ice-cold 75% ethanol for 18 h, stained with propidium iodide (PI) and then analyzed by flow cytometry (CantoII; Becton-Dickinson, USA) to detect apoptotic cells. A minimum of 10,000 events were analyzed in each experiment.

**Analysis of Bcl-2 and Bax mRNA.** Total RNA was extracted from the cultured cells (10⁷) cultured in the presence or absence of isatin for 48 h using a TRIzol RNA isolation kit (Life Technologies, Gaithersburg, MD, USA) and detected by reverse transcription-polymerase chain reaction (RT-PCR), according to the manufacturer's protocol. Primers were: for GAPDH, 5'-ACCACAGTCCATGCCATC-3' and 5'-TCCACACGCTTTGCTGTA-3' to give a product of 452 bp; Bcl-2, 5'-GGAGGATTGCTGCTTTTG-3' and 5'-GGTGCCGTTGACTAATAA-3' to give a product of 120 bp; Bax, 5'-TCAACAGGAGTGGAGCAG-3' and 5'-GTCAAGGCTAATGTTTCT-3' to give a product of 257 bp. Each RT-PCR assay was performed in triplicate. Primers specific for human GAPDH cDNA were added to a parallel reaction to standardize for variations in the PCR between samples. PCR products were resolved on a 1.0% agarose gel, visualized under UV light and quantified using a JS-380B Imager (Shanghai Peiqing, China).

**Western blot analysis.** MCF-7 cells (10⁷) were cultured in the presence or absence of isatin for 48 h. The cells were scraped and lysed in buffer (20 mmol/l Tris-HCl, pH 7.4, 137 mmol/l NaCl, 10% glycerol, 1% Nonidet P-40, 2 mmol/l sodium vanadate and 100 mmol/l sodium fluoride) for 20 min on ice, before centrifugation at 12,000 x g for 2 min. The protein concentration of the supernatants was determined using Bradford protein assay reagent (Bio-Rad), separated in 10% SDS-PAGE and blotted onto a PVDF membrane. The blots were blocked in non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature, before incubation with the monoclonal primary antibodies, anti-Bcl-2, anti-Bax and anti-inhibitor of caspase-activated DNase (ICAD) (1:1,000; New England Biolabs, Inc., Beverly, MA, USA) overnight at 4°C. The blots were washed for 3x5 min in TBS-T and then incubated in a 1:2,000 dilution of peroxidase-conjugated donkey anti-rabbit secondary antibodies for 2 h at room temperature. The blots were again washed for 3x5 min in TBS-T and proteins were detected using an enhanced chemiluminescence plus kit (Amersham Biosciences, Buckinghamshire, UK).

**Measurement of the mitochondrial membrane potential (ΔΨm).** Rhodamine 123 is widely used for mitochondrial staining due to its rapid cellular uptake and equilibration. Viable cells appear as a highly fluorescent population, whereas apoptotic cells exhibit a lower fluorescence (17). Cells were treated with isatin (50, 100, 200 µmol/l) for 48 h, washed with PBS and then incubated in PBS containing 5 µmol/l Rhodamine 123 at room temperature for 30 min. After two washes and a final resuspension in PBS, the fluorescence of the cells was measured by flow cytometry (CantoII; Becton-Dickinson, USA).

**Detection of cytochrome c release from the mitochondria to the cytosol.** Cytochrome c determination in cytosolic and mitochondrial fractions was performed using western blot analysis. The cells were harvested after the respective treatments and washed once with ice-cold PBS. For the isolation of mitochondria and cytosol, cells were sonicated in a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mmol/l NaCl, 175 mmol/l sucrose and 12.5 mmol/l EDTA and the cell extracts were centrifuged at 1,000 x g for 10 min to pellet the nuclei. The supernatant obtained was centrifuged at 18,000 x g for 30 min to pellet the mitochondria. The resulting supernatant was termed the cytosolic fraction. The pellet was lysed and the protein content was estimated in both fractions using Bradford's method. Equal amounts of protein were separated on 15% SDS-PAGE and electrophoretically transferred to a PVDF membrane. The membrane was then incubated in 5% non-fat milk in TBS for 2 h followed by overnight incubation with the primary antibody (Sigma, 1:1,000). The incubated membranes were extensively washed with TBST before incubation for 2 h with the secondary antibody. After extensive washing with TBST, the immune complexes were detected using an enhanced chemiluminescence detection kit.

**Caspase-9 and -3 assay.** Caspase-9 and -3 were analyzed by flow cytometry using CaspGLOW™ Flourescein Active caspase-9 staining kit (eBioscience, USA) and PE-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Biosciences, USA). Cells were collected and washed in cold PBS, resuspended at a concentration of 1x10⁶ cells/0.5 ml. Samples were washed twice with wash buffer, before FITC-LeHD-FMK (a specific inhibitor of caspase-9) (eBioscience, 1:300) and PE-conjugated monoclonal anti-active caspase-3 antibodies (BD Biosciences, 1:500) were added and the cells were incubated for 30 min at room temperature. The marked samples were analyzed by flow cytometry (CantoII; Becton-Dickinson).

**Statistical analysis.** Each experiment was performed at least three times. All data are expressed as the mean ± SD. Statistical analysis was performed using one-way ANOVA. A level of P<0.05 was considered to indicate a statistically significant difference.
Results

Effect of isatin on MCF-7 cell apoptosis. Apoptotic cells have special morphologic features such as cell shrinkage, chromatin condensation and margination as well as forming apoptotic bodies. Hoechst 33258 staining was used to determine whether the isatin-induced reduction in cell viability was attributable to the induction of apoptosis. As shown in Fig. 1A, the treatment of MCF-7 cells with isatin resulted in the induction of chromatin condensation and fragmentation, which was visualized as intense pycnotic bluish-white fluorescence within the cell nuclei. To further confirm the isatin-induced apoptosis, we detected it by flow cytometry. When cells were treated with isatin (50, 100 and 200 µmol/l) for 48 h, cell populations in the apoptotic phases increased from 11.25±2.6 and 15.63±2.40 to 23.47±5.06%, compared with 6.33±1.40% of apoptotic cells in the control (Fig. 1B).

Effect of isatin on Bcl-2 and Bax expression. Bcl-2 family members actively participate in the apoptotic process either via pro-apoptotic proteins such as Bax, or via anti-apoptotic ones including Bcl-2. The ratio of pro- and anti-apoptotic molecules is essential to apoptosis. Therefore, we studied the effect of isatin on Bax and Bcl-2 mRNA expression. The RT-PCR
results showed that isatin decreased the expression of Bcl-2 mRNA and while it did not modulate the expression of Bax mRNA (Fig. 2), the Bcl-2/Bax ratio was decreased. The Bcl-2 and Bax protein expression results from the western blot analysis were consistent with those from mRNA analysis (Fig. 3). These results demonstrate that isatin possesses a pro-apoptotic effect on MCF-7 cells.

**ΔΨm determination.** Disruption of mitochondrial integrity is one of the early events leading to apoptosis. Thus, when cells are stained with a potential-sensitive mitochondrial specific probe, the staining and the fluorescence intensity are directly correlated with the mitochondrial polarization status. To assess whether isatin affects mitochondrial function, changes in the ΔΨm were analyzed through the use of

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Figure 2. RT-PCR of Bcl-2 (A Bcl-2/AgAPDH) and Bax (A Bax/AgAPDH) mRNA relative expression in MCF-7 cells after 48 h of culture with isatin. The Gel electrophoresis is representative of three independent experiments (A) The densitometric quantification was normalized to GAPDH. (B) Data are shown as the mean ± SD for three independent experiments. *P<0.05 vs. control. M, DNA marker; lane 1, control cells; lanes 2-4, MCF-7 cells treated with 50, 100 and 200 µmol/l isatin, respectively.

Figure 3. Expression of Bcl-2 and Bax in MCF-7 cells after 48 h of treatment with isatin detected by western blot analysis. The immunoblots are representative of three independent experiments. (A) The densitometric quantification was normalized to β-actin. (B) Data are shown as the mean ± SD for three independent experiments. *P<0.05 vs. control. Lane 1, control cells; lanes 2-4, MCF-7 cells treated with 50, 100 and 200 µmol/l isatin, respectively.

Figure 4. Isatin-induced Dym Depolarization in MCF-7 cells (representative experiment).
a fluorescent mitochondrial dye, Rhodamine 123. As shown in Fig. 4, exposure to isatin for 48 h resulted in a decrease in the fluorescence intensity by ~77.00±2.27, 71.47±2.12 and 63.67±4.40% at 50, 100 and 200 µmol/l, respectively, which was lower than that in control MCF-7 cells (88.40±6.15%). This suggests that treatment with isatin for 48 h results in decreases in the ΔΨm.

Cytochrome c release from mitochondria to cytosol. Cytochrome c release from mitochondria is a critical step in the apoptotic cascade since this activates downstream caspases. To investigate cytochrome c results in isatin-treated cells, we conducted western blot analysis in both cytosolic and mitochondrial fractions. The results demonstrate a clear increase in cytosolic cytochrome c after treatment with isatin (at 50, 100, and 200 µmol/l). At the same time, a decrease in cytochrome c was detected in the mitochondrial fraction (Fig. 5).

Analysis of caspase-9 and -3 protein expression. We analyzed active caspase-9 and -3 in MCF-7 cells treated for 48 h with isatin at 0, 50, 100, 200 µmol/l by flow cytometry with specific inhibitors of caspase-9 and -3. As shown in Fig. 6, exposure to isatin for 48 h resulted in an increase in the expression of active caspase-9 by ~21.17±2.63, 31.77±3.56 and 38.73±4.30% at 50, 100 and 200 µmol/l, respectively, which was higher than that in control MCF-7 cells (13.07±2.81%). In addition, the expression of active caspase-3 was increased in isatin-treated cells, but not in control cells (Table I).

Western blot analysis of ICAD. Western blot analysis indicated that ICAD expression was downregulated when MCF-7 cells were treated with isatin (50, 100, 200 µmol/l) for 48 h (Fig. 7).

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Table I. Effect of isatin on caspase-3 activity in MCF-7 cells measured by flow cytometry.

<table>
<thead>
<tr>
<th>Group</th>
<th>Unactivated caspase-3</th>
<th>Activated caspase-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.43±2.96</td>
<td>9.40±2.76</td>
</tr>
<tr>
<td>Isatin</td>
<td></td>
<td></td>
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<tr>
<td>50 µmol/l</td>
<td>83.00±1.67</td>
<td>16.57±1.36</td>
</tr>
<tr>
<td>100 µmol/l</td>
<td>72.47±4.22</td>
<td>26.40±4.21</td>
</tr>
<tr>
<td>200 µmol/l</td>
<td>63.87±4.56</td>
<td>35.97±4.52</td>
</tr>
</tbody>
</table>

All values are presented as mean ± SD of one experiment performed in triplicate. *P<0.05 vs. control.
Discussion

The lack of selectivity of many anticancer agents and the occurrence of intrinsic or acquired resistance of tumors to chemotherapy are major obstacles in the treatment of cancer. Studies have shown that isatin and its analogs display diverse biological activities including anticancer activities as an endogenous molecule in humans and other mammals (18). Therefore, isatin has gained considerable attention due to its anticancer activities, and it is expected to be a novel candidate for low toxic tumor therapy (19,20). In this study, we used a commercially available isatin and its inhibitory activities on the growth of MCF-7 cells were assayed. The results demonstrated that isatin strongly induced human breast cancer cell line apoptosis in vitro.

In this context, we thoroughly investigated the molecular pathways induced by isatin in breast cancer cells. Based on morphologic observations, DNA fragmentation suggested that MCF-7 cell death induced by isatin involved an apoptotic mechanism. In addition, the apoptotic rate, analyzed by flow cytometry, further confirmed that isatin induced MCF-7 cell apoptosis in a concentration-dependent manner. Apoptosis is a regulated biological mode of cell death and includes two major pathways, the death receptor-mediated extrinsic pathway and the mitochondria-dependent intrinsic pathway (21). The Bcl-2 family of proteins, including both pro- and anti-apoptotic members, play important roles in controlling the mitochondria-dependent intrinsic pathway at critical checkpoints. However, studies have demonstrated that the anticancer effects of many currently available chemotherapeutic agents may be inhibited by upregulation of Bcl-2 expression which blocks the apoptotic pathway (22). A promising antisense strategy to downregulate Bcl-2 is under clinical evaluation for the treatment of melanoma (23). Therefore, we determined Bcl-2 and Bax proteins in MCF-7 cells following treatment with various concentrations of isatin. The results showed that, concomitant with an increase in isatin, Bcl-2 expression decreased and the ratio of Bcl-2 to Bax significantly decreased (P<0.05). These results indicated that the mitochondria-dependent intrinsic pathway is an important pathway in isatin-induced apoptosis. Previous studies have also demonstrated that the ratio of Bax/Bcl-2 sets the threshold of susceptibility to apoptosis in the mitochondrial pathway (24). Cytochrome c is one of a host of pro-death molecules located within mitochondria and is a universal feature of apoptosis. Studies have shown that the Bcl-2 family is closely involved in the regulation of cytochrome c release into the cytosol (25). The anti-apoptotic proteins such as Bcl-2 preserve the integrity of mitochondria and block the release of cytochrome c. Bax is located in the cytosol and can interact with the anti-apoptotic protein Bcl-2. In response to apoptotic signals, Bax translocates to the mitochondria and inserts into the outer mitochondrial membrane, heterodimerizing with Bcl-2 to abrogate the inhibition of apoptosis caused by Bcl-2 by promoting the release of cytochrome c into the cytosol. Our results showed that cytochrome c levels in the mitochondria were downregulated, suggesting that it was released into the cytosol. The release of cytochrome c from the mitochondria to the cytosol is an important step in the apoptotic pathway.

Caspases are a family of cysteases, which cleave protein substrates after their Asp residues and appear to be involved in regulating the activation of apoptotic signal transmission (26). It is well known that mitochondrial damage caused by apoptotic stimuli triggers the release of apoptogenic proteins including cytochrome c and Smac. Cytochrome c triggers the activation of caspases. Based on our current findings on the regulatory effects of isatin on decreased mitochondrial membrane potential and the release of cytochrome c, we hypothesized that caspase-9 and -3 may play important roles in isatin-induced apoptosis. The results of this study confirmed that caspase-9 and -3 activities were increased following isatin treatment. To further confirm the participation of caspase-3 in cell death, we examined the protein expression of ICAD. ICAD is digested by caspase-3, resulting in activation of caspase-activated DNase (CAD) and nuclear internucleosomal DNA fragmentation (27). Our results showed that the expression of ICAD was downregulated. Taken together, these results suggest that the caspase cascade plays a critical role in isatin-induced MCF-7 cell apoptosis.

In summary, isatin significantly inhibits the growth of MCF-7 cells in vitro. This anticancer mechanism may involve the deregulation of Bcl-2, a decrease in the ΔΨm and activation of caspase-9 and -3. These results strongly support the hypothesis that the mitochondrial pathway is involved in apoptosis. However, as relatively little research has focused on its in vitro effects, further study is required to confirm the anticancer effect of isatin.

Figure 7. Expression of ICAD in MCF-7 cells after 48 h of treatment with isatin. The immunoblot is representative of three independent experiments. (A) The densitometric quantification was normalized to β-actin. (B) Data are shown as the mean ± SD for three independent experiments. *P<0.05 vs. control. Lane 1, control cells; lanes 2–4, MCF-7 cells treated with 50, 100 and 200 µmol/l isatin, respectively.
References