Indirubin-3'-monoxime, a CDK Inhibitor Induces Growth Inhibition and Apoptosis-independent Up-regulation of Survivin in Transitional Cell Cancer

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Abstract. In traditional Chinese Medicine, the preparation Danggui Longhui Wan has been used for years in the treatment of chronic myelocytic leukemia. The compound indirubin has been shown to be the active constituent. A cell permeable derivative, indirubin-3'-monoxime, is a selective and potent inhibitor of cyclin-dependent kinases (cdk). The ability of indirubin-3'-monoxime to induce apoptosis and tumor cell death in transitional cell cancer cell lines was investigated here. The growth-inhibitory properties were evaluated by EZ4U, a cytotoxic assay; apoptosis induction was determined by immunoblotting of cleaved PARP and flow cytometry of Annexin-V/PI staining during treatment. To evaluate further the underlying molecular action of indirubin-3'-monoxime on the cell cycle, the levels of cdk-1 and survivin, a mitotic spindle checkpoint and apoptosis-regulating protein, respectively, were additionally determined by flow cytometry and immunoblotting. The results indicated that indirubin-3'-monoxime induced reversible growth arrest in all four cell lines and an increase of apoptosis in two of them. The treatment with indirubin-3'-monoxime increased the expression of survivin almost four times in the RT4 cells and more than doubled it in the RT112 and T24 cells. In the SUP cells, the expression of survivin increased more than seven-fold after 72-h incubation. No clear correlation between the low apoptosis induction rate and extent of survivin expression was found. Cdk expression was not significantly altered by indirubin-3'-monoxime. In summary, indirubin-3'-monoxime might be a promising candidate for targeted cancer therapy, however, its molecular action remains to be further evaluated.

Transitional cell carcinoma (TCC) is moderately sensitive to chemotherapy and, although a number of new agents have been developed in recent years, the prognosis for any patient with progressive or recurrent advanced bladder cancer remains generally poor (1). Chemotherapeutic treatment yields an overall median duration of response of approximately 8 months and a median survival of 1 year (2, 3). Therefore, the search for active agents for treatment of this disease continues. Recently, Danggui Longhui Wan, a mixture of eleven Chinese herbal medicines traditionally used against leukemia, was found to display anticancer activity. Qing Dai (Indigo naturalis), one of the ingredients, was found to be the active constituent (4-8). The so-called indirubins were discovered to be potent inhibitors of cdks, a family of key cell cycle regulators. Preclinical studies performed with indirubin and derivatives confirmed that these compounds exhibited good antitumor activity with low toxicity (9). Clinical trials showed that indirubin was active against chronic myelocytic leukemia (7, 10-15). One of the cell-permeable indirubin derivatives, indirubin-3'-monoxime, was recently shown to inhibit the proliferation of tumor cells in a concentration-dependent manner. This arrest was associated with an increase in the apoptosis rate of tumor cells. Furthermore, it was shown that indirubin-3'-monoxime led to a G2/M arrest in almost all cell types studied (9). Despite its primary therapeutic use in hematological malignancies, there are data that support further experimental evaluation in solid tumors (16, 17). In this study, it was investigated whether indirubin-3'-monoxime could induce apoptosis and tumor cell death in bladder cancer cells. Indirubin derivatives inhibit Stat3 signaling, thereby resulting in down-regulation of the anti-apoptotic proteins Mcl-1 and survivin (18). With the

Abbreviations: Ab, antibody; cdk, cyclin-dependent kinase; IAP, inhibitors of apoptosis proteins; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PI, propidium iodide; SDS, sodium dodecyl sulphate; TCC, transitional cell carcinoma.

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intent to explore possible reasons for apoptosis resistance, cdk-1 and survivin expression were analyzed under indirubin-3'-monoxime treatment.

Materials and Methods

Tumor cell lines and culture conditions. The human transitional cell carcinoma cell lines RT4 (grade 1), RT112 (grade 1), T24 (grade 3) and TCCSUP (grade 4) were obtained from ATCC (Rockville, MD, USA). All the tumor cell lines were maintained as adherent cells at 37°C in a 5% CO₂ humidified atmosphere in RPMI 1640 culture medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% non essential amino acids, 2 mM L-glutamine, 100 units/ml penicillin G, 100 ìg/ml streptomycin medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% non essential amino acids, 2 mM L-glutamine, 100 units/ml penicillin G, 100 ìg/ml streptomycin and a vitamin solution (Gibco BRL, Life Technologies Inc., Frederick, MD, USA). For experiments, 1x10⁶ tumor cells were seeded in 25-cm² culture flasks (Costar, Cambridge, USA) and incubated for 48 h to allow maximum confluence, thus providing optimal cell-cell contact. The cells were routinely subcultured by trypsinization [0.05% trypsin, 1 mM EDTA] (Gibco BRL).

Reagents. The cell-permeable indirubin-3'-monoxime (Alexis, San Diego, CA, USA) is a chemical derivative of indirubin. Stock solutions of indirubin-3'-monoxime were dissolved in dimethyl sulfoxide (DMSO) and RPMI culture medium (without phenol red) and stored at −80°C. The solubility of indirubin-3'-monoxime is limited to a 15 ìM solution.

Cytotoxicity assay. The EZ4U cell proliferation assay (Biomedica, Vienna, Austria) was used to assess cell viability on the principle of cellular reductive conversion of a tetrazolium salt into a red formazan dye which can be detected by a spectrophotometer. In brief, trypsinized tumor cells were resuspended in medium at 5x10⁵ cells/ml. One hundred microlitres of the cell suspension were distributed into each well of a 96-well flat-bottomed microtiter plate and each plate was incubated for 24 h to allow adherent cell growth. Following incubation, the culture medium was removed and 5, 10 or 15 ìM of indirubin-3'-monoxime was reconstituted in complete medium and incubated for 24, 48 or 72 h. The controls were assessed in parallel for each experiment. At the different measuring times, the EZ4U substrate was added and incubated for an additional 4 h. The absorbance was then read at 450 nm with 620 nm as reference using an automatic multiwell spectrophotometer (340 ATTC SLT, Crailsheim). Additionally, the absorbance from a blank of substrate without cells was subtracted from all sample results. The growth inhibition percentage was calculated using the background-corrected absorbance as follows: % Growth inhibition = [(1-A of experimental well)/A of positive control well] ×100.

Each experimental data point represents average values obtained from six replicates; each experiment was performed in duplicate.

Assessment of apoptosis by Annexin-V and propidium iodide. To evaluate the extent and time-course of apoptosis after incubating cells with indirubin-3'-monoxime, Annexin-V and propidium iodide (PI) (Apoptosis Detection Kit (Annexin V-FITC), Alexis) were used in a double stain technique. The cells were counterstained with PI as a vital dye to distinguish between apoptotic (Annexin V-positive, PI-negative) and necrotic (Annexin V-positive, PI-positive) cells, respectively. Following the procedure described above, controls and 3x10⁵ treated cells (all attached cells and detached cells in supernatant) were re-suspended in 200 ìl of culture medium without phenol red, incubated with 5 ìl FITC-conjugated Annexin-V and 10 ìl PI for 20 min at room temperature in the dark and were then immediately analyzed by flow cytometry (FACScan, BD Immunocytometry Systems, Mountain View, CA, USA). A minimum of 10,000 events was acquired for each sample.

Flow cytometric analysis of survivin. The expression of survivin in tumor cells was determined by flow cytometry in permeabilized cells. Briefly, the cells were harvested by rapid trypsinization to achieve the best detachment of cells from the culture flask without damage to membrane integrity (after removal of the culture medium, trypsin was applied for 20 sec only, was removed again and the cells were incubated for another 5 min without trypsin or medium before washing of the cells). To detect intracellular survivin, the cells were washed once with staining buffer (PBS without Ca²⁺ or Mg²⁺, 0.1% calf serum, 0.09% NaN₃, pH 7.4), fixed by suspending in Cytofix/Cytoperm solution (BD PharMingen) at 3x10⁶ cells/sample and kept at 4°C for 20 min. After centrifugation, the cells were permeabilized and washed with Perm/Wash solution (PharMingen) and were incubated in 100 ìl Perm/Wash solution with pre-immune rabbit serum, 5 ìl of FITC-labeled anti-survivin (R&D Systems, AF886), 5 ìl of FITC conjugated irrelevant anti-rabbit IgG1 mAb and anti-rabbit IgG cells for 30 min at 4°C. After washing in Perm/Wash solution twice again, the cells were resuspended in 500 ìl staining buffer and were kept at 4°C in the dark until analyzed in a FACScan flow cytometer. A minimum of 10,000 events were acquired for each sample.

Western blot analyses for poly(ADP-ribose)polymerase (PARP) and survivin. The total cellular contents of PARP, survivin and cdk-1 were analyzed by Western blotting to determine the expression of these proteins after treatment with indirubin-3'-monoxime. The cells were harvested by trypsin, washed with ice-cold PBS (pH 7.4, Biochrom) and lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM magnesium chloride, 5 mM EDTA and 1% Triton-X-100) supplemented with 1 mM PMSF (protease inhibitor phenylmethylsulphonyl fluoride) (Bohringer), 200 ìg aprotinin (Sigma) and 10 ìM leupeptin (Bohringer) for 20 min at 4°C. The lysate was centrifuged (14,000 rpm) for 10 min at 4°C and its protein content was determined using a protein assay kit. Samples containing 100 ìg of protein in loading buffer (0.312 M Tris-HCl, pH 6.8, 0.5% bromophenol blue, 10% sodium dodecyl sulphate (SDS), 25% glycerol, 100 mM dithioerythriol) were boiled for 10 min, then subjected to SDS-PAGE (12.5%) and transferred to a nitrocellulose transfer membrane (Millipore). Equal loading of each lane was confirmed by ponceau S staining (Sigma) and the membranes were blocked with 5% non-fat dry milk in TBS (50 mM Tris, pH 7.5, 150 mM sodium chloride) and incubated overnight at 4°C with mouse anti-survivin pAb ([1:150], R&D Systems, Germany), mouse anti-PARP mAb [1:200], mouse anti-cdk-1 [1:100] (all Abs from Santa Cruz Biotech, CA, USA), diluted in 2% TBS Tween 20. Unconjugated IgG1 mAb (Dianova, Germany), anti-mouse IgG (Cell Signaling) and IgG1 mAb (Dako, Denmark) were used as controls. Immunological complexes were visualized by enhanced chemiluminescence (Pierce, Europe BV) using horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Dako).
The statistical analysis of the EZ4U assay was determined by two-tailed unpaired \( t \)-test (\( p < 0.05 \) was considered significant) and the Jonckheere-Terpstra-Test (\( p < 0.01 \) was considered significant) using the statistical analysis program SPSS. The statistical evaluation of the FACS data was done using the FACScan software, version "CellQuest" (Becton Dickinson). All experiments were repeated as indicated and the results were expressed as the mean±SD.

Results

Cytotoxicity and apoptosis induction of indirubin-3'-monoxime. Indirubin-3'-monoxime, in a dose of more than 5 \( \mu \)M, significantly inhibited the proliferation of all TCC cell lines after 24 h (Figure 1). There were differences between the cell lines regarding their responsiveness to the drug, however, no relationship with the histological grading of the cells was revealed. The RT4 and T24 cells demonstrated no significant apoptosis during treatment with indirubin-3'-monoxime. In the RT112 cells, exposure to 10 \( \mu \)M indirubin-3'-monoxime resulted in 16% apoptosis after 24 h, 30% after 48 h and 37% after 72 h. Indirubin-3'-monoxime-treated SUP cells demonstrated an apoptosis of 20% after 48 and 72 h. At 24 h, no difference to the normal turnover rate of cells was observed. As in the cytotoxicity experiments, no consistent results were found regarding the apoptosis-inducing properties of indirubin-3'-monoxime and cell differentiation.

To confirm these rather unexpected results, another apoptosis assay was performed. PARP is a 116-kDa nuclear protein which is strongly activated by DNA strand breaks and plays a role in DNA repair as well as in other cellular processes, e.g., DNA replication, cell proliferation and differentiation. During apoptosis, ICE family members such as caspase-3 cleave PARP to yield 85-kDa and 25-kDa fragments. However, indirubin-3'-monoxime treatment demonstrated only minor PARP cleavage in all the examined cell lines (data not shown).
Figure 2. Mean fluorescence of survivin expression after 72-h exposure to 10 μM indirubin-3’-monoxime as evaluated by intracellular flow cytometry (specific survivin expression [red-colored peak], unspecific IgG antibody binding [green-colored peak], autofluorescence of cells [black-colored peak]).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean IgG fluorescence</th>
<th>Mean survivin fluorescence</th>
<th>Increase of expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT4</td>
<td>24.96</td>
<td>93.63</td>
<td>375%</td>
</tr>
<tr>
<td>RT112</td>
<td>28.88</td>
<td>59.83</td>
<td>210%</td>
</tr>
<tr>
<td>T24</td>
<td>43.69</td>
<td>91.16</td>
<td>209%</td>
</tr>
<tr>
<td>SUP</td>
<td>11.71</td>
<td>81.29</td>
<td>694%</td>
</tr>
</tbody>
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Expression and regulation of survivin. Normal cells either do not or only minimally express survivin (19). In our experiments, all cell lines expressed this protein, and an increase of protein expression during treatment with indirubin-3'-monoxime was shown in all TCC lines (Figure 2). The most impressive increase of more than seven-fold survivin expression was found in the SUP cells in and expression of survivin in RT4 was almost four times higher after treatment with indirubin-3'-monoxime. In the RT112 and T24 cells, this effect was not as distinct, but the expression of survivin more than doubled. In the immunoblotting experiments, this increase of survivin was verified by a visibly enhanced protein quantity (data not shown).

Expression and regulation of cdk-1. With the intent of exploring possible reasons for apoptosis, resistance against apoptosis or alterations in the cell cycle, cdk-1 expression was evaluated by immunoblotting. A clear cdk-1 expression (35 kDa protein) was found in all TCC cells. In comparison to the controls, the protein quantity of cdk-1 was not significantly altered after 72-h treatment with indirubin-3'-monoxime (Figure 3).

Discussion

The aim of this study was to evaluate indirubin-3'-monoxime, a cdk-inhibitor with origins in Chinese phytomedicine, as a potential candidate for bladder cancer therapy. The discovery of indirubin goes back to a traditional Chinese medicine preparation, Danggui Longhui Wan, comprising eleven herbal constituents. This preparation was, and still is, used traditionally for the treatment of various chronic diseases (16). Investigations revealed that the antileukemic agent was a minor byproduct of Indigo naturalis, the red-colored 3,2'-isomer indirubin (4-8). It was found that indirubin-compounds induce arrest in G2/M as well as in G1/S, in most cases followed by apoptosis (17). Furthermore, it was shown that indirubin has antimitotic properties with time-dependent alterations in the protein levels of cdk1, cyclin B and the cdk1/cyclin B complex (20). In addition to alterations in the cellular levels of G2/M cell cycle regulators, another possible mechanism of interference with the cell cycle machinery in tumor cells is the direct inhibition of the kinase activity of cdk/cyclin complexes. Accordingly, in G2/M-arrested MCF-7 breast cancer cells, a significant and dose-dependent inhibition of cdk-1 kinase activity was observed (17). Indirubins bind to the ATP binding site of cdks in an ATP-competitive manner (16). Indirubin derivatives directly block the Src-Stat3 signaling pathway in human breast and prostate cancer cells. The anti-apoptotic proteins Mcl-1 and survivin, which are encoded in the target genes of Stat3, were down-regulated by indirubin derivatives followed by induction of apoptosis (18). Indirubin has growth inhibitory activity in a wide spectrum of human tumor cells, such as the lung cancer cell lines LXFL529L and A549, the breast cancer cell line MCF-7, lung fibroblast cells CCL-39, the prostate cancer cell line PC-12, murine leukemic cells L1210, the human leukemic cell line K-562 and human myeloid leukemia HL-60 (9, 17, 21). Proliferation of most cell lines was inhibited by indirubin in a concentration-dependent manner. Only the human adenocarcinoma cell line HT-29-18-C1 remained insensitive (9). Pharmacokinetic and pharmacodynamic studies on humans are lacking. The clinical trials were performed in China more than 20 years ago. Despite the fact that detailed clinical trial design, end-points and biostatistics are not available, the Chinese trials reported on 314 patients suffering from chronic myelocytic and chronic granulocytic leukemia. Indirubin was given orally at a daily dosage of 150 - 450 mg. Complete remissions were observed in 82 cases (26%), partial remission in 105 cases (33%) and "beneficial" effects were found in 87 patients (28%). The treatment was seemingly well tolerated and did not induce major side-effects (7, 10-15).

Indirubin-3'-monoxime, a chemical derivative of indirubin, significantly inhibited the proliferation of all TCC cell lines at a dose of more than 5 µM, after 24 h. The TCC
cells treated with indirubin-3’-monoxime, however, either did not undergo apoptosis (RT4, T24), or did so only to a minor extent (RT112, SUP). These unexpected results were found by assessing apoptosis through Annexin-V/PI staining and were confirmed by examination of PARP cleavage. To further explore the possible underlying mechanisms, the expression of survivin in the TCC cells was examined in untreated as well as in indirubin-3’-monoxime-treated cells. Survivin is a member of the inhibitor of apoptosis protein (IAP) family, that has been implicated in both the control of cell division and inhibition of apoptosis. Specifically, its anti-apoptotic function seems to be related to the ability to directly or indirectly inhibit caspases. Survivin is selectively expressed in the most common human neoplasms and appears to be involved in tumor cell resistance to anticancer agents. Survivin is regulated in a highly cell cycle-dependent manner with a marked increase in the G2/M-phase (22). Upon examination of the survivin expression of the TCC cell lines, it was found that all cell lines expressed survivin, which might, at least partly, explain the anti-apoptotic resistance to indirubin-3’-monoxime. The RT4 and SUP cells demonstrated a strong, whereas T24 and RT112 a weaker protein expression of survivin. In a study by Nam et al. (18), Mcl-1 and survivin expressions were dramatically reduced in response to disruption of the Stat3 signaling and apoptosis was induced in breast cancer cells by several indirubin derivatives.

We expected that indirubin-3’-monoxime would induce apoptosis in the TCC cell lines, as it did in other cell lines of solid tumors, due to the reduction of Stat3 activity resulting in the down-regulation of the anti-apoptotic proteins such as Bcl-xL, Mcl-1 and survivin. However, this did not occur. Furthermore, during treatment with indirubin-3’-monoxime, the expression of survivin increased almost four times in the RT4 cells and more than doubled in the RT112 and T24 cells in our study. In the SUP cells, the expression of survivin even increased more than seven-fold after 72 h of incubation. No clear correlation between the low apoptosis induction rate and the extent of expression of survivin was found. Cdk expression was not significantly altered by indirubin-3’-monoxime. At present, the underlying reasons for these observations remain open and require intense research. From the methodological perspective, Nam et al. (18) performed immunoblot analysis of lysates from cells treated with 1 to 10 μM of the indirubin-derivate E804 for 24 h; in our experiments, intracellular flow cytometry was used. Unfortunately, negative or positive control experiments are not available. The mere fact of the increased expression of survivin in the TCC cells does not explain apoptosis resistance, as demonstrated in other experiments with these cell lines (23-25).

While much of the research on indirubin derivatives is in the preclinical phase, some encouraging results suggest that future use of these drugs might help to improve the outcome for patients with bladder cancer.

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References


