Induction of cell death in pancreatic ductal adenocarcinoma by indirubin 3’-oxime and 5-methoxyindirubin 3’-oxime in vitro and in vivo

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A B S T R A C T

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with a poor prognosis. To identify potential effective therapeutic drugs for PDAC, we established a screening system based on spheroid formation using 170#3 mouse PDAC cells with or without fibroblasts. We found that indirubin 3’-oxime (Indox) and 5-methoxyindirubin 3’-oxime (5MeOIndox) inhibited PDAC cell proliferation. Furthermore, PDAC xenograft growth was also inhibited in BALB/c nu/nu mice after administration of Indox and 5MeOIndox. Both phosphorylated CDK1 and cyclin B1 levels in 170#3 cells were significantly reduced by treatment with Indox and 5MeOIndox in vitro and in vivo. Cell cycle analysis revealed that 5MeOIndox, but not Indox, induced G2/M arrest. Annexin V–propidium iodide double-staining analysis demonstrated that Indox induced abundant non-apoptotic cell death of 170#3 cells, while 5MeOIndox predominantly induced early apoptosis, indicating that the cytotoxicity of 5MeOIndox is lower than that of Indox. These results suggest that one mechanism of 5MeOIndox is to induce G2/M arrest of PDAC cells via inhibition of CDK1/cyclin B1 levels, thereby leading to apoptosis. Our findings suggest 5MeOIndox as a potential useful anticancer agent in PDAC.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis and high mortality rate [33,34]. PDAC is often diagnosed at an advanced stage and inoperable patients require chemotherapy including gemcitabine (GEM) and S-1 [37]. The overall survival of patients with PDAC has been prolonged using recently developed combination chemotherapy [37], but PDAC is often resistant to chemotherapy. One conceivable reason for this chemoresistance is the reduced permeability of anticancer drugs caused by the presence of abundant desmoplastic stroma [22]. The close interactions between PDAC cells and stromal fibroblasts enhance the potential for cell proliferation, as well as invasion and metastasis [3]. In vitro analysis has indicated that some factors released from fibroblastic spheroids, which represent a more comparable condition to the body than 2D culture [26], promote tumor proliferation [38]. Therefore, the PDAC-fibroblast interactions should be taken into account in developing an alternative therapeutic approach for PDAC.

Indirubin is an active ingredient of Danggui Longhui Wan, a traditional Chinese herbal medicine used for the treatment of chronic myelogenous leukemia [40]. Indirubin and its derivatives competitively block the ATP-binding pocket in the catalytic domain of cell cycle-related kinases, such as cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3β [6,17,19,24]. Indirubin...
derivatives induce G2/M arrest in various cancer cells by inhibition of these enzymes [8,15,16], thereby inhibiting cell proliferation and partially inducing apoptosis [8,16]. The antitumor effects of indirubin derivatives in PDAC, however, are still poorly understood.

In this study, we established a three dimensional (3D) spheroid screening system using PDAC cells and fibroblasts to evaluate the anticancer activity of potential drug candidates.

Materials and methods

Anticancer drugs

Indirubin derivatives (Supplementary Fig. 1) were prepared using previously decreased methods [28].

Cell lines

The mouse PDAC cell line 170#3 was established from pancreatic cancer in a Ptf1a-cre, LSL-KrasG12D, Trp53 flox and Ink4a/Arf flox transgenic mouse [18]. Human PDAC cell lines MIA PaCa-2 and PAC-1 as well as mouse fibroblast NIH/3T3 cells were described previously [19].

Spheroid formation by three dimensional (3D) culture

Cells were maintained at 37 °C in an atmosphere consisting of 5% CO2 in D-MEM (Wako Pure Chemical Industries, Ltd. Osaka, Japan) containing 10% fetal bovine serum (Equitech-Bio Inc., Kerrville, TX, USA) and 1% penicillin/streptomycin. After treatment with 0.25% trypsin–EDTA, cells (4 × 10⁶/well) were gently mixed with indirubin derivatives and seeded onto a 3D culture plate that was coated with ultra-low attachment (Primersurface® 96U plate, Sumitomo Bakelite Co. Ltd, Tokyo, Japan). The cells were incubated for 3 days at 37 °C and 5% CO2, and then observed using phase-contrast microscopy. Four diameters per one spheroid were measured using ImageJ software, and the volume of the spheroids was calculated by the following formula: volume = 4/3πr³.

AlamarBlue® assay

At 72 h, 10 μL of AlamarBlue® Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA) was added to 100 μL of spheroid culture medium. The solution mixture was incubated for 24 h. Cell viability was measured in terms of absorbance at a wavelength of 550 nm and 595 nm using a microplate reader (TECAN Japan, Co. Ltd., Kanagawa, Japan).

Immunoblotting

Immunoblot analysis was performed as previously described [31]. Antibodies to CDK1 (phospho T161) (1:1000; ab194874: Abcam, Cambridge, UK), cyclin B1 (1:250; sc-752: Santa Cruz Biotechnology, Inc.), p-CDK1 (1:100), cyclin B1 (1:250), cleaved caspase-3 (CC3; 1:100; #9661: Cell Signaling Technology Japan, Tokyo, Japan), and CD31 (1:50; Abcam). The positive cells in at least four representative fields in a representative specimen were counted using Image J software.

Cell cycle analysis

Cell cycle analysis of 170#3 cells using a FC500 flow cytometer (Beckman Coulter, Brea, CA, U.S.A.) in the FL3 range was performed as described previously [14].

Annexin V-propidium iodide (PI) double-staining analysis

Apoptotic cells were detected with the Alexa Fluor® 488 Annexin V/Dead Cell apoptosis kit (Life Technologies Invitrogen, Carlsbad, CA, U.S.A.), as described previously [14]. Apoptosis was induced by 100 μM cisplatin as a positive control.

Statistical analyses

Statcel software version 4 (OMS Ltd., Saitama, Japan) was used for the statistical calculations [29]. Mann–Whitney’s U test was used to determine the significant difference in histopathological analysis. The significant difference of flow cytometric data was determined by one-way ANOVA followed by Bonferroni’s test.

Results

In vitro screening of indirubin derivatives

To evaluate the antitumor effect of indirubin derivatives, we established a spheroid screening system for PDAC cells and NIH/3T3 fibroblasts on a 3D culture plate. We used the more aggressive mouse PDAC cell line (170#3), which has a specific genetic background (KrasG12D, Trp53 flox and Ink4a/Arf flox) that makes it a suitable experimental cell system for considering future therapeutic approaches for inoperable patients with PDAC. We examined a panel of 18 indirubin derivatives (Supplementary Fig. 1) using the screening system as described in Methods, and the results revealed Indox and the methoxy derivatives at 5-, 6- and 7-positions (5MeOIndox, 6MeOIndox and 7MeOIndox, respectively) as candidate agents.

We next more closely examined the effects of the candidate agents on cell proliferation in dose-dependent experiments. Among the methoxy derivatives, Indox and 5MeOIndox exhibited an antiproliferative effect in 170#3 cells, but had no effect on NIH/3T3 fibroblasts (Supplementary Fig. 2). We found that 5MeOIndox dose-dependently inhibited the size of the co-cultured spheroids of 170#3 cells with NIH/3T3 cells, as well as 170#3 cells cultured alone (Fig. 1A and B). Importantly, no side effects as detected by the absence of cell damage was observed with low-dose treatment of either Indox or 5MeOIndox in NIH/3T3 fibroblasts. We also examined the antiproliferative effects of Indox and 5MeOIndox using the AlamarBlue® Assay, an oxidation-reduction indicator based on the detection of cell metabolic viability (Fig. 1C). Consistent with our previous observations, the AlamarBlue® Assay results demonstrated that Indox and 5MeOIndox reduced cell viability in both spheroids of 170#3 cells cultured alone and the co-cultured cluster of 170#3 cells with NIH/3T3 fibroblasts. In addition, Indox and 5MeOIndox appeared to inhibit the initial migration and coagulation of 170#3 cells and fibroblasts in round wells (Supplementary Fig. 3).

We also analyzed the effects of these agents in other human PDAC cell lines. The results revealed that Indox did not affect proliferation of PANC-1 and MIA PaCa-2 cells (Supplementary Fig. 4A and C), whereas high-dose 5MeOIndox suppressed spheroid proliferation of both cell lines (Supplementary Fig. 4B and D). Together these results suggest that while Indox showed no effect on human PDAC cells, 5MeOIndox was able to inhibit spheroid proliferation of both mouse and human PDAC cells as well as cocultured PDAC cells with fibroblasts.

Indox and 5MeOIndox inhibit proliferation of PDAC xenografts

We next investigated the antitumor effects of Indox and 5MeOIndox in a xenograft mouse model in vivo. After subcutaneous
transplantation of 170#3 cells into BALB/c nu/nu mice, the indirubin derivatives or vehicle (DMSO/PEG400) were injected intraperitoneally as described in Methods. Tumor growth from the transplanted 170#3 cells was inhibited by the administration of Indox and 5MeOIndox (Fig. 2A and B). We also observed a reduced tumor weight in mice that received injections of Indox and 5MeOIndox for 3 days. Bars, 500 μm. (B) The diameter of the spheroids (n = 3) was measured using ImageJ software and the volume was calculated using the formula 4/3πr³. (C) The cell viabilities were analyzed using the AlamarBlue® assay.

indicating that Indox and 5MeOIndox had no significant side effects (Fig. 2D).

Histopathologically, all xenografts were composed of sarcomatoid-like spindle PDAC cells rather than an epithelial phenotype (Fig. 3A). Given that studies have shown that indirubin derivatives induce G2/M arrest in various cancer cells, we examined mitotic cells. The number of mitotic cells was decreased in the xenografts from mice treated with Indox and 5MeOIndox (Fig. 3A).
and B). In addition, phosphorylation of ToPoIIa (Thr1343), a mitosis-specific event, was also reduced by the administration of Indox and 5MeOIndox. These data suggested that Indox and 5MeOIndox inhibit the cell cycle in PDAC cells.

5MeOIndox inhibits mitosis of PDAC by blocking of CDK1/cyclin B1 activities

Indirubin derivatives are potent inhibitors of CDK1/cyclin B1 activities and lead to G2/M arrest [8,16]. Thus, we next investigated the effect of indirubin derivatives on the cell cycle, specifically CDK1/cyclin B1 activities. A molecular docking simulation study revealed that indirubin derivatives competitively inhibit the ATP-binding pocket of CDK enzymes (Fig. 4A). Immunoblotting analysis showed that p-CDK1 level in 170#3 cells was reduced in cells treated with either Indox or 5MeOIndox (Fig. 4B). In addition, the expression level of cyclin B1 was downregulated. Similarly, in the xenografts treated with Indox or 5MeOIndox, we observed a decrease in the nuclear p-CDK1 expression levels and cytoplasmic cyclin B1 expression levels compared with controls (Fig. 4C and D). Cell cycle analysis using flow cytometry demonstrated that 5MeOIndox, but not Indox, induced significant G2/M arrest and increased the sub-G1 pre-apoptotic cell population (Fig. 5A and B). A similar effect on the cell cycle was also detected in human PDAC cells PANC-1 (Supplementary Fig. 5). In MIA PaCa-2 cells, treatment with 5MeOIndox induced a significant decrease of cells in

Fig. 2. Effect of Indox and 5MeOIndox on PDAC xenografts. (A and B) Mice received indirubin derivatives (0, 10, 20 and 40 mg/kg) in a mixture of DMSO and PEG400 every other day (a total of four times). Tumor volumes of PDAC xenografts were measured every day. (C) Weights of PDAC xenograft tumors (n = 6) at the final experimental endpoint are shown as mean ± SD. (D) Body weight of the mice was measured during the experimental period. *p < 0.05 vs. vehicle control.
G2/M phase, with an increase in sub-G1 phase cells (Supplementary Fig. 6). These results suggest that 5MeOIndox induces G2/M arrest of PDAC cell lines via inhibition of CDK1/cyclin B1 levels.

Indox and 5MeOIndox induce apoptosis of PDAC cells

We next examined the cell death in PDAC xenografts by the indirubin derivatives in more detail. Morphological cell death areas were observed in the tissues of xenografts in control mice, as well as mice treated with low-dose Indox and 5MeOIndox (Fig. 6A). Notably, we observed a decrease in cell death areas after treatment with 20–40 mg/kg Indox and 5MeOIndox according to decreasing tumor mass (Fig. 6B), with an increase in CC3 staining in a dose-dependent fashion. Annexin V-PI double-staining analysis showed that Indox induced late apoptosis and abundant non-apoptotic cell death of 170#3 PDAC cells in vitro (Fig. 7), while 5MeOIndox induced early apoptosis. These findings suggest that Indox and 5MeOIndox induce apoptosis of PDAC cells by different mechanisms.

Discussion

Patients with advanced and inoperable PDAC require chemotherapy using anticancer drugs such as GEM and S-1 [37]. However, these anticancer drugs are associated with side effects. Thus, the development of alternative anticancer drugs without side effects is urgently required. Furthermore, the PDAC-fibroblast interactions should be considered when developing new anticancer compounds because these interactions are associated with the enhancement of PDAC malignant potential as well as chemoresistance [3,22,38]. In the present study, we found that Indox and 5MeOIndox were effective in suppressing PDAC progression even in the presence of fibroblasts.

Indirubin derivatives and the related compounds have been reported to work as successful anticancer agents in various cancers including lung [2,41], breast [4], prostate [20], liver [9,13,35] and brain [14,39]. Although a few attempts have been made to treat pancreatic cancer using these compounds [5,21,27], the current study is the first to evaluate the antiproliferative effect of Indox and 5MeOIndox on PDAC cells. We previously generated a library of
indirubin derivatives and reported their biological activities, including anticancer effects [9,13,14,35]. Among the 18 indirubin derivatives, Indox and the three methoxy derivatives specifically inhibited spheroid formation in mouse PDAC 170#3 cells, but not in NIH/3T3 fibroblasts. Furthermore, 5MeOIndox exhibited greater antiproliferative activity in 170#3 cells compared with 6MeOIndox and 7MeOIndox. We observed similar findings in our previous report that showed that 5MeOIndox shows potent growth inhibitory effects in neuroblastoma cells such as SK-N-SH and NB-39 compared with 6MeOIndox and 7MeOIndox [28]. Although the precise mechanisms underlying the differential effects of indirubin derivatives remain unknown, differential affinities of the compounds for CDKs or other cell cycle-related proteins may be partly responsible.

Indirubin derivatives have the potential to inhibit CDKs by competitively binding to the ATP-binding site [6,19,24] and thereby

Fig. 4. Inhibition of p-CDK1 and cyclin B1 levels in PDAC xenografts by Indox and 5MeOIndox. (A) Binding feature of indirubin at the ATP-binding site of CDK. (B) Immunoblotting of p-CDK1 and cyclin B1 levels in PDAC 170#3 cells in vitro in response to Indox and 5MeOIndox treatments as indicated. (C) Immunostaining for p-CDK1 and cyclin B1 expression in PDAC xenografts treated with Indox and 5MeOIndox as indicated. (D) Quantification of staining presented in (C). Data are presented as mean ± SD from the four fields at 400× original magnification. *p < 0.05; **p < 0.01 vs. vehicle control.
induce G2/M arrest of cancer cells [8,15,16]. In our study, we observed a decrease in phosphorylated CDK1 and cyclin B1 levels in response to Indox and 5MeOIndox treatment in vitro. Nuclear p-CDK expression and cytoplasmic cyclin B1 expression were also decreased in the xenograft models treated with Indox and 5MeOIndox. In addition, the numbers of mitotic and M phase-specific p-ToPoIIα (Thr1343)-positive cells were reduced in the 170#3 xenografts treated with Indox and 5MeOIndox. Although a significant G2/M arrest was not induced by Indox in 170#3 cells in vitro, 5MeOIndox clearly induced G2/M arrest in both 170#3 and

Fig. 5. Cell cycle analysis of 170#3 cells treated with Indox and 5MeOIndox by flow cytometry. (A) Flow cytometry analysis results of 170#3 cells treated with 3 or 10 μM Indox or 5MeOIndox. Each column displays the percentages of the gated cell populations relative to the total cell population in each treatment condition. (B) Comparison of sub-G1, G0/G1, S, and G2/M phase populations in each treatment condition from (A). *p < 0.05; **p < 0.01 vs. vehicle control.
PANC-1 cells. Therefore, one likely mechanism of tumor growth inhibition by 5MeOIndox may be due to G2/M arrest via inhibition of p-CDK1/cyclin B1 activities, leading to early apoptosis.

Indirubin derivatives induce not only cell cycle arrest but also apoptosis in various cancer cells [20,23,25,32]. Our pathological analysis revealed cell death in the tissues of xenografts in the control mice as well as in the mice treated with low-dose Indox and 5MeOIndox. The ratio of the CC3-positive area relative to the cell death area was <20% in the low-dose experiments compared with approximately 10% in the control experiment. The CC3-positive cells were commonly located at the edge of the cell death areas, indicating that the main cell death areas might be ischemic central necrosis caused by massive tumor growth. In contrast, in xenografts in the high-dose experiment, CC3-positive apoptotic areas in the transplanted PDAC clusters were increased by the administration of Indox and 5MeOIndox and were located in both the edge of the cell death lesions and perivascular areas, indicating that these compounds induce apoptosis in vivo. Similar to our results, previous studies reported that Indox induces direct apoptosis in various cancer cell lines such as cervical (HeLa), colorectal (HCT116), hepatocellular (HepG2) and renal cell carcinomas (A498, Caki-1, Caki-2 and RENCA) [23,32], as well as chemically-induced lung cancer in a murine model [25]. 5MeOIndox induces apoptosis in human breast and prostate cancer cells [20]. Indox also induces polyploid and the sequential aneuploidy in human breast epithelial HBL-100 cells, finally leading to non-apoptotic cell death [7]. In mouse PDAC 170#3 cells, massive non-apoptotic cell death was also induced by Indox in vitro. In contrast, 5MeOIndox predominantly induced early apoptosis, suggesting that 5MeOIndox rather than Indox would be an effective compound to PDAC cells.

Recent studies reported that indirubin derivatives and the related compounds could inhibit tumor angiogenesis [11,39,42]. In the present study, areas of CD31-positive tumor vessels in the PDAC xenograft model were reduced by treatment with Indox and 5MeOIndox treatment (Supplementary Fig. 7). Inhibition of angiogenesis in tumor tissue by Indox and 5MeOIndox might be one of the mechanisms for growth inhibition and the induction of cell death. Although future investigations will need to elucidate the...
Fig. 7. Detection of apoptosis in 170#3 cells by Annexin V–propidium iodide (PI) double-staining analysis. Early and late apoptotic events of 170#3 cells treated with 3 or 10 μM Indox or 5MeOIndox were detected by flow cytometry. Top left quadrant (B1) indicates non-apoptotic cells; top right quadrant (B2) represents late apoptosis events; bottom left quadrant (B3) represents living cells; and bottom right quadrant (B4) represents early apoptosis cells. Graphs on bottom show quantification from the flow cytometry analysis according to cell population and treatment condition. *p < 0.05; **p < 0.01 vs. vehicle control by one-way analysis of variance.
details of the anticancer mechanism of Indox and 5MeOIndox, our findings suggest that 5MeOIndox may be an effective candidate therapeutic anticancer agent for PADC patients.

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**Conflict of interest statement**

None.

**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2017.03.031.

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