ISSN 2515-8260 Volume 09, Issue 03, 2022

ORIGINAL RESEARCH

Indirubin-3'-Oxime Eliminates Human Cholangiocarcinoma Through Apoptosis and Cell Cycle-Arrest

Parul Singh

Tutor, Department of Pharmacology, Medical College Baroda, Vadodara, Gujarat, India

Correspondence:
Parul Singh
Tutor, Department of Pharmacology, Medical College Baroda, Vadodara, Gujarat, India

ABSTRACT

Background: Cholangiocarcinoma (CCA) is one of the most prevalent diseases in the South Asia. Failing to determine this disease on time tends to be a great challenge in its diagnosis. In several human malignancies, indirubin-3'-oxime (I3O) has been found to decrease cell growth, cause cessation of cell cycle, and cell death.

Objective: The anticancer effects of I3O on human CCA cells will be demonstrated by exhibiting induced cessation of cell cycle and apoptosis in I3O-treated cells.

Methods: The viability, apoptosis of cells and cessation of cell cycle were all measured. Analysis using Western blotting, flow cytometry and immunofluorescence.

Results: According to the findings, in CCA cells, I3O treatment inhibits cell growth and induces cessation of cell cycle as well as caspase-dependent death.

Conclusion: These data imply that I3O, by regulating the cell cycle and generating apoptosis, could inhibit tumour growth and hence be a viable treatment drug for human CCA.

INTRODUCTION

Cholangiocarcinoma (CCA) is among the most common malignancies in the Southern parts of Asia (Amuamuta, et al [1]). As per previous studies, the incidences of CCA have escalated, with more than 7000 novel instances being reported each year (Blažević, et al [2]). These are extremely fatal diseases with early lymphatic system spread and distant metastases. Razumilava and Gores [3] mentions biliary-duct cysts, parasitic infection, hepatitis B or C infection, primary sclerosing cholangitis, hepatolithiasis and alcohol as factors responsible for CCA (Cheng, et al [4]). The failure to recognise CCA early and the disease's susceptibility to most chemotherapeutic drugs and radiation therapy are the key challenges in the treatment (Li, et al [5]). Regardless of the fact that surgery and curative liver transplantation are alternatives for some CCA patients, 5-year survival rates are still quite low (Li, et al [5]). Patients with metastatic or advanced CCA have recently been administered with chemotherapy, using cisplatin and/or gemcitabine.

Indirubin is an element of a plant named Indigo naturalis, which is used in traditional Chinese medicine for treating chronic conditions such chronic myelogenous leukaemia (Lee, et al [6]). Studies have found Indirubin and derivatives in human tumours for inhibiting cell growth, controlling the cell cycle, and causing apoptosis (Sano, et al [7]; Perabo, et al [8]; Lo and Chang [9], Hughes, et al [10]). Indirubin-3-monoxime (I3M) has better pharmaceutical characteristics and is less cytotoxic than indirubin (Choi, et al [11]). In addition, I3O has been illustrated in reducing cell growth, causing cessation of cell cycle, and inducing apoptosis in numerous human malignancies (Choi, et al [11]; Ahn, et al [12]; Santo, et al [13]). Early

reports were made on I3O's antitumor effects and inference in human leukaemia cells (Zhang and Liu [14]), demonstrating that I3O can be considered as a potential and safe anticancer drug. As a result, I3O is amongst the most effective indirubin derivatives for treating human malignancies, whilst its efficacy in anti-human CCA is unknown. In the present study, the anticancer effects of I3O on human CCA cells are demonstrated by exhibiting induced apoptosis and cessation of cell cycle in I3O-treated cells. Furthermore, we show that I3O therapy induces apoptosis through an intrinsic route. In human CCA cells, our findings imply that I3O could be a viable anticancer agent.

METHODOLOGY

CELL LINES AND CULTURES

OCUG-1 and NOZ are human gallbladder cancerous cells lines, whilst OZ and HuCCT1 are human bile duct melanoma cell lines. Dulbecco's modified Eagle's medium (Gibco) with 10% foetal bovine serum (FBS; Gibco) and RPMI1640 media were employed to sustain OCUG-1 and HuCCT1 cells respectively. NOZ and OZ cells were grown in Gibco's William's Medium E supplemented with 10% FBS. The samples were cultured at 37 degrees Celsius in 5% CO2.

CELL VIABILITY ASSAY

For 72, 48 and 24 hours, OCUG-1, NOZ, OZ and HuCCT1 cells had been treated with varying doses of I3O. All of the experiments employed less than 0.1 percent dimethyl sulfoxide (DMSO). The CCK-8 was used for testing the vitality of the cultivated cells. Every group's survival percentage was computed as follows:
(OD450 of treatment/OD450 of controls) x 100%

BRDU STAINING’

HuCCT1, OZ and NOZ cells were treated with, 12.5 and 5 μM I3O, correspondingly. After 24 hours, the cells were given 30 minutes of 40 g/ml BrdU treatment. They were then fixed in acid ethanol for about 10 minutes at -20°C. The cells are stimulated with Brdu PRIMARY ANTIBODY AND THE Alexa Fluor 488-conjugated anti-rabbit IgG (H + L) antibody after being treated with 2 N HCl at RT for 10 minutes. Cells were counterstained with Hochest 33258 before being observed under the microscope. BrdU positive cells were collected in random fields.

CELL CYCLE ANALYSIS

I3O concentrations of 12.5, 5 and 0 μM were being used for treating NOZ and HuCCT1 cells. The cells were extracted and frozen at 4°C with 100 percent methanol after being incubated for roughly 72, 48 and 24 hours. After that, the cells were cultured for 30 minutes with 2 mg/ml propidium iodide and 10 mg/ml RNase in the dark at room temperature. The DNA content was determined using a FACScan Flow Cytometer with ModFit LT 3.3 software. CDC25C, CDC2, cyclin A2, cyclin B1, and p21 antibodies were also used to establish the cell-cycle protein markers by utilising Western blotting.

ANALYSIS OF APOPTOSIS CELL DEATH

NOZ and HuCCT1 cells were given DMSO or I3O for 48 and 72 hours, respectively. The apoptotic cells were detected with the help of an Annexin V-FITC Apoptosis Detection Kit after incubation, as directed by the manufacturer. The FACScan Flow Cytometer was used to detect and assess the intensity of annexin V or PI fluorescent signals. The activation of caspase-9, caspase-8, caspase-3, poly (ADP-ribose) polymerase (PARP), Bax, caspase-12, and Bcl-xl was discovered by Western blotting to demonstrate the mechanism behind I3O-mediated apoptosis.

The apoptotic mechanism through the caspase-dependent pathway was confirmed using Z-VAD-FMK, a pan-caspase inhibitor. In addition, anti-Tom20 and anti-cytochrome c antibodies had been used for detecting cytochrome c and mitochondria, which were later evaluated using an Olympus FV1000 confocal laser scanning microscope. Rhodamine 123 dye was used to identify the membrane potential of mitochondria. NOZ cells were treated for forty eight hours with DMSO or I3O. For 30 minutes, Rhodamine 123 (5 M) was applied to the cells. The cells were extracted after incubation, and the intensity of Rhodamine 123 fluorescent signals was assessed with the help of a FACScan Flow Cytometer.

STATISTICAL ANALYSIS

The half maximum cytotoxic concentration was evaluated with the help of GraphPad Prism for Windows (CC50). The data for the current study was reported as mean SD, and the software for generating p-values was utilised in conjunction with a one-way analysis of variance analysis of the data. This was accomplished by employing Dunnett's comparative method. P-values of less than 0.5 were deemed statistically significant.

RESULTS

I3O INHIBITED GROWTH IN HUMAN CCA CELLS

Cell viability was measured using Cell Counting Kit 8 after human CCA cells were cultured in a media containing either dimethyl sulfoxide or I3O. After treatment for 24, 48, and 72 hours, the NOZ cells had one-half maximal inhibitory concentrations of I3O of 12.7, 9.1, and 4.4 μM, respectively. The maximal inhibitory concentrations of I3O for the OZ cells were .3, 3.2, and 4.7 μM, those for the OZ cells were 4.3, 3.2, and 4.7 μM (Figure 1C); and those for the OCUG-1 cells were >20 μM (Fig 1D).

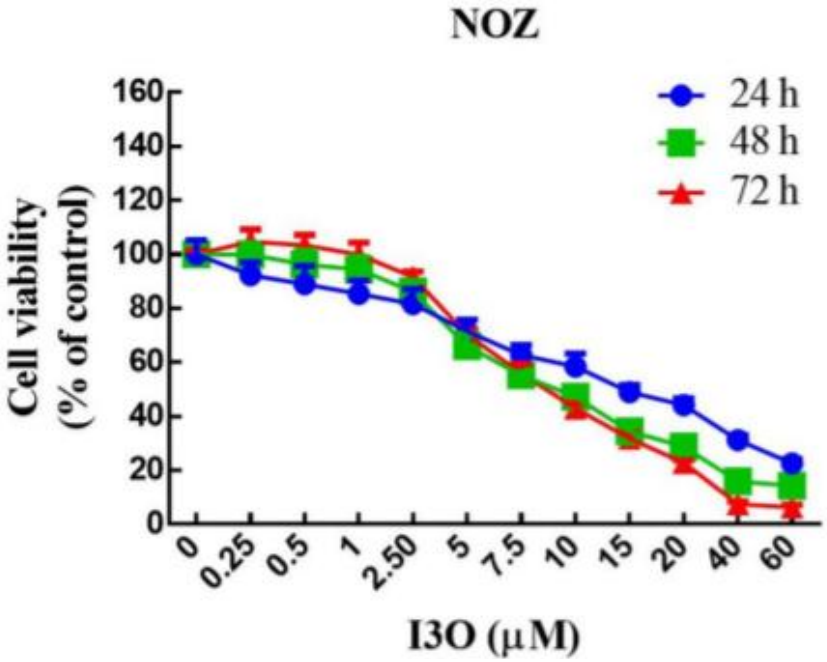

Figure 1(A) I3O suppressed the proliferation of human cholangiocarcinoma (CCA) cells [NOZ]

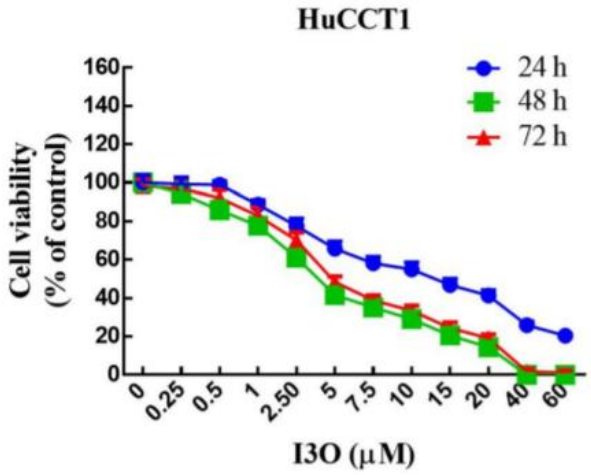

Figure 1 (B) I3O treatment inhibits NOZ, HuCCT1 and OZ cell proliferation [HuCCT1]

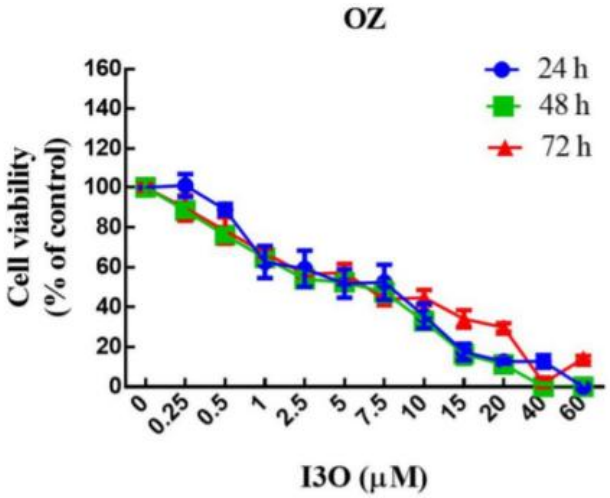

Figure 1 (C) I3O treatment inhibits NOZ, HuCCT1 and OZ cell proliferation [OZ]

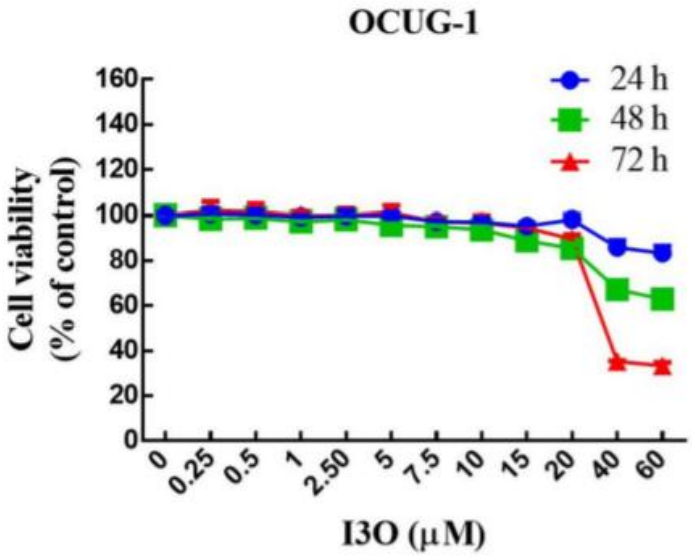

Figure 1 (D) I3O treatment inhibits NOZ, HuCCT1 and OZ cell proliferation [OCUG-1]

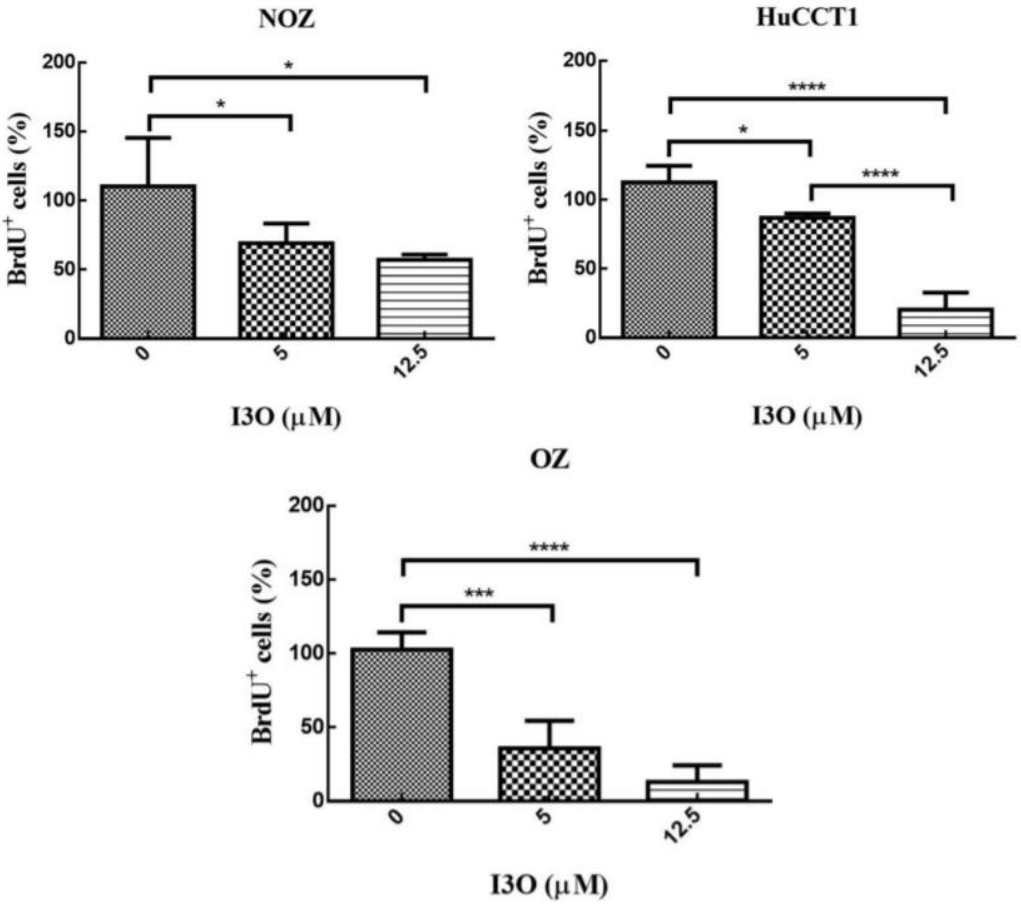

Figure 2. I3O treatment inhibits NOZ, HuCCT1 and OZ cell proliferation

After I3O treatment, cell viability in NOZ, HuCCT1, and OZ cells was substantially reduced (OZ > HuCCT1 NOZ). OZ cells were shown to be further sensitive to I3O treatment, while OCUG-1 cells were found to be further resistant. The BrdU label was evaluated by the immunofluorescent staining for further investigation if cell proliferation was suppressed following I3O treatment. Percentages of BrdU-positive cells in the I3O-treated group reduced in a dose-dependent manner in NOZ, HuCCT1, and OZ cells, according to BrdU staining data (Figure 2). After I3O treatment, OZ cells were more responsive than NOZ and HuCCT1 cells (Figure 2). Cell viability and BrdU staining results were identical. These findings manifested that I3O could stop human CCA cells from growing.

NOZ cells were incubated with either DMSO or I3O, and the cell-cycle was studied using flow cytometry to see if cell-cycle regulation altered I3O's suppressive effect on CCA cell proliferation. After I3O therapy, cessation of cell cycle was found at the G2/M phase. Furthermore, HuCCT1 cells corroborated this tendency. Furthermore, after I3O treatment, the expression of SubG1 increased in a dose- and time-dependent manner. The cell-cycle progression markers p21, CDC25C, CDC2, cyclin A2, and cyclin B1 were examined using immunoblotting to confirm that the G2/M phase was halted after I3O therapy.

The results had manifested that I3O boosted p21 expression while decreasing CDC25C, CDC2, cyclin A2 and B1 expression. These findings showed that I3O might cause cessation of cell cycle in NOZ cells during the G2/M phase. In addition, after I3O therapy, cell death was observed. SubG1 expression increased in NOZ cells treated with I3O, implying that I3O may trigger cell death in human CCA cells. NOZ cells were treated with 0, 5, and 12.5 M I3O, and apoptotic cells were measured using flow cytometry after 48 and 72 hours of incubation. The findings revealed that I3O caused apoptosis in a time- and dose-dependent

manner. Furthermore, I3O clearly triggered apoptosis in HuCCT1 cells, suggesting that apoptosis in anti-human CCAs might be induced by I3O treatment.

DISCUSSION

CCA, which comprises ICC, perihilar, and ECC CCA, is one of the most prevalent biliary cancers. After diagnosis, the median survival rate is twenty-four months (Farley, et al [16]). Participants with CCA have a dismal prognosis, and treatment choices are limited. Surgery or chemotherapy can improve the survival rate; however, the rates are still quite poor after 5 years (Valle, et al [17]; Razumilava and Gores [3]). As a consequence, novel diagnostic and/or therapeutic procedures for CCA management are critical. A derivative of indirubin i.e. I3O, has better pharmacological qualities than indirubin and is more secure for usage (Lo and Chang [9]).

In a recent work, we had discovered that I3O did not cause cytotoxicity in primary granulocytes and primary lymphocytes isolated from healthy donors' peripheral blood (Lee, et al [6]). I3O, on the contrary, showed antitumor effects in numerous human malignancies (Ahn, et al [12]; Blazevic, et al [2]; Lo and Chang [9]; Nam, et al [18]; Perabo, et al [8]). These findings showed that I3O is a safe and potentially effective treatment for human malignancies. We were the first to show that I3O could decrease cell growth, cause G2/M cessation of cell cycle, and activate apoptosis through an intrinsic mitochondrial route in this study.

The anticancer activities of I3O were tested using two human gallbladder carcinoma cell lines, OCUG-1 and NOZ, and two human bile duct carcinoma cell lines, HuCCT1 and OZ. We discovered that I3O had an anticancer effect on NOZ, OZ, and HuCCT1 cells (Figure 1 A, B, C and 2), with OZ cells being more sensitive and OCUG-1 cells being more resistant to I3O treatment than the others (Figure 1 A, B, C and 2). OCUG-1 was the only cell with a wild-type K-Ras, whereas the others had a K-Ras mutation (Yeung, et al [15]). Furthermore, when the K-Ras gene was mutated, CCA cells had a lot of Ras activity (Yeung, et al [15]). Ras' downstream signalling pathways, such as Erk, JNK, and p38, have also been discovered to have a role in cell proliferation, differentiation, and survival (Zhang and Liu [14]). I3O has been shown to suppress the activation of the Erk, JNK, and p38 pathways in a number of studies (Kim and Park [19]; Lee, et al [6]). Hence, whether K-Ras mutation is the cause of I3O sensitivity in human CCAs needs to be investigated further.

Indirubin has been identified as a cyclin-dependent kinase (CDK) and glycogen synthase kinase-3 (GSK-3) inhibitor (Choi, et al [11]; Liao and Leung [20]). Indirubin inhibits CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, and CDK5/p25, all of which are involved in cell cycle progression, and so suppresses tumour cell proliferation (Nam, et al [18]; Santo, et al [13]). Indirubin has been demonstrated to cause cessation of cell cycle at the G/S or G2/M stage in various tumour cells. I3O has previously been shown to diminish cell viability and induce cessation of cell cycle at the G2/M phase in chronic myelogenous leukaemia cells and human acute lymphoblastic leukaemia. Phase G2/M arrests were discovered in I3O-treated human CCA cells, and they were mediated by raised levels of p21 and decreased levels of cyclin B1, cyclin B2, CDC2, and CDC25C in NOZ cells.

CONCLUSION

Finally, it was discovered that I3P has beneficial effects on anti-human CCA activity, resulting in cessation of cell cycle, cell proliferation suppression, and cessation of cell cycle at the G2/M phase, as well as apoptosis. Furthermore, I3O has the potential to cause apoptosis via the caspase-dependent intrinsic mitochondrial route. These findings will aid in the study of I3O's antitumor mechanism in human CCAs and point to I3O as a possible anticancer drug for disease treatment.

REFERENCES

1. Amuamuta A, Plengsuriyakarn T, Na-Bangchang K. Anticholangiocarcinoma activity and toxicity of the Kaempferia galanga Linn. Rhizome ethanolic extract. BMC Complementary and Alternative Medicine. 2017 Dec;17(1):1-1.
2. Blažević T, Schaible AM, Weinhäupl K, Schachner D, Nikels F, Weinigel C, Barz D, Atanasov AG, Pergola C, Werz O, Dirsch VM. Indirubin-3′-monoxime exerts a dual mode of inhibition towards leukotriene-mediated vascular smooth muscle cell migration. Cardiovascular research. 2014 Mar 1;101(3):522-32.
3. Razumilava N, Gores GJ. Cholangiocarcinoma. The Lancet. 2014 Jun 21;383(9935):2168-79.
4. Cheng X, Merz KH, Vatter S, Zeller J, Muehlbeyer S, Thommet A, Christ J, Wölfl S, Eisenbrand G. Identification of a water-soluble indirubin derivative as potent inhibitor of insulin-like growth factor 1 receptor through structural modification of the parent natural molecule. Journal of Medicinal Chemistry. 2017 Jun 22;60(12):4949-62.
5. Li H, Hu B, Zhou ZQ, Guan J, Zhang ZY, Zhou GW. Hepatitis C virus infection and the risk of intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma: evidence from a systematic review and meta-analysis of 16 case-control studies. World Journal of Surgical Oncology. 2015 Dec;13(1):1-8.
6. Lee MY, Liu YW, Chen MH, Wu JY, Ho HY, Wang QF, Chuang JJ. Indirubin-3'-monoxime promotes autophagic and apoptotic death in JM1 human acute lymphoblastic leukemia cells and K562 human chronic myelogenous leukemia cells. Oncology reports. 2013 May 1;29(5):2072-8.
7. Sano M, Ichimaru Y, Kurita M, Hayashi E, Homma T, Saito H, Masuda S, Nemoto N, Hemmi A, Suzuki T, Miyairi S. Induction of cell death in pancreatic ductal adenocarcinoma by indirubin 3′-oxime and 5-methoxyindirubin 3′-oxime in vitro and in vivo. Cancer Letters. 2017 Jul 1; 397:72-82.
8. Perabo FG, Froessler C, Landwehrs G, Schmidt DH, Von Ruecker A, Wirger A, Mueller SC. Indirubin-3′-monoxime, a CDK inhibitor induces growth inhibition and apoptosis-independent up-regulation of survivin in transitional cell cancer. Anticancer research. 2006 May 1;26(3A):2129-35.
9. Lo WY, Chang NW. An indirubin derivative, indirubin-3′-monoxime suppresses oral cancer tumorigenesis through the downregulation of survivin. PLoS One. 2013 Aug 13;8(8): e70198.
10. Hughes T, O'Connor T, Techasen A, Namwat N, Loilome W, Andrews RH, Khuntikeo N, Yongvanit P, Sithithaworn P, Taylor-Robinson SD. Opisthorchiasis and cholangiocarcinoma in Southeast Asia: an unresolved problem. International journal of general medicine. 2017; 10:227.
11. Choi OM, Cho YH, Choi S, Lee SH, Seo SH, Kim HY, Han G, Min DS, Park T, Choi KY. The small molecule indirubin-3′-oxime activates Wnt/β-catenin signaling and inhibits adipocyte differentiation and obesity. International journal of obesity. 2014 Aug;38(8):1044-52.
12. Ahn MY, Kim TH, Kwon SM, Yoon HE, Kim HS, Kim JI, Kim YC, Kang KW, Ahn SG, Yoon JH. 5-Nitro-5′-hydroxy-indirubin-3′-oxime (AGM130), an indirubin-3′-oxime derivative, inhibits tumor growth by inducing apoptosis against non-small cell lung cancer in vitro and in vivo. European Journal of Pharmaceutical Sciences. 2015 Nov 15; 79:122-31.
13. Santo L, Siu KT, Raje N. Targeting cyclin-dependent kinases and cell cycle progression in human cancers. InSeminars in oncology 2015 Dec 1 (Vol. 42, No. 6, pp. 788-800). WB Saunders.

14. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell research. 2002 Mar;12(1):9-18.
15. Yeung Y, Lau DK, Chionh F, Tran H, Tse JW, Weickhardt AJ, Nikfarjam M, Scott AM, Tebbutt NC, Mariadason JM. K-Ras mutation and amplification status is predictive of resistance and high basal pAKT is predictive of sensitivity to everolimus in biliary tract cancer cell lines. Molecular oncology. 2017 Sep;11(9):1130-42.
16. Farley DR, Weaver AL, Nagorney DM. "Natural history" of unresected cholangiocarcinoma: patient outcome after noncurative intervention. InMayo Clinic Proceedings 1995 May 1 (Vol. 70, No. 5, pp. 425-429). Elsevier.
17. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, Madhusudan S, Iveson T, Hughes S, Pereira SP, Roughton M. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. New England Journal of Medicine. 2010 Apr 8;362(14):1273-81.
18. Nam S, Buettner R, Turkson J, Kim D, Cheng JQ, Muehlbeyer S, Hippe F, Vatter S, Merz KH, Eisenbrand G, Jove R. Indirubin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells. Proceedings of the National Academy of Sciences. 2005 Apr 26;102(17):5998-6003.
19. Kim SA, Kim YC, Kim SW, Lee SH, Min JJ, Ahn SG, Yoon JH. Antitumor activity of novel indirubin derivatives in rat tumor model. Clinical cancer research. 2007 Jan 1;13(1):253-9.
20. Liao XM, Leung KN. Indirubin-3'-oxime induces mitochondrial dysfunction and triggers growth inhibition and cell cycle arrest in human neuroblastoma cells. Oncology Reports. 2013 Jan 1;29(1):371-9.