Scientific Research and Essays Vol. 8(1), pp. 26-31, 4 January, 2013
Available online at http://www.academicjournals.org/SRE
DOI: 10.5897/SRE12.591
ISSN 1992-2248 ©2013 Academic Journals

Full Length Research Paper

Induction of cholangiocarcinoma cells apoptosis by an andrographolide analog

Narumol Bhummaphan[1], Jintapat Nateewattana[1], Kanoknetr Suksen[2], Rungnapha Saeeng[4], and Arthit Chairoungdua[1,2,3]*

[1]Toxicology Graduate Program and Center of Excellence on Environmental Health and Toxicology (EHT), Science and Technology Postgraduate Education and Research Development Office (PERDO), Faculty of Science, Mahidol University, Bangkok 10400, Thailand.
[2]Department of Physiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.
[3]Research Center of Transport Proteins for Medical Innovation, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.
[4]Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Burapha University, Chonburi 20131, Thailand.

Accepted 26 October, 2012

Cholangiocarcinoma (CCA) is a malignant tumor derived from bile duct epithelial cells that has high incidence in the Northeastern area of Thailand. It is associated with the infection of liver fluke (*Opisthorchis viverrini*). Chemotherapeutic treatment of CCA is often ineffective due to its resistance to drugs. Therefore, new effective agents for treatment of CCA are urgently required. RSPP 050 analog is a modified compound of andrographolide which has previously been reported to potently inhibit proliferation in many cancer cell lines. In the present study, we aim to investigate the anti-proliferative effect of RSPP 050 on cholangiocarcinoma cells (KKU-M213). Cell viability of KKU-M213 was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The IC_{50} values were 9.4±3.12 µM, 3.47±0.75 µM, and 3.44±0.5 µM at 24, 48 and 72 h, respectively. To investigate the induction of apoptosis in KKU-M213 cells by RSPP 050, APO-BrdU™ TUNEL assay was conducted. RSPP 050 markedly increased the number of apoptotic cells compared to the untreated control. By using 4'-6-diamidino-2-phenylindole (DAPI) staining, RSPP 050 at 10 µM for 24 h, increased the chromatin condensation about 25±0.12% compared with 0.1% DMSO treated cells (2±0.04%) and the percentage of nuclear fragmentation of 50±0.33% compared to 0.1% DMSO treated cells (4±0.01%). These results suggest that RSPP 050 potently inhibited proliferation of KKU-M213 cells by inducing apoptosis. It would be useful for further development as the therapeutic agent for treatment of cholangiocarcinoma.

Key words: Andrographolide analog, apoptosis, cholangiocarcinoma.

INTRODUCTION

Cholangiocarcinoma (CCA) is a common bile duct cancer, accounts for an approximately 15% of liver cancer (Wongkham et al., 2009). The highest incidence of cholangiocarcinoma has been reported in the Northeastern area of Thailand (Shaib and El-Serag, 2004). The risk factors of cholangiocarcinoma in this region have been reported to be associated with infection of liver fluke, *Opisthorchis viverrini* from eating uncooked food and poor sanitation practice (Sripa and Pairojkul, 2008). Cholangiocarcinoma has resulted in a high mortality rates and poor prognosis. At present, surgery is a potentially curative approach, which unfortunately have no improvement with long term survival (Wongkham et

*Corresponding author. E-mail: scacr@mahidol.ac.th. Tel: (66)2 201-5619. Fax: (66) 2 354-7154.

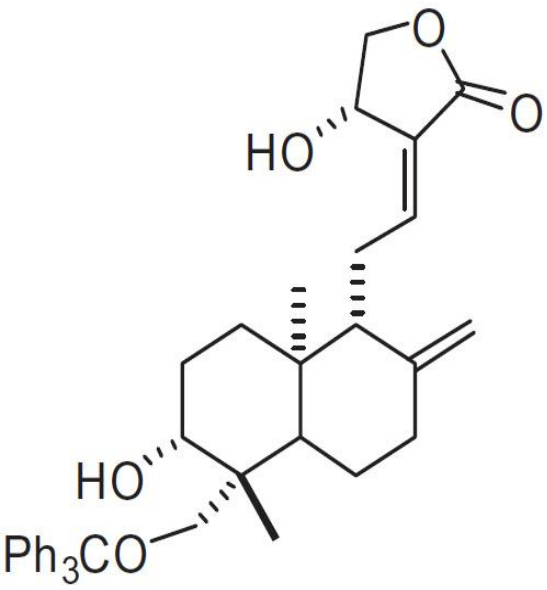

Figure 1. 19-*O*-triphenylmethylandrographolide (RSPP 050) was isolated and purified as previously described; compound 18 (Sirion et al., 2012).

al., 2009). In addition, the chemotherapy is ineffective (Tepsiri et al., 2005). Therefore, searching for the effective drugs with less adverse effect and high sensitivity to cholangiocarcinoma are needed.

Andrographolide is the main compound isolated from *Andrographis paniculata* Nees (Burm.f.) (family Acanthaceae) which is widely used in many Asian countries including Thailand. It has a number of pharmacological activities. In Thailand, Andrographolide has been used for the treatment of common cold, fever, tonsillitis, diarrhea and anti-inflammation (Varma et al., 2009). Recently, andrographolide has been reported to have anti-cancer activity in several cancer cell lines. Kumar et al. (2004) reported that *A. paniculata* extracted by dichloromethane inhibited HT29 (colon cancer) cell proliferation whereas the ethanol extract was cytotoxic to Jurkat (Human T-lymphocyte cells), HepG2 (hepatoma), PC-3 (prostate cancer), and Colon205 (colonic cancer) cells (Kumar et al., 2004). However, andrographolide has poor water solubility which makes it difficult to be applied in clinic and its potency and efficacy is quite low (Das et al., 2010). Thus, various semi-synthetic andrographolides were developed by several groups. The introduction of trityl group at C-19 position resulted in 14- and 12- folds increase in cytotoxicity in KB and LU-1 (Sirion et al., 2012). Interestingly, RSPP 050 or compound 18 exhibited higher cytotoxic activity than the potent anticancer drug, ellipticine in many cancer cell lines including P-388, KB, COL-2, MCF-7, LU-1 and ASK cells (Sirion et al., 2012), however, the effect and its mechanism of RSPP 050 on cholangiocarcinoma cells has not yet been investigated. In the present study, we determined the anti-proliferative activity of andrographolide analog RSPP 050 and its mechanism of action in cholangiocarcinoma cells (KKU-M213), cancer cells derived from Thai patient. In this study, we reported the potent antiproliferation of RSPP 050 on cholangiocarcinoma cell through activation apoptosis signaling pathway.

MATERIALS AND METHODS

Chemicals

An andrographolide analog (RSPP 050) was prepared according to the method for preparing compound 18 which was previously described by Sirion et al. (2012) (Figure 1). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Vinblastin, Dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), 4,6-Diamidine-2-Phenylindole dihydrochloride (DAPI) was purchased from Roche Diagnostics GmbH (Mannheim, Germany), Ham's F-12 medium, APO-BrdU™ TUNEL Assay Kit were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture

Human cholangiocarcinoma cells (KKU-M213) provided by Assoc. Prof. Banchob Sripa (Khon Kean University) were cultured in Ham's F-12 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37°C under humidified 5% CO_2 incubator.

Cell viability assay

Cell viability assay was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay. Cells were plated on 96-well plates at a density of 1×10^4 cells/well and incubated at 37°C under humidified 5% CO_2 incubator. After 24 h, cells were treated with various concentrations (0.5, 1, 5, 10, 20 and 30 µM) of the andrographolide analogue (RSPP 050) and various concentrations of Ellipticin (0.5, 1, 5, 10, 20 and 30 µM) for 24, 48 and 72 h. Cells were incubated with 0.5% MTT reagent and incubated at 37°C in 5% CO_2 incubator for 4 h. Then, culture medium was removed and 100% DMSO was added to stop the reaction before measurement at an absorbance 590 nm using FLUOStar OPTIMA microplate reader spectrophotometer (BMG LABTECH GmbH, Germany). The results were calculated as the % of cell viability.

Apo-BrdU™ TUNEL assays

Apoptotic cells were determined using APO-BrdU™ TUNEL Assay Kit and flow cytometry. Cells were plated on 60-mm dish at a density of 5×10^5 cells/dish. After 24 h, cells were treated with 10 µM RSPP 050, 0.1% DMSO and 10 µM Vinblastin for 24 h. Cells were resuspended with 1% formaldehyde and fixed overnight with 70%

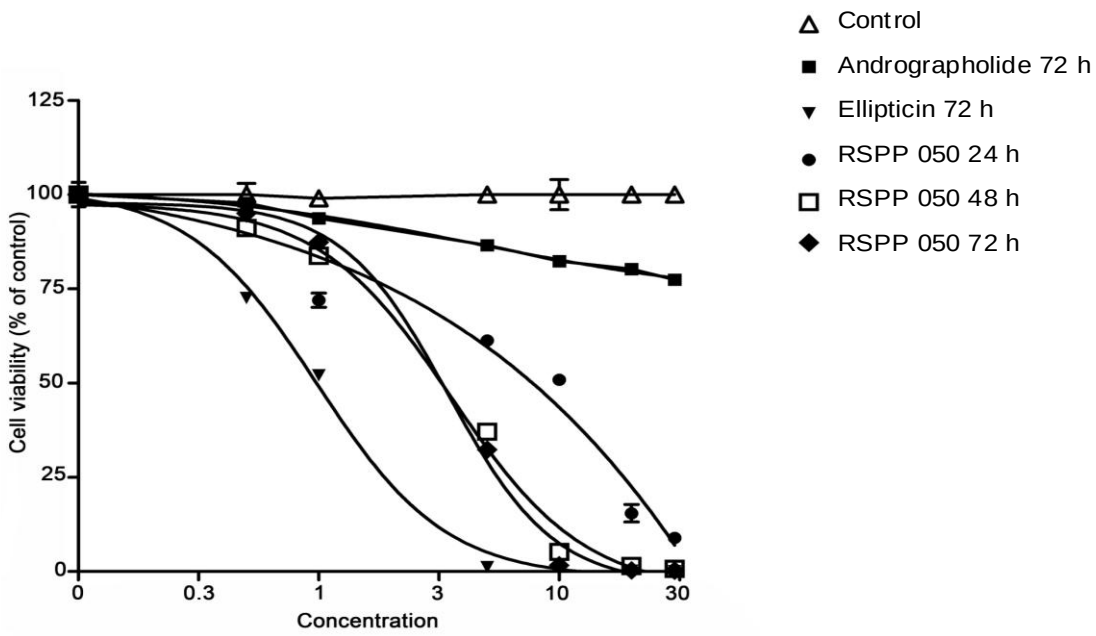

Figure 2. The anti-proliferative effect of RSPP 050 on KKU-M213 cells. Cells were treated with various concentrations of RSPP 050 (0.5, 1, 5, 10, 20 and 30 µM) for 24, 48 and 72 h. Cell viability was determined using MTT assay. Each value represents mean ±S.E.M. of three experiments performed in triplicates.

ethanol at 4°C. Then, cells were washed twice with washing buffer and incubated with 50 µl of DNA labeling solution containing 10 µl of terminal deoxynucleotidyl transferase reaction buffer, 0.75 µl of terminal deoxynucleotidyl transferase (TdT) enzyme, 8 µl of Br-dUTP, and 31.25 µl of dH_2O for 120 min at 37°C. Cell pellets were collected and incubated at room temperature in 100 µl of the antibody solution (5.0 µl of the Alexa Fluor® 488 dye-labeled anti-BrdU antibody in 95 µl of rinse buffer) for 30 min before incubating with propidium iodide/RNase A staining buffer for 30 min. BrdU stained cells were detected by flow cytometry (BD FACSCanto™).

DAPI staining

KKU-M213 cells were plated on 60-mm dish at a density of 5×10^5 cells/dish. After 24 h, cells were treated with 10 µM RSPP 050 and 0.1% DMSO for 12 h. Then, cells were stained with 5 µg/ml of DAPI for 20 min at 37°C. Chromatin condensation and nuclear fragmentation were examined under a fluorescent microscope (HB-10101 AF, Nikon, Japan). At least 1,000 stained cells were counted and calculated as percentage of chromatin condensation and nuclear fragmentation compared to those of the control cells.

RESULTS

Anti-proliferative effect of RSPP 050 on KKU-M213 cells

The anti-proliferative activity of RSPP 050 on KKU-M213 cells were evaluated. Cells were treated with various concentrations of RSPP 050 (0.5, 1, 5, 10, 20 and 30 µM) for 24, 48 and 72 h. Ellipticin, a potent anti-cancer drug was used as a positive control (72 h). As shown in Figure 2, treatment with andrographolide parent compound did not inhibit growth of KKU-M213 cells. However, RSPP 050 exhibited concentration and time-related anti-proliferative activity on these cholangiocarcinoma cells. The IC_{50} values of RSPP 050 were 9.4±3.12 µM, 3.47±0.75 µM and 3.44±0.5 µM at 24, 48 and 72 h, respectively. This result indicates that andrographolide analogue (RSPP 050) has a potential for further development as an anti-proliferative drug for cholangiocarcinoma treatment.

Apoptotic effect of RSPP 050 on KKU-M213 cells

To determine whether the anti-proliferative effect of RSPP 050 was related to apoptotic cell death, DNA fragmentation was evaluated using APO-BrdU™ TUNEL assay and DAPI staining. Following treatment with compound RSPP 050 at 10 µM for 24 h in KKU-M213 cells, the percentage of apoptotic cells increased at 32.3±0.05% compared to DMSO treated cells at 0.2±0.01%. Interestingly, RSPP 050 exerted the apoptotic effect higher than vinblastin (9.8±0.25%), a well known compound to induce apoptosis (Figure 3(A)).

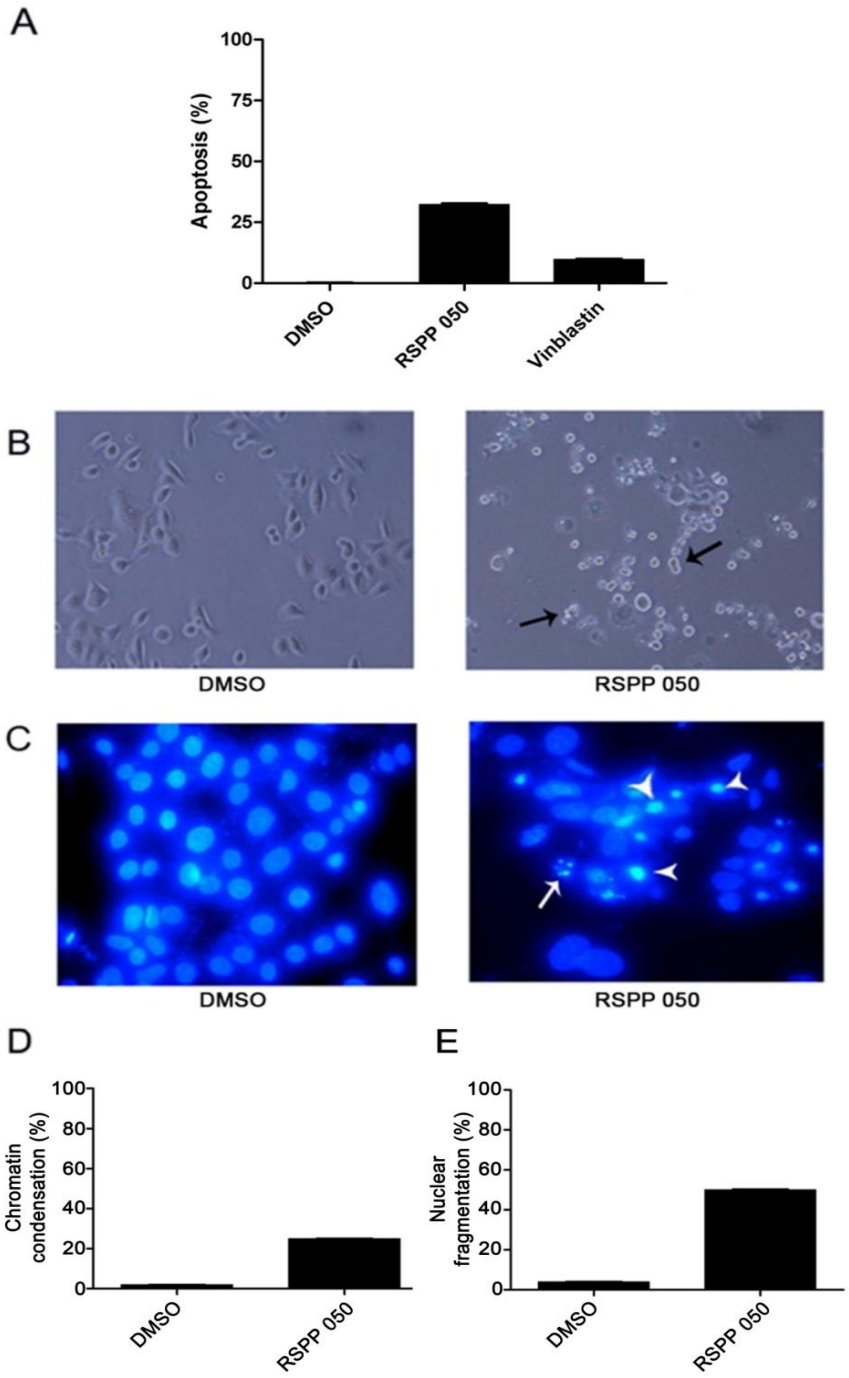

Figure 3. Apoptotic effect of RSPP 050 on KKU-M213 cells. (A) Cells were treated with 10 µM RSPP 050, 0.1% DMSO and 10 µM vinblastin for 24 h. The number of apoptotic cells were determined using APO-BrdU™ TUNEL assay, followed by flow cytometry. (B) Cell shrinkage and perinuclear apoptotic bodies (black arrow) were observed after RSPP 050 treatment at 10 µM for 24 h compared with normal cells. (C) Chromatin condensation and nuclear fragmentation were observed by DAPI staining and view under fluorescence microscope at magnification of 400X. (D-E) The percentage of chromatin condensation and nuclear fragmentation in KKU-M213 cells after treatment with 10 µM RSPP 050 for 12 h were shown. At least 1,000 cells were counted and calculated as % of chromatin condensation and nuclear fragmentation. Values are mean±S.E.M. (n=3).

These results indicate that anti-proliferative effect of RSPP 050 mediated through apoptosis signaling pathway. One of the hallmarks of apoptosis is chromatin condensation and nuclear fragmentation. As shown in Figure 3(B), cell numbers decreased after treatment with 10 µM RSPP 050 for 24 h. KKU-M213 cells treated with RSPP 050 exhibited the apoptotic bodies as indicated by changing in cell morphology from polygonal shapes in control cells to apoptotic-like cells. The characteristic of apoptotic features such as chromatin condensation and nuclear fragmentation were observed in KKU-M213 cells treatment with 10 µM RSPP 050 at 12 h. However, they were not present in the control cells (Figure 3(C)). The number of chromatin condensation and nuclear fragmentation were quantified as shown in Figure 3 (D, E). Treatment of RSPP 050 for 12 h resulted in a significant increase in chromatin condensation at 25±0.12% compared with 0.1% DMSO treated cells (2±0.04%) and the percentage of nuclear fragmentation at 50±0.33% compared with 0.1% DMSO treated cells (4±0.1%). Collectively, our results indicate that RSPP 050 inhibits KKU-M213 cells proliferation through activation apoptosis signaling pathway.

DISCUSSION

The present study demonstrated that the semisynthetic andrographolide analog (RSPP 050) exerted potent anti-proliferative effect on human cholangiocarcinoma cells; KKU-M213 which were derived from Thai CCA patient. RSPP 050 induced apoptosis was characterized by increase in the number of chromatin condensation and nuclear fragmentation. Thus, RSPP 050 has great potential to further develop as a therapeutic agent for cholangiocarcinoma treatment.

Many chemotherapeutic agents have shown the anti-proliferative effect via cell cycle arrested and induction of apoptosis (Lin et al., 2008). In the present study, RSPP 050 induced KKU-M213 cell death that displayed many characteristic features of apoptosis. Interestingly, the anti-proliferative effect of RSPP 050 was higher than that of vinblastin. In addition, flow cytometric analysis indicated that RSPP 050 arrested KKU-M213 cell proliferation at G1 phase of cell cycle accompanied with reduction in the percentage of cells at S and G2/M phase (Data not shown). The IC_{50} of RSPP 050 on KKU-M213 at 72 h was 3.4±0.5 µM which was comparable to IC_{50} values obtained for P-388 (murine leukemia cell line), KB (human epidermoid carcinoma), COL-2 (human colon cancer), MCF-7 (human breast cancer), LU-1 (human lung cancer) and ASK (rat glioma) cell lines in the earlier report (Sirion et al., 2012), suggesting the potential of compound for the treatment of several types of cancers.

The apoptotic effect of RSPP 050 was accompanied with chromatin condensation, nuclear fragmentation, and formation apoptotic bodies which are biochemical hallmarks of apoptosis (Jariyawat et al., 2011). These morphological changes of cells induced by apoptosis have been reported to correlate with various cellular components initiated by activated caspases family. Caspases are key proteins in the apoptotic signaling network (Bratton et al., 2000). The major roles of caspases involved the breakdown of structural nuclear protein and fragmentation of DNA during apoptosis. Moreover, the activities of caspases family are correlated with the functions of p53 which is known to induce cell cycle arrest and apoptosis. p53 induced apoptosis by interfering the expression of G1/S checkpoint proteins in response to DNA damage (Ciciarello et al., 2001). However, in the present study, the activation of the caspases activity and p53 functions which are the key regulators in the intrinsic pathway of apoptosis were not determined therefore further experiments are required.

In summary, we reported the anti-proliferative effect of RSPP 050 on cholangiocarcinoma cells (KKU-M213) which activated the apoptosis signaling pathway. Therefore, RSPP 050 has a potential to further development for cholangiocarcinoma treatment.

ACKNOWLEDGEMENTS

This research project is supported by Mahidol University, and a grant from the Center for Environmental Health, Toxicology and Management of Chemicals under Science and Technology Postgraduate Education and Research Development Office (PERDO), Ministry of Education. Special thanks to Assoc. Prof. Banchob Sripa, Department of Pathology, Faculty of Medicine and Liver Fluke and Cholangiocarcinoma Research Center, Khon Kean University, Thailand for providing the KKU-M213 cells used in this study.

REFERENCES

Bratton SB, MacFarlane M, Cain K, Cohen GM (2000). Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. Exp. Cell Res. 256(1):27-33.

Ciciarello M, Mangiacasale R, Casenghi M, Zaira LM, D'Angelo M, Soddu S, Lavia P, Cundari E (2001). p53 displacement from centrosomes and p53-mediated G1 arrest following transient inhibition of the mitotic spindle. J. Biol. Chem. 276(22):19205-19213.

Das B, Chowdhury C, Kumar D, Sen R, Roy R, Das P, Chatterjee M (2010). Synthesis, cytotoxicity, and structure-activity relationship (SAR) studies of andrographolide analogues as anti-cancer agent. Bioorg. Med. Chem. Lett. 20(23):6947-6950.

Jariyawat S, Thammapratip T, Suksen K, Wanitchakool P, Nateewattana J, Chairoungdua A, Suksamrarn A, Piyachaturawat P (2011). Induction of apoptosis in murine leukemia by diarylheptanoids from Curcuma comosa Roxb. Cell Biol. Toxicol. 27(6): 413-423.

Kumar RA, Sridevi K, Kumar NV, Nanduri S, Rajagopal S (2004). Anticancer and immunostimulatory compounds from *Andrographis paniculata*. J. Ethnopharmacol. 92(2-3): 291-295.

Lin SS, Huang HP, Yang JS, Wu JY, Hsia TC, Lin CC, Lin CW, Kuo CL, Gibson WW, Chung JG (2008). DNA damage and endoplasmic reticulum stress mediated curcumin-induced cell cycle arrest and apoptosis in human lung carcinoma A-549 cells through the activation caspases cascade- and mitochondrial-dependent pathway. Cancer Lett. 272:77-90.

Shaib Y, El-Serag HB (2004). The epidemiology of cholangiocarcinoma. Semin. Liver Dis. 24(2):115-125.

Sirion U, Kasemsook S, Suksen K, Piyachaturawat P, Suksamrarn A, Saeeng R (2012). New substituted C-19-andrographolide analogues with potent cytotoxic activities. Bioorg. Med. Chem. Lett. 22(1):49-52.

Sripa B, Pairojkul C (2008). Cholangiocarcinoma: Lessons from Thailand. Curr. Opin. Gastroenterol. 24(3):349-357.

Tepsiri N, Chaturat L, Sripa B, Namwat W, Wongkham S, Bhudhisawasdi V, Tassaneeyakul W (2005). Drug sensitivity and drug resistance profiles of human intrahepatic cholangiocarcinoma cell lines. World J. Gastroenterol. 11:2748-2753.

Varma A, Padh H, Shrivastava N (2009). Andrographolide: A New Plant-Derived Antineoplastic Entity on Horizon. Evid. Based Complem. Altern. Med. 815390:1-9.

Wongkham S, Junking M, Wongkham C, Sripa B, Chur in S, Araki N (2009). Suppression of galectin 3 expression enhances apoptosis and chemosensitivity in liver fluke associated cholangiocarcinoma. Cancer Sci. 100(11):2077-2084.