CHAPTER II
TNP-470, A METAP2 INHIBITOR, INHIBITS PROLIFERATION BY INDUCTION OF APOPTOSIS AND ENHANCES THE ANTI-TUMOR ACTIVITY OF CHEMOTHERPEUTIC DRUGS ON CHOLANGIOCARCINOMA CELL LINES

2.1 Introduction

Cholangiocarcinoma (CCA) is identified as a cancer that developing from biliary epithelium which well known as intrahepatic CCA (ICC) and extrahepatic CCA (ECC). Nowadays, the incidence of CCA is increasing over the world such as in Korea and China. Moreover, high incidence rate are still found in Thailand, particularly in the northeast part and its associated with OV infection up to 85 in 100,000 cases (Bragazzi et al., 2011). Owing to its lately state of diagnosis, thus there are many therapeutic regimens for treatment CCA patients such as surgery and adjuvant therapy for example chemo- and radio-therapy which have different response and survival rate of CCA patients. Many reports showed 5 years survival rate of the patients after resection (DeOliveira et al., 2013; Glazer et al., 2012). However, this regimen is still giving low 5 year survival, due to high recurrent rate. In addition, some patients can not undergo resection due to cancer metastasis. Thus, it might be potential to use with adjuvant therapy such as chemo- and radio- therapy. Chemotherapy is the treatment of cancer with drugs that can destroy cancer cells by impeding their growth. Due to the limitation of surgical strategy, chemotherapy has been used in an attempt to improve the survival and quality of life in patients with unresectable, recurrent and metastasis.

Many chemotherapeutic drugs such as gemcitabine, capecitabine, 5FU, folinic acid and irinotican, have been widely tested in clinical for CCA patients (Iwasa et al., 2012; Thongprasert et al., 2012). Nevertheless, chemotherapy is still giving low response rate and median survival time. Moreover, the frequent acquisition of drug-resistant phenotypes and occurrence of secondary malignancies associated with chemotherapy remain serious problems particularly the toxic effects of chemotherapy

still remain a major drawback in the treatment of CCA patients. Thus, there is a need for the development of new and alternative approaches to treat of CCA patients.

Molecular alterations associated with CCA have been explored by using different high-throughput techniques. Exploiting select molecular targets that are aberrantly expressed during carcinogenesis and metastasis of CCA is one approach being used to develop specific chemopreventive and therapeutic strategies. Accordingly, we applied a comparative genomics approach by using the serial analysis of gene expression (SAGE) database (http://cgap.nci.nih.gov/SAGE). A comparison of SAGE data of tissues obtained from CCA and normal liver indicated that several genes are up-regulated in CCA tissues. We focused on the methionine aminopeptidase2 (MetAP2) for further investigation.

MetAP2 is an enzyme which is vital for endothelial and tumor cell growth. Many reports demonstrated that MetAP2 plays a crucial role in development and progression of cancers. MetAP2 also plays an important role in the growth of tumors. And it has been consider as a main regulator of the proliferative and apoptotic pathways in mesothelioma cells (Catalano et al., 2001). Inhibition of MetAP2 action by its inhibitor also induced apoptosis in hepatoma cells (Sheen et al., 2005). Therefore, inhibition of MetAP2 activity may represent a potential mean for therapeutic intervention in several cancers.

The overexpression of MetAP2 has also been reported in CCA. MetAP2 was rarely expressed in normal bile duct epithelia. In contrast, MetAP2 was expressed more frequently in precancerous (hyperplasia and dysplasia) biliary epithelial cells and CCA tissues than in normal bile duct epithelia (Figure 2.1). This implied that MetAP2 may involve in the carcinogenesis and progression of CCA (Sawanyawisuth et al. 2007).

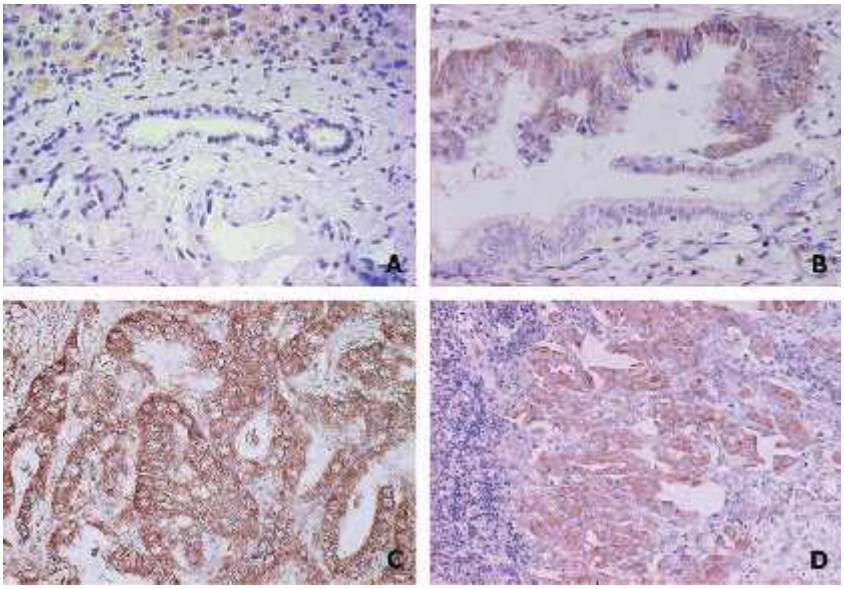

Figure 2.1 Immunohistochemical staining patterns of MetAP2 in human bile duct epithelium. A: normal biliary cells with negative staining, 40x magnification; B: hyperplastic and dysplastic bile duct epithelia with moderate staining, 20x magnification; C: well differentiated tubular CCA, 20x magnification and D: lymph node with metastatic CCA, with strongly positive staining, 10x magnification (Sawanyawisuth et al., 2007)

This study, we aimed to investigate the anti-tumor activity of TNP-470, the specific MetAP2 inhibitor, in CCA cell lines. The chemosensitizing effect of TNP-470 with four chemotherapeutic drugs including 5FU, cisplatin, doxorubicin and gemcitabine also was determined.

2.1.1 Anti-tumor effects of MetAP2 inhibitors and their clinical applications

Nowadays a series of drugs with different chemical structures have been identified as the potential targets for MetAP2 for example, fumagillin, sulphonamide and TNP-470. The actions of these drugs lead to mostly direct on the inhibition of cell growth and anti-angiogenesis (Hotz et al., 2001; Hou et al., 2009; Sawanyawisuth et al., 2012; Selvakumar et al., 2009). There are many reports revealed that fumagillin inactivated MetAP2 and significantly suppresses the

proliferation of different types of cancer cells including mesothelioma (Catalano et al., 2001) and cholangiocarcinoma (Sawanyawisuth et al., 2007). The anti-proliferative activity of the sulfonamide series had been shown to strongly correlate with their cellular activity inhibiting of MetAP2 enzyme function (Kawai et al., 2006; Wang et al., 2007). Supplementation of this compound induced cell arrested at G1 phase of cell cycle without apoptosis in neuroblastoma, prostate carcinoma, cell lymphoma and colon carcinoma (Wang et al., 2008). In spite of this, fumagillin treated endothelial cells were arrested in the G1 phase of cell cycle and also induced apoptosis by induction of early mitochondrial damage in mesothelioma cells (Catalano et al., 2001).

TNP-470 is a synthetic analog of fumagillin and identified as a specific of MetAP2 inhibitor. It has been widely tested as anti-cancer agent *in vitro*, *in vivo* and clinical trail. TNP-470 could suppress the growth of hepatocarcinoma cell by inducing p53 activation leading to elevation of p21 (CIP/WAF) expression, subsequently affected on growth arrest at G_1 phase (Mauriz et al., 2007). TNP-470 (>1µg/mL) also significantly suppressed the proliferation of the human pancreatic cancer cell lines (Hotz et al., 2001). The growth of human umbilical vein endothelial cell (HUVEC) also decreased at 100 pg/mL of TNP-470. This concentration of TNP-470 also induced cell cycle arrest of HUVEC at G_1 phase (Antoine et al., 1994; Hotz et al., 2001; Yeh et al., 2000; Zhang et al., 2000). In addition, TNP-470 was used as anti-cancer in several cancer cells as shown in table 2.1.

Table 2.1 Anti-tumor activity of TNP- 470; *in vitro*

Cell types	Results	References
Uterine carcinoma	Inhibit growth	Naganuma *et al.*, 2011
Neuroblatoma	Arrest cell cycle at G_1 phase	Wang *et al.*, 2008
Murine melanoma	Induce apoptosis	Okrój *et al.*, 2006
Pancreatic cancer	Inhibit growth	Hotz *et al.*, 2001

The *in vivo* study, supplementation of TNP-470 at 30 mg/kg for 5 weeks significantly suppressed xenograft Wilms tumor (83%) (Huang et al., 2004). It also suppressed the growth of human uterine carcinosarcoma xenografts (Emoto et al., 2003). In addition, TNP-470 not only suppresses tumor growth, but it was also proved as an anti-angiogenesis (Svensson et al., 2007). Down-regulation of VEGF secretion with inhibition of angiogenesis in HT1080 fibrosacroma tumor by TNP-470 (Kaya et al., 2001). Additionally, combination of TNP-470 and chemotherapeutic drug, irinotican, has been tested in HCT-116 human colon carcinoma xenograft in SCID mice model. The tumor size was significantly decreased when TNP-470 was given in combination with the drug (Wang et al., 2008).

Table 2.2 Anti-tumor activity of TNP- 470; *in vivo*

Tumor types	**Results**	**References**
Uterine carcinosarcroma	Suppress tumor growth	Naganuma *et al.*, 2011
Gioblastoma	Suppress tumor growth	Yao *et al.*, 2010
Murine neuroblastoma	Suppress tumor growth Induce apoptosis	Chesler *et al.*, 2007
Sarcoma	Suppress tumor growth	Kanamori *et al.*, 2007
Wilms tumor cells	Anti-angiogenesis	Huang *et al.*, 2004

TNP-470 was reported as the first anti-angiogenesis drug which applied in clinical trial. It was also used to treat several cancers including kaposi's sarcoma, renal cell carcinoma, brain cancer, breast cancer, and prostate cancer as shown in table 2.3. Administration of TNP-470 at dose range from 10-70 mg/m^2 over 1 hour infusion to patients with early AIDS associated Kaposi's for 24 weeks, was well-tolerated without dose-limiting toxicity (Bhargava et al., 1999). Similar effect on anti-tumor of clinical model by TNP-470 treatment for phase I of androgen independent prostate cancer, but patients were encountered by reverse effect as neurological toxic effect during treatment (Logothetis et al., 2001). Moreover, paclitaxel and TNP-470

were determined to be 225 mg/m^2 administered every 3 weeks and 60 mg/m^2 administered three times a week, respectively treatment lung cancer patient, the median survival duration was 297 days. The neurological symptoms observed in this and other clinical studies of TNP-470 are similar to those seen in patients receiving paclitaxel, however these adverse events was resolved within 2 weeks after discontinuation of TNP-470 treatment (Herbst et al., 2002).

Table 2.3 Effect of TNP- 470 in clinical trials

Cancer patients	Dose (mg/m^2)	n	RR (%)	References
Lung cancer	60	17	24	Tran *et al.*, 2004
	60	32	33	Herbst *et al.*, 2002
Kaposi's sarcoma	10-70	38	ND	Dezube *et al.*,1998
Cervical cancer	9.3 -71.2	18	ND	Kudelka *et al.*,1998
Renal carcinoma	60	33	3	Stadler *et al.*,1999
Prostate cancer	71	33	ND	Stadler *et al.*,1994

2.2 Materials and methods

2.2.1 CCA cell lines

All CCA cell lines (KKU-M213 and KKU-M214) were developed from primary tumor obtained from CCA patients by Associate Professor Bunchop Sripa; Liver Fluke and Chongiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kean University (Sripa et al., 2005). All cell lines are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum and 1% antimycotic-antibiotic (Gibco, Grand Island, NY) at 37 °C under the present of 5% CO_2.

2.2.2 Chemicals and reagents

All chemicals and reagents which were used in this experiment are list in Table 2.4.

Table 2.4 List of chemical and suppliers

Chemicals	Company, City, Country
Cell culture reagents	
- DMEM medium	Gibco (Invitrogen), NY, USA
- Fetal bovine serum (FBS)	Gibco (Invitrogen), NY, USA
- Antibiotic-Antimycotic	Gibco (Invitrogen), NY, USA
MTT	Invitrogen, NY, USA
TNP-470, Product Number : T1455	Sigma-Aldrich Pte Ltd, Singapore
TRIzol Reagent	Invitrogen, NY, USA
Protease inhibitor cocktails	Roche diagnostics , IN, USA
Rabbit anti caspase3	Cell signaling, Danvers, MA
Rabbit anti caspase9	Cell signaling, Danvers, MA
Rabbit anti Bax IgG	Santa Cruz Biotechnology, CA
Mouse anti Bcl-xL IgG	Santa Cruz Biotechnology, CA
Mouse anti p38 IgG	Santa Cruz Biotechnology, CA
Mouse anti pp38α/β IgG	Santa Cruz Biotechnology, CA
Mouse anti β-actin IgG	Sigma Aldrich, St. Louis, MO
Donkey anti rabbit IgG-HRP	GE healthcare, Piscataway, NJ
Goat anti mouse IgG-HRP	SB, Birmingham, AL
5-Fluorouracil (5FU), lot# 8008	BORYUNG PHARM.CO.,LTD, Korea
Cisplatin, lot# 1021	BORYUNG PHARM.CO.,LTD, Korea
Doxorubicin, lot# 1016	BORYUNG PHARM.CO.,LTD, Korea
Gemcitabine, lot# A5054476	Fresenius kabi Oncology LTD, India

2.2.3 Cell proliferation assay (MTT assay)

CCA cell lines at $3x10^3$ cells/100 µl were treated with TNP-470 or vehicle. The cell numbers was determined by MTT method. Briefly, after an incubation period, cells were washed and determined by adding equal volume of MTT into 10% of the culture medium then incubates for 4 hours at 37^oC. After incubated with MTT, 1% HCl in isopropanol is added to dissolve insoluble formazan, mixing by pipetting to disperse the MTT formazan. Spectrophotometrically measure absorbance at 540 nm and subtract by an absorbance at 620 nm using ELISA reader machine (Tecan Austria GmbH, Salburg, Austria).

2.2.4 Cell cycle and apoptotic analysis by flow cytometry

The effect of TNP-470 on cell cycle progression was assessed using flow cytometry method. CCA cells at 1×10^5 were grown in 6 wells plate with different concentration of TNP-470. Cells were harvested, washed with cold PBS, trypsinized and centrifuged. Cells were suspended in 50 µl cold PBS, added 450 µl cold ethanol and incubate for 1 hour at 4°C. After centrifugation at 110x g for 5 minutes, pellet was washed with cold PBS and suspended in 500 µl PBS then incubated with 5 µl RNase (20 µg/mL final concentration) at 37°C for 30 minutes. The cells were chilled on ice for 10 minutes and incubated with propidium iodide (50 µg/mL final concentration) for 1 hour in the dark. The cell cycle distribution was determined by flow cytometer and analyzed DNA histograms using FlowJo software (Tree Star, San Jose, CA, USA) for cell cycle analysis.

2.2.5 Western blot analysis

Protein preparation

1.5×10^5 CCA cells grown in 6 wells plate were washed with PBS and then scraped in RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% Tween-20, 1% Sodium deoxycholate, 0.1% SDS) containing 1x protease inhibitor cocktails (Roche Molecular Biochemicals). After centrifugation at 13,000 RPM for 15 minutes at 4^oC, the supernatant was measured for protein concentration against a standard curve that constructed by using 2 mg/mL of standard bovine serum albumin, undertaken by Bradford protein assay (Quick Start™ Bradford 1x Dye Reagent. BIO-RAD, USA) and kept at -20^oC until use.

SDS-PAGE and western blot analysis

Cell lysates were mixed with 4x SM; solubilising medium (125 mM Tris-HCl pH 6.8, 4% (w/v) 2-mercaptoethanol, 4% (w/v) SDS, 10% (v/v) glycerol and 0.2% (w/v) bromophenol blue) and boiled for 5 minutes. Equivalent amounts of protein (50 µg per lane) were resolved in 12% SDS-PAGE using vertical electrophoresis system (Laemmli, 1970). After electrophoresis, the gel is pre-equilibrated in transferring buffer (40 mM Tris, pH 7.4, 20 mM sodium acetate, 2 mM ethylene diamine tetraacetic acid (EDTA), 20% (v/v) methanol, and 0.05% SDS for 15 minutes then electro-transferred onto Polyvinylidene fluoride (PVDF) membrane (Bolt and Mahoney, 1997). The PVDF membrane is incubated with 3% BSA in 0.3% Tween in PBS pH 7.4, at 4^{o}C overnight to prevent non-specific binding and further incubated with primary antibody; rabbit anti-Caspase3 (1:1,000), rabbit anti-Caspase9 (1:1,000), rabbit anti-Bax (1:1,000), mouse anti-Bcl-xL (1:1,000), mouse anti p38 and pp38α/β (1:1000), mouse anti-PARP-1 (1:1,000) for overnight at 4ºC, and mouse anti-β-actin (1:40,000) for 1 hour at room temperature. After washing with 0.3% Tween in PBS pH 7.4 for 3 times, membranes are probed with HRP-conjugated antibody depending on primary antibody used. The membranes are washed for 3 times with 0.3% Tween in PBS before detection of peroxidase activity using ECL^{TM} Western blotting Analysis system (GE Healthcare, Buckinghamshire, UK) to develop the immunoreactive bands which are quantitated by Gel Pro Analyzer gel analysis and using ImageJ^{TM} analysis software.

2.2.6 Combination index

CCA cell lines (3 x10^{3} cells/well) were simultaneously incubated with TNP-470 (IC_{25}, IC_{50} and IC_{75}) and chemotherapeutic drugs including 5FU, cisplatin, doxorubicin and gemcitabine (IC_{25}, IC_{50} and IC_{75}) for 72 h. Cell numbers in treated and non-treated group were determined by MTT method. The combination index (CI) theorem of Chou-Talalay (Chou, 2010) offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations. Combination effects were the effects from the combination between TNP-470 (IC_{25}, IC_{50} and IC_{75}) and chemotherapeutic drug (IC_{25}, IC_{50} and IC_{75}). The most synergistic effect will be gained if the value of combination effect is less. The single agent was

compared to control. And the combination effect was compared to the effect of single drug for example the combination effect between IC_{25} of TNP-470 and individually $IC_{(25 \text{ or } 50 \text{ or } 75)}$ of chemotherapeutic drugs was compared to the effect of $IC_{(25 \text{ or } 50 \text{ or } 75)}$ of chemotherapeutic drugs alone.

All data were analysed using COMPUSYN Software (ComboSyn, Inc., NY, USA).

2.2.7 Statistic analysis

A statistical approach is applied to assess (parametric test). The difference of continuous data between two groups as treatment and non-treatment group was analyzed by unpaired t-test (parametric test). All analyses are performed using SPSS software version 16.0 (Chicago, IL. USA) and GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). $P < 0.05$ is considered to be statistically significant.

2.3 Results

2.3.1 TNP-470 exerted anti-proliferative effect on CCA cell lines

To invastigate the anti-proliferative effect of TNP-470, two CCA cell lines established from different histological types of CCA patients namely KKU-M213 (mixed papillary and non-papillary CCA) and KKU-M214 (well-differentiated CCA) were treated with various concentrations of TNP-470 (2.5, 5, 10 and 20 µg/mL). Cell treated with 0.005 % Dimethyl sulfoxide (DMSO) was used as a control. The result showed that TNP-470 significantly reduced growth of CCA cell lines in a dose and time dependent manner ($P < 0.05$). The IC_{50} of TNP-470 were 16.86± 0.9 µg/mL, 3.16± 0.6 µg/mL and 1.78± 0.8 µg/mL for KKU-M213 cells at 24, 48 and 72 h, respectively. For KKU-M214, the IC_{50} were 22± 0.9 µg/mL, 9.43± 0.8 µg/mL and 2.43± 0.5 µg/mL at 24, 48 and 72 h, respectively.

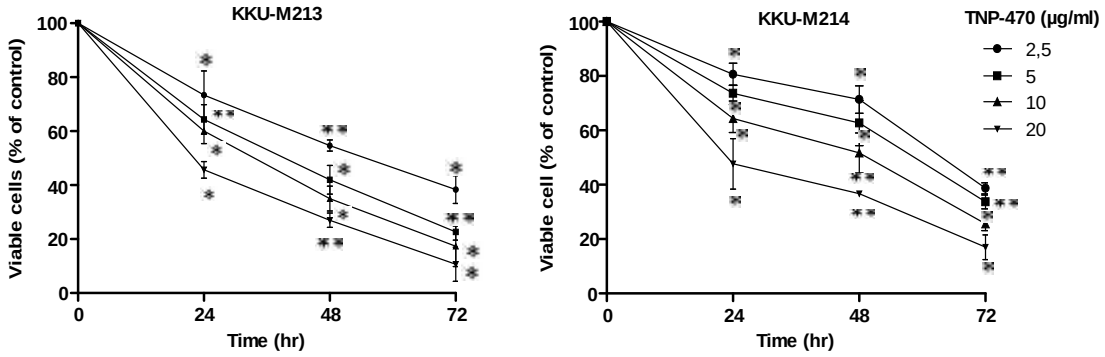

Figure 2.2 The effect of TNP-470 on the growth of CCA cell lines, KKU-M213 and KKU-M214 were cultured in the presence or absence of TNP-470 (2.5, 5, 10 and 20 µg/mL). Cell viability was measured by MTT assay comparing between treatment and vehicle (0.05% DMSO). *P < 0.05; **P < 0.01

2.3.2 Effect of TNP-470 on cell cycle and apoptosis of CCA cell lines

CCA cell lines, KKU-M213 and KKU-M214, were incubated for 48 hour with various concentrations of TNP-470 (0, 2.5, 5, 10 and 20 µg/mL, respectively). Cells were stained by propidium iodide then analyzed by flow cytometry. The histograms of cell cycle were plotted by using BD Biosciences FACSDiva software version 6.1.3. The results showed that TNP-470 did not affect on the cell cycle of CCA cells compared with control group (Figure 2.3).

However, the results showed that treated by various concentrations of TNP-470 significantly increased the apoptosis of KKU-M213 for 17, 21, 29 and 39 percent, respectively. TNP-470 also induced apoptosis in KKU-M214 for 16, 23, 43 and 51 percent, respectively ($P < 0.05$) as shown in figure 2.4.

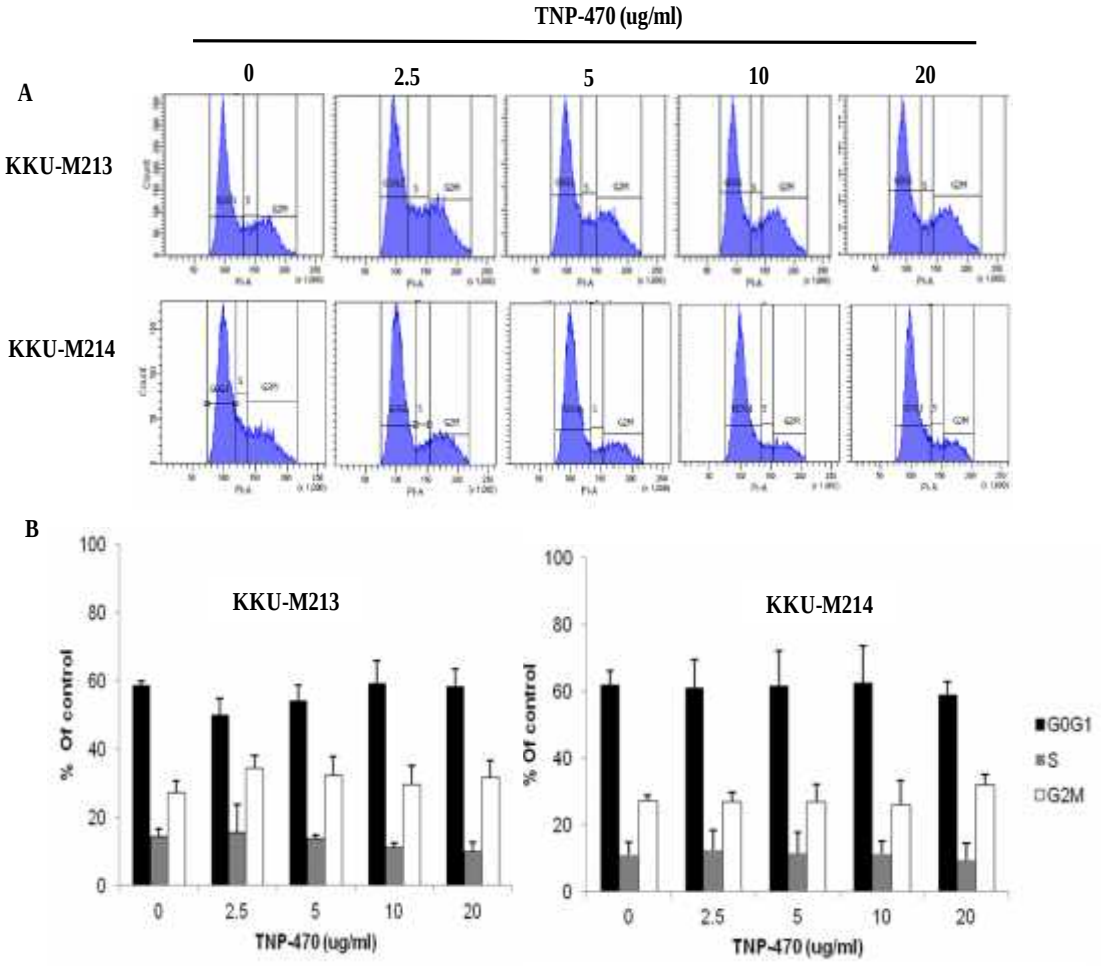

Figure 2.3 Effect of TNP-470 on cell cycle of CCA cell lines. (A) CCA cell lines, KKU-M213 and KKU-M214, were treated with TNP-470 for 48 hours and stained with propidium iodide, then analyzed by flow cytometry. (B) Relative cell numbers are illustrated at each phase of the cell cycle. The result showed the mean of three independent experiments. There was not statistical difference on the percentage of cell cycle in the presence of TNP-470

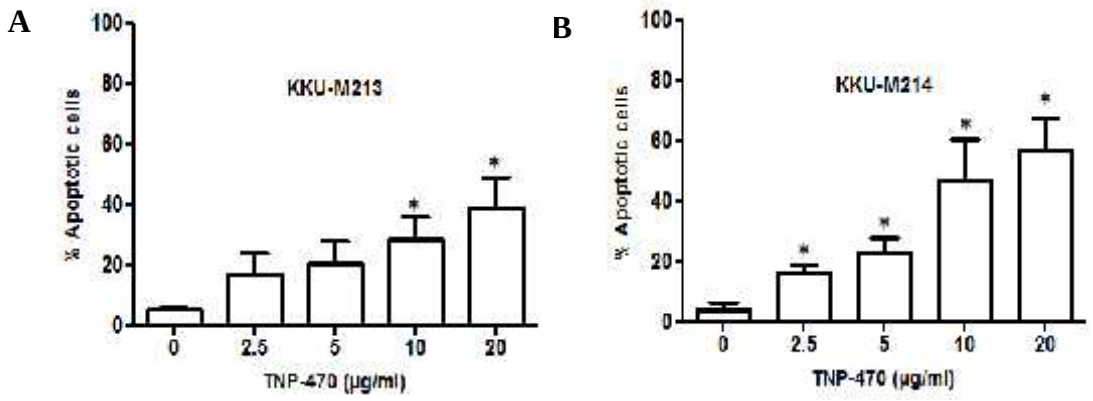

Figure 2.4 Effect of TNP-470 on apoptosis of CCA cell lines. (A) and (B) Percent of apoptotic cells in KKU-M213 and KKU-M214, respectively

2.3.3 Effect of TNP-470 on the expression of apoptotic regulatory proteins.

To investigate the mechanism by which TNP-470 induced apoptosis in CCA cell lines, KKU-M213 and KKU-M214 were treated with various concentrations of TNP-470 (2.5, 5, 10 and 20 µg/mL) for 48 hours. The expression of apoptotic regulatory proteins including caspase9 and caspase3, Bcl-xL, Bax, p38 and phosphorylated p38 were determined by western blot technique. TNP-470 treatment had no effect on the expression of p38. By contrast, the level of phosphorylated p38 was dose-dependent increased at the range of 2.5-10 µg/mL of TNP-470 (Figure. 2.5). The increase in p38 phosphorylation after TNP-470 treatment paralleled the induction of apoptosis (Figure 2.4), indicating that p38 pathway might be involved in the regulation of apoptosis. Since p38 phosphorylation was correlated with apoptosis induction in a TNP-470 dose-dependent manner, we hypothesized that the p38 pathway might mediate apoptotic signals in response to TNP-470. Next, we investigated the dose effect of TNP-470 on the levels of apoptosis-related molecules as shown in Figure 2.5, TNP-470 induced the pro-apoptotic protein, Bax, in a dose-dependent manner. In contrast, the anti-apoptotic protein, Bcl-xL, was decreased in a dose-dependent manner, resulting to activate the activity of caspase9 and caspase3, respectively, which leaded to induction of apoptosis in CCA cell lines.

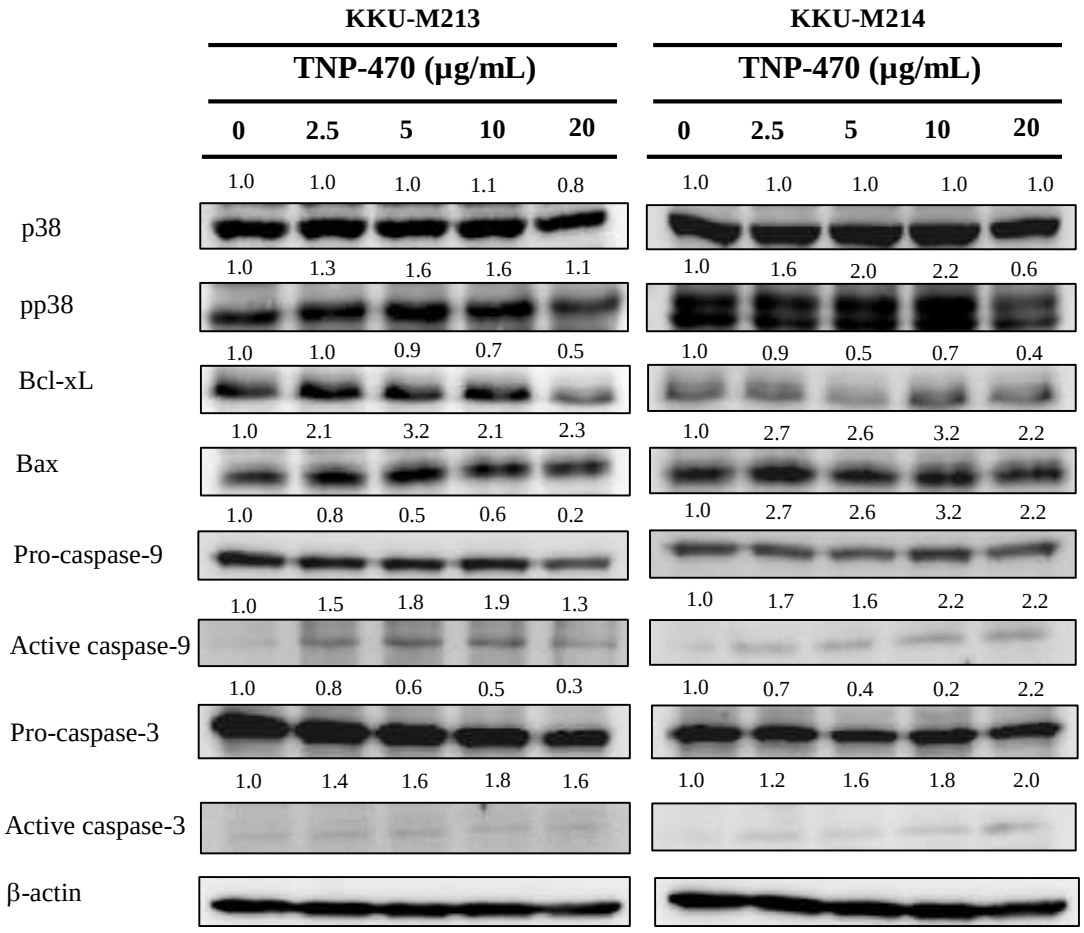

Figure 2.5 Effect of TNP-470 on the expression of apoptotic proteins; caspase9 and 3, Bcl-xL, Bax, p38, pp38 on CCA cell lines. The cells (1.5×10^5) were seeded to 6 well plates for 24 hours before treated by TNP-470 with various concentrations (2.5, 5, 10 and 20 µg/mL) or control (0.05% DMSO) for 48 hours. 50 µg of total proteins were collected after incubation. All samples were subjected to Western blot analysis for p38, pp38, Bcl-xL, Bax, caspase3 and caspase9. β-actin was used as internal control

2.3.4 TNP-470 enhances the anti-tumor activity of chemotherapeutic drugs on CCA cell lines

To examine the chemosensitizing effect of TNP-470 on CCA cell lines, KKU-M213 and KKU-M214 were treated with varying concentrations of chemotherapeutic drugs (5FU, cisplatin, doxorubicin and gemcitabine) at IC_{25}, IC_{50}

and IC_{75} with and without TNP-470 at IC_{25}, IC_{50} and IC_{75} for 72 h (Table 2.5). Cell viability was measured using MTT assay, and combination index was determined by Compusyn software. The results showed that combination of chemotherapeutic drugs with TNP-470 significantly inhibited growth of the two CCA cell lines compared to treatment with a single agent (Figure 2.6 and 2.7). In addition, supplementation of TNP-470 enhanced cytotoxicity of all chemotherapeutic drugs by giving synergistic effects in both KKU-M213 and KKU-M214 (CI< 1) as shown in table 2.6 and 2.7.

Table 2.5 IC (25, 50, 75) of TNP-470 and chemotherapeutic drugs at 72 hours on KKU-M213 and KKU-M214

CCA cells	**ICs**	**TNP-470 (µg/mL)**	**5FU (µM)**	**Cisplatin (µM)**	**Doxorubicin (µM)**	**Gemcitabine (µM)**
KKU-M213	IC_{25}	1.1	0.4	1	0.1	0.1
	IC_{50}	1.78	4	1.86	0.9	0.6
	IC_{75}	4.6	60	7.16	2.4	249.7
KKU-M214	IC_{25}	1.0	0.1	0.08	0.1	0.1
	IC_{50}	2.43	6.7	1.91	2.1	27.9
	IC_{75}	16.5	383.5	49.28	11	1396.4

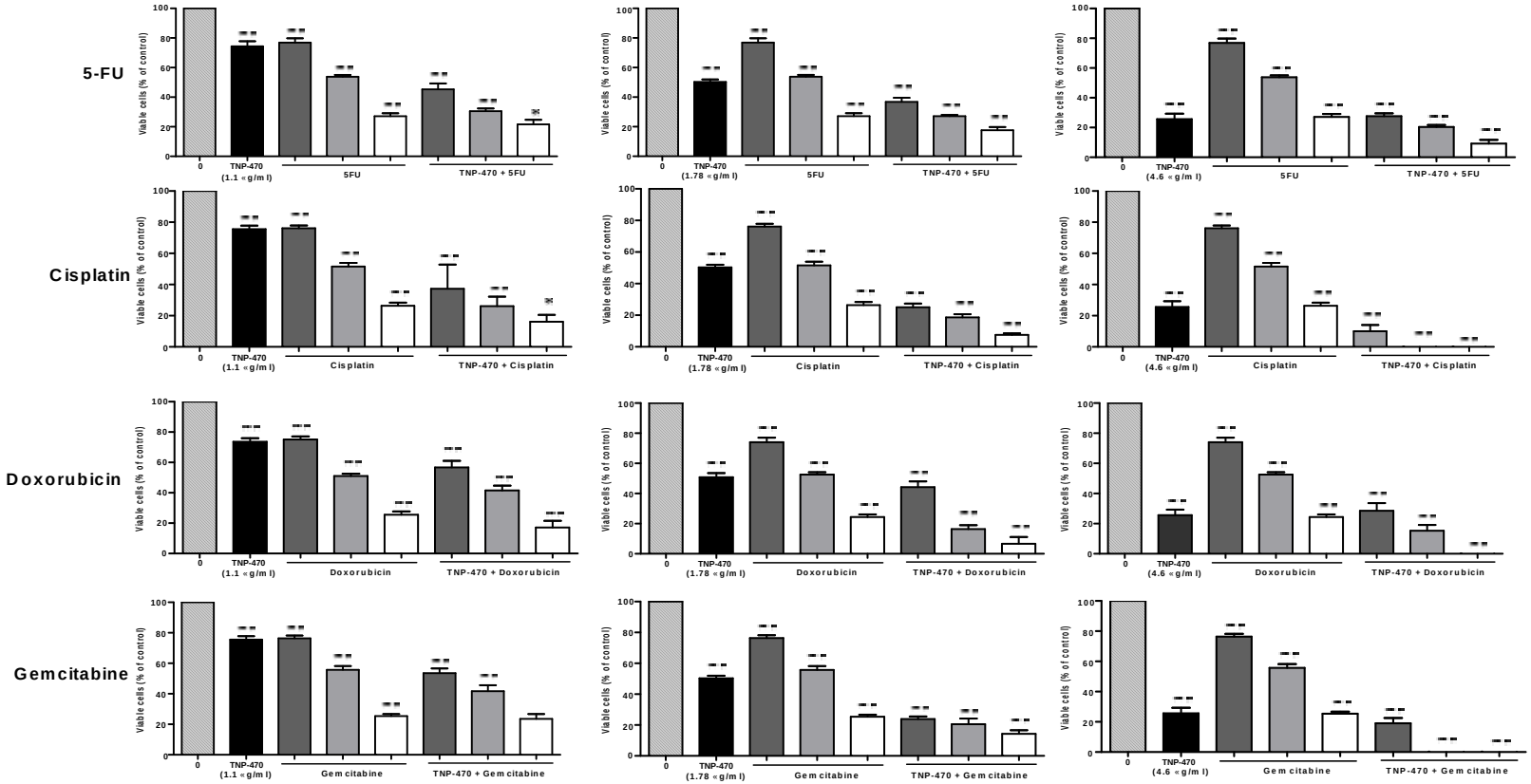

Figure 2.6 Synergistic cytotoxic effect of chemotherapeutic drugs and TNP-470 on KKU-M213, CCA cell line was treated with chemotherapeutic drugs at IC_{25}, IC_{50} and $IC_{75}$with and without TNP-470 at IC_{25}, IC_{50} and IC_{75}

*P < 0.05; **P < 0.01 (a = single agent test with control. b=IC_{25}+TNP-470 test with drug alone. c = IC_{50} + TNP-470 test with drug alone. d = IC_{75} + TNP-470 test with drug alone)

Table 2.6 Combination index of chemotherapeutic drugs and TNP-470 on KKU-M213

TNP-470 (µg/mL)	**5FU (µM)**	**Combination index (CI)**
1.1	0.4	0.38376
1.1	4.0	0.45058
1.1	60.0	0.28444
1.78	0.4	0.49480
1.78	4.0	0.23154
1.78	60.0	0.11912
4.6	0.4	0.71774
4.6	4.0	0.31580
TNP-470 (µg/mL)	**Cis (µM)**	**Combination index (CI)**
1.1	1.0	0.61465
1.1	1.86	0.50530
1.1	7.16	0.97241
1.78	1.0	0.39306
1.78	1.86	0.35402
1.78	7.16	0.19094
4.6	1.0	0.66000
4.6	1.86	0.23386
TNP-470 (µg/mL)	**Doxo (µM)**	**Combination index (CI)**
1.1	0.1	0.76346
1.1	0.9	0.56209
1.1	2.4	0.34386
1.78	0.1	0.80849
1.78	0.9	0.37992
1.78	2.4	0.21568
4.6	0.1	1.20784
4.6	0.9	0.76109

Table 2.6 Combination index of chemotherapeutic drugs and TNP-470 on KKU-M213 (Cont.)

TNP-470 (µg/mL)	Gem (µM)	Combination index (CI)
1.1	0.1	0.61387
1.1	0.6	0.44755
1.1	249.0	0.73005
1.78	0.1	0.38044
1.78	0.6	0.31146
1.78	249.0	0.27564
4.6	0.1	0.80406

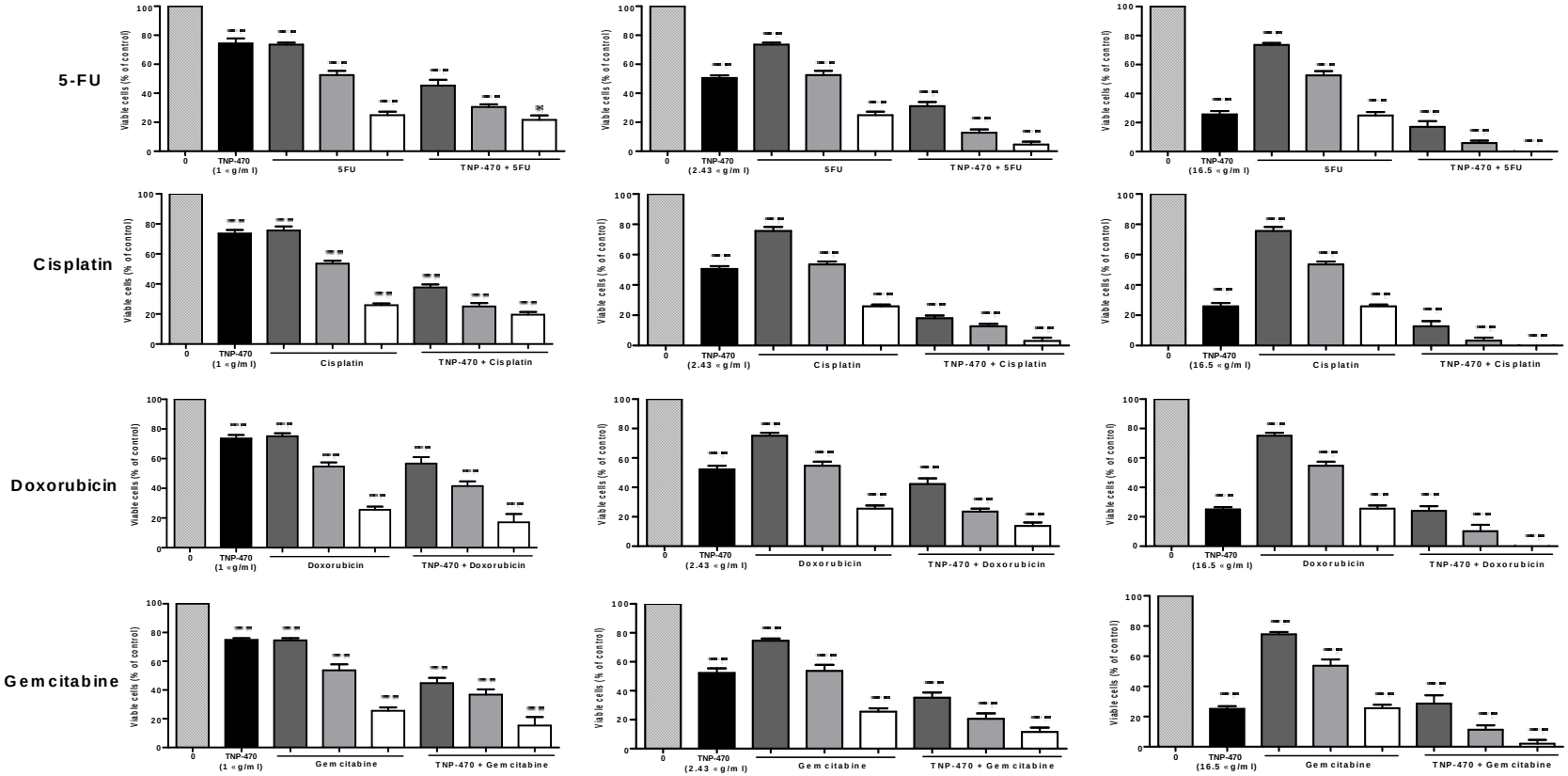

Figure 2.7 Synergistic cytotoxic effect of chemotherapeutic drugs and TNP-470 on KKU-M214, CCA cell line was treated with chemotherapeutic drugs at IC_{25}, IC_{50} and $IC_{75}$with and without TNP-470 at IC_{25}, IC_{50} and IC_{75}

*P < 0.05; **P < 0.01 (a = single agent test with control. b=IC_{25}+TNP-470 test with drug alone. c = IC_{50} + TNP-470 test with drug alone d = IC_{75} + TNP-470 test with drug alone).

Table 2.7 Combination index of chemotherapeutic drugs and TNP-470 on KKU-M214

TNP-470 (µg/mL)	**5FU (µM)**	**Combination index (CI)**
1.0	0.1	0.13846
1.0	6.7	0.15332
1.0	383.5	0.05684
2.43	0.1	0.22918
2.43	6.7	0.04992
2.43	383.5	0.01217
16.5	0.1	0.51929
16.5	6.7	0.10229
TNP-470 (µg/mL)	**Cis (µM)**	**Combination index (CI)**
1.0	0.08	0.15378
1.0	1.91	0.09602
1.0	49.28	0.41208
2.43	0.08	0.08463
2.43	1.91	0.05236
2.43	49.28	0.00620
16.5	0.08	0.33474
16.5	49.28	0.03797
TNP-470 (µg/mL)	**Doxo (µM)**	**Combination index (CI)**
1.0	0.1	0.55797
1.0	2.1	0.91486
1.0	11.0	1.28627
2.43	0.1	0.50870
2.43	2.1	0.23211
2.43	11.0	0.35601
16.5	0.1	0.95487
16.5	2.1	0.23289

Table 2.7 Combination index of chemotherapeutic drugs and TNP-470 on KKU-M214 (Cont.)

TNP-470 (µg/mL)	Gem (µM)	Combination index (CI)
1.0	0.1	0.22102
1.0	27.9	0.25295
1.0	1396.4	0.05032
2.34	0.1	0.28552
2.34	27.9	0.10919
2.34	1396.4	0.04929
16.5	0.1	1.36530
16.5	27.9	0.25553
16.5	1396.4	0.02044

2.4 Discussion

Methionine aminopeptidases (MetAPs) are bifunctional proteins that play a critical role in the regulation of post-translational processing and protein synthesis. These enzymes are responsible for the removal of methionine from the amino-terminus of newly synthesized proteins (Selvakumar et al., 2006). In eukaryotes, two proteins are known to possess MetAP activity, MetAP1 and MetAP2. However, MetAP2 has attracted much more attention than MetAP1 due to the role of MetAP2 in the growth of several cancers (Yin et al., 2012). Many reports documented that inhibition of MetAP2 action by its inhibitors were lethal for cancer cells.

In the current study, we used TNP-470, a MetAP2 inhibitor, to study the growth-inhibitory effects on CCA cells. TNP-470 significantly suppressed the growth of 2 CCA cell lines in a dose-dependent and time dependent manner. The results of our study had demonstrated the same effect of TNP-470 on anti-proliferation of human breast cancer cell lines (KPL-1, MDA-MB-231, and MKL-F) with IC_{50} at 25-35 µg/mL (Singh et al., 1997). However, the CCA cell lines were more sensitive to TNP-470 if comparing to above three breast cancer cell lines.

We next demonstrated the mechanism by which TNP-470 inhibited the growth of CCA cell lines was the induction of apoptosis. The results from flow cytrometry analysis demonstrated that TNP-470 had no affect to the cell cycle of CCA cell lines.

However, the significant amount of apoptotic cells was found under TNP-470 treatment in CCA cell lines. Similar observation is in accordance with previous reports in cancers of melanoma cell (Okrój et al., 2006), neuroblastoma (Wassberg et al., 1999) and colon cancer (Huang et al., 2003).

Furthermore, we examined the molecular mechanism by which TNP-470 induced apoptosis in CCA cell lines. The expression of apoptotic related proteins were determined. p38 is a class of mitogen activated protein kinases that are involved in cell differentiation, apoptosis (Hu et al., 2012; Ren et al., 2005) and autophagy (Grossi and Simone, 2012). Bcl-xL is a Bcl-2 family related gene that can function as a bcl-2 independent regulator of programmed cell death (Boise et al., 1993). Bax is classified as pro-apoptotic member of the Bcl-2 protein family (Oltval et al., 1993). A single copy of Bax induced apoptosis in the absence of Bcl-2. In contrast, overexpression of Bcl-2 still prevented apoptosis in the absence of Bax (Knudson and Korsmeyer, 1997). Caspase3 and caspase9 are a member of the cysteine protease family, which play a crucial role in apoptotic pathways by cleaving a variety of key cellular proteins (Alnemri et al., 1996).

In this report we have first demonstrated that TNP-470 is a potent stimulator of p38 activation. p38 MAP kinase activation is mediated by phosphorylation. There are some evidence for pro-apoptotic and anti-apoptotic role of p38 MAPKs, depending on the cell type and the stimuli (Lenassi and Plemenitaš, 2006; Porras and Guerrero, 2011). The ways by which p38 contributes to an enhanced pro-apoptotic response include different mechanisms such as the phosphorylation and translocation from the cytosol to mitochondria of proteins from the Bcl-2 family, such as Bax, which leads to the release of cytochrome c from the mitochondria (Gomez-Lazaro et al., 2007; Van Laethem et al., 2004). The results revealed that when p38 was phosphorylated, the expression of Bax and Bcl-xL were altered. However, the mechanism by which p38 induces Bax and inhibit Bcl-xL are presently unknown. Alteration of Bax and Bcl-xL on the mitochondria has been shown to reduce mitochondrial membrane potential, enhance cytochrome C release from the mitochondria, thus activating the caspase cascade (Emily et al., 2001; Finucane et al., 1999; Letai, 2011). Results obtained from the western blot analysis showed an increase in the activity of caspase3, confirming the activation of p38, after TNP-470

treatment, modulates the cellular levels of Bax /Bcl-xL and the subsequent activation of programmed cell death.

Recently, several studies reported that TNP-470 capable to sensitize cancer cells to chemotherapeutic agents. However, the chemosensitizing effect of TNP-470 in CCA cells is still unknown. Here we report for the first time that TNP-470 could substantially enhance the anti-tumor efficiency of four chemotherapeutic drugs (5FU, doxorubicin, cisplatin and gemcitabine) against CCA cells. In the current report, combinational index is calculated to determine the interaction between TNP-470 and chemotherapeutic drugs.

The results demonstrated that TNP-470 significantly enhanced the anti-tumor activity of chemotherapeutic drugs (5FU, doxorubicin, cisplatin and gemcitabine) in CCA cell lines, KKU-M213 and KKU-M214. The combination index showed synergistic effect (CI<1) in combination between TNP-470 and chemotherapeutic drugs. Similar observation was found in human colon adenocarcinoma cell line (Fan et al., 2002). TNP-470 increased the anti-tumor activity of cisplatin in pancreatic carcinoma (Shishido et al., 1998) and bladder cancer (Zhang et al., 2002), enhanced the anti-growth of 5FU in uterine carcinosarcoma (Naganuma et al., 2011) and colon cancer (Ogawa et al., 2000). In addition, TNP-470 also enhanced the anti-tumor activity of gemcitabine in bladder cancer cell (MURAMAKI et al., 2004) and paclitaxel in patients with solid tumor (Herbst et al., 2002) This information provides the probability of TNP-470 as potential chemosensitizer for clinical application.

In conclusion, we demonstrated that TNP-470 exhibits anti-tumor activity against human CCA cells by enhancing apoptosis through the activation of p38 pathway. In addition, supplementation of TNP-470 was also enhanced anti-proliferation of chemotherapeutic drugs by given synergistic effect on CCA cell lines. Up to our knowledge, we suggest that TNP-470 might be a potential adjuvant agent to enhance the effect of chemotherapeutic drugs for the treatment of CCA patients in the future.