RESEARCH ARTICLE

TNP-470, A Methionine Aminopeptidase-2 Inhibitor, Inhibits Cell Proliferation, Migration and Invasion of Human Cholangiocarcinoma Cells *In Vitro*

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Abstract

Methionine aminopeptidase-2 (MetAP2) is over-expressed in several cancers, including the cholangiocarcinoma (CCA). We reported previously suppressive effects of fumagillin, a MetAP2 inhibitor, on growth of CCA cell lines. In the present study, we evaluated the anti-proliferative and anti-invasive activities of TNP-470, a fumagillin analogue with higher MetAP2 inhibitory activity, on CCA cell lines (KKU-M213 and KKU-M214). TNP-470 significantly inhibited growth of both lines in a dose and time dependent fashion. Moreover, a sub-toxic dose of TNP-470 significantly reduced migration and invasion of CCA cells. Exploration of the molecular mechanisms by which TNP-470 inhibited growth and metastasis of CCA cell lines demonstrated expression of *c-MYC*, *MMP2* and *MMP9* to be decreased in TNP-470 treated cells. These results suggest that TNP-470 could be a potential therapeutic agent for CCA.

Keywords: TNP-470 - MetAP - MetAP2 - MMP9 - metastasis - angiogenesis inhibitor

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Introduction

Early detection of cholangiocarcinoma (CCA) is difficult since there are no specific symptoms during the early stages of tumor development. As a consequence, the majority of CCA patients present with advanced incurable disease and are not good candidates for curative surgery. Even in those who have undergone complete surgical resection, recurrence is common and the 5-year survival rate is unfavorable (Yamamoto et al., 2011). Novel treatment strategies directed against this malignancy are, therefore, urgently needed.

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 important role of MetAP2 in the growth of several cancers (Yin et al., 2012). Over-expression of MetAP2 was reported in several tumors, such as colon (Selvakumar et al., 2004), B-cell lymphoma (Kanno et al., 2002) and CCA (Sawanyawisuth et al., 2007).

Fumagillin is the first drug known to inhibit specifically MetAP2. At present, there are several MetAP2 inhibitors which are the analog of Fumagillin, e.g., ovalicin, TNP-470 and CDK731. Among these TNP-470 is more attractive, because it is being tested in clinical trials. TNP-470 exerts antitumor activity in endothelial cells and many cancer cells *in vitro* and *in vivo* by induction of cell cycle arrest (Yeh et al., 2000; Zhang et al., 2000), activating apoptosis (Konno, 1999; Zhang et al., 2002) and inhibiting tumor invasion (Qin et al., 1999). The potent enhancement of TNP-470 on the sensitivity and restoration of anticancer drugs in cancer cells has also been reported (Ogawa et al., 2000; Svensson et al, 2002). Moreover, a number of clinical trials of TNP-470 on androgen-independent prostate cancer (Logothetis et al., 2001), non-small-cell lung carcinoma (Herbst et al., 2002) and advanced squamous cell cancer of the cervix (Kudelka et al., 1997) have been reported.

MetAP2 was highly expressed in CCA patient samples and diminution of MetAP2 activity using fumagillin, a specific MetAP2 inhibitor, which effectively reduced cell proliferation of human CCA cell lines (Sawanyawisuth et al., 2007). In the present study, we determined the effect of TNP-470 on growth, migration and invasion of human CCA cell lines. The expression levels of *c-MYC*, *MMP2* and *MMP9* which are the key effectors of growth, migration and invasion processes (Sanceau et al., 2003; Prochownik 2004; Sun et al., 2008; Slack-Davis et al., 2009) were also explored.

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Materials and Methods

CCA cell lines

Two CCA cell lines (KKU-M213 and KKU-M214) were established from primary tumors of CCA patients (Sripa et al., 2005). The cell lines were 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.

Cell proliferation assay

MTT assays were applied to test cell viability. In brief, 3 x 10^3 cells per well were seeded in a 96-well plate and incubated with various concentration of TNP-470 for 24, 48, and 72 h at 37 ˚C, 5% CO_2. For comparison, cells cultured in the absence of TNP-470 were used as a control. After an incubation period, 10 µL MTT (0.5 mg⁄mL final concentration) was added to each well. After 4 h of additional incubation, 100 µL of 0.01 N HCl in isopropanol was added to dissolve the crystals. Absorption at 570 nm was determined by ELISA plate reader (Tecan Austria GmbH, Salburg, Austria).

Migration and Invasion assay

Migration activity of CCA cells was determined using Transwell® insert (8 µm pore size) (Corning, St. Louis, Mo). Briefly, 4 x 10^4 CCA cells in serum-free DMEM medium were seeded into the upper chamber and 10% FBS supplemented DMEM medium was applied at the lower compartment of the chamber. The KKU-M213 and KKU-M214 cells were incubated for 10 and 14 h, respectively. Cells in the upper surface of the filter were scraped off and the migrating cells underneath the filter were fixed with 4% paraformaldehyde for 15 min and stained with 0.4% sulforhodamine B (SRB) in 1% acetic acid. The number of migrating cells was counted under a microscope. Mean values of at least nine fields (10 x magnifications) per sample were determined. The cells were expressed as percentage of the control (untreated group)

For invasion assay, 4 x 10^4 CCA cells in serum-free DMEM medium were seeded into the upper chamber which was pre-coated with Matrigel (40 µg/insert). Complete medium was applied in the lower compartment of the chamber. The number of invading cells was analyzed the same as mentioned in the migration assay.

Real-time RT-PCR

Total RNA of KKU-M213 and KKU-M214 treated with 1.25 µg/mL of TNP-470 for 10 and 14 h, respectively was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of RNA was monitored using 1.5% denaturing agarose gel. A NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) was used to quantify the amount of RNA.

RNA (2 µg) was reverse-transcribed to cDNA using oligo-dT primers and quantitative real-time PCR for *c-MYC, MMP2* and *MMP9* were performed using a LightCycler 480 (Roche Applied Science, Indianapolis, IN). Briefly, the reaction mixture (10 µL) contained template DNA,forward and reverse primers and LightCycler® 480 SYBR Green I Master (Rox) (Roche Diagnostics, Mannheim, Germany). The PCR protocol consisted of an initial denaturation step of 95°C for 5 min, followed by 50 amplification cycles with 10 sec at 95°C, 10 sec at annealing temperature; 60°C and 3 sec at 72°C. β2-microglobulin (*B2M*) was used as an internal control, and cells treated with 0.08% DMSO was used as the vehicle control. Primer sequences are shown in Table 1. Gene expression difference levels were calculated using the $2^{-\Delta\Delta Ct}$ method for relative quantification and expressed as the fold change relatively to the untreated control (TNP-470 non-treated group).

Statistical analysis

The results were presented as a mean± SD for at least three separate experiments. Statistical significance was determined using the Student's T-test where $P < 0.05$ was considered statistically significant.

Results

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

To analyze the anti-proliferative effect of TNP-470, two CCA cell lines, established from different histological types of primary tumors from CCA patients 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. As compared to the control, TNP-470 significantly reduced growth of CCA cell lines in a dose and time dependent manner ($P<0.01$). No significant difference of apoptotic cell numbers was observed between cells treated with TNP-470 and the controls. 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 1).

TNP-470 inhibited migration and invasion of CCA cell lines

The sub-cytoxicity dose of TNP-470 (1.25 µg/mL) was used to determine the effect of TNP-470 on migration and invasion. CCA cells were treated with TNP-470 during the assays of KKU-M213 for 10 h and of KKU-M214 for 14 h. The results showed that TNP-470 significantly reduced the number of migrated cells and invaded cells as compared with the vehicle treated group. TNP-470 decreased the migrated cells of KKU-M213 to 26% and of

Table 1. Nucleotide Sequences of Primers used in this Study

Gene	Forward primer (5'to 3')	Reverse primer (5'to 3')
MMP2	GGTTCATTTGGCGGACTG	AGGCTGGTCAGTGGCTTG
MMP9	GGTGCCTTTGGACACGCA	CCCACTTGGTCCACCTGGTT
c-MYC	ACCACCAGCAGCGACTCTGA	GGGCTGTGAGGAGGTTTGCT
B2M	CTTCAATCTTTGCACTCAAAG	CTTGTGGAACAAAAATAAACC

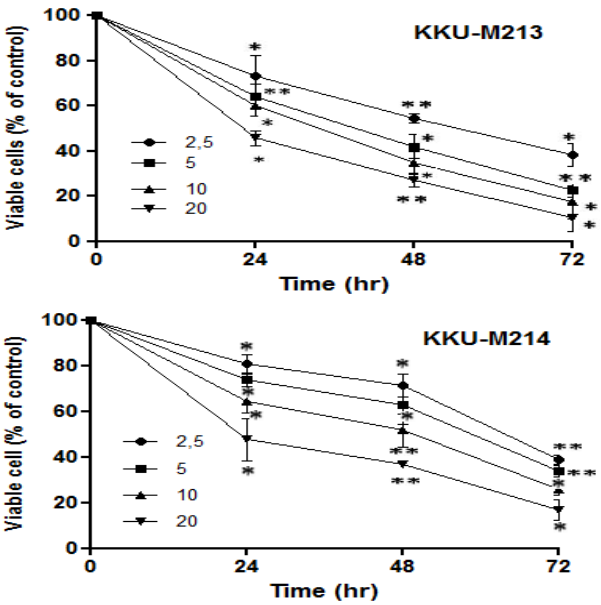

Figure 1. Growth Inhibition Effect of TNP-470 on CCA Cell Lines. CCA cell lines, KKUM213 and KKU-M214, were cultured in the presence of TNP-470 (2.5, 5, 10 and 20 μg/mL). Cell numbers were measured by MTT assay. TNP-470 significantly inhibited the growth of CCA cell lines in a dose dependent and time-dependent manner. *P < 0.05; **P < 0.01.

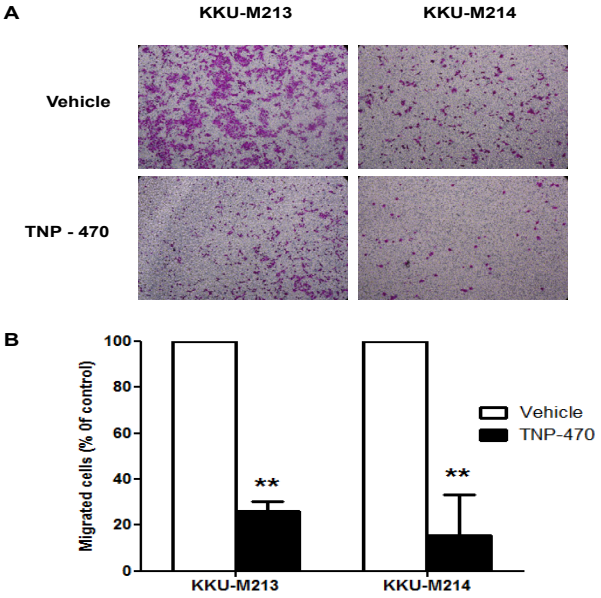

Figure 2. TNP-470 inhibited migration of CCA cell lines. CCA cell lines, KKUM213 and KKU-M214, were cultured in sub-cytoxicity dose of TNP-470 (1.25 μg/mL). The migration assay indicated that TNP-470 significantly inhibits the migration of CCA cell lines. Data are expressed as the means± standard deviations for duplicate assays. *P<0.05; **P<0.01

KKU-M214 to 11% (P < 0.01) (Figure 2). Similarly, TNP-470 also significantly affected cell invasion, the number of invaded cells was reduced to 25% in KKU-M213 (P < 0.01) and to 15% in KKU-M214 (P < 0.01) (Figure 3).

Expressions of growth and metastasis related genes were suppressed in TNP-470 treated cells

To investigate the inhibitory mechanism underlying growth, migration and invasion of TNP-470, the expression levels of c-MYC, MMP2 and MMP9, which are the key

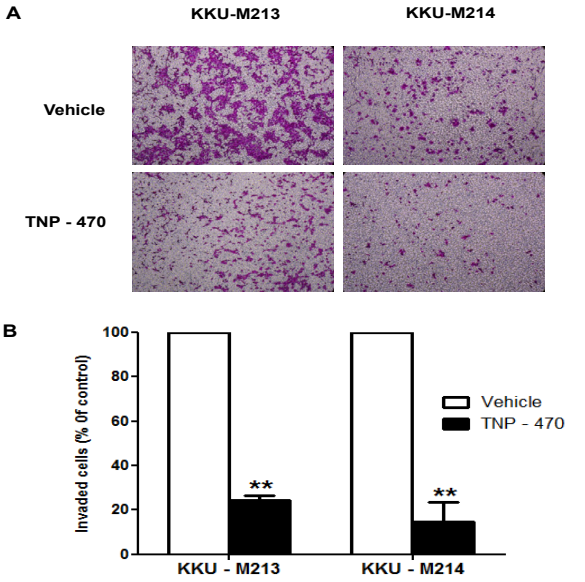

Figure 3. TNP-470 Inhibited Invasion of CCA Cell Lines. CCA cell lines, KKUM213 and KKU-M214, were cultured in sub-cytotoxicity dose of TNP-470 (1.25 μg/mL). The invasion assay indicated that TNP-470 significantly inhibits the migration of CCA cell lines. Data are expressed as the means± standard deviations for duplicate assays. *P < 0.05; **P < 0.01.

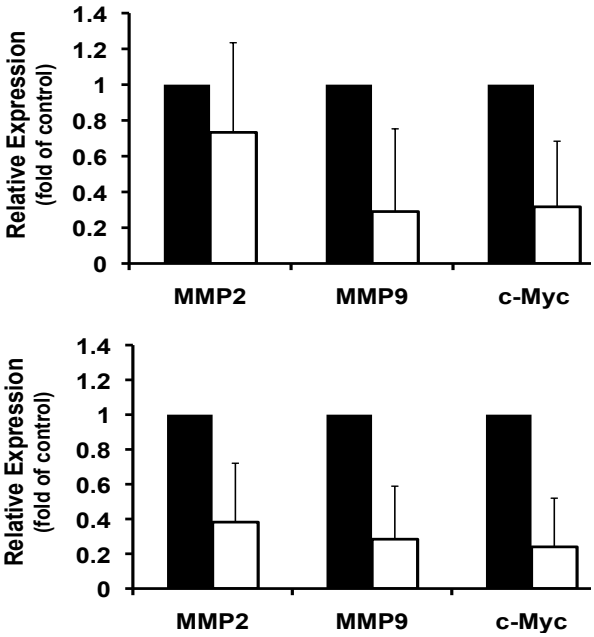

Figure 4. TNP-470 Inhibited the Expression of Growth and Metastasis Associated Genes. Cells were incubated without or with 1.25 μg/mL of TNP-470 for 10 h and 14 h in KKUM213 and KKU-M214, respectively. Down-regulation of genes related to growth and metastasis were found in TNP-470 treated cells. *P<0.05

effectors of growth, migration and invasion processes were determined in KKU-M213 and KKU-M214 treated with 1.25 μg/mL of TNP-470 for 10 and 14 h, respectively. The expression levels of the targeted genes were determined by real-time RT-PCR.

As shown in Figure 4, the relative expressions of *MMP2, MMP9, c-MYC* in TNP-470 treated cells were significantly suppressed compared to the vehicle treated cells.

Discussion

Inhibition of MetAP activity could affect the biological activity of protein, subcellular localization, and degradation which can interfere with the normal cell signal transduction and cell cycle progression. Higher levels of MetAP2 was shown in clinical specimens of CCA patients than normal cells, suggesting a greater dependence on this enzyme by malignant cells for their function and proliferation. Hence, reduction of MetAP2 activity may be more deleterious to cancer than normal cells. Based on this approach, we have previously shown that Fumagillin, a MetAP2 inhibitor, could significantly reduced growth of CCA cell lines. In the present study, we presented the anti-metastatic activity of TNP-470, an analog of Fumagillin, on migration and invasion of human CCA cell lines. The molecular mechanism related to TNP-470 action was also revealed.

The intracellular enzyme MetAP2 becomes a candidate target enzyme for cancer treatment due the association of MetAP2 with particular functions, e.g., cell growth, and angiogenesis (Catalano et al., 2001; Hou et al., 2009). TNP-470, a derivative of the natural product fumagillin, is a potent inhibitor of MetAP2. The safety and efficacy of TNP-470 for the treatment of solid tumors and arthritis have been shown in preclinical trials and several animal studies (Ingber et al., 1990; Shusterman et al., 2001). Of note, TNP-470 has been used in human clinical trials for several malignancy treatments, e.g., AIDs-related Kaposi's sarcoma (Moore et al., 2000), androgen-independent prostate cancer (Logothetis et al., 2001), non-small-cell lung carcinoma (Herbst et al., 2002) and advanced squamous cell cancer of the cervix (Kudelka et al., 1997).

In the present study, the anti-proliferative activity of TNP-470 was clearly shown in two human CCA cell lines-KKU-M213 and KKU-M214. Growth inhibition of TNP-470 was shown to behave in a dose and time dependent fashion and is more likely to be growth arrest rather than induction of apoptosis, as we did not observe the significant amount of apoptotic cells under TNP-470 treatment. The IC_{50} of TNP-470 differs among different tumors. Human CCA cells are more sensitive to TNP-470 compared to other reported cancer cell lines. The IC_{50} of TNP-470 on growth inhibition of CCA cells, at 72 h was 2-3 μg/mL whereas those of 3 different human breast cancer cell lines (KPL-1, MDA-MB-231, and MKL-F) were 25-35 μg/mL (Singh et al., 1997). TNP-470 reduced the growth of endothelial cells more extensively than those of tumor cells (Yamamoto et al., 1994; Kusaka, et al. 1994). These data indicated that TNP-470 also inhibits tumor angiogenesis and may explain why TNP-470 is more effective *in vivo* than *in vitro*.

`With regard to the mechanisms of anti-tumor activity of TNP-470, several reports have indicated that TNP-470 can inhibit the expression of genes involved in the cell cycle (Yeh et al., 2000; Zhang et al., 2000; Mauriz et al., 2007) and metastasis (Kurebayashi et al., 1994; Sedlakova et al., 1999; Kaya et al., 2001). In our current study, the suppression of *c-MYC, MMP2* and *MMP9* were found in TNP-470 treated cell. Down-regulation of these genes caused suppression of growth, migration and invasion in many cancer cells (Sanceau et al., 2003; Prochownik 2004; Sun et al., 2008; Slack-Davis et al., 2009). So far there is no report indicating that the protein products of these affected genes are true substrates of MetAP2, therefore, it is possible that the suppression of these genes may be the consequence of upstream molecules which are the direct substrates of MetAP2.

To date, cyclophilin A, glyceraldehyde-3-phosphate dehydrogenase and 14-3-3 γ are reported as the specific substrates of MetAP2 (Turk et al., 1999; Towbin et al., 2003). Our group has reported the up-regulation of CypA in majority of the CCA tissues (Obchoei et al., 2011). Suppression of CypA expression inhibited proliferation rate of CCA cell lines *in vitro* and reduced tumor growth in a xenografted nude mouse model. The effects of CypA on MMP-9 production and cell invasion via induction of nuclear factor-kB (NF-kB) activity were observed in human monocyte cell line-THP-1 (Yang et al., 2008) and pericytes (Bell et al., 2012). All this information raises the possibility that TNP-470 may exert its effect on cell growth, migration and invasion of CCA cells via CypA and NF-kB which in turn reduces the expression of MMP9.

In conclusion, we demonstrated that the anti-angiogenic agent, TNP-470, exhibits anti-tumor activity against human CCA cells by inhibition of growth, migration and invasion through the suppression of *c-MYC, MMP2* and *MMP9* expression. According to the high expression of MetAP2 in CCA patients' tissues and the current data reported in this study, we suggest that TNP-470 can be a useful candidate of anti-cancer drug for the treatment of CCA patients in the future.

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