Myosin light chain kinase inhibitor, ML-7, suppressed cholangiocarcinoma cell survival but not cell invasion and matrix metalloproteinase-2 secretion

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Abstract
Myosin regulatory light chain (MRLC) regulates myosin activity which underlies various activities of the cells including migration invasion and survival of cells. The function of MRLC is activated by phosphorylation catalyzed by myosin light chain kinase (MLCK). In this study, the role of MRLC in cholangiocarcinoma cells was investigated by inhibiting the MRLC activity using myosin light chain kinase, ML-7, a MLCK inhibitor. ML-7 reduced MRLC phosphorylation dose dependently, correlating with suppression of cell viability as determined by MTT assay. However, ML-7 was not significantly reduction of invasiveness and MMP-2 gelatinase activity at sub-toxic dosage. These data indicate that MRLC activity is vital to cell viability but not significantly reduce the metastatic properties in cholangiocarcinoma cells.

Keywords**:** *cholangiocarcinoma; invasion; MRLC; survival*

1. Introduction

Cholangiocarinoma (CCA) is a malignancy of the biliary tract. It can occur at any part along the intrahepatic or extrahepatic biliary tree excluding gallbladder and ampular of vater (Bartella, 2015). Epidemiological studies revealed geographical clusters of choloangiocarcinoma (CCA) in endemic regions of liver fluke *O. viverini* or its close relative (Kamsa-ard, 2018). Thailand is the highest incidence rate of cholangiocarcinoma of the world. The incidence rate of chologiocarcinoma and liver cancer between 1988 and 2012 was between 40.5 and 33.9 per 1000,000 in males and 16.3 and 12.9 per 100000 in females (Kamsa-ard, 2018). The progression of a tumor cell to invasive and metastatic growth is one of the major causes of poor clinical outcome, treatment failure and death in cancer patients. Metastasis of cancer is a multi-step process by dynamic reorganization of actin-myosin cytoskeleton and remodeling of the extracellular matrix which allowing the malignant cells leave the primary site and spread to distant locations throughout the body (Unbekandt, 2014). Contraction of actin-myosin cytoskeletal structures generates the mechanical force required for cell motility and invasion (Unbekandt, 2014). Myosin is one of the most important and abundant contractile proteins of the cell (Heissler SM, 2014). It's activity is regulated by myosin light chain kinase (MLCK), an enzyme which activates myosin by phosphorylating it on its regulatory light chain (MRLC) (Heissler SM, 2014). Suppression of MLCK has been shown to inhibit motility and invasion of rat prostatic adenocarcinoma and glioma cell line (Gillespie GY, 1999, Tohtong R, 2003). However, the metastasis potential of breast cancer cells is increased by the loss of MLCK (Kim DY, 2016). In addition, myosin regulation in cell survival have been report (Connell LE, 2005). The role of myosin II in Cholangiocarcinoma is still unclear. So, in this study, we investigated the role of myosin II by inhibition of MRLC phosphorylation in a Thai cholangiocarcinoma cell line by focus on metastasis and survival properties.

2. Experimental Objectives

We determined if myosin II, plays a role in regulating the invasiveness or survival properties in cancer cells by inhibiting myosin regulatory light chain (MRLC) function using myosin light chain kinase (MLCK) inhibitor, ML-7, and assess the effects on cell viability and invasiveness properties *in vitro* by using a human cholangiocarcinoma (KKU-100) cell line as a model.

3. Materials and Methods

3. 1 Cell line and cell culture

KKU-100 was developed from a 65-year-old Thai woman patient who was diagnosed of cholangiocarcinoma (Sripa 2005). The histopathological investigation confirmed a poorly differentiated tubular adenocarcinoma cell type of cholangiocarcinoma associated with *Opisthorchis viverrini*. This cell line was grown as a monolayer in HAM's-F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml of penicillin G and 100 ug/ml streptomycin. The cancer cells were incubated at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 in air.

3.2 Cytotoxicity assay

Approximately $5x10^3$ cells of KKU-100 were grown in 96-well plates overnight (Corning, New York, U.S.A.). After 24 hours, various concentration of 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7) (0, 0.1, 1, 10, 25, 50, 75, 100, 150, and 200 μM) were plated out in triplicate. The toxicity profiles of the compound were assessed using 3-(4, 5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) solution (USB, Cleveland, OH, U.S.A.) After 6 hours of incubation, MTT (5 mg/ml) was added to the individual wells, followed by an incubation for 4 hours at 37^{o}C in a humidified atmosphere containing 5% CO_2 before removal of the media. DMSO was then added into each well to solubilize the formazan crystals. The absorbance was read at 540 nm using ELISA microplate reader (Multiscan MCC/340p, version 2.20). The concentration which inhibited 50% of cellular growth (IC_{50} value) was determines by KALEIDAGRAPH program.

3.3 Western blot analysis

KKU-100 cells were seeded in 60 mm dish and treated with 0, 75, 150 μM of ML–7 for 6 hours. The total protein of the cells was extracted with urea glycerol lysis buffer. The protein lysates were centrifuged at 12,000 x g for 10 min. The proteins were separated by gel electrophoresis by using 25 V at 4^{o}C for 1.5 hours, transferred to nitrocellulose membranes, blocked with 5% skim milk in TBS-Tween buffer for 1 hour at room temperature. The membrane was incubated with primary antibody specific to myosin light chain II antibody (sigma) overnight at 4^{o}C and then incubated with horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature. The protein bands were visualized by autoradiography.

3.4 *In vitro* invasion Assay

Cancer cell invasiveness was determined using transwell chamber (Costar, Cambridge, MA, USA) coated with 0.3 □g/□l matrigel (Collaborative Research Inc. Bedford, MA, USA). Approximately $1x10^5$ cells in complete culture media containing various concentration of ML-7 were added to the upper compartment of the transwell. The lower part contains 600 □l of 10% FBS medium. After incubation of the chambers at 37 ^{o} C, 5% CO_2 atmosphere for 6 hr, non-invasive cells on the upper filter were removed using cotton swabs and the cells that have invaded into the lower surface were fixed with 25 % methanol for 30 min, and stain with 0.5% crystal violet for 1 hr. The number of invaded cells in five random x 10 microscopic fields were counted under microscope.

3.5 Gelatin zymography assay

Approximately, $1x10^5$ cells of KKU-100 were treated with various concentration of ML-7 in medium without FBS for 6 hours. The conditioned medium was assayed for gelatinase activity by gelatin zymography assay. Briefly, fivefold volume of the conditioned medium was mixed with 5x SDS-sample buffer before separation in 12% SDS-PAGE containing 1 mg/ml gelatin. After electrophoresis at 200 V at 4 ^oC for 1 h, the gel was washed in 2.5 % (w/v) TritonX – 100 twice, 30 min each time. The gel was then incubated in buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 1 □M $ZnCl_2$, 1% Triton X-100 and 0.02% NaN_3 for 18 hours in 37 ^oC water bath. The gel was then stained with 0.5% Coomassie blue R 250. Gelatinolytic activity was presented as clear bands the blue background of the coomassie-stained gel.

4. Results

4.1 The effects of ML-7 (MLCK inhibitor) on cell viability

Effect of MLCK inhibitor (ML-7) on *in vitro* cell viability was performed by using MTT assay and KKU-100 cell line as a model. These experiments were performed 3 times, each done in duplicates. The inhibitory concentration at 50% (IC_{50}) was analyzed by non-linear sigmoidal algorithms in KALEIDAGRAPH (version 4.0). Cell viability was reduced dose-dependently, showing an IC_{50} of 90 μM. At 200 μM almost cells died after 6 hours of incubation (Figure 1).

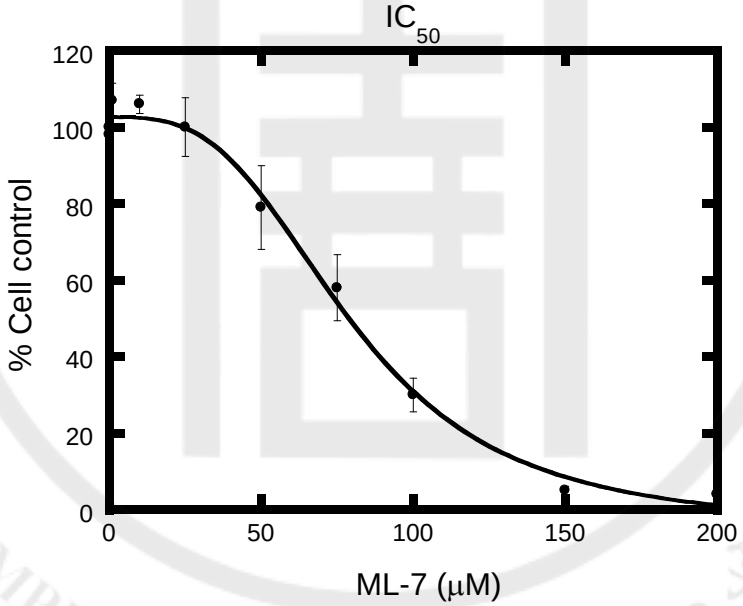

Figure 1. The IC_{50} of ML-7 treatment on KKU-100 analyzed by KALEIDAGRAPH

4.2 The effects of MLCK inhibitor on MRLC phosphorylation

Phosphorylated forms of the MRLC were analyzed by urea glycerol – PAGE which separate proteins on the basis of size and charge. The more phosphorylation (negatively–charge), the greater mobility of protein. Cholangiocarcinoma cell line (KKU 100) were treated with 0, 75, 150 μM of ML–7 for 6 hours at 37^oC, 5%CO_2 before the protein extract was prepared. Phosphorylated forms of MRLC appeared as 5 bands when probed with MRLC antibody. However, treatment with 75 and 150 □M of ML-7 resulted in a reduction of MRLC bands to 4 and 2 bands, respectively (Figure 2).

ML-7 0 75 150 μM

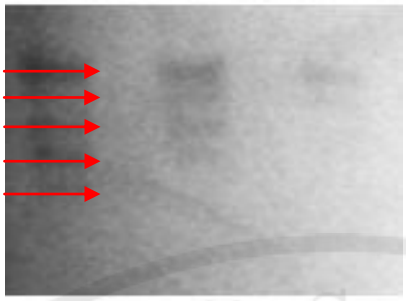

Figure 2. Immunoblot detection of MRLC phosphorylation after ML-7 treatment

4.3 The effects of myosin light chain kinase inhibitor (ML-7) on cancer cell invasiveness

Since Figure 1 showed that ML-7 reduced KKU-100 cell viability, we next determined if ML-7 had an effect on cell invasiveness, a key characteristics of metastatic cancer using *in vitro* Transwell assay. ML-7 up to 75 □M was used to treat KKU-100 cells, at which concentration cell viability was reduced to approximately 60%. However, cell invasion was not significantly reduced by ML-7 at a sub-toxic doses. ($p>0.05$, One-way ANOVA) (Figure 3).

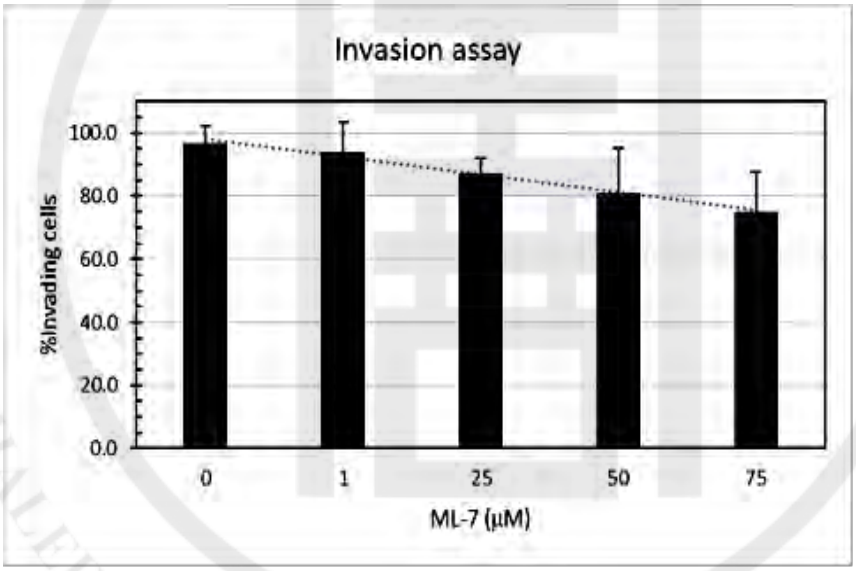

Figure 3. Effects of MLCK inhibitor (ML-7) on *in vitro* invasion of KKU-100 cell line.

4.4 Effects of MLCK inhibitor on MMP-2 secretion

Besides migration, extracellular matrix degradation is another key factor needed for cell invasiveness. We determined the levels of MMP-2 in the conditioned medium of KKU-100 cells using gelatin zymography. Although 50 and 75 □M ML-7 slightly reduced secreted MMP-2 level, this inhibitory effect was not statistically significant ($p>0.05$, One-way ANOVA) (Figure 4).

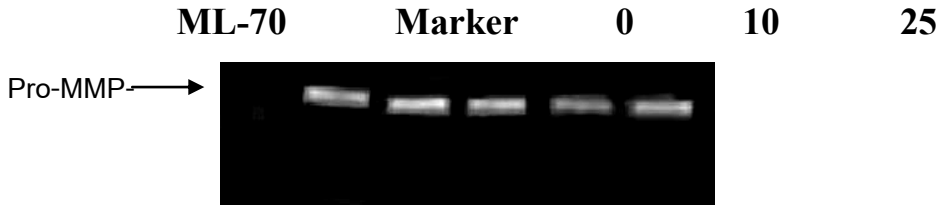

Figure 4 Gelatin Zymography of KKU-100 treated with ML –7. Each lane represents gelatinase activity after treated with 0, 10, 25, 50 and 75 □M of ML-7 respectively.

5. Discussion and conclusion

Myosins are involved in a myriad of functions such as muscle contraction, cytokinesis, adhesion, migration and survival (Heissle SM, 2014, Connell LE, 2005). The important of myosin regulation in cell function is depended by the multiple signal transduction pathways that control myosin regulatory light chain (MRLC) phosphorylation. In this study, we determined the role of MRLC using KKU-100, a Thai patient cholangiocarcinoma cell line as a model and using ML-7, a MLCK inhibitor for inhibition of MRLC phosphorylation. Our data indicated that ML-7 dose-dependently reduced MRLC phosphorylation and cell viability, but it not significantly reduce invasion and gelatinase activities of KKU-100 cell at sub-toxic doses. This finding is in contrast to previous report, where ML-7 significantly reduced the invasiveness of a prostate cancer cell line, MLL, at sub-toxic dose (Tohtong R, 2003). A studies of glioma cell line showed that ML-7 treatment resulted in a dose-dependent reduction of migration (Gillespie GY, 1999). However, the metastasis potential of breast cancer cells is increased by the loss of MLCK because changing of extracellular signal-regulated kinase and c-Jun N-terminal kinase signaling pathway (Kim DY, 2016). In addition, inhibition of myosin light chain kinase (MLCK) in breast epithelial cell and Ras-transformed breast epithelial cell can induce apoptosis (Connell LC, 2005). In lung cancer cell line, knockdown of myosin VI makes cancer cell susceptible to apoptosis upon DNA damage (Jung EJ, 2006). Our result supported that in a cholangiocarcinoma cell line, myosin II is important for cell survival but not curcial in metastasis properties. However, studied with more than one cholangiocarcinoma cell line and investigated more variety of signaling pathways during MLCK inhibition or molecular knockdown of MLCK to confirm this investigation is warranted.

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