RESEARCH ARTICLE

# Histone Deacetylases and their Inhibitors as Potential Therapeutic Drugs for Cholangiocarcinoma - Cell Line Findings

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## Abstract

**Histone deacetylation mediated by histone deacetylases (HDACs) has been reported as one of the epigenetic mechanisms associated with tumorigenesis. The poor responsiveness of anticancer drugs found with cholangiocarcinoma (CCA) leads to short survival rate. We aimed to investigate mRNA expression of HDACs class I and II, and the effect of HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA), in CCA *in vitro*. Expression of HDACs was studied in CCA cell lines (M213, M214 and KKU-100) and an immortal cholangiocyte (MMNK1) by semi-quantitative reverse transcription-PCR. SAHA and VPA, as well as a classical chemotherapeutic drug 5-fluorouracil (5-FU) were used in this study. Cell proliferation was determined by sulforhodamine assay. $IC_{50}$ and $IC_{20}$ were then analyzed for each agent and cell line. Moreover, synergistic potential of VPA or SAHA in combination with 5-FU at subtoxic dose ($IC_{20}$) of each agent was also evaluated. Statistic difference of HDACs expression or cell proliferation in each experimental condition was analyzed by Student's t-test. The result demonstrated that HDACs were expressed in all studied cell types. Both SAHA and VPA inhibited cell proliferation in a dose-dependent manner. Interestingly, KKU-100 which was less sensitive to classical chemotherapeutic 5-FU was highly sensitive to HDAC inhibitors. Simultaneous combination of subtoxic doses of HDAC inhibitors and 5-FU significantly inhibited cell proliferation in CCA cell lines compared to single agent treatment ($P \leq 0.01$), while sequentially combined treatments were less effective. The present study showed inhibitory effects of HDACIs on cell proliferation in CCA cell lines, with synergistic antitumor potential demonstrated by simultaneous combination of VPA or SAHA with 5-FU, suggesting a novel alternative therapeutic strategy in effective treatment of CCA.**

**Keywords:** Cholangiocarcinoma - histone deacetylase - inhibitor - epigenetics - cell lines

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## Introduction

For a fatal malignancy of biliary epithelium called cholangiocarcinoma (CCA), surgical resection is a potentially curative option but a vast majority of patients is mostly diagnosed at the late stages of the disease and showing poorer survival. Nonetheless, the first line treatment regimen is still an unmet need. This might lead to the fact that few CCA patients are suitable for the surgery as well as the limitation of other therapeutic modalities (Patel, 2006; Sia et al., 2013; Zabron et al., 2013). In addition, the palliative chemotherapy and radiotherapy have been used as adjuvant therapy for this cancer type. Various chemotherapeutic agents e.g. 5-fluorouracil (5-FU), gemcitabine, and cisplatin, either alone or in combination, have been widely used in CCA (Hejna et al., 1998; Khan et al., 2002; Thongprasert, 2005). However, the previous clinical studies have demonstrated that chemotherapeutic response rate of CCA is relatively poor with a partial response approximately 10-20% (Patt et al., 2001; Martin et al., 2003; Lee et al., 2004). Therefore, new agents and innovative approaches of therapy are important subjects focusing in effective treatment of CCA.

Histone deacetylases (HDACs) mediate deacetylation of acetylated histones resulting in chromatin condensation and transcriptional repression, particularly in tumor suppressor genes which are associated with tumorigenesis (Shukla et al., 2008). HDACs are divided into class I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 7, 9, and 10), class III (sirtuins; SIRTs), and class IV (HDAC11). The zinc-dependent enzymes, class I and II HDACs are widely studied and reported to be associated with cancer pathogenesis (Balasubramanian et al., 2009; Rikiishi, 2011). Moreover, they are found to be overexpressed in various types of cancer (Lin et al., 1998; Patra et al., 2001; Weichert et al., 2008; Fritsche et al., 2009; Mutze et al., 2010). Currently, there is little knowledge related to roles of histone modifications in carcinogenesis and

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pathogenesis of CCA (Limpaiboon, 2012).

HDAC inhibitors (HDACIs) inhibit the deacetylation of histones and weaken the histone-DNA interactions, thereby permitting a more relaxed conformation of chromatin which contributes to increasing gene transcription and enabling to induce differentiation, cell cycle arrest, and apoptosis of tumor cells (Vigushin et al., 2002). Recently, HDACIs are emerging as an exciting new class of potential anticancer agents for the treatment of solid and hematological malignancies with relative resistance in normal cells (Kelly et al., 2005). HDACIs exert their effect by triggering both mitochondria-mediated apoptosis and caspase-independent autophagic cell death (Shao et al., 2004). Various HDACIs have been under the multiple Phase I, II and III clinical trials in several human cancers, for instance trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA or vorinostat), LAQ-824/LBH 589, valproic acid (VPA), phenylbutyrate, and MS-275 (Pan et al., 2007). In addition, HDACIs have been shown to enhance efficacy of other treatment modalities, for example chemotherapy and radiotherapy by which HDACIs mediate sensitization of DNA-damaging agents via chromatin relaxation making it accessible for binding of DNA-damaging chemotherapeutic agents (Stiborova et al., 2012). Among HDACIs, SAHA has been shown the most promising prospective anticancer activity against many tumor types at well tolerated doses by the patients (Marks et al., 2005; Kumagai et al., 2007). It is the first HDACI approved by the U.S. Food and Drug Administration (FDA) to treat T cell cutaneous lymphoma (Sharma et al., 2010). Another interesting agent, VPA has been used in the treatment of epilepsy for almost three decades. It has been shown to have activity as a HDACI and anticancer agent widely used in several cancer models (Xia et al., 2006).

In the present study, the expression profile of *HDAC* class I and II genes in a panel of CCA cell lines as well as an immortal biliary epithelial cell line was investigated. In addition, the authors also determined the anticancer effect of HDACIs (SAHA and VPA) which inhibit class I and II HDACs on cell proliferation either as a single agent or combination with classical chemotherapeutic drug, 5-FU.

## Materials and Methods

*Cell lines and agents*

A panel of CCA derived cell lines (M213, M214 and KKU-100) and an immortal biliary cell line (MMNK1) were used in this study. All CCA cell lines were established in the Liver fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University (Khon Kaen, Thailand). MMNK1 was kindly provided by Dr. Naoya Kobayashi (Department of Gastroenterological Surgery, Transplant and Surgical Oncology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan). Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL, Ontario, Canada) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin (Gibco/Invitrogen, Grand Island, NY, USA). Cells were incubated at 37°C with humidified atmosphere of 5% $CO_2$.

Two HDACIs including VPA and SAHA were purchased from Cayman Chemical (Ann Arbor, MI, USA). Stock VPA solution (100 mM) was freshly prepared in sterile phosphate buffer saline (PBS). SAHA was dissolved in a 2:1 mixture of dimethyl sulfoxide (DMSO): PBS to prepare a stock solution (10 mM) and stored at -20°C until used. A classical chemotherapeutic agent 5-FU was purchased from Pharmachemie BV (Haarlem, Netherlands) and freshly prepared by diluting with culture medium into optimal concentration before used.

*RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)*

RNA was extracted from all cell lines using SV Total RNA Isolation System (Promega, Madison, WI, USA). For cDNA synthesis, 1 $\mu$g of total RNA was reverse transcribed in a 20 $\mu$L of PCR reaction using ImProm-II™ Reverse Transcription System (Promega) following the manufacturing protocol. Specific primers for expression profile of human HDACs (Class I and II) were designed from sequences available in the Genbank database using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) as detailed in Table 1. The housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize the amount of HDAC transcripts in each reaction as following primer sequences; 5'-ACAGTCCATGCCATCACTGCC-3' (forward) and 5'-GCCTGCTTCACCACCTTCTTG-3' (reverse) with amplicon size of 266 base pairs (bp). A total volume of 25 $\mu$L PCR reaction was performed using a G-Storm GS482 thermo cycler (GRI, Rayne, UK). PCR products were then separated on a 2.0% agarose

**Table 1. Sequences of Specific Primers for RT-PCR of Human HDACs Class I and II**

| HDACs | | Forward (5'-> 3') | Reverse (5'-> 3') | Product (bp) |
|---|---|---|---|---|
| Class I: | HDAC1 | ACCATGGTGACGGCGTGGAA | TGCCAGCCCCGATATCCCGTA | 120 |
| | HDAC2 | ACTGGCGGTTCAGTTGCTGG | AGCATGATGTAATCCTCCAGCCCA | 87 |
| | HDAC3 | TCCACTACGGAGCTGGACACCC | TCCTCGGAGTGGAAGCGGCA | 148 |
| | HDAC8 | CCCGAGTATGTCAGTATGTGTGA | TGCAGTGCATATGCTTCAA | 89 |
| Class II: | HDAC4 | ACCACATGCCCAGCACGGTGGA | AGTGAGAACTGGTGGTCCAGGCG | 100 |
| | HDAC5 | TGGTCCTAGTCTCCGCCGGG | AGTGGCCAAAACATCTGGCGGT | 90 |
| | HDAC6 | ACCAGGTGGGGATGCGGGAT | GCTGGAACTCGAGGGCGACTG | 78 |
| | HDAC7 | CGGCGCTGATGGGACCCAGGTGA | GGCTGAGGGCCTGGTGTGTCT | 99 |
| | HDAC9 | ACGAGAAAGGGCAGTGGCAAG | CCAGAGCTTGGGATGGC | 130 |
| | HDAC10 | CGGCTGCCAACGGGTTCTGT | AAGGACGCTGGGGTCATCCTCA | 155 |

gel electrophoresis, stained with ethidium bromide and visualized under UV illumination. PCR band intensity was evaluated for level of mRNA expression using software of GelAnalyzer (2010a). The RT-PCR amplification was carried out in duplicate for individual gene in each cell line.

*Cell proliferation assay*

Cell proliferation was determined by sulforhodamine B (SRB) assay based on the measurement of cellular protein content as previously described (Vichai et al., 2006). Briefly, cells ($1x10^4$ cells/well) were seeded in triplicate into a 96-well flat bottom plate and allowed to adhere for 24 hours (day 0). Then, 100 $\mu$L of medium containing varied concentrations of drugs were added to each well. In our study, these cell lines were treated with HDACIs as following final concentrations: 0, 0.5, 1, 2, 4, 8 and 12 mM of VPA as well as 0, 0.25, 0.5, 1, 2, 4, 8 and 12 $\mu$M of SAHA, respectively. Moreover, the panel of tested cell lines was also treated with varied concentrations of 5-FU (Pharmachemie BV) up to 80 $\mu$M or without as an untreated control. After 72-hour incubation, cell monolayer was fixed with 10% (W/V) trichloroacetic acid and stained with sulforhodamine B for 30 minutes, before washing repeatedly with 1% (V/V) acetic acid to remove excess dye. The protein-bound dye was dissolved in 10 mM Tris base solution for absorbance determination at 510 nm by a microplate reader (Tecan Ltd., Reading, UK). Percentage of cell proliferation was calculated ([mean $OD_{sample}$-mean $OD_{day0}$/mean $OD_{negative\ control}$-mean $OD_{day0}$]x100) and represented as means of at least three independent experiments±SD. Inhibitory concentration at 50% ($IC_{50}$) and 20% ($IC_{20}$) was then determined for each agent and cell line.

*Combined treatment of subtoxic doses of VPA or SAHA and 5-FU*

The potential of VPA or SAHA in combination with 5-FU in a subtoxic dose of each agent was also investigated. A subtoxic concentration ($IC_{20}$) of each agent against the immortal cholangiocyte MMNK1 was used in the combined treatments. The cell proliferation was then determined by SRB assay as described above.

*Statistical analysis*

The statistical analysis was performed using SPSS software (SPSS version 16.0, Chicago, IL). The difference of HDAC mRNA expression levels or cell proliferation in each experimental condition was analyzed by Student's t-test. Two-sided P≤0.01 is considered statistically significant.

## Results

*mRNA expression profile of HDAC class I and II in CCA cell lines and MMNK1*

To address whether histone deacetylation is one of epigenetic mechanisms mediated aberrantly silenced genes in CCA, the authors investigated mRNA expression profiles of *HDACs* class I and II in an immortal biliary cell MMNK1 and CCA cell lines using RT-PCR. The result showed that *HDACs* were expressed in all cells tested (Figure 1A). The relative mRNA expression of individual *HDAC/GAPDH* revealed no significant difference among a panel of CCA cell lines and MMNK1 (Student's t-test) (Figure 1B).

*HDAC inhibitors inhibit cell proliferation in CCA cell lines and MMNK1*

Since HDACs were expressed in all cells tested, inhibition of HDAC activities should affect tumor cell

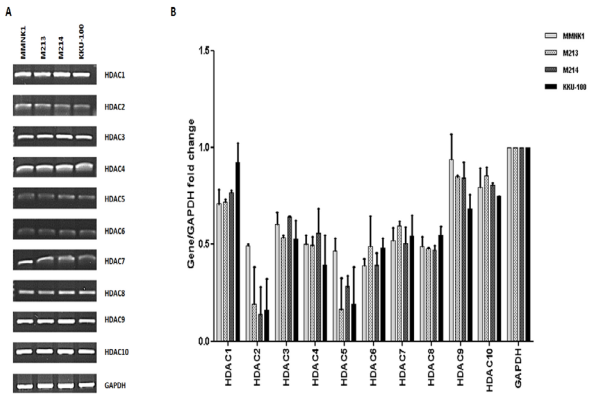


**Figure 1. RT-PCR Expression Analysis of HDAC Encoding Genes in MMNK1 and the Tested Panel of Human CCA Cell Lines (M213, M214 and KKU-100).** A) Representative result of RT-PCR products amplified by specific primers of HDACs profile. B) Most of HDACs encoding genes were less expressed than the housekeeping gene GAPDH. No significant difference of expression was found in any gene between a panel of CCA cell lines and MMNK1

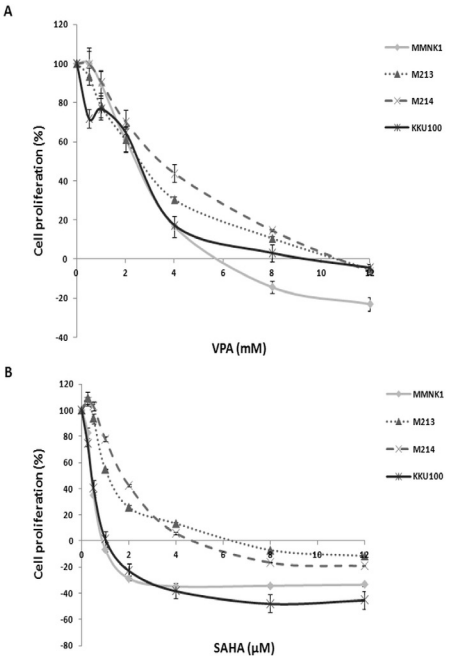


**Figure 2. HDACIs Induced Growth Inhibition in a Spectrum of Cell Lines.** Each cell line (MMNK1, M213, M214 or KKU-100) was seeded into 96-well flat bottom plates ($1x10^4$ cells/well) in triplicate and allowed to adhere for 24 hours. Then, cell lines were treated with HDACIs as following final concentrations; A. VPA: 0, 0.5, 1, 2, 4, 8 and 12 mM, respectively, or B. SAHA: 0, 0.25, 0.5, 1, 2, 4, 8 and 12 $\mu$M, respectively. After incubation period of 72 hours, cell proliferation was determined by sulforhodamine B assay

characteristics such as cell proliferation. Our study showed that VPA and SAHA could inhibit cell proliferation in a dose-dependent manner in human CCA cell lines (M213, M214 and KKU-100) and MMNK1 (Figures 2A and B). $IC_{50}$ and $IC_{20}$ (subtoxic dose) were determined for each agent and cell line (Table 2). The ranges of $IC_{50}$ of VPA and SAHA in all cell lines were 2.2-3.0 mM and 0.4-1.6 $\mu$M, respectively. Moreover, the subtoxic dose of both agents which rarely affected cell proliferation was determined as $IC_{20}$ values. The ranges of $IC_{20}$ were 0.8-1.3 mM of VPA and 0.2-0.7 $\mu$M of SAHA in all tested cells as shown in Table 2. In addition, the $IC_{20}$ and $IC_{50}$ of classical chemotherapeutic drug 5-FU were also evaluated in each cell line. The result showed that MMNK1 and KKU-100 with less sensitivity to 5-FU (rank 3 and 4) tended to be highly sensitive to HDACI treatments (rank 1 and 2) whereas highly sensitive to 5-FU cells, M213 and M214, were conversely responsive (Table 2).

*Combined treatments of subtoxic doses of VPA or SAHA and 5-FU effectively inhibit cell proliferation*

To study the synergistic potential of HDACIs and 5-FU as anticancer agents in CCA cell lines, the subtoxic doses of VPA, SAHA, and 5-FU at 1 mM, 0.25 $\mu$M, and 8 $\mu$M, respectively were used to determine their inhibition effect on cell proliferation either simultaneously (co-cultured for 72 hours) or sequentially combined treatment (36 hours of HDACIs followed by 36 hours of 5-FU). The result demonstrated that simultaneously combined treatments of subtoxic doses VPA and 5-FU, or SAHA and 5-FU were significantly decreased cell proliferation in CCA cell lines and an immortal cholangiocyte MMNK1 compared to single agent (5FU, VPA or SAHA) treatment ($P \leq 0.01$) as shown in Figure 3. Moreover, the sequential treatments of those tested drugs also significantly inhibited cell growth in M213 and M214 but not MMNK1 and KKU-100 when compared with single agent VPA or SAHA ($P \leq 0.01$) (Figure 3).

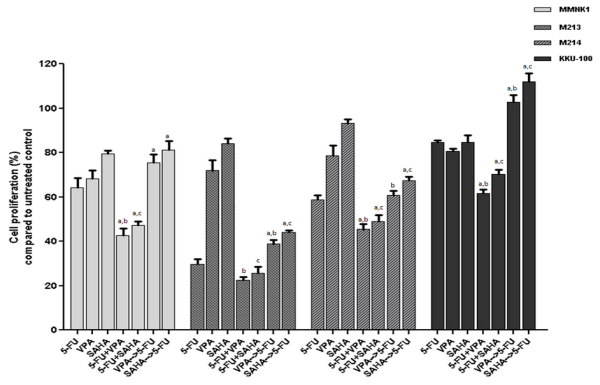


**Figure 3. Effect of Chemotherapeutic Treatments with Subtoxic Concentration of 5-FU, VPA or SAHA and Combinations on Cell Proliferation of MMNK1 and a Panel of CCA Cell Lines.** Simultaneously combined treatments of subtoxic doses of VPA (1 mM) and 5-FU (8 $\mu$M), or SAHA (0.25 $\mu$M) and 5-FU (8 $\mu$M) were significantly inhibited cell proliferation in CCA cell lines and MMNK1 compared to single agent used ($P \leq 0.01$). Moreover, sequentially combined treatments of those tested agents were also significantly inhibited proliferation of M213 and M214 but not MMNK1 and KKU-100 when compared with single VPA or SAHA used ($P \leq 0.01$). a, b, and c represents statistical significance with $P \leq 0.01$ versus single treatment with 5-FU, VPA, and SAHA, respectively

## Discussion

The present study provides expression profile of *HDACs* class I and II in human CCA and immortal biliary cell lines. Although our result did not show the different overexpression pattern of any HDAC analyzed in CCA compared to immortal cholangiocyte MMNK1, the result agreed with the previous report in which HDAC class I (HDAC1 and HDAC2) was expressed in both carcinoma and normal epithelial tissues (Yamaguchi et al., 2010). The previous study in intrahepatic CCA tissue samples showed that HDAC1 expression is significantly correlated with advance tumor stage, lymph node metastasis, and vascular invasion. Moreover, it has been found that patients with HDAC1 positive have 5-year survival and disease free survival shorter than HDAC1 negative group (Morine et al., 2012). The expression profile of HDACs in clinical samples is warranted to get insight into their epigenetically crucial role in CCA.

Our study showed the proliferation-inhibitory effect of VPA and SAHA on CCA cell lines and the immortal biliary epithelium in a dose-dependent manner. It supports the anticancer potential of these inhibitors in human CCA as well as previously reported in other cancers (Cinatl et al., 1997; Blaheta et al., 2002; Butler et al., 2002; Xia et al., 2006; Venkataramani et al., 2010; Chen et al., 2011; Yamaguchi et al., 2010; Iwahashi et al., 2011). In addition, our study showed that CCA cell lines with low sensitivity to 5-FU tended to be highly sensitive to VPA or SAHA whereas 5-FU sensitive CCA cell lines were conversely responsive to both HDACIs. This finding suggests that the application of VPA or SAHA should be potentially exploited in 5-FU resistant CCA. Moreover, simultaneously combined treatments of subtoxic concentration of HDACIs and 5-FU significantly inhibited cell proliferation in the panel of tested cell lines when compared to single agent used as confirmed with the previous study in CCA (Iwahashi et al., 2011) whereas sequentially combined treatments showed lower

**Table 2. $IC_{20}$ and $IC_{50}$ of VPA, SAHA and 5-FU Treated in MMNK1 and CCA Cell Lines**

| Cell line | VPA (mM) | | | SAHA ($\mu$M) | | | 5-FU ($\mu$M) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $IC_{20}$±SD | $IC_{50}$±SD | Rank* | $IC_{20}$±SD | $IC_{50}$±SD | Rank* | $IC_{20}$±SD | $IC_{50}$±SD | Rank* |
| MMNK1 | 1.12±0.11 | 2.19±0.15 | 1 | 0.20±0.02 | 0.39±0.02 | 2 | 7.93±0.36 | 24.66±2.02 | 3 |
| M213 | 1.02±0.04 | 2.48±0.08 | 3 | 0.56±0.04 | 1.40±0.07 | 3 | 1.99±0.23 | 9.01±0.83 | 1 |
| M214 | 1.26±0.12 | 2.96±0.18 | 4 | 0.71±0.04 | 1.59±0.06 | 4 | 2.45±0.40 | 12.57±1.68 | 2 |
| KKU-100 | 0.82±0.10 | 2.30±0.10 | 2 | 0.16±0.03 | 0.36±0.04 | 1 | 3.39±1.29 | 31.52±6.03 | 4 |

*Rank of sensitivity to drug treatment at $IC_{50}$

effectiveness. The synergistic antitumor effect of 5-FU by HDACIs could be explained by at least two proposed mechanisms; a synergistic increase in apoptosis and reduced expression of the 5-FU cellular target gene, thymidylate synthase, a key enzyme involved in DNA replication (Stiborova et al., 2012).

In conclusion, our current results demonstrated that an epigenetic regulation in term of HDACs and their role in histone modification could be an important molecular event in CCA. The expressions of *HDACs* class I and II were slightly varied among tested genes and cell lines. Moreover, our interesting finding showed decreased cell proliferation in HDACIs treated cells and the synergistic antitumor effect of the simultaneously combined treatments of VPA or SAHA with 5-FU in CCA cells. This suggested that further investigation of HDACIs in combination with classical chemotherapeutic drug is required in CCA researches which might lead to further clinical application in the patients.

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