

Histone deacetylase inhibitory activity of hydroxycapsaicin, a synthetic derivative of capsaicin, and its cytotoxic effects against human colon cancer cell lines

Thanaset SENAWONG^{1,3,4,*}, Paweena WONGPHAKHAM¹, Thussanee SAIWICHAI¹,
Chanokbhorn PHAOSIRI^{2,3}, Pakit KUMBOONMA²

¹Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand

²Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand

³Natural Products Research Unit, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

⁴Food and Products Chemical Analysis Research Group, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

Received: 23.09.2014

Accepted/Published Online: 07.12.2014

Printed: 15.06.2015

Abstract: Capsaicin possesses cytotoxic/anticancer activity and shares some common structural features, including a benzene ring and a long hydrophobic carbon tail, with the histone deacetylase (HDAC) inhibitors trichostatin A and suberoylanilide hydroxamic acid. The aims of this study were to investigate HDAC inhibitory and cytotoxic activities of a synthetic derivative of capsaicin, hydroxycapsaicin (6-hydroxy-N-(4-hydroxy-3-methoxybenzyl)-8-methylnonenamide), in colon cancer cell lines. Hydroxycapsaicin inhibited HDAC activity in vitro ($IC_{50} = 72 \mu\text{M}$) much more effectively than the prototype capsaicin ($IC_{50} > 13.1 \text{ mM}$) and also exhibited HDAC inhibitory activity in human cells (HeLa cells). MTT assay demonstrated that hydroxycapsaicin was less toxic than capsaicin against both cancer and noncancer cells; however, hydroxycapsaicin, with greater HDAC inhibitory activity, was more effective than capsaicin at inducing apoptosis in colon cancer cell lines, especially in HCT116 cells. Hydroxycapsaicin appeared to induce less apoptotic cell death than capsaicin in Vero cells. Moreover, only hydroxycapsaicin induced S-phase cell-cycle arrest in both HT29 and HCT116 cells. The current study demonstrates that hydroxycapsaicin can act as a novel HDAC inhibitor, which would lead to a promising strategy for the development of safe and effective chemotherapeutic drugs from the abundant natural capsaicin of chili pepper.

Key words: Capsaicin, apoptosis, cell-cycle arrest, cytotoxicity, HDAC inhibitor, colon cancer

1. Introduction

Chili peppers are commonly used as an ingredient in many cuisines and are eaten by many people around the world. Capsaicinoids, including capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin, are the major active components of chili peppers, which not only add spice to the food but also possess potential anticancer (Surh, 2002; Zhang et al., 2003; Oh and Lim, 2009; Choi et al., 2010a; Ghosh and Basu, 2010; Thoenissen et al., 2010; Yang et al., 2010; Jin et al., 2014), antiinflammatory (Lee et al., 2010), antioxidant (Nascimento et al., 2014), antiobesity (Reinbach et al., 2009; Saito and Yoneshiro, 2013), and analgesic (Knotkova et al., 2008) activities. Capsaicin, the most abundant capsaicinoid found in chili peppers, has been shown to bind vanilloid receptors on the cell membranes of various cell types (Jordt and Julius, 2002). Vanilloid receptors are a nonselective cation channel gated by noxious heat, vanilloids, and extracellular protons and are involved in the etiology of inflammatory pain, such as inflammatory

bowel disease, bladder inflammation, and cancer pain (Messegue et al., 2006). The role of vanilloid receptors in the cytotoxicity of capsaicin is not fully understood, but is likely that different cell lines respond in unique manners to stimulation of this receptor by capsaicin/ligand binding (Reilly et al., 2003). Although the anticancer activity of capsaicin has been extensively documented, there is evidence that some capsaicin metabolites may cause DNA damage, leading to carcinogenesis (Baez et al., 2010). Lui et al. (2012) demonstrated that capsaicin at low concentrations upregulated the expression of tumor-associated NADH oxidase in the human colon carcinoma cell line HCT116, in association with enhanced cell proliferation and migration. Furthermore, epidemiological data showed that people who consume chili peppers in large amounts have a higher risk of gastric cancer (Lopez-Carillo et al., 1994). Therefore, capsaicin has been considered a double-edged sword with both carcinopreventive and carcinogenic properties. Nonetheless, capsaicin is not mentioned in the current list of natural chemopreventive agents (Mehta,

* Correspondence: sthanaset@kku.ac.th

2014). Moreover, its high concentration requirement is a potential limitation for the use of capsaicin in cancer therapy (Luo et al., 2011). Accordingly, modification of capsaicin's chemical structure may overcome the drawbacks of this naturally available compound.

Recently, inhibition of histone deacetylase (HDAC) activity has been considered a promising mechanism for cancer therapy, especially for combination chemotherapy (Carew et al., 2008; Bots and Johnstone, 2009; Ma et al., 2009; Mark and Xu, 2009). In the last 15 years, a number of HDAC inhibitors from natural sources and from chemical libraries have been studied, particularly their anticancer activity or toxicity on various cancer cell lines (Mark and Xu, 2009). However, there is a continuous need to develop new HDAC inhibitors with better pharmacological efficacy and novel modes of action in comparison to known HDAC inhibitors. Based on the structural similarity of capsaicin to the potent HDAC inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), several capsaicin derivatives were synthesized (Kumboonma, 2010) in order to explore potential therapeutic drugs from capsaicin-based compounds. In this study, the HDAC inhibitory activity of a capsaicin derivative, hydroxycapsaicin (6-hydroxy-*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnonenamide), was evaluated comparatively with a prototype capsaicin. The cytotoxic effects of hydroxycapsaicin on growth, apoptosis, and cell-cycle progression of human colon cancer cell lines were investigated in order to validate its anticancer potency, which would lead to a future study on its possible use as an anticancer agent.

2. Materials and methods

2.1. Cell lines and reagents

HCT116 and HT29 cells (human colon cancer cells) were obtained from Dr Osamu Tetsu (University of California, San Francisco, USA) and Dr Pornticha Picha (National Cancer Institute, Thailand), respectively. Both cell lines possess capsaicin/vanilloid receptors on their cell membrane (Bromberg et al., 2013; de Jong et al., 2014). Vero cells (African green monkey kidney cells) obtained from Dr Sahapat Barusrux (Khon Kaen University, Thailand) were chosen for this study as they lack a capsaicin receptor (Creppy et al., 2000). Capsaicin was purchased from Sigma-Aldrich Corporation (USA). Hydroxycapsaicin was synthesized as previously described (Kumboonma, 2010).

2.2. Cell culture

HCT116, HT29, and Vero cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco-BRL) at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% CO₂.

2.3. Assessment of HDAC inhibition in vitro

Inhibition of HDAC activity in vitro was assessed using the Fluor-de-Lys HDAC activity assay kit (Biomol, Enzo Life Sciences International, Inc., USA). HeLa nuclear extract provided with the kit was used as a source of HDAC enzymes for in vitro study. The spectra Max Gemini XPS microplate spectrofluorometer (Molecular Devices, USA) was used to measure fluorescence signal with excitation at 360 nm and emission at 460 nm. A decrease in fluorescence signal indicated an inhibition of HDAC activity.

2.4. Assessment of HDAC inhibition in human cells

HeLa cells with high levels of HDAC expression were chosen as a cellular model for evaluation of HDAC inhibition in human cells. Cells (1×10^6 cells) were seeded into a 4.5-cm dish. After incubation for 24 h, cells were treated with hydroxycapsaicin (50–200 µg/mL) for 6 h. Cells treated with a pan HDAC inhibitor TSA (5 nM) and DMSO (0.37%) were used as positive and negative controls, respectively. Histone proteins were extracted from treated cells as previously described (Senawong et al., 2013). Acetylated histone H4 in HeLa cells was determined by acid-urea-Triton (AUT) polyacrylamide gel electrophoresis.

2.5. AUT X-100 polyacrylamide gel electrophoresis

To evaluate HDAC inhibition in human cells, AUT gel electrophoresis was used to measure hyperacetylation of histone H4 as described previously (Senawong et al., 2013). AUT gels were stained using the PageSilver Silver Staining Kit (Fermentas, Canada). The abundance of triacetylated histone H4 was quantified with Quantity One software (Bio-Rad, USA).

2.6. MTT assay

Determination of antiproliferative activity or cytotoxicity of hydroxycapsaicin and capsaicin was performed using MTT assay as described previously (Senawong et al., 2013) with some modifications. In brief, HT29, HCT116, and Vero cells were treated with hydroxycapsaicin and capsaicin at varied concentrations (25, 50, 100, 200, 400 µg/mL), and incubated for 24, 48, and 72 h. After replacing culture medium, the cells were treated with 10 µL of MTT for 2 h at 37 °C. The formazan dye was dissolved with DMSO and determined by measuring the absorbance at 550 nm, using the absorbance at 655 nm as a reference wavelength with a microplate reader (Bio-Rad Laboratories, USA). The production of formazan was proportional to the number of viable cells.

2.7. Analysis of apoptosis by flow cytometry

Apoptosis analysis was performed using Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Invitrogen Corporation, USA) according to the manufacturer's instructions. HT29, HCT116, and Vero cells (1×10^6 cells) were seeded into a 4.5-cm dish and treated with various concentrations of

hydroxycapsaicin or capsaicin (50, 100, 200 µg/mL) for 24 h. Cells were then harvested by trypsinization, washed with PBS, and resuspended in ice-cold annexin-binding buffer added to Alexa Fluor 488 annexin V and propidium iodide (PI) solutions. Cells were stained in the dark for 15 min at room temperature and analyzed by flow cytometry using a BD FACSCantoII Flow Cytometer (Becton Dickinson, USA).

2.8. Analysis of cell-cycle arrest by flow cytometry

Cell-cycle phase distribution was determined as previously described (Poolman and Brooks, 1998). Briefly, HT29, HCT116, and Vero cells were seeded into a 5.5-cm dish (1 × 10⁶ cells/dish), incubated for 24 h, and treated with hydroxycapsaicin and capsaicin (50, 100, and 200 µg/mL) or vehicle (DMSO, 0.37%) for 24 h. Cells were then harvested by trypsinization and washed once with PBS. Cells were fixed in 1 mL of ice-cold 70% ethanol for 30 min at 4 °C and then centrifuged at 340 × g for 5 min. The pellet was resuspended in 0.5 mL of PBS and subsequently added to 10 µL of PI staining solution (50 µg/mL of PI in PBS) and 0.1 mg/mL of RNase. After incubation at 37 °C for 40 min, the stained cells were subjected to flow cytometric analysis using a BD FACSCantoII flow cytometer (Becton Dickinson, Canada).

2.9. Statistical analysis

All quantitative data are expressed as means ± standard deviation (SD) calculated from 3 replicate experiments. All the statistical analysis was carried out by SPSS 11.5 for Windows (SPSS Corporation, USA). The significant difference between 2 groups was assessed by one-way ANOVA with Duncan's post hoc test. A probability value of P < 0.05 was set for statistical difference.

3. Results

3.1. Hydroxycapsaicin exhibited greater HDAC inhibitory activity than the prototype capsaicin in vitro

Capsaicin shares some structural characteristics, including a benzene ring and a long hydrophobic carbon tail, with pan HDAC inhibitors TSA and SAHA. We first investigated the in vitro HDAC inhibitory activity of capsaicin using HeLa nuclear extract as a source of HDAC enzymes; however, capsaicin showed little effect on HDAC inhibition in vitro, with an IC₅₀ value of >4001 µg/mL (>13,000 µM) (Table 1). Therefore, hydroxycapsaicin was synthesized by adding a hydroxyl group to a position near the end of a long hydrophobic carbon tail in order to mimic the chemical structure of SAHA and TSA (Kumboonma, 2010). As expected, hydroxycapsaicin exhibited HDAC inhibitory activity in vitro with an IC₅₀ value of 23.29 µg/mL (72 µM) (Table 1), indicating a greater HDAC inhibition potency than the prototype capsaicin. Nonetheless, HDAC inhibitory activity of hydroxycapsaicin was much less potent compared with that of the pan HDAC inhibitor, TSA (IC₅₀ = 0.049 µM).

3.2. HDAC inhibitory activity of hydroxycapsaicin in HeLa cells

Based on our findings from the in vitro study, we next investigated the HDAC inhibitory activity of hydroxycapsaicin in a cellular context. In general, HDAC inhibition causes accumulation of acetylated histone molecules. Therefore, hyperacetylation of histone H4 in HeLa cells was investigated upon treatment with hydroxycapsaicin. HeLa cells were chosen as a model system because they express many HDAC isozymes, including HDAC 1, 2, 3, 4, and 8 (Hassig et al., 1998; Hu et al., 2000; Fischle et al., 2002; Khan

Table 1. In vitro HDAC inhibitory activities of hydroxycapsaicin and capsaicin.

Compounds	Molecular weight (g/mol)	Chemical structure	IC ₅₀ values ^a	
			µM	µg/mL
TSA ^b	302.37		0.049	0.015
Capsaicin	305.42		>13,100	>4001
Hydroxycapsaicin	323.43		72	23.29

^aIC₅₀ values represent concentrations of the indicated compounds that inhibit 50% of HDAC activity.

^bTSA (trichostatin A) is a potent HDAC inhibitor.

et al., 2008). HDAC inhibition in HeLa cells was analyzed by AUT gel electrophoresis, whereby the cellular core histone H4 with different levels of acetylation can be separated. The profiles of histone H4 extracted from hydroxycapsaicin-, TSA-, or DMSO-treated HeLa cells are given in Figure 1. The addition of hydroxycapsaicin to cell cultures resulted in

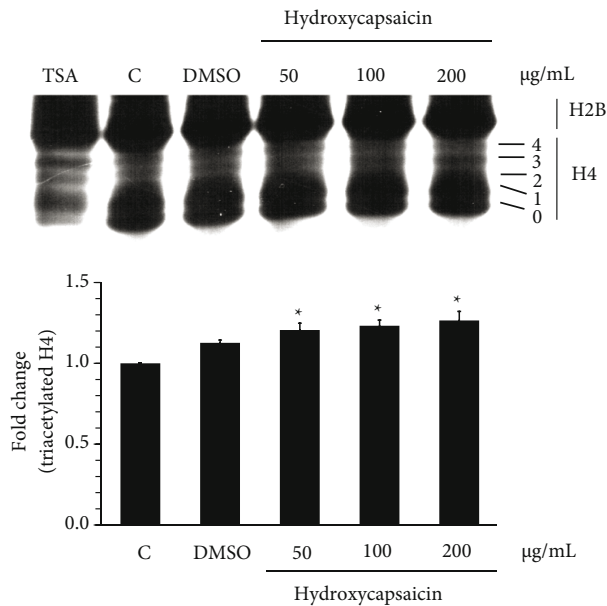


Figure 1. HDAC inhibitory activity of hydroxycapsaicin in human cells (HeLa cells). After 6 h of treatment, the level of acetylated histone H4 was determined by AUT gel electrophoresis as described in Section 2.5. Control (C) and DMSO represent the levels of acetylated histone H4 in untreated and solvent control treated cells, respectively. The TSA treatment was used as a positive control. The degree of histone acetylation of histone H4 is indicated as follows: 0, nonacetylated; 1, monoacetylated; 2, diacetylated; 3, triacetylated; and 4, tetraacetylated. The data shown are representative of 2 independent experiments performed in duplicate. Bar graph shows fold change of triacetylated histone H4 from untreated cells (C) was normalized to a value of 1.0. Asterisks indicate statistical significance ($P < 0.05$) compared with the solvent control treatment (DMSO).

the accumulation of hyperacetylated histone H4 molecules, specifically triacetylated H4 molecules, at the concentration of 200 µg/mL (618.37 µM). However, HDAC inhibition of hydroxycapsaicin in HeLa cells was less effective compared to that of a positive control TSA (5 nM), a very potent HDAC inhibitor. These results suggest that hydroxycapsaicin enters the cells and inhibits HDAC enzymes in the nucleus.

3.3. Effect of hydroxycapsaicin on proliferation of human colon cancer cell lines

Toxicity or antiproliferative activity of hydroxycapsaicin against human colon cancer cell lines (HCT116 and HT29 cells) and a cell line lacking capsaicin receptors (Vero cells) was investigated comparatively with capsaicin by MTT method, a tetrazolium-based colorimetric cell viability assay. As expected, both capsaicin and hydroxycapsaicin inhibited the growth of HT29, HCT116, and Vero cells in a concentration- and time-dependent manner (Table 2). Interestingly, both capsaicin and hydroxycapsaicin were slightly more toxic to Vero cells than to the colon cancer cell lines. Based on IC_{50} values at 24-, 48- and 72-h exposures, capsaicin was about twice as toxic as hydroxycapsaicin to all cell lines tested. The effect of hydroxycapsaicin on the growth of both HT29 and HCT116 cells was not significantly different.

3.4. Effect of hydroxycapsaicin on apoptosis induction in human colon cancer cell lines

Capsaicin inhibited the growth of several cancer cells via induction of both apoptosis (Lin et al., 2013; Jin et al., 2014) and autophagy (Oh and Lim, 2009; Choi et al., 2010b). However, cancer cell death via apoptosis induction is more preferable in cancer therapy due to the cytoprotective role of autophagy (Vazquez-Martin et al., 2009; Dalby et al., 2010). To confirm that induction of apoptosis underlies the antiproliferative activity of hydroxycapsaicin, its capacity to induce apoptosis in colon cancer cell lines was examined. Colon cancer cell lines and Vero cells were treated comparatively with different concentrations (50, 100, 150 mg/mL) of both hydroxycapsaicin and capsaicin for 24 h and then stained with Alexa Fluor 488-annexin V and PI, followed by

Table 2. Antiproliferative activities of hydroxycapsaicin and capsaicin against colon cancer cell lines.

Compounds	Molecular weight (Da)	IC_{50} values ^a (mean ± SD; n = 3; µg/mL)								
		Vero cells ^b			HT29 cells			HCT116 cells		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Capsaicin	305.42	68.60 ± 2.86	52.98 ± 1.88	51.19 ± 1.26	70.51 ± 1.44	67.13 ± 8.36	64.23 ± 1.26	57.15 ± 3.22	54.60 ± 2.17	53.28 ± 2.79
Hydroxycapsaicin	323.43	107.88 ± 5.17	103.37 ± 0.13	95.44 ± 3.95	134.36 ± 10.48	118.93 ± 0.55	114.26 ± 0.29	129.58 ± 2.04	127.46 ± 1.54	116.27 ± 0.66

^a IC_{50} values represent concentrations of the indicated compounds that inhibit 50% of cell proliferation.

^bA cell line lacking capsaicin receptors.

quantitative flow cytometric analysis. Surprisingly, capsaicin induced much greater levels of apoptotic cell death in Vero

cells than in HCT116 and HT29 cells (Figure 2). In contrast, hydroxycapsaicin induced minimal apoptotic cell death

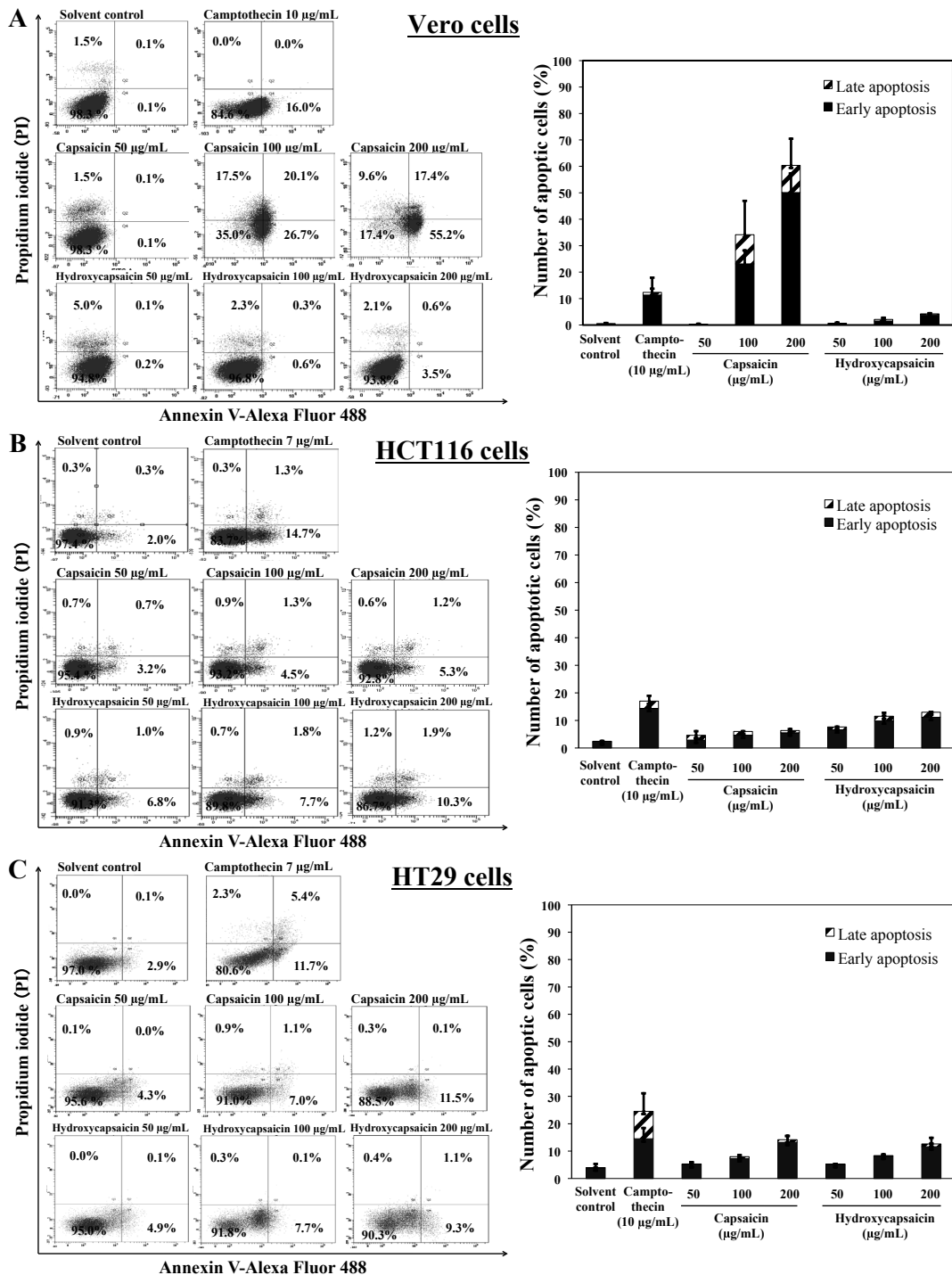


Figure 2. Apoptosis induction activities of capsaicin and hydroxycapsaicin against Vero cells and colon cancer cell lines. After 24 h of treatment with the indicated concentrations, Vero cells (A), human colorectal carcinoma cell line (HCT116 cells) (B), and human colon adenocarcinoma cell line (HT29 cells) (C) labeled with Alexa Fluor 488 annexin V and propidium iodide (PI) were analyzed by flow cytometry to determine the percentage of cells displaying an increase in early and late apoptosis. Bar graph shows the summarized data from 2 independent experiments performed in duplicate. Cells treated with DMSO (solvent control, 0.37%) and camptothecin (7–10 µg/mL) were used as negative and positive controls, respectively.

in Vero cells (Figure 2A), indicating that hydroxycapsaicin was much less toxic than capsaicin in a cell line lacking capsaicin/vanilloid receptors (Creppy et al., 2000). These results suggest that apoptosis induction by both capsaicin and hydroxycapsaicin in cancer cells may be influenced by a capsaicin/vanilloid receptor on the cell membrane. Although apoptosis induction of hydroxycapsaicin and capsaicin in HT29 cells was not significantly different (Figure 2C), apoptosis induction of hydroxycapsaicin in HCT116 cells was greater than that of capsaicin (Figure 2B).

3.5. Effect of hydroxycapsaicin on induction of cell-cycle arrest in human colon cancer cell lines

In addition to apoptosis induction, cell-cycle arrest may also underlie a growth inhibition effect of hydroxycapsaicin. The possible effect of hydroxycapsaicin on cell-cycle progression was investigated comparatively with capsaicin in both Vero cells and colon cancer cell lines. As shown in Figures 3A–3F, a significant dose-dependent alteration of cell numbers in different cell-cycle phases was observed. In both Vero cells and colon cancer cells, capsaicin treatments for 24 h caused substantial cell death/apoptosis with the dramatic increase of the sub-G0/G1 peak (Figures 3A, 3C, and 3E), whereas hydroxycapsaicin induced lower levels of cell death at high concentrations (Figures 3B, 3D, and 3F). In Vero cells, capsaicin treatments at 50 and 100 µg/mL for 24 h induced an increase of cells in the S-phase of the cell-cycle (Figure 3A). Interestingly, hydroxycapsaicin treatment at the highest concentration (200 µg/mL) for 24 h induced both an elevation of cell death and cell-cycle arrest at the S and G2/M phases in Vero cells (Figure 3B). In both HCT116 and HT29 cells, hydroxycapsaicin treatment caused cell-cycle arrest at the S-phase of the cell cycle (Figures 3D and 3F). In contrast, capsaicin treatment caused no cell-cycle arrest (Figures 3C and 3E) in these colon cancer cells.

4. Discussion

HDAC inhibition by HDAC inhibitors shows promising effects for cancer therapy (Mark and Xu, 2009). Although the link between HDAC inhibition and apoptosis induction is not fully understood, treatments with HDAC inhibitors usually cause a shift in the balance of pro- and antiapoptotic genes toward apoptosis (Carew et al., 2008). The cellular response to HDAC inhibition is complicated and appears to involve both transcriptional and nontranscriptional events. The substrates for HDAC enzymes are not only the histone proteins but also more than 50 nonhistone proteins, such as transcription factors, DNA repair proteins, DNA-binding proteins, chaperone proteins, and signal-transduction molecules (Ocker, 2010). Inhibition of HDAC enzymes promotes the acetylation of both histone and nonhistone proteins, resulting in downregulation of prosurvival proteins such as

Bcl-2 and Bcl-1, which maintain integrity of mitochondria (Rikiishi, 2011), and upregulation of proapoptotic proteins such as Bim, Bak, and Bax, which initiate intrinsic cell death pathways (Zhao et al., 2005; Zhang et al., 2006). In addition, hyperacetylation has been shown to stabilize p53 and activate cell-cycle arrest in cancer cells (Xu, 2006). However, the link between hydroxycapsaicin-induced hyperacetylation and induction of cell-cycle arrest and apoptosis in both HT29 and HCT116 cells (Figures 2 and 3) remains to be investigated. There are 11 family members of zinc-dependent HDACs (Martinez-Iglesias et al., 2008; Mark and Xu, 2009), which commonly have a narrow pocket and zinc (Zn^{2+}) ion in the catalytic site. The general structural characteristics of TSA and SAHA that strongly inhibit zinc-dependent HDAC isozymes include a zinc-binding moiety, an opposite capping group, and a straight-chain alkyl linker connecting the zinc-binding moiety and the capping group (Finnin et al., 1999; Mark and Xu, 2009). Based on its structural similarity with SAHA and TSA, we hypothesized that hydroxycapsaicin may have a similar orientation and binding mode as SAHA or TSA in the active site of zinc-dependent HDACs. Here we demonstrated that hydroxycapsaicin could inhibit HDAC activity both *in vitro* and in human cells. However, hydroxycapsaicin exhibited little effect on HDAC inhibition in a cellular model (Figure 1) and its HDAC inhibitory activity did not correspond well with the *in vitro* results (Table 1). The minimal HDAC inhibition by hydroxycapsaicin in HeLa cells may result from the existence of vanilloid receptor (TRPV1) on the plasma membrane of this cell line (Ahn et al., 2014), preventing some hydroxycapsaicin molecules from entering the cells.

The HDAC inhibitory activity of hydroxycapsaicin makes it more attractive in combination chemotherapy because HDAC enzymes are different chemotherapeutic drug targets than those of highly effective chemotherapeutic drugs such as cisplatin, 5-fluorouracil, and doxorubicin. For example, combination therapies using HDAC inhibitors vorinostat and sodium butyrate with aurora kinase inhibitors and doxorubicin, respectively, enhance activity against cancer cells (Cheriyath et al., 2011; Kretzner et al., 2011). Accordingly, further studies on specificity for specific HDAC family members and HDAC inhibition in more cancer cell lines of hydroxycapsaicin are of interest.

Hydroxycapsaicin was synthesized to provide a novel HDAC inhibitor and to overcome capsaicin's toxicity. Here we demonstrated that a novel HDAC inhibitor, hydroxycapsaicin, was about 2 times less toxic than the prototype capsaicin in both colon cancer cell lines and a cell line lacking capsaicin receptor (Table 2). Our preliminary data revealed that hydroxycapsaicin inhibited cell proliferation through induction of apoptosis (Figure 2) and cell-cycle arrest (Figure 3). Capsaicin was more toxic to

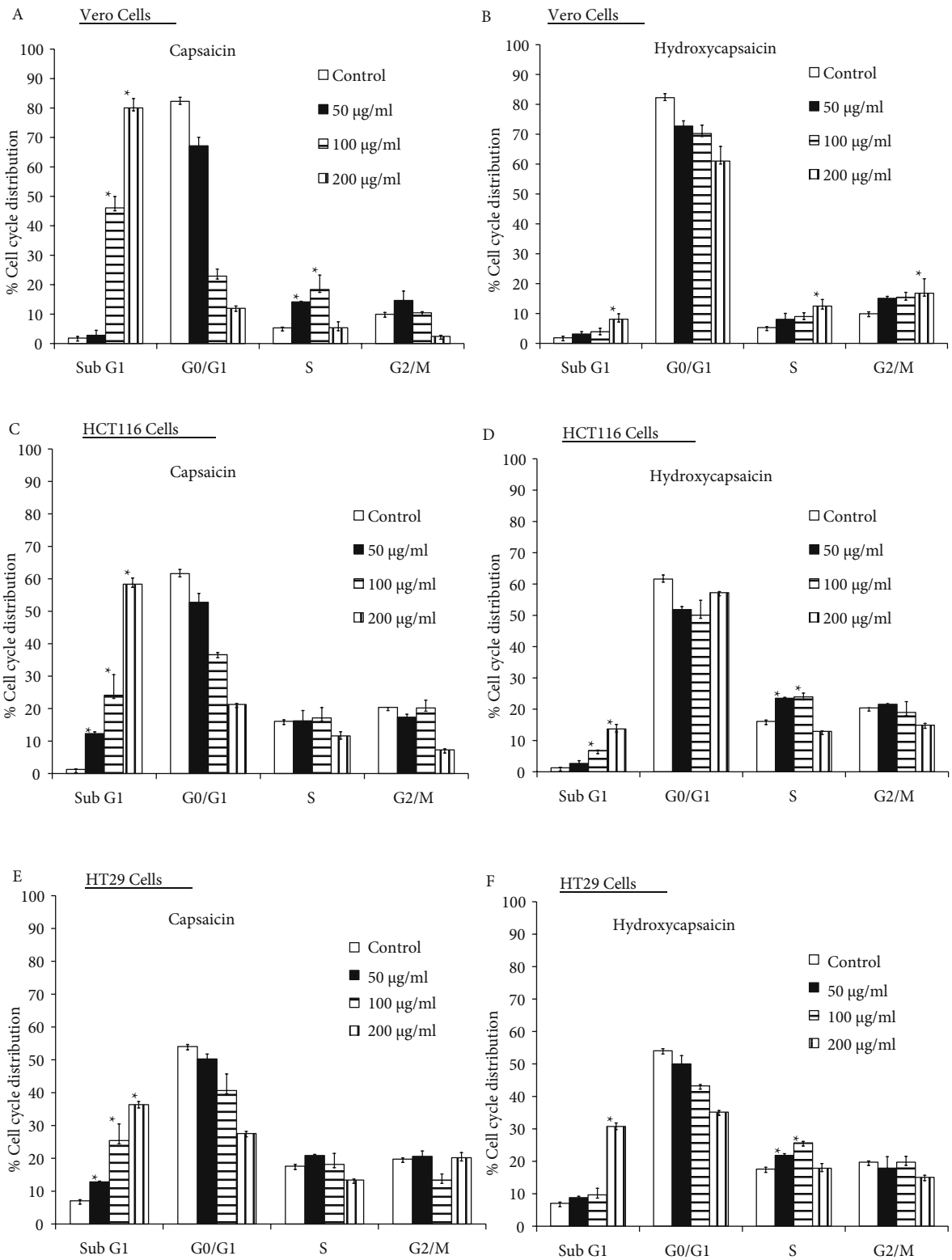


Figure 3. Induction of cell cycle arrest by capsaicin and hydroxycapsaicin in Vero cells and colon cancer cell lines. After 24 h of treatment with the indicated concentrations, Vero cells (A, B), human colorectal carcinoma cell line (HCT116 cells) (C, D), and human colon adenocarcinoma cell line (HT29 cells) (E, F) were stained with propidium iodide (PI) and analyzed by flow cytometry to determine the cellular phase distribution. The graph shows the summarized data from 2 independent experiments performed in duplicate. Cells treated with DMSO (0.37%) were used as a control. Asterisk indicates a significant difference ($P < 0.05$) compared with a control group.

the cells lacking capsaicin receptors (Vero cells) than those possessing capsaicin receptors (HT29 and HCT116 cells) (Figure 2; Table 2), suggesting that capsaicin toxicity might be affected by the capsaicin receptor. Capsaicin has been shown to inhibit cell proliferation via inhibition of protein synthesis (Creppy et al., 2000) and endoplasmic reticulum stress-induced autophagy/apoptosis (Oh and Lim, 2009). It is of interest to investigate whether hydroxycapsaicin may exert the same mechanisms as capsaicin to inhibit cell proliferation.

The aim of chemotherapy is to kill cancer cells by inducing apoptosis. In this study, we identified apoptotic cells by detecting the “eat-me” signal of apoptotic cells (Ravichandran, 2010), an externalized phosphatidylserine, using a combined annexin V/PI dual staining assay. Here we demonstrated that the effect of hydroxycapsaicin on apoptosis induction was greater than that of capsaicin in HCT116 cells (p53 wild type), but not different in HT29 cells (p53 mutant) (Figure 2). This apparent finding suggests that the presence of p53 may facilitate apoptosis induction by hydroxycapsaicin. However, the involvement of p53 in apoptosis induction by hydroxycapsaicin in HCT116 cells remains to be clarified.

In addition to apoptosis induction, cell-cycle arrest is also a mechanism to suppress cancer cell growth. However, the induction of cell-cycle arrest by capsaicin appeared to be specific to cell type and to play a limited role in some cancer cells, including KB cells (Lin et al., 2013). Consistently, in this study, cell-cycle arrest was not

observed upon capsaicin treatment in either HCT116 or HT29 cells (Figures 3C and 3E), in which the suppression of their growth may be more correlated with induction of cell death/apoptosis. Interestingly, hydroxycapsaicin, with HDAC inhibitory activity and less toxicity, suppressed the growth of both colon cancer cell lines, at least in part, via induction of cell-cycle arrest at the S-phase. However, the molecular mechanisms underlying hydroxycapsaicin-induced cell-cycle arrest at the S-phase in both colon cancer cell lines are unknown and further investigation is needed.

In conclusion, hydroxycapsaicin, a capsaicin-based HDAC inhibitor, exhibited HDAC inhibitory effects both in vitro and in cellular model. However, vanilloid receptors may limit the effect of its HDAC inhibitory activity in a cellular context. Exposure of both colon cancer cell lines (HCT 116 and HT29 cells) to hydroxycapsaicin reduced cell proliferation, induced apoptosis, and stimulated cell-cycle arrest at the S-phase. These findings suggest that hydroxycapsaicin possesses HDAC inhibitory and anticancer activities and may be a potential anticancer drug.

Acknowledgment

This work was supported by Khon Kaen University through a Researcher Training Grant (to TS). We are thankful to Professor Nison Sattayasai and Associate Professor Veerapol Kukongviriyapan, Khon Kaen University, Thailand, for technical advice and coordination for the use of microplate spectrofluorometer, respectively.

References

- Ahn S, Park J, An I, Jung SJ, Hwang J (2014). Transient receptor potential cation channel V1 (TRPV1) is degraded by starvation- and glucocorticoid-mediated autophagy. *Mol Cells* 37: 257–263.
- Bots M, Johnstone RW (2009). Rational combinations using HDAC inhibitors. *Clin Cancer Res* 15: 3970–3977.
- Bromberg Z, Goloubinoff P, Saidi Y, Weiss YG (2013). The membrane-associated transient receptor potential vanilloid channel is the central heat shock receptor controlling the cellular heat shock response in epithelial cells. *PLoS ONE* 8: e57149.
- Carew JS, Giles FJ, Nawrocki ST (2008). Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. *Cancer Lett* 269: 7–17.
- Cheriyath V, Kuhns MA, Kalaycio ME, Borden EC (2011). Potentiation of apoptosis by histone deacetylase inhibitors and doxorubicin combination: cytoplasmic cathepsin B as a mediator of apoptosis in multiple myeloma. *Brit J Cancer* 104: 957–967.
- Choi CH, Jung YK, Oh SH (2010a). Selective induction of catalase-mediated autophagy by dihydrocapsaicin in lung cell lines. *Free Radic Biol Med* 49: 245–257.
- Choi CH, Jung YK, Oh SH (2010b). Autophagy induction by capsaicin in malignant human breast cells is modulated by p38 and extracellular signal-regulated mitogen-activated protein kinases and retards cell death by suppressing endoplasmic reticulum stress-mediated apoptosis. *Mol Pharmacol* 78: 114–125.
- Creppy EE, Richeux F, Carratú MR, Cuomo V, Cochereau C, Ennamany R, Saboureau D (2000). Cytotoxicity of capsaicin in monkey kidney cells: lack of antagonistic effects of capsazepine and Ruthenium red. *Arch Toxicol* 74: 40–47.
- Dalby KN, Tekedereli I, Lopez-Berestein G, Ozpolat B (2010). Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. *Autophagy* 6: 322–329.
- de Jong PR, Takahashi N, Harris AR, Lee J, Bertin S, Jeffries J, Jung M, Duong J, Triano AI, Lee J et al. (2014). Ion channel TRPV1-dependent activation of PTP1B suppresses EGFR-associated intestinal tumorigenesis. *J Clin Invest* 124: 3793–3806.
- Finnin MS, Donigan JR, Cohen A, Richon VM, Rifkind RA, Marks PA, Breslow R, Pavletich NP (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* 401: 188–193.

- Fischle W, Dequiedt F, Hendzel MJ, Guenther MG, Lazar MA, Voelter W, Verdin E (2002). Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. *Mol Cell* 9: 45–57.
- Ghosh AK, Basu S (2010). Fas-associated factor 1 is a negative regulator in capsaicin induced cancer cell apoptosis. *Cancer Lett* 287: 142–149.
- Hassig CA, Tong JK, Fleischer TC, Owa T, Grable PG, Ayer DE, Schreiber SL (1998). A role for histone deacetylase activity in HDAC1 mediated transcriptional repression. *P Natl Acad Sci USA* 95: 3519–3524.
- Hu E, Chen Z, Fredrickson T, Zhu Y, Kirkpatrick R, Zhang GF, Johanson K, Sung CM, Liu R, Winkler J (2000). Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. *J Biol Chem* 275: 15254–15264.
- Jin J, Lin G, Huang H, Xu D, Yu H, Ma X, Zhu L, Ma D, Jiang H (2014). Capsaicin mediates cell cycle arrest and apoptosis in human colon cancer cells via stabilizing and activating p53. *Int J Biol Sci* 10: 285–295.
- Jordt SE, Julius D (2002). Molecular basis for species-specific sensitivity to “hot” chili peppers. *Cell* 108: 421–430.
- Khan N, Jeffers M, Kumar S, Hackett C, Boldog F, Khramtsov N, Qian X, Mills E, Bergs SC, Carey N et al. (2008). Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. *Biochem J* 409: 581–589.
- Knotkova H, Pappagallo M, Szallasi A (2008). Capsaicin (TRPV1 agonist) therapy for pain relief: farewell or revival? *Clin J Pain* 24: 142–154.
- Kretzner L, Scuto A, Dino PM, Kowolik CM, Wu J, Ventura P, Jove R, Forman SJ, Yen Y, Kirschbaum MH (2011). Combining histone deacetylase inhibitor vorinostat with aurora kinase inhibitors enhances lymphoma cell killing with repression of c-Myc, hTERT, and micro-RNA levels. *Cancer Res* 71: 3912–3920.
- Kumboonma P (2010). Isolation and structural modification of capsaicin/dihydrocapsaicin from *Capsicum annum* and biological activities. MSc, Khon Kaen University, Khon Kaen, Thailand.
- Lee EJ, Jeon MS, Kim BD, Kim JH, Kwon YG, Lee H, Lee YS, Yang JH, Kim TY (2010). Capsiate inhibits ultraviolet B-induced skin inflammation by inhibiting Src family kinases and epidermal growth factor receptor signaling. *Free Radic Biol Med* 48: 1133–1143.
- Lin CH, Lu WC, Wang CW, Chan YC, Chen MK (2013). Capsaicin induces cell cycle arrest and apoptosis in human KB cancer cells. *BMC Complement Altern Med* 2013: 13: 46.
- Liu NC, Hsieh PF, Hsieh MK, Zeng ZM, Cheng HL, Liao JW, Chueh PJ (2012). Capsaicin-mediated tNOX (ENOX2) up-regulation enhances cell proliferation and migration in vitro and in vivo. *J Agric Food Chem* 60: 2758–2765.
- Lopez-Carillo L, Avila MH, Dubrow R (1994). Chili pepper consumption and gastric cancer in Mexico: a case control study. *Am J Epidemiol* 139: 263–271.
- Luo XJ, Peng J, Li YJ (2011). Recent advances in the study on capsaicinoids and capsinoids. *Eur J Pharmacol* 650: 1–7.
- Ma X, Ezzeldin HH, Diasio RB (2009). Histone deacetylase inhibitors: current status and overview of recent clinical trials. *Drugs* 69: 1911–1934.
- Marks PA, Xu WS (2009). Histone deacetylase inhibitors: potential in cancer therapy. *J Cell Biochemistry* 107: 600–608.
- Martinez-Iglesias O, Ruiz-Llorente L, Sanchez-Martinez R, Garcia L, Zambrano A, Aranda A (2008). Histone deacetylase inhibitors: mechanism of action and therapeutic use in cancer. *Clin Transl Oncol* 10: 395–398.
- Mehta RG (2014). Current paradigms of cancer chemoprevention. *Turk J Biol* 38: 839–847.
- Messegueur A, Planells-Cases R, Ferrer-Montiel A (2006). Physiology and pharmacology of the vanilloid receptor. *Curr Neuropharmacol* 4: 1–15.
- Nascimento PL, Nascimento TC, Ramos NS, Silva GR, Gomes JE, Falcão RE, Moreira KA, Porto AL, Silva TM (2014). Quantification, antioxidant and antimicrobial activity of phenolics isolated from different extracts of *Capsicum frutescens* (pimenta malagueta). *Molecules* 19: 5434–5447.
- Ocker M (2010). Deacetylase inhibitors-focus on non-histone targets and effects. *World J Biol Chem* 1: 55–61.
- Oh S, Lim SC (2009). Endoplasmic reticulum stress-mediated autophagy/apoptosis induced by capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin is regulated by the extent of c-Jun NH2-terminal kinase/extracellular signal-regulated kinase activation in WI38 lung epithelial fibroblast cells. *J Pharmacol Exp Ther* 329: 112–122.
- Poolman RA, Brooks G (1998). Expressions and activities of cell cycle regulatory molecules during the transition from myocyte hyperplasia to hypertrophy. *J Mol Cell Cardiol* 30: 2121–2135.
- Ravichandran KS (2010). Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med* 207: 1807–1817.
- Reilly CA, Taylor JL, Lanza DL, Carr BA, Crouch DJ, Yost GS (2003). Capsaicinoids cause inflammation and epithelial cell death through activation of vanilloid receptors. *Toxicol Sci* 73: 170–181.
- Reinbach HC, Smeets A, Martinussen T, Moller P, Westerterp-Plantenga MS (2009). Effects of capsaicin, green tea and CH-19 sweet pepper on appetite and energy intake in humans in negative and positive energy balance. *Clin Nutr* 28: 260–265.
- Rikiishi H (2011). Autophagic and apoptotic effects of HDAC inhibitors on cancer cells. *J Biomed Biotechnol* 2011: 830260.
- Saito M, Yoneshiro T (2013). Capsinoids and related food ingredients activating brown fat thermogenesis and reducing body fat in humans. *Curr Opin Lipidol* 24: 71–77.

- Senawong T, Misuna S, Khaopha S, Nuchadomrong S, Sawatsitang P, Phaosiri C, Surapaitoon A, Sripa B (2013). Histone deacetylase (HDAC) inhibitory and antiproliferative activities of phenolic-rich extracts derived from the rhizome of *Hydnophytum formicarum* Jack.: sinapinic acid acts as HDAC inhibitor. *BMC Complement Altern Med* 2013; 13: 232.
- Surh YJ (2002). More than spice: capsaicin in hot chili peppers makes tumor cells commit suicide. *J Natl Cancer Inst* 94: 1263–1265.
- Thoennissen NH, O’Kelly J, Lu D, Iwanski GB, La DT, Abbassi S, Leiter A, Karlan B, Mehta R, Koeffler HP (2010). Capsaicin causes cell-cycle arrest and apoptosis in ER-positive and -negative breast cancer cells by modulating the EGFR/HER-2 pathway. *Oncogene* 29: 285–296.
- Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA (2009). Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab. *PLoS ONE* 4: e6251.
- Xu Y (2003). Regulation of p53 responses by post-translational modifications. *Cell Death Differ* 10: 400–403.
- Yang ZH, Wang XH, Wang HP, Hu LQ, Zheng XM, Li SW (2010). Capsaicin mediates cell death in bladder cancer T24 cells through reactive oxygen species production and mitochondrial depolarization. *Urology* 75: 735–741.
- Zhang J, Nagasaki M, Tanaka Y, Morikawa S (2003). Capsaicin inhibits growth of adult T-cell leukemia cells. *Leuk Res* 27: 275–283.
- Zhang Y, Adachi M, Kawamura R, Imai K (2006). Bmf is a possible mediator in histone deacetylase inhibitors FK228 and CBHA-induced apoptosis. *Cell Death Differ* 13: 129–140.
- Zhao Y, Tan J, Zhuang L, Jiang X, Liu ET, Yu Q (2005). Inhibitors of histone deacetylases target the Rb-E2F1 pathway for apoptosis induction through activation of proapoptotic protein Bim. *P Natl Acad Sci USA* 102: 16090–16095.