

# Anti-Tumorigenic Effects of Capsaicin in Colon Cancer

Seong-Ho Lee<sup>1,2\*</sup> and Ruth Clark<sup>1</sup><sup>1</sup>Department of Nutrition and Food Science, College of Agriculture and Natural Resources, University of Maryland, College Park, MD 20742, USA<sup>2</sup>Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996, USA**\*Correspondence to:**

Seong-Ho Lee  
Department of Nutrition and Food Science  
College of Agriculture and Natural Resources  
University of Maryland, College Park  
MD 20742, USA  
Tel: (301) 405-4532  
Fax: (301) 314-3313  
E-mail: [slee2000@umd.edu](mailto:slee2000@umd.edu)

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## Abstract

Colon cancer is one of most common malignancies in the world. Capsaicin is a major component of chili peppers and has been shown to possess anti-cancer activity against various types of cancers. There is currently a limited number of preclinical studies that test the anti-cancer effects of capsaicin in colon cancer. The first objective of the present study is to investigate whether capsaicin influences tumor formation using a colon cancer mouse model. The second objective is to elucidate the anti-cancer mechanism of capsaicin using an *in vitro* cell culture system. For the *in vivo* study, we treated *Apc<sup>Min/+</sup>* mice having truncated deletion of APC (adenomatous polyposis coli) with capsaicin. For the *in vitro* study, we exposed human colorectal cancer cell lines to different concentrations of capsaicin and performed western blot analysis. Oral gavage of capsaicin at a dose of 20 mg/kg body weight for 4 weeks tended to decrease the number of polyps ( $43.5 \pm 14.6$  vs  $27.8 \pm 8.8$ ,  $p = 0.114$ ) and tumor load ( $78.6 \pm 20.9$  vs  $47.4 \pm 21.3$ ,  $p = 0.083$ ) in the intestine of *Apc<sup>Min/+</sup>* mice. *In vitro* studies showed that, in human colon cancer cells, capsaicin induced phosphorylation of cyclin D1 at threonine 286 (T286) and decreased cyclin D1 expression in a dose- and time-dependent manner. In addition, capsaicin treatment increased intracellular ubiquitination of proteins in a dose-dependent manner and decreased the caspase-like activity of 20S proteasome significantly ( $p = 0.026$ ). This study identifies capsaicin as a potential anti-cancer agent that targets cyclin D1 degradation and proteasome activity in colon cancer.

## Keywords

Capsaicin, Colon cancer, Polyp, Cyclin D1, Proteasome

## Abbreviations

ACF: Aberrant crypt foci; APC: Adenomatous polyposis coli; CDK: Cyclin dependent kinase; FAP: Familial adenomatous polyposis; HA: Hemagglutinin; NAG-1: Non-steroidal anti-inflammatory drug-activated gene-1; TCF4: T-cell factor 4

## Introduction

Colon cancer is one of the leading malignant cancers in the world [1]. There are very strong correlations between incidence of colon cancer and eating patterns, in particular the consumption of a plant-based diet [2]. Capsaicin is a major component of chili peppers and produces a spicy taste. Recently, numerous *in vitro* studies have reported the anti-cancer activities of capsaicin in various cancer

models [3-14], although several studies claimed that capsaicin exhibited potential cancer promoting activities [15, 16]. Our group has previously demonstrated that capsaicin induced apoptosis and suppressed cell proliferation through NSAIDs-activated gene-1 (NAG-1) and  $\beta$ -catenin/TCF4-mediated pathways in human colon cancer cells [8, 17]. Recently, we reviewed the anti-cancer mechanisms by which capsaicin led to apoptosis and growth arrest, and inhibited metastasis and angiogenesis [18]. Cao S et al., has also reviewed the anti-cancer mechanisms of capsaicin based on different cancer types [19]. However, most published studies have been carried out using *in vitro* cell culture systems and there has been no *in vivo* study to test the efficacy of capsaicin in a colon cancer model.

Normal regulation of the cell cycle is essential for tissue development and homeostasis. Cyclins play a significant role in the regulation of the cell cycle. Different cyclins participate in different phases of the cell cycle with specificity and the synthesis of each cyclin is coordinated with DNA synthesis and cell division. Aberrant regulation of cyclins impairs DNA check points and leads to tumorigenesis. Type D cyclins are involved in the transition of the cell cycle from the G1 phase to the S phase and influence growth arrest, which is a promising strategy to control initiation and progression of cancer [20]. Cyclin D1 overexpression is observed in many types of cancers [20] and amount of cyclin D1 in the cells is determined by several mechanisms. One of these mechanisms is transcriptional regulation, which is affected by activators and repressors. Many oncogenic triggers, including growth factors and cytokines, induce transcription of cyclin D1 [21]. With regard to this mechanism, we also reported that transcriptional activity of cyclin D1 promoter is down regulated by dietary compounds such as capsaicin [8] and 6-gingerol [22]. Another mechanism to determine the amount of cellular cyclin D1 is proteosomal degradation. It has been shown that several anti-cancer compounds, including retinoic acid and curcumin, down regulate cyclin D1 through increasing protein instability and subsequent proteosomal degradation [23, 24].

The current study was designed to observe whether capsaicin affects intestinal tumorigenesis in an adenomatous polyposis coli (APC)-truncated colon cancer mouse model (*Apc<sup>Min/+</sup>*) and elucidate the anti-cancer mechanism involving cyclin D1 degradation. The current study demonstrate that capsaicin tended to reduce polyp formation in *Apc<sup>Min/+</sup>* mice and suppressed cyclin D1 expression via an increase of degradation in human colon cancer cells.

## Materials and Methods

### Materials

Capsaicin was purchased from Sigma (St. Louis, MO, USA) and dissolved in absolute ethanol to make 100 mM stock solution. Antibodies for cyclin D1, cyclin D3, and actin were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibodies for phospho-cyclin D1 (T286) and hemagglutinin (HA) were purchased from Cell Signaling (Beverly, MA, USA) and BioLegend (San Diego, CA, USA). Unless

otherwise specified, all chemicals and cell culture media were purchased from Fisher Scientific (Waltham, MA, USA) and VWR International (Radnor, PA, USA) and used without further purification.

### *In vivo* study

The *APC<sup>Min/+</sup>* mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The mice were bred and genotyped as previously described [25]. The mice (10 weeks old) were randomly assigned to two groups (four mice per treatment) and gavaged orally with 0.5% methylcellulose only (vehicle) or capsaicin in 0.5% methylcellulose at a dose of 20 mg/kg body weight every other day for 4 weeks. For the gavage, 62 mg of capsaicin (65% purity) was suspended in 10 mL of 0.5% methylcellulose and 100  $\mu$ L of suspension was gavaged into the mice (20 g body weight in average). A week after the final gavage, the mice were euthanized and the intestinal tract was collected and washed with ice-cold phosphate-buffered saline (PBS). Tumor numbers and sizes were measured under a stereoscopic microscope as previously performed [25]. All procedures for the *in vivo* study were approved by the Institutional Animal Care and Use Committee (protocol #:1676) at the University of Tennessee.

### Cell culture and measurement of cell proliferation

Human colon adenocarcinoma cells (SW480, HCT116, LoVo and Caco-2) were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained as previously described [17, 18]. Cell proliferation was measured using the Cell Proliferation Assay system (Promega, Madison, WI, USA) according to the manufacturer's instruction. The absorbance value at  $A_{490}$  was detected using an ELISA plate reader (Bio-Tek Instruments Inc, Winooski, VT, USA).

### Transient transfection

An HA-tagged ubiquitin expression vector was transfected into the cells using Lipofectamin 2000 (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instruction. Briefly, the cells ( $2 \times 10^5$  cell/well) were plated onto 6-well culture dish and maintained overnight. The next day the cells were incubated in media containing the DNA/Lipofectamin complex for an additional 48 hours.

### Western blot

After treatment with vehicle (ethanol) and different doses of capsaicin, the cells were lysed by incubation with radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease/phosphatase inhibitors cocktail. The cell debris was removed by centrifugation at  $10,000 \times g$  for 10 min at  $4^\circ\text{C}$  and the supernatant containing cellular proteins was collected. Next, 30  $\mu$ g of protein from the supernatant were loaded onto a SDS-polyacrylamide gel, separated with electrophoresis, and transferred to a nitrocellulose membrane. After overnight incubation with the target antibodies, the membranes were incubated with horse radish peroxidase (HRP)-conjugated IgG for 1 hour and visualized using ECL (Amersham Biosciences, Piscataway, NJ, USA).

## 20S Proteasome assay

20S proteasome assays were performed using a Proteasome-Glo Assay Systems Kit (Promega, Madison, WI, USA) as previously described [26]. Briefly, the cells were treated with vehicle (ethanol) and capsaicin in triplicates and 30  $\mu\text{g}$  of protein from each sample was loaded in a white-walled 96-well plate. Then, equal amounts of chymotrypsin-like, trypsin-like, and caspase-like substrates were added. After incubation for 1 hour at room temperature, the luminescence for the three activities was measured using an ELISA plate reader (Bio-Tek Instruments Inc, Winooski, VT, USA).

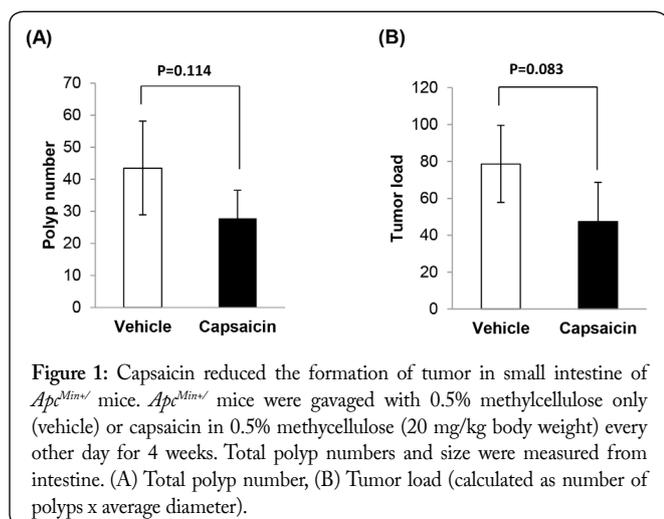
## Statistical analysis

Statistical analysis was performed using SPSS v18.0 with the Student *t* test.

## Results

### Capsaicin reduced the formation of tumor in the small intestine of *Apc<sup>Min/+</sup>* mice

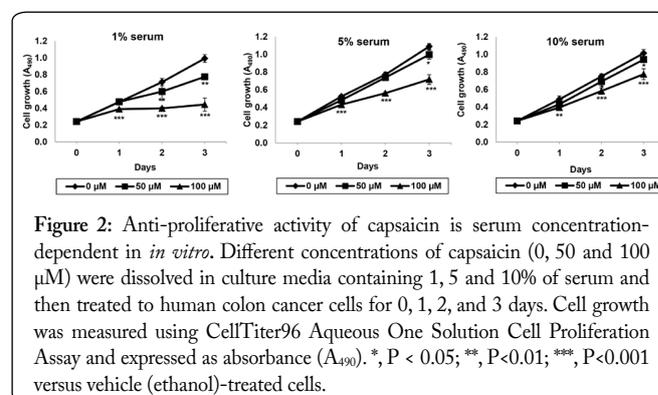
To investigate if capsaicin affects tumor formation, we used a genetically-induced colon cancer model (*Apc<sup>Min/+</sup>* mice). These mice produce a significant number of polyps due to expressing a truncated deletion of APC, which is an important tumor suppressor gene in the gastrointestinal tract. Ten-week old *Apc<sup>Min/+</sup>* mice were exposed to 0.5% methylcellulose only (vehicle) or capsaicin in 0.5% methylcellulose at a dose of 20 mg/kg body weight by oral gavage for 4 weeks. As shown in Figure 1, control mice produced 43.5 polyps on average, but capsaicin treatment reduced the average polyp number to 27.8, although the difference was not statistically significant ( $p = 0.114$ ). Tumor load (calculated as number of polyps  $\times$  average diameter) analysis showed a tendency for treatment with capsaicin to reduced polyp development, but the difference was not statistically significant ( $p = 0.083$ ).



### Anti-proliferative activity of capsaicin is serum concentration-dependent *in vitro*

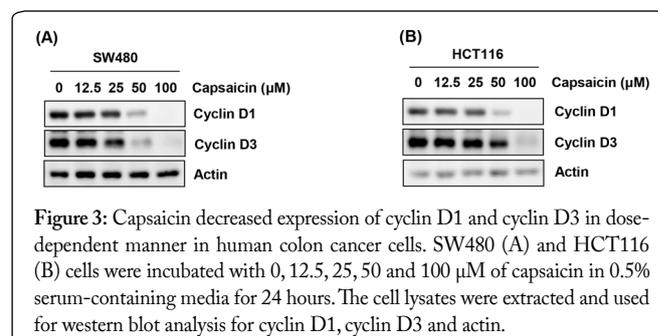
We further investigated whether a varied content of serum in the media affects capsaicin's anti-proliferative activity. As shown in Figure 2, capsaicin led to dramatic suppression of

proliferation in colon cancer cells incubated with a 1% serum-containing medium [Day 1:  $0.475 \pm 0.032$ ,  $0.478 \pm 0.021$  ( $p = 0.451$ ), and  $0.390 \pm 0.008$  ( $p < 0.001$ ); Day 2:  $0.712 \pm 0.043$ ,  $0.599 \pm 0.029$  ( $p = 0.002$ ), and  $0.399 \pm 0.017$  ( $p < 0.001$ ); Day 3:  $0.993 \pm 0.047$ ,  $0.774 \pm 0.106$  ( $p = 0.003$ ), and  $0.444 \pm 0.079$  ( $p < 0.001$ ) at 0, 50, and 100  $\mu\text{M}$  capsaicin, respectively]. However, the effects were weakened in cells treated with 5% serum-containing medium [Day 1:  $0.525 \pm 0.019$ ,  $0.484 \pm 0.007$  ( $p = 0.003$ ), and  $0.433 \pm 0.017$  ( $p < 0.001$ ); Day 2:  $0.773 \pm 0.029$ ,  $0.739 \pm 0.025$  ( $p = 0.062$ ), and  $0.562 \pm 0.014$  ( $p < 0.001$ ); Day 3:  $1.091 \pm 0.032$ ,  $0.998 \pm 0.051$  ( $p = 0.011$ ), and  $0.717 \pm 0.055$  ( $p < 0.001$ ) at 0, 50, and 100  $\mu\text{M}$  capsaicin, respectively] and 10% serum-containing medium [Day 1:  $0.487 \pm 0.040$ ,  $0.431 \pm 0.023$  ( $p = 0.027$ ), and  $0.395 \pm 0.012$  ( $p = 0.002$ ); Day 2:  $0.750 \pm 0.030$ ,  $0.692 \pm 0.050$  ( $p = 0.048$ ), and  $0.583 \pm 0.037$  ( $p < 0.001$ ); Day 3:  $1.014 \pm 0.042$ ,  $0.942 \pm 0.034$  ( $p = 0.018$ ), and  $0.775 \pm 0.062$  ( $p < 0.001$ ) at 0, 50, and 100  $\mu\text{M}$  capsaicin, respectively].



### Capsaicin decreased expression of cyclin D1 and cyclin D3 in a dose-dependent manner in human colon cancer cells

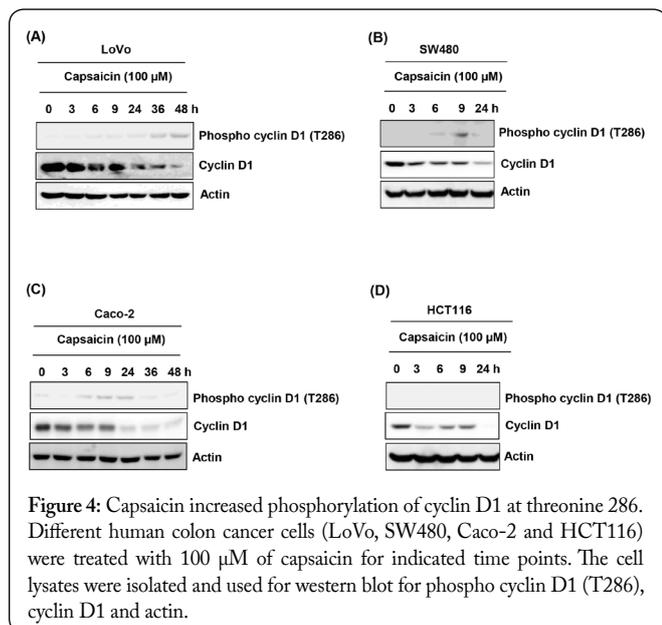
SW480 (*APC* mutant) and HCT116 (*APC* wild type) cells were incubated with different doses of capsaicin, followed by western blot analysis to measure expression of cyclin D1 and D3. As shown in Figure 3, the results indicate that capsaicin treatment reduces the expression of cyclin D1 and D3 in a dose-dependent and *APC*-independent manner.



### Capsaicin increased phosphorylation of cyclin D1 at threonine 286

We investigated if phosphorylation of cyclin D1 at threonine 286 is affected by capsaicin in four different types of human colon cancer cells with different genetic backgrounds. As a result, we found that capsaicin stimulated phosphorylation of cyclin D1 in LoVo, SW480, and Caco-2 cells, but not in

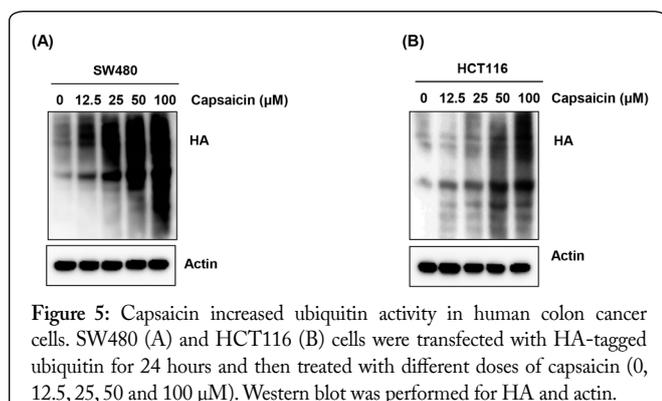
HCT116 cell and subsequently repressed expression of cyclin D1 in a time-dependent manner in this cell type (Figure 4).



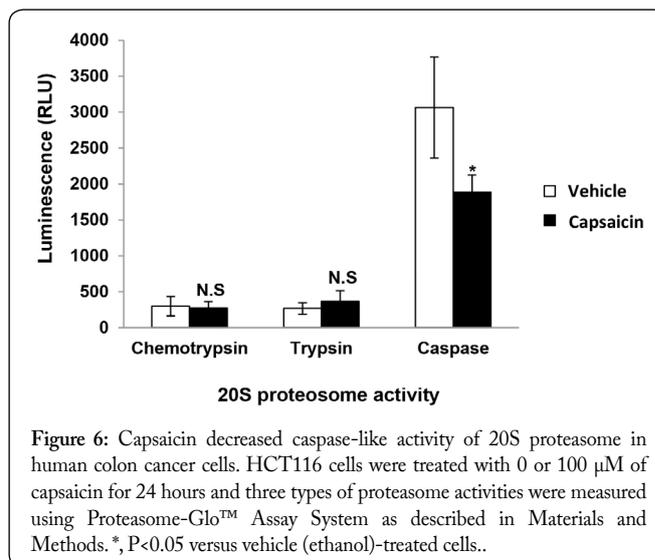
**Figure 4:** Capsaicin increased phosphorylation of cyclin D1 at threonine 286. Different human colon cancer cells (LoVo, SW480, Caco-2 and HCT116) were treated with 100  $\mu\text{M}$  of capsaicin for indicated time points. The cell lysates were isolated and used for western blot for phospho cyclin D1 (T286), cyclin D1 and actin.

### Capsaicin increased ubiquitination and decreased caspase-like activity of the 20S proteasome in human colon cancer cells

We tested the effects of capsaicin on cellular ubiquitination profiles. As shown in Figure 5, capsaicin increased the accumulation of ubiquitinated proteins for cells in a dose-dependent manner. Ubiquitin accumulation was more dramatic in SW480 cells compared to HCT116 cells, implying varying responsiveness to capsaicin. Next, we tested if capsaicin influences 20S proteasome activity because ubiquitinated proteins undergo proteasomal degradation. There are three major proteolytic activities, specifically chymotrypsin-like, trypsin-like, and caspase-like activities, within the 20S core. Therefore, we used a Proteasome-Glo Assay Systems that measure the above-described activities. As shown in Figure 6, capsaicin did not affect chymotrypsin- [298.3  $\pm$  135.2 vs 274.0  $\pm$  90.8 ( $p = 0.404$ )] or trypsin-like activity [270.7  $\pm$  81.1 vs 365.7  $\pm$  150.5 ( $p = 0.195$ )], but significantly reduced caspase-like activity [3064.3  $\pm$  704.9 vs 1889.0  $\pm$  235.5 ( $p = 0.026$ )].



**Figure 5:** Capsaicin increased ubiquitin activity in human colon cancer cells. SW480 (A) and HCT116 (B) cells were transfected with HA-tagged ubiquitin for 24 hours and then treated with different doses of capsaicin (0, 12.5, 25, 50 and 100  $\mu\text{M}$ ). Western blot was performed for HA and actin.



**Figure 6:** Capsaicin decreased caspase-like activity of 20S proteasome in human colon cancer cells. HCT116 cells were treated with 0 or 100  $\mu\text{M}$  of capsaicin for 24 hours and three types of proteasome activities were measured using Proteasome-Glo™ Assay System as described in Materials and Methods. \*,  $P < 0.05$  versus vehicle (ethanol)-treated cells.

## Discussion

Capsaicin is a major derivative of homovanillic acid and is abundant in spicy food. Recently, we reviewed the anti-cancer activities of capsaicin in various types of cancer including colon cancer [18]. Capsaicin induced apoptosis and growth arrest, and inhibited metastasis and angiogenesis through up- or down-regulation of complex cancer-associated genes and related signaling pathways. However, a lack of pre-clinical studies prohibits the practical use of capsaicin as an anti-cancer compound. Yoshitani SI, et al., reported that capsaicin treatment suppressed the formation of aberrant cryptic foci (ACF) in rats exposed to azoxymethane, which is colon-specific carcinogen [27]. However, ACF represent histological changes in the very early pre-neoplastic stage. Therefore, we employed an animal model with a more advanced staged tumor in order to test the anti-cancer effects of capsaicin. The mouse model used here has a truncated deletion in the *APC* gene and resembles human familial adenomatous polyposis (FAP) syndrome. These mice typically develop small adenomas or polyps in the small intestine. Although the difference was not statistically significant, treatment with capsaicin (20 mg/kg body weight) tended to prevent the formation or enlargement of polyps in mice, implying a potential anti-cancer activity of capsaicin in the early stages (adenoma) of colon cancer.

In our previous study, we observed that capsaicin treatment decreased proliferation of several colon cancer cell lines with different genetic backgrounds [8]. It is known that serum in culture media may affect the efficacy of test compounds due to the binding of test compounds to serum components. In fact, phenol compounds, including capsaicin, can bind to serum proteins and blunt their own efficacy [28, 29]. Based on the results from Figure 2, we recommend the use of culture media containing 0.5 or 1.0% of serum for the treatment with capsaicin.

Cyclin D1 and cyclin D3 are the major form of D type cyclins and play a significant role in the transition of the cell cycle from the G1 to S phase in collaboration with partner proteins, such as CDK4/6 (cyclin-dependent kinases 4/6) [20]. Deregulation and accumulation of cyclin D1 is an

important event in the development of various malignant cancers, including colon cancer. In most colon cancer tissues, cyclin D1 expression is constitutively high due to defective APC or  $\beta$ -catenin genes, which are the main upstream regulators of cyclin D1. However, Wnt/GSK3/ $\beta$ -catenin axis-independent regulation of cyclin D1 is also common in cancer. Therefore, we explored cyclin D1 degradation as an alternative anti-cancer mechanism for capsaicin. For the first time, our data revealed that capsaicin increased cyclin D1 phosphorylation at threonine 286, which is responsible for proteasomal degradation of cyclin D1 in human colon cancer cells [30]. This is consistent with the finding that capsaicin inhibited the G1 to S transition in the same type of colon cancer cells [31]. In terms of cyclin D1 phosphorylation, several kinases, including p38, GSK3, and Mirk, have been proposed phosphate donors in the mechanism of cyclin D1 phosphorylation. We speculate that Mirk could be the responsible kinase for cyclin D1 phosphorylation because there was no induction of phosphorylation in HCT116 cells (Figure 4D), which are Mirk null [32]. In addition, HCT116 cells may use a different mechanism for cyclin D1 down regulation, as opposed to threonine 286-mediated degradation.

The ubiquitin-proteasome pathway is an important regulatory system in determining protein stability and cancer development [33]. Many proteins, including cyclin D1, are initially conjugated with ubiquitin and then transferred to the 26S proteasome, which consists of a 20S core and a 19S regulatory complex [34]. In the current study we found that capsaicin increased the amount of ubiquitinated proteins in a dose-dependent manner (Figure 5). On the other hand, it is known that the 20S proteasome contains three major activities, chymotrypsin-, trypsin-, and caspase-like activities. Due to their unique biological functions, we measured each activity. Surprisingly, capsaicin did not affect chymotrypsin- or trypsin-like activities and significantly decreased caspase-like activity [ $3064.3 \pm 704.9$  vs  $1889.0 \pm 235.5$  ( $p = 0.026$ )] (Figure 6). This data is consistent with previous observation that capsaicin inhibited 20S proteasome activity in neuron cells [35]. To our knowledge, this is the first report that capsaicin specifically represses the caspase-like activity of the 20S proteasome in colon cancer cells. Based on our data, we speculate that capsaicin-induced cyclin D1 degradation and ubiquitination may be proteasome-independent. Further studies are required to address this hypothesis.

The capsaicin concentration used in the current study is similar to those used in several other studies [36–38]. As we discussed in previous studies [8, 17], the accurate blood concentration of capsaicin after intake is unknown. According to other studies, the blood concentration of capsaicin can reach up to 581 ng/mL (equivalent to 2  $\mu$ M) in rats after intravenous administration of capsaicin (2 mg/kg body weight) [39]. We believe that the relevance of *in vitro* doses should be estimated with careful consideration of several factors, such as bioavailability, potential active metabolites, and local concentration. For example, a much higher amount of capsaicin may reach colon because the local concentration of phytochemicals is much higher than in plasma [40].

On the other hand, increased autophagy has been proposed

as one of the anti-cancer mechanisms of capsaicin. High doses of capsaicin induce autophagy, apoptosis, and inhibition of autophagy sensitized capsaicin-induced apoptosis in bladder [41] and liver cancer cells [42]. Although the autophagic parameters were not measured in the current study, autophagy could be considered as a potential mechanism of capsaicin's anti-cancer activity in human colon cancer cells.

## Conclusion

The current study provides an important clue for potential use of capsaicin as an anti-cancer agent in colon cancer. *In vitro* data provide evidence that cyclin D1 degradation and 20S proteasome activity could be the mechanistic target for anti-cancer activity of capsaicin in human colon cancer.

## Conflict of Interest

There is no conflict of interest pertaining to this manuscript.

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