Induction of colon cancer cell death by 7-hydroxystaurosporine (UCN-01) is associated with increased p38 MAPK and decreased Bcl-x_L

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UCN-01, a selective inhibitor of protein kinase C, is known to inhibit the growth of cancer cells. Although it is currently undergoing clinical evaluation, information about its effect on human colon cancer is limited and the mechanism responsible is lacking. The objective of this study was to examine the cytotoxicity of UCN-01 to human colon cancer cells *in vitro* and its effect on the apoptotic molecules. HT-29, a radiation- and chemotherapy-resistant human colon cancer cell, was used in the study. Cell death/apoptosis was determined by the MTT assay and DNA fragmentation measurement. NF-κB activity was measured by an enzyme immunoassay method. Western blot was employed to examine the expression of relevant apoptotic molecules. The result showed that UCN-01 could induce apoptosis of human colon cancer cells in a time- and dose-dependent manner. It markedly reduced the expression of Bcl-x_L, but enhanced the level of p38 MAPK. In addition to Bcl-x_L and p38 MAPK, UCN-01 also increased both caspase-3 and peroxisome proliferator activated receptor γ protein levels. HT-29 cells transfected with exogenous Bcl-x_L showed a significant increase in NF-κB activity and prevented apoptosis induced by UCN-01. The overexpression of Bcl-x_L also reversed other relevant molecular changes observed in UCN-01-treated cells. In conclusion, UCN-01 exerted an antitumor effect in human colon cancer cells by inducing apoptosis. The mechanism responsible appeared to be related to reduction of Bcl-x_L and increased p38 MAPK. The overexpression of Bcl-x_L can significantly prevent apoptosis induced by UCN-01. *Anti-Cancer Drugs* 14:761–766 © 2003 Lippincott Williams & Wilkins.

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Introduction

The mitogen-activated protein kinases (MAPKs) are a family of protein kinases that transmit signals of stimuli from the cell membrane to the nucleus. In the nucleus, these enzymes activate various transcription factors, which have been implicated in many cellular processes such as proliferation, differentiation and apoptosis [1,2]. Therefore, these pathways could play a role in malignant transformation or malignant cell growth. The three relatively well-described pathways are the c-Jun N-terminal kinases (JNK), extracellular-regulated kinase (ERK) and p38 pathways. Each cascade is composed of at least three enzymes activated in series. The ERK1/2 pathway, the best-characterized of the group, is stimulated predominantly by growth factors, and plays an important role in cell growth and differentiation. The JNK1/2 are first identified by their ability to phosphorylate the c-Jun transcription factor following exposure to UV irradiation, growth factors or expression of transforming oncogenes. A third group of mammalian MAPKs collectively known as p38 MARK has been less well studied, but appears to be regulated in a fashion similar to that of JNK [3]. It has been reported that p38 MARK is downregulated in human colon cancer [4] and that its activity is suppressed by protein kinase C (PKC) [5]. Protein kinases participate in growth factors and oncogene product-dependent signal transduction pathways, and have emerged as key regulators of cell proliferation. Studies also demonstrate that PKC is implicated in colon carcinogenesis in humans and in rodent models [6].

UCN-01 is a selective inhibitor of PKC that inhibits the growth of human and murine tumor cells *in vitro* and *in vivo* [7–9]. It is a derivative of staurosporine, which is a natural product derived from fermentation extracts of a number of bacterial species. Staurosporine was initially recognized as a potent inhibitor of PKC, a Ca^{2+} and phospholipid-activated kinase. Different isoforms of PKC are activated in response to growth factors that act on receptor tyrosine kinases as well as 7-transmembrane

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domain receptors. Subsequent studies have revealed that staurosporine is a broad-acting kinase inhibitor with little specificity or selectivity for PKC. UCN-01 has greater selectivity for PKC as well as the ability to inhibit numerous other kinases [7–9]. Inhibition of PKC in UCN-01-treated cells is not critically or directly related to growth inhibition by various criteria, suggesting additional or mediators or targets as the basis for the anti-proliferative action of UCN-01. Generally speaking, UCN-01 can execute three distinct cellular effects *in vitro*: cell cycle arrest, induction of apoptosis and potentiation of DNA damage-related toxicity.

Although UCN-01 has been studied in various human cancers, reports on its effect on human colon cancer cells are limited. Further, the mechanism responsible for its anti-tumor effect is not fully known. Novel cancer treatment strategies are in development to target the molecular abnormalities that drive cancer cell proliferation and growth. The proliferation of colonic mucosa is positively associated with an increase in Bcl-x_L level [10]. The downregulation of Bcl-x_L by antisense RNA leads to diminished rates of cellular proliferation in prostate cancer cells [11]. The activity of p38 MAPK is linked to the cellular proliferation in a number of cells including intestinal epithelial cells [12] and, further, the proliferation is negatively regulated by p38 MAPK in some tumor cells such as pancreatic cancer cells [13]. The relationship between UCN-01 and p38 MAPK in human colon cancer has not been investigated before. Whether UCN-01 will affect the expression of Bcl-x_L is also unknown in human colon cancer cells.

Materials and methods

Reagents

Primary antibodies, Bcl-x_L, Bid, p38 MAPK, caspase-3, peroxisome proliferator activated receptor (PPAR)γ and β-tubulin, and the secondary antibody, IgG–horseradish peroxidase (HRP), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 and RPMI 1640 medium were supplied by Invitrogen (Carlsbad, CA). UCN-01 was kindly provided by the National Cancer Institute (Rockville, MD). Other reagents, if not otherwise indicated, were purchased from Sigma (St Louis, MO).

Cell culture

A human colon cancer cell line, HT-29, was purchased from ATCC (Rockville, MD), and cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and antibiotics.

Quantitation of cell death and apoptosis

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to quantify cell death/viability. Cell death was determined by DNA fragmentation measurement. Briefly, DNA fragments in the cytoplasm were labeled with BrdU. The BrdU-labeled DNA fragments were then detected using an ELISA kit from Roche (Mannheim, Germany). The assay was performed according to the manufacturer's instruction.

DNA transfection

Stable transfection was performed using cationic lipid reagent, Lipofectamine 2000. The experiment was carried out according to the manufacturer's instruction. Briefly, HT-29 cells (80–85% confluence) were transfected with 500 ng of plasmid DNA in serum-free conditions. After the cells were incubated for 6 h in serum-free medium containing DNA and Lipofectamine 2000, an equal volume of growth medium containing 20% serum was then added without removing the transfection mixture. The supernatant was changed with complete medium at 24 h following the start of transfection. At 72 h after transfection, the cells were passaged into the selective medium containing Geneticin-selective antibiotic, G418. The concentration of G418 for the selection was determined previously by dose–response assay. Positive stably transfected cells were selected by G418 (0.4 mg/ml) after 2 months.

Western blot analysis

Cell samples were homogenized with ice-cold PBS and then lysed in a solution containing 8 M urea, 0.1 M $Na_2H_2PO_4$ and 0.01 M Tris–HCl. Supernatants were obtained after centrifugation at 10 000 *g*. After boiling, proteins were separated on 10% SDS–polyacrylamide gels. Proteins were then electrophoretically transferred from the gel onto nitrocellulose membranes and the membranes were blocked for 1 h in PBS/Tween buffer containing 5% dry milk powder (fat free) at room temperature. The membranes were then incubated with a primary antibody for 1 h. After washing, the membranes were incubated with a secondary antibody, IgG–HRP. Finally, they were treated with the reagents in the chemiluminescent detection kit (ECL system; Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Anti-human β-tubulin antibody was used to detect human β-tubulin, which was used as a control for equal loading. The densities of the protein bands corresponding to the size were determined with a GSP-700 scanner with Quantity One image software (Hercules, CA). Quantitation of the protein was performed with a GSP-700 scanner with Quantity One supplied by same company.

Nuclear protein extraction and NF-κB activity assay

Nuclear protein was isolated according to the procedure described [14]. Briefly, HT-29 cells were harvested, washed in PBS and collected by centrifugation. The cell pellet was resuspended in 0.5 ml of 10 mM Tris–HCl (pH 7.5)/5 mM $MgCl_2$/0.05% (v/v) Triton X-100 and lysed

with 20 strokes in a homogenizer. The homogenate was centrifuged at 10 000 *g* for 15 min at 4°C. The pellet was obtained. The nuclei pellet volume was estimated and the pellet was resuspended in an equal volume of 10 mM Tris–HCl (pH 7.4)/5 mM $MgCl_2$, followed by the addition of 1 nuclei pellet volume of 1 M NaCl/10 mM Tris–HCl (pH 7.4)/4 mM $MgCl_2$. The lysing nucleus was left on ice for 30 min and then centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant (nuclear extract) was removed and 80% glycerol was added so the final glycerol concentration was 20% (v/v). The concentration of the nuclear protein was determined. NF-κB activity was measured by an enzyme immunoassay kit from Oxford Biomedical Research (Oxford, MI), which employed an oligonucleotide containing the DNA-binding NF-κB consensus sequence. If there is any NF-κB presented in the sample, it will specifically bind to the oligonucleotide coated on the plate and the DNA-bound NF-κB can be selectively recognized by the antibody to NF-κB subunit, p50 or p105.

Statistical analysis

All values were expressed as mean ± SEM. Drug effect was assessed using one-way ANOVA. Statistical comparisons between two groups were analyzed by the Student's *t*-test. A p value of less than 0.05 was taken as statistically significant. All statistical analyses were done by an InStat software (GraphPad Software, San Diego, CA).

Results

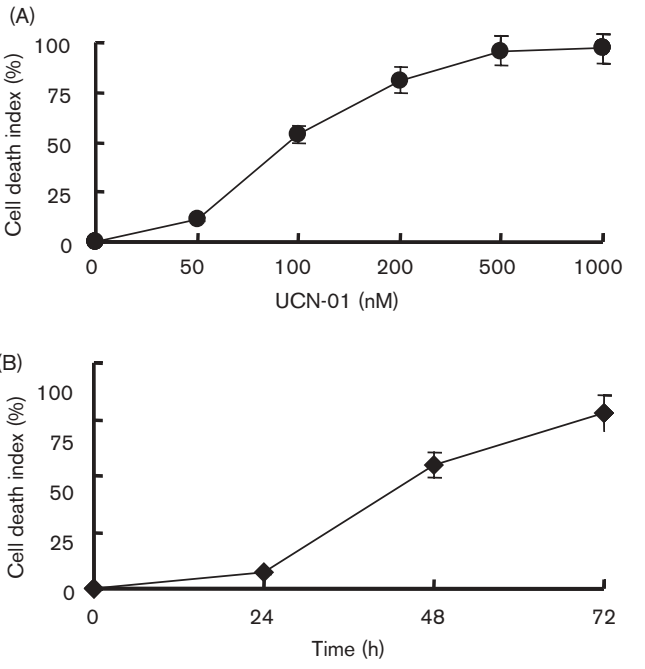
UCN-01 induced apoptosis

UCN-01 significantly induced cell death of HT-29 at the concentrations equal to or higher than 100 nM (Fig. 1A). The cell death appeared to be dose dependent. A plateau was reached when the concentration of UCN-01 was at 200 nM or above. The cell death was also induced in a time-dependent manner by UCN-01 (Fig. 1B). The cell death was apparent after the 48-h treatment with 100 nM UCN-01.

UCN-01 increased the expression of p38 MAPK but decreased the level of Bcl-x_L

The level of p38 MAPK protein in HT-29 cells was induced after treatment with 100 nM UCN-01 for 48 h, compared with that in the cells without UCN-01 treatment (Fig. 2). In contrast to p38 MAPK, the level of Bcl-x_L protein was much lower in HT-29 cells treated with UCN-01 than in those cells without the treatment (Fig. 2). The decrease was found to be nearly 1.5-fold. Accompanying the differential expression of p38 MAPK and Bcl-x_L, the protein level of PPARγ was elevated in the cells with treatment as compared with those without treatment (Fig. 2). Although there was only a slight increase in the level of 32-kDa caspase-3 (pro-enzyme) in the cells treated with UNC-01, the active caspase-3 (20 kDa) appeared only in the cells with treatment (Fig. 2).

Fig. 1



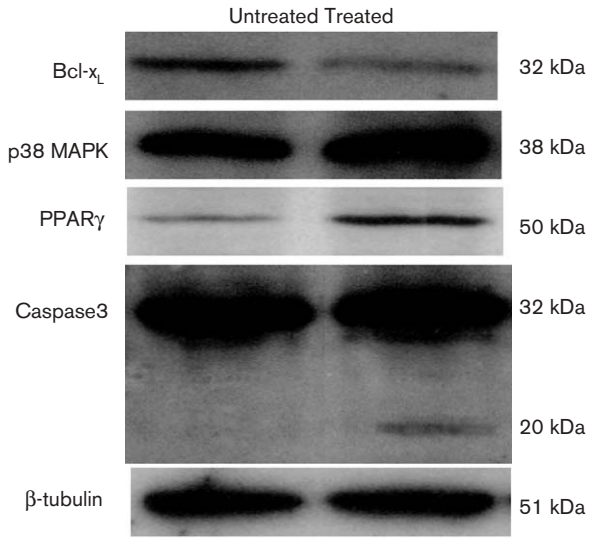
UCN-01 induced a time- and dose-dependent cell death. For the time course study, HT-29 cells were treated with 100 nM for different periods of time as indicated. For the dose–response experiment, HT-29 cells were exposed to several concentrations of UCN-01 for 48 h. The cell death was determined by the MTT assay. Control cells were treated with vehicle only. The result of the MTT assay was expressed as the cell death index, which was calculated by the following formula: $[1 - \text{absorbance}_{(\text{test well} - \text{background})}/\text{absorbance}_{(\text{control} - \text{background})}] \times 100\%$. Each experiment was repeated at least 3 times. $^{**}p < 0.01$, compared with non-treatment.

The expression of Bid protein was not different between the cells with treatment and those without (result not shown).

Overexpression of Bcl-x_L regulated the expression of p38 MAPK, caspase-3 and PPARγ

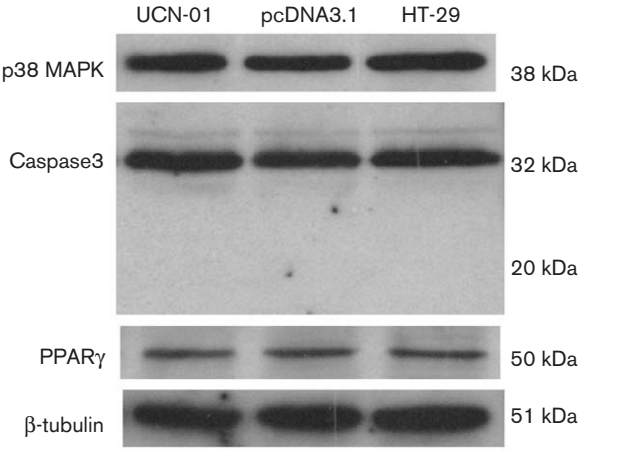
In order to examine how Bcl-x_L affects UCN-01-targeting molecules in HT-29 cells, we cloned the human Bcl-x_L gene into pcDNA3.1, a mammalian expression vector, and then stably transfected HT-29 cells with the Bcl-x_L gene. The transfected HT-29 cells expressed much higher levels of Bcl-x_L than those without Bcl-x_L transfection (data not shown). It was found that the level of p38 MARK in the cells with Bcl-x_L overexpression would no longer be altered by UCN-01 treatment. After UCN-01 treatment, the expression of p38 MARK in the cells transfected with Bcl-x_L was not significantly different from that in the cells transfected with pcDNA3.1 vector or without transfection (Fig. 3). Similarly, the alterations of caspase-3 and PPARγ induced by UCN-01 were no longer detected (Fig. 3).

Fig. 2



Effect of UCN-01 on the expression of p38 MAPK and Bcl-x_L proteins. HT-29 cells were treated with 100 nM UCN-01 for 48 h. At the end of treatment, the cell lysates were obtained for Western blot analysis. β-tubulin, a constitutively expressed protein, was used as a control for equal loading.

Fig. 3



Effect of Bcl-x_L overexpression on the expression of p38 MAPK, caspase-3 and PPARγ. HT-29 cells were stably transfected with the Bcl-x_L gene. After the establishment of the cells overexpressing Bcl-x_L, HT-29 cells were treated with 100 nM UCN-01. HT-29 cells without transfection and transfected with an empty pcDNA3.1 vector were used as controls. Cell lysates were isolated from the cells and used to determine the target protein expression. β-tubulin, a constitutively expressed protein, was used as a control for equal loading.

Overexpression of Bcl-x_L increased NF-κB activity and protected HT-29 cells from apoptosis

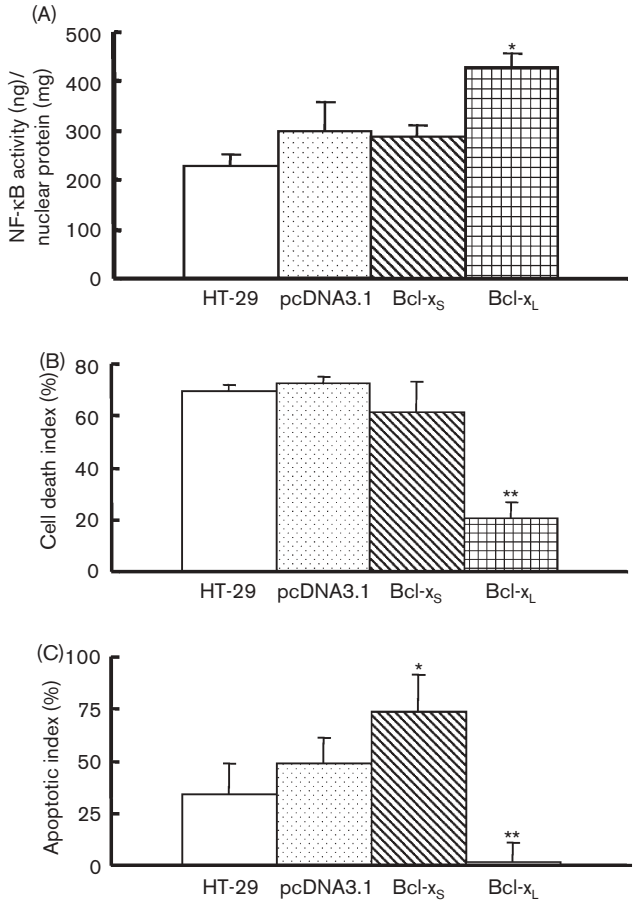
Overexpression of Bcl-x_L significantly increased the activity of NF-κB in HT-29 cells, whereas the overexpression of Bcl-x_S did not change the activity, compared with the cells transfected with pcDNA3.1 vector or without transfection (Fig. 4). Accompanying the change of NF-κB activity, the overexpression of Bcl-x_L was able to protect the cells from cell death induced by UCN-01, as evident by MTT assay and DNA fragmentation measurement (Fig. 4). The excess level of Bcl-x_S failed to either increase or decrease the cell death detected by MTT. However, it significantly increased DNA fragmentation induced by UCN-01 (Fig. 4).

Discussion

UCN-01, which is presently under clinical evaluation, has been shown to have an inhibitory effect on a variety of human tumor cells. The sensitivity of tumor cells to UCN-01 is varied, suggesting that different types of tumor cells may have their own specific molecules interacting with UCN-01 or that the mechanism responsible for the antitumor effect of UCN-01 may not be the same in different types of tumor cells. In human breast cancer, UCN-01 induces DNA double-strand breaks in p53 mutant tumor cells, but not in normal or $p53^-$ epithelial cells [15]. However, UCN-01 can also induce cell death in B cell chronic lymphocytic leukemia cells through a p53-independent mechanism [16]. In human thyroid cancer cells, our previous experiment demonstrates that the level of Bcl-2 is inversely related to the tumor cell susceptibility to UCN-01 [17]. In the present report, we show that apoptosis induced by UCN-01 is associated with an increase in p38 MAPK and a reduced level of Bcl-x_L in human colon cancer cells.

HT-29, a human colon cancer cell line, is frequently resistant to radiation and chemotherapeutic agents, which is thought to be due to the mutated p53 gene [18,19]. Despite the fact that HT-29 contains the mutated p53 gene, UCN-01 is still able to induce cell death, as shown in the present experiment. HT-29 cells underwent death in a dose- and time-dependent manner upon HT-29 treatment. The proliferation and growth of human colon cancer cells is known to be associated with PKC [6], as PKC is involved in DNA synthesis and cell survival pathways of colon cancer cells [20]. Overexpression of PKC confers resistance to tumor necrotic factor (TNF)-α- and paclitaxel-induced apoptosis in colon cancer cells [21]. Therefore, it is not surprising to observe that UCN-01, an inhibitor of PKC, significantly exerts an inhibitory effect on the growth of human colon cancer cells. Furthermore, the result is in agreement with previous studies in which the activity of PKC was suppressed by other agents such as soybean saponins and geraniol, a natural component of plant essential oils [22,23]. Using the strategy of antisense technology, Chakrabarty and Huang also demonstrated that the response of colon cancer cells to cytotoxic drugs was greatly improved when the cells were treated with an

Fig. 4



Overexpression of Bcl-x_L increased NF-κB activity and prevented apoptosis induced by UCN-01. HT-29 cells were stably transfected with the Bcl-x_L gene. After the establishment of the cells overexpressing Bcl-x_L, the cells were treated with 100 nM UCN-01. For NF-κB activity assay, the nuclear protein was isolated. NF-κB activity was measured by a commercially available ELISA kit (A). For cell death, the MTT assay was employed to determine the death of cells (B). The results of the MTT assay were expressed as the cell death index, which was calculated by the following formula: [1 – absorbance$_{(\text{test well} - \text{background})}$/absorbance$_{(\text{control} - \text{background})}$] × 100%. Cell apoptosis was determined by DNA fragmentation detection assay (C). DNA fragments from damaged cells were released into the culture supernatant and labeled by BrdU. The BrdU-labeled DNA was quantified using a monoclonal antibody against BrdU. The result of the DNA fragmentation detection assay was expressed as the apoptotic index, which was calculated by the following formula: [1 – absorbance$_{(\text{test well} - \text{background})}$/absorbance$_{(\text{control} - \text{background})}$] × 100%. Each point represents the mean of three independent experiments with triplicate wells. $^{**}p<0.01$, $^{*}p<0.05$ compared with the controls (not transfected and transfected with the empty vector).

antisense PKC expression vector, which was designed to downregulate the PKC level [24].

The activity of PKC is closely related to p38 MARK and Bcl-x_L, both of which were altered in human colon cancer cells treated with UCN-01 in the present study. p38 MARK can promote apoptosis by at least four channels. (i) It inhibits PKCζ activity, which potentiates apoptosis [25]. (ii) It participates in the induction of Fas ligand [26]. (iii) It mediates the apoptotic pathway of TNF-α–TNF receptor 1 by interacting with signal adaptor protein TRAF2 [27]. (iv) It is involved in nitric oxide-induced cell death [28]. However, whether all these channels are functional in the apoptosis of colon cancer cells induced by UCN-01 needs further investigation. Since the level of p38 MAPK is reduced in human colon cancer [4], a significant increase in p38 MAPK by UCN-01 indicates that such a reduction is reversible and thus p38 MAPK may serve as a potential target molecule for antitumor therapy of human colon cancer.

Bcl-x_L is an important anti-apoptotic Bcl-2 family member. Its level is often increased in human colon cancer cells [29,30], rendering it as one of the potential targets in tumor therapy. We found that UCN-01 reduced the level of Bcl-x_L in HT-29 cells and such a reduction is associated with a significant increase in cell death. We further demonstrated that the overexpression of Bcl-x_L could reverse the effect of UCN-01. This result is in agreement with the finding that the overexpression of Bcl-x_L inhibits apoptosis induced by the activation of p38 MAPK [31]. We also examined whether the overexpression of Bcl-x_S, a pro-apoptotic Bcl-2 family member, would affect UCN-01 treatment. It appeared that excess Bcl-x_S could significantly promote apoptosis of HT-29 cells, but it failed to increase the total number of cell death. Therefore, the overall effect of Bcl-x_S on UCN-01 antitumor treatment in human colon cancer cells may be limited. This assumption is supported by our finding that the overexpression of Bcl-x_S had little effect on NF-κB activity, while the overexpression of Bcl-x_L significantly elevated NF-κB activity. NF-κB plays a role in inhibition of apoptosis in a number of cells, including human colon cancer cells [32].

In addition to p38 MAPK and Bcl-x_L, the protein levels of caspase-3 and PPARγ were found increased in HT-29 cells treated with UCN-01. The pattern of caspase-3 alteration by UCN-01 is in line with the theory that the activation of caspase-3 happens through proteolytic degradation of a 32-kDa pro-enzyme into a 20-kDa activated form [33]. Caspase-3, an executioner caspase, plays a central role in apoptosis of tumor cells. Activated caspase-3 cleaves polypeptides that are involved in genome function such as poly(ADP)ribose polymerase, DNA-dependent protein kinase and ribonucleoproteins, and it cleaves proteins that are involved in regulatory functions of the cell cycle [34]. Therefore, activation of caspase-3 is generally considered as one biomarker of cell death or apoptosis. PPARγ is a ligand-activated nuclear transcriptional factor that is emerging as an important determinant of cell differentiation, proliferation and growth. The PPARγ promotes the suppression of DNA synthesis and induction of apoptosis of human colorectal

cells [35,36]. Although we have observed an increase in the expression of PPARγ protein in HT-29 cells treated with UCN-01, the exact role of PPARγ in UNC-01-induced apoptosis needs further study. Finally, our experiment showed that HT-29 cells with Bcl-x_L overexpression not only corrected the apoptosis induced by UCN-01, but also recovered the relevant molecular changes accompanying the apoptosis. The finding further supports the contention that Bcl-x_L plays a central role in UCN-01-induced apoptosis of human colon cancer cells.

In conclusion, the present study demonstrated that UCN-01 was able to induce apoptosis in human colon cancer cells and the mechanism responsible appeared to be related to a reduction of Bcl-x_L levels and an increase in p38 MAPK. The overexpression of Bcl-x_L can significantly prevent apoptosis induced by UCN-01.

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