Clitocine induces apoptosis and enhances the lethality of ABT-737 in human colon cancer cells by disrupting the interaction of Mcl-1 and Bak

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ABSTRACT

ABT-737 is a novel anti-apoptotic Bcl-2 family protein inhibitor with high affinity to Bcl-2, Bcl-xl and Bcl-w but relatively low affinity to Mcl-1/A1. Therefore, high level Mcl-1 usually confers human tumor cell resistance to ABT-737. At the present study, we observed that clitocine can induce apoptosis in six tested human colon cancer cell lines accompanied by suppression of Mcl-1. More interestingly, clitocine significantly enhances the ABT-737-mediated lethality by inducing apoptosis. At the molecular level we determined Mcl-1 is the potential target through which clitocine can sensitize human colon cancer cells to ABT-737 induced apoptosis. Knocking-down of Mcl-1 is sufficient to increase cancer cell susceptibility to ABT-737 while its over-expression can significantly reverse this susceptibility. We also determined that clitocine may activate Bak by disrupting the interaction between Mcl-1 and Bak to induce mitochondrial membrane permeabilization. Furthermore, silence of Bak with the specific siRNA effectively attenuates the apoptosis induction by co-treatment of clitocine and ABT-737. Finally, clitocine in combination with ABT-737 significantly suppress the xenograft growth in animal model. Collectively, our studies suggest clitocine can induce apoptosis and potentiate ABT-737 lethality in human colon cancer cells by disrupting the interaction of Mcl-1 and Bak to trigger apoptosis.

Introduction

Programmed cell death (apoptosis) is essential for normal development and tissue homeostasis in a healthy body, and its aberrant regulation contributes to multiple human diseases including cancer, autoimmunity, neurodegenerative disorders, and diabetes [1]. The complex interplay between pro-apoptotic and anti-apoptotic Bcl-2 family members extensively participates in the regulation of this interplay between pro-apoptotic and anti-apoptotic Bcl-2 family proteins to induce apoptosis in cancer cells, which acts by mimicking the ability of BH3-only protein Bad to dock to the hydrophobic groove of anti-apoptotic Bcl-2-family proteins, has been developed and is presently under preclinical and clinical evaluation [12,13].

ABT-737 exerts impressive anti-tumor activity as a single agent against many human cancer cells [14–16] and lowers the apoptotic threshold for chemotherapeutics by sensitizing cancer cells to several anti-cancer drugs such as TRAIL [17], BRAF inhibitors [18] and carboplatin [19]. However, ABT-737 has a low affinity for Mcl-1 [12] and therefore may exhibit limited cytotoxicity in cancer cells with high level Mcl-1 [20,21]. Mcl-1 is extensively expressed in many human cancer cells and acts as a critical survival factor [22,23] via negatively regulating the apoptosis by interacting with pro-apoptotic proteins such as Bak or Bax to inhibit their function [24,25]. Its elevated expression has been shown to confer cancer cell resistance to several chemotherapeutics including ABT-737-based therapy [26–28]. One candidate strategy to overcome the resistance of cancer cells to ABT-737 is to target Mcl-1 expression or functions by using other inhibitors [21,29].

Clitocine is a natural nucleoside extracted from wild mushroom and was observed to inhibit cancer cell proliferation as an inhibitor of adenosine kinase or by inducing apoptosis in several
human colon cancer cell lines [30–32]. Recently, we determined that clitocine could induce apoptosis in drug-resistant human hepato- 
toma cancer cells in vitro and inhibit tumor growth in vivo by down-
regulating Mcl-1 [33]. However, the anti-tumor activity of clitocine in human colon cancer cells has never been reported. Colorectal 
cancer cells usually show resistance to chemotherapeutics through 
Mcl-1 overexpression [34]. By suppressing Mcl-1, it is possible to 
overcome ABT-737 resistance in colorectal cancer cells [35,36]. 
Thereby, we hypothesized clitocine might be a sensitizer for ABT-
737 in human colon cancer cells. At the present study, we focused 
on determining whether clitocine could induce apoptosis as a single 
agent and additionally enhance ABT-737-mediated apoptosis in 
human colon cancer cells as well as its underlying mechanisms.

Materials and methods

Cell culture

Human colon cancer cells SW620, DLD1, LS411N, HCT116, RKO and Caco-2 used in this study were obtained from American Type Culture Collection, and cultured in DMEM medium (Invitrogen, Inc., Carlsbad, CA) with 5% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, in 37 °C humidified 5% CO2 incubator.

Reagents

Antibodies against caspase-3, Bcl-2, Bcl-xl, Bad, Bnip3, Bim, Mcl-1 as well as compound ABT-737 for in vitro assay were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against caspase 9, Bak, Puma were from Cell Signaling (Beverly, MA); antibody against active Bak was from Calbiochem (Merck KGaA, Darmstadt, Germany); antibody against caspase 8 was from R&D (Minneapolis, MN), JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide) was purchased from Molecular Probes (Eugene, OR, USA). Annexin-FITC was obtained from BD Biosciences Pharmingen (San Diego, CA). Lipofectamine 2000 reagent was purchased from Molecular Probes (Eugene, OR, USA). Annexin-V-FITC and propidium iodide were purchased from Sigma (St. Louis, MO). Clitocine was isolated and purified as described previously [32]. The purity of clitocine used in this study was 99% and the drug was dissolved in DMSO and absorbance at 570 nm was read by a microplate reader.

Cell viability assay

Cells were seeded in a 96-well plate and incubated for 24 h. After treatment, cells were incubated with 50 μL of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich Chemical) in PBS for 3 h according to the manufacturer’s instructions. The purple formazan was then solubilized by DMSO and absorbance at 570 nm was read by a microplate reader.

Assessment of apoptosis

After treatment, cells were stained with FITC-labeled Annexin V and propidium iodide (PI) and analyzed by flow cytometry (FACScanto, Becton Dickinson) as described previously [37]. Data acquisition and analysis were performed using the program MinWID 2.9 (Becton Dickinson). The percentage of apoptotic cell death was defined as the sum of early (Annexin V-positive and PI-negative cells) and late (Annexin V-positive and PI-positive cells) apoptosis.

Measurement of mitochondrial transmembrane potential (ΔΨm)

The ΔΨm was analyzed using the fluorescent cation dye JC-1. Briefly, cells were harvested and washed with PBS twice. The cells were resuspended in 500 μL PBS and 10 μM JC-1 for 15 min at 37 °C in the dark. Subsequently, cells were subjected to flow cytometry analysis. Data acquisition and analysis were performed using the program MinWID 2.9.

Western blotting analysis

Cells were incubated in total lysis buffer [2.1 μg/mL aprotinin, 0.5 μg/mL leupeptin, 4.9 mM/MgCl2, 1 mM/L orthovanadate, 1% Triton X100, and 1 mM/L phenylmethylsulfonyl fluoride]. Total lysate was separated in denaturing SDS-PAGE gel and transferred to a PVDF membrane (0.45 μM, Millipore). After blocking with 5% fat-free dry milk in PBS, the membranes were washed by PBS containing 0.1% Tween 20 and incubated with primary antibodies followed by respective horse-
radish peroxidase-conjugated secondary antibodies. Signals were visualized with enhanced chemiluminescence (Amersham Life Sciences, Inc., Buckinghamshire, UK). β-actin was used as loading control.

Clitocine inhibits colon cancer cell growth by inducing apoptosis

The cytotoxicity of clitocine on human colon cancer cell lines LS411N, SW620, Caco-2, HCT116, RKO and DLD1 was assessed by MTT assay. As shown in Fig. 1B, all cancer cell lines are sensitive to clitocine with the IC50s ranging from 0.3 μM to 1 μM. In the apoptotic assays, clitocine effectively induces discernable apoptosis in all cell lines in a dose-dependent manner which are evidenced by the in-
creased percentage of Annexin V-positive cells (Fig. 2A) and the cleavage of caspase and PARP (Fig. 2B) in the tested cell lines (LS411N
and SW620) after 48 h incubation. The expression profile of Bcl-2 family proteins was also determined and interestingly clitocine prefers to down-regulate Mcl-1 expression in both LS411N and SW620 cells even at a very low dose (0.3 μM, Fig. 2C). This finding suggests that clitocine might be a sensitizer of ABT-737 in human colon cancer cells due to the key role of Mcl-1 in the ABT-737 resistance of cancer cells.

**Clitocine potentiates ABT-737-induced apoptosis in human colon cancer cells**

At first, MTT assay was performed to determine the sensitivity of different colon cancer cell lines to ABT-737. All cell lines involved in the study are highly resistant to ABT-737 (Fig. 3A). Furthermore, Mcl-1 expression levels in these cell lines were also examined. As shown in Fig. 3B, Mcl-1 is highly expressed in RKO cells, moderately expressed in Caco-2 and DLD1 cells, while lowly expressed in LS411N, SW620 and HCT116 cells. The IC50s for ABT-737 in these cell lines are: > 25 μM (RKO), 19.7 μM (Caco-2), 18.78 μM (DLD1), 11.47 μM (LS411N), 12.24 μM (SW620) and 20.49 μM (HCT116) respectively. The expression profiles of Mcl-1 in these human colon cancer cells are consistent with their resistance to ABT-737.

Next, we examined whether combined treatment of clitocine and ABT-737 exerts enhanced lethality in colon cancer cells. After pretreatment with different concentrations of clitocine (0.1–0.8 μM) for 24 h, cancer cells were incubated with 5 μM ABT-737 for another 24 h and subjected to MTT assay. Interestingly, combination of clitocine and ABT-737 dramatically inhibits the cell growth in LS411N (Fig. 3C) and SW620 (Fig. 3D) cells. Similar results were observed in other colon cancer cell lines including Caco-2 (Fig. 3E), HCT116 (Fig. 3F), RKO (Fig. 3G) and DLD1 (Fig. 3H) cells. Furthermore, it looks like that 0.1 μM clitocine is enough to enhance the ABT-737 lethality in these cell lines. However, the most efficient concentration of clitocine is 0.2 μM especially in LS411N, SW620 and HCT116 cells as co-administration of 0.2 μM clitocine and 5 μM ABT-737 almost causes 70%–80% cell growth inhibition. Therefore, 0.2 μM clitocine and 5 μM ABT-737 were used in all subsequent experiments.

To confirm whether the sensitization effect of clitocine on ABT-737 involves apoptosis induction, LS411N, SW620 and HCT116 cell lines were subjected to flow cytometry analysis after cells were treated with clitocine, ABT-737 alone or in combination. As shown in Fig. 4A and B, clitocine dramatically enhances ABT-737-induced apoptosis in LS411N (the percentage of Annexin-V positive cells increases from 10.01% to 73.96%), SW620 (the percentage of Annexin-V positive cells increases from 12.74% to 82.01%) and HCT116 (the percentage of Annexin-V positive cells increases from 16.12% to 64.78%) cells. Co-treatment of clitocine and ABT-737 also increased the cleavage of caspase and PARP. As shown in Fig. 4C, co-treatment with clitocine and ABT-737 in LS411N and SW620 cells significantly increased the cleavage of caspase 3 and PARP. More interestingly, 0.2 μM clitocine alone significantly down-regulated Mcl-1 while ABT-737 elevated the Mcl-1 expression. However, 0.2 μM clotocine effectively inhibits the Mcl-1 expression and successfully attenuates the up-regulation of Mcl-1 induced by ABT-737 which suggests that clotocine might sensitize cancer cells to ABT-737 by targeting Mcl-1 (Fig. 4C).

**Mcl-1 is the target by which clotocine could enhance the apoptosis induced by ABT-737**

High level Mcl-1 confers cancer cell resistance to ABT-737 as the compound presents high affinity to Bcl-2 and Bcl-xl but not Mcl-1, and suppression of Mcl-1 can reverse such resistance [39]. Therefore, Mcl-1 might be a potential target for overcoming ABT-737 resistance in cancer cells. Here, we confirmed that knock-down of Mcl-1 by transfecting siRNA targeting Mcl-1 is sufficient to increase the sensitivity of human colon cancer cells LS411N and SW620 to ABT-737, which suggests that the sensitization effect of clotocine on ABT-737 in human colon cancer cells might be attributed to the suppression of Mcl-1 induced by clotocine. As shown in Fig. 5A, Western blotting assay indicated that siRNA-Mcl-1 successfully inhibited the Mcl-1 expression in SW620 and LS411N cells by about 50%, and flow cytometry analysis revealed that knock-down of Mcl-1 and ABT-737 treatment cooperatively induced apoptosis in both cell lines (the percentage of Annexin-V positive SW620 cells increased from 11.83% to 54.47%, and the percentage of Annexin-V positive LS411N cells increase from 4.51% to 58.33%).

To further confirm whether clotocine enhances ABT-737 lethality via inhibiting Mcl-1, LS411N and SW620 cells were transfected with Mcl-1 over-expression plasmid pcDNA3.1-Mcl-1 to enforce the exogenous Mcl-1 expression. After 24 h incubation, cells were pretreated with 0.2 μM clotocine and then incubated with 5 μM ABT-737 for another 24 h. As shown in Fig. 5B, Mcl-1 is highly expressed in cells transfected with pcDNA3.1-Mcl-1 compared with its control cells transfected with empty vector; more importantly, high level Mcl-1 significantly attenuates the apoptosis mediated by combination of clotocine and ABT-737. Taken together, clotocine overcomes resistance of human colon cancer cells to ABT-737 at least partially through inhibiting Mcl-1 expression.
Fig. 2. Clitocine induces apoptosis in human colon cancer cells accompanying Mcl-1 inhibition. (A), LS411N, SW620, Caco-2, HCT116, RKO and DLD1 cells were treated with indicated concentrations of clotocine for 48 h. After co-staining with Annexin V-FITC and PI, cells were analyzed by flow cytometry to measure the percentage of apoptotic cells. Apoptosis was quantified as Annexin V-positive cells. Data are presented as mean ± SD, n = 3. * P < 0.05 vs. control, ** P < 0.01 vs. control. (B and C), LS411N and SW620 cells were treated as A. Proteins were extracted and subjected to Western blotting assay to evaluate cleavage of caspase and PARP (B) as well as expression of Bcl-2 family proteins (C). C-Caspase, Cleavage-Caspase; C-PARP, Cleavage-PARP. Densitometric analysis of the protein bands was done using Image J (NIH). Data are presented as mean ± SD, n = 3. * P < 0.05 vs. control, ** P < 0.01 vs. control.
The activation of Bak is indispensable in the sensitization effect of clitocine on ABT-737

As mentioned above, ABT-737 can lower the threshold for apoptosis-induction in cancer cells by targeting Bcl-2 and Bcl-xl to initiate mitochondrial membrane permeabilization (MMP) [40]. However, cancer cells with high level Mcl-1 frequently exert resistance to ABT-737 as its low affinity to Mcl-1 [41]. Therefore, the mitochondrial membrane permeabilization was examined using JC-1 staining assay. After treatment with clitocine alone, ABT-737 alone or in combination, LS411N and SW620 cells were stained with JC-1 and subjected to flow cytometric analysis. As shown in Fig. 6, little influence was imposed on mitochondrial membrane permeabilization by clitocine or ABT-737 alone, while a combination of them dramatically induced MMP in both SW620 and LS411N cells (about 80% cells with low ΔΨm in two cell lines). This finding suggests that the intrinsic apoptotic pathway is involved in the sensitization effect of clitocine on ABT-737.

The activation of Bak was also examined using specific antibodies against active Bak by flow cytometry analysis due to its critical
role in mitochondrial membrane permeabilization [42]. As shown in Fig. 7A, conformational activation of Bak dramatically increased in LS411N and SW620 cells co-treated with clitocine and ABT-737 compared to cells treated with clitocine or ABT-737 alone. As previously mentioned Mcl-1 can directly bind to Bak and block its pro-apoptotic function in induction of mitochondrial membrane permeabilization [24,25], we supposed that clitocine might activate Bak via down-regulating Mcl-1. The immunoprecipitation assay was performed and the data revealed that clitocine indeed decreases the amount of Mcl-1 binding to Bak even under the presence of ABT-737 in SW620 cells (Fig. 7B). More interestingly, the knock-down of Bak mediated by siRNA targeting Bak effectively attenuated the apoptosis induced by the combination of clitocine and ABT-737 (Fig. 7C). Collectively, our data suggest that clitocine might sensitize ABT-737 by disrupting the interaction of Mcl-1 and Bak to initiate the intrinsic apoptotic pathway in human colon cancer cells.

Fig. 4. Clitocine sensitizes human colon cancer cells to ABT-737 via inducing apoptosis. (A and B), LS411N, SW620 and HCT116 cells were treated with vehicle or 0.2 μM clitocine (pretreated for 24 h) and then co-treated with 5 μM ABT-737 (treated for another 24 h). After total 48 h treatment, cells were subjected to Annexin V-FITC and PI staining. Flow cytometry assay was performed to detect the percentage of apoptotic cells. Data are presented as mean ± SD, n = 3. *P < 0.05 vs. control, **P < 0.01 vs. control. (C), Proteins were extracted in LS411N and SW620 cells treated as in A and assessed by Western blotting assay.
**Fig. 5.** Mcl-1 is the potential target by which clitocine can sensitize human colon cancer cells to ABT-737. (A), LS411N and SW620 cells were transfected with siRNA-negative control or siRNA-Mcl-1 and incubated for 24 h. The silence efficiency is confirmed by Western blotting assay. Then, cells were treated with 5 μM ABT-737 for another 24 h. After treatment, cells were co-stained with Annexin V-FITC and PI. Flow cytometry assay was performed to determine the apoptotic cell percentage. (B), LS411N and SW620 cells were transfected with empty vector pcDNA3.1 or pcDNA3.1-Mcl-1 and incubated for 24 h. Then, cells were pre-treated with 0.2 μM clitocine for 24 h followed by 5 μM ABT-737 treatment for another 24 h. After treatment, flow cytometry assay was performed to determine the apoptotic cell percentage by co-staining with Annexin V-FITC and PI. Densitometric analysis of the protein bands was done using Image J (NIH). Data are presented as mean ± SD, n = 3. * P < 0.05 vs. control, ** P < 0.01 vs. control.
The co-administration of clitocine and ABT-737 significantly suppresses the xenograft growth in vivo

To further determine the anticancer efficacy of clitocine in combination with ABT-737 treatment in vivo, LS411N cells were injected to nude mice, and the tumor-bearing mice were then treated with clitocine, ABT-737 alone, or in combination when the tumor reaches a size of approximately 100 mm³. Vehicle injection was used as control, and tumor growth was measured overtime. It is clear that single treatment with clitocine or ABT-737 cannot effectively inhibit xenograft growth (Fig. 8A). Amazingly, co-treatment with clitocine and ABT-737 could significantly suppress the xenograft growth by almost 50% (Fig. 8A). Meanwhile, the body weight of the mouse in each group keeps almost stable, which suggests that neither single nor combined treatment of clitocine or ABT-737 exerts obvious toxicity on animals in this study (Fig. 8B). Therefore, we determined that clitocine can strengthen the anti-cancer ability of ABT-737 not only in vitro but also in vivo.

Discussion

ABT-737 is a novel anti-apoptotic Bcl-2 family protein inhibitor with high affinity for Bcl-2/Bcl-xl/Bcl-w but relatively low affinity for Mcl-1/A1 [12]. Therefore, ABT-737 is less efficient in some tumor cells with high levels of Mcl-1 [43]. Increasing evidences indicated that targeting Mcl-1 is a potential strategy to reverse the ABT-737 resistance of tumor cells, and recently attentions have focused on discovering an inhibitor which can suppress the expression, promote degradation, or inhibit the function of Mcl-1 to overcome such resistance [39,44,45]. We previously reported that clitocine, a natural nucleotide extracted from wild mushroom, successfully induced apoptosis in several multidrug resistant human tumor cell lines by down-regulating Mcl-1 expression [33]. Therefore, we hypothesized that clitocine might be a novel sensitizer of ABT-737 against tumor cells. At the present study, the cytotoxicity and apoptosis-induction activity of clitocine was determined in six human colon cancer cell lines. Clitocine also effectively decreases the protein level of Mcl-1. More interestingly, clitocine significantly enhances the lethality of ABT-737 in human colon cancer cells both in vitro and in vivo.

At the present investigation, neither clitocine nor ABT-737 alone could induce apoptosis effectively, while combination of them dramatically induced apoptosis in human colon cancer cells accompanied by the cleavage of caspase 3 and PARP. Mcl-1 expression profile was also examined and 0.2 μM clitocine alone indeed down-regulated its expression. Significantly, ABT-737 enforced Mcl-1 expression discernibly but clitocine could override this function of the compound. It has been reported that Mcl-1 is a short-lived protein which can rapidly respond to environmental stimulus including apoptotic signal [46,47]. This might explain the result that ABT-737 treatment elevates the expression of Mcl-1 in the present study. Although how clitocine impacts Mcl-1 expression is still unclear, Mcl-1 can be highly regulated at both transcriptional and post-translational levels during apoptosis [48]. Interestingly, the compound effectively decreases Mcl-1 protein level with the mRNA unchanged (data not shown). These observations suggest clitocine might suppress Mcl-1 by promoting the protein degradation [28,46]. In the next step, more experiments will be performed to determine how clitocine impacts Mcl-1 expression.

Although it is reported that suppression of Mcl-1 by either inhibitors or genetic approaches can directly potentiate ABT-737 lethality [49], we reconfirmed that inhibition of Mcl-1 by siRNA can effectively increase the sensitivity of human colon cancer cells to ABT-737-induced apoptosis. More importantly, enforced exogenous Mcl-1 significantly attenuated apoptosis induced by co-treatment of clitocine and ABT-737. All these results support that...
Mcl-1 is a target by which clitocine can enhance sensitivity of human colon cancer cells to ABT-737 at least partially. Although the possibility of other potential mechanisms may also contribute to this sensitization function, there is no doubt that Mcl-1 suppression plays an important role in the sensitization effect of clitocine on ABT-737.

As an anti-apoptotic Bcl-2 family protein, Mcl-1 can repress apoptosis by interacting with pro-apoptotic proteins such as Bak or Bax to inhibit their activation and subsequently block mitochondrial membrane permeabilization [24,25]. Here, we observed that co-administration of clitocine and ABT-737 successfully activated Bak and initiated the mitochondrial membrane permeabilization as expected. The decreased level of Mcl-1 induced by clitocine might contribute to release and activate Bak which was initially sequestered by Mcl-1. This hypothesis was evidenced by immunoprecipitation assay by which it was determined that less Bak is detained by Mcl-1 under clitocine.
treatment. Furthermore, silence of Bak with specific siRNA targeting Bak efficiently neutralized the sensitization effect of clitocine on ABT-737 in human colon cancer cells. All these results indicated that the activation of Bak is a key step in the sensitization effect of clitocine on ABT-737.

In summary, the present study has illustrated that clitocine exhibits potent activity in apoptosis-induction as a single agent and increasing human colon carcinoma cell sensitivity to ABT-737-induced apoptosis as a sensitizer in vitro and in vivo. Inhibition of Mcl-1 expression induced by clitocine promotes the activation of Bak and cooperatively initiates the intrinsic apoptotic pathway in combination with ABT-737. Our findings warrant the further investigation of the potential of clitocine as an ideal adjunct agent in ABT-737-based therapy.

Conflict of interest

The authors declare that they have no conflict of interest.

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