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ARTICLE

Hispidulin Sensitizes Human Ovarian Cancer Cells to TRAIL-Induced Apoptosis by AMPK Activation Leading to Mcl-1 Block in Translation

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Whether hispidulin, a flavone from traditional Chinese medicine, can modulate the anticancer effects of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), the cytokine currently in clinical trials was investigated. In the present study, we found that hispidulin potentiated the TRAIL-induced apoptosis in human ovarian cancer cells and converted TRAIL-resistant cells to TRAIL-sensitive cells. When examined for its mechanism, we found that hispidulin was highly effective in activation of caspases 8 and caspase 3 and consequent poly(ADP-ribose) polymerase (PARP) cleavage. Moreover, we found that hispidulin downregulated the expression of Mcl-1, Bcl-2, and Bcl-xL. Whereas the downregulation of Bcl-2 and Bcl-xL was less pronounced, the downregulation of Mcl-1 was quite dramatic and was time-dependent. This sensitization is controlled through the adenosine monophosphate (AMP)-activated protein kinase (AMPK), which is the central energy-sensing system of the cell. Interestingly, we determined that AMPK is activated upon hispidulin treatment, resulting in mammalian target of rapamycin (mTOR) inhibition leading to Mcl-1 decrease. Therefore, our results show a novel mechanism for the sensitization to TRAIL-induced apoptosis linking hispidulin treatment to Mcl-1 downexpression. In addition, this study provides a rationale for the combined use of death receptor (DR) ligands with AMPK activators or mTOR inhibitors in the treatment of human cancers.

KEYWORDS: Hispidulin; Traditional Chinese medicine; TRAIL; AMPK; Mcl-1

INTRODUCTION

Ovarian cancer is the most lethal of gynecologic cancers, accounting for more than 15000 deaths annually (*1*). When ovarian cancer is diagnosed at early stages, the survival rate is close to 90%; however, nearly 75% of cases are diagnosed at an advanced stage, when the disease is widespread (*2*). This is primarily because ovarian cancer has few early or specific symptoms. Patients diagnosed with aggressive ovarian cancer are managed with surgical excision and chemotherapy, and the overall prognosis for patients remains poor (*3*). Therefore, an effective drug for ovarian cancer is required.

Recent studies have validated that many chemotherapeutic drugs eradicate cancer cells by inducing apoptosis (*4*). However, defects in apoptosis signaling not only contribute to tumorigenesis but are associated with the development of tumor chemoresistance. Any therapeutic strategy aimed at specifically triggering apoptosis in cancer cells has potential curative effects. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) has drawn considerable attention as an attractive molecule for cancer therapy because it selectively triggers apoptosis in transformed cells or malignant cells (*5*). TRAIL induces apoptosis through interacting with death receptor 4 (DR4; also termed TRAILR1) and death receptor 5 (DR5; also termed Apo2, TRAIL-R2, TRICK2, or Killer/DR5), leading to the recruitment

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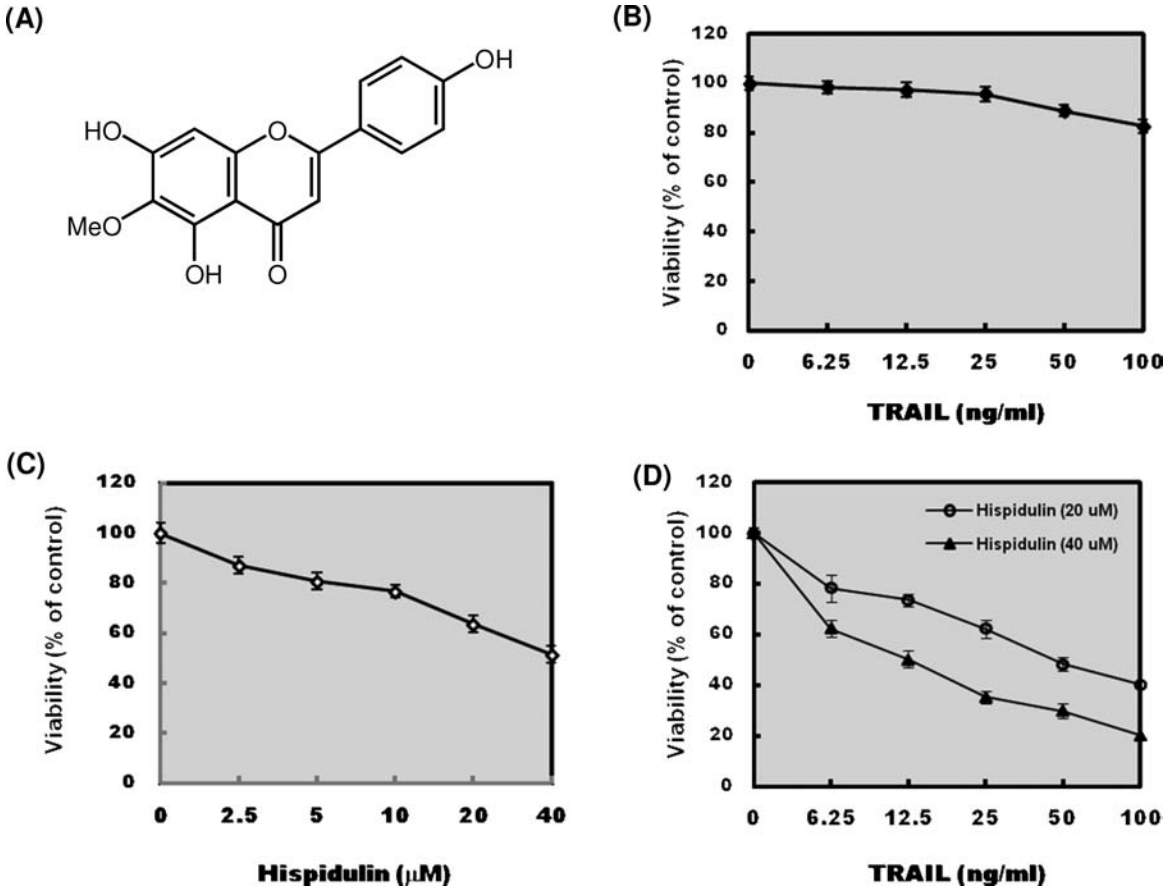

Figure 1. Hispidulin sensitizes SKOV3 cells to TRAIL. (**A**) Chemical structures of hispidulin. (**B**) SKOV3 cells were exposed to the indicated concentrations of TRAIL at 37 °C for 48 h. (**C**) SKOV3 cells were exposed to the indicated concentrations of hispidulin at 37 °C for 48 h. (**D**) SKOV3 cells were exposed to the indicated concentrations of TRAIL in the presence of 20 or 40 μM hispidulin at 37 °C for 48 h, respectively. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without of hispidulin as 100%. This experiment was repeated three times. Bar represents the SD.

of the Fas-associated protein with death domain (FADD) and initiator caspase-8 to the death-initiating signaling complex (DISC), resulting in enzymatic activation of caspase-8, which in turn activates the downstream caspase cascade (*6*). Although TRAIL exhibits killing effect selectively on tumor cells, resistance of cancer cells to TRAIL remains a major problem (*7*). Why or how cancer cells develop resistance to TRAIL is not clear, but numerous mechanisms have been reported including alteration of death receptors and overexpression of antiapoptotic proteins including FADD-like apoptosis regulator (c-FLIP), antiapoptotic Bcl-2 family proteins (e.g., Bcl-2, Bcl-xL and Mcl-1), and X-linked inhibitor of apoptosis (XIAP) (*7*). Therefore, TRAIL alone may not be sufficient for the treatment of various malignant tumor cells, and agents that can modulate some of these mechanisms of resistance to TRAIL have a potential in improving the cytokine therapy.

The AMP-activated protein kinase (AMPK) acts as a fuel gauge by monitoring cellular energy levels. AMPK controls process relative to tumor development, including proliferation, survival, cell cycle progression, and protein translation (*8*). The AMPK pathway is linked to tumor progression through regulation of the mammalian target of rapamycin (mTOR) pathway. AMPK activation suppresses the growth of a broad spectrum of cancers via mTOR, inhibits the proliferation of certain tumor cells, and can cooperate with other agents to induce apoptosis (*9*). The best-understood role of mTOR is to regulate protein translation by direct phosphorylation of key translation regulators, p70 ribosomal S6 kinase (p70-S6K), and eukaryotic initiation factor (eIF) 4E-binding protein1 (4E-BP1) (*9*). Recent studies have drawn increased interest in the use of mTOR inhibitors to treat various cancers (*10*).

Hispidulin (4′,5,7-trihydroxy-6-methoxyflavone) is a naturally occurring flavone commonly found in *Saussurea involucrata* Kar. et Kir., a rare traditional Chinese medicinal herb (*11*). Several in vitro studies have demonstrated its potent antioxidative, antifungal, anti-inflammatory, antimutagenic, and antineoplastic properties (*12*−*14*). In the present study, we investigate whether hispidulin could modulate TRAIL-induced apoptosis and ,if so, through what mechanism. We found that hispidulin can indeed enhance TRAIL-induced apoptosis in SKOV3 cells. This sensitization is mediated by AMPK activation and mTOR inhibition, culminating in the reduction of Mcl-1 levels through a block of its translation. Therefore, this AMPK−mTOR-dependent loss of Mcl-1 could explain how hispidulin enhances TRAIL-induced apoptosis, suggesting a rationale for the combined use of AMPK activators or mTOR inhibitors with DR ligands in the treatment of human cancers.

MATERIALS AND METHODS

Chemicals. MTT, compound c, PI, and antibodies for β-actin were purchased from Sigma (St. Louis, MO). Hispidulin was purchased from Tocris Bioscience (Bristol, UK). TRAIL was purchased from R&D Systems (Minneapolis, MN). Antibodies for phospho-mTOR (Ser2448), pohspho-4E-BP1 (Thr 37/46), phosphor-AMPK (Thr 172), phosphor-p70S6K (Thr389), Bcl-2, cleaved caspase-3, caspase-8, and PARP were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for

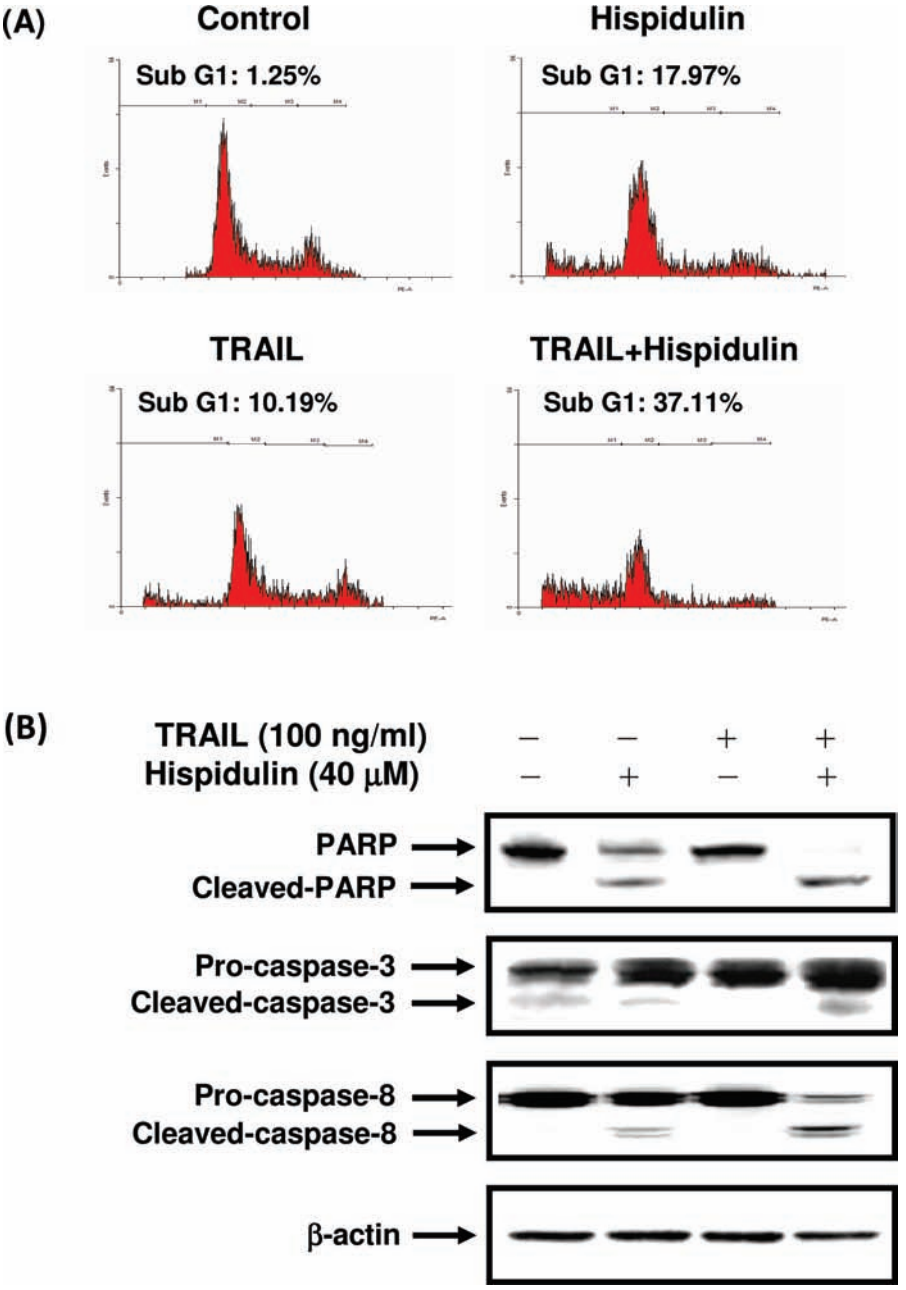

Figure 2. Hispidulin enhances TRAIL-induced apoptosis in SKOV3 cells. (**A**) SKOV3 cells were treated with TRAIL (100 ng/mL) in the absence or presence of hispidulin (40 μM) for 48 h. After harvesting, cells were analyzed for propidium iodide-stained DNA content by flow cytometry. (**B**) SKOV3 cells were treated with TRAIL (100 ng/mL) in the absence or presence of hispidulin (40 μM) for 48 h. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against cleavaged PARP, cleavaged caspase-3, cleavaged caspase-8, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments.

Bcl-XL and Mcl-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for mouse and rabbit conjugated with horseradish persdish peroxidase were purchased from Chemicon (Temecula, CA). Immobilon Western Chemiluminescent HRP Substrate was from Milliore Corporation (Billerica, MA).

Cell Culture. SKOV3 cells were purchased from American Type Culture Collection (Manassas, VA). SKOV3 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and were grown at 37 °C in a humidified atmosphere of 5% CO_2.

Cell Proliferation Assays. As described previously (*15*), the effects of hispidulin on cell proliferation were examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method.

Western Blot. Cells (2×10^6) were seeded onto a 100 mm tissue culture dish containing 10% FBS DMEM/F12 and cultured for 24 h. Then cells were incubated in 10% FBS DMEM/F12 treated with various agents as indicated in figure legends. After treatment, cells were placed on ice, washed with cold PBS, and lysed in lysis buffer. Western Blot was done as described previously (*16*). The intensity of the bands was scanned and quantified with NIH image software.

Cell Cycle Analysis. Cells (5×10^5) were cultured in 60 mm cell culture dish and incubated for 24 h. Then cells were harvested in a 15 mL tube, washed with PBS, resuspended in PBS, and fixed in 2 mL of iced 100% ethanol at −20 °C overnight. Cell pellets were collected by centrifugation, resuspended in 0.5 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at RT for 30 min. Then 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide−DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry (BD Biosciences, San Jose, CA).

Statistical Analysis. All valves were expressed as mean ± SD. Each value is the mean of at least three separate experiments in each group; Student's *t*-test was used for statistical comparison. Asterisks indicate that the values are significantly different from the control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

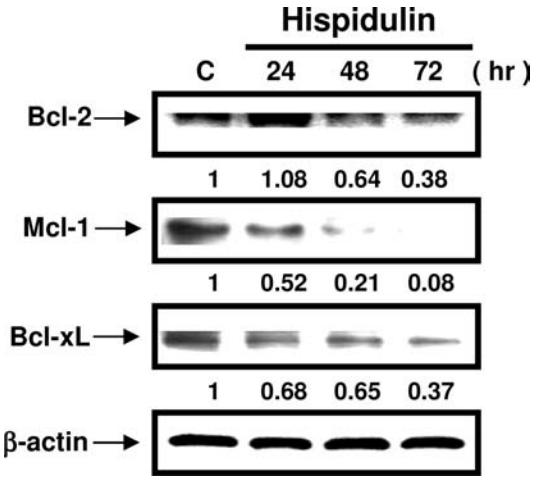

Figure 3. Hispidulin modulates antiapoptotic protein expression. SKOV3 cells were treated with 40 μM hispidulin for indicated time intervals. Whole cell extracts were prepared and analyzed by Western blotting using relevant antibodies against antiapoptotic proteins. Western blot data presented are representative of those obtained in at least three separate experiments.

RESULTS

Hispidulin Converted the TRAIL-Resistant SKOV3 Cells to TRAIL-Sensitive Cells. We examined first the sensitivity of human ovarian cancer SKOV3 cells to TRAIL. SKOV3 cells were treated for 48 h with different concentrations of TRAIL and then assessed for cell viability using the MTT method. Those results showed that SKOV3 cells were found to be highly resistant to TRAIL (**Figure 1B**). Whether hispidulin (**Figure 1A**) could sensitize the resistant SKOV3 cells to TRAIL was examined. Hispidulin alone had a minimal effect on cell viability (**Figure 1C**), but it significantly potentiated the cytotoxic effects of TRAIL in a dose-dependent manner (**Figure 1D**). Thus our results indicate that hispidulin converted the TRAIL-resistant SKOV3 cells to TRAIL-sensitive cells.

Hispidulin Enhances TRAIL-Induced Apoptosis. Next we determined whether hispidulin could potentiate TRAIL-induced apoptosis. We treated SKOV3 cells with TRAIL (100 ng/mL) in the absence or presence of hispidulin (40 μM) for 48 h. To reveal hispidulin enhancement of response, we examined apoptosis by determining the size of the sub-G1 cell pool. We found that TRAIL-induced apoptosis was increased from 10.19% to 37.11% in SKOV3 cells (**Figure 2A**). It is well-known that TRAIL-induced apoptosis requires the activation of caspases, which in turn cleave key protein substrates (*17*). TRAIL-induced activation of caspase-8 leads to activation of downstream caspases including caspase-3. To determine whether combined treatment with hispidulin and TRAIL leads to activation of caspases in TRAIL-resistant SKOV3 cells, the cells were treated

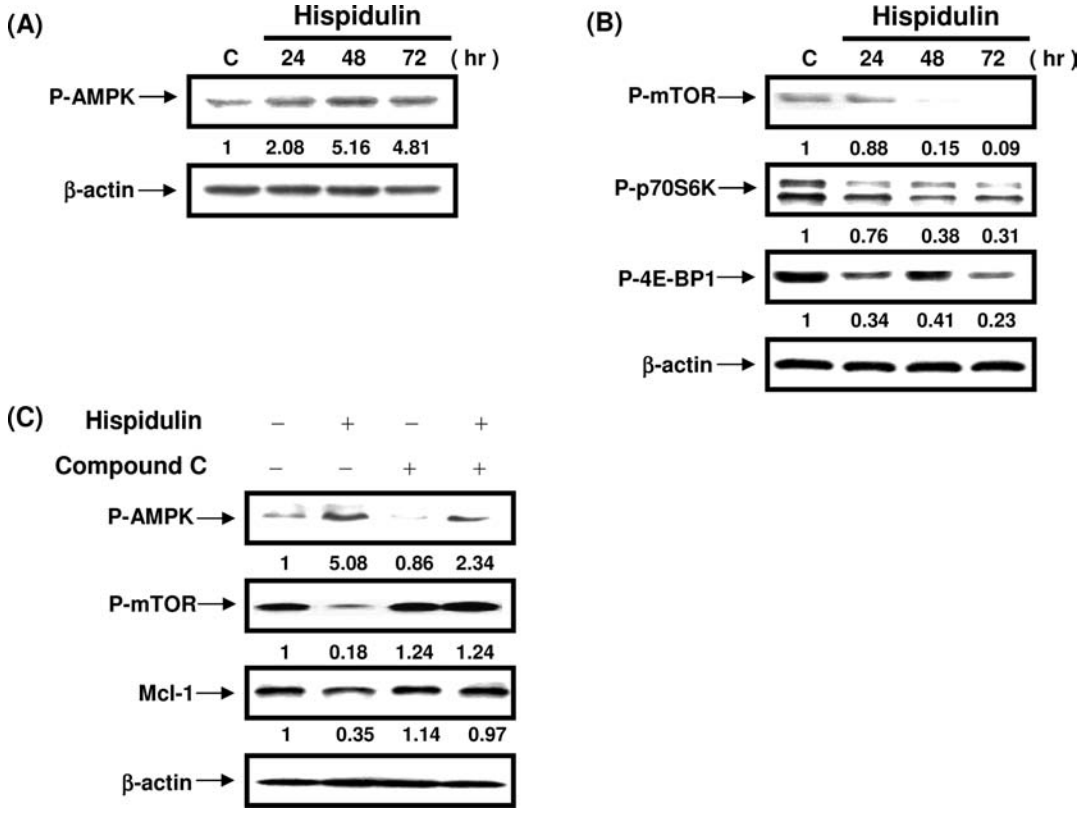

Figure 4. Sensitization to TRAIL-induced apoptosis by hispidulin is mediated by AMPK activation and Mcl-1 downregulation. (**A**) SKOV3 cells were treated with 40 μM hispidulin for indicated duration. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against phospho-AMPK (Thr172) and β-actin. (**B**) SKOV3 cells were treated with 40 μM hispidulin for indicated duration. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against phospho-mTOR (Ser2448), phospho-p70S6K (Thr389), phospho-4E-BP1 (Thr37/46), and β-actin. (**C**) SKOV3 cells were treated with 40 μM hispidulin in the absence or presence of compound c (5 μM) for 48 h. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against phospho-AMPK (Thr172), phospho-mTOR (Ser2448), Mcl-1, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments.

with TRAIL (100 ng/mL) in the absence or presence of hispidulin (40 μM), and their caspase activity was determined using Western blot analysis. As shown in **Figure 2B**, combined treatment with hispidulin and TRAIL induced the proteolytic processing of procaspase-8, and -3. Moreover, the combined treatment with hispidulin and TRAIL caused cleavage of PARP, a well-known endogenous substrate of caspase-3 (**Figure 2B**). These results indicate that hispidulin can enhance TRAIL-induced apoptosis. Moreover, our results strongly suggest that the potentiation of TRAIL-induced apoptosis by hispidulin is mediated through a caspase-dependent signaling cascade.

Sensitization to TRAIL-Induced Apoptosis by Hispidulin is Mediated by Mcl-1 Downregulation. Scaffidi et al. have previously shown that the mitochondrial pathway is required for DR-induced apoptosis in Jurkat cells (*18*). For this reason, we hypothesized that hispidulin could act on Bcl-2 family proteins. How hispidulin enhances TRAIL-induced apoptosis was investigated next. SKOV3 cells were treated with 40 μM hispidulin at 37 °C for different durations and then examined for expression of antiapoptotic proteins using relevant antibodies. Mcl-1 levels, but not Bcl-XL or Bcl-2 levels, were found to be largely altered (by 46−84%) upon hispidulin treatment (**Figure 3**). The results indicated that hispidulin downregulated the expression of Mcl-1, Bcl-2, and Bcl-xL. Whereas the downregulation of Bcl-2 and Bcl-xL was less pronounced, the downregulation of Mcl-1 was quite dramatic and was time-dependent.

Hispidulin Suppresses the Protein Synthesis of Mcl-1 by Activating AMPK. The previous study provides a rationale for the combined use of DR ligands with AMPK activators or mTOR inhibitors in the treatment of human cancers (*19*). We next identify whether hispidulin enhances TRAIL-induced apoptosis by activating AMPK in SKOV3 cells. SKOV3 cells were treated with 40 μM hispidulin at 37 °C for different durations. Western blot analysis indicated that hispidulin stimulates AMPK phosphorylation in a time-dependent manner (**Figure 4A**). The mTOR/4E-BP1 pathway controls the protein translation/synthesis in various types of cells (*7*). To determine whether hispidulin suppresses the protein synthesis by activating AMPK to inhibit mTOR pathway, phospho-mTOR and 4E-BP1 were detected by Western blotting. The phosphorylation of mTOR, p70S6K, and 4E-BP1 were decreased in a time-dependent manner (**Figure 4B**). We further identify whether hispidulin downregulates Mcl-1 expression by activating AMPK activity and then inhibits mTOR/4E-BP1 pathway. We added the compound c, an AMPK inhibitor, in the absence or presence of hispidulin. The AMPK activity was suppressed by compound c in the presence of hispidulin, and the mTOR activity and Mcl-1 expression were resumed in SKOV3 cells (**Figure 4C**). We hypothesized that hispidulin, by upregulating AMPK activity, would inhibit mTOR activation and downregulate Mcl-1 expression in SKOV3 cells.

Sensitization to TRAIL-Induced Apoptosis by Hispidulin Is Mediated by AMPK Activation. To test whether AMPK activation was involved in the observed sensitization to TRAIL-induced death, we used an AMPK inhibitor (compound C). SKOV3 cells were treated for 48 h with 40 μM hispidulin and 100 ng/mL TRAIL in the absence or presence of 5 μM of compound c and then assessed for cell viability using the MTT method. As presented in **Figure 5A**, compound C could recover sensitization to TRAIL by hispidulin. Moreover, compound C also could recover sensitization to TRAIL-induced apoptosis by hispidulin (**Figures 5B**). Our data suggested that sensitization to TRAIL-induced apoptosis by hispidulin occurred through AMPK activation.

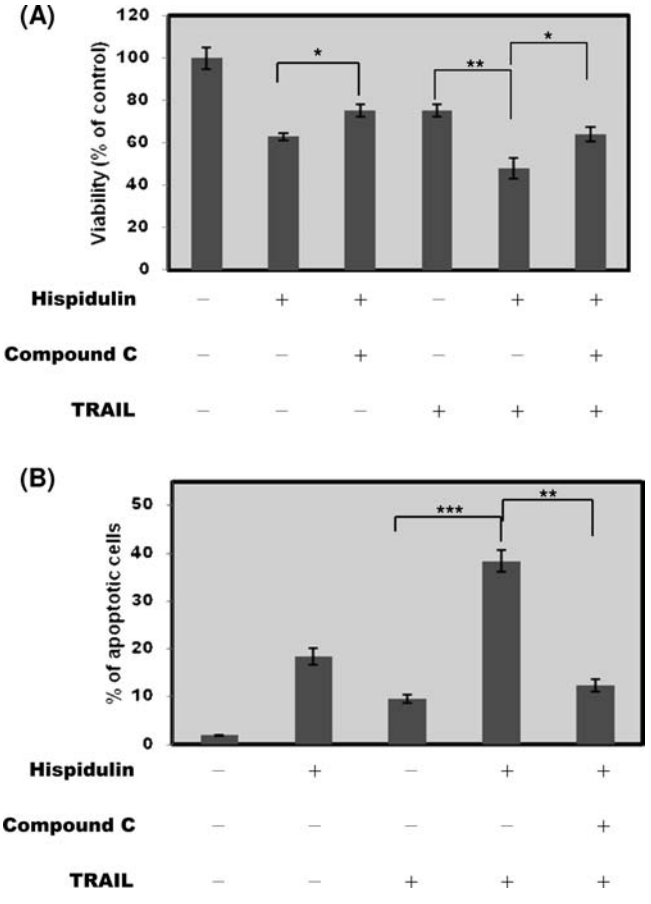

Figure 5. Hispidulin sensitizes SKOV3 cells to TRAIL-induced apoptosis by AMPK activation. (**A**) SKOV3 cells were treated with 40 μM hispidulin or 100 ng/mL TRAIL in the absence or presence of compound c (5 μM) for 48 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without of hispidulin as 100%. This experiment was repeated three times. Bar represents the SD *, $P < 0.05$; **, $P < 0.01$. (**B**) SKOV3 cells were treated with 40 μM hispidulin or 100 ng/mL TRAIL in the absence or presence of compound c (5 μM) for 48 h. After harvesting, cells were analyzed for propidium iodide-stained DNA content by flow cytometry. Bar represents the SD **, $P < 0.01$; ***, $P < 0.001$.

DISCUSSION

In recent years, TRAIL was identified as a powerful activator of apoptosis in tumor cells while sparing normal cells (*20*). However, 50% of all tumor cells are resistant to TRAIL-induced death for unclear reasons, suggesting that many patients will be nonresponders (*21*). Therefore, identification of the mechanism leading to increased sensitivity to the tumor-killing effects of DR ligand is of great importance. In the present study, we demonstrated that the combination of hispidulin and TRAIL synergistically induced apoptosis in TRAIL-resistant SKOV3 ovarian cancer cells. Interestingly, an implication of the AMPK−mTOR pathway in sensitizing SKOV3 cells to TRAIL-induced apoptosis was supported by the fact that (i) AMPK was found activated and mTOR inhibited under hispidulin treatment and (ii) an AMPK inhibitor (compound c) could prevent the sensitization to TRAIL-induced apoptosis under hispidulin treatment.

To understand where in the apoptotic pathway this sensitization occurred, we investigated mitochondrial implication in this context. We then demonstrated that the link between hispidulin treatment and the sensitization to TRAIL-induced apoptosis was through the antiapoptotic Bcl-2 member Mcl-1. Mcl-1, but no

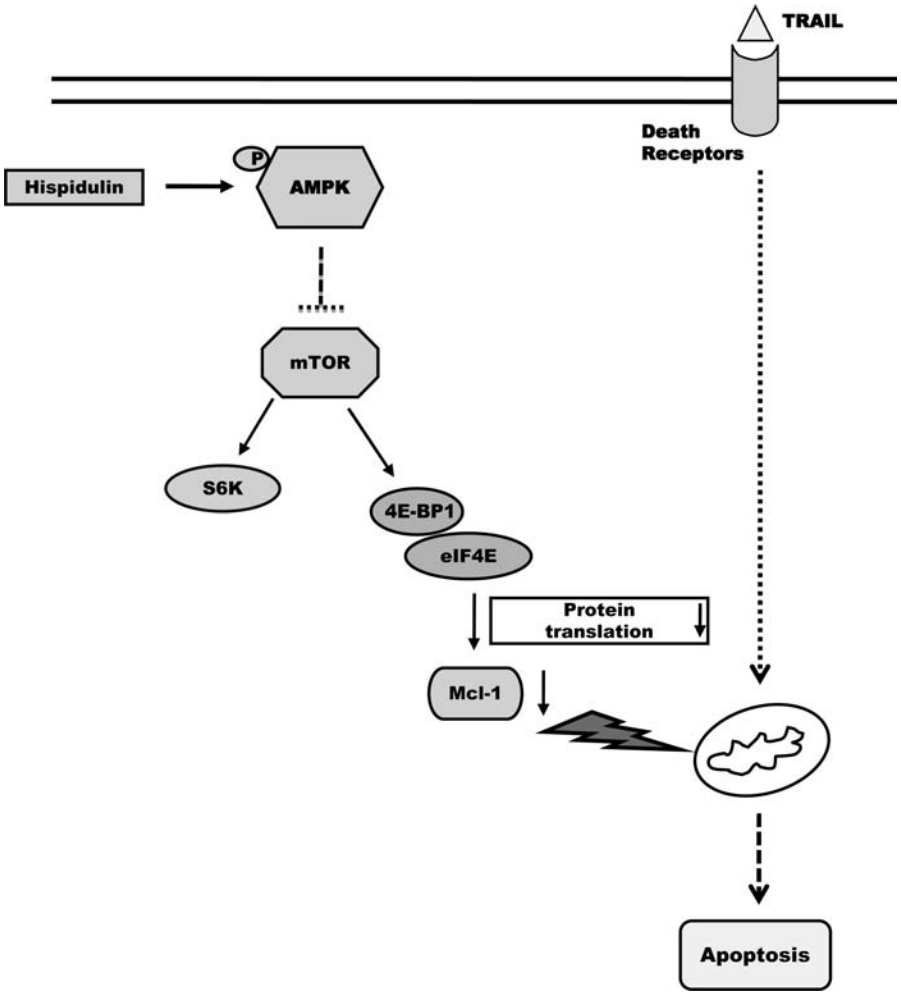

Figure 6. Model of AMPK pathway controlling Mcl-1 expression upon hispidulin treatment. Under hispidulin treatment, it upregulates AMPK activity. AMPK activation leads to mTOR inhibition, decrease in p70-S6 kKinase phosphorylation and stabilization of the 4E-BP1−eIF4E complex. Altogether this leads to an inhibition of translation resulting in Mcl-1 protein decrease. The decrease of this antiapoptotic member of Bcl-2 family sensitizes cells to TRAIL induced apoptosis via a mitochondrial amplification of the death cascade.

other Bcl-2 member tested, decreased by 46−84% under hispidulin treatment. An implication of the AMPK−mTOR pathway in Mcl-1 decrease was confirmed using chemical activators of this pathway. There is currently a high level of interest in signaling through mTOR. The role of mTOR in tumor acts as a sensor for energy, growth factors, and nutrients, all of which are required for protein translation. Activation of AMPK results in decrease mTOR signaling. Eukaryotic translation initiation factor 4E-BP1 is the downstream effector of mTOR, and through this effector mTOR controls the protein translation (*22*). Data presented here show that the inhibition of protein translation via AMPK−mTOR pathway by hispidulin in SKOV3 cells. Collectively, these findings strongly argue against the possibility that hispidulin enhances the sensitization to TRAIL-induced apoptosis act at the translational level to diminish Mcl-1 protein expression.

Mcl-1, an endogenous Bak inhibitor, is an antiapoptotic member of the Bcl-2 family. Recent studies have suggested that Mcl-1 decrease works in parallel with Puma and/or Bim to initiate apoptosis (*23*). Among the antiapoptotic members of the Bcl-2 family, Mcl-1 is of particular interest because it contains a G + C rich (> 70%) 5′ untranslated region (*24*), and given that Mcl-1 protein is highly regulated and rapidly degraded (*25*), inhibition of its synthesis would lead to a major decrease in protein level. Moreover, several studies are in support of Mcl-1 regulation at the translational level. In other contexts, Mcl-1 is a potential downstream target of eIF4E (*24*, *26*). Recently Wei et al. described the ability of rapamycin (mTOR inhibitor) to induce Mcl-1 but not Bcl-2 or Bcl-XL block in protein translation (*27*). Moreover, mir-29b was recently reported to regulate Mcl-1 expression, and its enforced expression made cancer cells sensitive to TRAIL (*28*).

In conclusion, AMPK is activated by hispidulin in SKOV3 cells. When this occurs, a key enzyme involved in protein synthesis, mTOR, is inhibited. Interestingly, hispidulin enhances the sensitization to TRAIL-induced apoptosis at the translational level to diminish Mcl-1 protein expression (**Figure 6**). Therefore, we could envision that a combined use of hispidulin with TRAIL may represent a very powerful method to selectively kill tumor cells.

ABBREVIATIONS USED

ACC, Acetyl-CoA carboxylase; AMPK, AMP-activated preotin kinase; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; 4E-BP1, eukaryotic initiation factor 4E-binding protein-1; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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