A novel STAT3 inhibitor HO-3867 induces cell apoptosis by reactive oxygen species-dependent endoplasmic reticulum stress in human pancreatic cancer cells

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Pancreatic cancer is the most commonly diagnosed malignancy among solid tumors and has shown an increasing trend year by year. Thus, there is an urgent need for the discovery of new anticancer drugs for the treatment of pancreatic cancer. In recent years, it has been reported that the compound HO-3867, a novel analog of the natural product curcumin, showed antitumor activity with low toxicity. However, the underlying mechanism of this compound’s attack on cancer cells is not very clear. In the present study, it was found that HO-3867 showed good antitumor activity at the concentration of 2 \(\mu\)mol/l in PANC-1 and BXPC-3 cells. Importantly, it was also found that HO-3867 treatment significantly induced reactive oxygen species (ROS) production in human pancreatic cancer cell lines, inducing PANC-1 and BXPC-3 cells. Co-treatment with the ROS scavenger, \(N\)-acetyl cysteine, partially abrogated HO-3867-induced cell apoptosis. The activation of mitogen-activated protein kinase and endoplasmic reticulum stress indicated a downstream event of ROS generation in mediating the anticancer effect of the HO-3867. In addition, independent of the ROS pathway, direct STAT3 inhibition was observed in HO-3867-induced cell apoptosis. Taken together, the results of this work suggest that both the ROS-dependent ER stress and STAT3 pathways were implicated in the cell apoptosis induced by the novel compound HO-3867. Anti-Cancer Drugs 00:000–000

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Introduction
Pancreatic cancer is the fourth leading cause of cancer-related deaths in the world and is clinically challenging, especially in East Asia [1,2]. Current treatment modalities for pancreatic cancer include surgery, radiotherapy, chemotherapy, and their combinations [3,4]. However, severe side effects and complications from current anticancer drugs have become a major problem in the clinical setting, which highlights the urgent need for novel, effective, and less toxic therapeutic approaches [5,6]. Therefore, novel effective and safe treatments need to be developed and tested [7,8].

Cancer cells have intrinsically higher levels of reactive oxygen species (ROS) and are under oxidative stress because of an imbalanced redox status in comparison with normal cells [9,10]. Elevated ROS levels also render cancer cells more sensitive to agents that further increase ROS and oxidative stress [11]. The signal transducer and activator of transcription 3 (STAT3) is constitutively activated in various human cancers and is a promising molecular target for the treatment of these cancers [12,13]. Many cancer cell lines and primary tumors, including pancreatic cancer, were reported to possess constitutively-active STAT3 (P-STAT3) that can lead to cellular transformation and ultimately tumorigenesis [14–16]. Previous studies suggested that HO-3867 acted as an inhibitor of STAT3, which could cause cancer cell apoptosis [17,18].

Here, it was found that the compound HO-3867 could induce cell apoptosis in pancreatic cancer cells by activating both ROS-dependent endoplasmic reticulum (ER) stress and inhibiting STAT3 pathways. Blocking ROS production by a specific inhibitor partly abolished the anticancer effect of HO-3867. Partial cell apoptosis caused by direct inhibition of STAT3 cannot be abolished by a specific inhibitor. These study results
suggested that HO-3867 could be a potential candidate for the treatment of pancreatic cancer.

Materials and methods
Reagents and cell culture
Human pancreatic cancer cell lines PANC-1 and BXPC-3 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cells were maintained in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO2. FITC Annexin-V apoptosis Detection Kit I and propidium iodide (PI) were purchased from BD Pharmingen (Franklin Lakes, New Jersey, USA). Antibodies including anti-GAPDH, goat anti-mouse IgG-HRP, anti-rabbit IgG-HRP, anti-Bcl-2, anti-Bax, anti-Cleaved PARP, and anti-Pro-casepase 3 were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Antibodies including anti-p-JNK, anti-JNK, anti-p-p38, anti-p-38, anti-p-ERK, anti-ERK, anti-P-STAT3, anti-STAT3, anti-p-perk, anti-p-eIF2α, anti-eIF2α, and anti-p-ATF4 were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). STAT3 siRNA was purchased from Gene-Pharma (Shanghai, China). HO-3867 was dissolved in DMSO to prepare a 50 mmol/l stock solution and stored at −20°C. The working dosages used in this study were 0.5, 1, and 2 μmol/l. Each working solution was freshly prepared in the basal medium with a final DMSO concentration of less than 0.1%.

Flow cytometric analysis of the apoptotic rate by Annexin V-FITC staining
For the detection of the apoptotic cells, the Annexin V assay was performed using Annexin V-FITC Apoptosis Detection Kits. PANC-1 cells were incubated with N-acetylcysteine (NAC), HO-3867, NAC, and HO-3867 for 24 h and then harvested and washed twice with cold PBS. Cells were resuspended in 500 μl binding buffer and then incubated for 10 min at room temperature in the dark after the addition of 5 μl Annexin V-FITC and 3 μl PI. The fluorescence was analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, California, USA).

Determination of cellular reactive oxygen species
Cellular ROS contents were measured by flow cytometry. Briefly, 5 x 10^5 cells were plated on 60 mm dishes, allowed to attach overnight, and then exposed to the compound HO-3867 for various durations of time. Cells were stained with 10 μmol/l DCFH-DA (Beyotime Biotech, Nantong, China) at 37°C for 30 min. Cells were collected and their fluorescence was analyzed using a FACS Calibur flow cytometer (BD Biosciences). In some experiments, cells were pretreated with 10 mmol/l NAC for 2 h before HO-3867 exposure and analysis of ROS generation.

Western blot analysis
Test cells were homogenized in protein lysate buffer and the protein concentration was determined after centrifugation at 12,000 rpm at 4°C for 10 min. After the sample loading buffer was added, all the protein samples were electrophoresed and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California, USA). The blots were blocked for 2 h at room temperature with fresh 5% nonfat milk in TBST and then incubated with a specific primary antibody in TBST overnight at 4°C. Following three washes with Tris-buffered saline, the nitrocellulose membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and visualized using enhanced chemiluminescence reagents (Bio-Rad Laboratories). The density of the immunoreactive bands was analyzed using Image J computer software (National Institute of Health, Bethesda, Maryland, USA).

Cell morphological assay
Test cells were seeded in six-well plates and treated with HO-3867 (2 μmol/l), HO-3867 (2 μmol/l) + NAC (10 μmol/l), and NAC (10 μmol/l). After 24 h and washing with PBS, the cells were observed using an inverted fluorescence microscope (Nikon, Tokyo, Japan) with 20× amplification.

Transient transfection of small interfering RNA
The cells were transfected with siRNA (50 pmol/ml) targeting STAT3 or nontargeted siRNA as a control using the lipofectamine 2000 reagent according to the manufacturer’s instructions. Subsequently, the transfected cells were washed and changed with complete media and used in further studies.

Statistical analysis
All of the experiments were conducted independently three times. The data are presented as means ± SE. The statistical significance of differences between groups was obtained using student’s t-test or analysis of variance multiple comparisons in GraphPad Pro (GraphPad, San Diego, California, USA). Differences were considered significant at P less than 0.05, 0.01, and 0.001.

Results
HO-3867-induced cell apoptosis in human pancreatic cancer cells
In this study, we first determined the pharmacological activities of HO-3867 in pancreatic cancer cell lines. The structure of the HO-3867 compound is shown in Fig. 1a. We evaluated the role of apoptosis in HO-3867-induced cell death using Annexin V-FITC/PI staining. As shown in Fig. 1b and c, HO-3867 treatment significantly decreased the viability of PANC-1 cells and BXPC-3 cells in a dose-dependent manner and the levels of apoptosis-associated proteins were also examined by western blot analysis in PANC-1 cells. As shown in
HO-3867-induced apoptosis in PANC-1 and BXPC-3 cells. (a) Molecular structures of curcumin and HO-3867. Induction of apoptosis in PANC-1 (b) and BXPC-3 (c) cells was determined by flow cytometry after treatment with HO-3867 (0.5, 1, and 2 μmol/l) for 24 h. Similar results were obtained in three independent experiments. (d) PANC-1 cells were treated with HO-3867 (0.5, 1, and 2 μmol/l) for 24 h and treated with HO-3867 (2 μmol/l) for different durations of (e). Whole-cell lysates were subjected to a western blot to assess the expression of cell apoptosis-related proteins. GAPDH was used as an internal control. Data represent similar results from three independent experiments.

Fig. 1d, HO-3867 treatment decreased the protein level of Bcl-2 and procaspase3 and increased the cleaved PARP in a dose-dependent manner, but had no effect on Bax expression. In addition, the protein level of Bcl-2 decreased in a time-dependent manner, which is shown in Fig. 1e.

**ROS overproduction mediated HO-3867-induced apoptosis in pancreatic cancer cells**

The implication of intracellular ROS generation in the anticancer effects of HO-3867 was investigated. The ROS level was assessed using the fluorescent probe DCFH-DA and detected with H₂O₂. Interestingly,
HO-3867 treatment significantly increased intracellular ROS generation in a time-dependent manner (Fig. 2a) in pancreatic cancer cell lines, including PANC-1 and BXPC-3 cells. In the following research, we chose PANC-1 cells because of their higher sensitivity compared with BXPC-3 cells. Co-treatment of PANC-1 cells with NAC, an ROS scavenger, significantly inhibited HO-3867-induced ROS generation (Fig. 2b). These data showed that HO-3867 could induce the accumulation of ROS in pancreatic cancer cells. We then examined whether increased ROS was required for cell apoptosis induced by HO-3867. Reversed cell apoptosis was also observed in HO-3867-treated cells through the morphological changes. Figure 2c shows that after treatment with HO-3867 for 24 h, the morphology of PANC-1 cells changed markedly in comparison with regular cancer cells. Under the microscope, it was observed that the cancer cells became round and clearly shriveled. Further examination was performed of the proapoptotic effect of HO-3867 on human pancreatic cancer cells using the Annexin V/PI staining assay. As shown in Fig. 2d and e, it can be seen that after a 24 h treatment with HO-3867 (2 μmol/l), the level of apoptosis of the PANC-1 cells was about 50%. A large proportion of this cell apoptosis abrogated when an ROS scavenger (NAC) was added together with the HO-3867. We also found that the reduction in the protein level of Bcl-2 induced by HO-3867 could be abrogated with the ROS scavenger (NAC) and the results are shown in Fig. 2f. These results suggested that the anticancer effect of HO-3867 is associated with the induction of ROS. Collectively, these results indicated that ROS generation plays a central role in HO-3867-induced cell apoptosis.

**HO-3867-induced ROS increased MAPK and ER stress, contributing toward HO-3867 lethality in pancreatic cancer cells**

Increased ROS levels and perturbation in the intracellular redox status increased the levels of unfolded proteins in the ER and induced an ER stress response [19,20]. Therefore, the expressions of ER stress-related proteins were examined, such as p-PERK, p-eIF2α, and ATF4, in HO-3867-treated pancreatic cancer cells. The time-course results indicated that HO-3867 (2 μmol/l) significantly activated ER stress. The expression levels of p-PERK, p-eIF2α, and ATF4 reached the peak at 0–3 h after treatment with PANC-1 cells (Fig. 3a). In addition, it was found that pretreatment with the antioxidant NAC completely blocked the expression of p-PERK and ATF4 in PANC-1 cells (Fig. 3b).

Some reports have indicated that ROS can activate ER stress through the mitogen-activated protein kinases (MAPK) pathway [21,22]. Therefore, an additional examination of the expressions of MAPK-related proteins, such as p-ERK, p-JNK, and p-P38, in HO-3867-treated pancreatic cancer cells was performed. It was found that the expression levels of these proteins reached a peak at 0–3 h after treatment, which meant that the MAPK pathway cloud was activated by HO-3867 in PANC-1 and BXPC-3 cells (Fig. 3c and d). Moreover, the expression of the MAPK-related proteins could be blocked when pretreatment was performed using the antioxidant NAC (Fig. 3e and f). These findings indicated that HO-3867-induced ROS could lead to activation of the MAPK pathways.

**Discussion**

In the previous studies, the curcumin derivative HO-3867 showed high bioavailability and considerable anticancer effects in human ovarian carcinoma cancer and endometrial cancer by inhibition of the STAT3 signaling pathway [17,18,23–25]. In the current work, another potential mechanism was found for HO-3867 in its anticancer effects in pancreatic cancer cells. This study showed that HO-3867 selectively induced ROS and apoptosis in pancreatic cancer cells in vitro.
Reactive oxygen species (ROS) overproduction mediated HO-3867-induced apoptosis in PANC-1 cells. (a) Intracellular ROS generation induced by HO-3867 as measured in human pancreatic cancer PANC-1 and BXPC-3 cells by staining with DCFH-DA (10 μmol/l) and flow cytometry analysis. (b) PANC-1 cells preincubated with 10 mmol/l N-acetyl cysteine (NAC) for 30 min before exposure to HO-3867 (2 μmol/l) for 3 h, intracellular ROS generation measured by flow cytometry. (c, d) Blocking of ROS generation abolished the cytotoxicity of HO-3867. PANC-1 cells were preincubated with or without 10 mmol/l NAC for 30 min before exposure to HO-3867 (2 μmol/l) for 24 h. The cell morphology was determined using an inverted microscope (c) and percentage of cell apoptosis was determined by Annexin-V/propidium iodide staining and flow cytometry (d). (e) Percentage of cell apoptosis was calculated and represented as the percent of control (**P < 0.001). (f) PANC-1 cells were preincubated with or without 10 mmol/l NAC for 30 min before exposure to HO-3867 (2 μmol/l) for 24 h and the expression of cell apoptosis-related proteins (Bcl-2 and Bax) was determined by western blot (*P < 0.05, **P < 0.01).
To the best of our knowledge, ER stress plays a critical role in the regulation of protein synthesis, folding, and trafficking [26]. Many papers have reported that a large number of signals could disrupt the ER function and induce ER stress [27]. In addition, the role of ROS in ER-stress-mediated apoptosis has been found in a variety of conditions.
of cell types [20,28]. This study is the first to show that HO-3867 treatment concomitantly induces ROS-mediated ER stress response and an observed extensive distension in the ER of PANC-1 cells treated with HO-3867. As expected, NAC pretreatment partly reversed these changes in ER induced by HO-3867. These data indicate that ROS production is the critical upstream regulator of HO-3867-induced ER stress in pancreatic cancer cells. All these data substantiate the notion that ROS production plays a crucial role in HO-3867's anticancer actions in human pancreatic cancer cells and also acts as an upstream signaling molecule involved
in HO-3867-induced activation of the ER stress signal pathway (Fig. 5).

MAPKs are serine–threonine protein kinases that play a major role in signal transduction from the cell surface to the nucleus [29,30]. Studies have shown that ROS are also involved in the regulation of different signal transduction pathways including MAP kinases and transcription factors [31,32]. Many studies have reported the regulation of ROS on MAPK activation in a variety of cancer cell lines and considerable evidence has suggested that JNK is primarily activated by various environmental stresses including oxidative stress [28]. According to the results of this study, in the PANC-1 and BXPC-3 cells, treatment with HO-3867 at a concentration of 2 µmol/l for 1–3 h increased the levels of all three pathways in MAPKs. Surprisingly, Fig. 3e and f indicated that ROS blockade completely inhibited HO-3867-induced phosphorylation of JNK, ERK, and P-P38. From these data, it can be concluded that HO-3867-induced apoptosis occurred through an ROS-dependent MAPK apoptotic pathway, indicating that ROS acts as an upstream signaling molecule involved in HO-3867-induced activation of the MAPK and ER stress pathway.

Many reports have shown that the compound HO-3867, as a STAT3 inhibitor, caused cancer cell apoptosis by directly inhibiting the STAT3 signaling pathway [17,18,23,24]. As far as is known, when the compound is added to the medium, many antitumor signaling pathways may be activated, thus inducing tumor cell apoptosis, such as the STAT3 signaling pathway, which has been reported, along with the ROS-dependent ER stress pathway found in this study. This study has uncovered another signaling pathway that is affected by HO-3867-induced apoptosis. In addition, it was also found that HO-3867 caused apoptosis of pancreatic cancer cells by directly inhibiting STAT3 (Fig. 4).

To date, it has been suggested that HO-3867-induced partial cell apoptosis through direct inhibition of STAT3 and played a role in the ROS-dependent ER stress pathway of cell apoptosis. In addition to being active in the STAT3 signaling pathway and the ROS-dependent ER stress pathway, it is expected that in the future, HO-3867 will be found to play a role in other signaling pathways. Throughout this work, there were challenges. Even though it was believed that HO-3867 could induce pancreatic cancer cells apoptosis by an ROS-dependent ER stress pathway, the mechanism of the role of HO-3867 was not very clear. Mitochondrial damage and ER stress were not explored in this study. Therefore, future work will focus on the mechanism of HO-3867-induced ROS activity.

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Conflicts of interest

There are no conflicts of interest.

References


