Research Square

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Heteronemin Promotes Iron-Dependent Cell Death in Pancreatic Cancer

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Abstract

As the source of several anticancer drugs, the marine environment is a treasure trove for the discovery of new drugs. In this study, a sesterterpenoid-type natural product heteronemin was investigated as a potential ferroptotic agent in the pancreatic cancer cell line (Panc-1). The effect of heteronemin on lipid peroxidation and autophagy- and ferritin-related protein expressions was examined using spectrophotometric and immunoblotting techniques, respectively. As well, several preclinical cell-based tests were used for the anticancer assessment. Results: Heteronemin at 55 nM concentration reduced cell viability by 50%. Heteronemin-induced cell death was reversed by a ferroptosis inhibitor, Ferrostatin-1. The levels of ferroptosis markers and malondialdehyde (MDA) were upregulated by heteronemin treatment while glutathione peroxidase-4 (GPX4) protein expression was downregulated. Also, significant alterations in ferritinophagy- and iron-related proteins (Atg5, Atg7, FTL, STEAP3, and DMT-1) were observed in Panc-1 cells ($p < 0.05$). Conclusions: The obtained results indicated that heteronemin exerted its pharmacological effect via triggering ferroptosis in pancreatic cancer. The potent cytotoxic effect of heteronemin suggested its potential development as a drug lead in the war against cancer.

Introduction

Pancreatic dysfunction can lead to several diseases including diabetes, pancreatitis, and cancer [1–3]. The vast majority (80–90%) of patients with pancreatic ductal adenocarcinoma (PDAC) have local metastases at the time of diagnosis [4, 5]. These patients do not benefit from surgery. Instead, they are treated by chemotherapeutic agents including a combination of gemcitabine or 5-fluorouracil plus leucovorin [6]. The high mortality rate of PDAC is observed due to its aggressive nature, early local and advanced metastasis, resistance to chemotherapeutics, and limited effective treatments [7, 8]. Chemotherapy and/or radiotherapy are insufficient for PDAC as cancer cells are resistant to apoptosis. There is an urgent need to identify new biomarkers for the early detection of pancreatic cancer and to identify new molecular targets involved in the progression of this aggressive disease.

Ferroptosis is an iron- and reactive oxygen species-dependent cell death pathway involves in several diseases i.e. ischemic organ damage, neurodegeneration, and cancer [9–17]. Unlike autophagy and apoptosis, ferroptosis is characterized by histological because of lipid peroxidation and decreased glutathione peroxidase-4 (GPX4) activity [18]. In cancer, ferroptosis can be considered as a tumor suppressor adaptive response. From this point of view, the regulation of ferroptosis could be a reasonable target for cancer cells that are resistant to the standard chemotherapy or molecular targeted therapies.

Most of the chemotherapeutic drugs are based on bioactive natural products [19, 20, 21]. Thus, the investigation of marine natural products against cancer has become a widespread approach among scientists. A considerable number of marine-derived products such as pachymatismin, bryostatins, and heteronemin have been shown to inhibit the proliferation of human cancer cells in the *in vitro* and *in vivo* models [22–24].

Heteronemin, a marine sesterterpenoid derivative isolated from the sponge *Hyrtios* sp., has attracted increasing interest due to its potent cytotoxic effect in cancer cell types [25–32]. Even though a recent study conducted by our laboratory reported its possible regulatory role in ferroptosis in hepatocellular carcinoma [25], it is unclear whether heteronemin induces ferroptosis in pancreatic cancer cells and if so, which pathway it uses when promoting ferroptosis. Herein, we evaluated ferroptosis and its related proteins as molecular targets of heteronemin for PDAC treatment.

Materials And Methods

Cell Culture and Treatments

Human pancreatic ductal adenocarcinoma cell line (Panc-1, CRL-1469™, RRID: CVCL_0480) was bought from the American Type Culture Collection (ATCC, Manassas, VA, USA). Immortalized human keratinocyte cell line (HaCaT) was kindly gifted by Prof. Çiğdem Yenisey of Aydın Adnan Menderes University. Cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2. For the experiments, the cells were treated with heteronemin at increasing concentrations (0.01-10 µM). HaCaT cell line was used to study the potential cytotoxicity of heteronemin on normal cells. GPX4 antibody (sc-166570, RRID:AB_2112427) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Atg5 antibody (Cat# 12994, RRID:AB_2630393), Atg7 antibody (Cat# 8558, RRID:AB_10831194), anti-rabbit IgG, HRP-linked antibody (Cat# 7074, RRID:AB_2099233) and anti-mouse IgG, HRP-linked antibody (Cat# 7076, RRID:AB_330924) were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). Ferritin light chain antibody (FNab03079), DMT1 (SLC11A2, FNab07905) and STEAP3 antibody (FNab08318) were purchased from Fine Test Wuhan Fine Biotech Corp. (Wuhan, China). All other compounds used in this study were purchased from Sigma-Aldrich (St. Louis, MO). Ethical approval is not applicable, because this article does not contain any studies with human or animal subjects.

Isolation of Heteronemin from the Sponge *Hippospongia* sp.

Heteronemin was separated from the marine sponge *Hippospongia sp.* following the same procedures in our previous report [27]. In short, samples were collected from coral reefs off the coast of Taitung, Taiwan by scuba diving at a depth of 20 m. Samples were freeze-dried and were extracted with EtOAc. Heteronemin was separated by a silica gel column with *n*-hexane-EtOAc (3:1) as the eluent solvent. The sample was further purified on HPLC. An LC-20A VP HPLC system (Shimadzu Inc., Tokyo, Japan) was used for analysis equipped with a quaternary pump (LC-20AT), an online degasser (DGU-14A), a photodiode-array detector (SPD-M20A), an autosampler (SIL-20AD), and data collection using ClassVP. Cosmosil 5C-18-MS-II column (5 µm, 150 × 4.6 mm I.D.) supplied by Nacalai Tesque, Inc. (Kyoto, Japan) used for liquid chromatography. The samples were injected (10 µL), and the mobile phase consisted of water (A) and acetonitrile (B). A gradient program was applied as follows, the initial elution condition was A:B (25:75, v/v), linearly changed to A:B (12:88, v/v) at 10 min, A:B (4:96, v/v) at 15 min. The percentage

of the mobile phase B increased linearly to 100% within 15 minutes and 210 nm was selected as the detection wavelength to collect the target compound.

Cell Viability Assay

Cellular viability was measured by MTS assay kit (CellTiter 96 Aqueous One Solution, Promega). Cells were incubated in Hank's Balanced Salt Solution (1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5.3 mM KCl, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 137.9 mM NaCl, 0.3 mM Na_2HPO_4, 5.6 mM D-glucose) containing increasing concentrations of heteronemin (0.001-10 μM) and/or Fer-1 (0.5 μM) dissolved in DMSO. MTS reagent was added to each well following 48 h exposure. The absorbance was measured at 490 nm with a microplate spectrophotometer. The selective-index was calculated by comparing heteronemin IC_{50} value in the HaCaT cell line against the IC_{50} value in Panc-1.

Colony Formation Assay

Cells were seeded at 1×10^3 cells/well in 6-well plates and were treated with heteronemin and/or Fer-1. The culture media were replaced with new media twice a week for two weeks. Crystal violet (Sigma-Aldrich, St. Louis, MO) was used to stain and make colonies visible. Then, colonies were photographed and counted in three independent wells.

Lipid peroxidation Assay

Lipid peroxidation product levels were evaluated by the method of Ohkawa et al. (1979) [33]. Briefly, the cells were incubated with heteronemin at an increasing concentration (0.01-1 μM) for 48 h. Then, the TBARS assay kit (Cayman Chemical) was used to measure malondialdehyde (MDA) levels in the sample at 532 nm.

Protein Analysis

Cells (3×10^5/well) were treated with two different concentrations of heteronemin (1 and 10 μM) for 48 h. The cells were harvested and were lysed in a buffer containing a protease/phosphatase inhibitor cocktail. The cellular lysates were analyzed by Western blot analysis as previously described [34].

Statistical Analysis

The obtained data were expressed as mean ± standard deviation (SD) from three-five independent experiments performed in triplicate. The Shapiro-Wilk normality test was used to determine whether the data were normally distributed. The statistical comparisons were estimated using one-way ANOVA followed by the Tukey test using GraphPad Prism (GraphPad Software, Inc). *p* values lower than 0.05 were regarded as statistically significant.

Results

Heteronemin significantly decreased cell viability of Panc-1 cells

We analyzed the cytotoxic activity of heteronemin in healthy immortalized human keratinocytes (HaCaT) and human pancreatic cancer cell line (Panc-1) using an MTS cell viability assay kit. Panc-1 and HaCaT cell lines treated with different concentrations of heteronemin showed IC_{50}values of 55 nM and 256 nM, respectively (Fig. 1). According to the IC_{50} values, Panc-1 cells were more susceptible to heteronemin than HaCaT cells after 48 h of treatment. The calculated SI value for heteronemin was 4.65. Next, we determined the ferroptotic potential of heteronemin. As shown in Fig. 2, ferroptosis inhibitor, Fer-1, significantly reversed cellular death induced by heteronemin. Accordingly, these observations indicated that the selectivity of heteronemin was high, and the cytotoxic activity of heteronemin depended on the induction of ferroptosis in Panc-1 cells.

Figure 1.

Figure 2.

Heteronemin inhibited colony formation of Panc-1 cells

Heteronemin reduced the colony-forming potential of Panc-1 cells in a concentration-dependent manner. Fer-1 treatment significantly increased colony numbers that were decreased by heteronemin. Nearly 90% increase in colony numbers was observed in Fer-1-treated cells (Fig. 3).

Figure 3.

Heteronemin increased lipid peroxidation and decreased GPX4 protein expression in Panc-1 cells

The concentration of lipid peroxidation final product, MDA, significantly increased in heteronemin-treated cells at higher concentrations compared with those untreated and cisplatin-treated cells. However, at low concentrations, no significant change in MDA level was observed (Fig. 4). Consistent with the elevated MDA levels, the protein analysis showed that GPX4 protein expression was significantly decreased following heteronemin (1 μM) treatment ($p < 0.05$).

Figure 4.

Heteronemin altered ferritinophagy- and iron-related protein expression

Autophagy is one of the mechanisms that promotes ferroptosis by breaking down ferritin inside the cell [35]. Several proteins that regulate autophagy can trigger ferroptotic process. Thus, we evaluated the effect of heteronemin on autophagy-related proteins, Atg5 and Atg7, in Panc-1 as well as ferritin light chain subunit (FTL). As shown in Fig. 5a, heteronemin upregulated Atg5 and Atg7 protein expression ($p < 0.05$). In addition, the protein expression of ferritin subunit, FTL, was downregulated at the highest concentration of heteronemin. Next, we determined the protein expression of divalent metal transporter-1 (DMT1) and six-transmembrane epithelial antigen of the prostate 3 (STEAP3) which are related to

divalent iron transport from endosome to cytosol and Fe^{3+} reduction, respectively. A significant increase in DMT1 and STEAP3 protein expression was observed following 48 h of heteronemin treatment ($p < 0.05$, Fig. 5b). These results suggested that heteronemin may trigger ferroptosis in pancreatic cancer cells by targeting iron transport and autophagy.

Figure 5.

Discussion

Natural products extracted from distinct species significantly contributed to the development of effective therapeutics against all types of diseases. In this context, the ocean is of immense importance as it has a large reservoir of marine species with their biologically active compounds possessing various activities including anticancer, anti-inflammatory, antimicrobial, and antioxidant [36–39]. Several marine-derived secondary metabolites such as alkaloids, terpenes, peptides, and steroids exhibit potent anticancer activities [40, 41].

In the present study, we focused on heteronemin and evaluated its ferroptotic potential in a pancreatic cancer model. As previously reported, heteronemin exhibited anticancer, anti-nutritional, antimicrobial, protein inhibitory, and antitubercular activities [42, 43]. In agreement with our findings, heteronemin reduced cell viability and proliferation in several cancer cell lines including leukemia, colon adenocarcinoma, breast cancer, and renal carcinoma at a concentration of less than one micromolar [31, 44]. In the present study, heteronemin showed potent cytotoxic activity against Panc-1 cells with IC_{50} of 55 nM. We observed a good selectivity profile with an SI value of 4.65 for heteronemin in pancreatic cancer cells following 48 h of treatment.

The most promising strategies for PDAC treatment are to inhibit mutated genes, such as KRAS, to regulate macromolecules that contribute to the disease progression, or to overcome chemoresistance [45]. The most used drugs approved by the FDA for pancreatic cancer are 5-fluorouracil, albumin-bound paclitaxel, cisplatin, gemcitabine, and FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan, oxaliplatin) [46]. These drugs have short half-lives and are usually given in higher and repeated doses which cause mild-to-moderate side effects [47–49]. Cisplatin is one of the agents used to treat pancreatic cancer. Severe side effects limit the therapeutic efficacy of cisplatin. Guo et al. (2018) reported that cisplatin inactivates GPX together with the induction of GSH depletion in cancer cells [50]. Thus, we decided to use cisplatin as the positive control to compare its effect with heteronemin in PDAC. Similar to the short-term cell survival findings, the long-term cell survival results monitored in a colony formation assay supported the inhibitory effect of heteronemin, which was comparable to cisplatin. Heteronemin-treated cells showed a reduced migration ability as the concentration increased and the results were comparable to cisplatin. These observations indicated that heteronemin selectively inhibited cell growth and the results were comparable to the clinically used anticancer drugs in pancreatic cancer cells.

Chemotherapeutic agents disrupt cell homeostasis via inhibiting DNA synthesis, increasing oxidative stress, arresting the cell cycle, and inducing cellular death mechanisms such as necrosis and apoptosis. Although Bcl-mediated apoptotic pathway and autophagy were reported to be induced by heteronemin in cancer cells [29, 31], the effect of heteronemin on other cellular death pathways was not fully elucidated.

Recently, ferroptotic cell death is widely investigated in cancer studies [51, 52]. Most of the clinically used chemotherapeutic drugs were found to induce ferroptosis as well as apoptosis [51, 53]. Similarly, we observed that heteronemin failed to stimulate cell death in the presence of a ferroptosis inhibitor, Fer-1. In agreement with the current data, we reported that heteronemin induced cellular death can be rescued by ferroptosis inhibitor in hepatocellular carcinoma cells [25]. These observations were critical for our further evaluation of heteronemin-induced ferroptosis in the present study. We hypothesized that heteronemin would regulate several pathways such as lipid peroxidation, iron transport, and iron storage to induce ferroptosis in cancer cells.

Increasing evidence demonstrated those numerous metabolic pathways contribute to ferroptosis through lipid-ROS production [54, 55]. Biochemical events including intracellular iron accumulation, and lipid peroxidation are critical for ferroptosis in cancer cells [56]. Pathways inducing ferroptosis are associated with the reduction of cysteine uptake through the inhibition of system X_c^- (SLC7A11), the reduction of GPX4 activity, and eventually the accumulation of intracellular lipid peroxides [57, 58]. GPX4 is a pivotal enzyme responsible for the detoxification of lipid peroxides and the progression of ferroptosis. Previously, the ability of heteronemin to reduce GPX4 protein expression was reported in hepatocellular carcinoma cell lines [25]. In our study, GPX4 protein expression was significantly decreased in response to heteronemin as well. The downregulation of GPX4 by heteronemin together with the increased MDA levels indicated that heteronemin successfully inhibited the lipid peroxidation product scavenging activity of GPX4 and promoted ferroptosis in pancreatic cancer cells.

One of the components that distinguish ferroptosis from other cell death mechanisms is iron metabolism. Free Fe^{2+} causes ferroptosis by catalyzing free radical formation via Fenton reaction. Biochemically, the reduction of Fe^{3+} to Fe^{2+} is catalyzed by STEAP3 in the endosome. Fe^{2+} is released into the cytoplasm via DMT1 [59]. Thus, any alteration in the expression of these proteins is critical for the labile iron pool and the consequent maintenance of iron homeostasis. Turcu et al. (2020) reported that blockade of DMT1 inhibits iron translocation which leads to lysosomal iron overload and ferroptosis in cancer stem cells [60]. However, the upregulation of STEAP3 and DMT1 in pancreatic cancer cells following heteronemin treatment indicated that the conversion of Fe^{3+} to Fe^{2+} as well as the release of free Fe^{2+} into cytoplasm may be triggered by heteronemin.

Ferritinophagy is defined as the degradation of ferritin, providing free Fe^{2+} for the cell, and contributing to ferroptosis as a source of unstable iron ions [10, 59]. Previously, Atg5 and Atg7 knockdown/knockout were demonstrated to block erastin-induced ferroptosis with decreased intracellular ferrous iron levels, and lipid peroxidation [35]. Conversely, upregulation of Atg5 and Atg7 protein expressions in response to heteronemin treatment with slightly decreased ferritin light chain (FTL) protein level and lipid peroxidation

may promote the induction of ferroptosis in cancer cells. Free iron accumulation inside the cell participated in the Fenton reaction to produce lipid peroxides, which was confirmed with the increased MDA levels.

Yang and Stockwell (2008) reported that cancer cells undergoing ferroptosis increased iron import and decreased iron storage when compared to other cells [18]. Thus, it can be suggested that heteronemin sensitizes tumor cells to ferroptosis by modulating iron metabolism. Reduced iron storage because of decreased FTL and increased autophagy-related protein expression in response to heteronemin may contribute to iron overload and eventually trigger ferroptosis in cancer cells.

Conclusions

Pancreatic cancers are resistant to the currently used drugs. To overcome drug-resistance mechanisms such as increased drug efflux, improved DNA repair, and impaired apoptosis; activating ferroptotic pathway is a state-of-the-art therapeutic strategy. Taken together, our present study demonstrated that heteronemin promoted ferroptosis in pancreatic cancer cells via the regulation of several proteins that possess critical roles in the progression of ferroptosis. We believe that our study will be of importance to understanding the heteronemin mechanism of action as a potential anticancer drug. Heteronemin itself, or its derivatives to be synthesized in the future with higher selectivity and affinity will be highly promising agents for patients suffering from pancreatic cancer.

Declarations

Author Contributions

All authors contributed to the study conception and design. Gizem Kaftan, Mümin Alper Erdoğan and Güliz Armagan performed cell culture studies and prepared figures. Mei-Chin Lu and Hung-Yu Linisolated heteronemin. The first draft of the manuscript was written by Güliz Armagan and Mohamed El-Shazly. Luciano Saso and Shou-Ping Shihrevised first draft. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

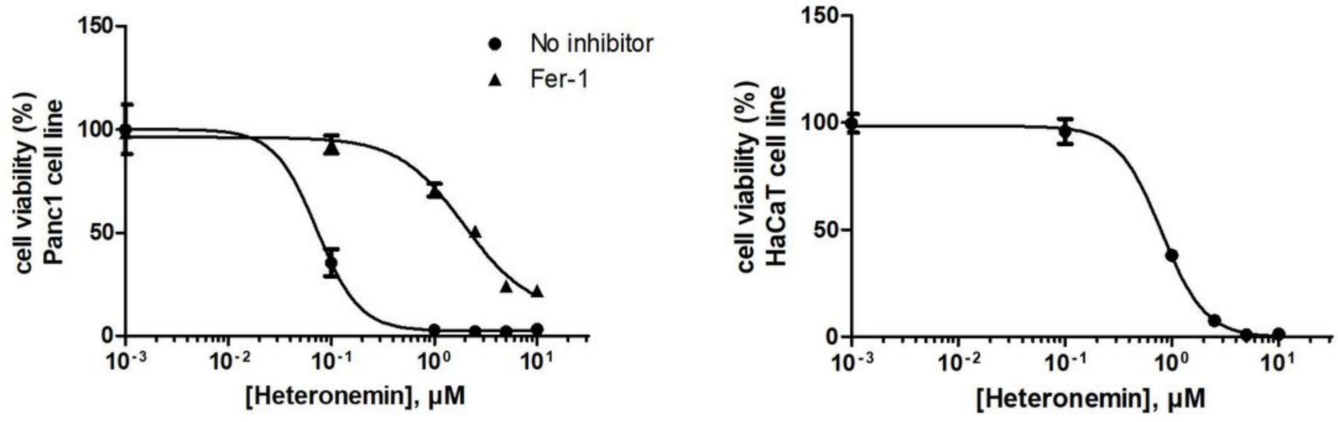

Figure 1

Antiproliferative effect of heteronemin on Panc-1 and HaCaT cell lines at increasing concentrations (0.001, 0.1, 1, 2.5, 5, 10 μM). The results are expressed as percentage survival after 48 h of exposure. IC_{50} values were calculated as 0.055 μM (Panc-1) and 0.256 μM (HaCaT) for each cell line

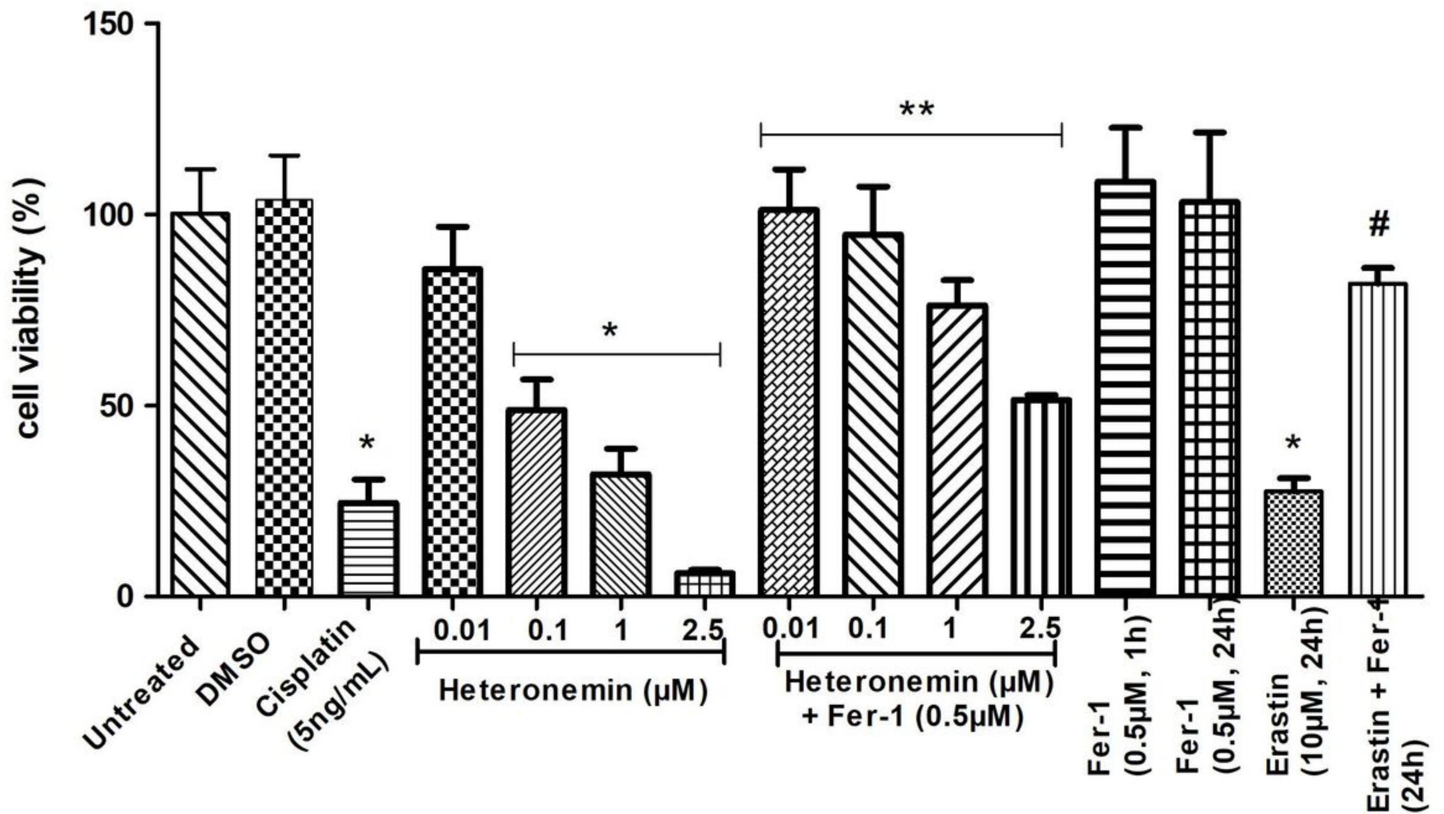

Figure 2

Ferroptosis inducer potential of heteronemin on Panc-1 in the presence of ferroptosis inhibitor, Fer-1 (0.5 μM). The results are expressed as percentage survival after 48 h of exposure. *$p < 0.05$ *vs*. untreated cells, **$p < 0.05$ *vs*. heteronemin only treated cells at the same concentration. $^{\#}p < 0.05$ *vs*. Erastin-treated cells

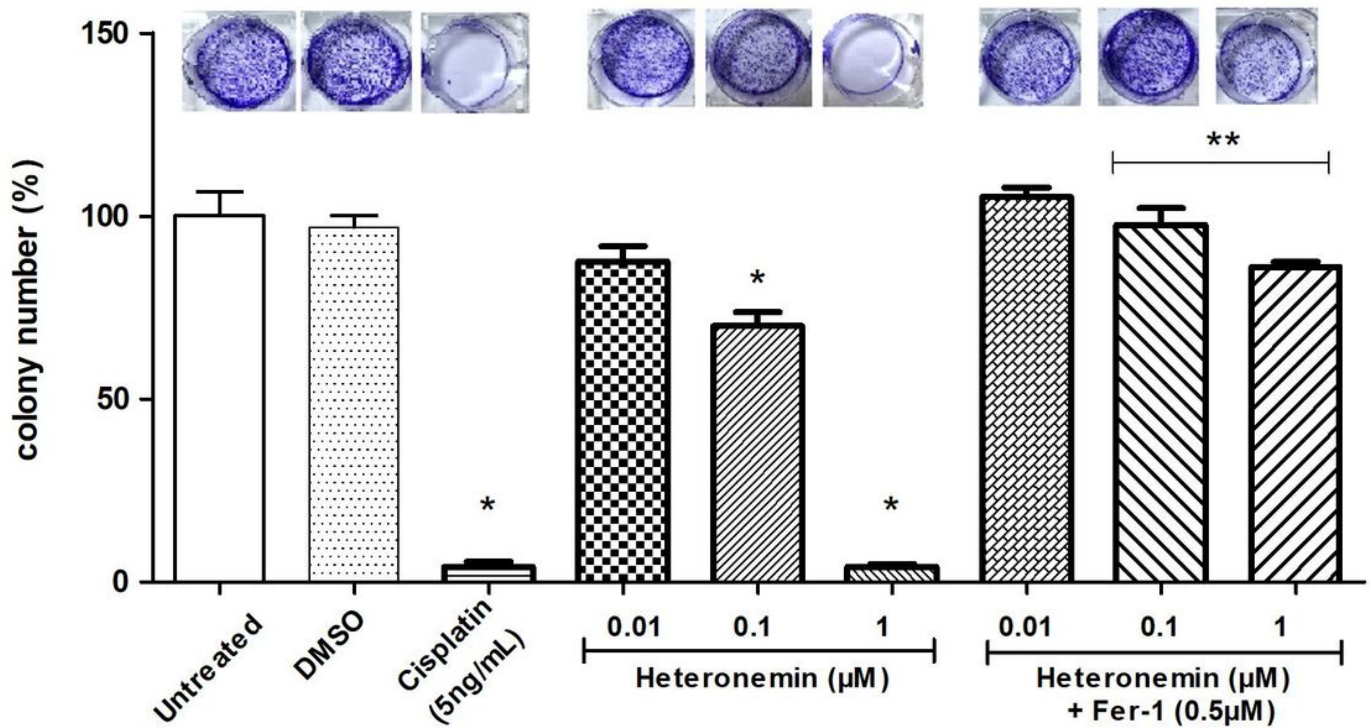

Figure 3

Colony formation analysis of Panc-1 cells treated with cisplatin (5ng/mL) heteronemin (0.01, 0.1 and 1 μM) and/or Fer-1 (0.5 μM) for 14 days. The bar graph represents the average of three biological replicates. Representative dishes stained with crystal violet. *$p < 0.05$ *vs.* untreated cells, **$p < 0.05$ *vs.* heteronemin only treated cells at the same concentration

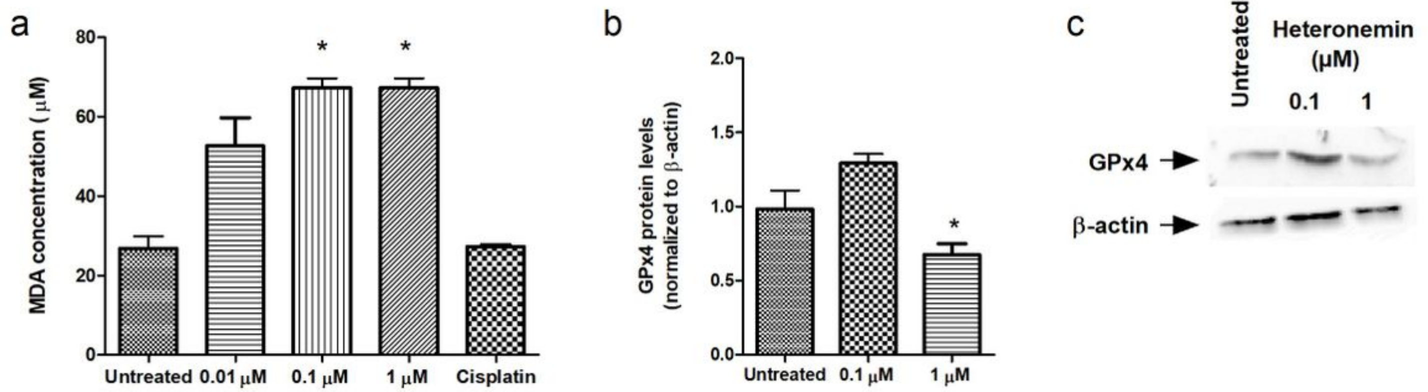

Figure 4

Heteronemin induced lipid peroxidation and decreased GPx4 protein expression in Panc-1 cells. (a) MDA concentration was measured in cisplatin- and heteronemin-treated cells. (b, c) Bar graph data represent the mean ± SD.; n = 3 independent experiments. Quantified band values of GPX4 were normalized to the corresponding *β*-actin signal. $^{*}p < 0.05$ *vs.* untreated cells

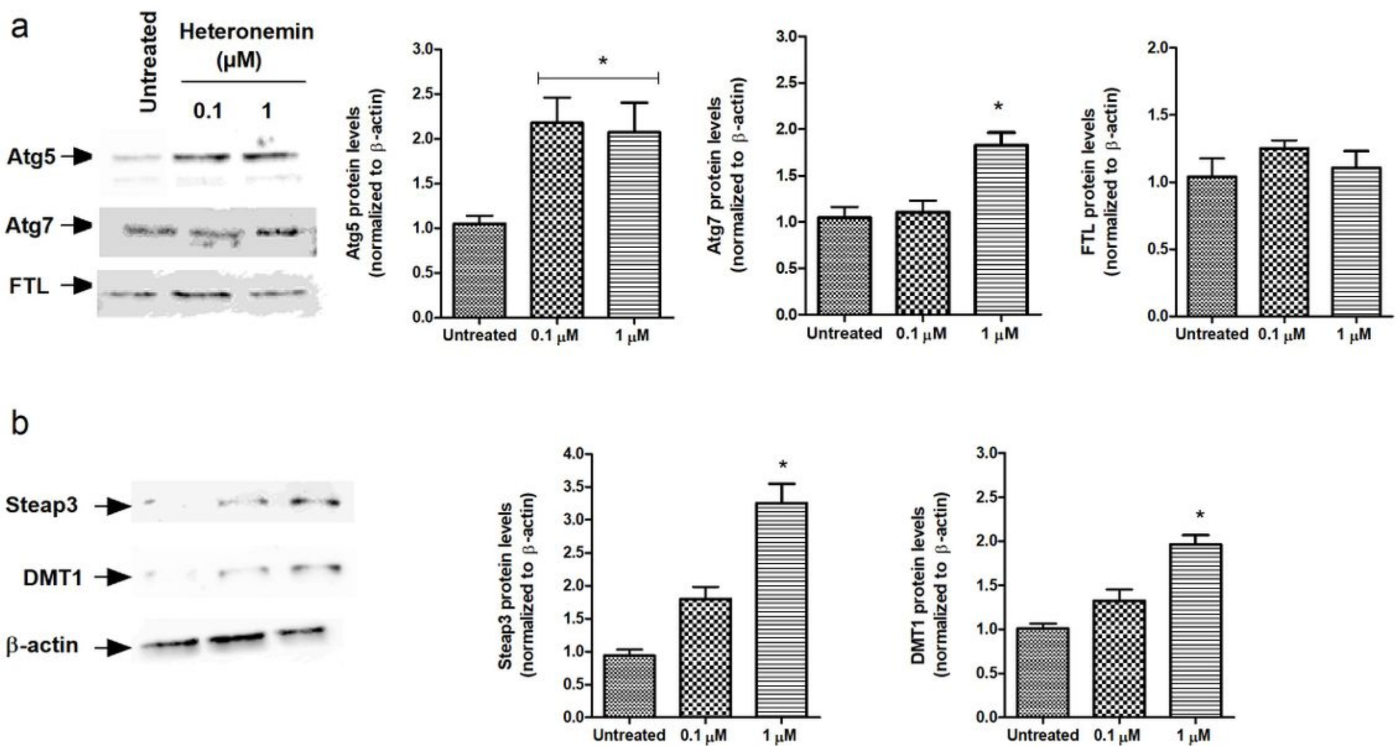

Figure 5

Alteration in (a) ferritinophagy- (Atg5, Atg7, FTL) and (b) iron-related (STEAP3, DMT1) protein levels following heteronemin treatment in Panc-1. Quantified band values were normalized to the corresponding

β-actin signal. Bar graph data represent the mean ± SD.; n = 3 independent experiments. *$p < 0.05$ *vs.* untreated cells