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Research Article

A Marine Terpenoid, Heteronemin, Induces Both the Apoptosis and Ferroptosis of Hepatocellular Carcinoma Cells and Involves the ROS and MAPK Pathways

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Hepatocellular carcinoma (HCC) is a leading cause of death, resulting in over 700 thousand deaths annually worldwide. Chemotherapy is the primary therapeutic strategy for patients with late-stage HCC. Heteronemin is a marine natural product isolated from *Hippospongia* sp. that has been found to protect against carcinogenesis in cholangiocarcinoma, prostate cancer, and acute myeloid leukemia. In this study, heteronemin was found to inhibit the proliferation of the HCC cell lines HA22T and HA59T and induce apoptosis via the caspase pathway. Heteronemin treatment also induced the formation of reactive oxygen species (ROS), which are associated with heteronemin-induced cell death, and to trigger ROS removal by mitochondrial SOD2 rather than cytosolic SOD1. The mitogen-activated protein kinase (MAPK) signaling pathway was associated with ROS-induced cell death, and heteronemin downregulated the expression of ERK, a MAPK that is associated with cell proliferation. Inhibitors of JNK and p38, which are MAPKs associated with apoptosis, restored heteronemin-induced cell death. In addition, heteronemin treatment reduced the expression of GPX4, a protein that inhibits ferroptosis, which is a novel form of nonapoptotic programmed cell death. Ferroptosis inhibitor treatment also restored heteronemin-induced cell death. Thus, with appropriate structural modification, heteronemin can act as a potent therapeutic against HCC.

1. Introduction

Natural products are the leading source of chemotherapy drugs [1–4]. Over 70% of the Earth's surface is covered by oceans, which have vast biodiversity and are the origin of life [5]. Natural marine products have been found to have bioactivity against cancer progression [6, 7]; for example, makaluvamines, a group of pyrroloiminoquinone alkaloids isolated from marine sponges, have been identified to induce DNA cleavage [8] and protect against skin cancer [9] and lung cancer [10]. Heteronemin is a metabolite found in the sponge *Hippospongia* sp. that exerts potent effects to inhibit carcinogenesis in cholangiocarcinoma [11], prostate cancer [12, 13], and acute myeloid leukemia (AML) [14]. Although the mechanism by which heteronemin inhibits cancer is not entirely clear, heteronemin has been found to regulate the Bcl-mediated apoptotic pathway [12, 15] and autophagy [15]. Topoisomerase II, which is associated with DNA replication [16], has also been found to be inhibited by the heteronemin treatment [12], and topoisomerase II inhibition is the mechanism underlying the effect of many clinical anticancer drugs, such as topotecan and irinotecan, which are topoisomerase I inhibitors [17, 18]. Therefore, heteronemin shows adequate potential as an anticancer agent.

Liver cancer is a leading cause of cancer-associated death around the world, particularly in Asia, and caused over 700 thousand deaths worldwide in 2018 [19, 20]. Approximately 80% of all liver cancer cases are classified as hepatocellular carcinoma (HCC) derived from hepatocytes [19]. Although many therapeutics for HCC, including surgery, organ transplantation, and chemotherapy [21], are available, chemotherapy is the major therapeutic strategy for advanced HCC patients [22]. Targeted therapy is a new approach to chemotherapy that utilizes small molecules or antibodies to target cancer-specific markers and results in cytotoxicity and cell death [23]. Heteronemin was found to target Ras signaling and downregulate NFκB, thus showing potential as a targeted therapeutic agent [14]. A major outcome of chemotherapy is apoptosis, which is the fundamental programmed cell death process [24]. Loss of apoptotic pathways commonly occurs in cancer and results in the survival of tumor cells. Therefore, chemotherapy often targets apoptosis [25]. In recent years, a novel form of programmed cell death called "ferroptosis," which is iron-dependent cell death that is associated with reactive oxygen species (ROS) and lipid peroxides, has been found to induce cell death and activate inflammation. It underlies the effect of many chemotherapeutic drugs, such as cisplatin [26] and sorafenib [27], which are the first-line treatment for advanced HCC [28].

ROS, including superoxide anions ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), play a vital role in chemotherapy and mediate several cellular pathways, including apoptosis and ferroptosis. ROS are also associated with the mitogen-activated protein kinase (MAPK) pathway, a conserved regulatory pathway that regulates signal transduction and is involved in several cellular processes, such as proliferation [29], differentiation [30], cell cycle arrest [29], survival [31], and death [32]. Extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 are the major MAPKs, and they respond to stimulation by regulating cell proliferation, apoptosis, ferroptosis, and inflammation [33]. In this study, we demonstrate that the anticancer effect of heteronemin on HCC is associated with ROS-associated MAPK activation and that heteronemin induces HCC death through apoptosis as well as ferroptosis.

2. Materials and Methods

2.1. Cell Culture. The human HCC lines HA22T/VGH (HA22T, #60168) and HA59T/VGH (HA59T, #60169) were purchased from the Bioresource Collection and Research Center (BCRC; Taiwan) and maintained in Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient Mixture (DMEM/F12, 3 : 2; Gibco; Waltham, MA, USA) supplemented with 8% fetal bovine serum (FBS; Gibco), 2 mM glutamine, and antibiotics at 37°C and 5% CO_2.

2.2. Cell Viability. Cell viability was measured with a trypan blue exclusion assay [34]. Briefly, the treated cells were exposed to 0.2% trypan blue reagent. Viable cells were not stained by the trypan blue dye, and the bright cells were counted as living cells.

2.3. Apoptosis Measurement. The HCC cell apoptosis was evaluated by annexin V/7AAD double staining. An apoptosis detection kit (Strong Biotech Corporation, Taipei, Taiwan) was used for annexin V/PI staining according to the manufacturer's instructions. Briefly, the treated cells were harvested, stained with annexin V/7AAD, and analyzed with an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo 7.6.1 software (Tree Star, Inc., Ashland, OR, USA).

2.4. Western Blot Analysis. To evaluate the changes in protein expression, western blotting was performed as follows. Briefly, cells were lysed with lysis buffer and centrifuged at 4°C. The protein concentration was determined by a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein lysates (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (PALL, Ann Arbor, MI, USA). The membranes were blocked with 5% nonfat milk in TBS-T buffer (TBS buffer containing 0.1% Tween 20) for one hour and incubated with primary antibodies such as Bax (AP1302a, Abgent, San Diego, CA, USA), ERK1/2 (GTX50868, GeneTex, Irvine, CA, USA), SOD1 (Ab13498, Abcam, Cambridge, UK, Eng.), SOD2 (Ab68155, Abcam), GPX4 (Sc-8007, Santa Cruz, Dallas, TX, USA), and β-actin (Sc-47778, Santa Cruz) as well as HRP-conjugated secondary antibodies. HRP luminescence was detected with an enhanced chemiluminescence (ECL) detection kit (Amersham Piscataway, NJ, USA).

2.5. ROS Detection. Briefly, 2′,7′-dichlorofluorescin diacetate (DCFDA) and dihydroethidium (DHE) were used to detect intracellular H_2O_2 and $O_2\bullet^-$ formation. Treated cells were incubated with 10 μM DCFDA or DHE for 20 minutes. After incubation, the cells were washed with phosphate-buffered saline (PBS) and analyzed by the FlowJo 7.6.1 software (Tree

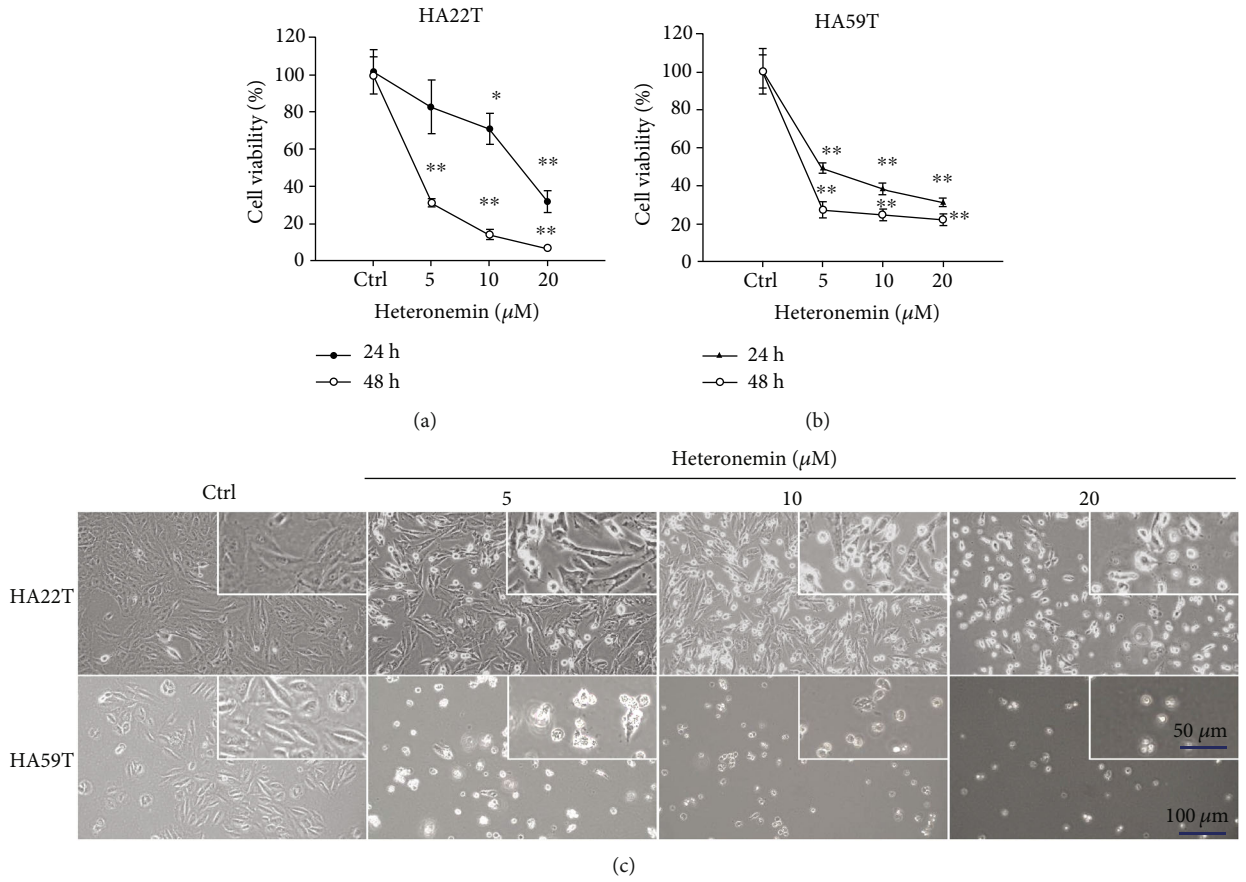


Figure 1: The cytotoxicity of heteronemin against HCC cell lines. The viability of (a) HA22T and (b) HA59T cells was determined 24 and 48 hours after the heteronemin treatment. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the control group; all data are presented as the mean ± S.D. of three independent experiments. (c) The morphological changes of HA22T and HA59T cells after 24 hours of heteronemin treatment. Magnification: 100x and 200x.

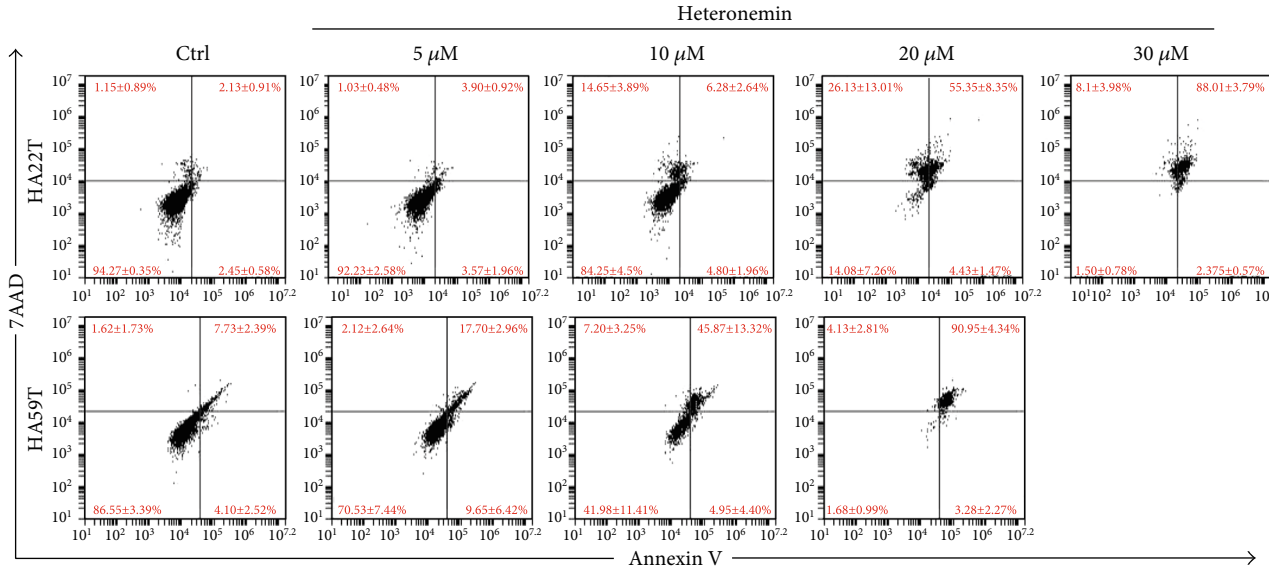
Star, Inc.) and SigmaPlot 11.0 software (Systat Software, San Jose, CA, USA).

2.6. Statistical Analysis. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) or Student's t-test at least in triplicate. $p < 0.05$ was considered significant.

3. Results

3.1. Heteronemin Modulates the Proliferation of HCC Cell Lines. The cytotoxicity of heteronemin, as a marine drug with potential anticancer effects, was measured in the HCC cell lines HA22T and HA59T. Significant cell death was observed in both HA22T and HA59T cells after the heteronemin treatment, and HA59T cells exhibited higher sensitivity to heteronemin (Figures 1(a) and 1(b)). The IC_{50} values of heteronemin after 24 hours of treatment were 10.4 and 5.25 μM, in HA22T and HA59T cells, respectively. The cell morphological change was also observed after the heteronemin treatment (Figure 1(c)). The results indicated the cytotoxicity of heteronemin in HCC.

3.2. Apoptosis Is a Major Regulatory Mechanism Underlying Heteronemin-Associated Programmed Cell Death. Apoptosis plays a vital role in the anticancer mechanism of most chemotherapy drugs, such as cisplatin and sorafenib [35, 36]. We stained cells with the apoptosis markers annexin V and 7-amino-actinomycin D (7AAD) to determine whether heteronemin induced apoptosis (Figure 2(a)). Over half of 20 μM heteronemin-treated HA22T and HA59T cells were apoptotic (annexin V^+) cells, including early-stage apoptotic cells and late-stage apoptotic cells, and HA59T cells were more sensitive than HA22T cells to the effects of heteronemin (Figures 2(b) and 2(c)). In addition, the numbers of annexin V^- and $7AAD^+$ nonapoptotic cells were increased in 20 μM heteronemin-treated HA22T cells and 10 μM heteronemin-treated HA59T cells (Figures 2(d) and 2(e)). The caspase family of proteins plays a vital role in apoptosis initiation and progression. To clarify the role of heteronemin-induced apoptosis, we inhibited caspase activity in HA22T and HA59T cells with the pan-caspase inhibitor Z-VAD-FMK. Approximately 20% of growth inhibited by heteronemin was restored by the Z-VAD-FMK treatment (Figures 2(f) and 2(g)). The apoptosis markers cleaved caspase-8, cleaved PARP-1, and Bax were upregulated, and the antiapoptotic protein Bcl2 was downregulated after the heteronemin treatment (Figures 2(h) and 2(i) and Supplementary Figure 1). These data suggested heteronemin showed anticancer potential by activating apoptosis to inhibit cancer growth and induce cell death.



(a)

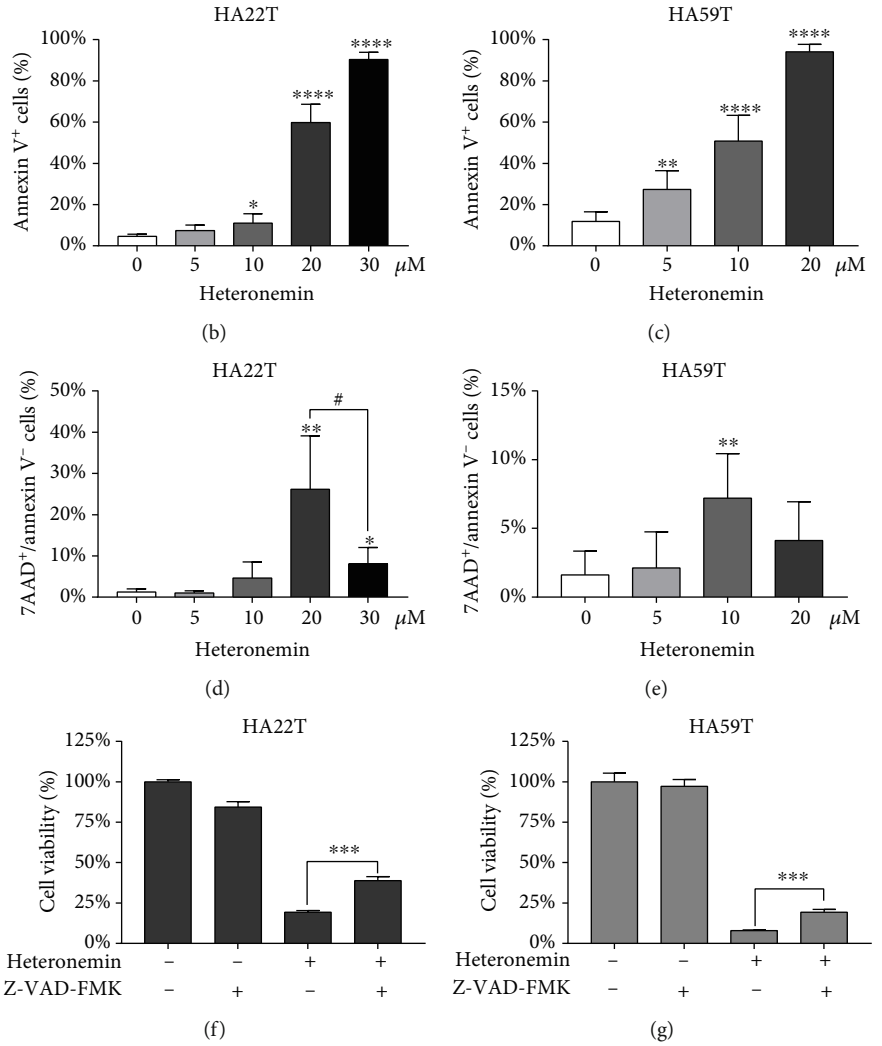


Figure 2: Continued.

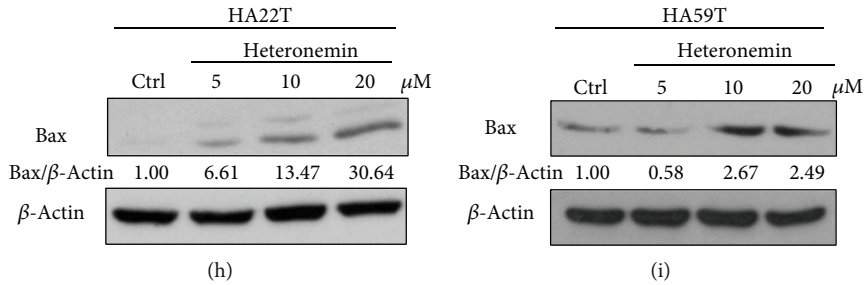


Figure 2: Heteronemin induces cell apoptosis via the caspase cascade. (a) HA22T and HA59T cells were treated with control or 5, 10, 20, or 30 μM heteronemin for 24 hours and stained with annexin V/7AAD to analyze apoptotic cells. (b) and (c) Quantification of apoptotic (annexin V^+) cells in (a). $^*p < 0.05$, $^{****}p < 0.0001$ compared with the control. (d) and (e) Quantification of nonapoptotic (annexin V^-/$7AAD^+$) cells in (a). $^{**}p < 0.01$ compared with the control. $\#p < 0.05$ compared with 20 μM and 30 μM heteronemin-treated cells. (f) and (g) Cell viability of HA22T and HA59T cells pretreated with 20 μM Z-VAD-FMK, a pan-caspase inhibitor, for 4 hours and treated with 20 μM heteronemin for 24 hours. $^{***}p < 0.001$. (h) and (i) Western blot analysis of the Bax expression in heteronemin-treated HA22T and HA59T cells. All data are presented as the mean ± S.D. of three independent experiments.

3.3. ROS Formation and MAPK/JNK Activation Play a Vital Role in Heteronemin-Mediated Cell Death. ROS are small molecules with high reactivity and play a vital role in many processes that maintain intracellular homeostasis, including autophagy and apoptosis [37, 38]. ROS, such as superoxide anions ($O_2\bullet^-$), hydroxyl radicals (OH•), and hydrogen peroxide (H_2O_2) [39], are primarily generated during the process of oxidative phosphorylation (OXPHOS), are elevated by many chemotherapeutics, and induce apoptosis [40]. ROS accumulation has been shown to activate the G protein axis, tyrosine kinase receptors, and the p53 pathway and to induce downstream biological pathways depending on the amount of ROS [41]. ROS accumulation also disrupts oxidative balance homeostasis and induces lipid peroxidation, resulting in ferroptosis, which is a novel programmed cell death induced by the disruption of the GSH/GSSH balance [42]. ROS accumulation has been observed in many studies on chemotherapeutic agents, such as 5-fluorouracil, erlotinib, and rituximab, and plays a vital anticancer role [43–45]. To confirm that ROS were formed after heteronemin treatment, 2′,7′-dichlorofluorescein diacetate (DCFDA) and dihydroethidium (DHE) were used to indicate H_2O_2 and $O_2\bullet^-$ formation, respectively. The number of H_2O_2 and $O_2\bullet^-$-positive cells was increased in HA22T and HA59T cells after heteronemin treatment (Figures 3(a)–3(d)). The superoxide dismutase family is associated with the removal of ROS and catalyzing ROS into water and oxygen. Heteronemin treatment downregulated the expression of SOD1 but upregulated the expression of SOD2 (Figures 3(e) and 3(f)). Furthermore, heteronemin-induced cell death was reversed after treatment with the ROS inhibitor N-acetyl-L-cysteine (NAC) (Figures 3(g) and 3(h)).

Many studies have demonstrated that ROS induce the MAPK signaling pathway and activate caspase-dependent apoptosis. Therefore, we next investigated the role of the MAPK/JNK axis in heteronemin-induced apoptosis. ERK 1/2, classical MAPKs that are activated by growth factors and play critical roles in cell proliferation and tumor progression [46], were downregulated in heteronemin-treated cells (Figures 4(a) and 4(b)). In contrast, the expression of the JNK downstream substrate c-Jun was upregulated, and SP600125, a JNK inhibitor, reversed the heteronemin-induced cell death (Figures 4(c) and 4(d) and Supplementary Figure 1). Additionally, treatment with the p38 inhibitor SB203580 restored the viability of HA22T and HA59T cells after the heteronemin treatment (Figures 4(e) and 4(f)). The results revealed that heteronemin treatment-induced cell death through inducing ROS formation and activating JNK/p38 MAPKs, resulting in cell apoptosis.

3.4. Ferroptosis, a Novel Form of Programmed Cell Death, Is Involved in Heteronemin-Induced Cell Death. Treatment with the caspase inhibitor Z-VAD-FMK or the p38 or JNK inhibitor reduced heteronemin-induced cell death by approximately only 20%. Heteronemin induced cell death not only through apoptosis but also through other forms of programmed cell death. Ferroptosis is a novel form of programmed cell death and is involved in cell death induced by many chemotherapeutics [26, 27]. GPX4 is a vital protein that protects against lipid peroxidation and inhibits ferroptosis initiation, and a reduction in the GPX4 expression is a critical feature of ferroptosis. Cells treated with heteronemin expressed lower levels of GPX4 protein (Figures 5(a) and 5(b)), showing that ferroptosis was involved in heteronemin-induced cell death. Additionally, the ferroptosis inhibitors ferrostatin and liproxstatin reversed heteronemin-induced cell death by approximately 15% (Figures 5(c)–5(f)). Interestingly, treatment with the ferroptosis inhibitor significantly decreased the number of late-stage apoptotic (annexin V^+/$7AAD^+$) cells and increased the proportion of healthy cells (Figure 5(g)). Therefore, like other drugs, heteronemin acts as a potential anticancer drug by inducing cell apoptosis and ferroptosis and may effectively suppress HCC progression.

4. Discussion

HCC is a severe disease that causes 700 thousand deaths annually worldwide [19, 20]. In this study, we demonstrated that heteronemin is an effective natural marine product that induces HCC cell proliferation and has potent anticancer

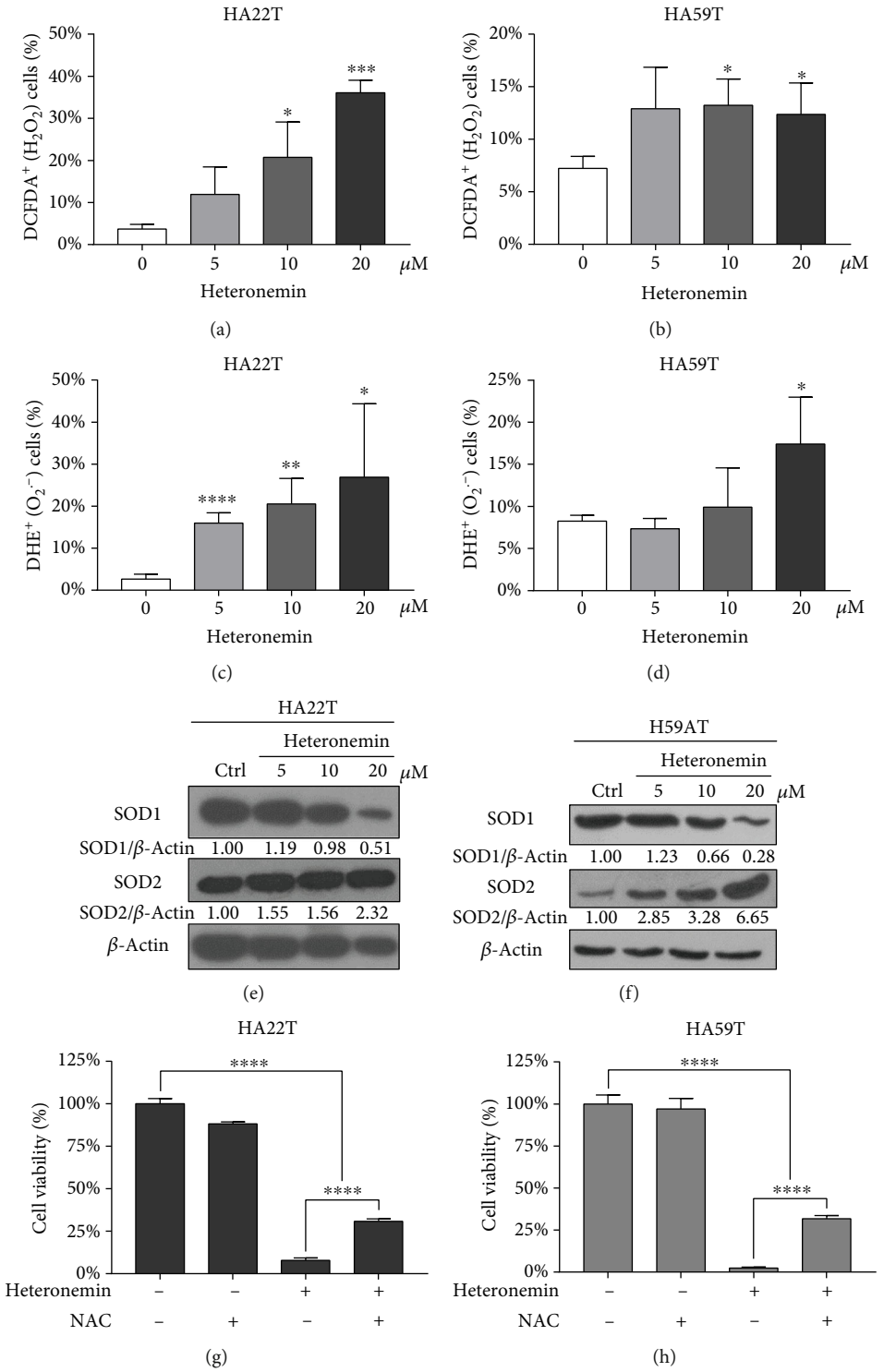


Figure 3: ROS formation is associated with heteronemin-induced cell death. The number of H_2O_2-positive cells in (a) HA22T and (b) HA59T was detected with DCFDA and analyzed by flow cytometry. In addition, $O_2\bullet^-$-positive cells in (c) HA22T and (d) HA59T cells were detected with DHE and analyzed by flow cytometry. Western blot analysis of the SOD1 and SOD2 expression in (e) HA22T and (f) HA59T cells after the heteronemin treatment. (g) HA22T and (h) HA59T cells were treated with NAC (10 mM) for 2 hours before being treated with 20 μM heteronemin, and cell viability was measured after 24 hours. All data are presented as the mean ± S.D. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$; all data are presented as the mean ± S.D. of three independent experiments.

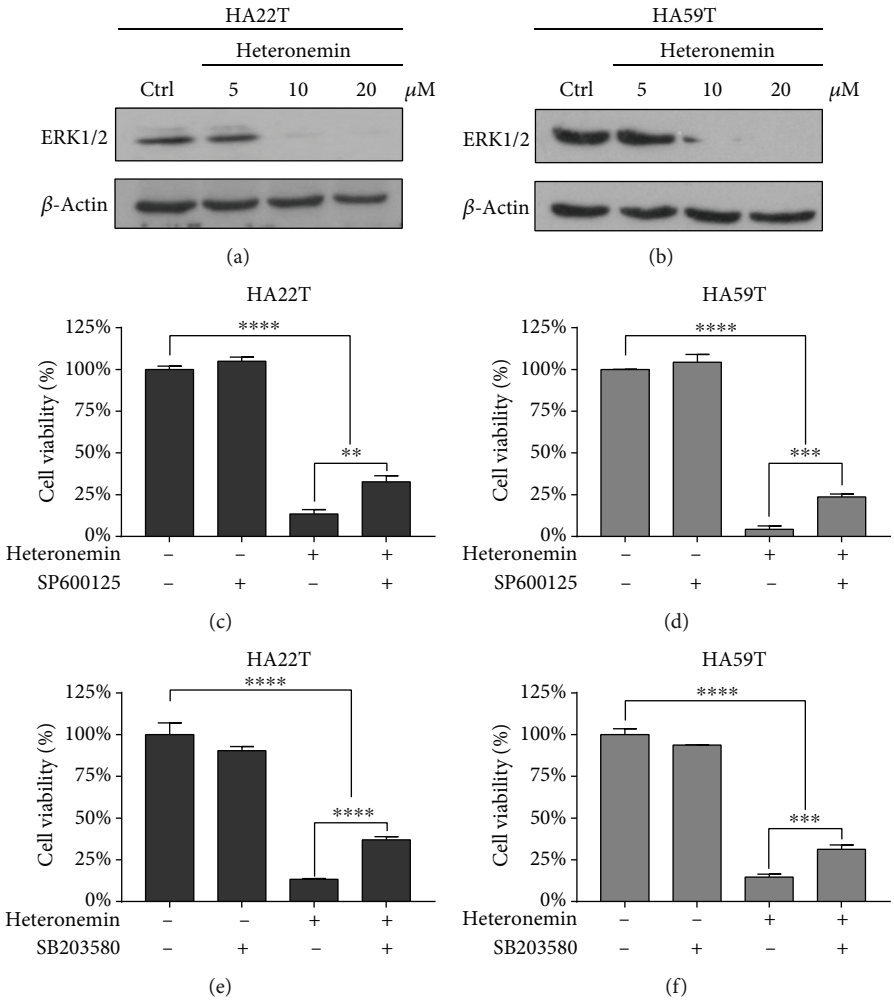


Figure 4: The MAPK signaling pathway regulates the heteronemin-mediated cell death. Western blot analysis of ERK1/2 expression in (a) HA22T and (b) HA59T cells after heteronemin treatment. (c) HA22T and (d) HA59T cells were pretreated with 30 μM SP600125, a JNK inhibitor, for 1 hour before being treated with 20 μM heteronemin, and cell viability was observed. (e) HA22T and (f) HA59T cells were pretreated with 30 μM SB203580, a p38 inhibitor, for 1 hour before being treated with 20 μM heteronemin, and cell viability was analyzed. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; all data are presented as the mean ± S.D. of three independent experiments.

potential. Heteronemin was first isolated from *Hyrtios erecta* by Kobayashi et al. in 1994 [47], but research showing that heteronemin induces apoptotic cell death by inhibiting NF-κB activation was not published until 2010 [5]. In recent years, heteronemin has been shown to have anticancer potential in several cancer types by inducing apoptosis, which is usually associated with oxidative stress [11, 12, 48, 49]. Here, we demonstrated that heteronemin has anticancer potential in HCC by inhibiting HA22T and HA59T cell growth and inducing cell apoptosis (Figures 1 and 2).

The ability of heteronemin to induce ROS formation was demonstrated in HCC cell lines (Figures 3(a) and 3(b)). Interestingly, the expression of SOD family proteins, which are essential for ROS removal, was found to be altered. After heteronemin treatment, SOD2 was overexpressed, and SOD1 was downregulated. Similar alterations in expression have been found in C_8-ceramide-induced apoptosis in lung cancer, and opposing alterations have been observed in breast cancer development [50, 51]. SOD1 is a Zn-Cu-associated dismutase located in the cytoplasm, and SOD2 is a Mn^{2+}-associated dismutase located in mitochondria [52]. As shown in Figures 3(e) and 3(f), SOD2 was upregulated, and SOD1 was downregulated in cells in response to heteronemin, showing that mitochondrial oxidative stress is harmful and suggesting that heteronemin may play a role in mitochondrial dysfunction. Consistently, heteronemin was previously found to induce mitochondrial dysfunction and apoptosis in leukemia [49].

The MAPK signaling transduction pathway plays a vital role in various physiological processes and responses to oxidative stress [33]. Three major MAPKs, namely, ERK, JNK, and p38, are involved in this signaling pathway and result in cell proliferation, autophagy, apoptosis, and inflammation. ERK-mediated MAPK signaling has been found to be triggered by stimulation with growth factors (such as epidermal growth factor (EGF) [53]), and the activation of the downstream RAS/RAF/MEK/ERK cascade results in cell proliferation [54]. This cascade is commonly dysregulated in many

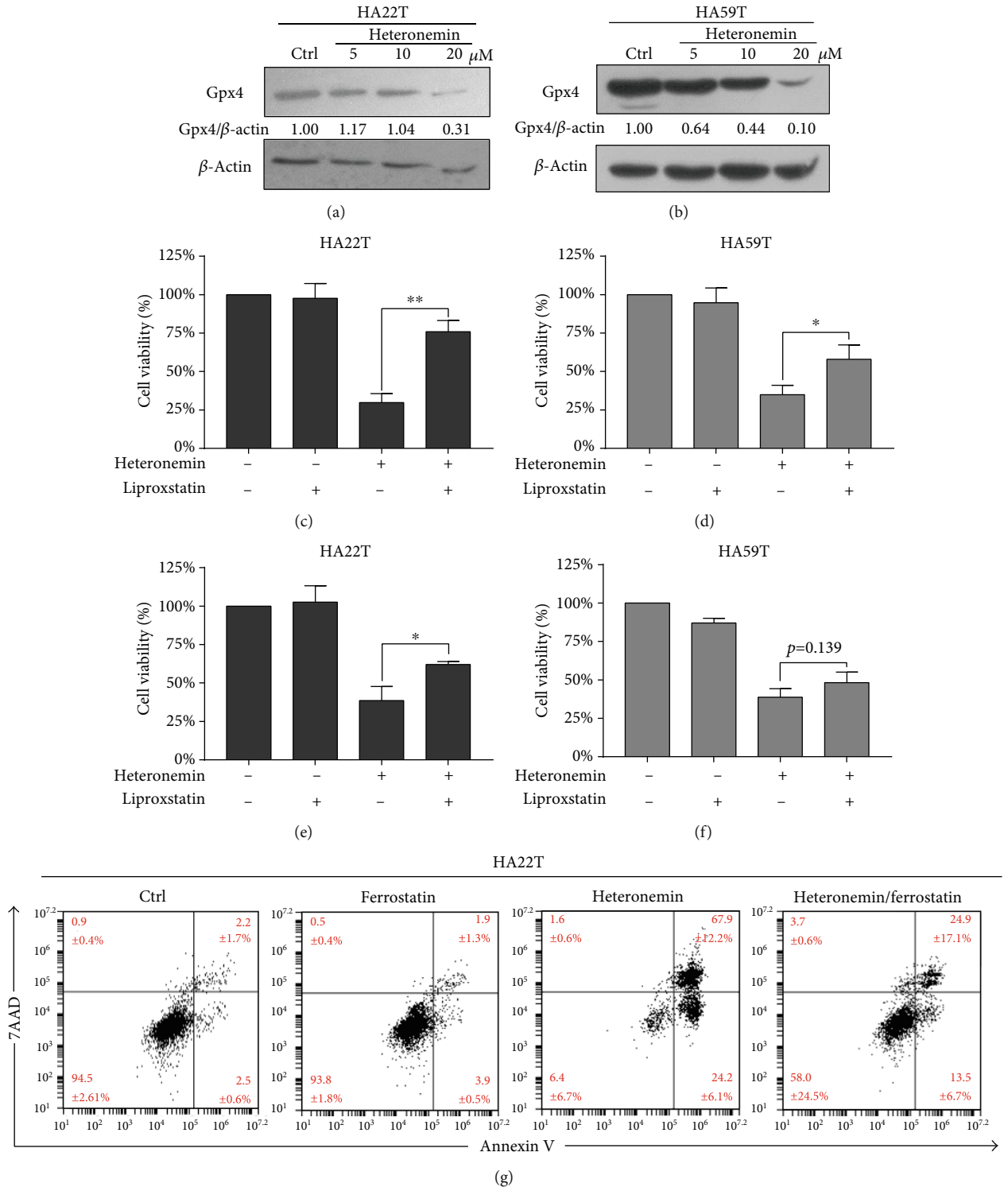


Figure 5: Heteronemin initiates ferroptosis, which is associated with heteronemin-induced cell death. Western blot analysis of ferroptosis markers and the reduction in GPX4 in (a) HA22T and (b) HA59T cells after heteronemin treatment. Liproxstatin and ferrostatin were used to determine the effect of ferroptosis on heteronemin-associated cell death. (c) HA22T and (d) HA59T cells were cotreated with 5 μM liproxstatin and 20 μM heteronemin, and cell viability was measured. (e) HA22T and (f) HA59T cells were cotreated with 15 μM ferrostatin and 20 μM heteronemin treatment, and cell viability was measured. (g) HA22T was cotreated with 15 μM ferrostatin and 20 μM heteronemin, and apoptosis was measured with annexin V/7AAD double staining. $^{*}p < 0.05$, $^{**}p < 0.01$; all data are presented as the mean ± S.D. of three independent experiments.

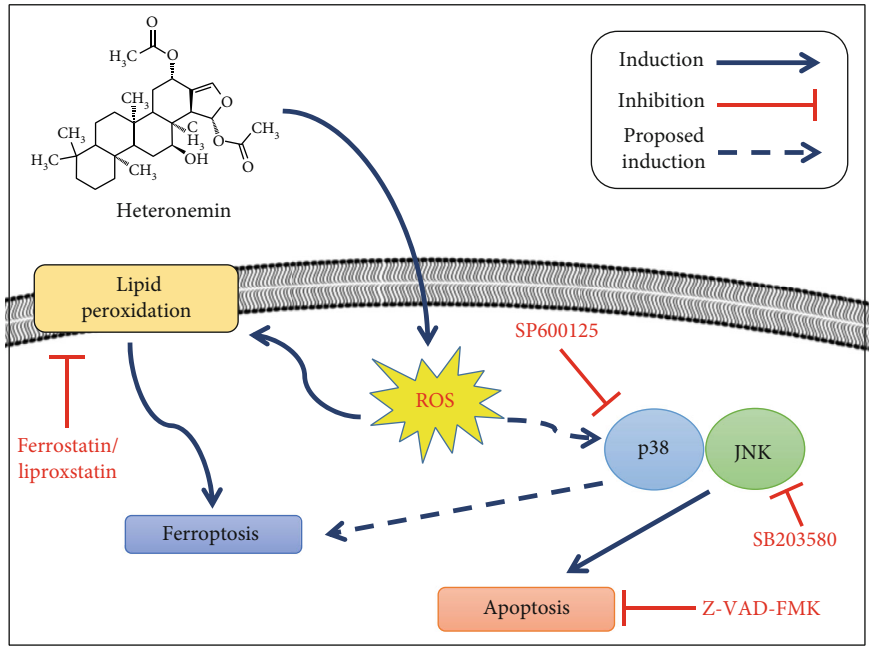


Figure 6: The potential anticancer mechanism of heteronemin. Heteronemin was found to induce ROS formation, resulting in p38/JNK activation and caspase-associated apoptosis and ferroptosis and leading to cancer cell death.

cancers [55, 56]. ROS-dependent JNK activation has been found to be a robust activator of apoptosis that induces Bcl-Bax signaling and is involved in caspase-dependent apoptosis [57–59]. The ROS/p38/p53 cascade is also a key regulator of cytochrome *c* release, and Bax-initiated caspase activation results in extrinsic and intrinsic (mitochondrial) apoptosis [60–62]. As shown in Figures 4(a) and 4(b), heteronemin effectively reduced the expression level of ERK. On the other hand, treatment with the p38 or JNK inhibitor reversed the cell death caused by heteronemin (Figures 4(c)–4(f)). The results suggested that heteronemin induced ROS formation and initiated apoptosis via the JNK/p38 MAPK signaling pathway.

Ferroptosis is a novel form of programmed cell death associated with oxidative stress, iron accumulation, and lipid peroxidation. Many clinical chemotherapy drugs have been found to not only initiate apoptosis but also induce ferroptosis and protect against cancer growth [63–65]. In addition, immunotherapy has also been found to regulate ferroptosis by enhancing the accumulation of lipid peroxides and regulating the expression of SLC3A2 and SLC7A11, the subunits of the chloride-dependent cystine-glutamate (xCT) antiporter system, which regulates redox homeostasis and oxidative stress [66] to inhibit lipid peroxidation and ferroptosis [67]. GPX4 is a phospholipid-hydroperoxide glutathione peroxidase that protects against lipid peroxidation and ferroptosis [68]. GPX4 is commonly inactivated during ferroptosis [69]. Heteronemin treatment downregulated GPX4, and the ferroptosis inhibitors liproxstatin and ferrostatin significantly reversed heteronemin-induced cell death (Figures 5(a)–5(f)). Interestingly, treatment with the ferroptosis inhibitors liproxstatin and ferrostatin reduced the level of late-stage apoptotic cell death (Figure 5(g)); previous research has shown that annexin V/PI-positive cells may be late-stage apoptotic cells, necroptotic cells, or ferroptotic cells [70–72]. The MAPK signaling pathway has also been found to be involved in ferroptosis initiation. In AML cells, the inhibition of MAPKs, especially p38 and JNK, but not ERK, results in AML insensitivity to erastin [73]. In addition, in 2018, Poursaitidis et al. [74] showed that inhibiting MAPK signaling protects lung cancer cells against ferroptosis. Consistently, MAPKs also play a vital role in heteronemin-induced ferroptosis.

Finally, we performed an animal experiment to validate the anticancer potential of heteronemin *in vivo* (data not shown). We treated mice with three different doses of heteronemin (1 mg/kg, 5 mg/kg, and 10 mg/kg), and tumor volume was significantly reduced after treatment with 1 mg/kg heteronemin; however, due to the cytotoxicity of heteronemin, the 5 mg/kg and 10 mg/kg doses of heteronemin were lethal, and even the mice treated with 1 mg/kg heteronemin died after two weeks of treatment. The results indicated that heteronemin is cytotoxic to HCC cells but also has severe side effects in mice. Thus, it is critical to determine the side effects of heteronemin. In addition, it is crucial to further investigate the cytotoxicity of heteronemin in healthy cells. In this experiment, heteronemin was administered via intraperitoneal injection, which caused the drug to spread to all organs of the mice. Hepatic arterial infusion chemotherapy (HAIC), which directly delivers drugs to tumors and minimizes systemic toxicity, is a feasible strategy for administering heteronemin [75].

5. Conclusions

In conclusion, heteronemin is an effective agent against HCC that induces HCC cell apoptosis and ferroptosis by inducing intracellular ROS formation and the p38/JNK MAPK

signaling pathway, revealing the potent MAPK-mediated crosstalk mechanism between apoptosis and ferroptosis (Figure 6).

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: The expression of apoptotic- and MAPK-associated proteins. HA22T cells were treated with indicated concentrations of heteronemin for 14 hours respectively, and the changes of protein expression was determined by Western blot assay. *(Supplementary Materials)*

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