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ORIGINAL ARTICLE

Anticancer activity of Honokiol against lymphoid malignant cells via activation of ROS-JNK and attenuation of Nrf2 and NF-κB

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Summary

Purpose: *To evaluate the effect of Honokiol (HK) in the ROS-JNK pro-apoptotic pathway and NF-κB, Nrf2 anti-apoptotic pathways, in order to seek a possible explanation for its anticancer efficacy.*

Methods: *The Raji and Molt4 cell lines were utilized for the determination of anticancer activity against lymphoid malignant cells. BALB/C nude mice, weighing 18-20g each and aged 4–5 weeks, were procured from the central animal house facility. For establishing non-Hodgkin lymphoma in BALB/C, the nude mice were subcutaneously administered 1×10^7 Raji cells, suspended in 0.2 mL sterile PBS on the back. The mice were then randomly divided into 3 groups (6 mice in each group). HK cytotoxicity was determined using the colorimetric MTT assay.*

Results: *In colorimetry-based MTT assay, the cytotoxicity of HK was determined at different time intervals, in lymphoid malignant Raji and Molt4 cell lines. HK exhibited prominent cytotoxicity against Raji cell lines with IC_{50} of 0.092 ± 0.021 μM. In Molt4 cells, the administration of HK caused significant cytotoxicity with IC_{50} of 0.521 ± 0.115 μM. The treatment of HK caused significant increase in the activity of reactive oxygen species (ROS) in Raji cells at various time intervals. Moreover, the level of NF-κB was significantly reduced in the presence of HK, which could be easily understood by a decreased level of p-65. Furthermore, in the presence of ROS inhibitor NAC (10mM) for 24 hrs, the JNK pathway was markedly activated, together with inhibition of NF-κB activity and a reduced level of Nrf2 expression. To further confirm the in vitro results by in vivo activity, HK was observed to inhibit the proliferation of Raji cells in vivo, which might be attributable to its inhibitory effect against the progression of the tumor ($p<0.05$).*

Conclusion: *The present study suggests that HK causes considerable induction of apoptosis in lymphoid malignant cells, both in vitro and in vivo, whereas the generation of ROS might serve as an underlying mechanism for inducing apoptosis.*

Key words: *anticancer activity, honokiol, NF-κB, Nrf2, ROS-JNK*

Introduction

Despite various advances in cancer therapies, this disease is still considered as a major cause of morbidity and mortality across the globe [1]. Since there are many types of cancer, almost every part of the body can be affected [2]. More than a hundred types of cancers exist. The uncontrolled growth of cancer cells is considered to be due to some underlying mechanisms with devastating effects [3]. These cells are able to invade the neighbouring normal healthy tissues and thereby disrupt their function and to metastasize to distant places [4]. The therapies used to treat or cure cancer are often associated with severe toxicity and adverse effects [5]. In this regard, plants or

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plant-based products are certainly promising for the development of novel anticancer agents with little or no toxicity [6]. Various studies have indicated the numerous roles of natural compounds, exhibiting excellent anticancer activity via attenuating key catalytic pathways [7-10].

Among the several established factors for the progression of cancer, the injury mediated via the generation of ROS has strong involvement in the cellular degeneration [11]. It has been established that cancer cells usually acquire and tolerate higher ROS activity than normal cells [12]. Apart from this, recent research has been focussing on the development of novel agents than are able to modulate the dysregulated oxidative pathways efficiently [13]. Various studies have indicated the role of Nrf2, a basic leucine zipper (bZIP) protein in the regulation of expression of antioxidant proteins, which protects cancer cells from the damages induced by ROS, anticancer drugs and other chemicals [14-17]. Therefore, downregulation of Nrf2 and the induction of ROS in cancer cells can lead to the initiation of apoptosis [18].

The pro-apoptotic mitogen-activated protein kinase (MAPK) pathways were reported to be significantly involved in the ROS-induced apoptosis [19]. The studies showed that C-Jun NH2-terminal kinase (JNK), from the MAP Kinases family, is able to trigger resistant cells against anticancer drug therapy, suggesting its role in drug-induced apoptosis [20]. NF-kB is another antiapoptotic factor found in elevated levels mainly in lymphoid malignant cells, which leads to resistance against apoptosis [21]. Therefore, drugs that are concurrently able to modulate the sustained ROS pro-apoptotic pathway and attenuate NF-κB and Nrf2 activity, may provide a beneficial effect in cancer therapy [22].

The present study illustrates the effect of HK in the ROS-JNK pro-apoptotic pathway and NF-κB, Nrf2 antiapoptotic pathways, for a possible explanation of its anticancer efficacy *in vitro* and *in vivo.*

Methods

Cell lines and experimental animals

The Raji and Molt4 cell lines were grown at 37°C in 5% CO_2 in RPMI 1640 medium, with 10% heat-inactivated FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. The BALB/C nude mice, weighing 18-20g each, at 4–5 weeks of age, were procured from the central animal house facility of the Hangzhou First Hospital and all the experiments were performed with prior approval of the Animal Care and Use Committee.

Induction of non-Hodgkin lymphoma animal model and treatment

For establishing non-Hodgkin lymphoma in mice, the experimental nude mice were subcutaneously administered with 1×10^7 Raji cells, suspended in 0.2 mL sterile PBS on the back. The mice were then randomly divided into 3 groups (6 mice in each group). The day of the first injection was recorded as day zero. Thus, counting from the initial day, the mice in the control group received saline for 10 days, while the treated group was administered HK (5mg/20g and 10mg/20g in the morning) on days 8-12 and 15-19. Using Vernier calliper, the size of the tumor was measured continuously from the beginning of the treatment and the growth curve has been analysed based on this. The weight of the mice was also registered on each alternate day. Finally, on day 20 of the experiment, the mice were sacrificed and the tumors were excised for Western blot analysis.

Cell viability assay

HK cytotoxicity was determined by using the colorimetric MTT assay. For this, the cells (1×10^5 cells/well) were seeded into 96-well plates containing 100 µl of the growth medium, in the absence or presence of increasing concentrations of HK, at 37°C in 5% CO_2 for 24 and 48 hrs. MTT (50 µl/well, 5 mg/ml in PBS) was then added and incubated for 4 hrs at 37°C. To dissolve the dark blue crystals of formazan 100 µl of DMSO were added. Later, the absorbance was measured in a microplate reader (ELX800, BioTEK, USA). The experiments were performed in triplicate. While the cytotoxicity was determined by plotting, the number of affected cells vs drug concentration and inhibition of growth was expressed as a percentage of the untreated controls. Thus, the IC_{50} was determined as the concentration that inhibited cell growth by 50% [23].

Analysis of apoptotic cells by flow cytometry

In brief, the cells (3×10^5) were grown in 25 cm^2 flasks and exposed to different concentrations of HK for different time intervals (6, 12, or 24 hrs). The whole system was washed twice with ice-cold PBS and then re-suspended in 500 µl binding buffer. The cells were further incubated with Annexin V-FITC and Propidium Iodide for 15 min at room temperature, in the absence of any interference of light. The stained cells were analyzed by using flow cytometry, using FACS Calibur (BD Biosciences, Oxford, UK) and Cell Quest (BD Biosciences) software.

Estimation of HK effect on ROS production

In brief, the ROS production in cells was determined by utilizing 2,7- dichlorodihydro fluorescein diacetate (DCFDA; Sigma-Aldrich, Dorset, UK). For

this, 4×10^5/ml cells were cultured in a culture dish and treated with HK for 6, 12 or 24 hrs. Later, the cells were collected and DCFDA was added to the cell suspension at a finishing concentration of 10 μM. After incubation in the dark at 37 °C for 30 min the cells were centrifuged and the pellet was washed twice with ice-cold PBS. The pellet was then re-suspended in FACS buffer and the fluorescence was analyzed with FACS Calibur (BD Biosciences, Oxford, UK) and CellQuest (BD Biosciences) software. DCFDA fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. High ROS producing cells were quantified for the determination of the percentage of ROS.

Western blot analysis

Whole protein (50 μg/lane) from each sample was resolved in 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a PVDF membrane (Millipore, UK) and blotted with various antibodies. Non-specific binding was avoided by blocking the nitrocellulose membrane with 5% skimmed milk in TBS-T for 1 hr. The 5% skimmed milk in TBS-T was also used to dilute primary antibodies (SAPK/JNK, rabbit polyclonal, 1:1000, CST; Phospho-SAPK/JNK, rabbit polyclonal 1:1000, Cell Signaling techonology; Phospho-c-jun, rabbit polyclonal, 1:500, Bioworld Technology Co., Ltd., c-jun, rabbit polyclonal, 1:1000, Santa Cruz; P65, rabbit polyclonal, 1:1000, Santa Cruz; Nrf2, rabbit polyclonal, 1:1000) and HRP-conjugated monoclonal secondary antibodies (1:5000; Amersham Pharmacia Biotech, NJ). The membranes were incubated with the primary antibodies overnight at 4°C and in the secondary antibody for 1 hr at room temperature. The quantity of protein loaded was verified by staining the same membranes with anti-β actin antibody (1:2000, Sigma-Aldrich, Dorset, UK). The signals were detected on X-ray films, using an ECL Western blotting detection kit (Amersham, Pharmacia Biotech, Piscataway, NJ, USA).

Statistics

All the results were analyzed by Student's t-test and one-way analysis of variance (ANOVA), using the SPSS 13.0 software package. The statistical significance was set at $p<0.05$.

Results

Cytotoxic effect of HK in Raji and Molt4 cells

In the colorimetry-based MTT assay, the cytotoxicity of HK was determined at different time intervals in lymphoid malignant Raji and Molt4 cell lines. The treatement with HK exhibited prominent cytotoxicity against Raji cell lines, with IC_{50} of 0.092±0.021 μM as calculated from Figure 1A. Moreover, in Molt4 cell line, the introduction of HK caused significant cytotoxic activity with considerable potency showing IC_{50} 0.521±0.115 μM against the Molt cells as seen in Figure 1B.

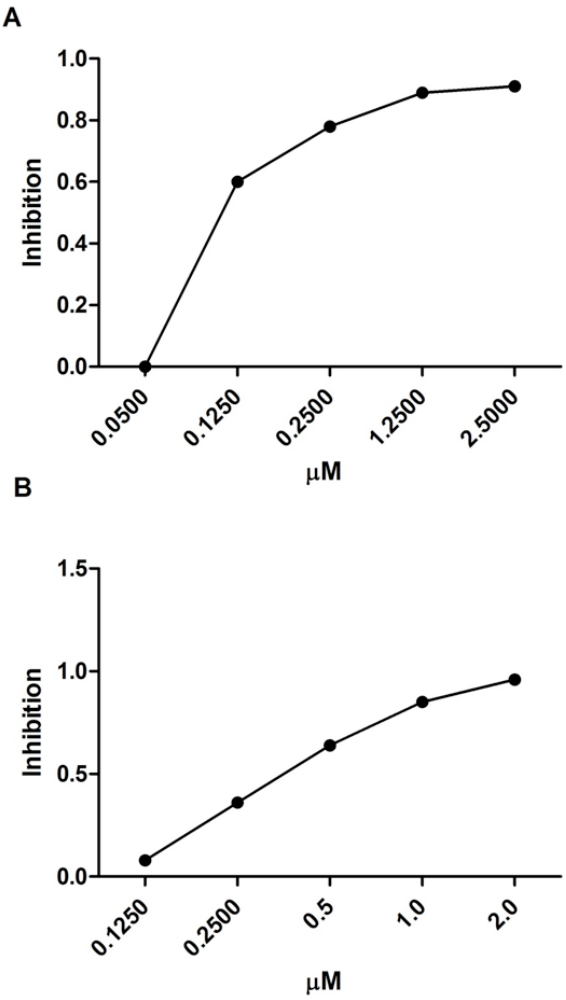

Figure 1. The effect of HK in Raji and Molt4 cells for the estimation of cytotoxicity. (**A**) In Raji cells at 72 hrs. (**B**) In Molt-4 cells at 24 hrs. The results show the dose-dependent effect of HK on the viability of Raji and Molt-4 cells.

In addition, the effect of HK on apoptosis of these cell lines was also determined and the results are presented in Figure 2. The study was conducted by using Annexin V-FITC/PI staining method at the 3.5 μM concentration of HK. This concentration has served as IC_{50} of HK against Raji cells at 24 hrs. It was found that the rate of apoptosis increased significally, mainly dependent on the duration of exposure (Figure 2A). As the exposure increased, the percentage of apoptosis also increased. In Molt4 cells, HK caused a significant induction of apoptosis in a concentration-dependent manner (Figure 2B).

Effect of HK on ROS in Raji cell line

Treatment of HK caused significant increase in the activity of ROS in Raji cells at various time intervals (Figure 3). It is noteworthy to mention that, as the time increased from 6 to 24 hrs, the level of ROS elevated significantly in comparison to control, a mechanism of action which could be used against lymphoid malignant cells. Thus, it could be suggested that, with increasing time of exposure to HK a direct increase on the activity of ROS was shown, while the highest ROS activity was detected at 24 hrs.

Effect of HK on the expression of Nrf2, NF-κB, and JNK pathways in Raji cells

As depicted in Figure 4A, the activity of ROS at 12 hrs was considerably increased, while with further increase of the exposure time, i.e., 24 hrs, the level of Nrf2 was significantly reduced. Therefore, the level of phosphorylated JNK and c-jun proteins was significantly elevated in Raji cells (Figure 4B). Moreover, as shown in Figure 4C, the level of NF-κB was significantly reduced, which can be easily understood by the decresed level of p-65.

Mechanistic analysis of the effect of HK on Nrf2, NF-κB, JNK pathways in Raji cells

As shown in Figure 5, in the presence of ROS inhibitor NAC (10mM) for 24 hrs, the JNK pathway was markedly activated, together with the inhibition of NF-κB activity and the reduced level of Nrf2 expression.

Effect of HK on the growth of xenografts derived from Raji cells

To further confirm the in vitro results by in vivo activity, a lymphoma mice model was used. The control group of mice received normal saline, while treated mice received the appropriate dose of HK as discussed above for 10 days. The survival rate confirmed that no mortality of the HK-treated mice occurred until the end of the experiment. As shown in Figure 6, the mean tumor volume of mice in the HK treated group was significantly smaller than in the control group ($p<0.05$), indicating that HK inhibits the proliferation of Raji cells in vivo.

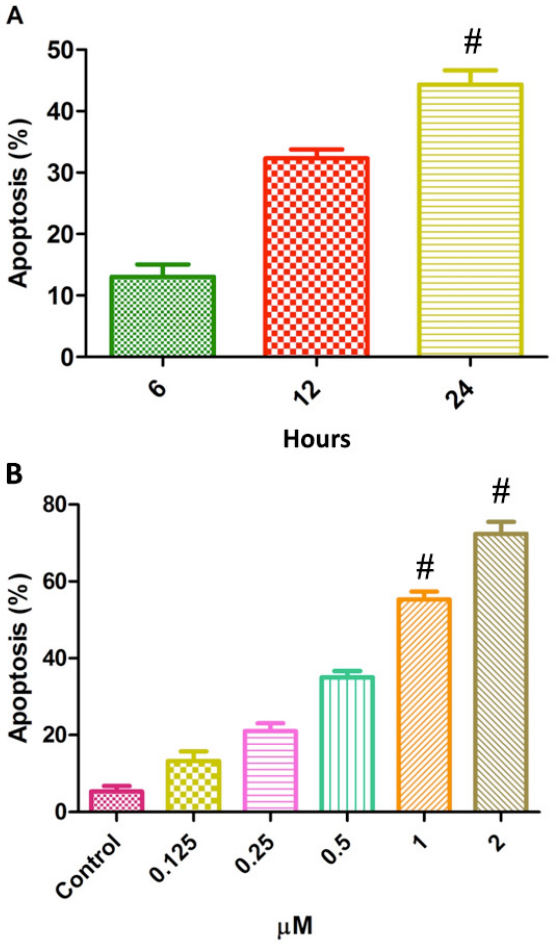

Figure 2. Induction of apoptosis under the influence of HK in lymphoid malignant Raji and Molt-4 cells, determined by flow cytometry. **A**: The apoptotic Raji cells were treated with HK at different time points (6, 12 and 24 hrs; n=3) and apoptosis was increased with increasing time of exposure. **B**: Molt-4 cells were treated with various concentrations of HK (0.125-2 μM; n=3) and the results show that the treatment exerted its effect in a concentration-dependent manner (#$p<0.05$).

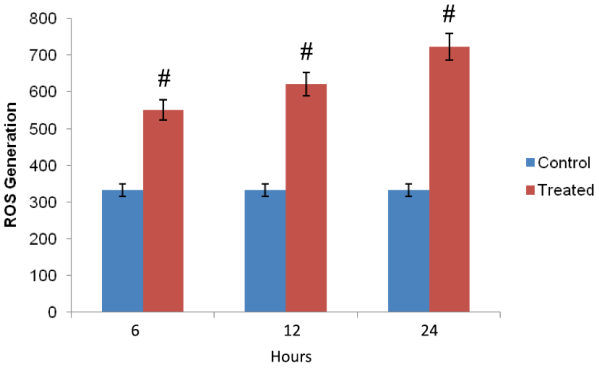

Figure 3. Effect of HK treatment on ROS generation at different time intervals (6, 12 and 24 hrs). The results confirmed that the ROS levels were considerably altered in the presence of HK (#$p<0.05$).

Discussion

This study represents the first report on the cytotoxic effect of HK in lymphoid malignant cells with detailed mechanistic analysis. It has been

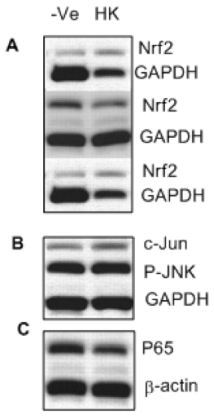

Figure 4. The effect of HK (3.5μM) on the expression levels and phosphorylation status of proteins in JNK (**B** after 6-hr treatment), as well as the expression in levels of Nrf2 (**A** after 24-hr treatment) and NF-κB pathways (**C** after 24-hr treatment) were detected by Western blot. The results indicated that the level of Nrf2 was greatly reduced on extending the treatment time while in the case of JNK its level has been considerably altered. Moreover, the expression of p-65 could be easily correlated with the level of NF-κB, suggesting it might also be affected in the presence of HK. -Ve: control.

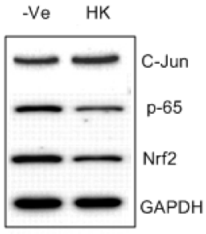

Figure 5. The effect of HK (3.5μM) on the expressions of c-jun, p65 and Nrf2 was detected by Western blot. The results showed that the level of these biomediators has been decreased significantly in the presence of HK. -Ve: control.

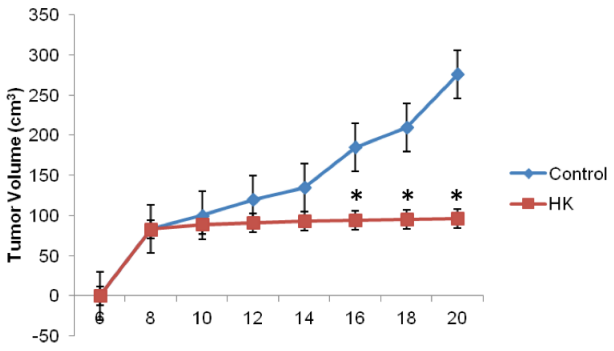

Figure 6. Tumor volume growth curve and effect of HK. The Figure indicates that as the treatment started the tumor volume also reduced accordingly (HK vs control, *p<0.05).

found that HK exerts significant inhibition of the mouse xenograft, along with induction of apoptotic markers.

The transcription factor Nrf2 was primarily considered as a key supervisor of intracellular antioxidants and phase-II detoxification enzymes and its level has been intensely elevated upon oxidative and redox stress–activated factors [24]. This activation will lead to decreased ROS. Various studies have indicated that Nrf2 is able to exert a protective effect on normal cells, against harmful stimuli and proved to be beneficial for adverse symptoms of cancer including trauma, inflammation and hemorrhage. Thereby, agents that are able to promote the level of Nrf2-inducing agents have been shown to result in decreased carcinogenesis in animal models and altered carcinogens' metabolism in humans as well [25]. In concordance with this, our results indicate that HK treatment causes a significant induction of ROS, which in turn could lead to the activation of Nrf2 in Raji cells as proved by Western blot analysis.

JNKs belong to the family of MAPK, known to play a major role in the regulation of multiple cellular processes. They control the development, differentiation, proliferation and apoptosis, in response to extracellular signals, metabolic status and environmental factors [26]. Various studies indicated that JNKs served as important mediators of stress and inflammation and have been connected with apoptotic responses to DNA damage and cytotoxic agents. Similarly, JNKs have been anticipated to play a role equally as tumor suppressors and mediators of cancer cells' propagation, existence and resistance to chemotherapy. Thus, the results of this study suggest that HK leads to a significant activation of JNKs.

Moreover, several authors have confirmed the role of NF-κB in the proliferation of cells by promoting various growth factors [27]. It leads to increased angiogenesis and metastasis through upregulation of various pro-inflammatory chemokines, including vascular endothelial growth factor (VEGF), IL-8 and metalloproteinases (MMPs) [28]. Therefore, when considering cancer therapy and drugs that suppress the NF-κB pathway, this might be crucial for cancer chemotherapy. The results of this study showed that, in Western Blot analysis, HK leads to significant attenutation of p-65, which serves as a key parameter for the generation of NF-κB in lymphoma Raji cells.

As a concluding remark, the present study suggests that HK causes induction of apoptosis

in lymphoid malignant cells, both in vitro and in vivo, whereas the generation of ROS might serve as an underlying mechanism for induced apoptosis. Moreover, ROS-related activation of the JNK pathway, as well as inhibition of NF-κB and Nrf2, might also contribute to the induction of apoptosis.

Conflict of interests

The authors declare no confict of interests.

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