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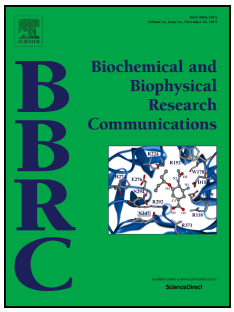

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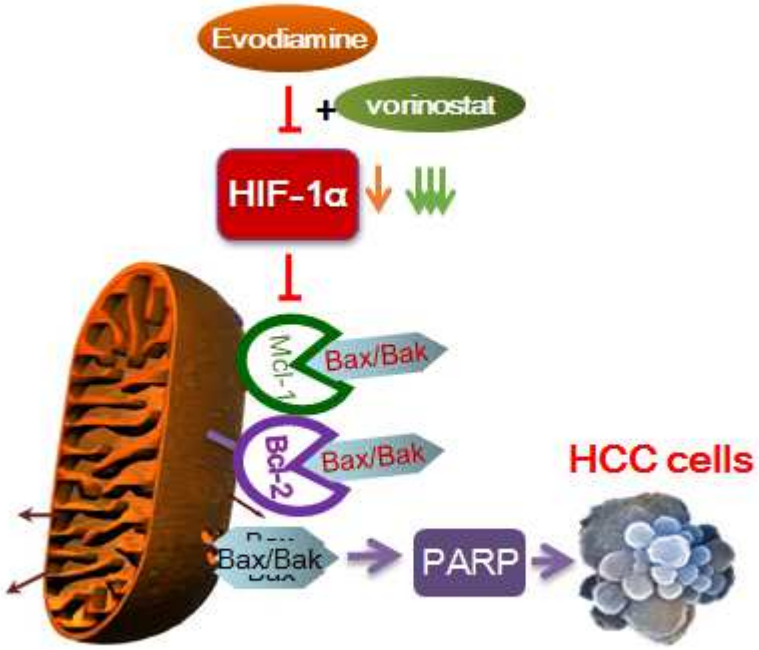

Evodiamine induces apoptosis and promotes hepatocellular carcinoma cell death induced by vorinostat via downregulating HIF-1α under hypoxia

Yang-ling Li[1], Ning-yu Zhang[2], Xiu Hu[2,3], Jia-ling Chen[2], Ming-jun Rao[4], Lin-wen Wu[2,3], Qing-yu Li[1], Bo Zhang[5], Wei Yan[1*], Chong Zhang[2*]

[1]Department of Clinical Pharmacology, Hangzhou First People's Hospital, Nanjing Medical University, Hangzhou, Zhejiang, China, 310006

[2]School of Medicine, Zhejiang University City College, Hangzhou, Zhejiang, China, 310015

[3]College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang, China, 310058

[4]Institute of Pharmacology, College of Pharmaceutical Sciences, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China, 311402

[5]Hangzhou Translational Medicine Research Center, Hangzhou First People's Hospital, Nanjing Medical University, Hangzhou, Zhejiang, China, 310006

[*]**Correspondence:** Chong Zhang and Wei Yan; Address: School of Medicine, Zhejiang University City College, No.51 Huzhou Street, Hangzhou, Zhejiang, China, 310015 and Department of Clinical Pharmacology, Hangzhou First People's Hospital, Nanjing Medical University, No.261 Huansha Road, Hangzhou, Zhejiang, China, 310006; E-mail: zhangchong@zucc.edu.cn, yanwei_115@163.com; Phone: 86-0571-88016565, Fax: 86-0571-88018442.

Abbreviations: hepatocellular carcinoma, HCC; Hypoxia-inducible factor 1α, HIF-1α; heat shock protein 90, Hsp90; hepatitis B virus, HBV; hepatitis C virus, HCV.

Abstract

Hypoxia promotes HCC progression and therapy resistance, and there is no systemic treatment for HCC patients after sorafenib resistance. Thus, it is urgent to develop potential therapeutic regimens for HCC patients by targeting hypoxia signaling. In this study, we showed that evodiamine might be a potential therapeutic medicine for HCC by suppressing HIF-1α. In addition, evodiamine could sensitize the anti-HCC effect of vorinostat in HCC cells under hypoxia. Furthermore, evodiamine plus vorinostat accelerated the degradation of HIF-1α in HCC cells under hypoxia. In general, evodiamine might be a potential therapeutic candidate for HCC patients, and evodiamine combining with vorinostat might be an attractive chemotherapy strategy for HCC treatment.

Keywords: Evodiamine; vorinostat; combination; hypoxia; HIF-1α; apoptosis

Introduction

Liver cancer is the second most common cause of death from cancer, and hepatocellular carcinoma (HCC) accounts for over 90% of all primary liver cancers occurring worldwide [1]. Sorafenib, the only approved targeted drug for HCC patients, is a multi-targeted tyrosine kinase inhibitor with activity against Raf kinase and several receptor tyrosine kinases [2]. In addition, sorafenib is an effective inhibitor of the HIF-1α/VEGFA pathway [3]. However, sorafenib treatment has limited anti-HCC efficacy due to cancer progression from the rapid development of acquired resistance [4]. Moreover, there is no systemic treatment for HCC patients whose disease progresses during sorafenib treatment [5].

HCC develops the ability of proliferation, metastasis, angiogenesis, radio-resistance and chemo-resistance under hypoxia. Furthermore, hypoxia also suppresses differentiation and apoptosis of HCC cells and consequently leads to tumor malignancy [6]. Hypoxia-inducible factor 1α (HIF-1α), activated under hypoxic conditions, is overexpressed in HCC cells and leads to angiogenesis and poor prognosis [7].

Vorinostat (suberoylanilide hydroxamic acid, SAHA), a histone deacetylase inhibitor, is US FDA-approved for the treatment of refractory cutaneous T-cell lymphoma [8]. Additionally, vorinostat is also extensively studied in hematological malignancies and is considered as a potent candidate for treating HCC [9,10]. Furthermore, vorinostat can enhance the anti-HCC activity of chemotherapeutic agents including sorafenib, 5-fluorouracil, TRAIL, etc. [11,12,13]. Vorinostat prevents the deacetylation of

histones, results in a loosened chromatin structure, promotes the acetylation of histones and numerous transcription factors [14]. Vorinostat increases the acetylation of heat shock protein 90 (Hsp90), represses the interaction between acetyl-Hsp90 and HIF-1α, and finally decreases HIF-1α transcriptional activity in HCC cells [15]. Evodiamine, an alkaloid extracted from Euodia rutaecarpa (Juss.) Benth, has the potential to be a therapeutic medicine for treating HCC [16]. The anti-cancer effect of evodiamine is associated with HIF-1α downregulation [17]. In this study, we showed that combining two anti-HIF-1α drugs (vorinostat plus evodiamine) could synergistically induce apoptosis and inhibit tumor proliferation in HCC cells under hypoxia. In addition, evodiamine plus vorinostat accelerated the degradation of HIF-1α in HCC cells under hypoxia. The potential to inhibit the expression of HIF-1α by combining evodiamine with vorinostat makes it an attractive chemotherapy strategy.

Materials and Methods

Materials

Evodiamine (catalog number: S2382) and vorinostat (catalog number: S1047) were purchased from Selleck Chemicals.

Cell culture

The human HCC cell lines (HepG2, Huh-7 and Hep3B) were obtained from Chinese Academy of Sciences (Shanghai Institutes for Biological Sciences, China). All the cells were maintained at 37 °C in 5 % (v/v) CO_2 using culture medium recommended by the supplier. HepG2 and Huh-7 cells were cultured in DMEM + 10% fetal bovine

serum (FBS). Hep3B cells were grown in MEM + 10% FBS. The hypoxic condition was achieved by placing HCC cells in a sealed hypoxia chamber equilibrated with a triple gas mixture of 1% O_2, 5% CO_2 and 94% N_2.

Cell viability assay

Cells were seeded on 96-well plates and incubated with indicated agents for 72 h. Cell viability was measured using sulforhodamine B (SRB) assay as described previously [18].

Clonogenic assays

Cells treated with evodiamine, vorinostat or both were plated in 35 mm dishes and incubated at 37°C for 14 days in hypoxic conditions. During the experiment, the compound-containing medium was replaced every 2 to 3 days. Finally, the colonies were scored and photographed.

Analysis of apoptosis by propidium iodide (PI) staining

Analysis of the sub-G1 phase after PI staining was used to assess apoptosis. Apoptosis was measured using PI staining as described previously [19].

DAPI staining

The apoptosis induced by evodiamine was detected by fluorescence microscopic analysis of cells with condensed and fragmented DNA assessed by DAPI staining [20].

Western blotting analysis

Whole-cell extracts for SDS–PAGE were prepared and western blotting analysis was performed as previously described [18]. The antibodies used for western blotting were

obtained from different resources: anti-HIF-1α antibody (catalog number: BD610959) was purchased from BD Transduction Laboratories; anti-Bcl-2 antibody (catalog number: sc-7382), anti-PARP antibody (catalog number: sc-7150), anti-procaspase-3 antibody (catalog number: sc-7148), anti-phospho-AKT(Ser-473) antibody (catalog number: sc-7985), anti-Mcl-1 antibody (catalog number: sc-819) and anti-GAPDH antibody (catalog number: sc-25778) were obtained from Santa Cruz Biotechnology.

Statistical analyses

Data were presented as mean ± SD of at least three independent experiments. Student's t-tests were used to determine the statistical significance of differences between the experiment conditions (*$p<0.05$; ** $p<0.01$). Combination index (CI) values were calculated using Calcusyn (Biosoft, Great Shelford, Cambridge, UK) and the mean CI values were chosen for presentation. A CI value < 0.9 indicated synergism; 0.9 to 1.10, additive; and >1.10, antagonism.

Results

Evodiamine showed anti-proliferation effect and induced apoptosis in HCC cells under hypoxia

Although evodiamine can inhibit cell viability and induce apoptosis in HCC cells under normoxia, whether evodiamine can exert anti-HCC activity under hypoxia needs further investigation [16,21]. Thus, we initiated the investigation by analyzing the cytotoxicity of evodiamine under hypoxia in three HCC cell lines. As shown in Figure 1A, evodiamine inhibited the proliferation of HCC cells in a dose-dependent manner under hypoxia. Then, we detected whether evodiamine could induce apoptosis in

HCC cells under hypoxia using DAPI staining. Figure 1B results showed that evodiamine could induce apoptosis in a dose-dependent manner under hypoxia. In addition, evodiamine could cause the increase of cleaved PARP under hypoxia, indicating that evodiamine could induce apoptosis in HCC cells under hypoxia (Figure 1B and C). Taken together, our data suggested that evodiamine could exert anti-HCC activity under hypoxia *in vitro*.

Evodiamine sensitized the anti-proliferation effect of vorinostat in HCC cells under hypoxia

Vorinostat is a potent candidate for treating HCC and effective against hypoxic cells. Thus, we next assessed whether evodiamine could sensitize the anti-proliferation effect of vorinostat in HCC cells under hypoxia. As shown in Figure 2A, our results showed that evodiamine plus vorinostat significantly inhibited the proliferation of HCC cells under hypoxia after treatment for 72 h, as compared to single agent treatment. In the meanwhile, CI value analysis revealed that evodiamine could synergize with vorinostat under hypoxia in HCC cell lines (CI<0.9). Furthermore, in colony formation assay, our data indicated that evodiamine could enhance the inhibition of HCC colony formation by vorinostat both in HepG2 and Huh-7 cells under hypoxia. Thus, we showed that evodiamine sensitized the anti-proliferation effect of vorinostat in HCC cells under hypoxia.

Evodiamine enhanced the apoptosis induced by vorinostat in HCC cells under hypoxia

Besides its synergistic anti-proliferative effect, evodiamine plus vorinostat significantly

induced apoptosis in HCC cells under hypoxia compared with either single agent (Figure 3A and B). Furthermore, western blotting detected cleaved-PARP and cleaved-caspase-3 confirmed that evodiamine plus vorinostat promoted HCC cell apoptosis under hypoxic condition (Figure 3C). As Bcl-2 family members are key regulators of apoptosis, we detected whether evodiamine plus vorinostat could induce apoptosis via regulating Bcl-2 family members in HCC cells under hypoxia. Figure 3C showed that evodiamine plus vorinostat could inhibit two anti-apoptotic Bcl-2 family members (Mcl-1 and Bcl-2). Thus, our findings suggested that evodiamine plus vorinostat promoted HCC cells apoptosis under hypoxia.

Evodiamine downregulated HIF-1α and evodiamine plus vorinostat accelerated the degradation of HIF-1α in HCC cells exposed to hypoxia

HIF-1α regulates multiple genes that contribute to tumor cell survival, hypoxia dramatically promoted HIF-1α protein expression. The level of HIF-1α protein declined in HCC cells after treated with evodiamine (Figure 4A). As shown in Figure 4B, the combination of evodiamine and vorinostat accelerated the degradation of HIF-1α in HCC cells under hypoxia. The potential to inhibit the expression of HIF-1α by combining evodiamine with vorinostat makes this an attractive chemotherapy strategy.

Discussion

Hypoxia is a common feature of HCC and promotes HCC proliferation, metabolism, angiogenesis, invasion, metastasis and therapy resistance [22]. Several studies reported that evodiamine exerts anti-proliferation induction of apoptosis in HCC cells

under normoxia [16,21,23]. However, the anti-HCC activity of evodiamine under hypoxia need to be further investigated. In this study, we firstly reported that evodiamine could inhibit cell proliferation and induce apoptosis in HCC cells under hypoxia. In addition, we also found that evodiamine could inhibit HIF-1α induced by hypoxia in HCC cells. Furthermore, HCC is mainly associated with chronic hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infections [24,25]. HBV and HCV infections also promote chronic inflammation, hepatic fibrosis or cirrhosis [26]. Evodiamine can repress hypoxia-induced inflammatory response and ameliorate liver fibrosis via TGF-β1/Smad pathway [27,28]. Collectively, previous reports and our study showed that evodiamine could target hypoxia signal, hepatic fibrosis, and inflammation response in HCC treatment. Thus, evodiamine might be a potential therapeutic medicine for HCC.

Vorinostat suppresses hypoxia signaling by decreasing nuclear/cytoplasmic HIF-1α expression [15]. In this study, we hypothesized two anti-HIF-1α drugs (vorinostat plus evodiamine) combination regimen might show synergistically anti-HCC activity under hypoxia. Indeed, our data showed that evodiamine plus vorinostat could significantly induce apoptosis and inhibit the proliferation of HCC cells under hypoxia. Furthermore, evodiamine plus vorinostat accelerated the degradation of HIF-1α in HCC cells under hypoxia. Activation of PI3K/AKT/mTOR pathway can upregulate the HIF-1α protein translation [29]. We found that evodiamine plus vorinostat suppressed AKT in HCC cells under hypoxia, indicating that PI3K/AKT/mTOR pathway was involved in HIF-1α inhibition induced by combination treatment. Thus, our results demonstrated that

evodiamine plus vorinostat might be promising as a novel therapeutic strategy for HCC by targeting HIF-1α. Loss of HIF-1α in endothelial cells disrupts hypoxia-induced VEGF expression which is essential for tumor metastasis [30]. Thus, evodiamine plus vorinostat might be an efficient combination regimen for HCC patients with metastatic cancer. Furthermore, pain in liver cancer patients is correlated with the high levels of HIF-1 and VEGF [31]. Our observations support the concept that further investigation of pain release in HCC patients treated with evodiamine plus vorinostat should be considered.

In general, our results provided some reasonable and basic research evidence for supporting a hypothesis that evodiamine might be a potential therapeutic candidate for HCC patients, and our results provided clear evidence that evodiamine could sensitize the anti-HCC effect of vorinostat via accelerating the degradation of HIF-1α under hypoxia.

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Conflicts of interest: The authors declare no potential conflicts of interest.

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Figure legends

Figure 1: Evodiamine inhibited cancer cells proliferation and induced apoptosis of HCC cells under hypoxia. (A) The HCC cells were treated with evodiamine for 72 h, and SRB was used to detect the proliferation of HCC cells under hypoxia. (B) HepG2 cells were incubated with evodiamine for 48 h, and DAPI staining was used to visualize the condensed and fragmented DNA in hepG2 cells under hypoxia. (C) HCC cells were incubated with evodiamine for 48 h, and the expression of PARP was detected under hypoxia.

Figure 2: Evodiamine plus vorinostat synergistically inhibited the anti-proliferation of HCC cells under hypoxia. (A) HCC cells were treated with evodiamine and/or vorinostat at indicated concentrations for 72h under hypoxia, and then the proliferation of HCC cells was measured by SRB. (B) HepG2 cells were incubated with evodiamine and/or vorinostat at indicated concentrations for 14 days under hypoxia, and dishes were then stained by crystal violet and colony numbers were counted. (C) Evodiamine plus vorinostat synergistically inhibited HepG2 cells colony formation under hypoxia. (D) Evodiamine plus vorinostat synergistically inhibited Huh-7 cells colony formation under hypoxia.

Figure 3: Evodiamine plus vorinostat synergistically induced apoptosis of HCC cells under hypoxia. Huh-7 and HepG2 cells were treated with evodiamine and/or vorinostat for 48 h under hypoxia. (A and B) PI staining were used to evaluate the apoptosis. (C) Western blotting was used to detect the expressions of Bcl-2, Mcl-1, caspase-3 and PARP.

Figure 4: Evodiamine downregulated HIF-1α and evodiamine plus vorinostat

accelerated the degradation of HIF-1α in HCC cells under hypoxia. (A) Huh-7 and HepG2 cells were treated with evodiamine at the indicated concentrations for 48 h under hypoxia. The expression of HIF-1α was evaluated by western blotting. (B) Huh-7 and HepG2 cells were treated with evodiamine and/or vorinostat for 48 h under hypoxia. The expressions of HIF-1α and p-AKT (ser-473) were evaluated by western blotting.

Figure 1

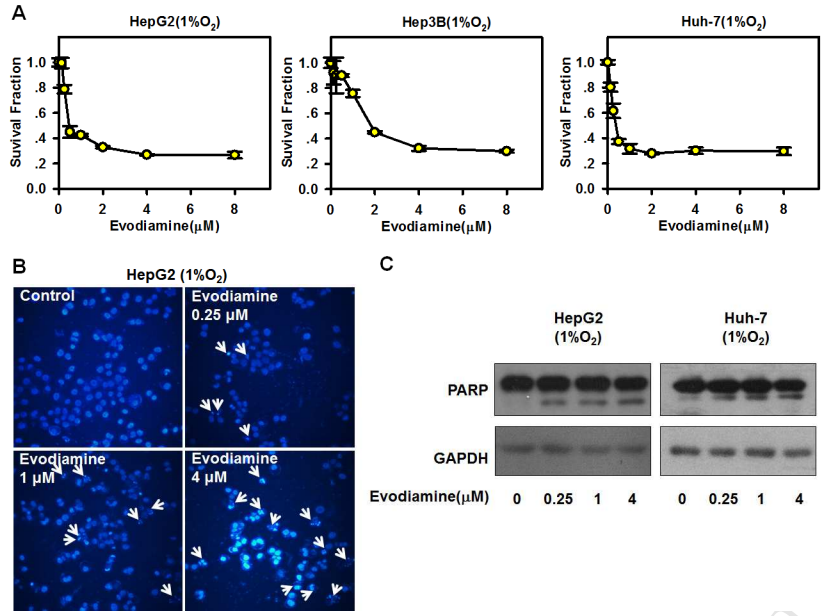


Figure 2

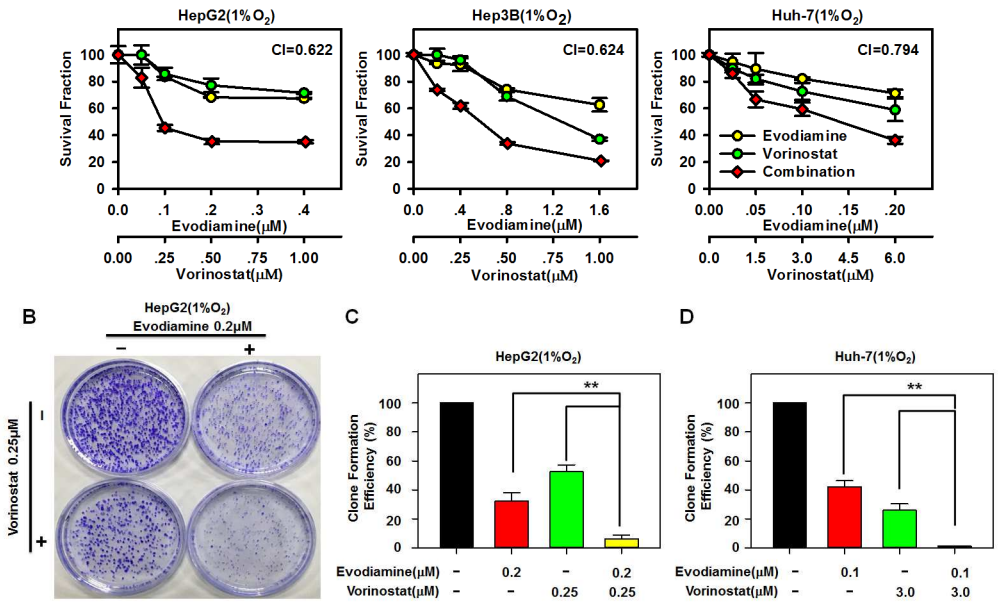


Figure 3

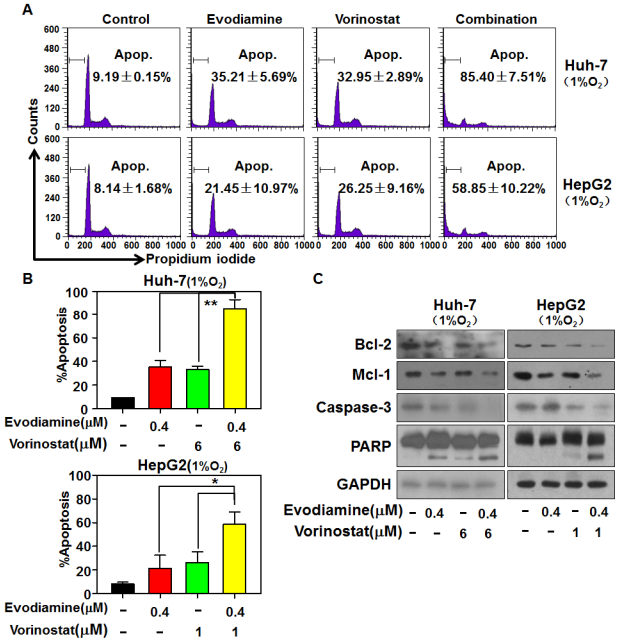
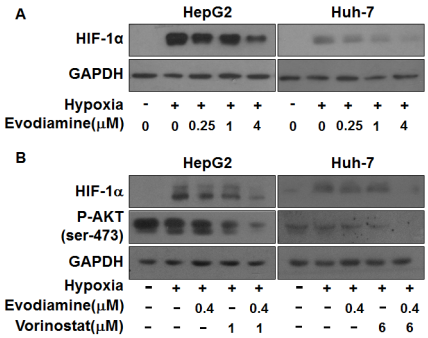


Figure 4



Highlights:

- evodiamine might be a potential therapeutic medicine for HCC by suppressing HIF-1α
- evodiamine could sensitize the anti-HCC effect of vorinostat under hypoxia
- evodiamine + vorinostat accelerated the degradation of HIF-1α