Biomedicine & Pharmacotherapy 97 (2018) 656–666

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

The evaluation of potent antitumor activities of shikonin coumarin-carboxylic acid, PMMB232 through HIF-1α-mediated apoptosis

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ARTICLE INFO

Keywords:
Apoptosis
Coumarin
HIF-1α
Microenvironment
Shikonin

ABSTRACT

In current study, a series of shikonin derivatives were synthesized and its anticancer activity was evaluated. As a result, **PMMB232** showed the best antiproliferation activity with an IC_{50} value of 3.25 ± 0.35 μM. Further, treatment of HeLa cells with a variety of concentrations of target drug resulted in dose-dependent event marked by apoptosis. What's more, the mitochondrial potential (Δym) analysis was consistent with the apoptosis result. In addition, PARP was involved in the progress of apoptosis revealed by western blotting. To identify the detailed role and mechanism of **PMMB232** in the progression of human cervical cancer, we detected the expression of HIF-1α and E-cadherin in HeLa cells. Results showed that expression of HIF-1α was downregulated, while E-cadherin protein was upregulated. Meanwhile, glycolysis related protein PDK1 was decreased in HeLa cells. Conversely, the expression of PDH-E1α was upregulated. Docking simulation results further indicate that **PMMB232** could be well bound to HIF-1α. Taken together, our data indicate that compound **PMMB232** could be developed as a potential anticancer agent.

1. Introduction

Over the past decade, the role of tumor microenvironment, as a pivotal factor influencing tumor resistance, has drawn the attention of researchers. Many components of tumor microenvironment have been identified, which are able to affect tumor sensitivity or resistance to chemotherapy-mediated apoptosis and play a major role in tumor survival and progression [1]. As well, tumors being surrounded by cellular environment is able to form hypoxic niches, which is associated with aberrantly accelerated proliferation of cancer cells. As is known, hypoxia is capable of stabilizing hypoxia-inducible factors (HIFs) which are promptly degraded by an E3-ubiquitin ligase, pVHL, under normoxic conditions [1–5]. As a result, adapting hypoxia promotes various key aspects of cancer progression, causing patients death [6–11]. HIF-1, a heterodimeric protein composed of a HIF-1β subunit in constitutive expression and an O_2-regulated HIF-1α subunit, is a major activated transcriptional factor in response to hypoxia [7,12,13]. In addition, its activity is primarily determined by hypoxia with inducing the stabilization of HIF-1α [11]. It has been reported that HIF-1α is overexpressed in a broad range of human cancer types, and increased levels of HIF-1α activity are often associated with increased tumor aggressiveness and therapeutic resistance [7,10,12]. Given the crucial role of HIF-1α in the association with resistance to drugs in a range of tumors, inhibiting the expression of HIF-1α could be more accurate or more reliable to predict the efficacy of chemotherapy in tumors.

Currently, evidence suggest that the activation of HIF-1α signalling pathway induces a vast array of gene products to control energy metabolism, glycolysis, invasion, angiogenesis, apoptosis and cell cycle [14]. As an example, E-cadherin, significant in metastatic colonization, can functionally impact on the changes of metastasis phenotype, which is related to HIF-1α-dependent gene profile involved in angiogenesis and anoikis resistance in breast carcinoma [15]. Rathinasamy Baskaran et al. showed that HIF-1α was required for hypoxia-mediated apoptosis by inhibiting the expression of PARP and up-regulating expression of caspase-3 and caspase-9 [16]. Other studies suggest that HIF-1α is associated with the regulation of p53 in apoptotic neurons [5,17,18].

Abbreviations: HIF-1α, hypoxia-inducible factor alpha 1; DMAP, 4 dimethyamino- pyridine; DCC, N, N' dicyclohexylcarbodiimide; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4 5-dimethyl-2-thiazolyl)-2 5-diphenyl-2-H-tetrazolium bromide; IC_{50}, Half of maximal inhibitory concentration; SAR, structure–activity relationship; PDB, protein data bank; Δψm, mitochondrial membrane potential; JC-1, 1H-Benzimidazolium 5 6-dichloro-2-[3-(5 6-dichloro-1 3-diethyl-1 3-dihydro-2H-benzimidazol-2-ylidene)-1-propenyl]-1 3-diethyl- iodide; PDK1, 3-phosphoinositide-dependent protein kinase-1; PDH-E1α, pyruvate dehydrogenase (lipoamide) alpha 1; PMSF, phenylmethanesulfonylfluoride; EDTA, ethylene diamine tetra-acetic acid; EMT, Epithelial-to-mesenchymal transition; DCFH-DA, 2′,7-dichlorofluoresceindiacetate; ROS, reactive oxygen species

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http://dx.doi.org/10.1016/j.biopha.2017.10.159
Received 28 June 2017; Received in revised form 30 September 2017; Accepted 28 October 2017

Energy production is linked intimately with almost all cellular events. Thus, targeting at HIF-1α to modulate hepatic glucose metabolism may provide a promising therapy against energy metabolism with the relation to diseases, such as diabetes, postsurgical liver dysfunction and even cancers [10,19,20]. Shikonin and its derivatives are active naphthoquinone compounds extracted from the root of a Chinese herbal medicine *Lithospermum erythrorhizon* [21–24]. Currently, numerous evidences suggest that shikonin induces apoptosis process by p53 directed mitochondrial pathway, and suppress the expression of the family members of anti-apoptotic Bcl-2 (B-cell lymphoma 2). Meanwhile, it also increases the activities of caspases, inactivates AKT pathway, induces cancer cell apoptosis [25–28]. These results, together with our earlier findings, indicate that shikonin may have high efficacy for preventing and treating cervical cancer in the future. However, there are not many investigations on its precise anticancer effect and mechanism of inducing apoptosis in cervical cancer cells by reducing HIF-1α. It will be important in the future to ask whether the effort of shikonin is efficient to inhibit hypoxia-inducible factors and break the functions of mitochondria. Ultimately, disrupting cellular energy metabolism leads to cells apoptosis. Whereas, owing to poor solubility and gigantic cytotoxic effects on non-cancer cells, it is hindered to develop as a new clinical anticancer agent.

More importantly, a lot of coumarin compounds as medicinal candidates with strong pharmacological activity, low toxicity and side-effect, little drug resistance, high bioavailability, broad spectrum and good curative effects were being actively developed [29–32]. Recent studies also highlighted that newly synthesized coumarin substituted derivatives showed good water- solubility [33–35]. This could be attributed to the formation of hydrogen bonds between coumarin groups and water molecules [33]. In addition, curcumin was considered to interrupt HIF-1α expression at protein levels, promote HIF-1α degradation and achieve anticancer agent potential by targeting at HIF-1α [36–38]. Based on the aforementioned results, we proposed that coumarin into shikonin by introducing aminobenzoic acids as bridges may improve its water solubility and cervical tumor targeting.

2. Materials and methods

2.1. Chemistry

All chemicals and reagents used in current study were analytical grade. 4-hydroxycoumarin was purchased from J & K SCIENTIFIC LTD (Shanghai, China). Shikonin, doxorubicin and all acids were purchased from Sigma Aldrich (St. Louis, MO). All the ^{1}H NMR spectra were recorded on a Bruker DPX 300 Spectrometer in $CDCl_3$ and chemical shifts (δ) were reported as parts per million (ppm). ESI–MS spectra were recorded a Mariner System 5304 Mass spectrometer. Melting points were determined on a XT4 MP apparatus (Taike Corp, Beijing, China). Thin layer chromatography (TLC) was performed on silica gel plates (Silica Gel 60 GF254) and visualized in UV light (254 nm). Optical rotations were measured with a Rudolph Research Analytical II automatic polarimeter, using a sodium lamp (589 nm). The general procedure for preparation of compounds **A229-A240**, **B229-B240** and **PMMB229-PMMB240** were appended **in the Supplementary data (S1.1)**.

2.2. Biology

In our study, we used the following materials: Annexin V-FITC cell apoptosis assay kit (#BA11100) was purchased from BIO-BOX (Nanjing, China). BCA protein assay kit (#23227) was available from Pierce (Rockford, IL, USA). PVDF membranes were recruited from Biosharp (Hefei, China). Anti-HIF-1α (#WL01607) and anti-E-Cadherin (#WL01482) were purchased from Wanleibio. (Shenyang, China) Anti-PDH-E1α Antibody (D-6) (#sc-377092), anti-p-PDH-E1α (#ab177461), anti-PARP (#G3014) and cleaved PARP (#I1013) antibody were purchased from Santa Cruz Biotechnology, inc. Anti-PDK1 (#E1A6018-1), GAPDH antibody (#E1A7021) and the secondary antibody (anti-mouse or anti-rabbit IgG) (#E1WP319 or #E1WP318) were purchased from Enogene (Nanjing, China). ECL Kit (#34077) was purchased from Thermo Scientific (USA). 3-(4, 5- Dimethylthiazol-2-yl) −2, 5-diphenyltetrazolium (MTT) were from Beyotime Institute of Biotechnology (Haimen, China). Fibronectin (#F1056) and laminin (#L2020) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2.1. Cell lines and culture conditions

Human cervical cell lines (HeLa), human lung adenocarcinoma epithelial cell line (A549), human breast cancer cell line (MCF-7, MDA-MB-231), human embryonic kidney cells (293T) and L0-2, human normal liver cell lines, were obtained from American Type Culture Collection (ATCC; Man-assas, USA). All cells were maintained in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, China), 100 U/mL penicillin and 100 μg mL^{-1} streptomycin (Sigma, Brazil) at 37 °C and 5% CO_2 at the beginning of each experiment.

2.2.2. MTT assay for cell viability

The cell viability of cancer cells was determined by MTT assay. Briefly, cells were seeded in 96-well plates at a density of 2 × 10^4 cells/well and then allowed to adhere for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. After that, cells were treated with various concentrations of test compounds (0.1, 1, 10 and 100 μM) with shikonin as positive reference. Following a 24 h incubation, a volume of 20 μL of PBS containing 4 mg mL^{-1} of MTT was added to each well. Besides, plates were incubated for a further 4 h, before they were centrifuged at 1500 rpm at 4 °C for 10 min, followed by the removal of the supernatant. Then, DMSO (150 μL) was added to each well for coloration, and the plates were subsequently shaken vigorously to ensure complete solubilization for 10 min at room temperature. The light absorption (OD, optical densities) were recorded on a BioTek ELx800 ELISA reader at a test wavelength of 570 nm and a reference wavelength of 650 nm. In all experiments, three replicate wells were used only for each drug concentration, while each assay was performed for at least three times. The effect of **PMMB229-240** on tumor cell viability was expressed by IC_{50} of each cell line and the results are presented in Table 2.

2.2.3. Cell apoptosis analysis

Cells were plated in 6-well plates (2 × 10^5 cells per well) and incubated at 37 °C for 24 h. Fresh medium containing a variety of concentrations of **PMMB232** were then added to culture dishes. Following 24 h incubation, the untreated cells (control) or cells treated were centrifuged with (2500 rpm at 4 °C for 10 min), rinsed twice with precooled phosphate buffered saline (PBS), and prepared for apoptosis analysis.

Cells were stained with FITC-conjugated Annexin V-FITC incubation for 10 min and then performed propidium iodide stain for another 10 min at room temperature in dark (Life Technologies). Flow cytometric analysis was conducted after supravital stain immediately.

2.2.4. Cell adhesion assay

Cells adhesion assay was performed using the method published by Lin et al. (Lin et al., 2015) with some modifications. Briefly, 96-well flat-bottom plates were coated with 50 uL fibronectin and laminin (10 ug mL^{-1}) in PBS overnight at 4 °C and then blocked with 0.2% BSA for 2 h at room temperature followed by three times washing. Then, HeLa cells, which had been presented by **PMMB232**, shikonin or doxorubicin for 24 h, were added to each well (2 × 10^5 per well) in triplicate, and incubated at 37 °C, 5% CO_2 for 40 min. Next, plates were washed twice with PBS to eliminate unbound cells. Cells remained adhering to the plated were determined by MTT assay. The binding cells were calculated by dividing the optical density of initial input cells. Each assay was carried out at least three times.