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Research paper

Design and characterization of α-lipoic acyl shikonin ester twin drugs as tubulin and PDK1 dual inhibitors

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ABSTRACT

Shikonin exhibits powerful anticancer activities for various cancer cells, but its poor solubility and strong toxicity hinder its development as clinical anticancer agent. We previously confirmed that shikonin and its derivatives can disturb mitosis through targeting tubulin. In this study, α-lipoic acid, the naturally-occurring co-factor of pyruvate dehydrogenase (PDH), was introduced into shikonin to design the twin drugs against both mitosis (tubulin) and glycolysis (PDK). 18 kinds of α-lipoic acid shikonin ester derivatives were achieved through three rounds of screening process performed by computer assistant drug design method, being designated as the outstanding compounds. Among them, **1c** displayed the most potent cytotoxicity towards cervical cancer cells (HeLa) with an IC_{50} value of 3.14 ± 0.58 μM and inhibited xenotransplanted tumor growth in a dose-dependent manner. Further pharmacologic study demonstrated that **1c** can cause cell cycle arrest in G2/M phase as tubulin polymerization inhibitor. Moreover, it also showed good PDK1 inhibitory activity, promoting PDH activity and forced HeLa cells to process more aerobic metabolism to undergo cell apoptosis. We reported here the first dual inhibitors of tubulin and PDK1 based on shikonin. It may form a basis for shikonin optimization through twin drug design framework for the discovery of new and potent shikonin derivatives in the study of targeted cancer therapy.

1. Introduction

It has been known for over half a century that tumor cells consume glucose at surprisingly higher rates compared to normal tissues. In addition, even in the presence of sufficient oxygen, tumors metabolize glucose *via* oxygen-independent aerobic glycolysis rather than *via* oxygen-dependent (more efficient) process of oxidative phosphorylation [1]. This phenomenon was known as "Warburg effect" and often considered to be the foundation for researching cancer metabolism to obtain effective anticancer therapies [2–4].

Pyruvate dehydrogenase complex (PDHC; E.C. 1.2.4.1) is a key enzyme series in metabolism which catalyzes the oxidative decarboxylation of pyruvate to produce acetyl-CoA thus linking glycolysis to tricarboxylic acid cycle [5–7]. PDHC activity is controlled by two regulatory enzymes; pyruvate dehydrogenase kinase (PDK), which phosphorylates, then inactivates the enzyme,

Abbreviations: CADD, computer assistant drug design; NOS, nitric oxide synthase; PKM2, pyruvate kinase isozymes M2; GLUT1, Glucose transporter 1; LA, alpha-lipoic acid; PDH, pyruvate dehydrogenase; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; PMSF, phenylmethanesulfonyl fluoride; PVDF, Poly(vinylidene fluoride); DAPI, 4′,6-diamidino-2-phenylindole; TLC, thin layer chromatography; DMSO, dimethylsulfoxide; NMR, nuclear magnetic resonance; BSA, albumin from bovine serum; PI, prodium iodide.

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and pyruvate dehydrogenase phosphatase (PDP), which dephosphorylates the enzyme to its active form [8,9]. PDK, a member of the GHKL ATPase/kinase superfamily, contains four mammalian isoforms (PDK1~4) in mitochondria characterized in terms of their differences in activity, tissue distribution and regulations [10]. Among these four isoforms, PDK1 is mostly associated with cancer malignancy by reducing PDC activity through phosphorylation of specific serine residues in E1α subunit of pyruvate dehydrogenase (PDH) [11–13]. It has been reported earlier that PDK1 was remarkably over-expressed in multiple clinical cancer specimens and has become popular pharmacological target for cancer chemotherapy [14–16].

Microtubules (MTs) are cytoskeletal filaments that are composed of α- and β-tubulin proteins [17]. The formation of microtubules is a dynamic process that involves the polymerization and de-polymerization of α- and β-tubulin heterodimers [18]. Disruption of the dynamic equilibrium blocks the cell division machinery at mitosis and leads to cell cycle arrest at metaphase, resulting in cell death [19]. Likewise, tubulin has become an important target for the design and development of new anti-cancer agents [20].

Shikonin is a major naphthoquinone compound found in the roots of *Lithospermum erythrorhizon* and exhibits powerful anti-cancer activities for various cancer cells [21–23]. Previous studies have shown that shikonin is a highly active functional molecule that can kill cancer cells effectively in different ways, like inducing cell apoptosis [23,24], inhibiting topoisomerase [25], inhibiting nitric oxide synthase (NOS) [26], etc. Our previous research has also confirmed that shikonin and its derivatives exhibit potent tubulin inhibitory activity [27–29]. They can disrupt the dynamic equilibrium of tubulin polymerization and de-polymerization during the process of mitosis, subsequently resulting in cell cycle arrest thus causing cell death [28–30].

Alpha-lipoic acid(LA), a naturally-occurring co-factor is involved in regulating metabolism. It is found to be present in a number of multi-enzyme complexes, including PDHC and has been widely reported to induce apoptosis in various cancer cell lines [31,32]. In cells, LA is reduced to dihydrolipoic acid, which scavenges various reactive oxygen species and regenerates other endogenous antioxidants without adverse side effects [33]. CPI-613, an analogue of LA, which has been approved by FDA for the treatment of relapse/refractory leukemia, can strongly disrupt mitochondrial metabolism, with selectivity for tumor cells in culture [34,35]. Furthermore, it also shows strong antitumor activity in *vivo* against human non-small lung and pancreatic cancer cells in xenograft models without any significant adverse effects [36].

Having two different pharmacophores, the nonsymmetrical twin drugs are expected to have two different pharmacological effects (dual action) [37]. To achieve outstanding compounds, a screening process of a 40,000 compounds scale was performed by computer assistant drug design (CADD) method. We attempted to screen at three levels preliminarily, the backbone, the derivation and the substitution. After the first round, we chose LA pattern as a relay point (Fig. 1). As shown in Fig. 1, a molecular hybridization strategy based on the natural product shikonin and LA analogues yield a scaffold which has three parts: i) a natural shikonin scaffold as an anticancer pharmacophore fragment against tubulin; ii) a naturally-occurring co-factor LA as the medium-chain and iii) various aromatic aldehyde units attached with LA fragment as another anticancer pharmacophore fragment against PDK1. To the best of our knowledge, such a design in shikonin structural modification is unprecedented. Based on CADD screening, we conducted further biochemical and pharmaceutical assays to evaluate whether or not the novel twin drugs, LA shikonin ester derivatives will provide a synergistic anticancer activity through the dual action at the two targets.

2. Methods

2.1. Cell lines and culture conditions

The cell lines used in this study were human hepatoma cell line (HepG2), human breast cancer cell line (MDA-MB-231), carcinoma of cervix cell line (HeLa), human lung adenocarcinoma epithelial cell line (A549), human embryonic kidney cell (293 T) and human liver cell (L02). They were obtained from State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University. Cells were maintained in DMEM/high glucose supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin and incubated at 37 °C in humidified atmosphere containing 5% CO_2.

2.2. Animals feeding

Female nude mice (5–7 weeks old) were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). Briefly, mice were fed with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12 h light-dark cycle. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nanjing University and approved by the Animal Ethics Committee of the Ministry of Science and Technology of China (2006). All efforts were made to minimize animal's suffering and to reduce the number of animals used.

2.3. Anti-proliferation assay

The anti-proliferative activity of the prepared compounds against five cancer cell lines, i.e HepG2, MDA -MB-231, HeLa, A549 and two normal cell lines, i.e L02 and 293 T was evaluated as described previously [38]. Target tumor cell lines were grown to log phase in DMEM medium supplemented with 10% fetal bovine serum. After dilution to 2×10^4 cells mL^{-1} with the complete medium, 100 μL of the obtained cell suspension was added to each well of 96-well flat bottom plates and then allowed to adhere for 12 h at 37 °C, 5% CO_2 atmosphere. Tested samples at pre-set concentrations (0.1, 1, 10, 100 μM) were added to 96-well flat bottom plates with shikonin, colchicine and DCA as positive references. After 24 h exposure period, 20 μL of PBS containing 2.5 mg mL^{-1} of MTT was added to each well. Plates were then incubated for further 4 h and then centrifuged with 1500 rpm at 4 °C for 10 min followed by supernatant removal. Afterwards, 150 μL of DMSO was added to each well to dissolve purple crystal. The plates were shaken for 10 min at room temperature to ensure maximum solubility. The absorbance was measured and recorded by ELISA reader (ELx800, BioTek, USA) at a test wavelength of 570 nm. Each experiment had three replicates and was repeated thrice. The IC_{50} values defined as concentrations that caused 50% loss of cell viability were calculated by Origin 7.5.

2.4. Flow cytometric analysis of apoptosis

2.4.1. Annexin V-APC/propidium iodide (PI) assay

Briefly, HeLa cells were seeded in 6-well plates (1×10^5 cells per well) for 12 h and then treated with **1c** (0, 1, 2 and 4 μM) for 24 h or treated with 6 μM **1c** for 0, 12, 24 and 36 h. Cells were then collected, washed twice with PBS, stained with 5 μL of Annexin V-FITC and 2.5 μL of PI (5 μg mL^{-1}) in 500 μL of 1× binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$) for 30 min at room temperature in the dark. Apoptotic cells were quantified using a FACScan cytofluorometer (PT. Madagasi Brosa Inc. Jl. Batang Hari No. 73, Propinsi Sumatera Utara, Indonesia). Statistical analysis was done using Flowjo 7.6.1 software.