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Bioassay guided identification of small chaperone proteins α-crystallin and Hsp27 inhibitors from Copaiba oil

Rati Lama [a], Bo Zhong [a], Daniel G. Kulman [a], Bin Su [a,b,*]

[a] Department of Chemistry, College of Sciences and Health Professions, Cleveland State University, 2121 Euclid Ave., Cleveland, OH 44115, USA
[b] Center for Gene Regulation in Health and Disease, College of Sciences & Health Professions, Cleveland State University, 2121 Euclid Ave., Cleveland, OH 44115, USA

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ABSTRACT

Over-expression of small chaperone proteins in cancer tissue contributes to the resistance of chemotherapy. Targeting these small chaperones including α-crystallin and heat shock protein 27 (Hsp27) is a promising strategy for cancer treatment. Hardwickiic acid (HA), a clerodane diterpenoid from Copaiba oil has been reported to inhibit Hsp27. We expect to identify new small chaperone inhibitors from Copaiba oil that is abundant of diterpenoids. In the current study, cytotoxicity and anti-chaperone assay guided isolation of Copaiba oil led to two major fractions (non-acidic and acidic components) and seven sub-fractions from the acidic components. The non-acidic components and one sub-fraction showed significant cytotoxicity in prostate cancer cells. Four sub-fractions exhibited potent anti-chaperone activity. Three chemical components were identified from these sub-fractions including copalic acid, hardwickiic acid and 3-acetoxycopalic acid. All three compounds inhibited the chaperone activities of α-crystallin and Hsp27. In addition, these compounds enhanced the anti-proliferative activity of the chemotherapeutic agent carboplatin in LNCaP cells. 3-Acetoxycopalic acid slightly decreased the level of Hsp27 client protein signal transducer and activator of transcription 3 (Stat3) in PC3 cells. Overall, several diterpenoids were identified to be small chaperone inhibitors and could be used as lead compounds for the development of more potent derivatives.

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1. Introduction

Copaiba oil, which is obtained from the trees of the *Copaifera* species, is composed of a mixture of sesquiterpenes and diterpenes (Leandro et al., 2012). More than 75% of the composition of Copaiba oil was found to be sesquiterpens. Most sesquiterpens present in Copaiba oil are hydrocarbons, alcohols and epoxides. Diterpenes, the minor components in Copaiba oil, were identified to be various diterpenic carboxylic acids including copalic acid, *ent*-agathic acid, kaurenoic acid, hardwickiic acid and others (Leandro et al., 2012; Soares et al., 2013; Souza Barbosa et al., 2013). Copaiba oil is of interest because of its wide application in cosmetics and pharmaceutical industries. Particularly, it has been used as a traditional medicine for wound healing and general inflammation (Estevao et al., 2013; Leandro et al., 2012; Pieri et al., 2012; Santos et al., 2013). In recent years, studies of Copaiba oil and its components have revealed their potential anti-cancer activity. The Copaiba oil from *C. multijuga* species and its fraction have been reported to significantly inhibit tumor growth in mice (Lima et al., 2003). Sesquiterpenes such as β-caryophyllene and β-elemene that are the major components of Copaiba oil showed anti-proliferative activity against several cancer cell lines. In particular, β-caryophyllene exhibited synergistic effects by increasing anti-cancer activity of the chemotherapeutic agent paclitaxel (Leandro et al., 2012; Legault and Pichette, 2007; Lima et al., 2003). As another important class of natural products detected in Copaiba oil, diterpenic acids mainly show antibacterial activities (Leandro et al., 2012; Pieri et al., 2012; Santos et al., 2012, 2013). Interestingly, a recent study by Feiella, etc. revealed that hard-wickiic acid, one of the diterpenic acids existing in Copaiba oil, inhibits heat shock protein 27 (Hsp27) chaperone activity (Faiella et al., 2012).

The over-expression of Hsp27 in cancer tissues is correlated with anti-cancer drug resistance, which makes it a promising molecular target for drug development (Andrieu et al., 2010; Arts et al., 1999; Concannon et al., 2003; Garrido et al., 2006). Hsp27

* Corresponding author at: Department of Chemistry, College of Sciences and Health Professions, Cleveland State University, 2121 Euclid Ave., Cleveland, OH 44115, USA. Tel.: +1 216 687 9219; fax: +1 216 687 9298.
E-mail addresses: B.su@csuohio.edu, subin624@hotmail.com (B. Su).

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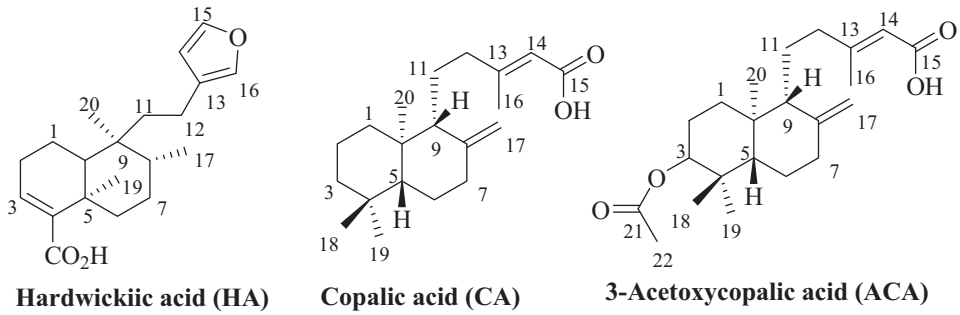

Fig. 1. Structures of hardwickiic acid (HA), copalic acid (CA), and 3-acetoxycopalic acid (ACA).

blocks the apoptosis of cancer cells induced by anti-cancer agents through its molecular chaperone functions. Hsp27 directly interacts with its client proteins affecting multiple steps in apoptosis, including cytochrome c release, formation of apoptosome complex, and caspase-activation (Andrieu et al., 2010; Bruey et al., 2000; Concannon et al., 2003; Garrido et al., 1999). Preclinical studies have reported that antisense oligonucleotides (ASO) or siRNA decrease Hsp27 expression to suppress tumor growth (Baylot et al., 2011; Cayado-Gutierrez et al., 2013). However, these strategies are associated with difficulties in drug delivery. Small molecule Hsp27 inhibitors without administration problems will be more desirable in cancer treatment. In addition to Hsp27, another small chaperone protein, α-crystallin has been reported to play a cytoprotective role in apoptotic cancer cells as well (Arrigo et al., 2007; Hamann et al., 2013). α-Crystallin targeting agents may sensitize cancer cells to apoptosis inducing agents. Overall, small chaperone inhibitors are expected to have a broad application in cancer treatment.

The discovery of Hsp27 inhibitory activity of hardwickiic acid raised our interest in Copaiba oil, specifically to its diterpene components (Faiella et al., 2012). The inhibition of small chaperone protein by hardwickiic acid may contribute to the anti-cancer activity of Copaiba oil. We speculate that other diterpenic acids in Copaiba oil might interfere with small chaperone proteins as well. Since the diterpene components share similar structures. On the other hand, small chaperones have been identified as novel anti-cancer targets (Zoubeidi and Gleave, 2012). However, so far very few inhibitors of the small chaperones have been discovered. Copaiba oil may be a good recourse to isolate lead compounds that inhibit small chaperone proteins. To elucidate the anti-cancer mechanism of Copaiba oil and search for small molecule chaperone protein inhibitors, the oil resin was initially separated into two major fractions including sesquiterpenes and diterpenic acids. The diterpenic acid components were further fractionated. The two major fractions from Copaiba oil and the seven sub-fractions from diterpenes were examined for their potential anti-cancer and anti-small chaperone activities. Four sub-fractions showed potent anti-small chaperone activities. Further isolation of the individual components in the active sub-fractions followed by structural elucidation and biological activity examination led to the identification of several small chaperone inhibitors including, copalic acid, hardwickiic acid and 3-acetoxycopalic acid (Fig. 1).

2. Materials and methods

2.1. Chemistry

Crude Copaiba oil was manufactured by Natural Joint Solutions Inc. (FL). Human recombinant insulin and α-crystallin from bovine eye lens were from Sigma–Aldrich. Human recombinant Hsp27 were from Biosciences. Other commercial chemicals and solvents were used as received without further purification. Thin layer chromatography was performed on silica gel TLC plates with fluorescence indicator 254 nm (Fluka). Flash column chromatography was performed using silica gel 60 Å (BDH, 40–63 μM). Mass spectra were obtained on the Bruker ion-trap mass spectrometer at Cleveland State University MS facility Center. All the ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer using $CDCl_3$ as solvent. Chemical shifts (δ) for ^{1}H NMR spectra were reported in parts per million to residual solvent protons.

2.1.1. General procedure for fractionation of crude Copaiba oil

Crude Copaiba oil (2.3 g) in ethyl ether (10 ml) was extracted twice with aqueous 5% KOH (10 ml). The organic layer was washed with water and dried over anhydrous sodium sulfate, then concentrated under vacuum to give non-acidic fraction (**B**) with a yield around 65%. The combined aqueous layers were neutralized with 3 N HCl and then extracted with ethyl ether. The organic layers were washed with water and dried over anhydrous sodium sulfate, then concentrated under vacuum to give acidic fraction (**C**) with yield around 28%. 0.66 g Fraction **C** was subjected to column chromatograph using gradient elution with pure hexane, hexane/ethyl acetate (95:5), hexane/ethyl acetate (90:10), hexane/ethyl acetate (80:20), to give seven subfractions (**D–J**) with yields of 39%, 21%, 8%, 5%, 5%, 12%, 4% respectively. Fraction **H** contains one single component, which is 3-acetoxycopalic acid. ^{1}H NMR δ 5.689 (1H, s), 4.899 (1H, s), 4.543 (2H, m), 2.433 (1H, m), 2.341 (1H, m), 2.188 (3H, s), 2.074 (3H, s), 2.014 (2H, m), 1.824–1.742 (3H, m), 1.704–1.505 (4H, m), 1.423 (1H, m), 1.285 (1H, m), 1.200 (1H, m), 0.896 (3H, s), 0.870 (3H, s), 0.739 (3H, s); ESI-MS calculated for $C_{22}H_{33}O_4$ $[M-H]^-$ 361.2, found 361.2.

2.1.2. General procedure for isolation of copalic acid and hardwickiic acid

To a solution of fraction **E** (0.436 g), 4-methoxyphenol (0.178 g, 1.434 mmol), dicyclohexyl carbodiimide (0.325 g, 1.577 mmol) in dichloromethane was added 4-dimethylaminopyridine (0.192 g, 1.577 mmol) and stirred at room temperature for 4 h. The insoluble solid was filtered. The filtrate was concentrated under vacuum and the residue was purified by column chromatograph to yield 0.36 g 4-methoxyphenol ester of copalic acid. 0.2 g 4-methoxyphenol ester of copalic acid in the mixture of 3 N aqueous NaOH and ethanol (1:1) was refluxed for 1 h. The reaction mixture was neutralized by addition of 3 N HCl and subsequently extracted with ethyl acetate. The organic layer was concentrated under vacuum followed by purification by column chromatograph to yield 0.12 g copalic acid. ^{1}H NMR δ 5.697 (1H, s), 4.873 (1H, s), 4.516 (1H, s), 2.414 (1H, m), 2.344 (1H, m), 2.195 (3H, s), 2.054–1.955 (2H, m), 1.782–1.700 (2H, m), 1.636–1.410 (6H, m), 1.344 (1H, m), 1.207 (1H, m), 1.110 (1H, s), 1.022 (1H, m), 0.896 (3H, s), 0.826 (3H, s), 0.708 (3H, s); ESI-MS calculated for $C_{20}H_{31}O_2$ $[M-H]^-$ 303.2, found 303.4.

Isolation of hardwickiic acid from fraction **F** followed the similar procedure with a yield close to 15%. ^{1}H NMR δ 7.373 (1H,