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Research paper

Novel oxime-bearing coumarin derivatives act as potent Nrf2/ARE activators *in vitro* and in mouse model

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ABSTRACT

We have designed and synthesized certain novel oxime- and amide-bearing coumarin derivatives as nuclear factor erythroid 2 p45-related factor 2 (Nrf2) activators. The potency of these compounds was measured by antioxidant responsive element (ARE)-driven luciferase activity, level of Nrf2-related cytoprotective genes and proteins, and antioxidant activity. Among them, (*Z*)-3-(2-(hydroxyimino)-2-phenylethoxy)-2*H*-chromen-2-one (**17a**) was the most active, and more potent than the positive *t*-BHQ in the induction of ARE-driven luciferase activity. Exposure of HSC-3 cells to various concentrations of **17a** strongly increased Nrf2 nuclear translocation and the expression level of Nrf2-mediated cytoprotective proteins in a concentration-dependent manner. HSC-3 cells pretreated with **17a** significantly reduced *t*-BOOH-induced oxidative stress. In the animal experiment, Nrf2-mediated cytoprotective proteins, such as aldo-keto reductase 1 subunit C-1 (AKR1C1), glutathione reductase (GR), and heme oxygenase (HO-1), were obviously elevated in the liver of **17a**-treated mice than that of control. These results suggested that novel oxime-bearing coumarin **17a** is able to activate Nrf2/ARE pathway *in vivo* and are therefore seen as a promising candidate for further investigation.

1. Introduction

The induction of the antioxidant proteins and metabolizing/detoxifying enzymes has been considered as an effective way to protect cells against acute and chronic cell injury provoked by environmental stresses [1,2]. Nuclear factor erythroid 2 p45-related factor 2 (Nrf2), a member of the Cap'n'Collar (CNC) family of transcription factors that share a highly conserved basic region-leucine zipper (bZIP) structure, mainly regulates transcriptional activation through antioxidant responsive element (ARE, 5′-(A/G) TGACNNNGC(A/G)-3′) [3], which is a *cis*-acting regulatory element in promoter regions of several cytoprotective genes. Nrf2 is ubiquitously expressed in all human organs at low transactivating levels due to tight regulation by Kelch-like ECH associating protein 1

Abbreviations: ABCC3, ATP-binding cassette, subfamily C, member 1; AKR1C1, aldo-keto reductase 1 subunit C-1; AKR1C2, aldo-keto reductase 1 subunit C-2; AKR1C3, aldo-keto reductase 1 subunit C-3; ARE, antioxidant responsive element; *t*-BHQ, *tert*-butylhydroquinone; *t*-BOOH, *tert*-butyl hydroperoxide; CAPE, caffeic acid phenethyl ester; GCLC, γ-glutamyl cysteine synthetase catalytic subunit; GCLM, γ-glutamyl cysteine synthetase modifier subunit; GR, glutathione reductase; HO-1, heme oxygenase-1; Keap 1, Kelch-like ECH associating protein 1; NQO1, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2 p45-related factor 2; ROS, reactive oxygen species.

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(Keap 1), an actin-binding protein, which is a substrate adaptor protein for a Cullin3-based E3 ubiquitin ligase [4]. Under basal condition, Nrf2 is constantly targeted for Keap 1-mediated ubiquitination and subsequent proteasomal degradation to maintain low Nrf2 protein levels. Upon activation, the enzymatic activity of the Keap-Cullin3 E3 ubiquitin ligase is compromised, resulting in stabilization of Nrf2 and activation of Nrf2 downstream antioxidant proteins-, phase II metabolizing/detoxifying enzymes-, and phase III APT-dependent drug efflux pumps-encoded genes [5–7]. Therefore, the Nrf2/ARE pathway has been highlighted to be the most important regulators of cytoprotective responses to oxidative and/or electrophilic stresses, which is believed to play a critical role in the development of many disease, such as cancer [5,7], Alzheimer's [8], Parkinson's [8], multiple sclerosis [8], chronic kidney disease [9], chronic obstructive pulmonary disease [10], and inflammatory bowel disease [11].

The vast number of both natural and synthetic compounds able to induce Nrf2/ARE pathway were identified, including Michael reaction acceptors [12], sulforaphane [13,14], dithiolethione [15,16], curcumin [17], caffeic acid phenethyl ester (CAPE) [18], chalcones [19,20], and vinyl sufones [21]. Notably, one Nrf2 activator, dimethyl fumarate, was approved by the Food and Drug Administration (FDA) for the treatment of multiple sclerosis. In addition, bardoxolone methyl, a synthetic oleanane triterpenoid, is under clinical investigation of the treatment of pulmonary hypertension. These results encouraged us to further discover novel Nrf2 activators for clinical application.

Coumarin constitutes a class of natural phytochemicals found in many plant species. Certain coumarin derivatives such as novobiocin (an antibiotic), dicoumarol (an anticoagulant), and warfarin (a rodent poison) were found to be biologically active. Therefore, extensive studies on coumarin derivatives have continuously attracted much attention [22–28]. For the past few years, we have also synthesized a number of coumarin derivatives and evaluated for their cytotoxic and antiplatelet activities [29–34]. It is notable that coumarin bears a α,β-unsaturated ketone moiety, which is a common functional group of certain Nrf2 activators such as curcumin, CAPE, and chalcone (Fig. 1). The present report describes the synthesis of novel oxime- and amide-bearing coumarins and evaluate for their Nrf2 activating activities.

2. Chemistry

The preparation of oxime-bearing coumarin derivatives is illustrated in Scheme 1. Alkylation of 3-hydroxycoumarin (**1**) with 2-(bromoacetyl)naphthalene under basic conditions gave 3-(2-(naphthalen-2-yl)-2-oxoethoxy)-2*H*-chromen-2-one (**9e**) in a 94% yield. The same procedure was applied to convert compounds **2**, **3**, and **4** to compounds **10e**, **11e**, and **12e**, respectively. Preparation of compounds **5**–**8**, **9a**–**d**, **10a**–**d**, **11a**–**d**, and **12a**–**d** were previously reported [29–34].

Treatment of 3-(2-oxopropoxy)-2*H*-chromen-2-one (**5**) with NH_2OH afforded exclusively (*E*)-3-(2-(hydroxyimino)propoxy)-2*H*-chromen-2-one (**13**) in a 89% yield. The configuration of the oxime moiety was determined by through-space nuclear Overhauser effect spectroscopy (NOESY) which revealed a coupling connectivity to CH_3 protons. In addition, the ^{13}C NMR signal for OCH_2 of the (*E*)-form oxime derivative was shifted downfield [35,36] to approximately 70 ppm (δ = 70.5 ppm for (*E*)-**13**). Accordingly, reaction of the ketone derivatives, **6**–**8**, with NH_2OH afforded their respective *E*-form oximes, **14**–**16**. However, treatment of 3-(2-oxo-2-phenylethoxy)-2*H*-chromen-2-one (**9a**) with NH_2OH under the same reaction conditions gave exclusively (*Z*)-3-(2-(hydroxyimino)-2-phenylethoxy)-2*H*-chromen-2-one (**17a**) in a 93% yield. The ^{13}C NMR signal for OCH_2 of the (Z)-form oxime derivative was shifted upfield to approximately 60 ppm [35,36] (δ = 59.6 ppm for (*Z*)-**17a**). The same synthetic procedures were applied for the synthesis of (*Z*)-**17b**–**e** from **9b**–**e** respectively; (*Z*)-**18a**–**e** from **10a**–**e** respectively; (*Z*)-**19a**–**e** from **11a**–**e** respectively; (*Z*)-**20a**–**e** from **12a**–**e** respectively.

The preparation of amide-containing coumarins is illustrated in Scheme 2. Alkylation of 3-hydroxycoumarin (**1**) with 2-bromoacetophenone under basic conditions gave 3-(2-oxo-2-phenylethoxy)-2*H*-chromen-2-one (**9a**) which was then treated with H_2SO_4 and NaN_3 to afford 2-(2-oxo-2*H*-chromen-3-yloxy)-*N*-phenylacetamide (**21a**) in a good overall yield. The same synthetic procedures were applied for the synthesis of *N*-(4-fluorophenyl) counterpart **21b**, *N*-(4-methoxyphenyl) counterpart **21c**, *N*-(4-biphenyl-4-yl) counterpart **21d**, and *N*-(naphthalen-2-yl) counterpart **21e** from their respective ketone precursors **9b**, **9c**, **9d**, and **9e** which in turn was prepared *via* alkylation of **1**. Accordingly, compounds **22a**–**e**, **23a**–**e**, and **24a**–**e** were also prepared by the same reaction sequences from their respective ketones **10a**–**e**, **11a**–**e**, and **12a**–**e** which in turn were prepared *via* alkylation of 4-hydroxycoumarin (**2**), 6-hydroxycoumarin (**3**), and 7-hydroxycoumarin (**4**) respectively. Structures of newly synthesized compounds were confirmed by NMR spectra and elementary analysis.

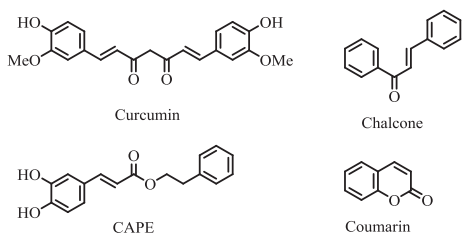

Fig. 1. Structures of curcumin, chalcone, CAPE, and coumarin.

3. Results and discussion

3.1. Novel coumarin derivatives induced ARE-driven luciferase activity in HSC3-ARE9 cells

In this study, we used a stable ARE-driven reporter system to screening the potential Nrf2 activators [37]. Luciferase activities were determined after cells treated with test compounds at a concentration of 50 μM for 24 h. *tert*-Butylhydroquinone (*t*-BHQ), a well-known Nrf2 activator in ARE-controlled gene transcription, showed two-fold induction of ARE-driven luciferase activity, was used as positive control. All the hydroxycoumarins **1**–**4** and their respective 2-oxopropoxy derivatives **5**–**8** showed no induction of luciferase activity as shown in Table 1. Although 3-(2-oxopropoxy)-2*H*-chromen-2-one (**5**) was inactive, replacement of the methyl group with a phenyl ring significantly enhanced activity (1.72-fold induction for compound **9a** *vs* 0.92-fold induction for **5**). For 3-substituted coumarin derivatives, the potency decreased in an order **9a** (R = Ph; 1.72-fold) > **9b** (R = 4-F-Ph, 1.64-fold) > **9c** (R = 4-MeOPh, 1.54-fold) indicated substitution at phenyl ring is unfavorable especially the electron-donating group such as the methoxy group. The same trends were observed in 6-substituted coumarins (**11a** > **11b** > **11c**) and 7-substituted coumarins (**12a** > **12b** > **12c**). Among a total of 24 oxime-bearing coumarin derivatives tested, methyl group substituted derivatives **13**–**16** exhibited 1.37–1.68 fold induction of luciferase activity which was less active than that of *t*-BHQ (Table 1). Replacement of the methyl group with a phenyl ring significantly enhanced activity (3.45-fold induction for compound **17a** *vs* 1.63-fold induction for **13**). For 3-