Orobanone analogues from acid-promoted aromatization rearrangement of curcumol inhibit hypoxia-inducible factor-1 (HIF-1) in cell-based reporter assays

Yue-qing Li, Guang-zhe Li, Yi Dong, Xu Ma, Hui-juan Dong, Qian-qian Wu, Wei-jie Zhao

School of Pharmaceutical Science and Technology, Dalian University of Technology, No. 2 Linggong Road, Dalian 116024, China
State Key Laboratory of Fine Chemicals, Dalian University of Technology, No. 2 Linggong Road, Dalian 116024, China

ABSTRACT

In this paper, the mechanism of orobanone analogues formation via aromatization rearrangement of curcumol was minutely explored. Aromatization of curcumol with acetone under acidic condition was selected as the model reaction. The formation of a stable aromatic system was the driving force for this reaction. Based on the model reaction, other four new orobanone analogues were prepared through curcumol reacting with different carbonyl compounds. The results showed that the stability of carbocation, which was generated from the carbonyl compounds, and the steric hindrance were main factors affecting the aromatization. We also synthesized the analogue of aromaticane B using compound 2. In vitro anti-proliferative activity of some derivatives were tested by MTT assay. Two derivatives showed weak anti-tumor effect on two cancer cell lines (HepG2 and MCF7) under normoxia. Four orobanone analogue 2, 5, 6 and 9 significantly inhibited hypoxia-induced HIF-1 luciferase reporter activity in HeLa cells with the IC50 values of 13.6, 6.6, 2.4 and 18.2 μM, respectively.

1. Introduction

Curcumae rhizome is a kind of Chinese traditional medicine, which is the dry rhizome of Curcuma phaeocaulis Val., Curcuma kwangsiensis or Curcuma wenyujin [1,2]. It has a high content of essential oil. Curcumol (1) is one of the major active ingredients of the essential oil, nominated (3S-(3α, 3aα, 5α, 6α, 8a-β))-Octahydro-3-methyl-8-methylene-5-(1-methylethyl)-6H-3a,6-epoxy azulen-6-ol (Fig. 1). Curcumol has been reported with many bioactivities, such as antifungal activity [3], anti-proliferative activity [4–6], anti-inflammation [7], and anti-virus, etc. Recently, orobanone isolated from Curcuma zedoaria showed antibiotic effect on Bacillus cereus (Fig. 1) [8]. Similarly, as the orobanone analogues, Phaeocaulisins D and phaeocaulisins L, isolated from rhizomes of Curcuma phaeocaulis, exhibited inhibitory activity against lipopolysaccharide-induced NO production in RAW 264.7 macrophages. Phaeocaulisins L showed moderate activity (IC50 54.27 ± 4.23 μM) compared to phaeocaulisins D (IC50 5.9 μM) (Fig. 1) [9,10]. Aromaticane B isolated from Curcuma aromatica showed notable antioxidant effects on oxidative injury induced by H2O2 [11].

So far, all the tested orobanone and its analogues are natural products except compound 2 (Fig. 1). Almost thirty years ago, compound 2, an analogue of orobanone with antitumor activity, was prepared by the reaction of curcumol with HBr in acetone [12,13]. As we know, there is none report of the synthesis of orobanone. Meanwhile, the main skeleton of orobanone is 2, 3-dihydroazulen-6(1H)-one, of which the available synthetic methods are very limited, including intramolecular cyclization [14–18], intermolecular addition [19,20], local oxidation [21] and aromatization after the elimination of epoxy bridge [22]. It was interesting that curcumol can be converted into another kind of natural product analogues by the aromatization rearrangement catalyzed by proton acid. Due to the similar antifungal [3,6] and anti-inflammatory activities [7,9,10] between orobanone analogues and curcumol, it was assumed that orobanone and/or its analogues have anti-tumor effect, which had not been reported. Thus the reaction of curcumol with different carbonyl compounds might provide us the opportunity to synthesize more orobanones analogues and be meaningful to find more anti-proliferative candidates.

The model reaction in our study was that curcumol reacted with acetone catalyzed by hydrobromic acid. Based on two important intermediates, we proposed the mechanism of the model reaction. Successionally, the reaction of curcumol with different carbonyl compounds was studied and other four new orobanone analogues were...
Hypoxia inducible factor-1 (HIF-1) plays pivotal roles by regulating angiogenesis, invasion, metastasis, glucose uptake, and cell survival during hypoxic tumor microenvironment [23]. Natural products have been widely explored as HIF-1 inhibitors with particular effectiveness in cancerous diseases [24,25]. Recent researches about the anti-tumor activity of curcumol have demonstrated that curcumol can inhibit cancer cell proliferation [4–6,26–30], induce cell cycle arrest [31,32], induce apoptosis [5,30,32–34] and suppress cancer cell metastasis [35,36] via different pathways and targets. It was reported that MAPK [5] and PI3K-Akt [27,31,35] were involved in the signaling pathways suppressed by curcumol. Most importantly, both signaling pathways are related to the downregulation of HIF-1 transcription and expression [37]. Herein, we hypothesized that curcumol and the orobanone analogues may have anti-tumor effect via inhibiting HIF-1 expression.

2. Results and discussion

2.1. Chemistry

Aromatization of curcumol in acetone under the catalysis of hydrogen bromide has been reported [12,13]. However, the mechanism of this reaction has never been reported. In our study, since the difficulty in preparing acetone-hydrogen bromide solution, hydrobromic acid was used. Unexpectedly, this change caused lower yield and more complicated post-processing compared to acetone-hydrogen bromide solution. When the mixture of acetone and hydrobromic acid was dehydrated by phosphorus pentoxide in succession to sodium sulfate, the final product was crystallized from the brick-red oily solution at room temperature in a reasonable yield (53–75%).

The aromatization rearrangement process monitored by TLC indicated that several intermediate compounds were involved. First, compound 3, a yellow crystalline, was isolated by column chromatography. Compound 3, with two double bonds in the five-membered ring, was an isomeric compound of compound 2. Therefore, we can confirm that double bond shift occurred during the process (Scheme 2). Interestingly, the increased unsaturation made the seven-membered rings possible to form a stable aromatic system. The result indicated that unsaturated bonds of the molecule had a further shift under the influence of proton. All the unsaturated bonds moved to seven-membered rings leading to the generation of compound 2. It was believed that the formation of a stable aromatic system was the driving force for this reaction. In other words, the process of aromatization promoted the occurrence of the reaction.

From structures of compound 2 and 3, we could figure out that curcumol underwent ring-opening of oxygen-bridge first. However whether acetone participated in the ring-opening was not clear. The reaction of curcumol catalyzed by hydrobromic acid in THF without acetone was studied as well. Compound 4 is the main product. The probable ring-opening route was shown below (Scheme 3).

Since there are two exocyclic double-bonds, compound 4 is prone to aromatization by rearrangement. This means that the reaction tends to produce a product with the largest number of alkyl groups. Comparing compound 3 with compound 4, we speculated a very complex process (Scheme 4). First, the double bond migration occurred under the catalysis of protonic acid. Second, carbocation was formed by a union of a carbonyl compound and a proton, which attacked π electrons of double bond. This electrophilic addition reaction was a key step in the process, which generated new carbon-carbon bond and carbocation simultaneously. The carbocation further underwent E 1 elimination and a double bond was formed subsequently. These two steps resembled the electrophilic substitution reaction on benzene. After that, a new hydroxyl group was formed through addition-elimination reaction. Under the catalysis of a proton, the hydroxyl group left in the form of a water molecule. This process formed another carbocation. Then a new carbon-carbon double bond was formed through E1 elimination. At last, compound 3 was generated by double bond migration.

Based on the model reaction, a series of different carbonyl compounds were selected to react with curcumol. Several new trienone compounds 5–8 were prepared (Table 1). In the process, we noticed that the reaction of other carbonyl compounds needed longer time and produced more by-products with strong acid under reflux. We also found that the reaction did not take place between curcumol and alicyclic ketones except for acetone. Alicyclic ketones did not participate in the reaction.
not carry out the reaction either. Aromatic aldehydes with electron-donating group can carry out the reaction smoothly. But aromatic ketones did not undergo this reaction.

Interestingly, the analogue of aromatocane B was synthesized based on the aromatization rearrangement of curcumol accidentally (Scheme 5). The mechanism of this reaction needs further study.

According to the assumption, carbonyl compounds are supposed to be transformed into carbocations during the reaction. The stability of carbocation is the key factor in this reaction. Therefore, compounds such as benzaldehyde can carry out the reaction especially when there are electron-donating groups on the benzene ring. Carbocation formed from alicyclic ketones is less stable and unable to participate in the aromatization of curcumol. In addition, although they can form a stable carbocation, benzophenone and acetophenone cannot undergo this kind of reaction due to the bigger steric hindrance (Table S1).

The structures of 2 and 6 have been confirmed by X-ray analysis (Fig. 2). Suitable crystal of compound 2 grew at room temperature from a solution of normal hexane, and suitable crystal of compound 6 grew at room temperature from a solution of ethyl acetate. Since there are two chiral carbon atoms in compound 2 or compound 6, the single crystal is composed of their enantiomers. This could be additional powerful proof of the supposed double bond migration process.

### Table 1

<table>
<thead>
<tr>
<th>Carbonyl compounds</th>
<th>Reaction time (h)</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>2</td>
<td>2</td>
<td>75%</td>
</tr>
<tr>
<td>O-hydroxybenzaldehyde</td>
<td>8</td>
<td>5</td>
<td>59%</td>
</tr>
<tr>
<td>Vanillin</td>
<td>8</td>
<td>6</td>
<td>59%</td>
</tr>
<tr>
<td>2,4-dimethoxybenzaldehyde</td>
<td>10</td>
<td>7</td>
<td>53%</td>
</tr>
<tr>
<td>p-hydroxybenzaldehyde</td>
<td>10</td>
<td>8</td>
<td>53%</td>
</tr>
</tbody>
</table>

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2.2. Effect of compounds on cell proliferation

The anti-inflammatory activity of natural orobanone analogues is similar to curcumol [9,10]. Curcumol is an antiproliferative agent [4–6,21–26]. It was reported that compound 2 had anti-tumor activity [12]. Therefore, we tested the in vitro anti-proliferative activity of the products to figure out whether the orobanone analogues reserved the anti-tumor activity under normoxia. After the first primary screening, three derivatives were selected and tested for their cytotoxic activity against two cancer cell lines (HepG2 and MCF7) by the MTT assay (Fig. S1). Notably, both compound 5 and 6 showed a better result than curcumol in the assay (Table 2). However, there was no selective inhibition between HepG2 and L-O2 by the orobanone analogues we tested. Surprisingly, the survival rates of the cancer cells were high at the low concentrations (< 50.0 μM) of compound 5 and 6. When it was at 10.0 μM, compound 5 induced hormesis. However, the survival rates of cancer cells declined quickly at the concentrations from 50.0 μM to 100.0 μM of compound 5 and 6 (Fig. S1). The dose-response curves of compound 5 and 6 were abnormal, possibly due to as yet undetermined feedback, off-target, or non-specific mechanisms. There is no self-aggregation phenomenon when the analogues are under the high concentrations during the experiment and predicted by Aggregator Advisor [38].

2.3. Inhibition of HIF-1 transcription

We tested curcumol and the orobanone analogues about their inhibition of HIF-1 transcriptional activity (Table 3), reported phenoxacytanilide 10 by Nakamura’s groups as reference substance (see Fig. 3). For the first time, we found curcumol could inhibit HIF-1 transcription with IC50 of 68.5 ± 6.19 µM, which was identical with our speculation. The orobanone analogues showed better inhibition than curcumol, especially for compound 6 that was synthesized by curcumol and vanillin [40]. The inhibitory activity of compound 6 is nearly three times of that of reference compound 10. The result indicated that the aromatization of curcumol was beneficial to improve the inhibition of HIF-1 transcription. The aromatic substituents on C2 also elevated the compounds’ inhibition on HIF-1 transcription. Nevertheless, the hydroxylation of C1 did not show significant effect. Therefore, 2-aromatic substituted orobanone analogues may become a novel kind of HIF-1 inhibitors.

3. Conclusions

We herein synthesized six orobanone analogues. Five of the products were synthesized through the mechanism of aromatization...
rearrangement of curcumol we proposed in this paper. We supposed the stability of carboxylation generated from the carbonyl compounds and the steric hindrance of carbonyl compound were main factors that affect the aromatization. The activity tests revealed the orobanone analogues products had lower IC50 concentration than curcumol, which demonstrated the analogue preserved the antimtorum activity. Notably, curcumol and orobanone analogues inhibited HIF-1 transcription under hypoxia. Orobamone’s aromaticity and 2-aromatic substituents contribute to the orobanone analogues’ improved activity against HIF-1 transcription. Interestingly, the synthesis of compound 9 was based on compound 2 accidently, which may be used as a novel method of hydroxylation and needs further exploring. Further detailed mechanistic studies of HIF-1 inhibition by compound 6 is currently under investigation in our research group.

4. Experimental section

4.1. Chemistry

4.1.1. General

Solvents and chemicals were reagent grade or better and obtained from commercial sources. NMR spectra were recorded on Bruker Advance II spectrometer. Analytical thin layer chromatography (TLC) was performed on silica gel plates produced by Qingdao Haiyang Chemical Co. Ltd. (200 × 200 mm, 0.2-0.25 mm) with F254 indicator. Detection: under 254 nm UV light or color development by spraying with vanillin-concentrated sulfuric acid reagent. Compound 2 has been reported with limited spectroscopic data.

4.1.2. Typical procedure for the aromatization arrangement of curcumol

Method A: 8 ml 40% of hydrobromic acid solution was added to 80 ml acetone and dehydrated by anhydrous sodium sulfate for 3 h. Phosphorus pentoxide was used to further dehydrate for another 15 min. After filtering the desiccant, 5.0 g curcumol was dissolved in the filtrate and heated to reflux for 3 h. The reaction solution was neutralized by saturated solution of sodium bicarbonate and then extracted by ethyl acetate. The combined extract was dried by anhydrous sodium sulfate. Ethyl acetate was removed under vacuum. The residue was brown oil. Hereafter this is designated as method A.

Method B: The solution of 0.50 g curcumol and carbonyl compounds (molar ratio 1:1) in 30 ml THF was mixed with 5 ml 40% of hydrobromic acid. The mixture was heated to reflux for 8 h. The reaction solution was neutralized by saturated solution of sodium bicarbonate and then extracted by ethyl acetate. The combined extract was dried by anhydrous sodium sulfate. Ethyl acetate was removed under vacuum. The residue is oil-like substance and purified by silica gel chromatograph. Hereafter this is designated as method B.

4.1.2.1. 2,3-dihydro-1,4-dimethyl-2-(4-hydroxy-3-methoxybenzyl)-7-isopropyl-azulen-6(1H)-one (4).

The substrate was salicylaldehyde. Compound 4 was prepared according to method B in 59% yield (0.4 g). White solid; mp 163–164 °C; δ 7.80, 8.16, C18H26O4: 322.1942, found: 322.1933. (m/z) HRMS (EI) 

4.1.2.2. 4,5-dihydro-3,8-dimethyl-2,5-diisopropyl-azulen-6(1H)-one (3).

Compound 3 was prepared according to method A. The reaction time was shortened to 1 hr. The crude product was purified by silica gel chromatograph to afford compound 3 (20%). Yellow crystal; mp 140–142 °C; δ 7.08–7.14, 2.85(1H, s, H-8), 6.99(1H, s, H-8), 6.97–7.08(1H, s, H-7a), 7.01–7.06(1H, d, J = 13.6, H-14 or H-15), 0.91(3H, d, J = 6.4, H-16 or H-17); 13C NMR (100 MHz, CDCl3): δ 210.5(C-6), 158.6(C-7), 151.9(C-9), 141.3(C-10), 138.9(C-5), 130.6(C-11), 124.9(C-7), 54.8(C-5), 39.9(C-1), 25.7(C-11), 25.0(C-18), 23.5(C-4), 23.0, 22.9(C-12, C-13), 21.2, 20.1(C-16, C-17), 10.8(C-14); HRMS (EI, m/z): [M + Na]+ calcd for C14H23O2: 258.1995, found: 258.1984.

4.1.2.3. (S)-3,8-dimethyl-5-isopropyl-2,4,5,7-tetraydroazulen-6(1H)-one (4).

1.00 g curcumol was completely dissolved in 30 ml THF and mixed with 5 ml 40% of hydrobromic acid. The mixture was heated to reflux for 4 hr. The reaction solution was neutralized by saturated solution of sodium bicarbonate and extracted with ethyl acetate. The combined extract was dried by anhydrous sodium sulfate. Ethyl acetate was evaporated under vacuum. The residue was a yellow oil-like substance. The residue was purified by silica gel chromatograph to afford compound 4 in a 60% yield. Colorless oil; 1H NMR (400 MHz, CDCl3): δ 3.79(1H, d, J = 13.6, H-14 or H-15), 2.57(1H, dd, J = 14.2, 3.2 Hz, H-4), 2.34–2.41(5H, m, H-1, H-2, H-4, H-12, H-15), 1.76(6H, s, H-11, H-15), 0.98(3H, d, J = 6.8, H-16 or H-17), 0.91(3H, d, J = 6.4, H-16 or H-17); 13C NMR (100 MHz, CDCl3): δ 210.5(C-6), 143.8(C-9), 141.7(C-3), 135.4(C-10), 113.8(C-8), 57.2(C-5), 48.1(C-7), 35.5(C-2), 30.9(C-12), 28.0(C-1), 23.7(C-4), 22.8(C-15), 18.8, 20.8(C-13, C-14), 14.8(C-11); HRMS (EI, m/z): [M + Na]+ calcd for C14H23O2: 218.1676, found: 218.1676.
B in 53% yield (0.41 g). The reaction time was 10 h and the substrate was 2,4-dimethylbenzaldehyde. Yellow oil; χ20 = 5.4 (c 0.31, CHCl3). 1H NMR (400 MHz, CDCl3): δ 7.90 (1H, s, H-8), 7.02 (1H, d, J = 2.4 Hz, H-3′), 7.01 (1H, dd, J = 8.4, J = 2.4 Hz, H-5′), 6.46 (1H, s, H-5), 6.34 (1H, d, J = 8.4 Hz, H-6′), 3.80 (3H, s, -OCH3), 3.79 (3H, s, -OCH3), 3.48 (1H, hept, J = 6.4 Hz, H-13), 2.90 (1H, dd, J = 17.6, 8.0 Hz, H-16a), 2.77 (1H, dd, J = 13.6, 6.0 Hz, H-3a), 2.75–2.90 (1H, m, H-1), 2.57 (1H, dd, J = 13.6, 6.0 Hz, H-3b), 2.55 (1H, dd, J = 17.6, 8.0 Hz, H-16b), 2.18–2.28 (1H, m, H-2), 2.17 (3H, s, H-12), 1.31 (3H, d, J = 6.8 Hz, H-11), 1.18 (3H, d, J = 6.8 Hz, H-14 or H-15), 1.17 (3H, d, J = 6.8 Hz, H-14 or H-15). 13C NMR (100 MHz, CDCl3): δ 185.3, 159.4, 158.5, 158.2, 149.5, 145.1, 145.0, 138.9, 130.6, 130.0, 121.2, 103.9, 98.5, 55.3, 55.2, 50.4, 44.3, 40.4, 34.4, 29.9, 25.1, 22.6, 22.5, 19.6. HRMS (EI) (m/z): [M]+ calcd for C23H28O3, M = 352.45, colorless crystal 0.35 × 0.29 × 0.24 mm, a = 9.618 (5) Å, b = 12.634 (7) Å, c = 13.196 (7) Å, α = 90.00′, β = 93.834 (8)′, γ = 90.00′, V = 1599.9 (15) Å3, ρcalcd = 1.073 g cm−3, μ = 0.08 mm−1, Z = 4, monoclinic, space group P2(1)/a, λ = 0.71073 Å, T = 293 (2) K, 6688 measured reflections, 2813 independent reflections (Rint = 0.018), and 2207 observed reflections (I > 2σ(I)), 172 refined parameters, R = 0.052, wR2 (F2) = 0.158, max(min) residual electron density 0.21 (−0.25) e Å−3, hydrogen atoms calculated and refined as riding atoms.

4.1.3. X-ray crystal structure analysis for compound 2. Formula C18H26O2, M = 258.39, colorless crystal 0.35 × 0.29 × 0.24 mm, a = 9.441 (2) Å, b = 10.674 (3) Å, c = 11.047 (3) Å, α = 108.023′ (4), β = 85.520 (4)′, γ = 85.735 (4)′, V = 975.4 (Å3), 3 Å3, hydrogen atoms calculated and refined as riding atoms.

4.2. Biology

4.2.1. MTT assay for cell growth inhibition

The inhibition of the title compounds against HepG2 human liver cancer cells and L-O2 human liver cells were evaluated using a standard MTT-based colorimetric assay. Five thousand corresponding cells per well were seeded into 96-well plates and incubated at 37 °C, 5%CO2 for 24 h. Then the medium was dispensed into wells to maintain the final concentration as 100, 90, 80, 70, 60 and 50 μmol/L. Each concentration was in triplicate. After 48 h incubation, cell survival was determined by the addition of 20 μL MTT (Sigma Aldrich, St. Louis, USA) work solution (5 mg/mL MTT dissolved in Phosphate Buffer Solution (PBS)). After post-incubation at 37 °C for 4 h, the medium was discarded following by adding 100 μL DMSO (SigmahAldrich, St. Louis, USA). The plates were then vortexed for 10 min for complete dissolution. The optical absorbance was measured at 570 nm. The data represented the mean of three independent experiments in triplicate and were expressed as mean ± SD. The IC50 value was defined as the concentration at which 50% of the cells could survive. HeLa cells were incubated for 60 h with various concentrations (400 nM to 100 μM) of compounds under the normoxic condition, and viable cells were tested by the MTT assay. The drug concentration required to inhibit cell growth by 50% (IC50) was determined from semi-logarithmic dose–response plots, and results represent means ± SD of triplicate samples.

4.2.2. Cell-based reporter assays for hypoxia-inducible factor-1 (HIF-1) inhibition

HeLa cells expressing HRE-dependent firefly luciferase reporter construct (HRE-Luc) and constitutively expressing CMV-driven Renilla luciferase reporter with SureFECT Transfection Reagent were established with Cignal™ Lentivector System (SABiosciences, Frederick, MD) according to the manufacturer’s instructions. The consensus sequence of HRE was 5′-TAGTGTGCT-3′ from the erythropoietin gene. Cells stably expressing the HRE-reporter gene were selected with puromycin. The cells were incubated for 12 h with or without drugs under the normoxic or hypoxic condition (1% O2). After incubation, the luciferase assay was performed using a Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. The drug concentration was obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44(1223)336–033; E-mail:deposit@ ccdc.cam.ac.uk].
required to inhibit the relative light units by 50% (IC50) was determined from semi-logarithmic dose–response plots, and the results represent means ± SD of triplicate samples.

Acknowledgements

We gratefully acknowledge the financial support of the Young Teachers Program of Dalian University of Technology. The bioactivity evaluation part of this work was supported by the National Science Foundation for Young Scientists of China (No. 21606036).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.013.