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NOTE

Biological synthesis and anti-inflammatory activity of arylalkylamine

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Abstract Hydroxycinnamic acid amides (HCAAs) are natural compounds with antifungal, anticancer, and anti-inflammatory activities. Extraction from plants and chemical synthesis have been the major approaches to obtain these compounds. We used a biological method to synthesize HCAA derivatives (arylalkylamines). Two genes, *SHT* encoding serotonin *N*-hydroxycinnamoyl transferase and 4CL encoding 4-coumaroyl-CoA ligase, were introduced into *Escherichia coli*. Using this *E. coli* transformant as a biocatalyst, 24 arylalkylamines were synthesized. The anti-inflammatory activities of five synthesized compounds, including *N*-*p*-coumaroyl phenethylamine, *N*-caffeoyl phenethylamine, *N*-*p*-coumaroyl 3-phenylpropylamine, *N*-*p*-coumaroyl 4-phenylbutylamine, and *N*-*p*-coumaroyl 4-methoxyphenethylamine, were measured. Among them, *N*-*p*-coumaroyl 4-phenylbutylamine showed the best anti-inflammatory activity.

Keywords Anti-inflammatory activity · Hydroxycinnamic acid amides · Metabolic engineering

Introduction

Hydroxycinnamic acids (HCAs; also known as phenyl-propanoids) are the major constituents of diverse phenolic compounds found in plants, including lignin, flavonoids, and tannins [1]. HCAs also form conjugates with other molecules such as amines, sugars, and organic acids. HCA amides (HCAAs) are formed by the reaction between HCAs and amines such as phenethylamine, tyramine, tryptamine, or dopamine [2, 3]. HCAAs have been isolated from plants and their structures were determined [2, 4]. Some phenolic amides, such as dihydro-*N*-caffeoyltyramine trans-*N*-feruloyloctopamin, trans-*N*-caffeoyltyramine, and cis-*N*-caffeoyltyramine, exhibit antifungal activity [5]. Furthermore, the production of some phenolic HCAAs is increased upon infection of pathogens and wounding. Therefore, they are considered to mediate host defenses [6, 7].

The biological synthesis of HCAAs is mediated by BAHD (benzyl alcohol *O*-acetyltransferase, anthocyanin *O*-hydroxycinnamoyltransferase, *N*-hydroxycinnamoyl/ benzoyltransferase, and deacetylvindoline 4-*O*-acetyl-transferase) family enzymes [8]. Phenolic amides, such as conjugates between HCAs and phenethylamine or tyramine, are synthesized by tyramine hydroxycinnamoyl transferase [9] and serotonin hydroxycinnamoyl transferase (SHT) [10], both of which are *N*-hydroxycinnamoyl/ben-zoyltransferases. Tyramine hydroxycinnamoyl transferase and SHT use several acyl group donors and acceptors. For example, SHT uses diverse hydroxycinnamoyl-CoAs such as caffeoyl-, feruloyl-, *p*-coumaroyl-, and cinnamoyl-CoA as donors. Serotonin is the best acceptor followed by phenethylamine, dopamine, tryptamine, and tyramine when feruloyl-CoA is used as the acyl donor [10]. Activation of acyl donors is achieved by attaching coenzyme A (CoA) to HCA via 4-coumaroyl-CoA ligase (4CL). 4CL from *Arabidopsis thaliana* and *Oryza sativa* uses various HCAs as substrates [11, 12]. These previous studies suggested that diverse amine conjugates could be synthesized by taking advantage of the promiscuity of these enzymes.

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HCAAs show several biological activities including antioxidant, antifungal, anticancer, and anti-inflammatory effects [5, 13–15]. They are isolated from plants and some found in wolfberry or corn bran, such as *N*-cafferoyl tyramine, *N*-caffeoyl dopamine, *N*-feruloyl phenethylamine, dicoumaroyl-putrescine, and diferuloylputrescine, exhibit anti-inflammatory properties [2, 16, 17]. HCAAs can also be synthesized chemically and their anti-inflammatory activities have been evaluated [18].

In this report, we have described the synthesis of 24 arylalkylamines using engineered *Escherichia coli*. Among these 24 compounds, the anti-inflammatory activities of five (*N*-*p*-coumaroyl 4-methoxyphenethylamine, *N*-*p*-coumaroyl 3-phenylpropylamine, and *N*-*p*-coumaroyl 4-phenylbutylamine) were examined.

Materials and methods

Chemicals

4-Methoxyphenethylamine, 3-phenylpropylamine, 4-phenylbutylamine, and phenethylamine were purchased from Sigma–Aldrich (Gyeonggi-do, Republic of Korea). HCA derivatives (*p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, caffeic acid, ferulic acid, cinnamic acid, 3-methoxycinnamic acid, 4-methoxycinnamic acid, 2,4-dimethoxycinnamic acid, 3,4-dimethoxycinnamic acid, sinapic acid, and 3,4,5-trimethoxycinnamic acid) were also purchased from Sigma–Aldrich.

Curcumin and lipopolysaccharide (LPS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Penicillin/streptomycin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum and Dulbecco's phosphate-buffered saline were obtained from Gibco (Grand Island, NY, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA) kit was obtained from BD Biosciences (Franklin Lakes, NJ, USA).

Synthesis of chemicals

4CL and SHT were cloned into the pCDF duet vector as reported previously (pC-4CL-SHT) [19]. The resulting construct was transformed into *E. coli* BL21 (DE3). After overnight culture, the transformant was inoculated into fresh Luria–Bertani broth containing 50 μg/mL spectinomycin. The transformant was grown at 37 °C to an absorbance value of 1.0 at 600 nm. Genes in the transformant were induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside to the culture medium, which was then incubated at 18 °C for 16 h. The cells were harvested and resuspended in M9 medium. To examine the relative conversion rate of each compound, 1 mM substrate was added to the medium and it was incubated at 30 °C.

The reaction products were analyzed using high-performance liquid chromatography [19, 20]. The structures of *N*-HC phenethylamine derivatives were determined using proton nuclear magnetic resonance (NMR) spectroscopy [21]. Spectral features were: *N*-*p*-coumaroyl 4-methoxyphenethylamine, ^{1}H NMR δ (ppm) in acetone-*d*6: 2.80 (2H, *m*, H-10), 3.49 (2H, *m*, H-9), 3.75 (3H, s, H-14), 6.46 (1H, *d*, $J = 15.7$ Hz, H-6), 6.85 (4H, *d*, $J = 8.5$ Hz, H-2, H-13), 7.15 (2H, *d*, $J = 8.6$ Hz, H-12), 7.41 (2H, *d*, $J = 8.6$ Hz, H-3), 7.45 (1H, *d*, $J = 16.0$ Hz, H-5); *N*-*p*-coumaroyl 3-phenylpropylamine, ^{1}H NMR δ (ppm) in acetone-*d*6: 1.85 (2H, quintet, $J = 7.5$ Hz, H-10), 2.67 (2H, *t*, $J = 7.7$ Hz, H-11), 3.49 (2H, *m*, H-9), 6.47 (1H, *d*, $J = 15.7$ Hz, H-6), 6.85 (2H, *d*, $J = 8.7$ Hz, H-2), 7.16 (1H, *m*, H-15), 7.25 (4H, *m*, H-13, H-14), 7.42 (2H, *d*, $J = 8.6$ Hz, H-3), 7.45 (1H, *d*, $J = 15.6$ Hz, H-5); *N*-*p*-coumaroyl 4-phenylbutylamine, ^{1}H NMR δ (ppm) in acetone-*d*6: 1.57 (2H, *m*, H-10), 1.66 (2H, *m*, H-11), 2.64 (2H, *t*, $J = 7.6$ Hz, H-12), 3.33 (2H, *m*, H-9), 6.46 (1H, *d*, $J = 15.8$ Hz, H-6), 6.85 (2H, *d*, $J = 8.6$ Hz, H-2), 7.15 (1H, *m*, H-16), 7.20 (2H, *m*, H-14), 7.25 (2H, *m*, H-15), 7.41 (2H, *d*, $J = 8.6$ Hz, H-3), 7.44 (*d*, $J = 16.0$ Hz, H-5). Quantification of each product was carried out using *p*-coumaric acid as a standard. Triplicate experiments were performed, and error bars indicate the standard deviations.

Cell viability

The murine macrophage cell line (RAW 264.7) was maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and penicillin/streptomycin at 37 °C under a humidified atmosphere of 5% CO_2. RAW 264.7 cells were plated in 96-well plates at a density of 3×10^4 cells per well 24 h prior to treatment. Five different compounds [*N*-*p*-coumaroyl phenethylamine, *N*-caffeoyl phenethylamine, *N*-*p*-coumaroyl 3-phenylpropylamine, *N*-*p*-coumaroyl 4-phenylbutylamine, and *N*-*p*-coumaroyl 4-methoxyphenethylamine; (Table 1)] were added to the cells at 0, 1, 10, and 25 μM. After incubation for 24 h, the culture medium was removed completely and CCK-8 solution was added 1:10 (v/v) with fresh culture medium. After further incubation for 1 h at 37 °C, the absorbance of the supernatant was measured at 450 nm using a SpectraMAX microplate reader (Molecular Devices, Sunnyvale, CA, USA) according to manufacturer's protocol. To examine cell viability in detail, two compounds were selected (*N*-*p*-coumaroyl 4-methoxyphenethylamine and *N*-*p*-coumaroyl 4-phenylbutylamine) and added to cells at 0, 1, 2, 5, and 10 μM. After 24 h incubation, cell viability was assessed as described above.

Table 1 Concentrations of compounds used for assessing cell viability and anti-inflammatory activity

	Full name	Final concentration (μM)	Final concentration of DMSO (%)
Non	No treat	–	–
Cur	Curcumin	10	0.1
I	*N-p*-coumaroyl 4-phenylbutylamine	10	0.01
II	*N*-caffeoyl phenethylamine	1	0.001
III	*N-p*-coumaroyl 3-phenylpropylamine	10	0.1
IV	*N-p*-coumaroyl 4-phenylbutylamine	1	0.01
V	*N-p*-coumaroyl 4-methoxyphenylethylamine	1	0.1

Cytokine release study

RAW 264.7 cells were plated in 6-well plates at a density of 6×10^5 cells per well 24 h prior to treatment. After cells were pre-incubated with each of six compounds (curcumin, *N-p*-coumaroyl phenethylamine, *N*-caffeoyl phenethylamine, *N-p*-coumaroyl 3-phenylpropylamine, *N-p*-coumaroyl 4-phenylbutylamine, and *N-p*-coumaroyl 4-methoxyphenethylamine) for 6 h at different concentrations as shown in Table 1, LPS (1 μg/mL) was added to the cells for 18 h. The cells were then pelleted by centrifugation at 13,000 rpm for 10 min at 4 °C. The amount of TNF-α released into the supernatants was measured using a mouse TNF-α ELISA kit according to manufacturer's protocol. To examine TNF-α induction in detail, two compounds (*N-p*-coumaroyl 4-methoxyphenethylamine and *N-p*-coumaroyl 4-phenylbutylamine) were added to cells at 1, 2, 5, and 10 μM for 6 h prior to co-incubation with LPS (100 ng/mL) for a further 18 h. Released TNF-α levels were measured quantitatively by an ELISA as described above.

Results and discussion

Biological synthesis of arylalkylamines

E. coli harboring pC-4CL-SHT synthesized six different HCA-phenethylamine derivatives from eight HCAs tested [19]. This indicated that SHT and 4CL used diverse substrates. We investigated whether SHT could use phenethylamine derivatives. To examine whether *E. coli* harboring pC-4CL-SHT could use 4-methoxyphenethylamine, 3-phenylpropylamine, and 4-phenylbutylamine as acyl group acceptors, and *p*-coumaric acid as an acyl group donor to produce arylalkylamines, the culture filtrate was analyzed using high-performance liquid chromatography. A new peak was observed from each reaction combination (Fig. 1). The molecular masses of the products of 4-methoxyphenethylamine, 3-phenylpropylamine, and 4-phenylbutylamine were 297.666, 281.621, and 295.643 Da, respectively. These were the expected molecular masses of each product. We also determined the structure of each product using NMR (see below).

Twelve acyl group donors (HCA derivatives) along with three phenethylamine derivatives were also tested as substrates. One acyl group acceptor and one acyl group donor were fed at a time to *E. coli* expressing pC-4CL-SHT. Among the 36 donor and acceptor combinations tested, over 27 products were synthesized. Each acyl group acceptor showed a similar preference; HCA derivatives containing 4-hydroxy or 4-methoxy were better substrates than those containing 2-hydroxy, 3-hydroxy, or 3-methoxy groups. HCAs containing more than two methoxy groups (2,4-dimethoxycinnamic acid, 3,4-dimethoxycinnamic acid, sinapic acid, and 3,4,5-trimethoxycinnamic acid) were poor substrates (Table 2).

To confirm that the reaction products had the expected structures, the structures of three compounds (each acyl acceptor and *p*-coumaric acid conjugate) were determined using NMR. Three compounds were synthesized by feeding *p*-coumaric acid and each amine at 1 mM. We synthesized 799.1 μM *N-p*-coumaroyl 4-methoxyphenethylamine, 730.2 μM *N-p*-coumaroyl 3-phenylpropylamine, and 868.3 μM *N-p*-coumaroyl 4-phenylbutylamine.

The synthesized compounds were purified as described in An et al. [22]. The structure of each compound was determined using NMR. In the ¹H NMR spectrum of *N-p*-coumaroyl 4-methoxyphenethylamine, there were eight proton peaks. One was a methoxy signal (3.75 ppm, *s*, 3H) and two showing an aryl region were H-9 and H-10. Five peaks were evident in the aromatic region. Among them, two at 7.45 and 6.46 ppm showed doublets with approximately 16 Hz of coupling constants. Therefore, they were easily assigned as H-5 and H-6. Integration of the other three peaks at 7.41, 7.15, and 6.85 ppm revealed they were 2H, 2H, and 4H, respectively. All eight protons of the phenyl groups were evident. The ¹H NMR assignments of

Fig. 1 High-performance liquid chromatograms of synthesized arylalkylamines. (*A*) Four substrates were used for the synthesis of arylalkylamines: 4 M, 4-methoxyphenethylamine; *3P*, 3-phenylpropylamine; *4P*, 4-phenylbutylamine; and CA, *p*-coumaric acid. (*B*) Reaction product (P1) from *p*-coumaric acid and 4-methoxyphenethylamine. (*C*) Reaction product (P2) from *p*-coumaric acid and 3-phenylpropylamine. (*D*) Reaction product (P3) from *p*-coumaric acid and 4-phenylbutylamine
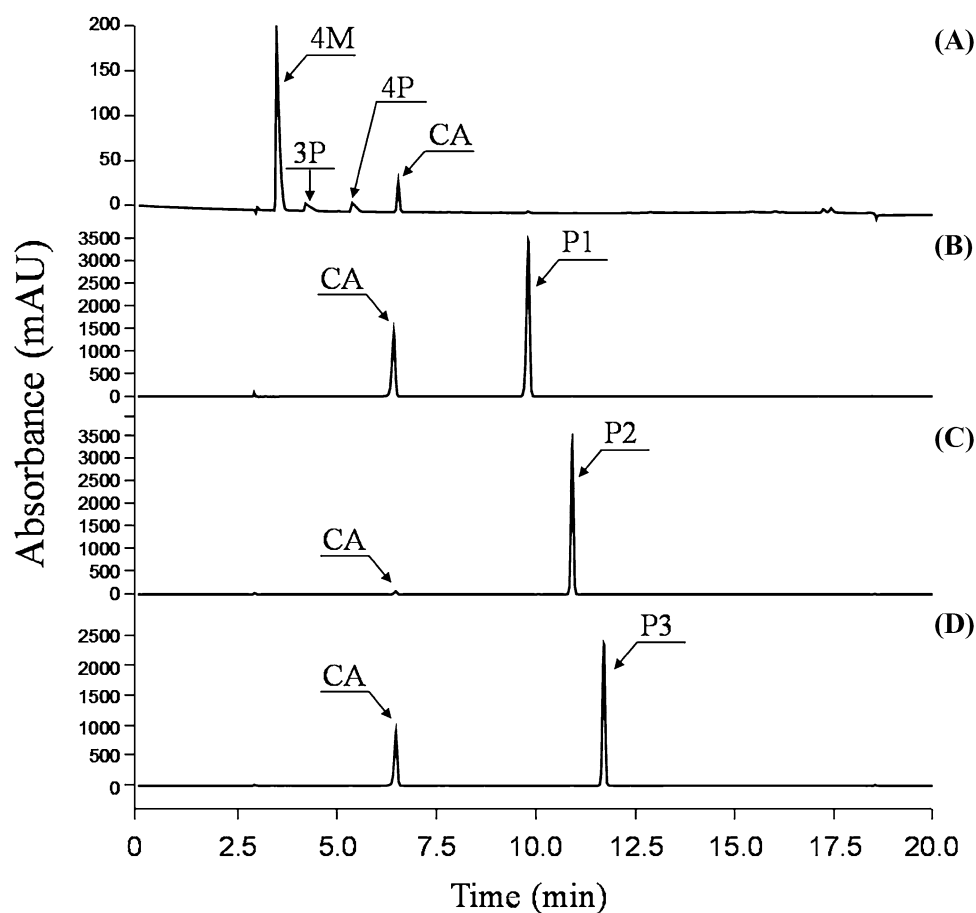
Table 2 Percentage conversion rates of various hydroxycinnamic acids upon reaction with 4-methoxyphenethylamine, 3-phenyl-1-propylamine, and 4-phenylbutylamine using *E. coli* harboring pC-4CL-SHT

	4-Methoxyphenethylamine	3-Phenylpropylamine	4-Phenylbutylamine
p-coumaric acid	75.7	98.5	79.8
m-coumaric acid	68.5	78.4	49.8
o-coumaric acid	56.3	72.2	21.6
Caffeic acid	56.2	62.9	53.8
Ferulic acid	98.9	100	100
Cinnamic acid	71.8	87.5	79.5
3-Methoxycinnamic acid	100[a]	99.5	99.3
4-Methoxycinnamic acid	28.1	22.5	21.1
2,4-Dimethoxycinnamic acid	2.4	1.7	2.3
3,4-Dimethoxycinnamic acid	3.3	1.9	3.4
Sinapic acid	0	0	0
3,4,5-Trimethoxycinnamic acid	0	0	0

[a] The conversion rate was the percentage of arylalkylamines synthesized for 3 h from 1 mM of substrates.

N-p-coumaroyl 3-phenylpropylamine and *N-p*-coumaroyl 4-phenylbutylamine were the same as above. The four peaks of H-2, H-3, H-4, and H-5 exactly overlapped with those of *N-p*-coumaroyl 4-methoxyphenethylamine.

Therefore, structures of the three reaction products were determined to be *N-p*-coumaroyl 4-methoxyphenethylamine, *N-p*-coumaroyl 3-phenylpropylamine, and *N-p*-coumaroyl 4-phenylbutylamine, respectively.

Toxicity and anti-inflammatory activity of five synthesized compounds

We tested the toxicity and anti-inflammatory activity of the five synthesized compounds (N-p-coumaroyl phenethylamine, N-caffeoyl phenethylamine, N-p-coumaroyl 3-phenylpropylamine, N-p-coumaroyl 4-phenyl butylamine, and N-p-coumaroyl 4-methoxyphenethylamine) by the CCK-8 assay and measuring TNF-α release, respectively. None of the compounds showed any significant cytotoxicity up to 10 μM (Fig. 2A). N-p-Coumaroyl phenethylamine, N-caffeoyl phenethylamine, and N-p-coumaroyl 4-methoxyphenethylamine showed negligible inhibition of TNF-α release following treatment with 1 μg/mL LPS at concentrations of 10 and 1 μM. However, N-p-coumaroyl 3-phenylpropylamine and N-p-coumaroyl 4-phenylbutylamine showed approximately 24.6 and 22.6% inhibition at 10 and 1 μM, respectively (Fig. 2B).

We further investigated the effects of N-p-coumaroyl 4-phenylbutylamine and N-p-coumaroyl 4-methoxyphenethylamine using four different concentrations of each compound to test their cytotoxicity and the anti-inflammatory activity at a lower LPS concentration (100 ng/mL). Neither compound was cytotoxic up to 10 μM (Fig. 2C). The amount of TNF-α released was decreased to 68.0 ± 3.2 and $82.1 \pm 3.9\%$ by treatment with 10 μM N-p-coumaroyl 4-phenylbutylamine and N-p-coumaroyl 4-methoxyphenethylamine, respectively (Fig. 2D).

To date, most studies of HCAA involved changing the side chain in phenolic amides and using these compounds to test several activities. For example, Lee et al. [18] synthesized 15 HCAA amides, all of which had different side chains on the phenol ring, and tested their anti-inflammatory activities. Wolfberry contains diverse HCAAs, and 15 were chemically synthesized. Five of the
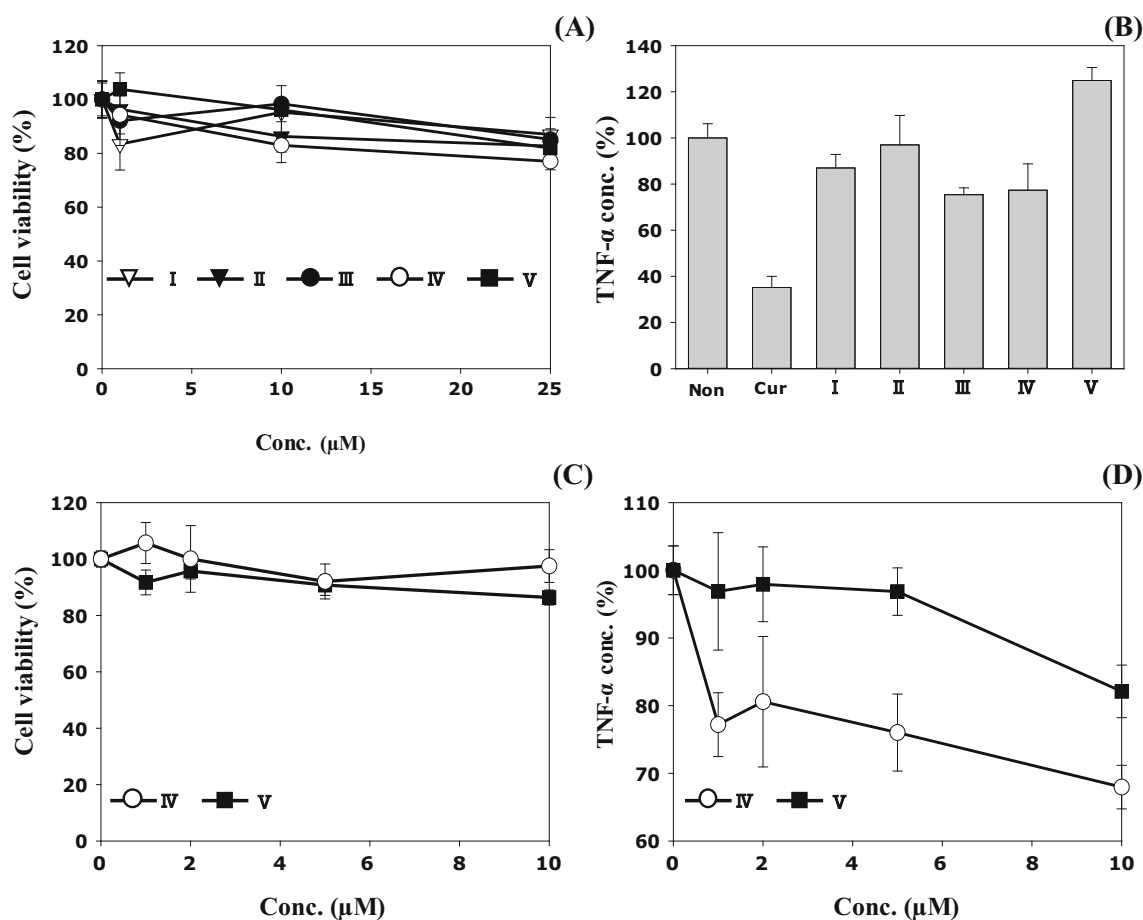
Fig. 2 Cytotoxicity and anti-inflammatory activity of five arylalkylamines. (A) Cell viability after treatment with the arylalkylamines. (B) Tumor necrosis factor (TNF)-α release following treatment with 1 μg/mL lipopolysaccharide and the arylalkylamines. Non, no treatment; cur, 10 μM curcumin; I, 10 μM N-p-coumaroyl 4-phenylbutylamine; II, 1 μM N-caffeoyl phenethylamine; III, 10 μM N-p-coumaroyl 3-phenylpropylamine; IV, 1 μM N-p-coumaroyl 4-phenyl butylamine; V, 1 μM N-p-coumaroyl 4-methoxyphenylethylamine. (C) Cell viability after treatment with N-p-coumaroyl 4-phenylbutylamine (IV) and N-p-coumaroyl 4-methoxyphenylethylamine (V) at different concentrations. (D) Tumor necrosis factor (TNF)-α release following treatment with 100 ng/mL lipopolysaccharide and N-p-coumaroyl 4-phenylbutylamine (IV) and N-p-coumaroyl 4-methoxyphenylethylamine (V) at different concentrations

synthesized HCAAs showed anti-inflammatory activity [17]. These HCAAs had diverse amine moieties such as tyramine, dopamine, and phenethylamine, and higher concentrations (25 and 50 μM) of each compound were used. Biological synthesis of arylalkylamines has been tried previously [19, 23], but these studies were limited to synthesizing arylalkylamines that had diverse phenolic side chains. In the current study, we changed not only the phenolic side chain in the amines, but also the carbon chain length of the amines using engineered *E. coli,* and tested their anti-inflammatory activity. This was the first attempt to alter the chain length of the phenolic moiety in the amine.

Previous studies showed that caffeic and ferulic acid moieties of HCAAs showed better anti-inflammatory activity than *p*-coumaric acid. Our study revealed that increasing the length of the alkyl group of the phenyl amine, such as 4-phenylbutylamine, yielded a better anti-inflammatory compound. Our initial screening involved synthesizing *N*-feruloyl 4-phenylbutylamine, and *N*-cafferoyl 4-phenylbutylamine. The anti-inflammatory activity of these compounds is intriguing and should be examined further.

Author contribution Authors M. K. Song and S. J. Lee contributed equally to this work.

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