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Full paper

Curcumin and its demethoxy derivatives possess p300 HAT inhibitory activity and suppress hypertrophic responses in cardiomyocytes

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ABSTRACT

The natural compound, curcumin (CUR), possesses several pharmacological properties, including p300-specific histone acetyltransferase (HAT) inhibitory activity. In our previous study, we demonstrated that CUR could prevent the development of cardiac hypertrophy by inhibiting p300-HAT activity. Other major curcuminoids isolated from *Curcuma longa* including demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) are structural analogs of CUR. In present study, we first confirmed the effect of these three curcuminoid analogs on p300-HAT activity and cardiomyocyte hypertrophy.

Our results showed that DMC and BDMC inhibited p300-HAT activity and cardiomyocyte hypertrophy to almost the same extent as CUR. As the three compounds have structural differences in methoxy groups at the 3-position of their phenol rings, our results suggest that these methoxy groups are not involved in the inhibitory effects on p300-HAT activity and cardiac hypertrophy. These findings provide useful insights into the structure–activity relationship and biological activity of curcuminoids for p300-HAT activity and cardiomyocyte hypertrophy.

Introduction

As morbidity and mortality rates for heart failure remain high in developed countries, new therapies for heart failure are required to reduce these rates and the cost of their associated medical care. The heart works as a pump, circulating blood via its rhythmic contractions. The cardiomyocytes that constitutes the heart have the ability to proliferate in the fetal stage but lose this ability after birth. Various kinds of hemodynamic stress that affect the heart, such as hypertension and myocardial infarction, lead to cardiomyocyte hypertrophy, in an attempt to maintain cardiac function. However, this compensatory response of cardiomyocytes degrades and cardiac function diminishes, leading to heart failure.[1,2] The established drugs for heart failure, such as β-blockers, angiotensin-converting enzyme inhibitors, and angiotensin II receptor blockers, target neurohumoral factors and receptors on the cell membrane.[3,4] However, even with the use of these drugs, patients with severe heart failure have a short life expectancy,[5] and new therapies for heart failure are urgently required.

Signals of hemodynamic stress finally reach the common nuclear pathways of cardiomyocytes and activated a transcriptional coactivator, p300. The histone acetyltransferase (HAT) activity of

Abbreviations: CUR, curcumin; HAT, histone acetyltransferase; DMC, demethoxycurcumin; BDMC, bisdemethoxycurcumin; DMSO, dimethyl sulfoxide; GST, glutathione-S-transferase; PE, phenylephrine; SS, salt saline; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide.

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p300 has been found to play a crucial role in the progression of cardiac hypertrophy and the development of heart failure. Therefore, HAT activity of p300 is a potential new therapeutic target for heart failure.[6,7] Balasubramanyam et al. reported that curcumin (CUR), derived from the plant *Curcuma longa*, is a specific inhibitor of p300-HAT activity.[8] A polyphenol, CUR has long been used in traditional Ayurvedic and Chinese herbal medicines and as a spice in Indian and Chinese food. In previous studies, we demonstrated that CUR significantly attenuated cardiomyocyte hypertrophy, diminished cardiac dysfunction, and prevented the development of heart failure in two different heart failure animal models.[9,10] These findings suggested that CUR, an inexpensive and safe polyphenol, might serve as a new therapy for heart failure.

CUR (1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione) is a natural compound made up of 2 hydroxyl groups, 2 phenyl groups, 2 methoxy groups, and a β-diketone moiety. The β-diketone moiety undergoes keto–enol tautomerism, and CUR often occurs in an enol form when a liquid state.[11,12] In addition to CUR, curcuminoids include demethoxycurcumin (DMC), which has a single methoxy group, and bisdemethoxycurcumin (BDMC), which has no methoxy groups (Fig. 1). Typical commerically available curcuminoids consist of 77% CUR, 17% DMC, and 3% BDMC.[13] Like CUR, DMC and BDMC also have various physiological activities, such as antioxidant,[14–16] anticancer,[17–19] and anti-inflammatoryactivities.[16,17,19] However, studies have revealed that the potency of these activities differs among CUR, DMC, and BDMC. Nevertheless, these studies have not examined the effect of different curcuminoids on p300 HAT activity and hypertrophic response. In this study, we performed a comparative study of curcuminoids to determine their ability to inhibit p300 HAT activity and hypertrophic response.

Material and methods

Material

CUR, DMC, and BDMC were purchased from Nagara Science Corporation (Gifu, Japan). These curcuminoids were prepared as 100 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at −20 °C.

Measurement of p300 HAT activity in vitro

Gene fragments containing the catalytic domain of human p300-HAT (amino acids 1284–1673) were subcloned into pGEX-6p1 (GE Healthcare, USA). Glutathione-S-transferase (GST) fusion protein was prepared from the BL21 strain of *E. coli* and purified by affinity chromatography using glutathione Sepharose 4B. Five micrograms of core histones from Calf Thymuscarf (Worthington, USA) was incubated in HAT assay buffer (50 mM Tris–HCl of pH 8.0, 10% (v/v) glycerol, 1 mM dithiothreitol, and 0.1 mM EDTA-Na^2 of pH 8.0) at room temperature for 30 min with or without the recombinant p300-HAT domain in the presence or absence of curcuminoids. Then, acetyl-CoA was added and the mixtures were further incubated for another 30 min. The reaction mixture was subjected to SDS-PAGE followed by Western immunoblotting using rabbit polyclonal anti-acetyl-H3K9 antibody and rabbit polyclonal anti-histone H3 antibody (Cell Signaling Technology, USA). The signals were detected with a C-DiGit Chemiluminescent Western Blot Scanner (LI-COR, USA). The ratio of acetylated histone H3K9 to total histone H3 was quantified using Image Studio LITE software (LI-COR, USA).

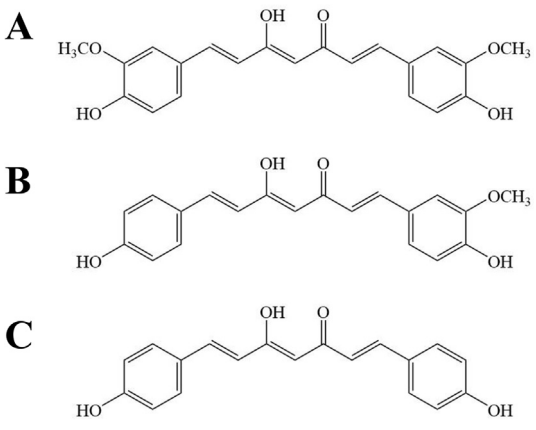

Fig. 1. Structure of curcuminoids. A, curcumin (CUR). B, demethoxycurcumin (DMC). C, bisdemethoxycurcumin (BDMC).

Cell culture and histone acetylation analysis

All procedures were performed in conformity with the Guide for the Care and Use of Laboratory Animals of the University of Shizuoka and the protocol was approved by the Institutional Animal Care and Use Committee of the University of Shizuoka. Isolation of primary neonatal rat cardiomyocytes has been previously described.[20] Cardiomyocytes were pretreated with either 10 μM curcuminoids or DMSO as its solvent for 2 h, and then stimulated with 30 μM phenylephrine (PE) or salt saline (SS) as negative control. Forty-eight hours after incubation, the core histones from these cells were isolated by acid extraction as previously described.[21] The samples were subjected to SDS-PAGE, followed by western blotting.

RNA extraction and quantified real-time PCR (RT-PCR)

Total RNA from cardiomyocytes was isolated, reverse-transcribed, and amplified as previously described.[22] Briefly, total RNA from cardiomyocytes was isolated using TRI Reagent (MRC Inc, USA) and subjected to reverse transcription using a ReverTra Ace qPCR RT Master Mix (TOYOBO, Japan). RT-PCR was performed as previously described.[22] An ABI 7500 real-time PCR system (Applied Biosystems, USA) was used with SYBR Green (TOYOBO, Japan) for the purpose. The primer pairs of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and 18S have been previously described.[23–25] Values were normalized to 18S and represented using the ΔCt method.

Measurement of cardiomyocyte surface area

Cardiomyocytes cultured in flask-style chambers with glass slides (Thermo, USA) were fixed in 3.7% paraformaldehyde and stained with anti-β-myosin heavy chain (β-MHC) antibody (Leica, USA) as previously described.[26] Then, the surface areas of 50 cardiomyocytes were measured semiautomatically using an image analyzer (Image J v1.46).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were performed by analysis of variance (ANOVA), followed by Tukey's multiple-comparison test; $p < 0.05$ was considered statistically significant.

Results

To evaluate the inhibitory effect of curcuminoids against p300-HAT activity, an *in vitro* HAT assay was performed using recombinant p300-HAT domain with 20 and 60 μM of CUR, DMC, and BDMC, and after 2 h, samples were subjected to immunoblotting with the antibody against acetylated forms of histone H3K9 or total histone 3 (Fig. 2A). The results showed that p300-induced acetylation of H3K9 was dose-dependently suppressed by CUR, DMC, and BDMC treatments. High dose (60 μM) of DMC and BDMC significantly inhibited the acetylation of H3K9, and to the same extent as those of CUR. These findings indicate that these three curcuminoids have similar inhibitory effects on p300 HAT activity *in vitro*.

Next, to examine the effect of curcuminoids on PE-induced histone acetylation in primary cardiomyocytes prepared from neonatal rats, the cells were stimulated with saline or 30 μM PE, an α1-adrenergic agonist, in the presence or absence of curcuminoids (10 μM), and after 48 h, protein extracts from these cells were subjected to immunoblotting with the antibody against acetylated forms of H3K9 (Fig. 3A). The results showed that DMC and BDMC significantly repressed PE-induced acetylation of H3K9 to the same extent as CUR although CUR, DMC, and BDMC did not affect the basal level of H3K9-acetylation in cardiomyocytes.

Finally, to examine the effects of curcuminoids on hypertrophic responses in primary cardiomyocytes, these cells were stimulated with saline or 30 μM PE in the presence or absence of curcuminoids (10 μM), and after 48 h, the cells were stained with an antibody against cardiac MHC (Fig. 4A). The results showed that CUR, DMC, and BDMC treatments did not change morphology of cardiomyocyte, such as cell surface are and myofibrillar organization.

CUR, DMC, and BDMC significantly inhibited the PE-induced increase in myocardial cell-surface area to a similar extent (Fig. 4B). After that, the effects of curcumioids on PE-induced expression of ANF and BNP were examined using real-time RT-PCR. The upregulated expression of them is a well-established marker of myocardial cell hypertrophy. DMC and BDMC significantly inhibited the PE-induced up-regulation of the expression of endogenous ANF (Fig. 4C) and BNP (Fig. 4D) mRNAs, and to the same extent as CUR.

Discussion

Our previous studies have shown both that the HAT activity of p300 is a pharmacological target for heart failure therapy, and that CUR is an inhibitor of the HAT activity of p300. In the present study, we examined the structure–activity relationship of CUR in p300 HAT activity. Three curcuminoids with different structures were

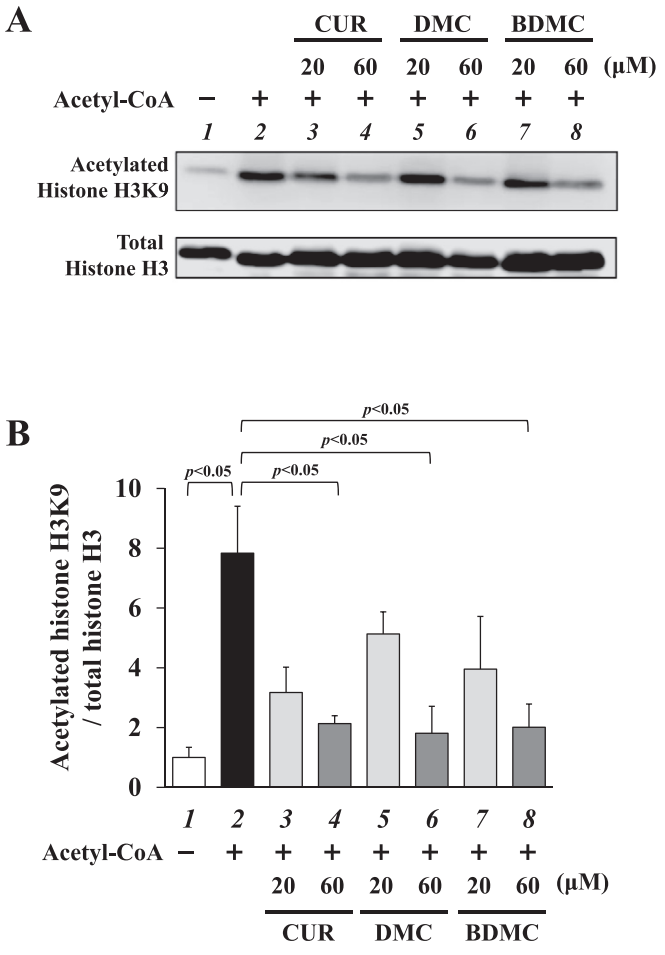

Fig. 2. Curcuminoids inhibit p300-HAT activity *in vitro*. A, *In vitro* HAT assays were performed with a recombinant p300 HAT domain in the presence of CUR, DMC, and BDMC (20, 60 μM) using core histones (2 μg). Acetylated histone H3K9 and total histone H3 were detected by western blotting. B, The ratio of acetylated histone H3K9 to total histone H3 was quantified by densitometry with the use of Image Studio LITE software (LI-COR). The data are shown as mean ± SEM. All data were based on three independent experiments.

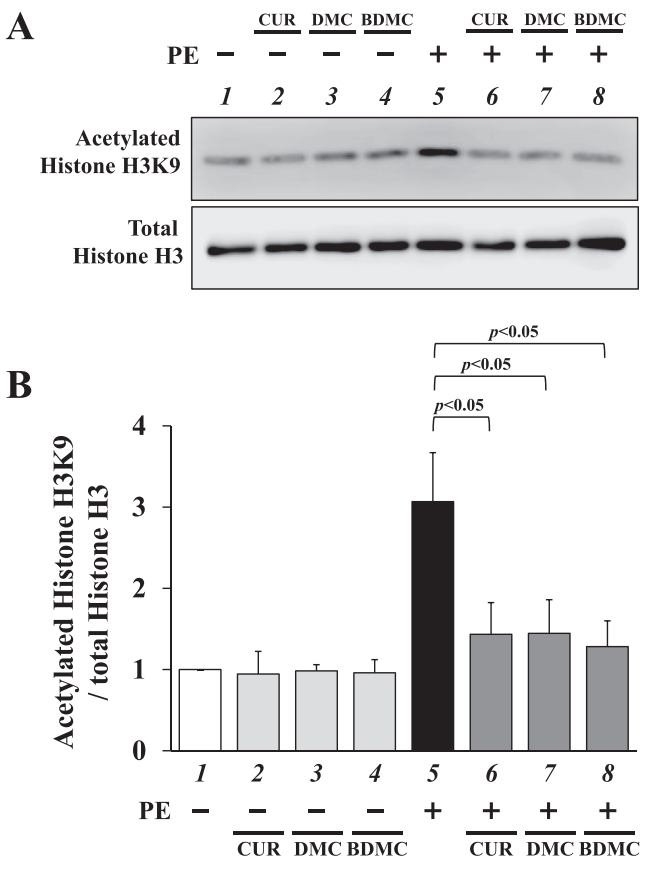

Fig. 3. Curcuminoids prevent phenylephrine-induced acetylation of histone H3K9 in cardiomyocytes. A, Cultured cardiomyocytes were treated with CUR, DMC, and BDMC (10 μM) in the presence or absence of PE (30 μM) for 48 h. Histone extracts from these cells were subjected to western blotting for acetylated histone H3K9 or total histone H3 as indicated. B, The amounts of acetylated H3K9 and total H3 were quantified. The data are shown as mean ± SEM. All data were based on three independent experiments.

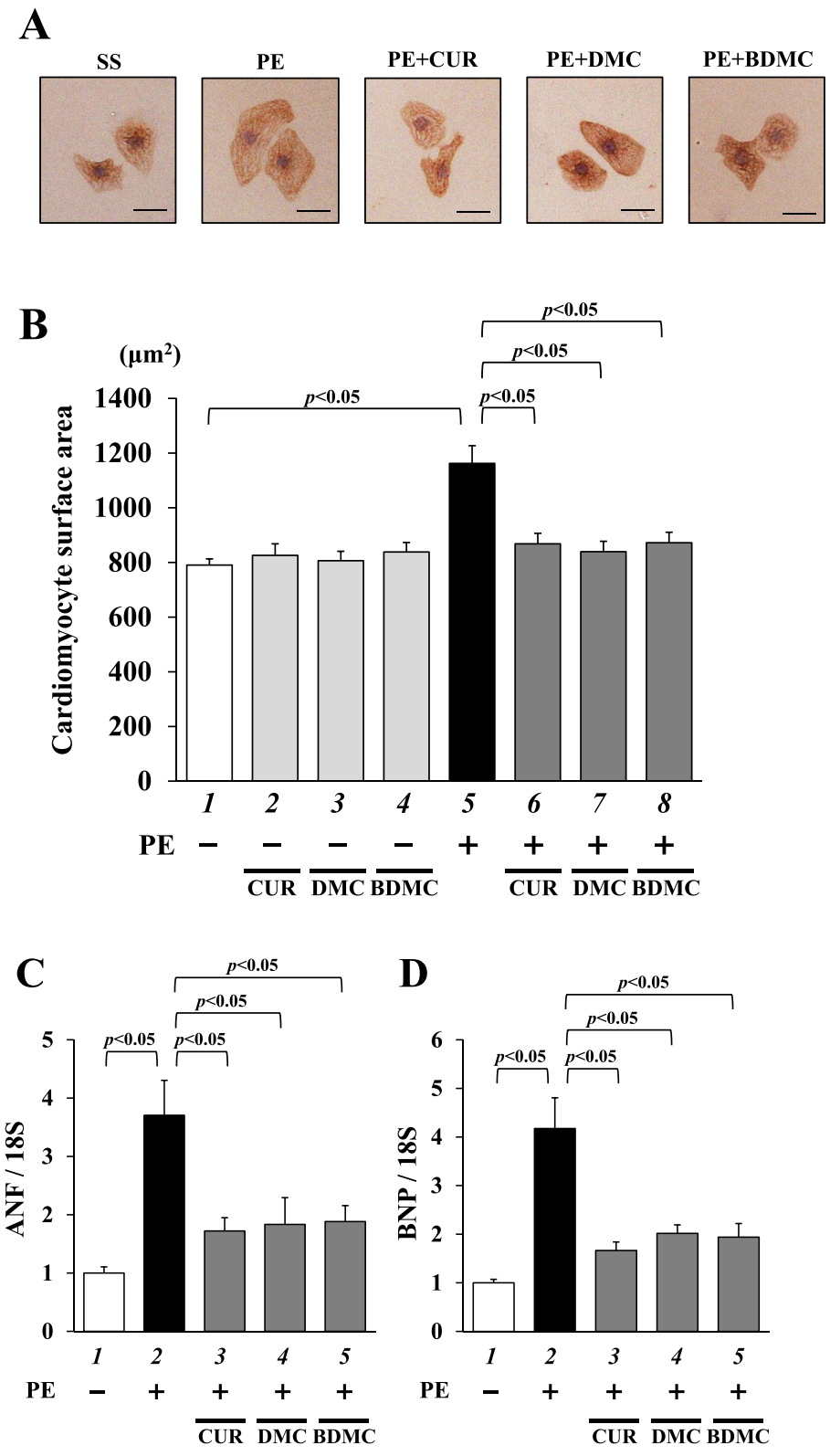

Fig. 4. Curcuminoids attenuate phenylephrine-induced hypertrophic responses in cardiomyocytes. A, Cardiomyocytes were incubated with CUR, DMC, and BDMC (10 μM) in the presence or absence of PE (30 μM) for 48 h. These cells were subjected to immunocytochemistry using the primary antibody against β-MHC, followed by staining with a secondary antibody conjugated with peroxidase (brown signals). B, The cell surface areas were quantified using ImageJ software. The data were based on three independent experiments and are presented as mean ± SEM. Scale bar indicates 20 μm. C and D, Cultured cardiomyocytes were stimulated with saline or PE (30 μM) in the presence of CUR, DMC, and BDMC (10 μM) for 48 h. Total RNA from these cardiomyocytes was subjected to real-time RT-PCR. The amounts of transcripts for the ANF (C) and BNP (D) genes were normalized by that of the 18S gene. The data were based on three independent experiments, each carried out in duplicate, and shown as mean ± SEM.

used to examine the effect of CUR on the inhibition of p300 HAT activity and hypertrophic responses. The results revealed no differences between CUR, DMC, and BDMC in terms of their inhibition of p300 HAT activity and hypertrophic responses. As these curcuminoids differ in the presence of absence of methoxy groups at the 3 position of their phenyl groups at either end of their molecules, it is safe to assume that these methoxy groups are not associated with the inhibition of p300-HAT activity or with the inhibition of hypertrophic responses.

Numerous studies have examined differences in the physiologic activity of these curcuminoids. A typical physiological activity of CUR is antioxidant activity. The structure–activity relationship of curcuminoids on antioxidant activity has been demonstrated.[14–16] Tetrahydrocurcumin, which hydrogenated the two double bonds conjugated with β-diketone of curcumin, was showed higher or comparable scavenging potency as curcumin toward all of the tested free radicals.[15] The unshared electron pair on the oxygen atoms in the hydroxyl group attached to both of the CUR phenyl groups readily delocalizes, so these hydroxyl groups are associated with antioxidant activity.[16] 5-chloro CUR, a CUR derivative with a chlorine atom replacing one of the oxygen atoms on β-diketone moiety, has stronger antioxidant activity than CUR or ascorbic acid.[27] These findings suggest that the antioxidant activity of CUR is attributable to the hydroxyl group attached to each phenyl group and the β-diketone moiety.

Differences have also been found between the three compounds in their anti-cancer and anti-inflammation activity. In an experiment inducing skin cancer in mice with the carcinogen 12-O-tetradecanoylphorbol-13-acetate (TPA), CUR and DMC inhibited carcinogenesis to the same extent, while BDMC had weaker inhibitory activity.[28] In addition, BDMC has been shown to inhibit cancer cell invasion to the same or a greater extent than DMC, but CUR inhibited cancer cell invasion to a lesser extent.[18] The Studies that have been done shown no differences in the ability of CUR, DMC, and BDMC to inhibit the growth of various types of cultured cancer cells. The inhibition of particular processes, such as carcinogenesis, proliferation, or metastasis, differs depending on the form of the curcuminoid, so the appropriate curcuminoid for cancer treatment may differ depending on the process that is being targeted.[19] On the other hand, CUR, DMC, and BDMC have also been shown to inhibit TPA-induced inflammation in the ears of mice to the same extent.[28] Nuclear factor-kappa B (NF-kB), which regulates various cytokines and plays a crucial role in inflammation, is most potently inhibited by CUR, followed by DMC, and then BDMC.[18,19] In summary, the 3′ methoxy groups of CUR are not associated with inhibition of p300 HAT activity, or inhibition of cardiomyocyte hypertrophy, suggesting the therapeutic effect of curcumin on heart failure may dependent p300-specific HAT inhibition.

p300-specific HAT inhibitors other than CUR have also been previously found. C646 was identified by *in silico* screening based on protein conformations.[29] C646 competes with acetyl-CoA to bind to p300, thus inhibiting its activity. CUR is unique in comparison to other HAT inhibitors. It binds to p300 at sites other than its sites of catalytic domain, altering the conformation of its HAT domain and inhibiting the binding of p300 to acetyl-CoA and substrates.[8] Thus, it is difficult to determine the structure–activity relationship of CUR based on conformation of the HAT domain. CUR is a β-diketone, and the β-diketone moiety of CUR results in its keto–enol tautomerism. Based on the results of the present study, the keto–enol tautomerism of curcumin, but not the methoxy group at the 3 position of the CUR phenyl group, may be associated with binding to the HAT domain of p300 and conformational changes at its sites of catalytic domain. These findings should be helpful for determining the structure–activity relationship of CUR and for developing a more specific and effective CUR derivative. Further experiments would be required to clarify whether β-diketone moiety of curcumin associate with the inhibition of p300-HAT activity and hypertrophic responses.

CUR, DMC, and BDMC exhibit strong or weak physiologic activity depending on the target molecule. The present study found no differences in the inhibition of p300 HAT activity by CUR, DMC, and BDMC or in the ability of those curcuminoids to inhibit hypertrophic responses. Because numerous factors are associated with the onset and progression of heart failure, further experiments should be conducted with animal models to investigate the differences between these curcuminoids. CUR has been found to be effective in preventing cardiac hypertrophy and the development of heart failure in animals, so CUR is expected to be applicable in clinical settings as well.[10,30–33] The present study has also revealed that the methoxy groups of CUR are not associated with the inhibition of p300 HAT activity, bringing us a step closer to elucidating the mechanism by which CUR inhibits p300. Further studies of the structure–activity relationship of CUR may lead to the development of novel therapies for heart failure.

Conflicts of interest

All authors declare no conflicts of interest associated with this manuscript.

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