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Original Research Article

Determination of activities of human carbonic anhydrase II inhibitors from curcumin analogs

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Abstract

Purpose: *To evaluate the activities of new curcumin analogs as carbonic anhydrase II (CA-II) inhibitor.*
Methods: *Carbonic anhydrase II (CA-II) inhibition was determined by each ligand capability to inhibit the esterase activity of CA-II using 4-NPA as a substrate in 96-well plates. Dimethyl sulfoxide was used to dissolve each curcumin analog compound, and then diluted with biological buffer. They were then mixed with CA-II solution and to start the reaction, 4-NPA was added. Hydrolysis of the substrate was evaluated at 405 nm after incubation for 2 h at 25 °C. The IC_{50} value of compounds with inhibitory activity higher than 40 % was then evaluated. Molecular docking was also used to predict enzyme-inhibitor interaction.*
Results: *Eight new curcumin analogs were potent to inhibit CA-II activity with IC_{50} values ranging from 7.92 ± 0.54 to 72.31 ± 2.21 µmol; the lowest value was exhibited by (3E,5E)-3,5-bis[(2-hydroxyphenyl)methylidene]piperidin-4-one (a1). Molecular docking analysis revealed that this molecule formed hydrogen bonds with Thr199, Thr200 and Gln92 at the active site of CA-II.*
Conclusion: *These curcumin analogs have inhibitory potential against CA-II; (3E, 5E)-3,5-bis[(2-hydroxyphenyl)methylidene]piperidin-4-one (a1) has the highest inhibitory activity and may be useful in the development of CA-II inhibitors for glaucoma treatment.*

Keywords: *Carbonic anhydrase II inhibitor, Curcumin analogs, Molecular docking*

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INTRODUCTION

The general function of carbonic anhydrase (CA) is to catalyze the reversible conversion of carbon dioxide and water to a bicarbonate ion and a proton [1–3]. Misregulation of this enzyme has been linked to many diseases, such as glaucoma, cancer, and epilepsy [4,5]. The isozyme CA-II is known to be one of the most efficient in CO_2 hydration [2]. Sulfonamide compounds, which are known as strong inhibitors of CA-II, have been used as commercial drugs to treat glaucoma [6]. However, these drugs produce many undesired side effects. Therefore, intensive search for novel drugs is ongoing, through synthesis of new derivatives of known drugs or from new molecular bases. Systematic search for new molecular bases is usually undertaken by exploring compounds from natural products [7,8].

Curcumin and related compounds show promising result as a molecular base on which to design novel inhibitors of CA-II [9]. Due to the

amounts and the variety of curcuminoids from natural sources are limited, methods of synthesizing curcumin and its analogs have been explored [10].

In the present study, 44 novel curcumin analogs, previously synthesized using microwave irradiation [10], were evaluated for their potential as inhibitors of human CA-II. The basic structure of all 44 curcumin analogs is depicted in Figure 1.

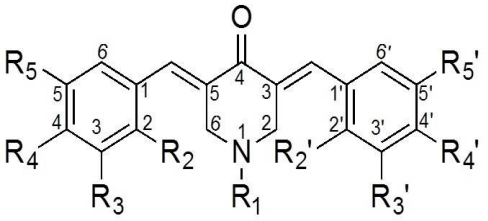

Figure 1: Basic structure of curcumin analogs

Table 1: List of ligand molecules

Compound	Structure	Inhibition (%)
a1	R_1=H, R_2=R_2'=OH	81.44
a2	R_1=H, R_2=R_2'=Cl	8.89
a3	R_1=H, R_2=R_2'=OCH_3	49.37
a4	R_1=H, R_3=R_3'=OH	11.43
a5	R_1=H, R_3=R_3'=Br	14.90
a7	R_1=H, R_3=R_3'=OCH_3	n.a.
a8	R_1=H, R_4=R_4'=OH	n.a.
a9	R_1=H, R_4=R_4'=Cl	n.a.
a10	R_1=H, R_4=R_4'=OCH_3	0.00
a11	R_1=H, R_4=R_4'=$N(CH_3)_2$	7.56
a12	R_1=H, R_2=R_3=R_2'=R_3'=OCH_3	0.68
a13	R_1=H, R_2=R_5=R_2'=R_5'=OCH_3	n.a.
a14	R_1=H, R_3=R_4=R_3'=R_4'=OCH_3,	0.00
a15	R_1=H, R_2=R_4=R_5=R_2'=R_4'=R_5'=OCH_3	0.00
a16	R_1=H, R_3=R_4=R_5=R_3'=R_4'=R_5'=OCH_3	0.00
b1	R_1=CH_3, R_2= R_2'=OH	0.00
b2	R_1=CH_3, R_2=R_2'=Cl	8.02
b3	R_1=CH_3, R_2=R_2'=OCH_3	0.00
b4	R_1=CH_3, R_3=R_3'=OH	17.71
b6	R_1=CH_3, R_5=R_5'=Br	18.42
b7	R_1=CH_3, R_3=R_3'=OCH_3	n.a.
b8	R_1=CH_3, R_4= R_4'=OH	3.79
b9	R_1=CH_3, R_4=R_4'=Cl	40.60
b10	R_1=CH_3, R_4=R_4'=OCH_3	0.00
b11	R_1=CH_3, R_4=R_4'=$N(CH_3)_2$	0.00
b12	R_1=CH_3, R_2=R_3=R_2'=R_3'=OCH_3	15.62
b13	R_1=CH_3, R_2=R_5=R_2'=R_5'=OCH_3	12.41
b14	R_1=CH_3, R_3=R_4=R_3'=R_4'=OCH_3	0.00
b15	R_1=CH_3, R_2=R_4=R_5=R_2'=R_4'=R_5'=OCH_3	14.85
b16	R_1=CH_3, R_3=R_4=R_5=R_3'=R_4'=R_5'=OCH_3	12.30
c1	R_1=C_7H_7, R_2=R_2'=OH	27.09
c2	R_1=C_7H_7, R_2=R_2'=Cl	22.20
c3	R_1=C_7H_7, R_2=R_2'=OCH_3	0.00
c4	R_1=C_7H_7, R_3=R_3'=OH	19.09
c6	R_1=C_7H_7, R_5=R_5'=Br	40.65
c7	R_1=C_7H_7, R_3=R_3'=OCH_3	48.66
c8	R_1=C_7H_7, R_4=R_4'=OH	19.09
c9	R_1=C_7H_7, R_4=R_4'=Cl	41.32
c10	R_1=C_7H_7, R_4=R_4'=OCH_3	58.50
c11	R_1=C_7H_7, R_4=R_4'= $N(CH_3)_2$	0.00
c12	R_1=C_7H_7, R_2=R_3=R_2'=R_3'=OCH_3	53.45
c13	R_1=C_7H_7, R_2=R_5=R_2'=R_5'=OCH_3	43.25
c14	R_1=C_7H_7, R_3=R_4=R_3'=R_4'=OCH_3	0.00
c16	R_1=C_7H_7, R_3=R_4=R_5=R_3'=R_4'=R_5'=OCH_3	n.a.

EXPERIMENTAL

Materials

All inhibitors were synthesized using microwave irradiation methods, as described in a previous study [10]. The side groups of each inhibitor are shown in Table **1**.

CA-II inhibition assay

Measurement of the reduction in esterase activity on the substrate p-nitrophenyl acetate (4-NPA) was used to perform CA II inhibition assay [11]. The assay was divided into two sections—a pre-test and an evaluation test. The former was performed to screen inhibitor molecules that have at least a 40 % inhibitory effect, while the latter was undertaken by measuring IC_{50} values for the molecules selected in the previous test.

For comparison, the inhibitory activity of a commercial inhibitor, acetazolamide, which is known as a strong inhibitor of CA-II activity was used as standard [7,12,13]. All inhibitor samples were prepared by first dissolving them in dimethyl sulfoxide (DMSO). All inhibitor samples were prepared by first dissolving them in dimethyl sulfoxide (DMSO). To dilute the samples, MOPS buffer at concentration 50 mM (pH 6.9) was used. They were then added with CAII solution. Esterase activity was measured by adding 4-NPA substrate to the mixture and incubating it for 2 h at 25 °C. The hydrolysis of the substrate was then evaluated at 405 nm using a Biorad Elisa Reader. The percent inhibition for each sample was calculated as.

Inhibition (%) = {(C - T)/C}100 …… (1)

where C (i.e., control) = total enzyme activity without inhibitor and T (i.e., test sample) = activity in the presence of test compound.

Inhibitory activity was expressed as IC_{50} values (the concentration at which 50 % of the enzyme activity inhibited), which were calculated using Microsoft Excel 2011 from a dose-response curve obtained using at least five concentrations (ranging from 1–10 µg/mL) of the inhibitor and carried out in triplicate.

Molecular docking

The initial structure of CA-II was obtained from the protein data bank (PDB), accession code 2Q38 [14]. Redocking LSA503 to the CA-II crystal structure was performed to derive docking parameters that will be used for the docking simulation with curcumin analogs. All ligand structures were prepared by the Marvin Sketch program [15]. Molecular docking simulations were observed by Autodock Vina and a genetic algorithm was used for exploring ligand poses inside the active site of CA-II [16]. Schematic representations of the ligand poses and interactions were generated by LigPlot programs [17].

Statistical analysis

Measurements were carried out in triplicate and the results presented as mean ± SEM. Statistical analysis was performed by t-test using Microsoft Excel 2011, and $p < 0.05$ was considered statistically significant.

RESULTS

CA-II inhibition

In order to evaluate the potential of the 44 curcumin analogs listed in Table 1 to inhibit CA-II activity, we first measured the CA-II activity in the absence and presence of each analog. The level of inhibition was expressed as the percentage difference in CA-II activity before and after the addition of the inhibitor (Table 2). Compounds a7, a8, a9, a10, a13, a14, a15, a16, b1, b3, b7, b10, b11, b14, c3, c11, c14, and c16 showed no inhibitory activities; compounds a2, a4, a5, a11, a12, b2, b4, b6, b8, b12, b13, b15, b16, c1, c2, c4, c8 exhibited inhibition below 40 %. Only eight

Table 2: List of ligand molecules and their inhibitory activity against CA-II

Compound	Inhibition (%)	Compound	Inhibition (%)	Compound	Inhibition (%)
a1	81.44	b1	-	c1	27.09
a2	8.89	b2	8.02	c2	22.20
a3	49.37	b3	-	c3	-
a4	11.43	b4	17.71	c4	19.09
a5	14.90	b6	18.42	c6	40.65
a7	-	b7	-	c7	48.66
a8	-	b8	3.79	c8	19.09
a9	-	b9	40.60	c9	41.32
a10	-	b10	-	c10	58.50
a11	7.56	b11	-	c11	-
a12	0.68	b12	15.62	c12	53.45
a13	-	b13	12.41	c13	43.25
a14	-	b14	-	c14	-
a15	-	b15	14.85	c16	-
a16	-	b16	12.30		

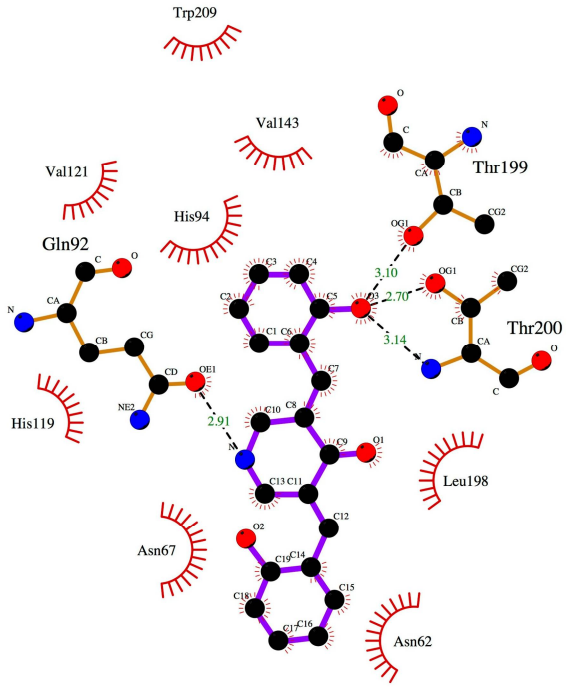

Figure 2: Schematic representation of the binding mode of a1 to the CA-II active site

analogs exhibited inhibition levels greater than 40 %: a1 (81.44 %), a3 (49.63 %), b9 (40.60 %), c7 (48.66 %), c10 (58.50 %), c6 (40.65 %), c12 (53.45 %), and c13 (43.25 %). Therefore, only these eight compounds were further evaluated by measurement of their IC_{50} values.

The IC_{50} values of these analogs ranged from 7.92 ± 0.54 µM to 72.31 ± 2.21 µM (

Table **2:** List of ligand molecules and their inhibitory activity against CA-II

Compound	Inhibition (%)	Compound	Inhibition (%)	Compound	Inhibition (%)
a1	81.44	b1	-	c1	27.09
a2	8.89	b2	8.02	c2	22.20
a3	49.37	b3	-	c3	-
a4	11.43	b4	17.71	c4	19.09
a5	14.90	b6	18.42	c6	40.65
a7	-	b7	-	c7	48.66
a8	-	b8	3.79	c8	19.09
a9	-	b9	40.60	c9	41.32
a10	-	b10	-	c10	58.50
a11	7.56	b11	-	c11	-
a12	0.68	b12	15.62	c12	53.45
a13	-	b13	12.41	c13	43.25
a14	-	b14	-	c14	-
a15	-	b15	14.85	c16	-
a16	-	b16	12.30		

). The compound with the lowest IC_{50} value, i.e., the most potent inhibitor among all of the assayed curcumin analogs, was compound a1, (3E, 5E)-3,5-bis[(2-hydroxyphenyl) methylidene]-piperidin-4-one.

Table 3: Inhibition of CA-II with curcumin analogs

Compound	IC_{50} (µM)
a1	7.92±0.54

c12	12.37±0.78
c10	15.80 ±0.76
c13	18.13±0.17
c6	21.85±1.97
c7	30.45±0.94
a3	38.31±0.46
b9	72.31±2.21

Molecular docking results

A schematic representation of the binding mode of a1 to CA-II, as found during the molecular docking studies is shown in Figure 2.

DISCUSSION

Based on the above analysis, we observed a correlation between the position of the hydroxyl group and the inhibition potential of the curcumin analogs. The presence of a hydroxyl group at R_2 (ortho position) in a1 molecule apparently cause increasing of inhibition against CA-II compared to the presence of hydroxyl group at R_3 as in a4, b4, and c4; or at R_4 as in a8, b8, and c8. Substitution of the hydroxyl group at the R_2 position with more rigid groups, such as methoxy and benzyl groups, decreased the inhibitory capacity of the molecules. However, compounds c1 and b1 with a hydroxyl group at R_2 exhibited contrasting results with a1, and both molecules showed low levels of inhibition towards CA-II activity. This suggests that the hydroxyl group may not be the only contributor to the inhibitory capacity of the curcumin analogs. Other types of interactions may also play roles that strengthen interactions with the enzyme.

In order to further investigate how the a1 molecule exhibited its strong inhibitory capacity against CA-II activity, we performed a molecular docking simulation using Autodock Vina. The docking result gave insight into the role of some of the functional groups in the a1 molecule that contribute to strengthening its interactions at the molecular level with residues inside the active site of the enzyme. We found that the oxygen atom of the hydroxyl group attached at the R_2 position of a1 strongly interacted via hydrogen bonding with the Thr199 and Thr200 residues that are located near the entrance of the active site cavity.

Another hydrogen bond occurred between the N atom at the R_1 position of a1 and Gln92. We propose that interaction of the N atom at R_1 has a dominant effect on the inhibitory activity of CA-II compared with the hydroxyl interaction at the ortho position. In addition, hydrophobic interactions among nonpolar moieties of the a1 molecule with the nearest nonpolar residues also contributed to stabilizing the position of the a1 molecule inside the CA-II active site. Interestingly, a Zn^{2+} ion was not directly involved in the stabilization of the a1 molecule, while most inhibitor molecules, such as the CA-II inhibitor acetazolamide, are strongly bound to this ion [12].

Previously, curcumin has been shown to inhibit CA-II [9]. In this study, eight new curcumin analogs also showed to effectively inhibit CA-II at the micromolar range.

CONCLUSION

The findings of this study indicate that curcumin analogs can be used as inhibitors of CA-II. Eight of the compounds demonstrated the potential to inhibit CA-II activity. The most effective inhibitor found in the present study is (3E, 5E)-3,5-bis[(2-hydroxyphenyl) methylidene]piperidin-4-one, which is also supported by molecular docking results. This curcumin analog strongly interacts with the residues Thr199, Thr200 and Gln92 in the active site of CA-II. Thus, these findings show facilitate the development of novel CA-II inhibitors for glaucoma treatment.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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