Bioorganic & Medicinal Chemistry 17 (2009) 5219–5228

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Molecular modifications on carboxylic acid derivatives as potent histone deacetylase inhibitors: Activity and docking studies

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ARTICLE INFO

Article history:
Received 20 December 2008
Revised 1 April 2009
Accepted 17 May 2009
Available online 23 May 2009

Keywords:
HDAC inhibitors
Molecular docking
Caffeic acid derivatives
Chlorogenic acid
Curcumin
Carboxylic acid derivatives

ABSTRACT

In the light of known HDAC inhibitors, 33 carboxylic acid derivatives were tested to understand the structural requirements for HDAC inhibition activity. Several modifications were applied to develop the structure–activity relationships of carboxylic acid HDAC inhibitors. HDAC inhibition activities were investigated in vitro by using HeLa nuclear extract in a fluorimetric assay. Molecular docking was also carried out for the human HDAC8 enzyme in order to predict inhibition activity and the 3D poses of inhibitor–enzyme complexes. Of these compounds, caffeic acid derivatives such as chlorogenic acid and curcumin were found to be highly potent compared to sodium butyrate, which is a well-known HDAC inhibitor.

1. Introduction

Histone acetylation and deacetylation play an essential role in the epigenetic regulation of gene expression. Histone deacetylases (HDAC) are a group of zinc-binding metalloenzymes that catalyze the removal of acetyl groups from histone tails. Deacetylation results in the tighter wrapping of DNA around the histone core leading to chromatin condensation so the accessibility of transcription factors and gene expression decreases. To date, 18 HDAC enzymes have been identified and grouped into three classes based on homology to yeast HDACs. Class I (HDAC1, 2, 3, 8) and II (HDAC4, 5, 6, 7, 9, 10) have both histone and non-histone protein targets but class III (Sir2 family) does not have histones as primary targets.[1,2] Because of the widespread biological effects, the hypothesis is that the inhibition of these enzymes will create a new therapeutic approach for many diseases, including neurodegenerative, hereditary and inflammatory diseases and cancer.[3–10] Recently, many compounds have been identified that inhibit the activities of HDAC I and II. HDAC inhibitors block the activity of HDAC enzymes leading to the accumulation of acetylated histones. They alter the expression of 7–10% of genes and induce cell growth arrest, differentiation and/or apoptosis.[4,11,12] Therefore, HDACs are popular targets in drug development and HDAC inhibitors are potential drug candidates for many diseases.

During the past 15 years, a number of structurally diverse HDAC inhibitors have been identified; with few exceptions these can be divided into structural classes including hydroximates, cyclic tetrapeptides, benzamides, electrophilic ketones and carboxylic acids.[11] Despite their different chemical structures, these agents have a common pharmacophore consisting of three components: a metal-binding functional group to coordinate the binding to the Zn^{2+} ion in the active site of the HDAC enzyme, a hydrocarbon linker to fill out the narrow tunnel, and a capping group to interact with the amino acids near the entrance of the active site (Fig. 1).[13] The molecular design of the new HDAC inhibitors is based on the modification of the metal-binding functional group, capping group and the hydrocarbon linker. Recent publication of the X-ray crystal structure of human HDAC8 enzyme and histone deacetylase-like protein (HDLP), a bacterial HDAC homologue, has brought about the opportunity to validate the key elements of inhibitor–enzyme interactions and for molecular docking studies to develop new HDAC inhibitors via rational design.[14–17]

HDAC inhibitors have different activities based on their metal-binding group and the most potent class is hydroxamates.[18] Although the hydroxamate class is effective in nanomolar dose

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doi:10.1016/j.bmc.2009.05.042

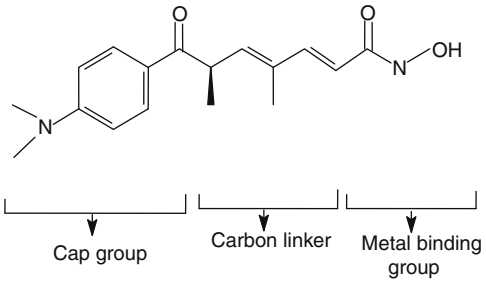


Figure 1. The common structure of HDAC inhibitors.

(TSA IC_{50}: 12 nM and SAHA IC_{50}: 110–370 nM), it has problems such as poor pharmacokinetic properties, severe toxicities and non-selectivity.[4] Thus there has been a growing interest in developing selective non-hydroxamate class inhibitors with less toxicity and improved pharmacokinetic properties.[19]

The carboxylic acid class is the least investigated inhibitor group probably due to poor HDAC inhibition activity.[11,13] Although the inhibition mechanism of this group is not clear, the carboxylic acid group is thought to be a metal-binding functional group.[18,19] A limited number of compounds such as BA (butyric acid), PBA (phenylbutyric acid) and VPA (valproic acid) are identified as carboxylic acid class HDAC inhibitors. All inhibitors are in phase trials with high micromolar doses (Fig. 2)[20–22] for the treatment of cancer and neurodegenerative disorders such as spinal muscular atrophy (SMA) which is characterized by loss of α-motor neurons and is the most common inherited genetic disease that is lethal to infants.[23] Hence BA, PBA and VPA are old drugs, which have anti-proliferative, anti-cancer and anti-convulsant effects, and their toxicity studies have been completed.[20,21]

Most carboxylic acids reported to date have simple alkyl chains and the structure–activity relationship studies for this group have been very limited. Therefore, advances are needed in designing new carboxylic acid derivatives with improved HDAC inhibition activity. Altering the functional groups, modifying the capping group and the hydrocarbon linker is an approach to enrich and optimize HDAC inhibition activity.[18,24] In addition to altering structures, molecular docking studies are another powerful tool for the discovery of specific HDAC inhibitors, their binding requirements and mode of protein–ligand interactions.[24]

In this paper we describe our efforts to establish the structural requirements and structure–activity relationships on carboxylic acid derivative HDAC inhibitors. Molecular modifications, docking studies and HDAC inhibition activities of designed compounds are reported.

2. Results and discussion

2.1. Compound design

Carboxylic acid derivatives were designed as BA, VPA and PBA analogs by focusing on branching and altering the length of the alkyl chain (compound nos.: **1**–**4**), creating points of unsaturation along the alkyl chain (restriction of conformation) (compound nos.: **5**, **14**), the insertion of hydroxyl and/or extra carboxylic groups into the alkyl chain or phenyl ring (compound nos.: **8**–**12**, **15**–**16**, **19**–**28**), amino acid and heterocyclic analogy (compound nos.: **6**–**7**, **17**–**18**), isosteric replacement of the methylene groups (compound no.: **13**) and an examination of the alternative zinc-binding groups (compound nos.: **18**–**21**, **29**–**33**) to develop the structure–activity relationship of this group and potent inhibitors. The structures of the derivatives are given in Tables 1–4.

2.2. In vitro HDAC ınhibition activity screening

Thirty three designed compounds were screened in vitro by using HeLa nuclear extract in a fluorimetric assay at 50 and 500 μM concentrations. Sodium butyrate (NaBA) was used as the reference compound because of its well established HDAC inhibition activity to compare the effects of molecular modifications on HDAC inhibition activities. The HDAC inhibition activity of salt and acidic forms of carboxylic acids were analyzed to be sure that there were no activity differences between these two forms; accordingly, the acidic forms have been used for the subsequent screening test.

Nine of the 33 compounds have HDAC inhibition activity (Tables 1–4). Mandelic acid, valeric acid and 8-hydroxyquinoline showed comparable activity, while propionic, isobutyric, ferulic and caffeic acids showed almost the same activity with NaBA. Among all screened compounds, chlorogenic acid and curcumin were found to be most effective at 50 and 500 μM concentrations.

The HDAC inhibition activity of 2-hydroxybutyric acid, sodium lactate, succinic acid, citric acid, tartaric acid, mandelic acid and benzylic acid (Tables 1 and 2, compound nos.: **8**–**12**, **15**–**16**) were tested to examine if α-hydroxylation and/or replication of the carboxyl group increased the activity (probably due to improved chelating properties). But these modifications also resulted in a reduction or loss of activity. Hippuric acid (Table 2, compound no.: **13**) and indol-2-carboxylic acid (Table 2, compound no.: **18**) obtained by isosteric replacement of the methylene ($-CH_2-$) groups in the PBA with amino group (–NH–) and carbonyl group (–C═O) and the heterocyclic analogy again showed a considerable reduction or loss in activity. Incorporation of a double bond in the alkyl chain resulted in inactive compounds such as sorbic acid (Table 1, compound no.: **5**) and cinnamic acid (Table 2, compound no.: **14**) by activity screening test whereas the prediction of the binding free energy of cinnamic acid (ΔG: −11.03 kcal/mol) by docking studies showed that this compound has an inhibition value which is even better than other compounds (Table 5).

This observation encouraged us to focus on cinnamic acids like phenolic compounds. Caffeic acid (Table 3, compound no.: **22**) is one of the molecules that best fits to this definition. It is a naturally occurring phenolic compound which has been shown to act as a carcinogenic inhibitor and also known as an antioxidant.[25] The HDAC inhibition activity of caffeic acid is found to be equal to NaBA. Therefore, modifications in the caffeic acid molecule seem to be important to describe the structure–activity relationship and also find active molecules. The double bond in the alkyl chain and the length of the alkyl chain ($n = 2$) looks to be critical for the activity since, dihydroxyphenylacetic acid, dihydroxybenzoic acid

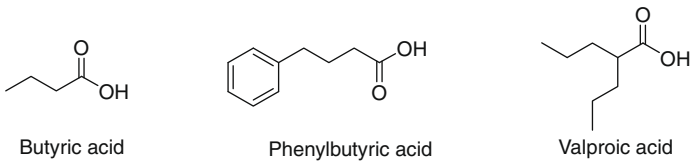
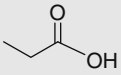
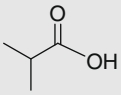
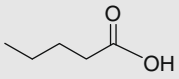
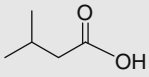
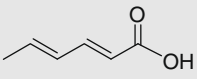
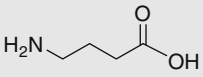
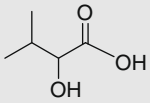
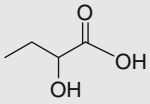
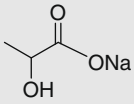
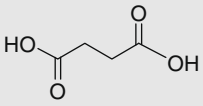
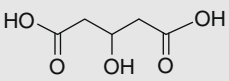
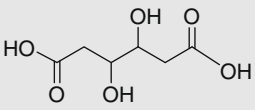


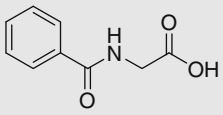
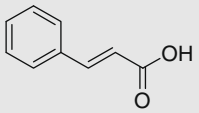
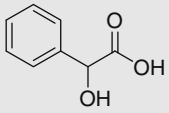
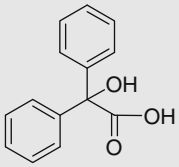
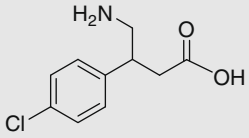
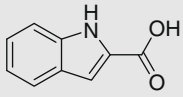
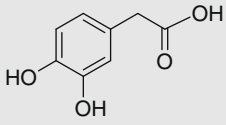
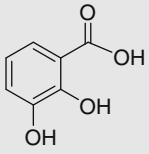
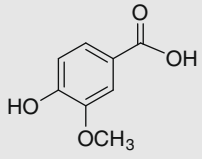
Figure 2. Examples of carboxylic acid group HDAC inhibitors.

Table 1
Molecular modifications of butyric and valproic acid analogs and HDAC inhibition activities

Compound no.	Name	Structure	Remaining HDAC activity (%)
1	Propionic acid		80
2	Isobutyric acid		80
3	Valeric acid		83
4	Isovaleric acid		100
5	Sorbic acid		91
6	GABA		100
7	L-Valine		100
8	2-Hydroxybutyric acid		96
9	Sodium lactate		100
10	Succinic acid		100
11	Citric acid		100
12	Tartaric acid		90

0–80%: more active than NaBA, 80–85%: comparable activity with NaBA, 85–100%: less active than NaBA.

Table 2
Molecular modifications of phenylacetic acid and phenylbutyric acid analogs and HDAC inhibition activities

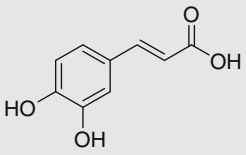
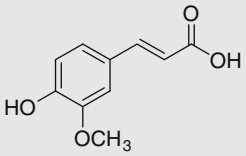
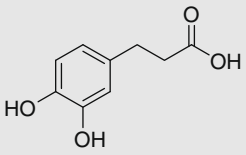
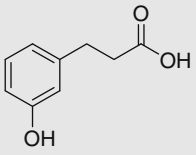
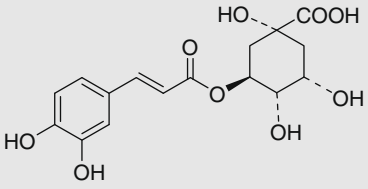
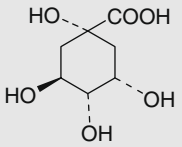
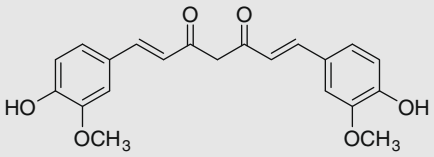
Compound no.	Name	Structure	Remaining HDAC activity (%)
13	Hippuric acid		100
14	Cinnamic acid		95
15	Mandelic acid		85
16	Benzylic acid		100
17	Baclofen		98
18	Indol-2-carboxylic acid		97
19	3,4-Dihydroxyphenyl acetic acid		90
20	2,3-Dihydroxybenzoic acid		98
21	Vanilic acid		99

0–80%: more active than NaBA, 80–85%: comparable activity with NaBA, 85–100%: less active than NaBA.

and dihydrocaffeic acid (Tables 2 and 3, compound nos.: **19**–**21**, **24**) were found to be inactive. Loss in the activity was also observed at 3-hydroxy cinnamic acid (Table 3, compound no.: **25**) upon the removal of one of the phenolic hydroxyl (*p*-) group on the caffeic acid. Since ferulic acid (Table 3, compound no.: **23**) was found to be as active as caffeic acid, it can be concluded that the two phenolic groups are not crucial but 3,4-disubstitution is important for the activity.

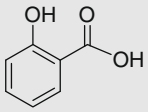
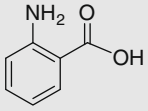
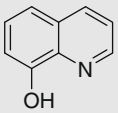
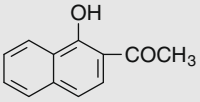
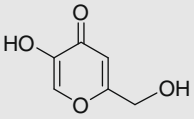
Therefore, two of the well-known natural caffeic acid derivatives were investigated by keeping the main structure of caffeic acid. One of them is chlorogenic acid (Table 3, compound no.: **26**), an ester of caffeic acid and quinic acid (Table 3, compound no.: **27**), a major phenolic compound in coffee, and the other is curcumin (Table 3, compound no.: **28**), which has a polyphenolic structure and is the principal curcuminoid of the Indian curry spice turmeric.[26] Both of the compounds, chlorogenic acid and curcumin

Table 3
Molecular modifications of caffeic acid analogs and HDAC inhibition activities

Compound no.	Name	Structure	Remaining HDAC activity (%)
22	Caffeic acid (3,4-dihydroxy-cinnamic acid)		80
23	Ferulic acid (3-methoxycaffeic acid)		80
24	Dihydrocaffeic acid		86
25	3-Hydroxycinnamic acid		96
26	Chlorogenic acid		40
27	D-(−)-Quinic acid		96
28	Curcumin (diferuloylmethane)		52

0–80%: more active than NaBA, 80–85%: comparable activity with NaBA, 85–100%: less active than NaBA.

Table 4
Compounds with alternative chelating groups and HDAC inhibition activities

Compound no.	Name	Structure	Remaining HDAC activity (%)
29	Salicylic acid		92
30	Antranilic acid (2-aminobenzoic acid)		100
31	8-hydroxyquinoline		85
32	α-Hydroxyacetonaphthone		100
33	Kojic acid		100

0–80%: more active than NaBA, 80–85%: comparable activity with NaBA, 85–100%: less active than NaBA.

Table 5
AutoDock 4.01 estimated free energies of binding (ΔG) and inhibition constant (K_i) values

Inhibitor	Free energy of binding (ΔG kcal/mol)	Inhibition constant (K_i)
Cinnamic acid	−11.03	8.21 nM
Chlorogenic acid	−9.37	135 nM
TSA[a]	−8.59	504 nM
Curcumin	−8.55	539 nM
SAHA[a]	−7.65	2.47 μM
Phenyl butyric acid[a]	−7.58	2.80 μM
Sodium phenylbutyrate[a]	−7.09	6.34 μM
GABA	−6.94	8.19 μM
Caffeic acid	−6.77	10.84 μM
Butyric acid[a]	−5.27	136 μM
Sodium butyrate[a]	−4.65	365 μM
Valproic acid[a]	−4.41	564 μM

[a] Known HDAC inhibitors.

showed the highest inhibition activity among all compounds; the activity is detected by the HDAC inhibition activity test (Tables 1–4, Fig. 3). Previous studies indicated that curcumin reduces histone acetylation via inhibited HAT activity.[27,28] Here we reported that curcumin also has HDAC inhibition activity. Our results showed that not only curcumin but also other caffeic acid derivatives such as chlorogenic acid and ferulic acid has HDAC inhibition activity as well. On the other hand, chlorogenic and caffeic acids are two common catechol-containing coffee polyphenols shown to be strong inhibitors of DNA methylation, which is one of the post-translational modifications involving transcriptional regulation.[29] It is known that DNA methylation and histone acetylation are linked to each other and they involve epigenetic gene regulation.

The activity of quinic acid was also tested to see if it conributes to the activity of chlorogenic acid but it was found to be inactive. Additionally salicylic acid (Table 4, compound no.: **29**), antranilic acid (Table 4, compound no.: **30**), kojic acid (Table 4, compound no.: **33**), α-hydroxyacetonaphthone (Table 4, compound no.: **32**) and 8-hydroxyquinoline (Table 4, compound no.: **31**) were also investigated as alternative zinc-chelating groups but none of them showed noticeable activity.

Since chlorogenic acid and curcumin, which are caffeic acid derivatives, were found to be the most effective compounds, IC_{50} values of these three compounds were determined in vitro in HeLa nuclear extracts (Fig. 3). These compounds showed HDAC inhibition activity in a concentration dependent manner. IC_{50} values of curcumin (IC_{50}: 115 μM.) and chlorogenic acid (IC_{50}: 375 μM) were at micromolar doses, which are less than IC_{50} values of known carboxylic acid inhibitors such as NaBA (IC_{50}: 0.80 mM) and VPA (IC_{50}: 7.24 mM).[4] However caffeic acid (IC_{50}: 2.54 mM) showed inhibition at millimolar doses. HDAC inhibitors differ in their potency and isoenzyme selectivity so half-maximum inhibition can be different among cell types because of tissue-specific expression. Therefore the isoenzyme selectivity of curcumin and chlorogenic acid should be investigated for the exact comparison of potencies.

2.3. Molecular docking studies

In order to obtain more insight into the binding mode, to predict the potential compounds and to obtain additional validations for experimental results, molecular docking studies were performed for chlorogenic acid, curcumin and caffeic acid. Because these are cinnamic acids like phenolic compounds, cinnamic acid was also investigated even though it has no HDAC inhibition activity in screening test. Well-known HDAC inhibitors such as TSA, SAHA, PBA, NaPBA, VPA, NaBA and BA were analyzed to compare the calculated free energy of binding (ΔG) and inhibition constants (K_i) for each enzyme–inhibitor complex. The molecular docking study results of butyric acid, cinnamic acid, chlorogenic acid, curcumin and caffeic acid are shown in Figures 4–8.

Visual inspection of the pose of butyric acid, which is a well-known HDAC inhibitor, into the HDAC8 binding site revealed that its carboxyl group approached the zinc ion as close as possible making ionic interaction with zinc (Fig. 4). His180, His143, Gln 263 and His142 are the other residues near the cofactor zinc ion in contact with the carboxyl group of the inhibitor. In addition to these interactions Gly 140, Gly 304 and Trp141 residues are the surrounding side chains of the tail of the butyric acid.

The binding mode of the cinnamic acid with HDAC8 observed at the end of docking simulation is shown in Figure 5. Carboxylic acid strongly interacts with the zinc ion, resulting in high inhibition potency. Phe152 and His180 pack against each other to form the wall of the active site cavity. Phe208 is the other residue making a close π–π interaction with the phenyl moiety of the ligand.

Analysis of the molecular docking results of chlorogenic acid in the complex with HDAC8 showed that the phenolic ring system of the ligand was oriented towards the entrance cavity surrounded by Arg37, Pro35 and Ile34 (Fig. 6). There are two important interactions considered as hydrogen bonds; the first one is in between the backbone carbonyl group of Tyr100 and the hydroxyl group of the cyclohexyl group (1.89 Å), and the second, hydrogen bond was observed between the Pro35 backbone carbonyl group and the phenolic oxygen of the inhibitor (1.97 Å). It is interesting to

note that chlorogenic acid does not make any ionic complex with zinc and still has considerably low binding energy (−9.37 kcal/mol). The high inhibition constant of chlorogenic acid towards HDAC8 could be justified by these two strong hydrogen bonds.

The binding mode of curcumin is shown in Figure 7. Analysis of the molecular docking results of curcumin in complex with HDAC8 revealed that the ligand adopted a stable binding pose extended towards the entrance cavity. Likewise chlorogenic acid curcumin does not interact with the zinc ion located at the bottom of the cavity. The inhibitor snugly fits the active site making various close contacts with the active site residues including Arg37, Pro35, Ile34 and Phe152. In addition to these weak interactions there are two hydrogen bonds; one between Asp29 carbonyl group and the hydroxy group of the ligand (2.46 Å) and the second one is in between Tyr100 backbone carbonyl group and the phenolic oxygen of the ligand (1.80 Å). Much of the low energy binding contribution (−8.55 kcal/mol) resulted from these two hydrogen bonds.

In Figure 8, visual analysis showed that the carboxyl group of the caffeic acid is oriented toward the zinc ion at the bottom of

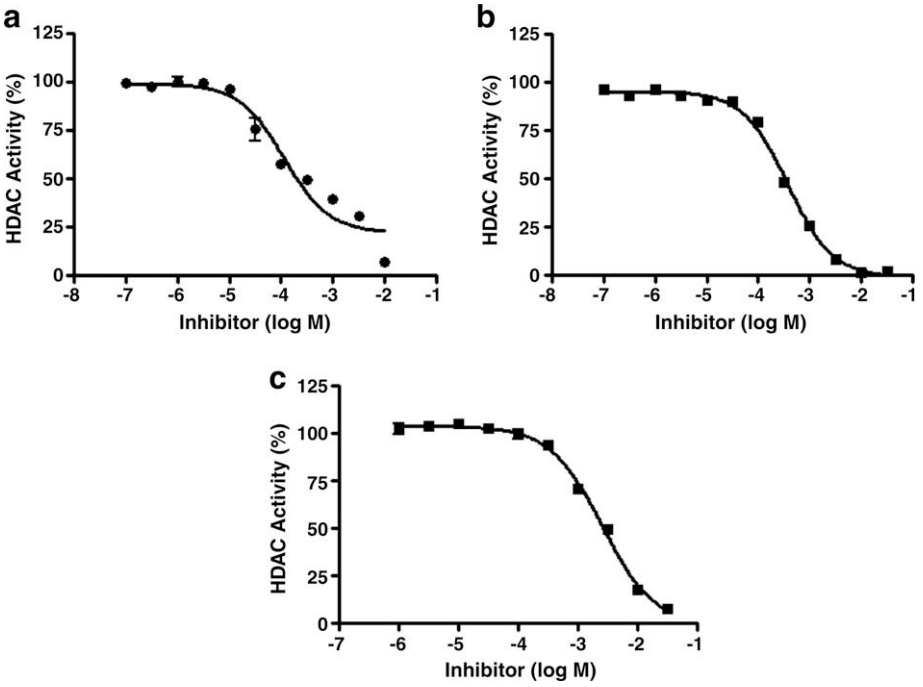


Figure 3. Half-maximum inhibition of HDAC activity in HeLa nuclear extracts by (a) curcumin (IC_{50}: 115 μM), (b) chlorogenic acid (IC_{50}: 375 μM), (c) caffeic acid (IC_{50}: 2.54 mM). Dose–response curves for the inhibition of HDACs in HeLa nuclear extracts were determined by non-linear regression analysis. Each concentration was performed in triplicate. The error bars represent standard error.

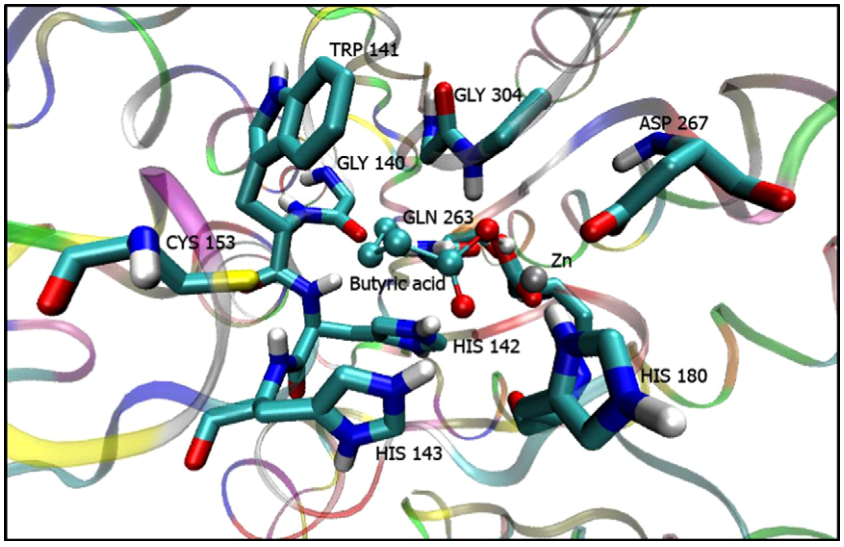


Figure 4. Binding mode of butyric acid into HDAC8 binding cavity. For clarity, only interacting important residues are displayed in ligorice style. The butyric acid was designated in CPK style, and part of the enzyme in the background was visualized in New Ribbon style using the VMD (Visual Molecular Dynamics) program.

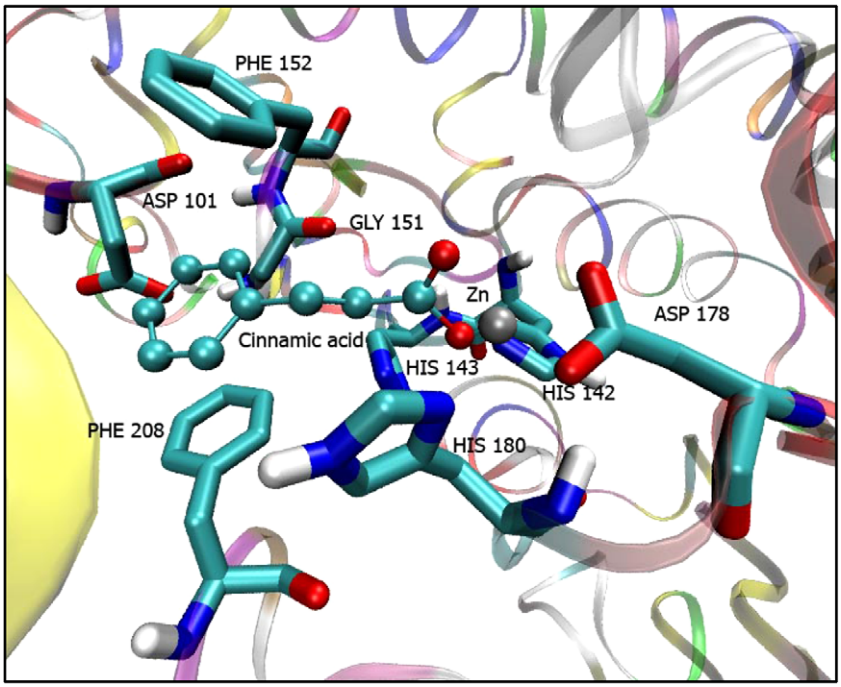


Figure 5. View of cinnamic acid in the active site cavity of HDAC8. The figure was generated using VMD.

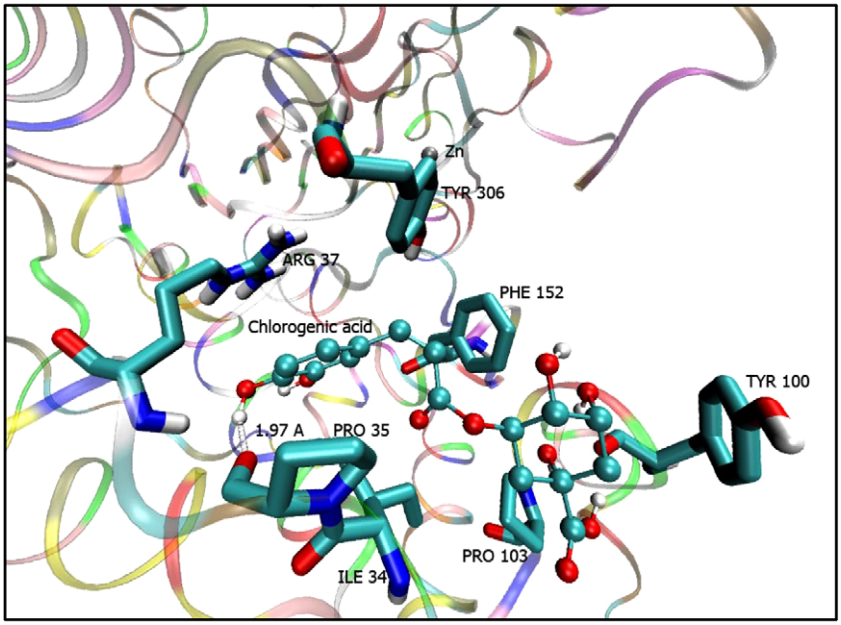


Figure 6. Binding mode of chlorogenic acid into the HDAC8 active site. The picture was generated using VMD.

the active site cavity making some close interactions with Tyr306 and Phe152 side chains. In addition to these interactions, another very strong hydrogen bond between the hydroxy group of the caffeic acid and the backbone carbonyl group of Asp29 reinforced the total free energy of the binding (−6.77 kcal/mol) of the caffeic acid.

In order to see the binding poses and binding interactions of these types of compounds in detail their enzyme ligand complexes were studied with both the ADT (AutoDockTools) and VMD programs. The docking studies analysis of inhibitors into the active site of HDAC8 provided well-clustered solutions. The computational results of our study correlated with the observed experimental values in good agreement with the exception of cinnamic acid. According to the computational results, cinnamic acid has a lower binding energy than that of chlorogenic acid, curcumin and caffeic acid although it has no HDAC inhibition activity. Such a difference between in vitro and in silico experiments is not rare and is acceptable.[30,31] Our docking studies revealed that the binding energy of caffeic acid derivatives is not based on the ionic interaction between Zn of HDAC enzymes and the ligand but mainly H-bonds in nature.

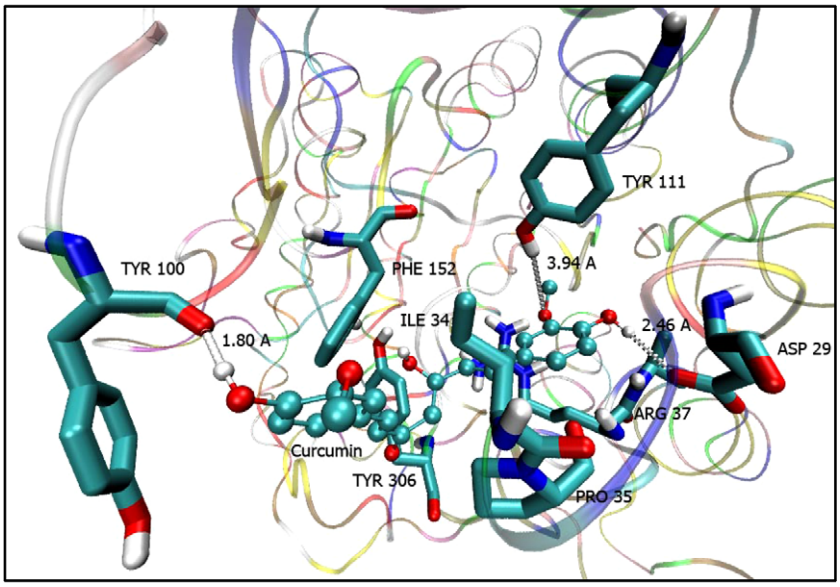


Figure 7. The interacting mode of curcumin with HDAC8. The curcumin was designated in CPK style, and part of the enzyme in the background was visualized in New Ribbon style using the VMD program.

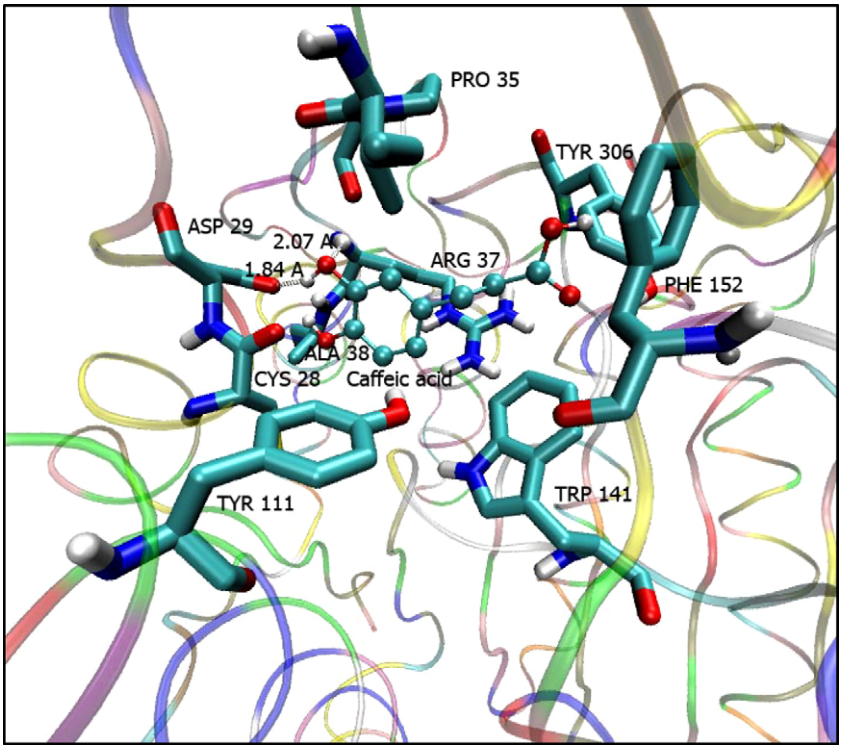


Figure 8. The binding mode of caffeic acid in the active site of HDAC8.

In our study, molecular modifications of BA and VPA analogues (compound nos.: **1**–**4**) were initially designed with branching and different chain lengths. While propionic and isobutyric acids were almost equipotent, valeric and isovaleric acids resulted in a loss of activity compared to NaBA, meaning that increasing/decreasing the length of alkyl chain or branching in the alkyl chain in this group did not increase the inhibition activity (Table 1). Besides, the HDAC inhibition activity screening of amino acid analogs such as GABA, L-valine and baclofen led to a loss of the activity but the calculated free energy of binding of GABA (ΔG: −6.94 kcal/mol) is better than BA (ΔG: −5.27 kcal/mol) (Table 5).

3. Conclusion

This study was performed to determine the structural requirements of carboxylic acid derivatives for HDAC inhibition activity, and to discover potent compounds or a promising lead structure. For this purpose thirty three compounds were tested for HDAC

inhibition by activity screening tests and molecular docking studies.

The docking studies provided us with invaluable data to figure out the observed experimental results which allowed us to estimate the free energy of binding, the binding mode, and the inhibition constant, all of which are promising tools for the discovery of new, active inhibitors useful as pharmacological agents. Overall, the results of this work will be useful in the rational design of novel selective and potent HDAC inhibitors.

None of the conventional modifications in the alkyl/aryl alkyl group of butyric acid, phenylbutyric acid or valproic acid for structure–activity relationship studies resulted in improved activities compared to NaBA. Among the tested compounds, chlorogenic acid and curcumin, showed potent HDAC inhibition activities. However caffeic acid itself showed only moderate activity. Half-maximum HDAC inhibition values were found at micromolar levels for curcumin and chlorogenic acid (IC_{50}: 115 μM; IC_{50}: 375 μM, respectively), but at a millimolar level for caffeic acid (IC_{50}: 2.54 mM). Nonetheless, caffeic acid may be considered a promising lead structure for HDAC inhibitors that have the potential to provide new therapeutics for diseases such as neurodegenerative, hereditary, and inflammatory diseases and cancer.

4. Experimental

4.1. Chemicals

HDAC inhibitors were purchased as follows: NaBa (Merck), curcumin (Sigma), chlorogenic acid (Sigma), caffeic acid (Sigma), ferulic acid (Aldrich), GABA (Sigma), cinnamic acid (Fluka), salicylic acid (Merck), sorbic acid (Merck), tartaric acid (Merck), citric acid (Merck), succinic acid (Merck), hippuric acid (Merck), isovaleric acid (Fluka), *n*-valeric acid (Fluka), propionic acid (Merck), isobutyric acid (Fluka), cojic acid (Aldrich), α-hydroxy-β-acetonaphtane (Aldrich), *o*-aminobenzoic acid (Merck), 8-hydroxykinoline (Sigma), mandelic acid (Aldrich), 2,3-dihydroxybenzoic acid (Aldrich), vanilic acid (Fluka), sodium lactate (Sigma), 2-hydroxybutyric acid (Aldrich), L-valine (Sigma), benzylic acid (Sigma), indole-2-carboxylic acid (Fluka), 3-hydroxy cinnamic acid (Fluka), D-(−)-quinic acid (Aldrich), dihydrocaffeic acid (Fluka), 3,4-dihydroxysiphenyl acetic acid (Fluka), baclofen (Merck). All compounds were dissolved in either 100% dimethylsulfoxide (Applichem) or distilled water prior their use in HDAC inhibition activity screening test.

4.2. In vitro HDAC inhibition activity screening

HDAC inhibition activity and half maximum HDAC inhibition (IC_{50}) of compounds were investigated by a fluorimetric assay (BioVision™) according to the manufacturer's protocol. The inhibitor candidate was mixed with HeLa nuclear extract which contains variety of HDAC enzymes. HDAC fluorometric substrate [Boc-Lys(Ac)-AMC], which comprises an acetylated side chain was added to the inhibitor and HeLa nuclear extract mixture. Deacetylation sensitized the substrate, and treatment with the lysine developer produced the fluorophore. Fluorescence was measured with fluorescence plate reader (Molecular Devices Spectramax M2) at excitation 350 nm and emission 440 nm. TSA was used as negative control according to the protocol and NaBA was used as reference compound in all tests as well. All compounds were screened in triplicate.

4.3. Data analyses

The fluorescence signal (arbitrary fluorescence unit, AFU) versus concentration of the compounds was analyzed. HDAC inhibition activity was assessed according to the decrease in fluorescence signal. 500 μM concentration, which showed efficient inhibition was chosen for calculations of remaining HDAC activity. The values of treated samples were normalized to nontreated ones which were set as 100%. The threshold value which belongs to NaBA was determined as 80% and the less active compounds were excluded.

For detection of half maximum HDAC inhibition (IC_{50}), 20 concentrations (10^{-11}–10^{-2} M) were applied to HeLa nuclear extracts. Dose–response curves of curcumin, caffeic acid and chlorogenic acid treatments were analysis by non-linear regression analysis using GraphPad Prism 4.0. Lower concentrations (10^{-11}–10^{-7} M) that give plateau were excluded. The top value of HDAC inhibition was set as 100% and IC_{50} values were determined.

4.4. Molecular docking

4.4.1. Protein setup

The crystal structures of human histone deacetylase HDAC8 [PDB entry code: 1T64, complexed with the inhibitor trichostatin A (TSA), Resolution: 1.90 Å][32] was obtained from the Protein Data Bank (http://www.rcsb.org).[33] All the water and all non-interacting ions were removed together with their irreversible inhibitor of TSA.

In order to relieve the crystal structure tension and to make the protein available to use in the Autodock docking simulation program, all missing hydrogens/side chain atoms were added. The obtained structure was minimized. The AutoDockTools (version 1.5.1) (ADT),[34] graphical user interface, program was employed to setup the enzymes: all hydrogens were added, Gasteiger[35] charges were calculated and non-polar hydrogens were merged to carbon atoms. For macromolecules, generated pdbqt files were saved.

4.4.2. Ligand setups

The 3D structures of ligand molecules were built, optimized (PM3) level), and saved in pdb format with the aid of the molecular modelling program Spartan (Wavefunction Inc.).[36] The AutoDockTools[37] package was also employed here to generate the docking input files of ligands. The resulting files were saved as pdbqt files.

4.4.3. Docking

AutoDock 4.01,[37,38] was employed for all docking calculations. The AutoDockTools (ADT) generated input files were used in dockings. In all docking a grid box size of 80 × 80 × 80 points in *x*, *y* and *z* directions was built, and because the location of the inhibitor in the complex was known, the maps were centred on Zn atom in the catalytic site of the protein. A grid spacing of 0.375 Å (approximately one forth of the length of carbon–carbon covalent bond) and a distances-dependent function of the dielectric constant were used for the calculation of the energetic map. Ten runs were generated by using Lamarckian genetic algorithm searches. Default settings were used with an initial population of 50 randomly placed individuals, a maximum number of 2.5×10^7 energy evaluations, and a maximum number of 2.7×10^4 generations. A mutation rate of 0.02 and a crossover rate of 0.8 were chosen. Results differing by less than 0.5 Å in positional root-mean-square deviation (RMSD) were clustered together and the results of the most favourable free energy of binding were selected as the resultant complex structures. All calculations were carried out on an IBM Intellistation Z Pro Intel Xeon 5160 machine of intel core duo processor at 2 × 3 GHz with 4 GB of RAM. The resultant structure files were analyzed using ADT[34] and VMD[39] (Visual Molecular Dynamics) visualization programs.

Acknowledgement

This study was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK, Project number: 105G014).

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