Original Article

Novel hydroxamates potentiated *in vitro* activity of fluconazole against *Candida albicans*

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Abstract

A set of 12 novel hydroxamate compounds (NHCs), structurally designed as inhibitors of histone deacetylase (HDAC) enzyme, were synthesized at our facility. These were adamantane derivatives with *N*-hydroxyacetamide as pharmacophore, and each of these compounds was tested for potentiating activity on fluconazole. The concentration of fluconazole which completely inhibited (concentration of complete inhibition [CCI]) the growth of *Candida albicans* ATCC 90028 and *C. albicans* ATCC 64550 was determined by micro-dilution method in the absence and presence of NHCs. The CCI of fluconazole without the NHC combination was 64 μg/ml and 1024 μg/ml against *C. albicans* ATCC 90028 and *C. albicans* ATCC 64550, respectively. The majority of the NHCs potentiated the fluconazole activity markedly as CCI of fluconazole against *C. albicans* ATCC 90028 reduced to 0.25 μg/ml. Similarly, CCI of fluconazole against *C. albicans* ATCC 64550 reduced to 4–8 μg/ml in combination with majority of NHCs while the best activity was displayed by the compound 1 with a reduction of CCI to 0.5 μg/ml. The study results revealed the potential usage of hydroxamate derivatives, structurally designed as HDAC inhibitors to enhance the activity of fluconazole against *C. albicans*.

Key words: Antifungals, candidiasis, fluconazole, histone deacetylase inhibitors, hydroxamates

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen which resides as normal mucous flora in many humans. Conditions such as immunodeficiency, broad-spectrum antibacterial therapy, and endocrine abnormalities are predisposing factors for mucosal (oropharyngeal,

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vaginal, and urinary tract), skin, nail, and systemic infections caused by *C. albicans*.[1] Antifungal azoles especially fluconazole remained the mainstay of therapy against these infections. Azoles exert their antifungal activity primarily by inhibiting Lanosterol 14 alpha-demethylase (encoded by *erg11* gene), an enzyme involved in an essential step in the biosynthesis of ergosterol, which results in fungistatic effect.[2] Antibiotic susceptibility of *Candida* strains is determined by broth method as recommended by the Clinical and Laboratory Standards Institute (CLSI) where the end-point is significant inhibition of growth relative to growth control after 48 h of incubation rather than complete growth inhibition or clear end-point.[3] Some of the *C. albicans* strains typically show trailing growth at concentrations, many fold above the azole minimum inhibitory concentrations (MICs) as determined by CLSI method which is of a concern in interpretation of susceptibility data.[4] Such strains though considered sensitive to azoles *in vitro* might not respond to treatment resulting in clinical failures. In addition, the surviving fungal cells may act as reservoirs for the resistance development.[1]

Inhibitors of histone deacetylase (HDAC) enzymes have emerged as an important therapeutic option for the treatment of cancers.[5] HDAC inhibitors (HDACIs) have also been investigated for antifungal applications, especially as potentiating agents for azoles.[6-8] HDACIs potentiated the *in vitro* activity of azoles against *C. albicans* by synergistic mechanism of action. *Candida* strains upon exposure to azoles were shown to upregulate expression of a set of genes which included *erg11* gene.[9] Smith and Edlind, 2002, revealed that trichostatin A, a HDACI, markedly decreased the upregulation of the *erg11* gene in *Candida* strains that were exposed to fluconazole.[1] The resulting benefit was the arrest of trailing growth of *Candida* strains and potentiation of fluconazole activity. These findings have paved the way for exploring HDACIs as synergetic agents to therapeutically potentiate the efficacy of azoles. The recent FDA approval for two HDACIs for use as anticancer agents has positive impact on such explorative investigations.[10]

Chemically, HDACIs belong to various groups such as hydroxamates, cyclic peptides, aliphatic acids, and benzamides.[10] Hydroxamates are the popular HDACIs and some of these such as vorinostat, belinostat, and panobinostat have demonstrated their efficacy in hematologic malignancies. The structural requirements for hydroxamate HDACIs have been explained based on crystallographic studies,[11,12] which were a capping group, a carbon linker, and a metal-binding moiety [Figure 1]. The cap occludes the entrance of the HDAC enzyme active site, metal-binding moiety chelates the zinc ion in the active site, and linker connects these two functional groups.

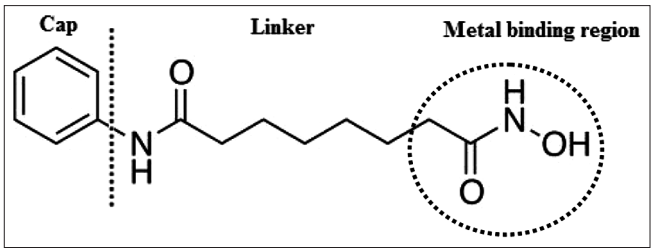


Figure 1: Example of hydroxamate histone deacetylase inhibitors (Vorinostat). The structural design consists of a capping group, a carbon linker and a metal binding moiety

At our facility, we synthesized several classes of HDACIs as antiproliferative agents against tumor cells. Among them, we reported some adamantane-based hydroxamates which were active at nanomolar concentrations in inhibiting HDAC enzymes and showing significant tumor growth inhibition in lung carcinoma xenograft model.[13] Based on this input, we have designed and synthesized a set of 12 novel hydroxamate compounds (NHCs) as adamantane derivatives with *N*-hydroxyacetamide as pharmacophore and evaluated them for their potentiating effect on activity of fluconazole against *C. albicans*.

MATERIALS AND METHODS

Chemistry

Reagents and solvents used, unless stated otherwise, were of commercially available LR grade quality and were used without further purification. Column chromatography was performed on silica gel eluting with MeOH in CH_2Cl_2. Melting points were determined on a Boetius melting point apparatus PHMK05 and were uncorrected. All substances were analyzed with an Agilent 1100 series high-performance liquid chromatography/Mass selective detector system. Purities were ascertained using the area percentage method on the ultraviolet trace recorded at a wavelength of 254 nm and found to be >95%. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker ARX 400 NMR spectrometer (Billerica, MA, USA). Chemical shifts (δ) are in parts per million (ppm) relative to $Si(CH_3)_4$, and coupling constants (*J*) are in hertz. The NMR solvent used was either $CDCl_3$ or dimethyl sulfoxide (DMSO).

General procedure

It involves synthesis of 2-(4-[3-(1-adamantyl) propoxy] phenyl)-*N*-hydroxyacetamide [Figure 2].

Step I

Preparation of 1-(3-bromopropyl)adamantane

A suspension of 1-(3-hydroxypropyl) adamantane (1.4 g, 7.2 mmol/L) in hydrobromic acid (13 mL, 72 mmol/L) and concentration sulfuric acid (1 ml) was heated to 80°C for 24 h. Reaction mixture was diluted with water (150 ml)

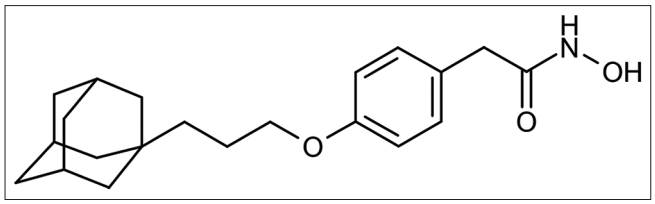


Figure 2: 2-(4-[3-(1-adamantyl) propoxy] phenyl)-*N*-hydroxyacetamide

and extracted with ethyl acetate (100 ml × 2). Combined organic layer was dried over sodium sulfate, concentrated and dried to get 1-(3-bromopropyl) adamantane (1.25 g, 67% yield).

Step II

Preparation of methyl (4-(3-[1-adamantyl] propyloxy)phenyl)acetate

To a solution of 1-(3-bromopropyl) adamantane (0.31 g, 1.2 mmol/L) and methyl (4-hydroxyphenyl) acetate (0.2 g, 1.2 mmol/L) in DMF (3 mL) was added potassium carbonate (0.5 g, 3.6 mmol/L) under stirring. Reaction mixture was heated to 80°C for 3 h and then water was added (70 ml). The aqueous layer was extracted with ethyl acetate (70 ml × 2). Combined ethyl acetate layer was washed with water (50 ml × 3), dried over Na_2SO_4, concentrated to remove solvent, and dried. Crude product was purified by column chromatography using 2% ethyl acetate in hexane as an eluent to give methyl (4-(3-[1-adamantyl] propyloxy) phenyl) acetate (0.255 g, 62% yield).

Step III

Preparation of 2-(4-[3-(1-adamantyl)propoxy] phenyl)-N-hydroxyacetamide (Compound 1)

Potassium hydroxide (0.74 g, 13.3 mmol/L), dissolved in methanol (1 ml), was added to hydroxylamine hydrochloride (0.925 g 13.3 mmol/L), suspended in methanol (1 ml). Potassium chloride salt formed was filtered. The filtrate was added to methyl (4-(3-[1-adamantyl] propyloxy) phenyl) acetate (0.255 g, 0.74 mmol/L) and stirred for 15 min. Methanol was removed completely. Pasty mass obtained was diluted with water (30 mL) and pH was adjusted to 7 using dilute acetic acid. Solid obtained was filtered and dried. Crude compound was purified by flash chromatography using 12% methanol in dichloromethane as an eluent to give the title compound (0.090 g, 35% yield). ^{1}H NMR (DMSO-d_6) δ (ppm): 1.13–1.17 (2H, m, –CH_2), 1.46 (6H, s, –CH_2), 1.58–1.68 (8H, m, –CH_2), 1.92 (3H, s, −CH), 3.18 (2H, s, –CH_2), 3.86–3.89 (2H, t, –CH_2), 6.82–6.84 (2H, d, Ar-H), 7.13–7.15 (2H, d, Ar-H), 8.78 (1H, s, –OH) 10.58 (1H, s, –NH). MS m/z: 344.1 (M^+ +1).

Using the same methodology, remaining compounds were prepared. The penultimate ester compound was prepared using peptide coupling reagent for amide linker, CDI for carbamate linker, and Wittig reaction followed by catalytic hydrogenation for the aliphatic linker. Detailed experimental procedures were mentioned in our earlier publication.[14]

2-(2-[4-(1-adamantyl) butyl]-1, 3-thiazol-4-yl)-N-hydroxyacetamide (Compound 2)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.23–1.32 (4H, m, –CH_2), 1.43–1.67 (14H, m, adamantyl-H), 1.90 (3H, s, adamantyl-H and –CH_2), 2.99–2.92 (2H, t, –CH_2), 3.39 (2H, s, CH_2), 7.19 (1H, s, Ar-H), 8.85 (1H, s, –NH), 10.6 (1H, s, –OH) MS m/z: 348.9 (M^+ +1).

3-(1-Adamantyl) propyl {4-[2-(hydroxyamino)-2-oxoethyl]-1,3-thiazol-2-yl} carbamate (Compound 3)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.00–1.15 (2H, m, –CH_2) 1.44 (6H, s, –CH_2), 1.53–1.72 (8H, m, –CH_2), 1.92 (3H, s, –CH), 3.29 (2H, s, –CH_2), 4.08–4.12 (2H, t, –CH_2), 6.84 (1H, s, Ar-H), 8.84 (1H, s, –NH), 10.57 (1H, s, –OH), 11.63 (1H, s, –NH). MS m/z: 394.1 (M^+ +1).

3-(tricyclo[3.3.1.0^{3,7}]non-3-yl) propyl {4-[2-(hydroxyamino)-2-oxoethyl]-1,3-thiazol-2-yl}) carbamate (Compound 4)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.50–1.67 (14H, m, –CH_2), 1.92–1.95 (1H, m, –CH), 2.16 (2H, s, –CH), 3.32 (2H, s, –CH_2), 4.14–4.20 (2H, t, –CH_2), 8.81 (1H, s, Ar-H), 10.54 (1H, s, –NH), 11.61 (1H, s, –OH). MS m/z: 380.1(M^+ +1).

(Tricyclo[3.3.1.0^{3,7}]non-3-ylmethyl {4-[2-(hydroxyamino)-2-oxoethyl]-1,3-thiazol-2-yl}) carbamate (Compound 5)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.73–1.77 (10H, m, –CH_2), 2.09–2.15 (3H, m, –CH), 3.34 (2H, s, –CH_2), 4.19 (2H, s, –CH_2), 6.84 (1H, s, Ar-H), 8.83 (1H, s, –NH), 10.56 (1H, s, –OH). 11.65 (1H, s, –NH). MS m/z: 352.1(M^+ +1).

N-(4-[2-(hydroxyamino)-2-oxoethyl]-1,3-thiazol-2-yl)-3-(tricyclo[3.3.1.0^{3,7}]non-3-yl) propanamide (Compound 6)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.48–1.52 (8H, m, –CH_2), 1.67–1.96 (2H, t, –CH_2), 1.77–1.81 (2H, t, –CH_2), 1.94–1.97 (1H, t, –CH), 2.15 (2H, s, –CH), 2.39–2.43 (2H, t, –CH_2), 3.32 (2H, s, –CH_2), 6.84 (1H, s, Ar-H), 8.83 (1H, s, –NH), 10.57 (1H, s, –OH), 12.08 (1H, s, –NH). MS m/z: 350.1 (M^+ +1).

3-(tricyclo[3.3.1.0^{3,7}]non-3-yl) propyl {4-[2-(hydroxyamino)-2-oxoethyl]-phenyl} carbamate (Compound 7)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.56–1.67 (14H, m, –CH_2), 1.93–1.96 (1H, t, –CH), 2.16 (2H, s, –CH), 3.19 (2H, s, –CH_2), 4.05–4.11 (2H, m, –CH_2), 7.12–7.14 (2H, d, Ar-H),

7.35–7.37 (2H, d, Ar-H), 8.78 (1H, s, –NH), 9.52 (1H, s, –OH), 10.59 (1H, s, –NH). MS m/z: 390.2 ($M^+ + NH_4^+$).

2-(4-{[3-(1-adamantylpropyl)amino] sulfonyl} phenyl)-N-hydroxyacetamide (Compound 8)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.25–1.33 (4H, m, –CH_2), 1.35–1.65 (13H, m, adamantyl-H), 1.89 (3H, s, adamantyl-H), 2.62–2.647 (2H, m, –CH_2), 3.4 (2H, s, –CH_2), 7.44–7.46 (2H, d, Ar-H), 7.48–7.51 (1H, t, –NH) 7.69–7.72 (2H, d, Ar-H) 8.89 (1H, s, –NH), 10.72 (1H, s, −OH) MS m/z: 407.1 (M^+ +1).

2-Adamantylethyl (4-[2-(hydroxylamino)-2-oxoethyl]-1,3-thiazol-2-yl) carbamate (Compound 9)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.47–1.50 (2H, d, –CH_2), 1.69–1.71 (6H, d, –CH_2), 1.75–1.77 (5H, m, –CH_2 and −CH), 1.83–1.86 (4H, d, –CH_2), 3.29 (2H, s, –CH_2), 4.17–4.18 (2H, t, –CH_2), 6.84 (1H, s, Ar-H), 8.84 (1H, m, –NH), 10.57 (1H, s –OH), 11.65 (1H, s, –NH). MS m/z: 380.1 (M^+ +1).

2-(4-[4-(1-adamantyl) butyl] phenyl)-N-hydroxyacetamide (Compound 10)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.24–1.26 (4H, m, –CH_2), 1.42–1.67 (14H, m, adamantyl-H), 1.90 (3H, s, adamantyl-H and –CH_2), 2.50–2.54 (2H, m, –CH_2), 3.22 (2H, s, –CH_2), 7.08–7.10 (2H, d, Ar-H), 7.13–7.15 (2H, d, Ar-H). MS m/z: 340.2 (M^+ –1).

2-(4-[4-(1-adamantyl) propyl] phenyl)-N-hydroxyacetamide (Compound 11)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.03–1.05 (2H, t, –CH_2), 1.41–1.66 (14H, m, adamantyl-H), 1.89 (3H, s, adamantyl-H and –CH_2), 2.47–2.53 (2H, m, –CH_2), 3.22 (2H, s, –CH_2), 7.08–7.10 (2H, d, Ar-H), 7.13–7.15 (2H, d, Ar-H) 8.79 (1H, s, –NH), 10.6 (1H, s, –OH) MS m/z: 328.2 (M^+ +1).

2-(4-[3-(1-adamantyl) propanamino] phenyl)-N-hydroxyacetamide (Compound 12)

^{1}H NMR (DMSO-d_6) δ (ppm): 1.11–1.17 (2H, m, –CH_2), 1.44–1.49 (8H, m, –CH_2), 1.57–1.68 (6H, m, –CH_2), 1.91 (3H, s, –CH), 2.88–2.93 (2H, m, –CH_2), 3.06 (2H, s, –CH_2), 5.38–5.41 (1H, m, –NH) 6.44–6.46 (2H, d, Ar-H), 6.92–6.94 (2H, d, Ar-H), 8.71 (1H, s, –OH) 10.50 (1H, s, –NH). MS m/z: 343.1 (M^+ +1).

Potentiating activity of novel hydroxamate compounds on fluconazole

In vitro activity of fluconazole was determined in combination with NHCs against two *C. albicans* strains. For this purpose, we followed the same experimental procedure (broth microdilution) as guided by the CLSI,[3] but used the concentration of complete inhibition (CCI), where no visible turbidity as the end-point.[15] The CCI of fluconazole was determined in combination with NHCs at 1 μg/ml against *C. albicans* ATCC 90028 and at 4 μg/ml against *C. albicans* ATCC 64550.

RESULTS

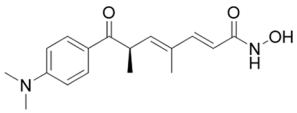
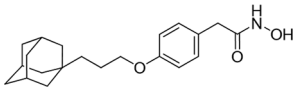
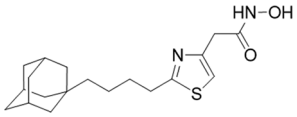
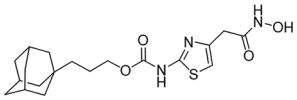
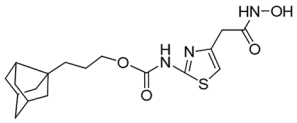
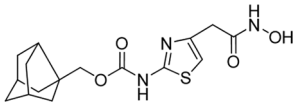
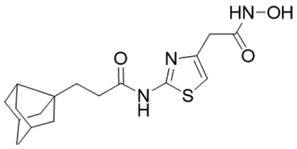
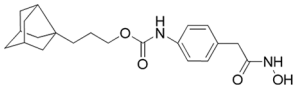
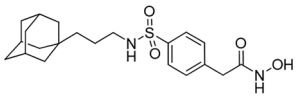
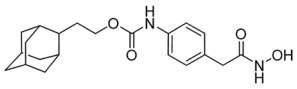
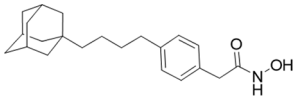
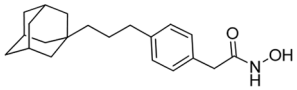
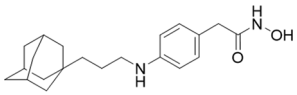
The CCI of fluconazole without the NHC combination was 64 μg/ml and 1024 μg/ml against *C. albicans* ATCC 90028 and *C. albicans* ATCC 64550, respectively. The molecular weight, molecular structure of NHCs, and the CCI of fluconazole in combination with NHCs are presented in Table 1. The majority of the NHCs potentiated the fluconazole activity markedly as CCI of fluconazole against *C. albicans* ATCC 90028 reduced to as low as 0.25 μg/ml. Similarly, CCI of fluconazole against *C. albicans* ATCC 64550, reduced to 4–8 μg/ml in combination with majority of NHCs while the best activity was displayed by compound 1 [Figure 2] with a reduction of CCI, to 0.5 μg/ml. The known HDACI, trichostatin, was used as internal control.

DISCUSSION

The objective of the study was to demonstrate potentiating activity of NHCs on fluconazole by arresting the trailing growth of *C. albicans* strains in micro-broth dilution method. For this reason, we used CCI, as measure of *in vitro* activity of fluconazole, where a complete inhibition of candidial growth was observed. Fluconazole exhibited CCI of 64 μg/ml and 1024 μg/ml against *C. albicans* ATCC 90028 and *C. albicans* ATCC 64550, respectively. Such higher values were due to trailing growth observed after 48 h incubation.

The tested NHCs possessed the *N*-hydroxy-2-(thiazol-4-yl) acetamide or *N*-hydroxy-2-(phenyl)acetamide as metal binding moiety, fused tricyclic moiety (adamantane or noradamantane) as cap group and varied aliphatic linkers. Except Compounds 2 and 9, the tested NHCs exhibited potentiating activity against both the *Candida* strains with several fold reduction in CCI of fluconazole. Despite using varied linkers, the CCI values of these NHCs varied in a narrow range of 0.25–1 μg/ml against *C. albicans* ATCC 90028 and slightly broader range of 4–32 μg/ml (except compound 1) against *C. albicans* ATCC 64550. However, a significant potentiating activity was achieved with Compound 1 which was the analog of Compound 10 with the replacement of butylene by propyloxy linker. Compound 1 potentiated the CCI of fluconazole to 0.25–0.5 μg/ml against both the tested strains This amounts a maximum reduction of CCI of fluconazole by 256 times against *C. albicans* ATCC 90028 and by 2048 times against *C. albicans* ATCC 64550. The results

Table 1: Concentration of complete inhibition of fluconazole in combination with histone deacetylase inhibitors against *Candida albicans*

Compound number	Molecular structure	Molecular weight	*Fluconazole + HDACI CCI (μg/ml)*	
			C. albicans ATCC 90028#	*C. albicans* ATCC 64550*
Trichostatin		302.37	0.25	16
1		343.46	0.25	≤0.5
2		348.5	>4	8
3		393.5	1	8
4		379.47	0.25	8
5		351.42	0.5	16
6		349.45	0.25	32
7		372.47	0.5	8
8		406.54	0.25	8
9		372.46	>4	8
10		341.5	0.25	4
11		327.5	0.25	4
12		342.48	0.25	8

#The CCI of fluconazole as single agent against *C. albicans* ATCC 90028 was 64 μg/ml, *The CCI of fluconazole as single agent against *C. albicans* ATCC 64550 was 1024 μg/ml. *C. albicans*: *Candida albicans*, HDACI: Histone deacetylase inhibitors, CCI: Concentration of complete inhibition

of the study corroborated with structural requirements of HDACIs, demonstrated by Bieliauskas *et al.* 2007.[11] To establish the mode of action NHCs as fungal HDACs, we have used semi-purified fungal HDACs (pan HDAC) and purified Hos2[16] to assess the inhibitory activity of NHCs (data not shown).

Results of the study ascertained that HDACIs such as hydroxamates provide a therapeutic option to potentiate the activity of fluconazole against *C. albicans*.

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Conflicts of interest

There are no conflicts of interest.

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