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Novel Mannich Bases Derived from 2-Substituted Benzimidazole and (Thio)Hydantoin Moieties as Potent Histone Deacetylase 6 (HDAC6) Inhibitors

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Abstract: Novel Mannich bases derived from 2-substituted benzimidazole and (thio) hydantoin moieties, were synthesized as histone deacetylase 6 (HDAC6) inhibitors with potential cytotoxic activity. All derivatives were tested *In vitro* against HDAC6 enzyme. **IR**, **^{1}H NMR, ^{13}C NMR** and mass spectroscopy confirmed the structure of synthesized compounds. All tested compounds significantly inhibited HDAC6 at nanomolar level. Compound **2c** was the most potent presenting significant HDAC6 inhibitory activity (IC_{50} = 97.35± 5.7 nM), nearly equipotent to SAHA reference drug (IC_{50} = 91.73 ± 5.4 nM). *In vitro* cytotoxicity study was also carried out. Compound **2c** exhibited potent cytotoxic activity against the tested cells (CCRF-CEM and MOLT-4) showing one digit micromolar IC_{50}s. Compound **2c** (IC_{50} = 3.66±0.22 uM) was 2-fold more active than SAHA reference drug (IC_{50} = 6.8±0.41 uM) against MOLT-4 cell line. Furthermore, a docking study demonstrated the ability of target Mannich bases to achieve an excellent fitting inside the binding site of HDAC6 enzyme.

Keywords: benzimidazole; (thio)hydantoin; HDAC6; Mannich bases; cytotoxicity.

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1. INTRODUCTION

Leukemia is a lethal type of cancer that leads to hematopoietic malignancies with relatively poor (20-40%) patients' survival. Even though the exploration for various therapy, leukemia still the sixth leading cause for death in USA[1]. Recently, a lot of work has been directed to epigenetic changes that accompanied the development of hematopoietic malignancies[2]. These changes play essential roles in regulating gene expression that can eventually induce oncogenic neoplasms[3]. Among these epigenetic changes is the modification of histone acetylation which can account for alteration of DNA accessibility and chromatin structure [4]. Histone acetylation is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes[5].

Among HDAC family, HDAC6 is a unique Zn^{+2}-dependent enzyme which plays a vital role in microtubule dynamics and so affects cell proliferation and survival. In addition, HDAC6 acts on nonhistone substrates eg. Hsp90 and peroxiredoxin. HDAC6 has a direct effect on cellular activities including motility, growth of cells, migrations and adhesion[6]. Furthermore, HDAC6 is highly expressed during the development of tumors [7,8]. It has also been reported that inhibition of HDAC6 resulted in apoptosis. Therefore, HDAC6 has been considered as an attractive target for designing potent anticancer candidates. Recently, several pan-HDAC inhibitors eg SAHA (suberoylanilide hydroxamic acid), were-approved by FDA (Food and Drug Administration) for the management of hematological cancers[9,10]. HDAC6 inhibitors eg. tubastatin A[11] and citarinostat (ACY-241)[12] have also been reported. Structurally, HDAC inhibitors have common pharmacophoric features: cap-linker-zinc binding group (ZBG) (**Figure.1**).

Benzimidazole scaffold has attracted considerable attention for the discovery and development of antitumor candidates[13,14]. Several benzimidazole–containing drugs such as nocodazole and veliparp

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have been clinically approved for cancer treatment [15]. Moreover, the HDAC inhibitory activity of benzimidazole derivatives has been reported by several research groups[16] (**Figure.2**).

On the other hand, hydantoin and thiohydantoin derivatives are five membered heterocyclic compounds that attracted much attention in medicinal chemistry. Hydantoin-based compounds have been reported to exhibit considerable anticancer activities [17]. On the other hand, owing to their chelating properties, hydantoin and thiohydantoin have also been recognized to inhibit different metalloenzymes like matrix metalloproteinase[18] and tryosinase[19]. For these observations, we assumed that hydantoin and thiohydantoin moieties might be considered as non-hydroxamate zinc chelating groups essential for HDAC inhibitory activity.

In the present study, novel Mannich bases derived from 2-substituted benzimidazole and (thio)hydantoin moieties were developed as HDAC6 inhibitors (**Figure.3**). Following the basic requirements for designing HDAC inhibitors, the benzimidazole nucleus is utilized as a capping moiety that interacts with the enzyme external surface. Whereas, the (thio) hydantoin moiety represents non-hydroxamate ZBG that can chelate Zn^{2+} metal at HDAC enzyme binding site. The benzimidazole and the (thio) hydantoin moieties were separated by a CH_2 linker. Different substituents (R= H, CH_3, Ph) were also added to the 2-posoition of benzimidazole ring to discover substituent effect on HDAC inhibition. In this work, all derivatives were screened against HDAC6. In vitro cytotoxicity and docking study were also conducted.

2. METHODS

2.1. Chemistry

For general remarks, see supplementary file. The staring compounds **1a-c** were prepared as illustrated in the literature reviews [20, 21].

Synthesis of benzimidazole derivatives (1a-c)

A mixture of *o*-phenylenediamine (0.1 mol) and the corresponding acids, formic acid, acetic acid and benzoic acid (0.1 mol) in 4 N HCl (15 ml), was refluxed for 6-8 h. The reaction mixture was neutralized with 10% sodium hydroxide solution. The precipitated product was filtered, dried and crystallized from absolute ethanol. **Yield** 80-90%.

Synthesis of Mannich bases 2a-f:

A new series of Mannich bases were prepared according to reported procedures[22-25]. A mixture of benzimidazole derivatives **1a-c** (2 mmol), and 38% formaldehyde solution (2 mmol) was added into the solution of hydantoin or thiohydantoin (2 mmol) in absolute ethanol (10 ml). The reaction mixture was refluxed for 3-4 h. The precipitated products were filtered, dried and crystallized from 99% ethanol.

5-((1*H*-benzo[*d*]imidazol-1-yl)methyl)imidazolidine-2,4-dione (2a)

Yield:(73.90%); **m.p.** 190- 191 °C; **IR (KBr)** (cm^{-1}): 3220, 3190 (2NH), 1780, 1698 (2C=O); **^{1}H NMR** (DMSO-d_6) δ (ppm): 3.97 (d, 1H, CH_2, J = 3.9 Hz), 4.07(d, 1H, CH_2, J = 3.9 Hz), 4.72- 4.76 (m, 1H, CH), 6.28 (s, 1H, NH, D_2O exchangeable), 6.42 (s, 1H, NH, D_2O exchangeable), 7.20-7.31 (m, 2H, benzimidazole-H), 7.72 (d, 1H, benzimidazole-H, J = 8 Hz), 8.21 (d, 1H, benzimidazole-H, J = 7.6 Hz), 8.33 (s, 1H, benzimidazole-H); **^{13}C NMR** (DMSO-d_6) δ (ppm): 48.25 (CH_2), 60.79 (CH), 111.07, 119.97, 123.29, 123.32, 133.74, 144.70, 158.81(N-C=N), 169.84, 174.36 (2C=O); **MS *m/z* (%)**: 230.11 (M^+, 8.17), 132.69 (1.37), 131.18 (2.27), 100.21(100); **Analysis** % for $C_{11}H_{10}N_4O_2$ (230) Calcd. (Found) C, 57.39 (57.13), H, 4.38 (4.09), N, 24.34 (23.99).

5-((2-methyl-1*H*-benzo[*d*]imidazol-1-yl)methyl) imidazolidine-2,4-dione (2b)

Yield:(60.93%); **m.p.** 90- 92 °C; **IR (KBr)** (cm^{-1}): 3317, 3300 (2NH), 1774, 1693 (2C=O); **^{1}H NMR** (DMSO-d_6) δ (ppm): 2.60 (s, 3H, CH_3) 4.01- 4.11 (m, 2H, CH_2,), 4.56-4.77 (m, 1H, CH), 7.16-7.24 (m, 2H, benzimidazole-H), 7.54-7.58 (m, 2H, benzimidazole-H), 8.11 (s, 1H, NH, D_2O exchangeable), 10.92 (s, 1H, NH, D_2O exchangeable); **^{13}C NMR** (DMSO-d_6) δ (ppm): 13.65 (CH_3), 47.70 (CH_2), 61.39 (CH), 110.47, 118.62, 122.62, 122.9, 134.86, 142.2, 158.83 (N-C=N), 169.84, 172.36 (2C=O); **MS *m/z* (%)**: 244.22 (M^+, 9.01), 241.62 (1.32), 130.42 (100); **Analysis** % for $C_{12}H_{12}N_4O_2$ (244) Calcd. (Found) C, 59.01 (58.81), H, 4.95 (4.63), N, 22.94 (23.14).

5-((2-phenyl-1*H*-benzo[*d*]imidazol-1-yl)methyl) imidazolidine-2,4-dione (2c)

Yield:(75.60%); **m.p.** 280- 282 °C; **IR (KBr)** (cm^{-1}): 3209 (2NH), 1766, 1693 (2C=O); **^{1}H NMR** (DMSO-d_6) δ (ppm): 4.00-4.06 (m, 2H, CH_2), 4.73-4.77 (m, 1H, CH), 7.27-7.31 (m, 2H, benzimidazole-H), 7.49-7.54 (t, 1H, phenyl-H), 7.56 (s, 1H, NH, D_2O exchangeable), 7.57 (s, 1H, NH, D_2O exchangeable), 7.58-7.69 (m, 2H, phenyl-H), 7.98 (d, 2H, benzimidazole-H, J = 8.4 Hz), 8.24-8.27 (d, 2H, phenyl-H, J = 8 Hz)); **^{13}C NMR** (DMSO-d_6) δ (ppm): 50.55 (CH_2), 60.27 (CH), 115.34, 123.42, 127.30, 129.04, 129.10, 129.20, 129.28, 129.57, 129.75, 131.08, 131.23, 138.20, 151.15 (N-C=N), 167.82, 172.36 (2C=O); **MS *m/z* (%)**: 306.11 (M^+, 17.72), 268.82 (1.42), 64.25 (100); **Analysis** % for $C_{17}H_{14}N_4O_2$ (306) Calcd. (Found) C, 66.66 (67.05), H, 4.61 (4.39), N, 18.29 (18.59).

5-((1*H*-benzo[*d*]imidazol-1-yl)methyl)-2-thioxoimi -dazolidin-4-one (2d)

Yield:(80.50%); **m.p.** 108- 110 °C; **IR (KBr)** (cm^{-1}): 3143, 3101 (2NH), 1755, 1616 (2C=O), 1199 (C=S); **^{1}H NMR** (DMSO-d_6) δ (ppm): 3.38-3.46 (m, 2H, CH_2), 4.55- 4.99 (m, 1H, CH), 6.78 (s, 1H, NH, D_2O exchangeable), 7.22-7.31 (m, 2H, benzimidazole-H), 7.69 (d, 2H, benzimidazole-H, J = 8 Hz), 8.29 (s, 1H, benzimidazole-H), 10.01 (s, 1H, NH, D_2O exchangeable); **^{13}C NMR** (DMSO-d_6) δ (ppm): 50.58 (CH_2), 67.80 (CH), 111.38, 119.82, 122.25, 122.89, 133.75, 144.11, 144.34 (N-C=N),174.36 (C=O), 181.78 (C=S); **MS *m/z* (%)**: 246.46 (M^+, 12.94), 244.21 (4), 240.47 (4.40), 64.40(100); **Analysis** % for $C_{11}H_{10}N_4OS$ (246) Calcd. (Found) C, 53.64 (53.98), H, 4.09 (3.80), N, 22.75 (23.06).

5-((2-methyl-1*H*-benzo[*d*]imidazol-1-yl)methyl)-2-thioxoimidazolidin-4-one (2e)

Yield:(69.45%); **m.p.** 139-140 °C; **IR (KBr)** (cm^{-1}): 3120 (2NH), 1755, 1616 (2C=O), 1149 (C=S); **^{1}H NMR** (DMSO-d_6) δ (ppm): 2.56 (s, 3H, CH_3) 3.61-3.82 (m, 2H, CH_2,), 4.82-4.99 (m, 1H, CH), 6.66 (s, 1H, NH, D_2O exchangeable), 7.16-7.24 (m, 2H, benzimidazole-H), 7.53-7.57 (m, 2H, benzimidazole-$H_{4,7}$), 10.01 (s, H, NH, D_2O exchangeable); **^{13}C NMR** (DMSO-d_6) δ (ppm): 13.74 (CH_3), 60.27 (CH_2), 66.35 (CH), 110.74, 118.32, 122.14, 122.65, 134.99, 142.02, 152.25 (N-C=N),174.16 (C=O), 181.43 (C=S); **MS *m/z* (%)**: 260.41(M^+, 3.32), 257.29 (3.66), 41.27 (100); **Analysis** % for $C_{12}H_{12}N_4OS$ (260) Calcd. (Found) C, 55.37 (55.69), H, 4.65 (5.03), N, 21.52 (21.14).

5-((2-phenyl-1*H*-benzo[*d*]imidazol-1-yl)methyl)-2-thioxoimidazolidin-4-one (2f)

Yield:(59.30%); **m.p.** 158- 160 °C; **IR (KBr)** (cm^{-1}): 3232, 3217 (2NH), 1755, 1681 (2C=O), 1180 (C=S); **^{1}H NMR** (DMSO-d_6) δ (ppm): 3.56-3.83 (m, 2H, CH_2), 4.83- 4.97 (m, 1H, CH), 6.37 (s, 1H, NH, D_2O exchangeable), 7.27-7.30 (m, 2H, benzimidazole-H), 7.49-7.68 (m, 3H, phenyl-H), 7.97 (d, 2H, benzimidazole-H, J = 7.4 Hz), 8.24 (d, 2H, phenyl-H, J = 7 Hz), 10.01 (s, 1H, NH, D_2O exchangeable); **^{13}C NMR** (DMSO-d_6) δ (ppm): 55.20 (CH_2), 69.60 (CH), 115.14, 124.08, 127.58, 127.88, 129.04, 129.34, 129.69, 129.73,131.20, 131.69, 133.34, 136.88, 150.68 (N-C=N), 167.79 (C=O), 173.90 (C=S); **MS *m/z* (%)**: 322.88 (M^+, 6.11), 318.77(5.11), 59.15 (100); **Analysis** % for $C_{17}H_{14}N_4OS$ (322) Calcd. (Found) C, 63.34 (62.98), H, 4.38 (4.75), N, 17.38 (17.67).

2.1.2. In vitro HDAC6 inhibition assay:

HDAC6 inhibitory activity was measured using BioVision's HDAC6 inhibitor screening kit using SAHA as a reference drug[26]. For details, see supplementary file.

2.1.3. Anticancer activity:

The anticancer activity of compound **2c** against CCRF-CEM and MOLT-4 cancer cells was estimated using MTT-based assay kit, Sigma [27-29]. For details, see supplementary file**.**

2.2. Figures, Tables and Schemes

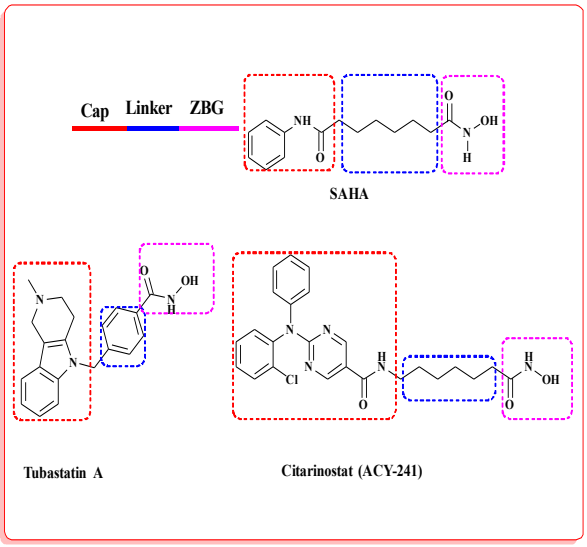


Figure 1. reported histone deacetylase inhibitors

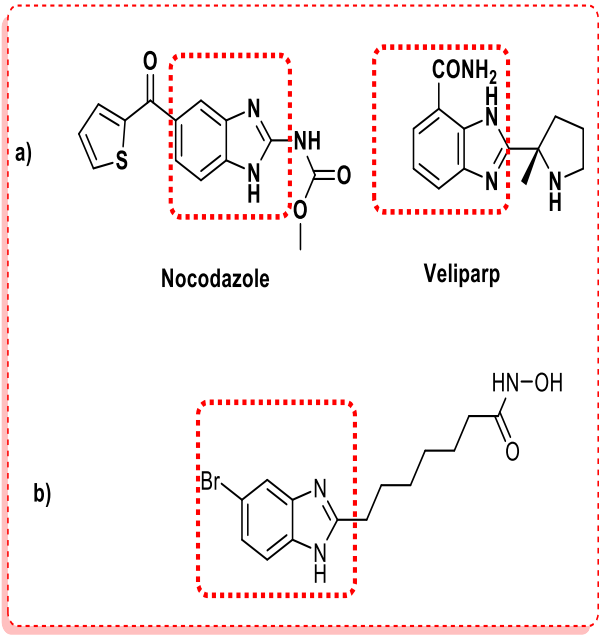


Figure2. a) Clinically approved enzimidazole -containing anticancer drugs.
b) Reported benzimidazole as HDAC inhibitors.

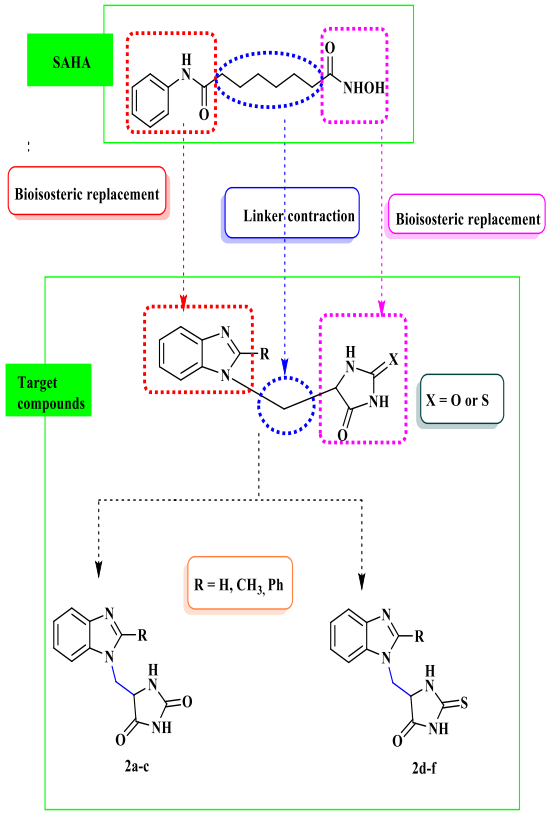
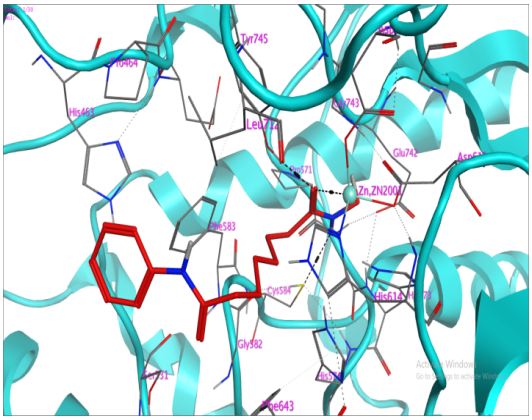


Figure 3. Design of target compounds as HDAC inhibitors.



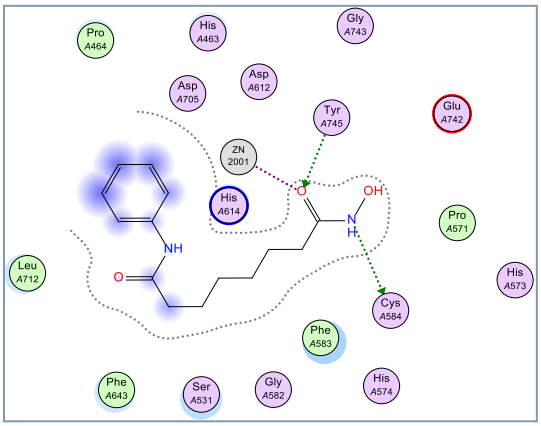
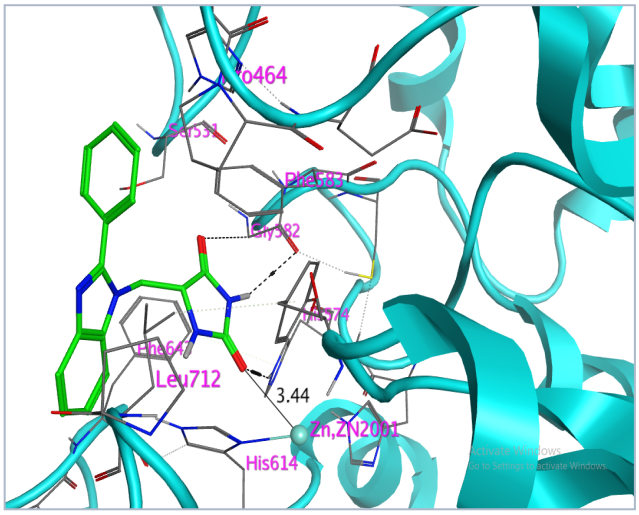


Figure 4a. 2D and **3D** of SAHA docked into the active site of HDAC6.



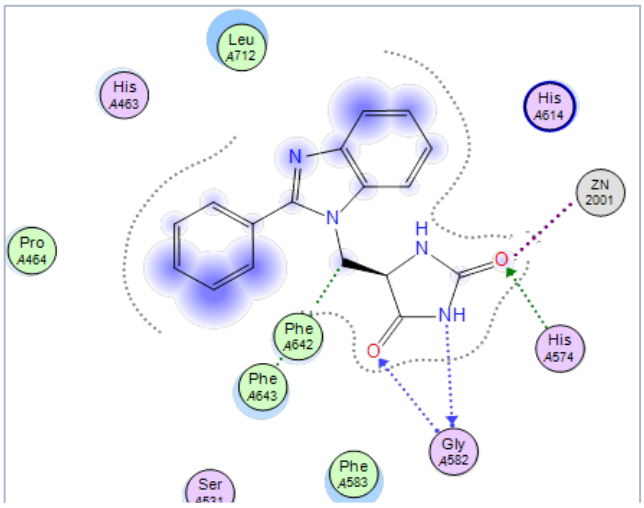


Figure 4b. 2D and **3D** of compound **2c** docked into the active site of HDAC6.

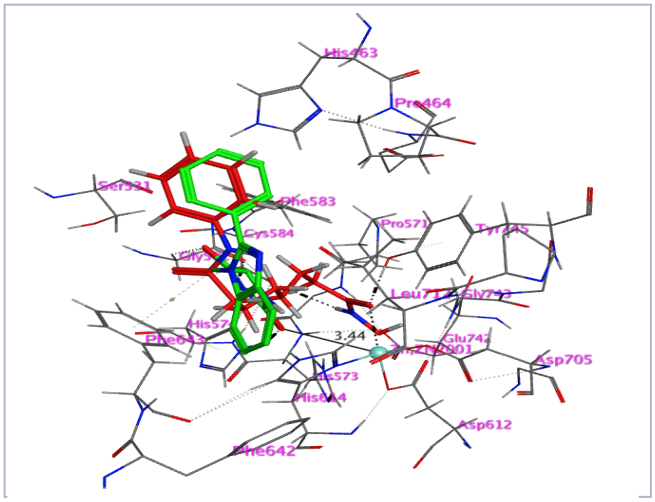


Figure 4c. The overlay of docked **2c** (green color) and SAHA (red color) within the active site of HDAC6.

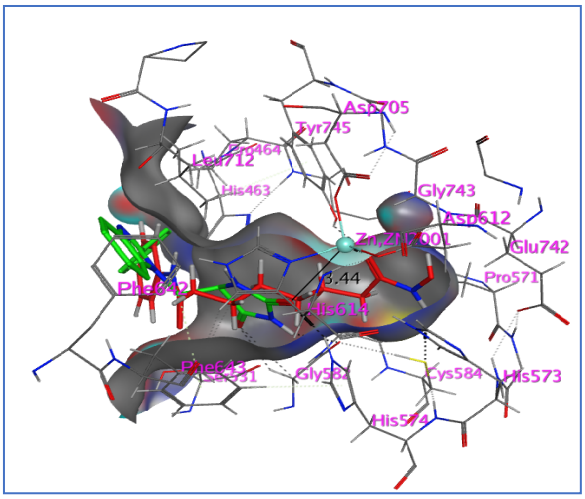


Figure 4d. Side view for compound **2c** (green colour) and **SAHA** (red colour) inside the binding pocket of **HDAC6.**

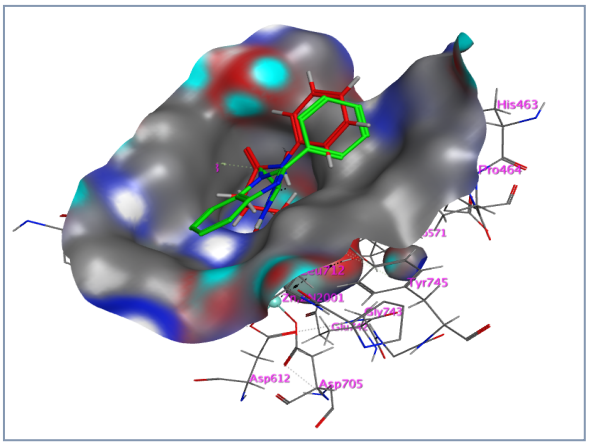
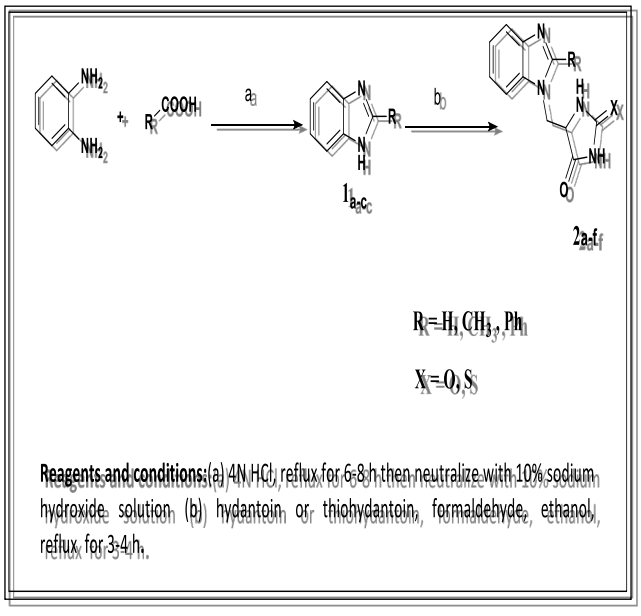


Figure 4e. Top view for compound **2c** (green colour) and **SAHA** (red colour) inside the binding pocket of **HDAC6**



Scheme 1: Synthesis of target Mannich bases **2a-f**.

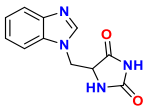
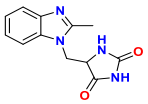
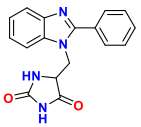
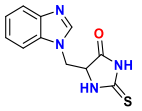
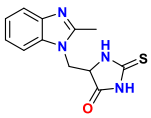
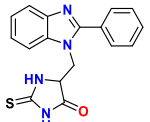
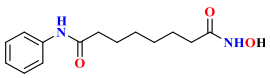
Comp. No.	Structure	IC_{50} (nM)
2a		279.59± 16
2b		166.09± 9.7
2c		97.35± 5.7
2d		209.27± 12
2e		386.38± 23
2f		179.73± 11
SAHA		**91.73±5.44**

Table 2: ***In vitro*** **anticancer activity** against two human leukaemia cancer cell lines, CCRF-CEM and MOLT-4

Comp. No.	Cytotoxicity	IC_{50} (uM)
	CCRF-CEM	MOLT-4
2c	**9.67±0.58**	**3.66±0.22**
SAHA	**3.83±0.23**	**6.8±0.41**

Table 3: Docking results for binding interaction of SAHA and **2c** in HDAC6 binding site.

Table 1: HDAC6 inhibitory activity of target compounds.

Comp. No	Docking score (Kcal/ mol)	No. of H bonds	Amino acid residues (bond length Å)	Atom of compound
SAHA	- 6.64	2	Cys 584 (4.07)	NH of hydroxymate
			Tyr 745 (2.65)	C=O of hydroxymate
			Zn (2.63)	O of C=O of hydroymate
			Leu 712	Phenyl capping group
			Ser 531	
			Phe 583	
			Phe 643	
			His 614	
			Pro 464	
2c	-4.68	3	His574 (2.89)	C=O of hydantoin
			Gly 582 (3.23)	C=O of hydantoin
			Gly 582 (3.36)	NH of hydantoin
			Phe 643 (4.45)	CH_2 of the linker moiety
			Zn (3.44)	O of hydantoin
			Leu 712	2-phenyl benzimidazole capping group
			Ser 531	
			Phe 583	
			Phe 642	
			Phe 643	
			His 614	
			His 463	
			Pro 464	

3. RESULTS

3.1 Chemistry

In the present work, 2-substituted-benzimidazoles **1a-c** were synthesized via the reaction of *o*-phenylenediamine with the appropriate carboxylic acid following reported procedure [20,21] **(Scheme 1).**

It is worth noting that Mannich reaction has a great application in medicinal chemistry as Mannich bases have interesting biological activities eg. anticancer, antibacterial, antiviral and anti-inflammatory[22]. Additionally, aminomethylation of drugs may improve their delivery in the human body and increase the hydrophilic properties through addition of a polar functional group[22]. Following reported procedures[23-25], benzimidazoles **1a-c** were reacted with hydantoin or thiohydantoin and formaldehyde solution to give Mannich bases **2a-f** through aminoalkylation reaction **(Scheme 1)**.

3.2 HDAC6 assay:

Mannich bases **2a-f**, were evaluated against HDAC6 using SAHA as a reference standard (**Table 1).** All derivatives efficiently suppressed HDAC6 activity showing IC_{50} values in the nanomolar range (97.356 to 368.38 nM), relative to SAHA reference drug (IC_{50} = 91.732 ± 5.4 nM). Compound **2c** was the most active against HDAC6 enzyme showing a significant HDAC6 inhibitory activity (IC_{50} = 97.356± 5.7 nM), nearly equipotent to SAHA (IC_{50} = 91.732 ± 5.4 nM). Analogues **2b, 2d** and **2f** revealed half potency of SAHA. While, compounds **2a** and **2e** were the least potent against HDAC6 enzyme.

***In vitro* anticancer activity:**

As mentioned before, SAHA was approved by FDA for the treatment of various hematological cancers including acute myeloid leukemia and multiple myeloma, thus its effectiveness against leukemia is mainly assigned to HDAC inhibition. Thus, compound 2c; the most potent compound against HDAC6, was exposed to anticancer evaluation against two human leukemia cancer cells; CCRF-CEM and MOLT-4 using MTT assay [27] **(Table 2).**

4. DISCUSSION

4.1 Chemistry

IR spectra for compounds **2a-c** showed sharp absorption bands ranging from 1780 to 1616 cm^{-1} correlated with the presence of C=O groups. Whereas, thiohydantoin derivatives **2d-f** also clearly depicted C=S stretching at 1199, 1149 and 1180 cm^{-1} respectively.

Moreover, the ^{1}HNMR analysis for **2a** revealed two doublets at δ 3.97 and δ 4.07 ppm ascertaining the presence of CH_2 protons in addition to a multiplet in the range of δ 4.72- 4.76 ppm indicating CH proton. ^{1}HNMR spectra for **2b,c** and **2d-f** showed two muliplets in the range of δ 3.61-4.01and δ 4.56-4.82 ppm for CH_2 and CH protons, respectively.

Furthermore, ^{13}C NMR spectra of **2a-c** and **2d-f** exhibited two signals around δ 48.25 - δ 60.27 and δ 60.27 - δ 69.60 ppm, corresponding to the two aliphatic carbons CH_2 and CH, respectively.

4.2 HDAC6 assay:

4.2.1 Structure-activity relationship:

Like all reported HDAC inhibitors, the designed compounds have general characteristic structural features necessary for inhibiting HDAC6 enzymes. Herein, these pharmacophoric features represented by benzimidazole capping moiety bearing different substitution on 2-position, CH_2 linker and ZBG that contains either O or S atom for binding with Zn^{+2} ion in the enzyme active site.

Regarding substituents on 2-position of benzimidazole ring, the addition of methyl group in compound **2b** (IC_{50} =166.09± 9.7 nM) resulted in an apparent increase in HDAC6 inhibition, comparing to unsubstituted analogue **2a** (IC_{50} =279.59± 16 nM). Moreover, a 3-fold increase in HDAC6 inhibition was observed for phenyl benzimidazole derivative **2c** (IC_{50} = 97.35± 5.7nM). Similarly, for thiohydantoin compounds **2d-f,** phenyl benzimidazole **2f** was much more potent than its unsubstituted (**2d)** and methyl (**2e)** analogues.

It is worth mentioning that the type of atom (X= O, S) on ZBG has an effective role in HDAC6 inhibitory activity of title compounds. It was noted that the hydantoin derivatives **2a,c (X=O)** were more potent than their corresponding thiohydantoin analogues **2d-f (X=S).**

4.3 In vitro anticancer activity:

Compound **2c** exhibited potent cytotoxic activity against the two tested cell lines showing one-digit micromolar IC_{50}s. Noticeably, compound **2c** (IC_{50} = 3.66±0.22 uM) was 2-fold more active than SAHA reference drug (IC_{50} = 6.8±0.41 uM) against MOLT-4 cell line. In addition, compound **2c** revealed half potency of SAHA against CCRF-CEM cells with IC_{50}s of 9.67±0.58 and 3.83±0.23 uM, respectively. It was also noted that MOLT-4 cell line was 2-fold more sensitive to compound **2c** than CCRF-CEM cell line.

4.4 Docking study:

In light of the well-established knowledge of the pharmacophoric parameters (cap, linker and ZBG) that are essential for HDAC6 inhibitory activity, a docking simulation was performed to clarify the interaction of the most promising compound (**2c**) with HDAC6. The molecular docking study was carried out using MOE 2014.0901 in HDAC6 isoenzyme that is co-crystallized with SAHA (PDB code: 5EEI)[30]. As speculated, compound **2c** was approached to the narrow channel of the binding site and chelated with Zn^{+2} ion at the bottom of catalytic site of HDAC6 (**Fig 4a-e**). In addition, compound **2c** formed three hydrogen bonds with the side chain residue **His 574** and the backbone residue **Gly 582** (**Table 3**). Furthermore, the 2-phenyl benzimidazole capping group formed additional hydrophobic contacts with important residues within the active pocket of HDAC6, including **Leu 712, Ser 531, Phe 583, Phe 642, Phe 643, His 614, His 463** and **Pro 464** that could contribute to significant improvement in compound activity**.** Accordingly, the removal or replacement of 2-phenyl benzimidazole with 2-methyl benzimidazole induces a great drop in HDAC6 inhibitory activity. This illustrates the important role of phenyl group in benzimidazole capping moiety. Moreover, CH_2 moiety of the linker was able to form an arene-H interaction with **Phe 643.** The obtained binding mode is observably matched with the pervious SAR study and could be considered as a valid explanation for the potent HDAC6 inhibition provoked by compound **2c**.

5. CONCLUSIONS

New 2-substituted benzimidazole (thio)hydantoin Mannich bases were synthesized as HDAC6 inhibitors. All derivatives inhibited HDAC6 at nanomolar level. Compound **2c** was the most potent and displayed HDAC6 inhibition equal to SAHA. In addition, **2c** was 2-fold more active than SAHA against MOLT-4 leukemia cells.

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List of Abbreviations: SAHA (suberoylanilide hydroxamic acid), FDA (Food and Drug Administration); HDAC6 (histone deacetylase 6), ZBG (zinc binding group).

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