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Short communication

Appraisal of GABA and PABA as linker: Design and synthesis of novel benzamide based histone deacetylase inhibitors

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ABSTRACT

Histone deacetylase inhibitors have been actively explored as a new generation of chemotherapeutics for cancers, generally known as epigenetic therapeutics. Two novel series of N-(2-amino-phenyl)-4-{[(2/3/4-substituted-phenylcarbamoyl)-methyl]-amino}-butyramide and N-(2-amino-phenyl)-4-{[(2/3/4-substituted-phenylcarbamoyl)-methyl]-amino}benzamide were designed and synthesized as novel histone deacetylase inhibitors. The anticancer potential of the compounds were determined *in-vitro* using MTT assay against HCT-116 and U251 (glioma) cell lines and histone deacetylase inhibitory assay. The synthesized compounds were investigated for anti-tumor activity against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice. The efforts were also made to ascertain structure–activity relationships among test compounds. The results of the present studying represents appraisal of γ-aminobutyric acid (GABA) and *para*-aminobenzoic acid (PABA) as linker moiety for development of newer benzamide based histone deacetylase inhibitor.

1. Introduction

The epigenetic changes play a vital role in tumorigenesis. The acetylation and deacetylation of histones have been found to support to a substantial degree to epigenetic regulation of gene expression [1,2]. Histone deacetylase inhibitors (HDACi) are a novel class of chemotherapeutic agent that control gene expression by increasing the acetylation of histones, leading to induction of chromatin relaxation and alteration of gene expression [3,4]. Recent findings indicate that HDACi selectively induce growth arrest, differentiation and apoptosis in tumor cells *via* transcriptional activation of a small set of genes that control cell proliferation and cell cycle development like p21 [5,6].

Entinostat (MS-275, SNDX-275) is a moderately potent benzamide based HDACi, which is in phase II clinical trial. MS-275 was found to be an inhibitor of class I HDACs with a high affinity for HDAC1 (IC_{50} = 0.368 μM) and HDAC3 (IC_{50} = 0.501 μM) but relatively weak inhibition of HDAC8 (IC_{50} = 63.4 μM). MS-275 inhibits class II HDAC4, HDAC6 and HDAC10 with IC_{50} of 10.7 μM, >100 μM and 50.1 μM, respectively. The IC_{50} value of MS-275 toward Class HDACIII SIRT1 was found more than 100 μM [7]. The mechanism inducing cell death in cell lines appears to involve generation of Reactive Oxygen Species (ROS). MS-275 has demonstrated antiproliferative action in an array of in-vitro human cancer cell lines including breast, ovary, colon, myeloma, pancrease and leukemia. It has also exhibited in vivo anti-tumor activity in a quite of adult and pediatric orthotopic tumor xenograft models following oral administration. Various reports of clinical trials in patients with refractory solid tumors or refractory hematologic malignancies have indicated that MS-275 is well tolerated and exhibited potential anti-tumor activity. MGCD-0103 and CS-055 are other benzamide based HDACi, which are in phase II clinical trial. WO2004035525-Ii is an example of patented benzamide based HDACi with potent biological activity [8–11]. (Fig. 1).

HDACi generally conform to a broadly accepted pharmacophore. The crystallographic studies for the design of valuable HDACi have pointed out three structural requirements: a cap group (A) that provides interaction with the pocket entrance, a terminal group (B) that can bind to the zinc ion at the bottom of the active site, also

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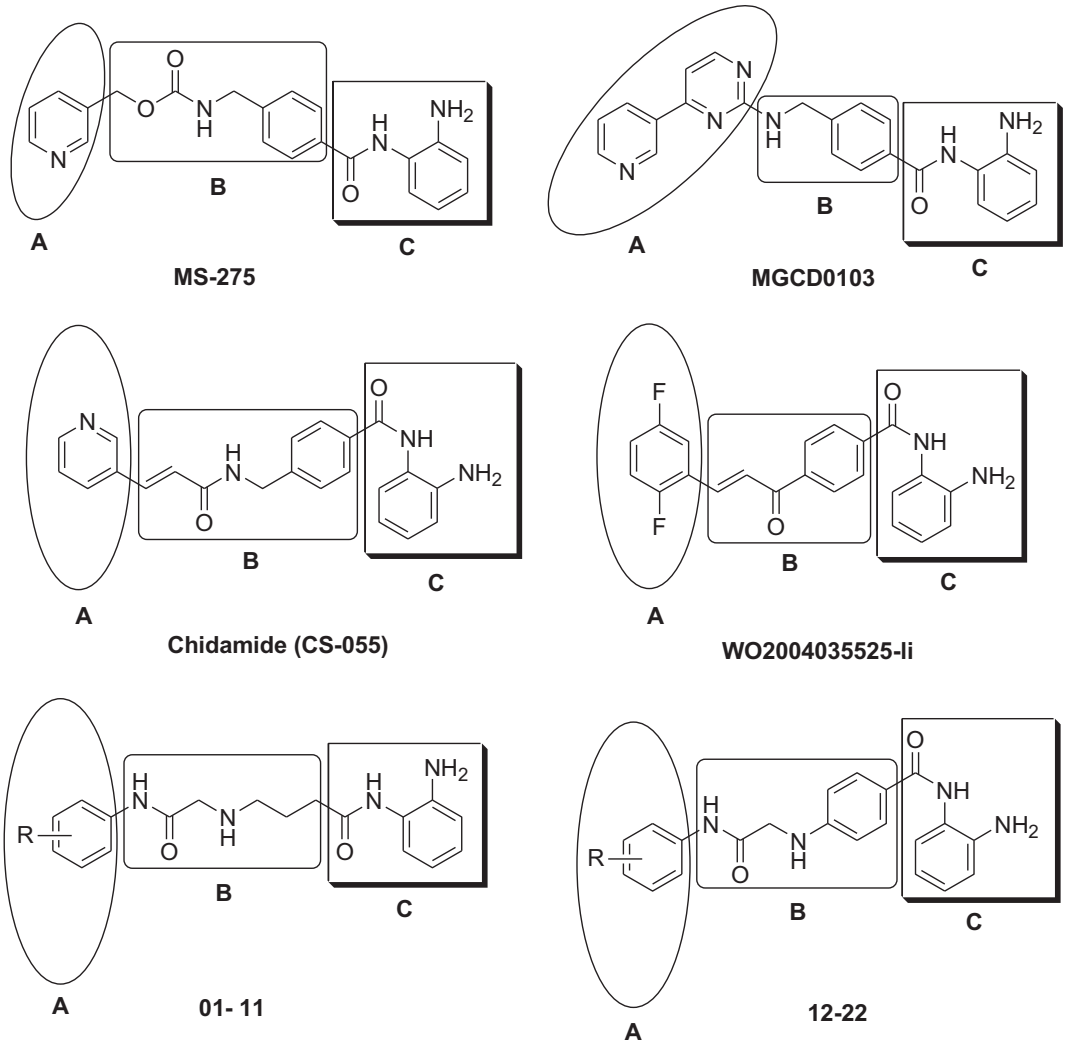


Fig. 1. Structural features present in clinically established benzamides and synthesized analogs 01–22. A = surface recognition moiety, B = Linker; C = zinc binding group.

known as zinc binding group (ZBG), and between both, a hydrophobic linker (C) fitting the tube-like portion of the binding pocket [12,13] (Fig. 1) The optimum chain length between cap and zinc binding group for HDACi activity has been found 7–8 atoms [14]. Present studies report the evaluation of γ-aminobutyric acid (GABA) and *para*-aminobenzoic acid (PABA) as linker in benzamide based HDACi.

2. Chemistry

The substituted anilines **I (i–xi)** were reacted with chloroacetylchloride resulting in the formation of N-(4-substituted-phenyl)-2-chloro-acetamide **II (i–xi)**. 4-{[(4-substituted-phenyl-carbamoyl)-methyl]-amino}-butyric acid **III (i–xi)** were prepared by condensation of **II (i–xi)** with GABA in ethanol in the presence of triethylamine [15]. The reaction of **III (i–xi)** with 1,2-pheny lenediamine in the presence of CDI and THF resulted in the formation of final benzamide analogs *i.e.,* N-(2-amino-phenyl)-4-{[(4-substituted-phenylcarbamoyl)-methyl]-amino}-butyramides **(01–11)** (Scheme 1). Another almost similar reaction scheme was also performed in which PABA was employed in place of GABA resulted in the preparation of N-(2-amino-phenyl)-4-{[(4-substituted-phenylcarbamoyl)-methyl]-amino}-benzamides **(12–22)** (Scheme 1). On critical observation, it is clear that the use of GABA/PABA has resulted in the formation of linker in the title compounds.

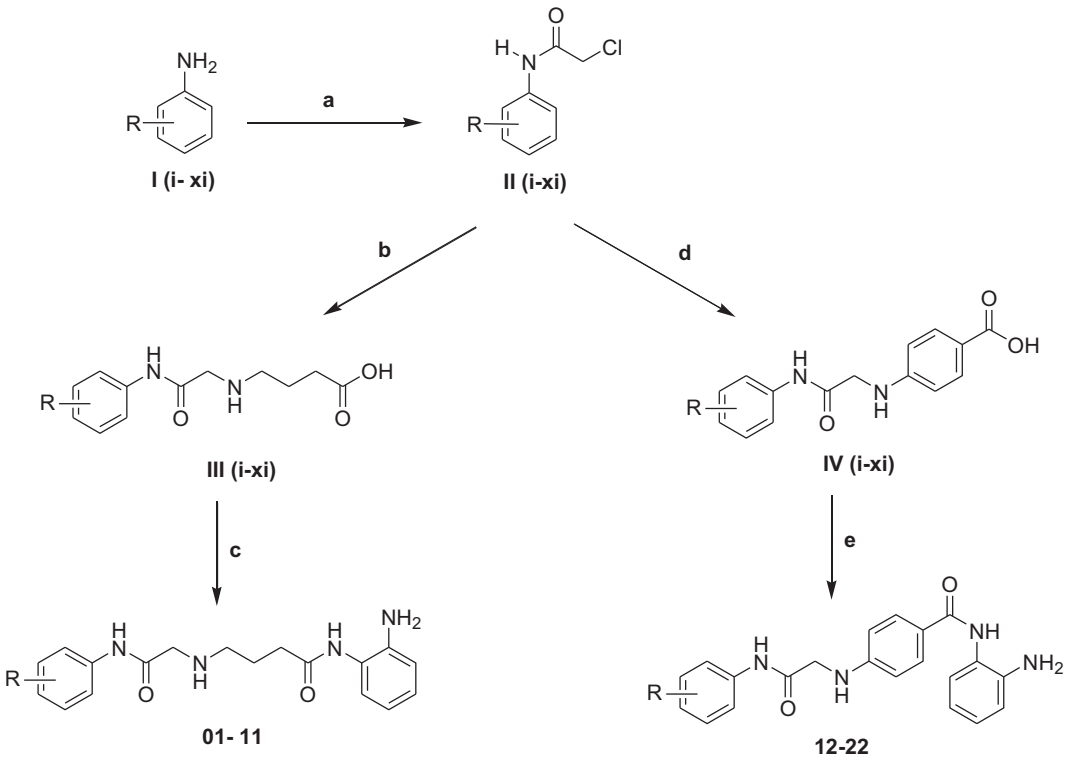
3. Pharmacology

All the synthesized compounds were tested for their ability to inhibit HDAC-1 activity and antiproliferative activity by MTT assays [16]. The test compounds were evaluated *in-vivo* for their anticancer activity against EAC cells in Swiss albino mice as per reported procedure [17].

4. Result and discussion

The structures of the title compounds were elucidated on the basis of elemental analysis, IR, ^{1}H NMR and mass spectral data. Elemental analyses for C, H and N were found within ±0.4% of the theoretical value. The spectroscopic data (IR, ^{1}H NMR and mass spectroscopy) of title compounds were in conformity with the assigned structure.

All the synthesized compounds were tested for their ability to inhibit HDAC-1 activity, antiproliferative activity by MTT assays and *in-vivo* anticancer activity against EAC cells. The HDAC-1 was chosen since it is widely implicated in both transcriptional repression and chromatin remodeling. Test compounds exhibited



Compound Code	R	Compound Code	R	Compound Code	R
I (i)	H	**1**	H	**12**	H
I (ii)	4-Br	**2**	4-Br	**13**	4-Br
I (iii)	2-Cl	**3**	2-Cl	**14**	2-Cl
I (iv)	3-Cl	**4**	3-Cl	**15**	3-Cl
I (v)	4-Cl	**5**	4-Cl	**16**	4-Cl
I (vi)	2- NO_2	**6**	2- NO_2	**17**	2- NO_2
I (vii)	3- NO_2	**7**	3- NO_2	**18**	3- NO_2
I (viii)	4- NO_2	**8**	4- NO_2	**19**	4- NO_2
I (ix)	4-OCH_3	**9**	4-OCH_3	**20**	4-OCH_3
I (x)	4-CH_3	**10**	4-CH_3	**21**	4-CH_3
I (xi)	2,5-Cl	**11**	2,5-Cl	**22**	2,5-Cl

Scheme 1. Synthesis of GABA and PABA-based benzamides. Reaction conditions: (a) $ClCH_2COCl$, CH_2Cl_2, 4–6 h stirring, 59–82%; (b) C_2H_5OH, $(C_2H_5)_3N$, GABA, 6–7 h reflux, 53–76%; (c) CDI, THF, 2 h stirring at 60–65 °C, 1,2-phenylenediamine, TFA, 9–10 h stirring, 58–71%; (d) C_2H_5OH, $(C_2H_5)_3N$, PABA, 8–9 h reflux, 54–66%; (e) CDI, THF, 2 h stirring at 60–65 °C, 1,2-phenylenediamine, TFA, 11–14 h stirring 56–68%.

significant activity in HDAC-1 inhibitory assay indicating that inhibition of histone deacetylase-1 might be one of the basis for anticancer activity. Inhibition of HDAC-1 causes apoptosis leading to anticancer activity [18]. The antiproliferative activity of test compounds was evaluated by MTT assays using HCT-116, U251 (glioma) cells [16]. HCT-116 cells were chosen for MTT assays because they have a high level of HDAC expression [19,20]. The test compounds were evaluated *in-vivo* for their anticancer activity against EAC cells in Swiss albino mice as per reported procedure [17]. The Mitomycin-C was chosen as standard drug in *in-vivo* anticancer studies as its mechasnism of action involves apoptosis [21]. The tumor weight inhibition (TWI) and tumor cell inhibition (TCI) were measured as parameters for anticancer evaluation.

The results of *in-vitro* anticancer studies shows that among GABA based benzamide analogs **(1–11)**, compound **8** exhibited maximum HDAC inhibitory activity with an $IC_{50} = 0.9$ μM in HDAC-1 assay. The compound **8** displayed $GI_{50} = 0.6$ μM and $GI_{50} = 9.4$ μM against HCT-116 and U251 (glioma) cell lines, respectively. On the other hand, compound **19** was found to be most active among PABA based benzamide analogs **(12–22)**, with HDAC inhibitory activity IC_{50} = 0.7 μM in HDAC-1 assay. The compound **19** showed $GI_{50} = 0.6$ μM and $GI_{50} = 8.2$ μM against HCT-116 and U251 (glioma) cell lines, respectively (Table 1). In terms of potency of synthesized benzamide analogs, almost similar results were observed during *in-vivo* anticancer studies against EAC cells in Swiss albino mice. The compound **8** (%TWI = 78.5; %TCI = 76.5) and **19** (%TWI = 83.3; % TCI = 77.5) were found most potent in GABA and PABA series of compounds, respectively (Table 2).

The title compounds were designed and synthesized with two points of diversity *i.e.*, variation of substituents on the surface recognition moiety (phenyl ring) and variation of linker. It is the surface recognition moiety along with linker that is responsible for variation in anticancer activity among different test compounds.

On going through structure–activity relationship, it has been observed that compounds bearing the groups like nitro, chloro and bromo on phenyl ring possess high potency in *in-vitro* and *in-vivo* anticancer evaluation. On the other hand, replacement of these groups with methyl groups resulted in compounds with lesser antiproliferative activity. On comparison of results, it has been found that anticancer activity of test compounds changes on varying *para*-substituted group on aryl moiety as follows: nitro > chloro > bromo > methoxy > methyl > no substitution. It is to be

Table 1
Outcomes of HDAC-1 inhibition and antiproliferative assays.

Compound	HDAC inhibitory activity[a] (IC_{50} (μM))	HCT-116[a] (μM)			U251 (Glioma)[a] (μM)		
		GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
1	9.2	70.4	>100	>100	>100	>100	>100
2	1.7	7.7	55.5	>100	10.4	80.0	>100
3	2.9	21.8	>100	>100	28.6	>100	>100
4	1.8	11.6	75.0	>100	19.5	>100	>100
5	1.4	5.5	45.0	>100	11.4	75.0	>100
6	3.1	2.8	32.0	>100	32.0	>100	>100
7	2.3	0.9	22.5	>100	15.0	93.5	>100
8	0.9	0.6	8.5	>100	9.4	66.0	>100
9	3.6	8.6	44.0	>100	34.0	>100	>100
10	7.5	17.9	>100	>100	57.0	>100	>100
11	8.9	31	78.0	>100	44.0	>100	>100
12	7.4	45.3	>100	>100	65.0	>100	>100
13	1.6	6.8	47.5	>100	10.8	45.0	>100
14	2.9	9.5	>100	>100	24.2	>100	>100
15	2.5	11.5	>100	>100	15.4	88.0	>100
16	1.0	4.2	62.5	>100	9.4	62.5	>100
17	2.0	3.5	25.0	>100	18.0	83.0	>100
18	1.4	1.1	9.0	>100	13.5	52.0	>100
19	0.7	0.6	6.5	>100	8.2	42.5	>100
20	3.1	7.5	38.0	>100	25.5	>100	>100
21	4.5	12.6	>100	>100	84.0	>100	>100
22	8.6	7.9	66.0	>100	38.6	>100	>100
MS-275	0.5	0.6	>100	>100	6.5	40	>100

Abbreviations: GI_{50} = Growth Inhibition at 50%; TGI = Tumor Growth Inhibition; LC_{50} = Lethal Concentration at 50%.
[a] Values are means of observation recorded in two experiments.

noted that *para*-nitro analogs *i.e.*, **8** and **19** showed greater potency than *ortho*-nitro substituted analogs *i.e.*, **6** and **17** or *meta*-nitro substituted compound *i.e.*, **7** and **18** in anticancer evaluation studies. The position of the substitution had dramatic effects on the HDAC activity. In a similar pattern analogs with *ortho*- and *meta*-chloro substitution were found to be lesser active than *para*-chloro analogs. This might be attributed to unfavorable steric effect of *meta*- and *ortho*-substitution or to exceeding the optimum lipophilic value of the substituents.

The optimum length of linker is one of the prime requirements for a benzamide based HDACi to exhibit anti-tumor activity. The presence of anticancer activity in title compounds may be attributed to the optimum length of linker *i.e.*, 7–8 atoms incorporated in the benzamide analogs from GABA and PABA. An overview of the results of anticancer evaluation shows that PABA based benzamide analogs were found to possess more activity than corresponding GABA based benzamide analogs. Thus it is clear that PABA provides better linker than that contributed by GABA in the design of HDACi.

5. Conclusion

The structure of the title compounds fulfilled all the three pharmacophoric structural requirements for HDAC inhibitors *i.e.*, benzamide group as zinc binding group, propylamino-acetamide moiety (in GABA based analogs)/tolyl-amino-acetamide (in PABA based analogs) as linker and phenyl moiety as surface recognition cap group. All the title compounds exhibited varied degree of anticancer activity and some compounds showed anticancer activity comparable to standard drug employed in respective studies. The presence of different substitutions in phenyl ring might have facilitated stronger cap group interactions with the amino acid side chains at the entrance of the HDAC active site. The optimum length of linker might have made possible the binding of benzamide group with zinc ion at the bottom of the active site.

In conclusion, we have designed and synthesized two novel series of benzamide analogs possessing linker derived from GABA or PABA for development of newer benzamide based HDACi. Present studies indicate that PABA provides better linker than that contributed by GABA in the design of HDACi. These results represent a remarkable step regarding the discovery of novel benzamide based HDACi.

6. Experimental protocols

6.1. Chemistry

All the chemicals and solvents employed in this study were procured from E-Merck (Germany), Aldrich (Germany), Himedia

Table 2
Anticancer activities of GABA and PABA based benzamide analogs in Swiss albino mice.

Compound	Average wt of Ascitic fluid (control)	Average wt. of Ascitic fluid (Test)[a]	% inhibition of Ascitic fluid (TWI)	Average no of cells/ml in control[a] × 10^5	Average no. of cells/ml Test[a] × 10^5	% inhibition of ascitic cell (TCI)
1	2.8 (±0.3)	2.1 (±0.4)	25.0	310.6 (±5.8)	251.2 (±4.7)	19.1
2	4.2 (±0.2)	1.1 (±0.2)	73.8	474.1 (±6.5)	150.8 (±5.8)	68.7
3	2.8 (±0.7)	2.2 (±0.3)	21.4	353.7 (±5.8)	308.5 (±4.2)	12.7
4	2.8 (±0.2)	1.9 (±0.5)	32.1	382.5 (±8.6)	296.2 (±3.3)	22.5
5	4.4 (±0.5)	1.2 (±0.6)	72.7	511.1 (±3.4)	127.4 (±4.7)	75.0
6	2.9 (±0.2)	1.7 (±0.8)	41.3	347.3 (±7.2)	310.8 (±2.5)	10.5
7	3.2 (±0.3)	1.9 (±0.2)	40.6	454.7 (±6.5)	290.6 (±5.8)	36.0
8	4.2 (±0.5)	0.9 (±0.4)	78.5	522.1 (±5.8)	122.6 (±4.2)	76.5
9	3.6 (±0.1)	1.3 (±0.5)	63.8	470.4 (±3.4)	172.5 (±6.5)	63.3
10	2.8 (±0.8)	1.6 ± 0.64)	42.8	366.9 (±8.6)	297.2 (±2.5)	18.9
11	3.4 (±0.4)	1.7 (±0.2)	50.0	442.6 (±6.5)	217.3 (±3.3)	50.9
12	2.7 (±0.5)	2.2 (±0.3)	18.5	292.4 (±5.8)	203.1 (±2.5)	30.5
13	3.8 (±0.6)	0.9 (±0.7)	76.3	516.5 (±3.4)	136.5 (±4.2)	73.5
14	3.0 (±0.2)	1.7 (±0.5)	43.3	439.6 (±5.3)	301.5 (±4.7)	31.4
15	3.8 (±0.8)	1.2 (±0.2)	68.4	465.3 (±7.2)	224.7 (±6.5)	51.7
16	3.9 (±0.6)	0.8 (±0.7)	79.4	490.2 (±5.8)	130.8 (±2.5)	73.3
17	3.1 (±0.2)	2.7 (±0.4)	12.9	322.6 (±5.3)	259.0 (±5.8)	19.7
18	3.4 (±0.5)	1.1 (±0.6)	67.6	444.3 (±6.5)	264.7 (±6.5)	40.4
19	4.2 (±0.3)	0.7 (±0.2)	83.3	508.8 (±8.6)	114.4 (±3.3)	77.5
20	3.3 (±0.2)	1.1 (±0.)	66.6	459.4 (±5.8)	172.9 (±2.5)	62.3
21	3.0 (±0.4)	1.7(±0.5)	43.3	361.7 (±7.2)	270.2 (±4.2)	25.2
22	3.6 (±0.6)	1.5 (±0.5)	58.3	443.9 (±8.6)	194.1 (±6.5)	56.2
MMC[b]	3.9 (±0.2)	0.0 (±0.0)	100	463.2 (±8.4)	0.0 (±0.0)	100.0

Abbreviations: GABA = γ-aminobutyric acid; PABA = *para*-aminobenzoic acid; TWI = Tumor Weight Inhibtion; TCI = Tumor Cell Inhibtion; MMC = Mitomycin-C.
[a] Observation shown are mean of 6 readings and data in parenthesis indicates standard error of mean.
[b] Mitomycin-C at a dose level of 1 mg/kg body weight was employed as standard, which showed 100% inhibition.

(India) and Spectrochem Pvt Ltd (India). Melting points were determined by open capillary method and are uncorrected. The IR spectra were recorded on a Perkin Elmer IR spectrophotometer (KBr disc) (Perkin Elmer, Beaconsfield, UK), NMR spectra on a Bruker DRX-300 NMR spectrometer (DMSO-d_6, TMS) (Bruker Bioscience, Billerica, MA, USA) and the electrospray mass spectra on a Micromass Quattro II triple-quadrupole mass spectrometer (Methanol) (Micromass, Manchester, UK). The title compounds were prepared using the synthetic strategy described in Scheme 1.

The N-(4-substituted-phenyl)-2-chloro-acetamide **II (i–xi)** and 4-{[(4-substituted-phenylcarbamoyl)-methyl]-amino}-butyric acid **III (i–xi)** were prepared according to the procedure reported [15].

*6.1.1. General procedure for synthesis of N-(4-substituted-phenyl)-2-chloro-acetamide II (**i–xi**)*

Substituted aniline (0.01 mol) **I (i–xi)** and Dichloromethane (20 ml) were mixed in a conical flask and Chloroacetylchloride (1.02 ml; 0.01 M) in a separating funnel was added drop by drop to the conical flask. The reaction mixture was stirred for 4–6 h resulting in the formation of white precipitate. The product obtained was filtered, and washed with a little cold water and recrystallized from water or from methanol or ethanol either alone or dilute with water.

Compound **II (i)**: Yield 82%, m.p. 86–88 °C. IR (cm^{-1}) (KBr) 3048.4 (Aromatic C–H str), 1600.4 & 1503.7 (Aromatic C–C str), 1670.24 (C=O str of amide), 3463.92 (N–H str of amide), 750.26 (C–Cl); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.95–7.68 (m, 5H, ArH), 8.16 (s, 1H, NH), 4.47 (s, 2H, CH_2); ESI-MS (Methanol) *m/z* 170.05 ($[M + H]^+$).

Compounds: II(ii) yield 77% m.p. 110–112 °C; **II(iii)** 60%, 93–95 °C; **II(iv)** 62%, 93–94 °C; **II(v)** 60%, 94–96 °C; **II(vi)** 59%, 121–122 °C; **II(vii)** 63%, 122–124 °C **II(viii)** 66%, 122–125 °C; **II(ix)** 67%, 136–137 °C; **II(x)** 64%, 116–118 °C; **II(xi)** 62%, 131–133 °C.

*6.1.2. General procedure for synthesis of 4-{[(4-substituted-phenylcarbamoyl)-methyl]-amino}-butyric acid III (**i–xi**)*

4-Amino butanoic acid (GABA) was dissolved in ethyl alcohol followed by addition of **II (i–xi)** (0.01 M) and triethylamine (0.01 mol). The reaction mixture was refluxed for 6–7 h. The reaction mixture was poured on crushed ice. The resulting solid was separated, dried, and recrystallized from ethanol.

Compound **III (i)**: Yield 76%, m.p. 195–197 °C. IR (cm^{-1}) (KBr) 3063.92 (Aromatic C–H str), 1601.85 & 1502.47 (Aromatic C–C str), 1674.77 (C=O str of amide), 3468.82 (N–H str of amide),, 2894.33 (C–H str alkane), 1710.25 (C–O str of COOH), 2728.43 (O–H str of COOH); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.90–7.62 (m, 5H, ArH), 8.11 (s, 1H, NH), 3.53 (d, 2H, $COCH_2NH$), 2.18 (q, 2H, $NHCH_2CH_2CH_2COOH$), 1.75 (p, 2H, $NHCH_2CH_2CH_2COOH$), 2.33 (t, 2H, $NHCH_2CH_2CH_2COOH$), 10.78 (s, 2H, COOH); ESI-MS (Methanol) *m/z* 237.16 ($[M + H]^+$).

Compounds: III(ii) yield 72% m.p. 122–124 °C; **III(iii)** 55%, 96–98 °C; **III(iv)** 53%, 95–98 °C; **III(v)** 56%, 97–99 °C; **III(vi)** 62%, 104–106 °C; **III(vii)** 61%, 105–106 °C **III(viii)** 66%, 104–107 °C; **III(ix)** 60%, 123–125 °C; **III(x)** 59%, 108–110 °C; **III(xi)** 57%, 116–118 °C.

*6.1.3. General procedure for synthesis of 4-{[(4-substituted-phenylcarbamoyl)-methyl]-amino}-benzoic acid IV (**i–xi**)*

4-Amino benzoic acid (PABA) (0.01 mol) was dissolved in ethyl alcohol followed by addition of **III (i–xi)** (0.01 M) and triethylamine (0.1 mol). The reaction mixture was refluxed for 7–8 h. The solution was poured on crushed ice. The resulting solid was separated, dried, and recrystallized from ethanol.

Compound **IV (i)**: Yield 62%, m.p. 224–226 °C. IR (cm^{-1}) (KBr) 3082.75 (Aromatic C–H str), 1602.60 & 1501.55 (Aromatic C–C str), 1670.68 (C=O str of amide), 3462.59 (N–H str of amide), 1715.27 (C–O str of COOH), 2703.94 (O–H str of COOH); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.67–7.84 (m, 9H, ArH), 8.14 (s, 1H, NH), 3.76 (d, 2H, $COCH_2NH$), 4.30 (t, 1H, $COCH_2NH$), 10.85 (s, 2H, COOH); ESI-MS (Methanol) *m/z* 271.12 ($[M + H]^+$).

Compounds: IV(ii) yield 56% m.p. 139–141 °C; **IV(iii)** 62%, 111–113 °C; **IV(iv)** 58%, 110–112 °C; **IV(v)** 63%, 111–114 °C; **IV(vi)** 60%, 127–129 °C; **IV(vii)** 57%, 127–128 °C **IV(viii)** 64%, 126–128 °C; **IV(ix)** 66%, 138–140 °C; **IV(x)** 54%, 120–122 °C; **IV(xi)** 59%, 145–146 °C.

*6.1.4. General procedure for synthesis of N-(2-amino-phenyl)-4-{[(4-substituted-phenylcarbamoyl)-methyl]-amino}-butyramides (**01–11**)*

Compound **III (i–xi)** (0.01 M) was added to 100 ml THF followed by proportion wise addition of CDI (0.01 M) at room temperature. The reaction mixture was stirred for 2 h at 60–65 °C. 1,2-phenylenediamine (0.01 M) and trifluoroacetic acid (0.01 M) were added to reaction mixture followed by stirring for 9–10 h. The solvent was evaporated under vacuum and crude product was stirred in a mixture of hexane and water (2:5 v/v) for 1 h and filtered and dried. The crude product was recrystallized using dichloromethane.

Compound **1**: Yield 63%, m.p. 109–112 °C. IR (cm^{-1}) (KBr) 3063.11 (aromatic C–H str), 1600.47 & 1501.81 (aromatic C–C str), 1674.68 (C=O str of amide), 3433.19 (N–H str of amide), 3365.38 (N–H str amino group), 2893.62 (aliphatic C–H str), 1442.64 (aliphatic C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.65–7.61 (m, 9H, ArH), 9.94 (s, 1H, NH), 3.55 (d, 2H, $COCH_2NH$), 2.58 (q, 2H, $NHCH_2CH_2CH_2CO$), 1.75 (p, 2H, $NHCH_2CH_2CH_2CO$), 2.31 (t, 2H, $NHCH_2CH_2CH_2CO$), 4.97 (s, 2H, NH_2); ESI-MS *m/z* 327.18 ($[M + H]^+$).

Compound **2**: Yield 69%, m.p. 124–126 °C; IR (cm^{-1}) (KBr) 3048.78 (aromatic C–H str), 1601.22 & 1500.73 (aromatic C–C str), 1677.24 (C=O str of amide), 3429.90 (N–H str of amide), 3357.48 (N–H str amino group), 2898.71 (aliphatic C–H str), 1436.83 (aliphatic C–H def), 824.59 (para-disubstituted benzene C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.41–7.63 (m, 8H, ArH), 9.86 (s, 1H, NH), 3.51 (d, 2H, $COCH_2NH$), 2.56 (q, 2H, $NHCH_2CH_2CH_2CO$), 1.77 (p, 2H, $NHCH_2CH_2CH_2CO$), 2.28 (t, 2H, $NHCH_2CH_2CH_2CO$), 4.94 (s, 2H, NH_2); ESI-MS *m/z* 405.10 ($[M + H]^+$).

Compound **3**: Yield 59%, m.p. 118–120 °C; IR (cm^{-1}) (KBr) 3036.35 (aromatic C–H str), 1600.48 & 1502.85 (aromatic C–C str), 1679.04 (C=O str of amide), 3432.62 (N–H str of amide), 3353.93 (N–H str amino group), 2893.14 (aliphatic C–H str), 1439.13 (aliphatic C–H def), 750.48 (ortho-disubstituted C–C def), 789.25 (C–Cl str); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.45–7.66 (m, 8H, ArH), 9.83 (s, 1H, NH), 3.54 (d, 2H, $COCH_2NH$), 2.58 (q, 2H, $NHCH_2CH_2CH_2CO$), 1.75 (p, 2H, $NHCH_2CH_2CH_2CO$), 2.29 (t, 2H, $NHCH_2CH_2CH_2CO$), 4.92 (s, 2H, NH_2); ESI-MS *m/z* 361.14 ($[M + H]^+$).

Compound **4**: Yield 62%, m.p. 117–119 °C; IR (cm^{-1}) (KBr) 3040.22 (aromatic C–H str), 1603.13 & 1503.46 (aromatic C–C str), 1672.97 (C=O str of amide), 3425.71 (N–H str of amide), 3350.54 (N–H str amino group), 2898.26 (aliphatic C–H str), 1444.18 (aliphatic C–H def), 700.94, 770.83 (meta-disubstituted C–H def), 785.92 (C–Cl str); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.47–7.66 (m, 8H, ArH), 9.91 (s, 1H, NH), 3.54 (d, 2H, $COCH_2NH$), 2.59 (q, 2H, $NHCH_2CH_2CH_2CO$), 1.77 (p, 2H, $NHCH_2CH_2CH_2CO$), 2.32 (t, 2H, $NHCH_2CH_2CH_2CO$), 4.88 (s, 2H, NH_2); ESI-MS *m/z* 361.14 ($[M + H]^+$).

Compound **5**: Yield 64%, m.p. 118–121 °C; IR (cm^{-1}) (KBr) 3034.45 (aromatic C–H str), 1601.73 & 1502.83 (aromatic C–C str), 1674.42 (C=O str of amide), 3427.38 (N–H str of amide), 3356.02 (N–H str amino group), 2890.42 (aliphatic C–H str), 1446.62 (aliphatic C–H def), 828.93 (para-disubstituted benzene C–H def),

780.84 (C–Cl str); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.46–7.62 (m, 8H, ArH), 9.86 (s, 1H, NH), 3.52 (d, 2H, COC$\underline{H}_2$NH), 2.55 (q, 2H, NHC$\underline{H}_2$CH$_2$CH$_2$CO), 1.74 (p, 2H, NHCH$_2$C$\underline{H}_2$CH$_2$CO), 2.33 (t, 2H, NHCH$_2$CH$_2$C$\underline{H}_2$CO), 4.94 (s, 2H, NH$_2$); ESI-MS *m/z* 361.14 ([M + H]$^+$).

Compound **6**: Yield 68%, m.p. 137–139 °C; IR (cm^{-1}) (KBr) 3043.49 (aromatic C–H str), 1600.89 & 1501.54 (aromatic C–C str), 1672.82 (C=O str of amide), 3423.18 (N–H str of amide), 3353.42 (N–H str amino group), 2888.58 (aliphatic C–H str), 1440.39 (aliphatic C–H def), 1521.68 & 1365.82 (N=O str of Ar–NO$_2$ group), 753.31 (ortho-disubstituted C–C def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.42–8.06 (m, 8H, ArH), 9.87 (s, 1H, NH), 3.55 (d, 2H, COC$\underline{H}_2$NH), 2.57 (q, 2H, NHC$\underline{H}_2$CH$_2$CH$_2$CO), 1.75 (p, 2H, NHCH$_2$C$\underline{H}_2$CH$_2$CO), 2.36 (t, 2H, NHCH$_2$CH$_2$C$\underline{H}_2$CO), 4.89 (s, 2H, NH$_2$); ESI-MS *m/z* 372.16 ([M + H]$^+$).

Compound **7**: Yield 66%, m.p. 138–139 °C; IR (cm^{-1}) (KBr) 3056.42 (aromatic C–H str), 1601.48 & 1502.11 (aromatic C–C str), 1675.27 (C=O str of amide), 3425.74 (N–H str of amide), 3351.31 (N–H str amino group), 2885.03 (aliphatic C–H str), 1442.72 (aliphatic C–H def), 1524.50 & 1368.64 (N=O str of Ar–NO$_2$ group), 703.64, 775.28 (meta-disubstituted C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.45–8.10 (m, 8H, ArH), 9.88 (s, 1H, NH), 3.57 (d, 2H, COC$\underline{H}_2$NH), 2.54 (q, 2H, NHC$\underline{H}_2$CH$_2$CH$_2$CO), 1.73 (p, 2H, NHCH$_2$C$\underline{H}_2$CH$_2$CO), 2.30 (t, 2H, NHCH$_2$CH$_2$C$\underline{H}_2$CO), 4.92 (s, 2H, NH$_2$); ESI-MS *m/z* 372.16 ([M + H]$^+$).

Compound **8**: Yield 71%, m.p. 137–140 °C; IR (cm^{-1}) (KBr) 3060.85 (aromatic C–H str), 1600.52 & 1501.28 (aromatic C–C str), 1672.94 (C=O str of amide), 3434.39 (N–H str of amide), 3355.85 (N–H str amino group), 2882.47 (aliphatic C–H str), 1438.61 (aliphatic C–H def), 1519.31 & 1360.36 (N=O str of Ar–NO$_2$ group), 825.39 (para-disubstituted benzene C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.40–8.02 (m, 8H, ArH), 9.84 (s, 1H, NH), 3.55 (d, 2H, COC$\underline{H}_2$NH), 2.58 (q, 2H, NHC$\underline{H}_2$CH$_2$CH$_2$CO), 1.74 (p, 2H, NHCH$_2$C$\underline{H}_2$CH$_2$CO), 2.28 (t, 2H, NHCH$_2$CH$_2$C$\underline{H}_2$CO), 4.91 (s, 2H, NH$_2$); ESI-MS m/*z* 372.16 ([M + H]$^+$).

Compound **9**: Yield 65%, m.p. 145–147 °C; IR (cm^{-1}) (KBr) 3067.39 (aromatic C–H str), 1601.74 & 1500.52 (aromatic C–C str), 1670.97 (C=O str of amide), 3438.11 (N–H str of amide), 3351.24 (N–H str amino group), 2880.63 (aliphatic C–H str), 1432.74 (aliphatic C–H def), 832.19 (para-disubstituted benzene C–H def), 1258.42 (C-O of OCH$_3$ group); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.42–7.53 (m, 8H, ArH), 9.92 (s, 1H, NH), 3.59 (d, 2H, COC$\underline{H}_2$NH), 2.57 (q, 2H, NHC$\underline{H}_2$CH$_2$CH$_2$CO), 1.72 (p, 2H, NHCH$_2$C$\underline{H}_2$CH$_2$CO), 2.32 (t, 2H, NHCH$_2$CH$_2$C$\underline{H}_2$CO), 4.84 (s, 2H, NH$_2$), 3.74 (s, 3H, OCH$_3$); ESI-MS *m/z* 357.23 ([M + H]$^+$).

Compound **10**: Yield 60%, m.p. 131–133 °C; IR (cm^{-1}) (KBr) 3053.41 (aromatic C–H str), 1600.58 & 1503.46 (aromatic C–C str), 1673.82 (C=O str of amide), 3432.75 (N–H str of amide), 3355.57 (N–H str amino group), 2886.18 (aliphatic C–H str), 1436.92 (aliphatic C–H def), 835.72 (para-disubstituted benzene C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.46–7.58 (m, 8H, ArH), 9.86 (s, 1H, NH), 3.54 (d, 2H, COC$\underline{H}_2$NH), 2.58 (q, 2H, NHC$\underline{H}_2$CH$_2$CH$_2$CO), 1.75 (p, 2H, NHCH$_2$C$\underline{H}_2$CH$_2$CO), 2.36 (t, 2H, NHCH$_2$CH$_2$C$\underline{H}_2$CO), 4.94 (s, 2H, NH$_2$), 2.42 (s, 3H, CH$_3$); ESI-MS *m/z* 341.15 ([M + H]$^+$).

Compound **11**: Yield 58%, m.p. 115–116 °C; IR (cm^{-1}) (KBr) 3048.73 (aromatic C–H str), 1601.52 & 1502.88 (aromatic C–C str), 1678.28 (C=O str of amide), 3430.52 (N–H str of amide), 3351.39 (N–H str amino group), 2893.38 (aliphatic C–H str), 1434.81 (aliphatic C–H def), 748.59 (ortho-disubstituted C–C def), 708.21, 776.04 (meta-disubstituted C–H def), 805.16 (C–Cl str); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.42–7.55 (m, 7H, ArH), 9.90 (s, 1H, NH), 3.56 (d, 2H, COC$\underline{H}_2$NH), 2.62 (q, 2H, NHC$\underline{H}_2$CH$_2$CH$_2$CO), 1.79 (p, 2H, NHCH$_2$C$\underline{H}_2$CH$_2$CO), 2.38 (t, 2H, NHCH$_2$CH$_2$C$\underline{H}_2$CO), 4.94 (s, 2H, NH$_2$); ESI-MS *m/z* 395.10 ([M + H]$^+$).

*6.1.5. General procedure for synthesis of N-(2-amino-phenyl)-4-{[(4-substituted-phenylcarbamoyl)-methyl]-amino}-benzamides (**12**–**22**)*

Compound **IV (i–xi)** (0.01 M) was added to 100 ml THF followed by proportion wise addition of CDI (0.01 M) at room temperature. The reaction mixture was stirred for 2 h at 60–65 °C. 1,2-phenylenediamine (0.01 M) and trifluoroacetic acid (0.01 M) were added to reaction mixture followed by stirring for 11–14 h. The solvent was evaporated under vacuum and crude product was stirred in a mixture of hexane and water (2:5 v/v) for 1 h and filtered and dried. The crude product was recrystallized using dichloromethane.

Compound **12**: Yield 60%, m.p. 124–126 °C. IR (cm^{-1}) (KBr) 3054.29 (aromatic C–H str), 1597.16 & 1502.51 (aromatic C–C str), 1670.83 (C=O str of amide), 3431.62 (N–H str of amide), 3361.42 (N–H str amino group), 2887.70 (aliphatic C–H str), 1438.81 (aliphatic C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.52–7.75 (m, 13H, ArH), 9.84 (s, 1H, NH), 3.76 (d, 2H, COC$\underline{H}_2$NH), 4.18 (d, 2H, COCH$_2$N$\underline{H}$), 4.88 (s, 2H, NH$_2$); ESI-MS *m/z* 361.18 ([M + H]$^+$).

Compound **13**: Yield 58%, m.p. 138–140 °C; IR (cm^{-1}) (KBr) 3062.92 (aromatic C–H str), 1603.41 & 1501.56 (aromatic C–C str), 1679.04 (C=O str of amide), 3435.72 (N–H str of amide), 3359.37 (N–H str amino group), 2885.52 (aliphatic C–H str), 1442.80 (aliphatic C–H def), 828.29 (para-disubstituted benzene C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.48–7.58 (m, 12H, ArH), 9.88 (s, 1H, NH), 3.79 (d, 2H, COC$\underline{H}_2$NH), 4.19 (d, 2H, COCH$_2$N$\underline{H}$), 4.93 (s, 2H, NH$_2$); ESI-MS *m/z* 439.10 ([M + H]$^+$).

Compound **14**: Yield 59%, m.p. 147–148 °C; IR (cm^{-1}) (KBr) 3048.77 (aromatic C–H str), 1603.94 & 1503.41 (aromatic C–C str), 1671.64 (C=O str of amide), 3431.12 (N–H str of amide), 3358.40 (N–H str amino group), 2886.63 (aliphatic C–H str), 1433.25 (aliphatic C–H def), 753.35 (ortho-disubstituted C–C def), 793.69 (C–Cl str); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.40–7.62 (m, 12H, ArH), 9.91 (s, 1H, NH), 3.76 (d, 2H, COC$\underline{H}_2$NH), 4.16 (d, 2H, COCH$_2$N$\underline{H}$), 4.95 (s, 2H, NH$_2$); ESI-MS *m/z* 395.12 ([M + H]$^+$).

Compound **15**: Yield 57%, m.p. 146–148 °C; IR (cm^{-1}) (KBr) 3053.84 (aromatic C–H str), 1600.38 & 1499.10 (aromatic C–C str), 1670.84 (C=O str of amide), 3428.65 (N–H str of amide), 3348.91 (N–H str amino group), 2892.74 (aliphatic C–H str), 1443.96 (aliphatic C–H def), 705.16, 771.44 (meta-disubstituted C–H def), 785.52 (C–Cl str); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.45–7.66 (m, 12H, ArH), 9.90 (s, 1H, NH), 3.81 (d, 2H, COC$\underline{H}_2$NH), 4.14 (d, 2H, COCH$_2$N$\underline{H}$), 4.90 (s, 2H, NH$_2$); ESI-MS *m/z* 395.12 ([M + H]$^+$).

Compound **16**: Yield 62%, m.p. 147–149 °C; IR (cm^{-1}) (KBr) 3038.39 (aromatic C–H str), 1600.82 & 1501.81 (aromatic C–C str), 1679.21 (C=O str of amide), 3429.39 (N–H str of amide), 3361.72 (N–H str amino group), 2897.31 (aliphatic C–H str), 1451.89 (aliphatic C–H def), 829.11 (para-disubstituted benzene C–H def), 784.97 (C–Cl str); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.49–7.29 (m, 12H, ArH), 9.85 (s, 1H, NH), 3.84 (d, 2H, COC$\underline{H}_2$NH), 4.17 (d, 2H, COCH$_2$N$\underline{H}$), 4.92 (s, 2H, NH$_2$); ESI-MS *m/z* 395.12 ([M + H]$^+$).

Compound **17**: Yield 67%, m.p. 156–158 °C; IR (cm^{-1}) (KBr) 3038.35 (aromatic C–H str), 1602.75 & 1502.92 (aromatic C–C str), 1677.52 (C=O str of amide), 3417.96 (N–H str of amide), 3347.84 (N–H str amino group), 2895.42 (aliphatic C–H str), 1446.28 (aliphatic C–H def), 1524.29 & 1366.85 (N=O str of Ar–NO$_2$ group), 757.96 (ortho-disubstituted C–C def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.56–8.14 (m, 12H, ArH), 9.87 (s, 1H, NH), 3.83 (d, 2H, COC$\underline{H}_2$NH), 4.19 (d, 2H, COCH$_2$N$\underline{H}$), 4.90 (s, 2H, NH$_2$); ESI-MS *m/z* 406.11 ([M + H]$^+$).

Compound **18**: Yield 65%, m.p. 155–158 °C; IR (cm^{-1}) (KBr) 3062.52 (aromatic C–H str), 1600.31 & 1508.59 (aromatic C–C str),

1678.92 (C=O str of amide), 3422.27 (N–H str of amide), 3356.62 (N–H str amino group), 2874.83 (aliphatic C–H str), 1438.78 (aliphatic C–H def), 1522.95 & 1362.89 (N=O str of Ar–NO_2 group), 704.82, 779.11 (meta-disubstituted C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.40–8.42 (m, 12H, ArH), 9.95 (s, 1H, NH), 3.88 (d, 2H, $COCH_2NH$), 4.17 (d, 2H, $COCH_2NH$), 4.92 (s, 2H, NH_2); ESI-MS *m/z* 406.11 ($[M + H]^+$).

Compound **19**: Yield 68%, m.p. 156–159 °C; IR (cm^{-1}) (KBr) 3056.93 (aromatic C–H str), 1601.32 & 1504.86 (aromatic C–C str), 1677.35 (C=O str of amide), 3439.27 (N–H str of amide), 3359.36 (N–H str amino group), 2884.25 (aliphatic C–H str), 1439.84 (aliphatic C–H def), 1523.81 & 1366.17 (N=O str of Ar–NO_2 group), 820.63 (para-disubstituted benzene C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.49–8.17 (m, 12H, ArH), 9.88 (s, 1H, NH), 3.84 (d, 2H, $COCH_2NH$), 4.11 (d, 2H, $COCH_2NH$), 4.86 (s, 2H, NH_2); ESI-MS *m/z* 406.11 ($[M + H]^+$).

Compound **20**: Yield 63%, m.p. 168–170 °C; IR (cm^{-1}) (KBr) 3073.47 (aromatic C–H str), 1602.84 & 1501.47 (aromatic C–C str), 1671.34 (C=O str of amide), 3439.31 (N–H str of amide), 3355.07 (N–H str amino group), 2883.73 (aliphatic C–H str), 1434.11 (aliphatic C–H def), 835.61 (para-disubstituted benzene C–H def), 1262.83 (C–O of OCH_3 group); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.82–7.58 (m, 12H, ArH), 9.85 (s, 1H, NH), 3.86 (d, 2H, $COCH_2NH$), 4.14 (d, 2H, $COCH_2NH$), 4.91 (s, 2H, NH_2), 3.65 (s, 3H, OCH_3); ESI-MS *m/z* 391.22 ($[M + H]^+$).

Compound **21**: Yield 62%, m.p. 140–142 °C; IR (cm^{-1}) (KBr) 3058.81 (aromatic C–H str), 1602.75 & 1505.92 (aromatic C–C str), 1678.52 (C=O str of amide), 3437.51 (N–H str of amide), 3348.33 (N–H str amino group), 2889.86 (aliphatic C–H str), 1438.52 (aliphatic C–H def), 837.82 (para-disubstituted benzene C–H def); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.52–7.78 (m, 12H, ArH), 9.80 (s, 1H, NH), 3.83 (d, 2H, $COCH_2NH$), 4.19 (d, 2H, $COCH_2NH$), 4.95 (s, 2H, NH_2), 2.42 (s, 3H, CH_3); ESI-MS *m/z* 375.18 ($[M + H]^+$).

Compound **22**: Yield 56%, m.p. 135–137 °C; IR (cm^{-1}) (KBr) 3052.79 (aromatic C–H str), 1604.80 & 1505.41 (aromatic C–C str), 1657.94 (C=O str of amide), 3435.36 (N–H str of amide), 3355.98 (N–H str amino group), 2890.32 (aliphatic C–H str), 1437.94 (aliphatic C–H def), 750.07 (ortho-disubstituted C–C def), 710.43, 775.61 (meta-disubstituted C–H def), 807.58 (C–Cl str); ^{1}H NMR (300 MHz, DMSO-d_6, TMS, δ ppm): 6.65–7.58 (m, 11H, ArH), 9.85 (s, 1H, NH), 3.87 (d, 2H, $COCH_2NH$), 4.15 (d, 2H, $COCH_2NH$), 4.90 (s, 2H, NH_2); ESI-MS *m/z* 429.05 ($[M + H]^+$).

6.2. Biological activity

All the synthesized compounds were tested for their ability to inhibit HDAC-1 activity and antiproliferative activity by MTT assays [16]. The test compounds were evaluated *in-vivo* for their anticancer activity against EAC cells in Swiss albino mice as per reported procedure [17].

HDAC1 inhibitory assay: The HDAC inhibitory activity of test compounds was assessed using the commercially available HDAC fluorescence activity assay/drug discovery (*Fluor de Lys*) kit (AK-511, BIOMOL Research Laboratories) according to the supplier's protocol.

Cell line studies: The antiproliferative activity of test compounds was evaluated by MTT assays using HCT-116 and U251 (glioma) cell lines [16]. Cells were incubated with different concentrations of the extract for 2 days in a 96 well plate, after which the live cells which did not take in stain and dead cells which took in stain were counted. For counting the cell suspension was mixed with an equal volume of trypan blue and was counted. The Growth Inhibition at 50% (GI_{50}), Tumor Growth Inhibition (TGI) and Lethal Concentration at 50% (LC_{50}) were measured as parameter for anticancer evaluation.

***In-vivo* anticancer evaluation** [17]: EAC cells were maintained *in-vivo* in Swiss albino mice (18–20 g; 10 week old), by passage after every 10 days. EAC cells 9 days old were employed for anticancer evaluation of test compounds. Mitomycin-C at a dose level of 1 mg/kg body weight was used as standard, which showed 100% inhibition. The compounds were dissolved/suspended in phosphate buffered saline with pH 7.2. EAC cells were collected from the donor mouse and suspended in sterile isotonic saline. The EAC cells were counted under the microscope and adjusted to 10×10^6 cells/mL. On day zero, 0.1 mL of EAC cells per 10-g body weight of the animal was injected (*ip*) followed by a day for incubation to permit proliferation of the cells. Seven doses of compound (0.2 mmol/kg, 0.1 mL per 10-g body weight) were injected *ip* from the first day up to the seventh day with 24-h intervals. Control group of animals received only vehicle. On eighth day, food and water were withdrawn 6 h before sacrificing the animals. All the animals were sacrificed; peritoneal fluid was collected for cell count. All the fluid in the peritoneal cavity was wiped off with absorbent cotton. The weight of the animals was measured before sacrificing and after removing the fluid from their peritoneal cavity. The difference in weight is considered as tumor weight. The percentage inhibition of cell count was calculated by equation as follows: Percentage inhibition of Ascitic cell (TCI) = $[(1 - T/C) \times 100]$; Percentage inhibition of Ascitic fluid (TWI) = $[(1 - T/C) \times 100]$.

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