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Original article

Synthesis of amidine and bis amidine derivatives and their evaluation for anti-inflammatory and anticancer activity

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ABSTRACT

2-Cyanopyridine (**1a**), 4-cyanopyridine (**1b**), 2-cyanopyrazine (**1c**) on condensation with mono amines (**2a**–**c**) and diamines (**4a**–**c**) in the presence of sodium methoxide as catalyst gave amidine derivatives (**3a**–**i**) and bis amidine derivatives (**5a**–**i**) in good yields. All these compounds were fully characterized by spectroscopic means and elemental analysis. On screening for anti-inflammatory activity and for *in vitro* anticancer activity compounds **5c** and **5d** exhibited good anti-inflammatory activity whereas compounds **5d** breast (T47D), **5h**, **5i** lung (NCI H-522), **5i** colon (HCT-15), **3c**, **3h**, **5i** ovary (PA-1) and **3c**, **5b**, **5h** liver (HepG2) exhibited good anticancer activity.

1. Introduction

Inflammatory diseases such as asthma, allergy, arthritis, multiple sclerosis etc. are quite common form which human beings suffer worldwide. For the treatment of inflammatory diseases various anti-inflammatory drugs i.e. indomethacin, diclofenac, aspirin, ibuprofen, nimisulide, celecoxib and roficoxib etc. are available in the market [1]. These drugs cannot be used continuously for long time as they can cause serious side effects such as ulceration, gastrointestinal bleeding and heart strock [2,3]. Another disease which is responsible for many deaths every year is cancer. At present various research groups worldwide are involved in search of safer anti-inflammatory and anticancer agents [4–7]. Amidine and bis amidine derivatives possessing anti-inflammatory [8–10], antimicrobial [11], antiparasitic [12], antibacterial [13], antiprotozoal [14], antimalarial [15], anticancer [16–22], anti HIV [23], antidegenerative [24], and urokinase inhibitor [25] activities are well documented in literature. Amidine derivatives also act as drug carrier [26] and as starting materials for various heterocyclic molecules [27].

Tempted by wide variety of biological activities exhibited by amidine and bis amidine derivatives and in continuation [28–31] of our efforts in search of potent molecules exhibiting anti-inflammatory and anticancer activities we have synthesized a number of amidine and bis amidine derivatives and screened them for anti-inflammatory and anticancer activities which we wish to report in this paper.

2. Results and discussion

2.1. Chemistry

Amidine and bis amidine derivatives can be synthesized by condensation of nitrile with mono and diamines, but in most of the cases activation of nitrile is needed [32–38]. 2-Cyanopyridine (**1a**) 4-cyanopyridine (**1b**) and 2-cyanopyrazine (**1c**) were first allowed to react with sodium methoxide by stirring at room temperature for 2 h using absolute methanol as solvent of reaction to give insitu intermediate [39] **1** (Scheme 1). Intermediate **1** (Scheme 1) undergoes substitution reaction with various mono and diamines to give amidine and bis amidine derivatives in good yields. Condensation of 2-cyanopyridine (**1a**), 4-cyanopyridine (**1b**) and 2-cyanopyrazine (**1c**) with 4-(2-aminoethyl) morpholine (**2a**), 3-(aminomethyl) pyridine (**2b**) and 1-(2-aminoethyl) piperazine (**2c**) in equimolar ratio and in the presence of sodium methoxide by refluxing for 8 h using absolute methanol as solvent of reaction gave

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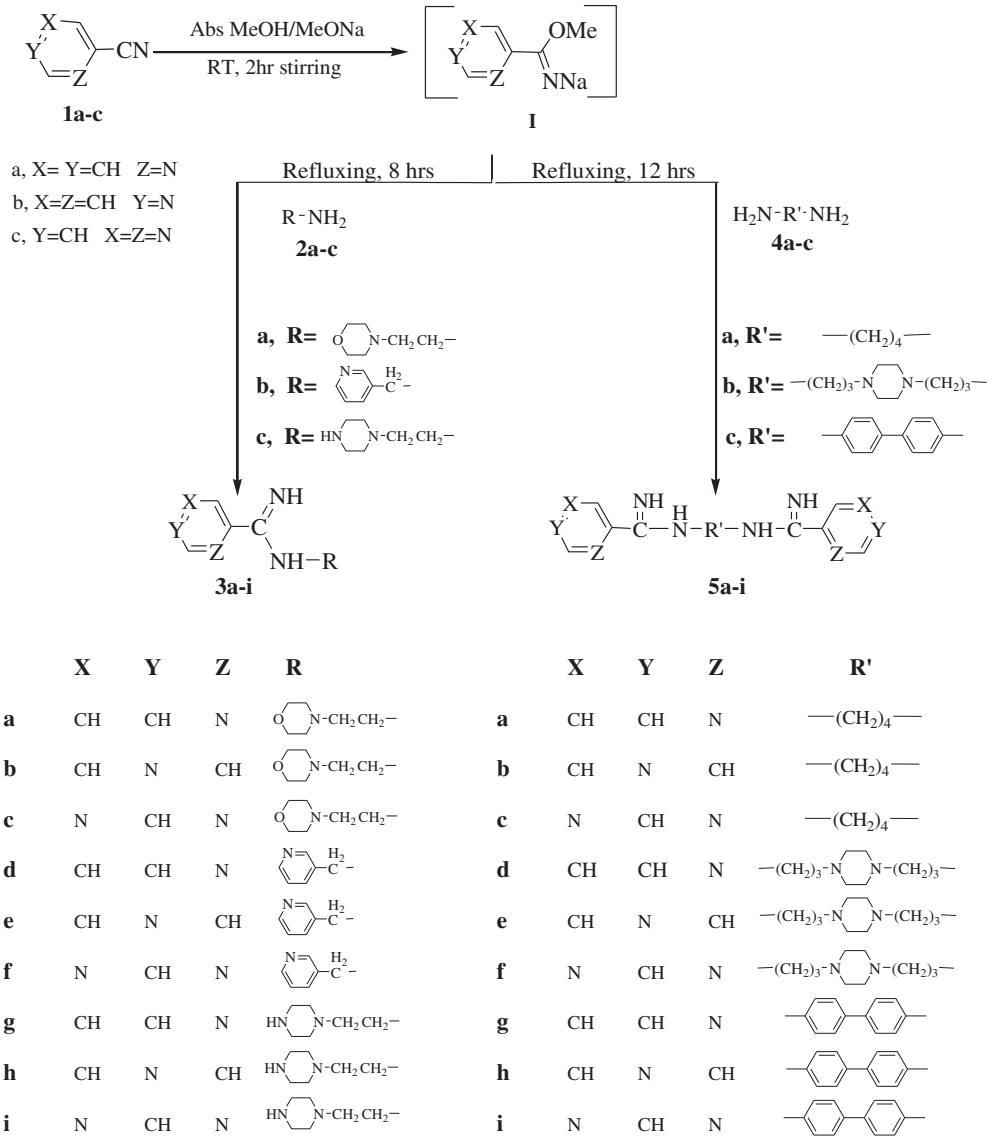


	X	Y	Z	R
a	CH	CH	N	
b	CH	N	CH	
c	N	CH	N	
d	CH	CH	N	
e	CH	N	CH	
f	N	CH	N	
g	CH	CH	N	
h	CH	N	CH	
i	N	CH	N	

	X	Y	Z	R'
a	CH	CH	N	
b	CH	N	CH	
c	N	CH	N	
d	CH	CH	N	
e	CH	N	CH	
f	N	CH	N	
g	CH	CH	N	
h	CH	N	CH	
i	N	CH	N	

Scheme 1. Synthesis of amidine **3a**–**i** and bis amidine **5a**–**i** derivatives.

amidine derivatives (**3a**–**i**) (Scheme 1) in good yields. All the compounds were purified by crystallization and structures assigned to (**3a**–**i**) are fully supported by spectral data i.e. IR, ^{1}H NMR, ^{13}C NMR and GC–MS and elemental analysis reported in experimental section of this paper.

Synthesis of bis amidine derivatives was carried out by condensation of (**1a**–**1c**) with 1,4-diaminobutane (**4a**), 1,4-bis(3-aminopropyl) piperazine (**4b**) and benzidine (**4c**) in 2:1 molar ratio, in the presence of sodium methoxide, by refluxing for 12 h using absolute methanol as solvent of reaction. This condensation reaction gave bis amidine derivatives (**5a**–**i**) in good yields. All the compounds were purified by crystallization. Spectral (IR, ^{1}H NMR, ^{13}C NMR and GC–MS) data and elemental analysis of **5a**–**i** (Scheme 1) reported in experimental section of this paper is in agreement with structures assigned to them.

2.2. Biological results

Purified and fully characterized amidine derivatives (**3a**–**i**) and bis amidine derivatives (**5a**–**i**) were screened for anti-inflammatory activity [40] using carrageenan induced paw oedema model. All the compounds were administered orally (p.o) and assayed at a dose of at 50 mg/kg body weight. Standard drug used for comparison was ibuprofen. Results of pharmacological evaluation are summarized in Table 1. A look at Table 1 indicates that bis amidine derivatives **5c** and **5d** possess good anti-inflammatory activity i.e. 37% and 38% at 50 mg/kg p.o. as compared to standard drug ibuprofen which exhibited 39% activity at 50 mg/kg p.o.

Amidine (**3a**–**i**) and bis amidine derivatives (**5a**–**i**) were screened *in vitro* for anticancer activity [41] against five human cancer cell lines i.e. breast (T47D), lung (NCI H-522), colon (HCT-15), ovary (PA-1) and liver (HepG2) at a concentration of 1×10^{-5} M concentration and results are summarized in Table 1. A look at Table 1 indicates that compounds **5d** breast (T47D), **5h**, **5i** lung (NCI H-522), **5i** colon (HCT-15), **3c**, **3h**, **5i** ovary (PA-1) and **3c**, **5b**, **5h** liver (HepG2) exhibited good anticancer activities against various cancer cell lines mentioned above. Compounds **3c**, **3h**, **5b**, **5d**, **5h** and **5i** which exhibited good anticancer activity against various cell lines were further studied and their IC_{50} values were determined. These IC_{50} values are summarized in Table 2. IC_{50} value for all above mentioned compounds for normal cell COS-1 is also reported in Table 2.

Table 1
Anti-inflammatory and *in vitro* anticancer activity of amidine and bis amidine **3a**–**i** and **5a**–**i** derivatives.

Comp No.	Anti-inflammatory activity (%) at 50 mg/kg p.o	[a]Anticancer activity (% growth inhibition) at a concentration of 1×10^{-5} M				
		Breast T47D	Lung NCl H-522	Colon HCT-15	Ovary PA-1	Liver HepG2
3a	00	21	31	12	43	40
3b	28	12	11	17	43	09
3c	15	13	35	06	**51**	**52**
3d	10	23	09	23	36	42
3e	00	22	07	22	21	20
3f	18	23	11	03	23	26
3g	11	14	08	24	40	27
3h	00	06	17	13	**46**	10
3i	20	22	09	28	36	23
5a	14	29	38	05	27	26
5b	15	27	20	05	10	**50**
5c	**37**	19	05	09	15	17
5d	**38**	**39**	39	31	31	34
5e	31	02	31	09	13	06
5f	27	28	15	12	26	22
5g	26	02	32	16	37	23
5h	15	12	36	03	07	**48**
5i	31	14	**43**	**45**	43	37
Ibuprofen	39	–	–	–	–	–
[b]**5-FU**	–	15	13	19	22	32
[c]**CYC-PHO**	–	09	11	04	12	18
[d]**CYC-HEXI**	–	11	09	16	34	18

Bold values represent compounds showing good anti-inflammatory and good anticancer activity.
[a] Compounds tested in triplicate, data expressed as mean value of three independent experiments.
[b] 5-FU 5-Fluorouracil.
[c] CYC-PHO Cyclophosphamide.
[d] CYC-HEXI Cycloheximide.

2.3. Structure activity relationship

Two series of compounds i.e. amidines (**3a**–**i**) and bis amidines (**5a**–**i**) are screened for anti-inflammatory and anticancer activities. Two compounds of bis amidine series i.e. **5c** and **5d** exhibited good anti-inflammatory activity whereas compounds **3c**, **3h**, **5b**, **5d**, **5h** and **5i** exhibited good anticancer activity. A look at the structures of compounds showing good anti-inflammatory and anticancer activity, we can say that pyridine and pyrazine derivatives do possess sites for interaction with the targets but compounds **5c**, **5d**; **3c**, **3h**, **5b**, **5d**, **5h** and **5i** have structures which can effectively interact with the targets both from electronic and stereochemical point of view and hence possess good anti-inflammatory and anticancer activity.

3. Conclusion

2-Cyanopyridine, 4-cyanopyridine and 2-cyanopyrazine interact with mono and diamines in the presence of sodium methoxide to give amidine (**3a**–**i**) and bis amidine (**5a**–**i**) derivatives. Compounds **5c** and **5d** exhibited good anti-inflammatory and **3c**, **3h**, **5b**, **5d**, **5h**, **5i** exhibited good anticancer activity.

4. Experimental protocols

4.1. General

Melting points (mp) were determined on a JSGW apparatus and are uncorrected. IR spectra were recorded using a Perkin Elmer 1600 FT spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker WH-500 spectrometer at a *ca* 5–15% (*w*/*v*) solution in DMSO-d_6, and D_2O. GC–MS was recorded on Perkin Elmer Clarus 500 gas chromatograph where built in MS detector was used. Elemental analysis was carried out on a Vario EL III elementor. Thin layer chromatography (TLC) was performed on silica gel G for TLC (Merck) and spots were visualized by iodine vapor or by irradiation with ultraviolet light (short wave length, 254 nm). During interpretation of ^{13}C NMR a few abbreviations has been used which are morp (morpholine), py (pyridine), pyra (pyrazine), piper (piperazine) and Ph (phenyl).

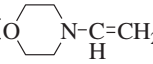
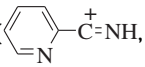
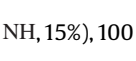
Table 2
IC_{50} values[a,b] of *in vitro* antitumor activity of active compounds.

Comp No.	IC_{50} (μm)					
	Breast T47D	Lung NCI H-522	Colon HCT-15	Ovary PA-1	Liver HepG2	Normal Cell COS-1
3c	55.38 ± 1.81	18.25 ± 1.16	78.30 ± 12.94	**13.55 ± 1.11**	**9.40 ± 2.45**	173 ± 5.98
3h	184.13 ± 14.8	35.30 ± 4.56	35.37 ± 4.61	**19.28 ± 1.87**	191.16 ± 9.87	147.1 ± 11.30
5b	42.95 ± 4.8	36.62 ± 4.75	87.8 ± 5.32	49.18 ± 3.09	**11.176 ± 2.65**	246.2 ± 9.38
5d	**22.52 ± 1.59**	21.98 ± 1.34	25.09 ± 5.51	39.70 ± 3.40	20.77 ± 4.46	203 ± 17.102
5h	34.09 ± 0.72	**13.43 ± 2.89**	123.76 ± 9.45	42.45 ± 3.61	**11.92 ± 1.17**	50 ± 10.76
5i	62.41 ± 5.4	**14.05 ± 2.57**	**11.92 ± 0.60**	**17.08 ± 0.70**	20.33 ± 2.22	100.3 ± 6.43
5-FU	51.8 ± 2.34	56.76 ± 3.4	45.01 ± 1.45	39.5 ± 4.32	29.87 ± 1.82	110 ± 8.98
CYC-PHO	70.1 ± 2.32	67.9 ± 3.09	74.32 ± 4.98	64.12 ± 5.43	55.3 ± 3.59	125.43 ± 9.24
CYC-HEXI	65.13 ± 7.31	60.1 ± 5.34	54.13 ± 4.65	40.6 ± 2.09	57.12 ± 4.65	128.31 ± 7.89

[a] 50% growth inhibition as determined by MTT assay (24 h drug exposure).
[b] Compounds tested in triplicate, data expressed as mean value ± SD of three independent experiments.

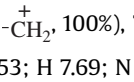
4.2. General procedure for the synthesis of amidine derivatives [3]

4.2.1. Synthesis of N-(2-morpholin-4-yl-ethyl)-pyridin-2-carboxamidine (3a)

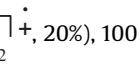
Sodium metal (23 mg) was dissolved in absolute methanol (20 ml) and was labeled as sodium methoxide solution in methanol. 2-Cyanopyridine (0.210 ml, 2 mmol) was dissolved in absolute methanol (10 ml) and to it was added sodium methoxide solution (0.5 ml) prepared above, the reaction contents were stirred at room temperature for 2 h. 4-(2-Aminoethyl) morpholine (0.26 ml, 2 mmol) was added to the reaction mixture. The reaction contents were heated under reflux for 8 h. Solvent was removed under reduced pressure and to the residue left behind was added diethyl ether, solid so obtained was filtered and washed with diethyl ether to give crude product. This crude product was purified by crystallization from ethyl acetate/methanol to give pure *N*-(2-morpholin-4-yl-ethyl)-pyridine-2-carboxamidine (**3a**). Yield 370 mg (79%) mp 125–126 °C. IR (KBr) ν_{max}: 3428 (NH), 1646 (–C=N–), cm^{-1}. ^1H NMR (500 MHz; DMSO-d_6) δ (ppm): 2.46 (bs, 4H, 2 × CH_2), 2.574–2.602 (t, 2H, J = 7 Hz, $-CH_2-$), 3.294–3.322 (t, 2H, J = 7 Hz, $-CH_2-$), 3.576–3.594 (t, 4H, J = 4.5 Hz, $-CH_2-$), 6.724 (bs, 2H, NH + NH, exch), 7.454–7.478 (q, 1H, J = 5.5 Hz, 6.5 Hz, Ar), 7.853–7.884 (t, 1H, J = 7.5 Hz, Ar), 8.118–8.133 (d, 1H, J = 7.5 Hz, Ar), 8.563–8.572 (d, 1H, J = 4.5 Hz, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 36.21 (morp), 53.45 (morp), 58.32 (morp), 66.30 (morp), 121.59 (py), 123.85 (py), 137.93 (py), 148.64 (py), 151.23 (py) and 163.87 (amidine). GC–MS *m*/*z* 235 (MH^+, 0.17%), 148 ( , 6%), 113 (, 65%), 105 ( , 15%), 100 ( , 100%), 78 (, 12%). Anal. Calcd for $C_{12}H_{18}N_4O$ C 61.53; H 7.69; N 23.93%. Found C 61.50; H 7.70; N 23.90%.

Similarly were synthesized other amidine derivatives **3b–i**. Physical constants and spectral data of **3b–i** are summarized below.

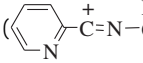
4.2.2. N-(2-Morpholin-4-yl-ethyl)-isonicotinamidine (3b)

Solvent of crystallization ethyl acetate/methanol. Yield 80%. mp 122–125 °C. IR (KBr) ν_{max}: 3402 (NH), 1657 (–C=N–), 1551 & 1456 (Ar) cm^{-1}. ^1H NMR (500 MHz; D_2O) δ (ppm): 2.359–2.388 (t, 4H, J = 7 Hz, 2 × CH_2), 2.669–2.698 (t, 2H, J = 7 Hz, CH_2), 3.224–3.231 (d, 2H, J = 3.5 Hz, $-CH_2-$), 3.657 (bs, 4H, 2 × CH_2), 7.652–7.664 (t, 2H, py), 8.617–8.628 (t, 2H, py). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 43.66 (morp), 53.13 (morp), 58.77 (morp), 66.14 (morp), 121.18 (py), 143.42 (py), 149.65 (py) and 164.67 (amidine). GC–MS *m*/*z* 234 (M^+, 0.65%), 100 ( , 100%), 78 (, 12.59%). Anal. Calcd. for $C_{12}H_{18}N_4O$ C 61.53; H 7.69; N 23.93%. Found C 61.55; H 7.67; N 23.95%.

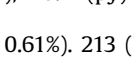
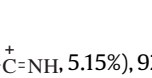
4.2.3. N-(2-Morpholin-4-yl-ethyl)-pyrazine-2-carboxamidine (3c)

Solvent of crystallization ethyl acetate/methanol. Yield 83%. mp 135–137 °C. IR (KBr) ν_{max}: 3432 & 3331 (NH), 1646 (–C=N–), 1595 & 1469 (Ar) cm^{-1}. ^1H NMR (500 MHz; D_2O) δ (ppm): 2.559 (s, 4H, 2 × CH_2), 2.683 (s, 2H, CH_2), 3.401 (s, 2H, CH_2), 3.689 (s, 4H, 2 × CH_2), 8.601–8.630 (d, 2H, J = 14.5 Hz, Ar), 8.998 (s, 1H, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 36.82 (morp), 54.05 (morp), 59.57 (morp), 66.65 (morp), 143.09 (pyra), 143.27 (pyra), 145.16 (pyra), 147.31 (pyra) and 164.22 (amidine). GC–MS *m*/*z* 236 (M + 1, 0.5%), 113 ( , 20%), 100 (, 100%) Anal. Calcd. for $C_{11}H_{17}N_5O$ C 56.17; H 7.23; N 29.78%. Found C 57.20; H 7.25; N 29.95%.

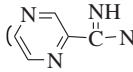
4.2.4. N-(Pyridin-3-ylmethyl)-pyridine-2-carboxamidine (3d)

Yield 76%. semisolid. IR (KBr) ν_{max}: 3444 & 3379 (NH), 1664 (–C=N–), 1526 & 1469 (Ar) cm^{-1}. ^1H NMR (500 MHz; D_2O) δ (ppm): 4.483 (s, 2H, CH_2), 7.247–7.273 (m, 1H, Ar), 7.434–7.475 (m, 1H, Ar), 7.656–7.679 (m, 1H, Ar), 7.820–7.838 (m, 2H, Ar), 8.265–8.277 (q, 1H, Ar), 8.336–8.352 (t, 1H, Ar), 8.433–8.462 (m, 1H, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 43.16 (CH_2), 123.21 (py), 127.83 (py), 129.10 (py), 132.70 (py), 134.74 (py), 137.90 (py), 139.31 (py), 147.48 (py), 148.72 (py), 151.22 (py) and 164.62 (amidine). GC–MS *m*/*z* 212 (M^+, 30%), 196 ( , 100%), 134 (, 7%), 92 (, 6%), Anal. Calcd. for $C_{12}H_{12}N_4$ C 67.92; H 5.66; N 26.41%. Found C 67.97; H 5.70; N 26.45%.

4.2.5. N-(Pyridin-3-ylmethyl)-isonicotinamidine (3e)

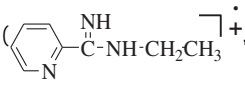
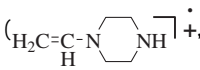
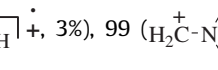
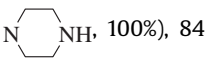
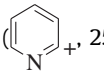
Solvent of crystallization ethyl acetate/methanol. Yield 72%. mp 278 °C. IR (KBr) ν_{max}: 3425 (NH), 1586 (–C=N–), 1547 & 1485 (Ar) cm^{-1}. ^1H NMR (500 MHz; D_2O) δ (ppm): 3.855 (s, 2H, CH_2), 7.391–7.416 (q, 1H, J = 5 Hz, 7.5 Hz, Ar), 7.675–7.687 (q, 1H, J = 1.5 Hz, 4.5 Hz, Ar), 7.795–7.811 (d, 1H, J = 8 Hz, Ar), 8.392–8.402 (q, 2H, J = 1.5 Hz, 3 Hz, py), 8.444–8.447 (d, 1H, J = 1.5 Hz, py), 8.550–8.562 (q, 2H, J = 1.5 Hz, 4.5 Hz, py). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 43.14 (CH_2), 119.41 (py), 123.23 (py), 125.31 (py), 134.74 (py), 139.22 (py), 147.46 (py), 148.74 (py) 150.70 (py) and 164.32 (amidine). GC–MS *m*/*z* 212 (M^+, 0.61%). 213 (MH^+, 18.15%), 107 ( , 100%), 105 ( , 5.15%), 92 (, 11.32%), 79 (, 85.62%), 78 (, 70.35%) Anal. Calcd. for $C_{12}H_{12}N_4$ C 67.92; H 5.66; N 26.41%. Found C 67.98; H 5.68; N 26.40%.

4.2.6. N-(Pyridin-3-ylmethyl)-pyrazine-2-carboxamidine (3f)

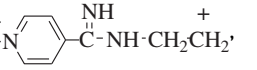
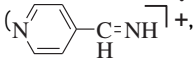
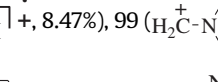
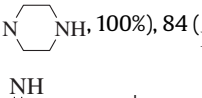
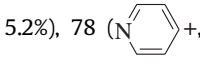
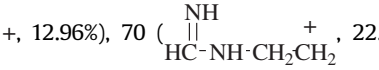
Solvent of crystallization ethyl acetate/methanol. Yield 81%. mp 135–136 °C. IR (KBr) ν_{max}: 3437 & 3334 (NH), 1655 (–C=N–), 1599, 1574 & 1476 (Ar) cm^{-1}. ^1H NMR (500 MHz; D_2O) δ (ppm): 4.474 (s, 2H, CH_2), 7.348–7.374 (q, 1H, J = 5 Hz, 10 Hz, Ar), 7.789–7.804 (d, 1H, J = 7.5 Hz, Ar), 8.348–8.358 (t, 1H, Ar), 8.456–8.459 (d, 1H, J = 1.5 Hz, Ar), 8.605–8.636 (m, 2H, Ar), 9.031–9.033 (d, 1H, J = 1.0 Hz, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 43.21 (CH_2), 123.34 (py), 135.22 (py), 136.82 (py), 142.54 (py), 143.04 (py), 145.36 (pyra), 147.03 (pyra), 147.49 (pyra), 148.95 (pyra) and 164.32 (amidine). GC–MS *m*/*z* 213 (M^+, 8%), 135 ( , 7%), 106 (, 11%), 105 (, 5%), 79 (, 5%), 78 (, 25%). Anal. Calcd. for $C_{11}H_{11}N_5$ C 61.97; H 5.16; N 32.86%. Found C 61.70; H 5.18; N 32.89%.

4.2.7. N-(2-Piperazin-1-yl-ethyl)-pyridine-2-carboxamidine (3g)

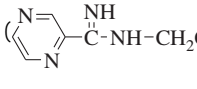
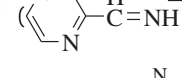
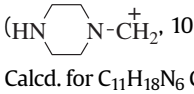
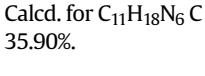
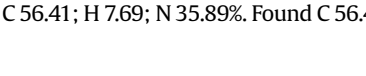
Yield 79%. Semisolid. IR (KBr) ν_{max}: 3391 (NH), 1647 (–C=N–), 1586 & 1464 (Ar) cm^{-1}. ^1H NMR (500 MHz; D_2O) δ (ppm): 2.461–2.475 (d, 1H, J = 7 Hz, one H of CH_2), 2.535 (bs, 4H, 2 × CH_2), 2.706–2.732 (t, 1H, J = 6.5 Hz, one H of CH_2), 2.812 (bs, 4H, 2 × CH_2), 3.526–3.570 (q, 2H,

J = 7 Hz, 15 Hz, CH_2), 7.556–7.623 (m, 1H, Ar), 7.883–7.996 (m, 2H, Ar), 8.532–8.605 (m, 1H, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 38.35 (piper), 45.59 (piper), 54.33 (piper), 61.79 (piper), 127.57 (py), 128.88 (py), 132.71 (py), 137.68 (py) 151.03 (py) and 164.91 (amidine). No molecular ion peak. *m/z* 149 ( , 20%), 112 ( , 3%), 99 ( , 100%), 84 ( , 15%), 78 ( , 25%) Anal. Calcd. for $C_{12}H_{19}N_5$ C 61.80; H 8.15; N 30.04%. Found C 61.82; H 8.13; N 30.08%.

*4.2.8. N-(2-Piperazin-1-yl-ethyl)-isonicotinamidine (**3h**)*

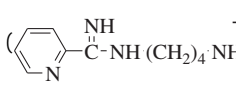
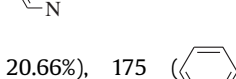
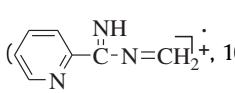
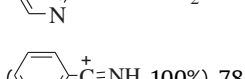
Yield 75%. Semisolid. IR (KBr) ν_{max}: 3397 (NH), 1681 (–C=N–), 1550 & 1470 (Ar) cm^{-1}. ^{1}H NMR (500 MHz; D_2O) δ (ppm): 2.395–2.629 (m, 6H, 3 × CH_2), 2.741–2.765 (t, 4H, 2 × CH_2), 3.471–3.485 (t, 2H, CH_2), 7.592–7.641 (m, 2H, py), 8.554–8.580 (m, 2H, py). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 39.13 (piper), 45.62 (piper), 54.43 (piper), 61.87 (piper), 125.48 (py), 144.29 (py), 150.63 (py) and 163.98 (amidine). GC–MS *m/z* 234 (MH^+, 0.2%), 149 ( , 5.15%), 106 ( , 8.47%), 99 ( , 100%), 84 ( , 5.2%), 78 ( , 12.96%), 70 ( , 22.60%). Anal. Calcd. for $C_{12}H_{19}N_5$ C 61.80; H 8.15; N 30.04%. Found C 61.82; H 8.13; N 30.06%.

*4.2.9. N-(2-Piperazin-1-yl-ethyl)-pyrazine-2-carboxamidine (**3i**)*

Yield 80%. Semisolid. IR (KBr) ν_{max}: 3431 (NH), 1633 (–C=N–), 1606, 1548 (Ar) cm^{-1}. ^{1}H NMR (500 MHz; D_2O) δ (ppm): 2.474–2.864 (m, 9H, 4 × CH_2 + one H of CH_2), 3.331–3.474 (m, 3H, 1 × CH_2 + one H of CH_2), 8.633–8.703 (m, 2H, Ar), 9.013–9.029 (d, 1H, J = 8 Hz, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 35.95 (piper), 45.18 (piper), 53.64 (piper), 57.16 (piper), 143.52 (pyra), 144.52 (pyra), 147.30 (pyra), 162.67 (pyra) and 165.14 (amidine). GC–MS *m/z* 235 ($M+1^{\cdot}$, 5%), 150 ( , 12%), 107 ( , 6%), 99 ( , 100%), 84 ( , 11%), 79 ( , 8%). Anal. Calcd. for $C_{11}H_{18}N_6$ C 56.41; H 7.69; N 35.89%. Found C 56.42; H 7.70; N 35.90%.

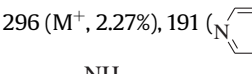
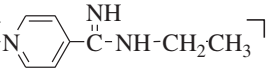
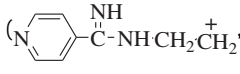
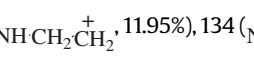
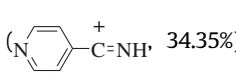
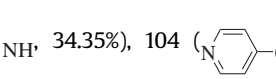
*4.3. General procedure for synthesis of bis amidine derivatives [**5**]*

*4.3.1. Synthesis of N-(2-pyridineimidoylamino-butyl)-pyridine-2-carboxamidine (**5a**)*

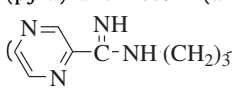
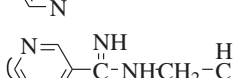
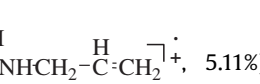
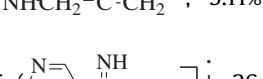
2-Cyanopyridine (0.420 ml; 4 mmol) was dissolved in absolute methanol (20 ml) and to it was added sodium methoxide solution in methanol (1.0 ml) (previously prepared). The reaction contents were stirred at room temperature for 2 h 1,4-diaminobutane (0.18 ml, 2 mmol) was added to the reaction mixture. Reaction contents were heated under reflux for 12 h. Solvent was removed under reduced pressure. Crude product so obtained was washed with diethyl ether and then with ethyl acetate to give thick mass. Solvent traces from this thick mass was removed by applying high vacuum for 15 min to give semisolid product i.e. *N*-(2-pyridineimidoylamino-butyl)-pyridine-2-carboxamidine (**5a**). Yield 480 mg (81%). IR (KBr) ν_{max}: 3438 (NH), 1637 (–C=N–), 1570, 1488 (Ar) cm^{-1}. ^{1}H NMR (500 MHz; DMSO + D_2O) δ (ppm): 1.648–1.676 (d, 4H, 2 × CH_2), 3.166–3.246 (m, 4H, 2 × CH_2), 7.484–7.509 (m, 2H, Ar), 7.867–7.901 (m, 2H, Ar), 8.005–8.040 (q, 2H, Ar), 8.565–8.574 (d, 2H, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 28.28 (CH_2), 29.96 (CH_2), 41.12 (CH_2), 46.34 (CH_2), 127.69 (py), 128.90 (py), 132.51 (py), 137.78 (py) 151.04 (py) and 163.07 (amidine). GC–MS *m/z* 296 (M^+, 3.60%), 192 ( , 33.07%), 176 (, 20.66%), 175 ( , 15.69%), 133 ( , 10.83%), 132 (, 36.19%), 105 ( , 100%), 78 (, 70.29%). Anal. Calcd. for $C_{16}H_{20}N_6$ C 64.86; H 6.75; N 28.37%. Found C 64.88; H 6.78; N 28.39%.

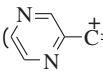
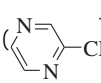
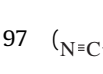
Similarly were synthesized other bis amidine derivatives **5b**–**i**. Physical constants and spectral data of 5**b**–**i** are summarized below.

*4.3.2. N-(4-Isonicotinimidoylamino-butyl)-isonicotinamidine (**5b**)*

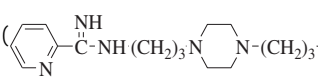
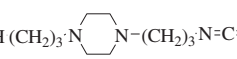
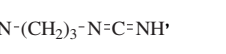
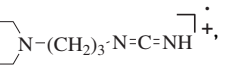
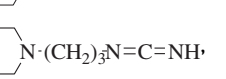
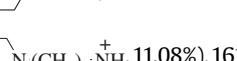
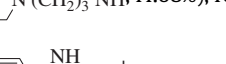
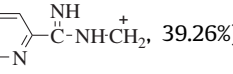
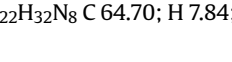
Yield 78%. mp 240–242 °C. IR (KBr) ν_{max}: 3400 (NH), 1673 (–C=N–), 1600 &1545 (Ar) cm^{-1}. ^{1}H NMR (500 MHz; D_2O) δ (ppm): 1.489–1.535 (d, 4H, 2 × CH_2), 2.714–2.741 (t, 2H, CH_2), 3.264–3.289 (t, 2H, CH_2), 7.672–7.739 (m, 4H, py), 8.673–8.721 (dd, 4H, py). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 27.92 (CH_2), 30.08 (CH_2), 45.63 (CH_2), 46.12 (CH_2), 125.65 (py), 149.93 (py) and 150.72 (py), 163.34 (amidine). GC–MS *m/z* 296 (M^+, 2.27%), 191 ( , 14.24%), 149 ( , 29.05%), 148 ( , 11.95%), 134 ( , 15.09%), 105 ( , 34.35%), 104 ( , 43.28%), 77 (, 98.56%). Anal. Calcd. for $C_{16}H_{20}N_6$ C 64.86; H 6.75; N 28.37%. Found C 64.88; H 6.79; N 28.40%.

*4.3.3. N-(2-Pyrazineimidoylamino-butyl)-pyrazine-2-carboxamidine (**5c**)*

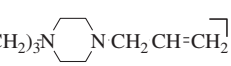
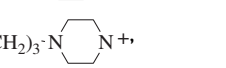
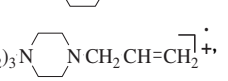
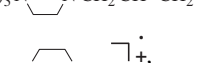
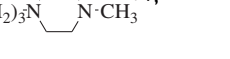
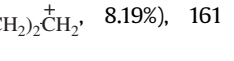
Yield 83%, mp 157–160 °C, IR (KBr) ν_{max}: 3423 & 3336 (NH), 1648 (–C=N–), 1598 &1474 (Ar) cm^{-1}. ^{1}H NMR (500 MHz; DMSO-d_6) δ (ppm): 1.738 (s, 4H, 2 × CH_2), 3.305 (s, 4H, 2 × CH_2), 8.567–8.575 (t, 2H, pyrazine), 8.613–8.618 (d, 2H, J = 2.5 Hz, pyrazine), 8.959–8.961 (d, 2H, J = 1 Hz, pyrazine). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 26.73 (CH_2), 44.52 (CH_2), 142.17 (pyra), 143.19 (pyra), 145.47 (pyra), 146.27 (pyra) and 163.12 (amidine). GC–MS *m/z* 298 (M^+, 3.70%), 176 ( , 9.12%), 163 ( , 5.11%), 149 ( , 4.05%), 135 ( , 26.37%), 134 (,

8.13%), 122 ( , 3.97%), 106 ( , 13.26%), 105 ( , 50.75%), 97 ( , 21.49%), 96 ( , 100%). Anal. calcd. for $C_{14}H_{18}N_8$ C 56.37; H 6.04; N 37.58%. Found C 56.39; H 6.01; N 37.60%.

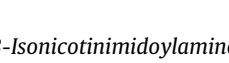
4.3.4. N-{3-[4-(2-Pyridineimidoylamino-propyl)-piperazin-1-yl]-propyl}-pyridine-2-carboxamidine (**5d**)

Yield 78%. mp 138–140 °C. IR (KBr) ν_{max}: 3436 & 3279 (NH), 1678 (–C=N–), 1587, 1568 & 1520 (Ar) cm^{-1}. ^1H NMR (500 MHz; DMSO-d_6 + D_2O) δ (ppm): 1.056–1.084 (t, 2H, J = 7 Hz, CH_2), 1.672–1.790 (m, 4H, 2 × CH_2), 2.362–2.386 (t, 8H, J = 6 Hz, 4 × CH_2), 3.334–3.392 (m, 6H, 3 × CH_2), 7.545–7.568 (t, 2H, J = 6 Hz, Ar), 7.957–8.017 (m, 4H, Ar), 8.604–8.613 (d, 2H, J = 4.5 Hz, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 25.30 (CH_2), 52.26 (CH_2), 56.15 (CH_2), 121.80 (py), 125.60 (py), 137.87 (py), 148.43 (py), 149.45 (py) and 164.24 (amidine). GC–MS m/z 408 (M^+, 6.64%), 329 ( , 7.62%), 251 ( , 14.47%), 210 ( , 11.08%), 209 ( , 67.82%), 208 ( , 38.45%), 183 ( , 11.08%), 161 ( , 45.48%), 134 ( , 39.26%), 105 ( , 10.73%). Anal. Calcd. for $C_{22}H_{32}N_8$ C 64.70; H 7.84; N 27.45%. Found C 64.73; H 7.80; N 27.46%.

4.3.5. N-{3-[4-(3-Isonicotinimidoylamino-propyl)-piperazin-1-yl]-propyl}-isonicotinamidine (**5e**)

Yield 82%. mp 159–160 °C. IR (KBr) ν_{max}: 3421 (NH), 1661 (–C=N–), 1541 (Ar) cm^{-1}. ^1H NMR (500 MHz; DMSO-d_6) δ (ppm): 1.723–1.749 (t, 4H, J = 6.5 Hz, 2 × CH_2), 2.042–2.373 (m, 12H, 6 × CH_2), 3.123–3.147 (t, 4H, J = 6 Hz, 2 × CH_2), 7.705–7.714 (d, 4H, J = 4.5 Hz, py), 8.595–8.607 (d, 4H, J = 6 Hz, py). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 28.92 (CH_2), 53.48 (CH_2), 56.62 (CH_2), 121.52 (py), 144.64 (py), 150.14 (py) and 164.08 (amidine). GC–MS m/z 408 (M^+, 1.75%), 287 ( , 4.40%), 246 ( , 3.14%), 210 ( , 4.42%), 184 ( , 27.06%), 162 ( , 8.19%), 161 ( , 8.60%), 160 (, 18.67%), 157 (, 14.00%), 148 (, 47.32%), 142 ($(H_2N{\cdot}(CH_2)_3)_2N^+$, 13.68%), 141 (, 9.36%), 127 (, 14.73%), 126 (, 32.96%), 111 (, 14.32%), 104 (, 44.10%), 78 (, 5.77%), 77 (, 21.97%), 56 (, 100%). Anal. Calcd. for $C_{22}H_{32}N_8$ C 64.70; H 7.84; N 27.45%. Found C 64.72; H 7.80; N 27.43%.

4.3.6. N-{3-[4-(3-Pyrazinimidoylamino-propyl)-piperazin-1-yl]-propyl}-pyrazine-2-carboxamidine (**5f**)

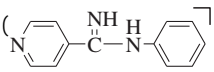
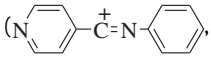
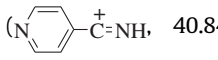
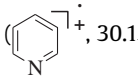
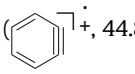
Yield 85%. mp 153–155 °C. IR (KBr) ν_{max}: 3446 & 3311 (NH), 1649 (–C=N–), 1595, 1525 & 1469 (Ar) cm^{-1}. ^1H NMR (500 MHz; D_2O) δ (ppm): 1.736–1.796 (m, 4H, 2 × CH_2), 1.943–2.751 (m, 12H, 6 × CH_2), 3.218–3.245 (t, 4H, J = 7 Hz, 2 × CH_2), 8.567–8.606 (m, 4H, pyrazine), 8.953–8.955 (d, 2H, J = 1.0 Hz, pyrazine). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 27.87 (CH_2), 53.57 (CH_2), 56.61 (CH_2), 140.44 (pyra), 143.65 (pyra), 144.70 (pyra), 145.07 (pyra), 147.52 (pyra) and 164.18 (amidine). GC–MS m/z 410 (M^+, 4.69%), 163 (, 11.18%), 149 (, 70.70%), 136 (, 9.87%), 121 (, 21.54%), 105 (, 3.37%), 78 ( , 100%). Anal. Calcd. for $C_{20}H_{30}N_{10}$ C 58.53; H 7.31; N 34.14%. Found C 58.55; H 7.33; N 34.10%.

4.3.7. N-(4′-Pyridinimidoylamino-biphenyl-4-yl)-pyridine-2-carboxamidine (**5g**)

Yield 74%. mp 232–234 °C. IR (KBr) ν_{max}: 3467 & 3356 (NH), 1639 (–C=N–), 1561 & 1492 (Ar) cm^{-1}. ^1H NMR (500 MHz; DMSO-d_6) δ (ppm): 4.994 (s, 4H, 4 × NH, exch), 6.571–6.588 (d, 4H, J = 8.5 Hz, Ar), 7.192–7.209 (d, 4H, J = 8.5 Hz, Ar), 7.746–7.774 (m, 2H, py), 8.058–8.079 (m, 4H, py), 8.768–8.780 (m, 2H, py). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 114.37 (Ph), 117.38 (Ph), 126.02 (py), 127.66 (py), 128.93 (Ph), 132.67 (Ph), 137.75 (py), 146.77 (py) 151.07 (py) and 163.96 (amidine). GC–MS m/z 392 (M^+, 1.56%), 104 (, 9.36%), 78 (, 72.94%). Anal. Calcd. for $C_{24}H_{20}N_6$ C 73.46; H 5.10; N 21.42%. Found C 73.42; H 5.08; N 21.40%.

4.3.8. N-(4′-Isonicotinimidoylamino-biphenyl-4-yl)-isonicotinamidine (**5h**)

Yield 76%. mp 238–240 °C. IR (KBr) ν_{max}: 3469 & 3367 (NH), 1614 (–C=N–), 1546 & 1494 (Ar) cm^{-1}. ^1H NMR (500 MHz; DMSO-d_6) δ (ppm): 5.329 (s, 2H, 2 × NH, exch), 6.739–6.816 (m, 6H, Ar),

7.062–7.075 (d, 2H, $J = 6.5$ Hz, Ar), 7.362–7.378 (t, 2H, Ar), 7.510–7.527 (t, 2H, Ar), 7.671–7.687 (t, 2H, Ar), 8.092 (bs, 2H, 2 × NH, exch), 8.851–8.857 (d, 2H, $J = 3$ Hz, Ar). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 114.36 (Ph), 116.72 (Ph), 119.40 (Ph), 125.87 (py), 128.79 (Ph), 146.81 (py), 150.70 (py) and 164.23 (amidine). GC–MS m/z 392 (M^+, 3.48%), 197 ( , 10.49%), 181 ( , 2.02%), 105 ( , 40.84%), 79 ( , 30.13%), 76 ( , 44.82%). Anal. Calcd. for $C_{24}H_{20}N_6$ C 73.46; H 5.10; N 21.42%. Found C 73.40; H 5.08; N 21.41%.

4.3.9. *N-(4′-Pyrazinimidoylamino-biphenyl-4-yl)-pyrazine-2-carboxamidine (**5i**)*

Yield 76%. mp > 300 °C. IR (KBr) ν_{max}: 3500 & 3384 (NH), 1624 (–C=N–), 1596, 1558 & 1490 (Ar) cm^{-1}. ^{1}H NMR (500 MHz; DMSO-d_6) δ (ppm): 6.715 (bs, 4H, 4 × NH, exch), 7.015–7.064 (q, 4H, $J = 6.5$ Hz, 16 Hz, Ar), 7.668–7.684 (t, 4H, Ar), 8.727–8.730 (d, 2H, $J = 1.5$ Hz, pyrazine), 8.820–8.830 (t, 2H, $J = 2.5$ Hz, pyrazine), 9.492–9.500 (d, 2H, $J = 4$ Hz, pyrazine). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 114.34 (Ph), 115.73 (Ph), 125.97 (Ph), 129.82 (Ph), 140.87 (pyra), 145.68 (pyra), 148.07 (pyra) and 148.51 (pyra), 164.33 (amidine). Anal. Calcd. for $C_{22}H_{18}N_8$ C 67.00; H 4.56; N 28.42%. Found C 67.03; H 4.52; N 28.43%.

4.4. Anti-inflammatory activity

Paw oedema inhibition test was used on albino rats of Charles Foster by adopting the method of Winter et al. [40]. Groups of five animals of both sexes (body weight 120–160 g), excluding pregnant females, were given a dose of test compound. Thirty minute later, 0.20 mL of 1% freshly prepared carrageenan suspension in 0.9% NaCl solution was injected subcutaneously into the planter aponeurosis of the hind paw and the volume was measured by a water plethysmometer apparatus and then measured again 1–3 h later. The mean increase of paw volume at each interval was compared with that of control group (five rats treated with carrageenan but not with test compound) at the same intervals and percent inhibition value calculated by the formula given below.

$$\%\ \text{anti} - \text{inflammatory activity} = [1 - D_t/D_c] \times 100$$

D_t and D_c are paw volumes of oedema in tested and control groups, respectively.

4.5. In vitro cytotoxicity against human cancer cell lines

Human breast (T47D), lung (NCI H-522), colon (HCT-15), ovary (PA-1) and liver (HepG2) cancer cell lines were obtained from National Center for Cell Science (NCCS), Pune, India. Cells were grown in tissue culture flask in complete growth medium (RPMI-1640 medium with 2 mM glutamine, pH 7.4 supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin) in a carbon dioxide incubator (37 °C, 5% CO_2, 90% RH). All cell culture reagents were from GIBCO (Invitrogen, USA). Penicillin, streptomycin, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5diphenyl-2H tetrazolium bromide), cell culture grade DMSO, 5-fluorouracil (5-FU), cyclophosphamide and actidione (cycloheximide) were from Himedia (Mumbai, India).

MTT assay was carried out as described in literature [41]. In brief, 5×10^3 cells in 200 μl of medium were seeded in 96-well plates (Griener, Germany). Serial dilutions of compound initially ranging from 0 to 100 μM in DMSO were added to the monolayer. The final DMSO concentration for all dilutions was 0.1% which was used as vehicle control. The cultures were assayed after 24 h by the addition of 50 μl of 5 mg/ml MTT and incubating for another 4 h at 37 °C. The MTT-containing medium was aspirated and 200 μl of DMSO (Himedia, Mumbai, India) and 25 μl of Sorensen glycine buffer (0.1 M glycine and 0.1 M NaCl, pH 10.5) were added to lyse the cells and solubilize the water insoluble formazone. Absorbance of the lysates was determined on a Fluostar optima (BMG Labtech, Germany) microplate reader at 570 nm.

The percentage inhibition was calculated as

$$\frac{\text{Mean OD of vehicle treated cells(negative control)} - \text{Mean OD of treated cells} \times 100}{\text{Mean OD of vehicle treated cells(negative control)}}$$

The IC_{50} values were calculated using graph pad prism, version 5.02 software (Graph Pad Software Inc., CA, USA).

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