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MEDICINAL CHEMISTRY RESEARCH

SYNTHESIS, ANTIVIRAL AND ANTIBACTERIAL ACTIVITIES OF ISATIN MANNICH BASES

Dharmarajan Sriram*, Tanushree Ratan Bal, Perumal Yogeeswari

Medicinal Chemistry Research Laboratory, Pharmacy Group,
Birla Institute of Technology and Science, Pilani – 333031, India

Abstract. HIV is the most significant risk factor for many opportunistic infections like tuberculosis, hepatitis, bacterial infections etc. We designed an isatin lead compound as a novel non-nucleoside reverse transcriptase inhibitor with broad-spectrum chemotherapeutic properties for the effective treatment of AIDS and AIDS-related opportunistic infections. Compound 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[[N^4-[3'-(4'-amino-5'-trimethoxybenzyl pyrimidin-2'-yl)imino-1'-(5-chloroisatinyl)]methyl]-N^1-piperazinyl]-3-quinoline carboxylic acid (14) emerged as the most potent broad-spectrum chemotherapeutic agent active against HIV, HCV, *M. tuberculosis* and various pathogenic bacteria.

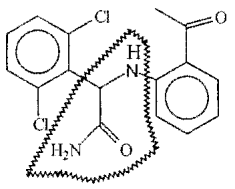
Acquired immunodeficiency syndrome (AIDS), due to infection with the human immunodeficiency virus (HIV), has become a worldwide epidemic [1]. HIV infection, which targets the monocytes expressing surface CD4 receptors, eventually produces profound defects in cell-mediated immunity [2]. Overtime, infection leads to severe depletion of CD4 T-lymphocytes (T-cells) resulting in opportunistic infections (OIs) like tuberculosis (TB), fungal, viral, protozoal and neoplastic diseases and, ultimately, death. TB is the most common OI in people with AIDS and it is the leading cause of death among people with AIDS. Co-infection of hepatitis C virus (HCV) and HIV is quite common, mainly because these infections share the same parenteral, sexual and

Corresponding author: e-mail: pyogee@bits-pilani.ac.in

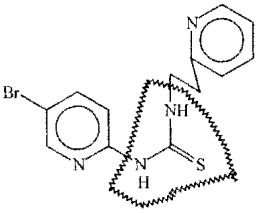
vertical routes of transmission [3]. Although classical OIs are now rarely seen, the toxicity of antiretroviral drugs, as well as liver diseases caused by HCV, represents an increasing cause of morbidity and mortality among HIV-positive patients. Predisposing liver damage results in a higher rate of hepatotoxicity by antiretroviral drugs, which may limit the benefit of HIV treatment in some individuals [4]. Logically, it appears that an ideal drug for HIV/AIDS patients should suppress HIV replication, thereby acting as an anti-HIV drug, as well as possess efficacy against OIs like TB, hepatitis and other bacterial infections. Earlier works in our laboratory have led to the identification of various isatinimino derivatives exhibiting broad-spectrum chemotherapeutic properties [5]. Continuing to develop broad-spectrum chemotherapeutics, we undertook the present study to design, synthesize and evaluate isatin analogues, which may suppress HIV replication in addition to inhibiting opportunistic microorganisms.

Design

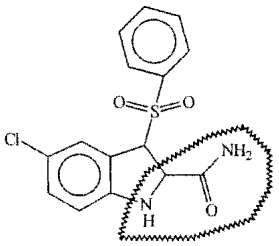
To qualify as a non-nucleoside reverse transcriptase inhibitor (NNRTI), the compound should interact specifically with a non-substrate binding site of the reverse transcriptase (RT) of HIV-1, and inhibit the replication of HIV-1 at a concentration that is significantly lower than the concentration typically observed to affect normal cell viability [6]. Based on this concept, more than thirty different classes of NNRTI's may be considered [7]. Although the NNRTI's seemingly belong to widely diverging classes of compounds, closer inspection reveals that most have some common features, that is a carboxamide or (thio) urea entity ('body'), surrounded by two hydrophobic, mostly aryl moieties ('wings'), one of which is often substituted by a halogen.



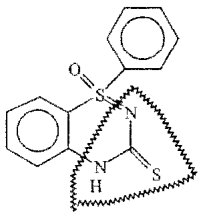
Loviride



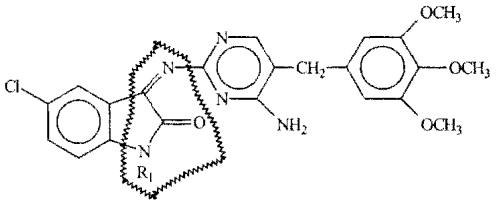
Trovirdine



Indole Carboxamide



Benzothiadiazine-1-oxide



Lead Compound

Thus, the overall structure may be considered analogous to a butterfly with a hydrophilic center ('body') and two hydrophobic outskirts ('wings'). In the present study, the isatin analogues are designed in accordance to this hypothesis. The iminocarbamoyl moiety (-N=C-CO-N-) constitutes the 'body' and the aryl isatin ring and the pyrimidine derivative constitute the 'wings' as depicted in the figure. The distance between the hydrophilic center (A) and hydrophobic outskirts (B and C) and the angle between the two aryl rings (B and C) were measured using Tripos Alchemy 2000 software from the energy minimized structures using the MM3 program. The lead compounds (**1-16**) were found to comply within the specification of the pharmacophoric distance map (Table 1).

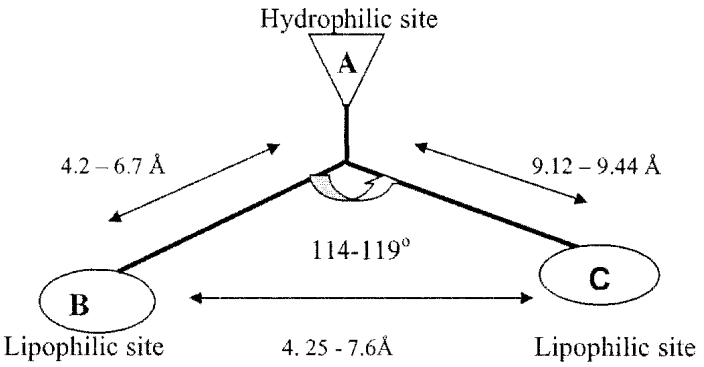
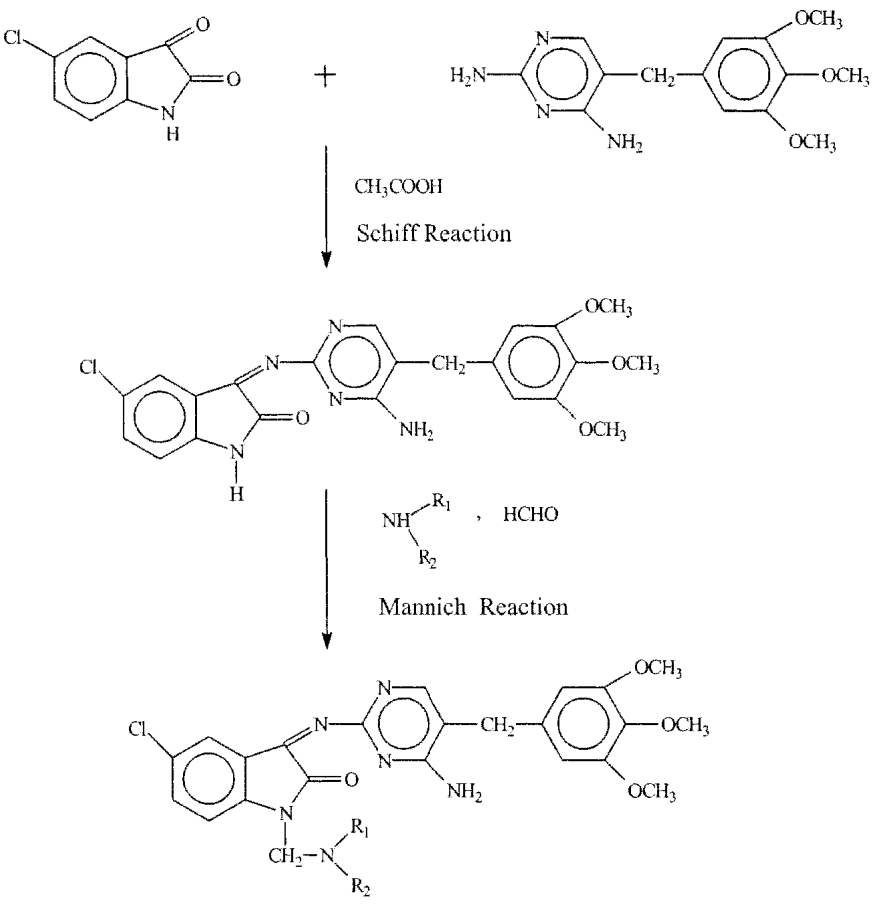


Table 1. The distance between the pharmacophoric functional groups of Anti-HIV drugs and the lead compound

DRUGS	AB (in Å)	BC (in Å)	CA (in Å)	ANGLE BAC
LOVIRIDE	4.36-5.19	4.25-6.25	9.4	119°
TROVIRDINE	4.22-6.67	6.53-7.6	9.44	117.5°
INDOLE CARBOXAMIDE	4.20-6.72	4.44-7.08	9.44	118°
BENZOTHIADIAZINE-1-OXIDE	4.25-6.60	4.86-7.02	9.12	114.9°
RANGE	4.20-6.72	4.25-7.6	9.12-9.44	114.9-119°
LEAD COMPOUND (1-16)	**4.23-5.34**	**4.07-6.63**	**9.113**	**116.7°**

Synthesis

The synthesis of various isatin Mannich bases was achieved in two steps (Scheme 1)[8]. 5-Chloroisatin was condensed with 5-trimethoxybenzyl-2,4-diamino pyrimidine in the presence of glacial acetic acid to form a Schiff's base. The N-Mannich bases of the above Schiff's base were synthesized by condensing the acidic imino group of isatin with formaldehyde and various secondary amines. All compounds gave satisfactory elemental analysis. IR and ^{1}H-NMR spectra were consistent with the assigned structures.



1-16

Scheme 1. Protocol for the synthesized compounds

Results and Discussion

The synthesized compounds were evaluated for their inhibitory effect on the replication of HIV-1 in MT-4 and CEM cell lines (Table 2) [9]. In the MT-4 cell lines, compound **14** was found to be the most active against replication of HIV-1 with EC_{50} of 9.4 μg /ml respectively and their selectivity index ($SI=CC_{50}/EC_{50}$) was found to be more than 19 with maximum protection of 101%. Other compounds (**1**, **2**, **3**, **7**, **12** and **15**) showed maximum protection of 59%-95% with SI of

approximately 3. In the T4 lymphocytes (CEM cell lines), the compounds showed marked anti-HIV activity (20-48%) at a concentration below their toxicity threshold. The loss of activity might be due to degeneration or rapid metabolism in the culture conditions used in the screening procedure.

Table 2. Anti-HIV, Anti-HCV and Antimycobacterial activity

	Anti-HIV activity						Anti-HCV activity at 50 µg/ml		Anti mycobacterial activity
	MT-4 cell line			CEM cell line			Cell growth (%)	Viral RNA replication (%)	% Inhibition
	EC_{50}[a]	CC_{50}[b]	% Protection	EC_{50}[a]	CC_{50}[b]	% Protection			
1	10.4	44.1	95.1	> 37.1	37.1	20.98	65	81	97
2	16.1	62.6	73.2	NT	NT	NT	48	84	67
3	23.6	79.1	64.1	> 98.4	98.4	20.69	72	67	53
4	> 36.1	36.1	41.8	> 94.7	94.7	22.33	79	66	50
5	> 39.2	39.2	20.9	> 34.4	34.4	23.00	67	96	44
6	>38.6	38.6	32.6	> 40.1	40.1	22.64	56	99	40
7	56.1	65.6	59.6	> 71.0	71.0	22.98	64	80	35
8	> 60.6	60.6	30.2	> 51.0	51.0	23.38	71	91	38
9	> 80.7	80.7	48.6	> 88.0	88.0	21.09	83	71	35
10	> 32.6	32.6	10.7	> 23.3	23.3	21.93	71	100	33
11	> 39.2	39.2	16.6	> 29.4	29.4	23.55	101	89	23
12	32.6	97.3	62.6	> 83.8	> 83.8	20.7	112	89	10
13	> 67.4	67.4	41.7	> 46.3	46.3	22.07	108	81	15
14	9.4	186.6	101	> 200	> 200	33.3	109	86	100
15	17.2	36.9	89	> 18.7	18.7	48.3	85	60	100
16	NT	NT	NT	> 67.0	67.0	20.04	NT	NT	100

NT indicates not tested

[a]50% Effective concentration, or concentration required to inhibit HIV-1 induced cytopathicity in cell lines by 50%.

[b]50% Cytotoxic concentration, or concentration required to reduce the viability of mock-infected cell lines by 50%.

One compound (**14**) was evaluated for the inhibitory effects on the HIV-1 RT enzyme[10] and the IC_{50} values were found to be 18.4 ± 2.4 µM. The *in-vitro* IC_{50} values for HIV-1 RT with Poly (vC) oligo (dG) as the template / primer were significantly higher than the corresponding EC_{50} values

for inhibition of the cytopathic effect of HIV-1 in MT-4 cell culture. This discrepancy is not unusual for NNRTI's as it may reflect the differences between the *in vitro* HIV-1 RT assay, in which a synthetic template/primer is used, and cellular systems[11].

The synthesized compounds were also evaluated for their inhibition of HCV viral RNA replication in HUH-7 cells at 50μg/ ml [12], and the results are presented in Table 2. Among these, three compounds (**11**, **13** and **14**) were found to be non-toxic to Huh-7 cells (cell growth of > 100%) and inhibited HCV viral RNA replication by about 81-89%. Three compounds (**5**, **6** and **10**) inhibited viral replication more than 95% but they were toxic to Huh-7 cells.

Table 3. *In vitro* antibacterial activity (MIC's in μg/ml)

Microorganisms*	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
a	1250	1250	625	1250	1250	1250	625	2500	1250
b	1250	1250	1250	625	1250	1250	1250	2500	1250
c	1250	1250	1250	1250	1250	1250	1250	2500	1250
d	39.06	39.06	19.53	39.06	19.53	19.53	9.76	9.76	4.88
e	1.22	39.06	39.06	39.06	19.53	39.06	19.53	39.06	19.531
f	625	625	625	625	1250	1250	625	2500	1250
g	625	1250	625	625	1250	1250	625	2500	1250
h	1250	2500	1250	1250	156.25	2500	1250	2500	1250
i	4.88	4.88	9.76	2.44	9.76	9.76	4.88	2.44	1.22
j	1250	1250	625	625	625	1250	1250	2500	1250
k	312.5	625	312.5	625	625	1250	625	1250	312.5
l	1.22	0.6103	1.22	4.88	0.61	4.88	4.88	39.06	1.22
m	19.53	19.53	39.06	9.76	1.22	2.44	39.06	4.88	0.6103
n	625	1250	1250	625	1250	1250	625	2500	1250
o	625	1250	1250	625	1250	625	625	2500	625
p	625	1250	625	625	1250	1250	625	2500	1250
q	625	1250	312.5	625	1250	1250	625	2500	1250
r	1250	1250	312.5	625	625	1250	625	2500	1250
s	625	625	78.125	625	1250	625	312.5	625	156.25
t	625	1250	1250	625	1250	1250	625	2500	1250
u	625	625	625	625	1250	1250	625	2500	1250
v	1250	1250	312.5	625	1250	1250	625	2500	1250
w	625	1250	312.5	625	625	1250	625	625	312.5
x	1250	1250	1250	625	1250	1250	625	2500	1250

*a. *K. ozaenae;* b. *K. pneumoniae;* c. *S. sonnei;* d. *Plesiomonas;* e. *S. boydii;* f. *M. morganii;* g. *S. aureus;* h. *P. aeroginosa;* i. *V. mimicus;* j. *V. fluvialis;* k. *V. cholerae 0139;* l. *V. cholerae 01;* m. *V. parahaemolyticus;* n. *E. Coli NCTC10418;* o. *E. tarda;* p. *P. vulgaris;* q. *P. mirabilis;* r. *S. typhimurium;* s. *S. paratyphi A;* t. *S. typhi;* u. *S. enteritidis;* v. *C. ferundii;* w. *Enterobacter;* x. *B. megatherius*

The synthesized compounds were also screened against *M. tuberculosis* strain $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium initially at 6.25 μg/ml (Table 2) [13]. Three compounds (**14-16**) showed complete inhibition (100%) of *M. tuberculosis* in the primary screening. In the secondary screening level the actual minimum inhibitory concentration (MIC) and cytotoxicity in VERO cells of these three compounds were determined. The MIC's of two compounds (**14** and **16**) were found to be 3.13 μg/ml, while compound **15** was found to be 1.56 μg/ml. Compounds (**14-16**) were not cytotoxic to VERO cells up to 62.5 μg/ml.

All compounds were evaluated for their *in-vitro* antibacterial activity against 24 pathogenic bacteria by conventional agar dilution procedures,[14] the results of which are summarized in Table 3 and 4. The data for ciprofloxacin, lomefloxacin and gatifloxacin were included for comparison. The antibacterial activity data demonstrated all the test compounds to show mild to moderate activity against the tested bacteria. The most sensitive organisms were *Plesiomonas, Shig. boydii. Vibrio mimicus, V. cholerae 01, V. cholerae 0139* and *V. parahaemolyticus*. These compounds inhibited them at a concentration of less than 50 μg/ml. Compound **14**, containing a ciprofloxacin moiety at the N-1 position, was found to be more active than ciprofloxacin itself against eight tested bacteria. When compared to lomefloxacin, compound **15** (lomefloxacin derivative) was found to be more active against 14 tested bacteria. Compound **16** bearing gatifloxacin at the N-1 position was found to be more active than gatifloxacin against 9 tested bacteria.

In-vivo antibacterial activity of some selected compounds against an experimentally induced infection of mice after oral administration [15] are presented in Table 5, along with the *in-vitro* activity against the infecting organism *E. coli* NCTC 10418. Ciprofloxacin and lomefloxacin were used as reference compounds. Compound **14** was found to be 2 times more active (ED_{50}: 0.62 mg/kg body weight) than ciprofloxacin (ED_{50}: 1.25 mg/kg) while compound **15** was slightly more active (ED_{50}: 1.25 mg/kg) than lomefloxacin (ED_{50}: 1.87 mg/kg) in the tests.

In summary seven of the sixteen new derivatives developed in this study showed inhibition against replication of HIV-1 in MT-4 cells with an EC_{50} ranging from 9.4-56.1μg/ml. Thirteen compounds were active against HCV RNA replication showing 80% inhibition at 50 μg/ml. One compound inhibited *M. tuberculosis* H37Rv with an MIC of 1.56 μg/ml. Three compounds

showed high activity against various pathogenic bacteria. Among the synthesized compounds, **14** emerged as an excellent broad-spectrum chemotherapeutic candidate.

Table 4. *In vitro* antibacterial activity (MIC's in μg/ml)

#*	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Cipro**	**Lome**	**Gati**
a	1250	625	1250	625	4.88	19.531	0.0381	0.15	1.22	0.08
b	625	1250	1250	1250	0.31	9.765	2.44	0.04	2.44	0.08
c	625	1250	1250	1250	0.31	4.88	0.152	0.04	39.06	0.08
d	0.6103	9.76	19.53	39.06	1.22	1.22	0.0381	0.04	1.22	2.44
e	0.6103	2.44	4.88	4.88	2.44	0.3051	0.0381	0.04	9.77	1.22
f	2500	312.5	1250	1250	1.22	0.1526	0.0381	0.04	1.22	0.02
g	156.25	1250	312.5	1250	4.88	0.0763	0.0190	0.04	0.61	0.02
h	2500	1250	1250	1250	1.22	0.1526	0.0190	0.15	4.88	0.15
i	0.6103	1.22	0.6103	1.22	0.0095	0.0381	0.0190	0.04	1.22	0.15
j	1250	1250	1250	1250	0.0095	0.0190	0.0190	0.04	1.22	0.02
k	312.5	312.5	625	1250	0.019	0.0381	0.1526	0.04	2.44	0.02
l	1.22	9.76	4.88	4.88	4.88	0.0095	0.6103	0.04	0.02	0.02
m	2.44	1.22	1.22	2.44	0.0763	0.0047	0.0763	0.04	39.06	9.77
n	2500	1250	625	1250	0.0381	0.1526	0.0763	0.02	0.61	0.02
o	1250	1250	1250	1250	0.0190	0.0763	0.0763	0.04	4.88	0.02
p	1250	625	625	1250	0.0095	0.1526	0.0763	0.04	0.61	0.02
q	2500	1250	625	1250	0.0190	0.0763	0.0763	0.04	2.44	0.02
r	2500	1250	1250	1250	0.0047	0.0381	0.0381	0.04	4.88	0.02
s	156.25	312.5	625	312.5	0.1526	0.0190	0.1526	0.04	0.61	0.02
t	1250	1250	1250	1250	0.3051	0.0190	0.0190	0.04	9.77	0.02
u	1250	1250	625	1250	0.6103	0.0095	0.0190	0.04	19.53	0.02
v	1250	625	625	312.5	1.22	0.0047	0.0095	0.04	19.53	0.08
w	1250	1250	625	1250	0.0381	0.0095	0.0190	0.04	19.53	0.02
x	1250	1250	625	625	0.0190	0.0190	0.0190	0.04	19.53	0.08

*a. *K. ozaenae;* b. *K. pneumoniae;* c. *S. sonnei;* d. *Plesiomonas;* e. *S. boydii;* f. *M. morganii;* g. *S. aureus;* h. *P. aeroginosa;* i. *V. mimicus;* j. *V. fluvialis;* k. *V. cholerae 0139;* l. *V. cholerae 01;* m. *V. parahaemolyticus;* n. *E. Coli NCTC10418;* o. *E. tarda;* p. *P. vulgaris;* q. *P. mirabilis;* r. *S. typhimurium;* s. *S. paratyphi A;* t. *S. typhi;* u. *S. enteritidis;* v. *C. ferundii;* w. *Enterobacter;* x. *B. megatherius*

Table 5: *In vivo* antibacterial study on *E. coli* NCTC 10419 strain

Compound	*In Vitro* MIC (in μg / ml)	*In Vivo* EC_{50} (in mg / Kg body wt.)
14	0.0381	0.62
15	0.1526	1.25
Ciprofloxacin	0.0190	1.25
Lomefloxacin	0.6103	1.87

Experimental

Chemistry

Melting points were determined in an open end capillary tube on a Büchi 530 melting point apparatus and are uncorrected. Infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded using Jasco IR Report 100 (KBr) and Bruker Avance (300MHz) instruments, respectively. Chemical shifts are reported in parts per million (ppm) using tetramethyl silane (TMS) as an internal standard. Elemental analyses (C, H, and N) were undertaken with a Perkin-Elmer model 240C analyzer. The homogeneity of the compounds was monitored by ascending thin layer chromatography (TLC) on silicagel-G (Merck) coated aluminium plates, visualized by iodine vapour. Developing solvents were chloroform-methanol (9:1). The pharmacophoric distance map and log P values were determined using Alchemy-2000 and Scilog P softwares (Tripos Co.).

Synthesis of (3-{[4'-amino-5(3'', 4'', 5''- trimethoxybenzyl) pyrimidin-2'-yl]} imino}-5-chloro-1, 3-dihydro-2H-indol-2-one)

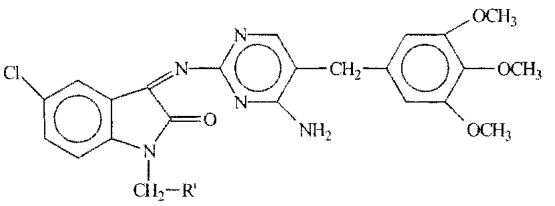
Equimolar quantities (0.01 mole) of 5-bromoisatin and 5-(3',4',5'-trimethoxybenzyl)-2,4-diaminopyrimidine were dissolved in warm ethanol containing 1 ml of glacial acetic acid. The reaction mixture was irradiated in an unmodified domestic microwave oven at 80% intensity with 30 sec/cycle for 3 minutes and set aside. The resultant solid was washed with dilute ethanol dried and recrystallized from ethanol-chloroform mixture. Yield 84.2%; m.p.: 192 °C; IR (KBr) : 3300,

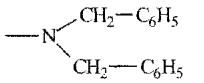
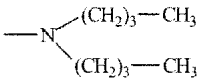
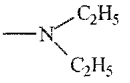
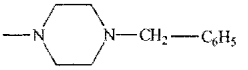
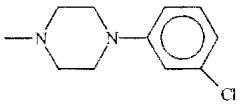
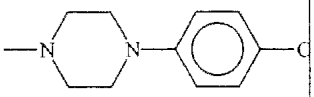
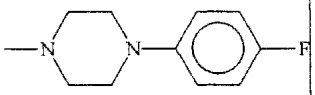
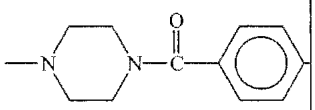
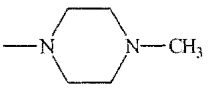
2050, 1660, 1625,1586 cm^{-1}; ^{1}H-NMR (CDCl3) δ (ppm): 3.18 (s, 2H, CH_2), 3.7 (s, 9H, -OCH_3), 5.6 (s, 2H, NH_2), 6.7-7.2 (m, 6H, Ar-H), 10.7(s, 1H, -NH).

General procedure for the preparation of Mannich bases

To a suspension of 3-{[4'-amino-5-(3'', 4'', 5''- trimethoxybenzyl) pyrimidin-2'-yl]} imino}-5-chloro-1,3-dihydro-2H-indol-2-one (0.02mol) in ethanol was added appropriate secondary amines (0.02 mole) and 37% formaldehyde (0.5 ml) and irradiated in a microwave oven at an intensity of 80% with 30sec/cycle. The number of cycle depended on the completion of the reaction, which was checked by TLC. The reaction timing varied from 1.5-3 min. The solution obtained after the completion of the reaction was kept at 0°C for 30 min and the resulting precipitate was recrystallized from a mixture of DMF and water.

Table 6. Physical constants of the synthesized compounds **1-9**

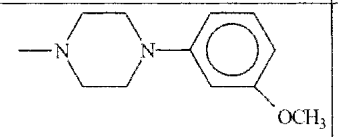
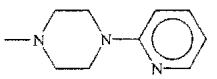
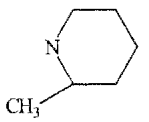
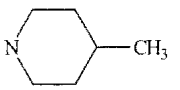
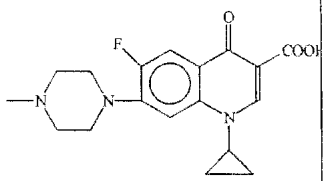
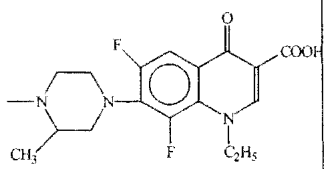
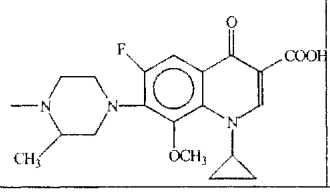


	R'	Molecular Formula	Molecular Weight	Yield (%)	M.P. (°C)	Log P
1		$C_{37}H_{35}N_6O_4Cl$	663.16	72.20	122	9.28
2		$C_{31}H_{39}N_6O_4Cl$	595.13	68.25	108	7.98
3		$C_{27}H_{31}N_6O_4Cl$	539.02	65.80	100	4.38
4		$C_{34}H_{36}N_7O_4Cl$	642.14	73.50	109	8.45
5		$C_{33}H_{33}N_7O_4Cl_2$	662.56	71.65	81	8.55
6		$C_{33}H_{33}N_7O_4Cl_2$	662.56	70.80	77	8.55
7		$C_{33}H_{33}N_7O_4ClF$	646.11	68.75	86	8.23
8		$C_{34}H_{33}N_7O_5ClF$	674.12	66.00	72	8.03
9		$C_{28}H_{32}N_7O_4Cl$	566.05	66.50	94	3.45

(3-{[4'-Amino-5'-(3'', 4'', 5''- trimethoxybenzyl) pyrimidin-2'-yl]} imino}-5-chloro-1-[(diethylamino) methyl]-1,3-dihydro-2H-indol-2-one) (3)

Yield: 65.8%; m.p.: 100°C ; IR (KBr) : 3010, 2850, 2840, 1730, 1616, 1506, 1236, 1129 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.86 (t, 6H, CH_3 of C_2H_5), 3.2 (s, 2H, CH_2 of benzyl), 3.7 (s, 9H, $-OCH_3$), 4.2 (q, 4H, CH_2 of C_2H_5), 5.2 (s, 2H, $-NCH_2N-$), 5.6 (s, 2H, NH_2), 6.8-7.28 (m, 6H, Ar-H) ; Calculated for $C_{27}H_{31}N_6O_4Cl$: C, 60.16; H, 5.8; N, 15.59; found: C, 60.18; H, 5.69; N, 15.60.

Table 7. Physical constants of the synthesized compounds **10-16**

	R'	Molecular Formula	Molecular Weight	Yield (%)	M.P. (°C)	Log P
10		$C_{34}H_{36}N_7O_5Cl$	658.14	67.20	92	8.25
11		$C_{32}H_{33}N_8O_4Cl$	629.10	74.40	84	8.02
12		$C_{29}H_{33}N_6O_4Cl$	565.06	71.50	102	4.70
13		$C_{29}H_{33}N_6O_4C$	565.06	70.62	98	4.97
14		$C_{40}H_{38}N_8O_7FCl$	797.23	67.00	247	5.06
15		$C_{40}H_{39}N_8O_7F_2Cl$	817.23	64.00	>250	5.66
16		$C_{42}H_{42}N_8O_8FCl$	841.28	68.00	140	5.72

(3-{[4'-Amino-5'-(3'', 4'', 5''- trimethoxybenzyl) pyrimidin-2'-yl]} imino}-5-chloro-1- [(3-chlorophenyl piperazinyl) methyl]-1,3-dihydro-2H-indol-2-one) (5)

Yield: 71.65%; m.p.: 81°C ; IR (KBr) : 3010, 2856, 2830, 1720, 1620, 1500, 1240, cm ; 1H-NMR ($CDCl_3$) δ (ppm): 3.17 (s, 2H, CH_2 of trimethoxybenzyl), 3.65 (s, 9H, $-OCH_3$), 3.9 -4.1 (m, 8H, piperazine-H), 5.2 (s, 2H, $-NCH_2N-$), 5.65 (s, 2H, NH_2), 6.67-7.82 (m, 10H, Ar-H) ; Calculated for $C_{33}H_{33}N_7O_4Cl_2$: C, 59.82; H, 5.02; N, 14.8; found: C, 60.04; H, 5.11; N, 14.72

1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[[N4-[3'-(4'-amino-5'- trimethoxybenzyl pyrimidin-2'-yl)imino-1'-(5-chloroisatinyl)] methyl] N'-piperazinyl]-3-quinoline carboxylic acid (14)

Yield: 67%; m.p.: 247° C ; IR (KBr) : 3010, 2850, 2840, 1736, 1620, 1506, 1236, 1125 cm ; 1H-NMR ($CDCl_3$) δ (ppm): 0.88-1.12 (m, 4H, cyclopropyl-H), 3.2 (s, 2H, CH_2 of benzyl), 3.5 (m, 1H, cyclopropyl-H), 3.60 (s, 9H, $-OCH_3$), 3.7-4.1 (m, 8H, -piperazine-H), 5.1 (s, 2H, $-NCH_2N$), 5.8 (s, 2H, NH_2), 6.58-8.66 (m, 9H, Ar-H), 8.6 (s, 1H, C_2-H) ; Calculated for $C_{40}H_{38}N_6O_7FCl$: C, 60.26; H, 4.8; N, 14.06; found: C, 60.19; H, 4.71; N, 14.13.

Biology

Anti-HIV screening in MT-4 cells

The compounds were tested for anti-HIV activity against replication of HIV-1 (III B) in MT-4 cells. The MT-4 cells were grown in RPMI-1640 DM (Dutch modification) medium (Flow lab, Irvine, Scotland), supplemented with 10% (v/v) heat-inactivated calf serum and 20 μg/mL gentamicin (E. Merck, Darmstadt, Germany). HIV-1 (III B) was obtained from the culture supernatant of HIV-1 infected MT-4 cell lines and the virus stocks were stored at –70 °C until used. Anti- HIV assays were carried out in microtitre plates filled with 100 μL of medium and 25μL volumes of compounds in triplicate so as to allow simultaneous evaluation of their effects on HIV and mock infected cells. Fifty microlitres of HIV at 100 CCID50 medium were added to either the HIV infected or mock infected part of the microtitre tray. The cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. Five days after infection the viability of mock and HIV-infected cells were examined spectrophotometrically by the MTT method.

In CEM cells

Candidate agents were dissolved in dimethylsulfoxide, and then diluted 1:100 in cell culture medium before preparing serial half- log10 dilutions. T4 lymphocytes (CEM cell-line) were added and after a brief interval HIV-1 was added, resulting in a final dilution of 1:200 of the compound. Cell lines without HIV-1, but with the compound served as a toxicity control, and infected (HIV-1) and uninfected cells without the compound served as basic controls. Cultures were incubated at 37°C in a 5% carbon dioxide atmosphere for 6 days. The tetrazolium salt, XTT was added to all the wells, and cultures were incubated to allow formazan color development by viable cells. Individual wells were analyzed spectrophotometrically to quantitative formazan production and, in addition, were viewed microscopically for detection of viable cells and confirmation of protective activity

HIV-1RT assay

The reaction mixture (50µl) contained 50mMTris–HCl (pH 7.8), 5mM dithiothreitol, 30 mM glutathione, 50 µM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 µg of bovine serum albumin, an appropriate concentration of the radiolabelled substrate $^{[3H]}$dGTP, 0.1 mM poly(vC)·oligo(dG) as the template/primer, 0.06% Triton X-100, 10 µl of inhibitor solution (containing various concentrations of compounds), and 1 µl of RT preparation. The reaction mixtures were incubated at 37 °C for 15 min, at which time 100 µl of calf thymus DNA (150 µg/ml), 2 ml of $Na_4P_2O_7$ (0.1 M in 1 M HCl), and 2 ml of trichloroacetic acid (10% v/v) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analyzed for radioactivity. For the experiments in which 50% inhibitory concentration (IC_{50}) of each test compound was determined, a fixed concentration of 2.5 µM $^{[3H]}$dGTP was used.

Antiviral and cytotoxicity assays for HCV

Cell culture

Huh-7 cells the subgenomic HCV replicon BM4-5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum, 1% l-glutamine, 1% l-pyruvate, 1% penicillin and 1% streptomycin supplemented with 500 mg/mL G418 (Geneticin, Invitrogen). Cells were passaged every 4 days.

Cytotoxicity

Huh-7 cells were respectively seeded at a density of3 $X10^{-4}$ cells/well in 96-well plates for the cell-viability assay, or at a density of $6X10^{-5}$ cells/well in six-well plates for the antiviral assay. Sixteen hours post seeding, cells were treated with the compounds at 50µg/mL for 3 days. The administration of each drug was renewed each day. Other drugs, including ribavirin (ICN Pharmaceuticals, USA), and interferon alpha- 2b (IntronA) were used in the same conditions as positive controls. At the end of treatment, cell viability assays were performed with the 96-well plates using Neutral Red assay (Sigma).

Antiviral assay

Total RNA (tRNA) was extracted from six-well plates with the 'Extract All' reagent (Eurobio), which is a mixture of guanidinium thiocyanate–phenol–chloroform. Northern Blot analysis was then performed using the NorthernMaxTM-Gly (Ambion) kit, following manufacturer's instruction. Ten micrograms of tRNA was denatured in glyoxal buffer at 50°C for 30 min and separated by agarose gel electrophoresis, then transferred for 12 h onto a charged nylon membrane (Biodyne B, Merck Eurolab). Hybridisation was carried out with three different [32P]CTP-labelled riboprobes obtained by *in-vitro* transcription (Promega). These probes were complementary to the NS5A region of the HCV genome, and to the cellular gene GAPDH, respectively. First, the blot was hybridized with two riboprobes directed against the negative strand of HCV RNA and the GAPDH mRNA, respectively. After one night of hybridization at 68°C, the membrane was washed then exposed to X-ray film and a phosphor screen for quantitative analysis. The amount of GAPDH mRNA was used as an internal loading control to standardize the amount of HCV RNA detected. The same membrane was subsequently hybridized with a negative-sense riboprobe to determine the level of HCV-positive strand RNA using the same approach.

Antimycobacterial screening

Primary screening was conducted at 6.25 µg/ml against *Mycobacterium tuberculosis* strain $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium using a broth micro dilution assay, the Microplate Alamar Blue Assay (MABA) [14].

In vitro antibacterial activity

Compounds were evaluated for their *in-vitro* antibacterial activity against 28 pathogenic bacteria procured from the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, India. The agar dilution method was performed using Mueller-Hinton agar (Hi-Media) medium. Suspensions of each microorganism were prepared to contain approximately 10^6 colony forming units (cfu/ml) and applied to plates with serially diluted compounds in DMF to be tested and incubated at 37°C overnight (approx. 18-20 h). The minimum inhibitory concentration (MIC) was considered to be the lowest concentration that completely inhibited growth on agar plates, disregarding a single colony or a faint haze caused by the inoculums.

In vivo antibacterial activity (mouse protection test)

The *in-vivo* antibacterial activity of the test compounds was determined in CF-strain male mice (20–25 g body weight, six per group). The mice were infected intraperitoneally with a suspension containing an amount of the indicated organism slightly greater than its lethal dose 100 (LD_{100}). The mice were treated orally (p.o.) with a specific amount of the test compound administered at 1 and 4 h after infection. ED_{50} values were calculated by interpolation among survival rates in each group after a week. They express the total dose of compound (mg/kg) required to protect 50% of the mice from an experimentally induced lethal systemic infection of the indicated organism.

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