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Design, synthesis and biological evaluation of novel non-nucleoside HIV-1 reverse transcriptase inhibitors with broad-spectrum chemotherapeutic properties

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Abstract—Acquired immunodeficiency syndrome (AIDS) results from infection by the retrovirus, human immunodeficiency virus (HIV). HIV is the most significant risk factor for many opportunistic infections like tuberculosis, hepatitis, bacterial infections, etc. In this paper, we designed aminopyrimidinimino 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-ethyl-6-fluoro-1,4-dihydro-4-oxo-7[[N^4-[3′-(4′-amino-5′-trimethoxybenzylpyrimidin-2′-yl)imino-1′-(5-fluoroisatin-yl)]methyl]-N^1-piperazinyl]-3-quinoline carboxylic acid (**12**) emerged as the most potent broad-spectrum chemotherapeutic agent active against HIV, HCV, *Mycobacterium tuberculosis* and various pathogenic bacteria.

1. Introduction

Since first reported in the 1980s, acquired immunodeficiency syndrome (AIDS), which is caused by the human immunodeficiency virus (HIV) and results in life threatening opportunistic infections and malignancies, has spread rapidly through the human population and become a major worldwide pandemic.[1,2] The global AIDS epidemic claimed more than three million lives in 2003, and ca. 40 million people were living with HIV or AIDS at the end of that same year.[3] The HIV infection, which targets the monocytes expressing surface CD4 receptors, eventually produces profound defects in cell-mediated immunity.[4] 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 killer of people with AIDS. The coinfection by hepatitis C virus (HCV) and HIV is quite common, mainly because these infections share the same parenteral, sexual and vertical routes of transmission.[5] Although classical OIs are now rarely seen, the toxicity of anti-retroviral drugs as well as liver diseases caused by HCV represent an increasing cause of morbidity and mortality among HIV-positive persons. Predisposing liver damage favours a higher rate of hepatotoxicity of anti-retroviral drugs, which can limit the benefit of HIV treatment in some individuals.[6] Through logic and orderly thinking, it appears that an ideal drug for HIV/AIDS patients should suppress HIV replication thereby acting as anti-HIV drug and also should treat OIs like TB, hepatitis and other bacterial infections. Earlier works in our laboratory have identified various isatinimino derivatives exhibiting broad-spectrum chemotherapeutic properties.[7] As a continuation to our effort in developing broad-spectrum chemotherapeutics, we undertook the present study to design, synthesize and evaluate aminopyrimidinimino isatin analogues, which could suppress HIV replication and also inhibit the opportunistic microorganisms.

2. Chemistry

2.1. Design

To qualify as a non-nucleoside reverse transcriptase inhibitors (NNRTI), the compound should interact specifically with a non-substrate binding site of the reverse

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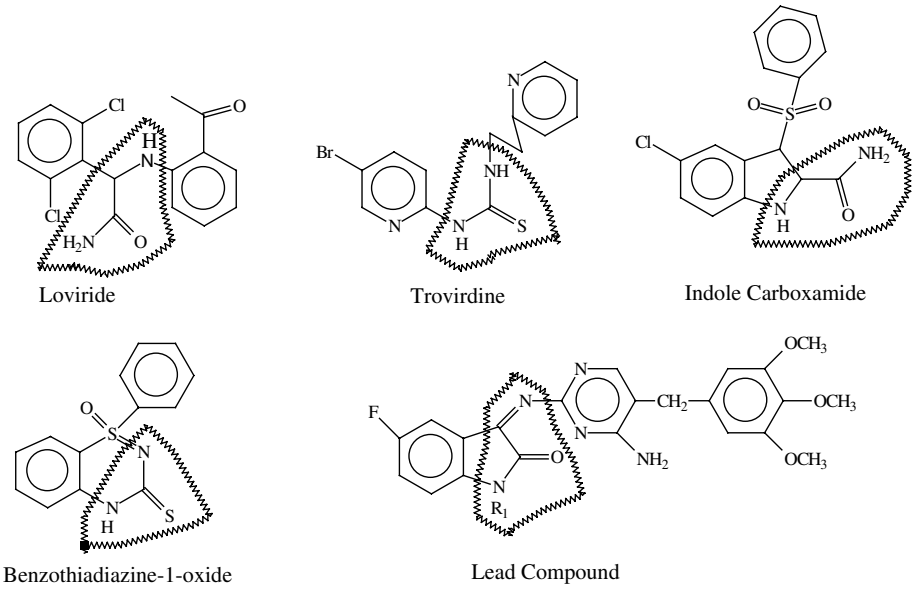


Figure 1. Existing NNRTI's and lead compound.

transcriptase (RT) of HIV-1, and inhibit the replication of HIV-1 at a concentration that is significantly lower than the concentration required to affect normal cell viability.[8] Based on these premises, more than 30 different classes of NNRTI's could be considered.[9] Although the NNRTI's seemingly belong to widely diverging classes of compounds, closer inspection reveals that most have some features in common, that is, a carboxamide, or (thio) urea entity ('body'), surrounded by two hydrophobic, mostly aryl moieties ('wings'), one of which is quite often substituted by a halogen group (Fig. 1). Thus, the overall structure may be considered reminiscent of a butterfly with hydrophilic centre ('body') and two hydrophobic outskirts ('wing'). In the present study, the aminopyrimidinimino isatin analogues are designed in accord to this hypothesis. The iminocarbamoyl moiety (–N=C–CO–N–) constitutes the 'body' and the aryl ring of isatin and the pyrimidine derivative constitute the 'wings' as depicted in Figure 1. The distance between the hydrophilic centre (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[10] from the energy minimized structures using MM3 program. The lead compound was found to comply within the specification of the pharmacophoric distance map (Fig. 2 and Table 1).

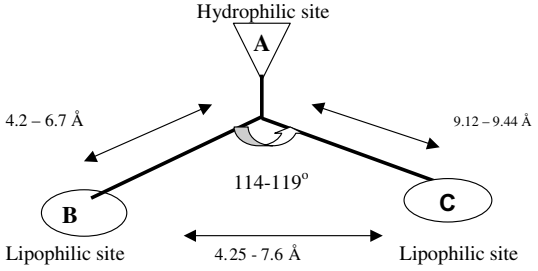


Figure 2. Schematic representation of a butterfly-like configuration of NNRTI'S and the pharmacophoric distance map.

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

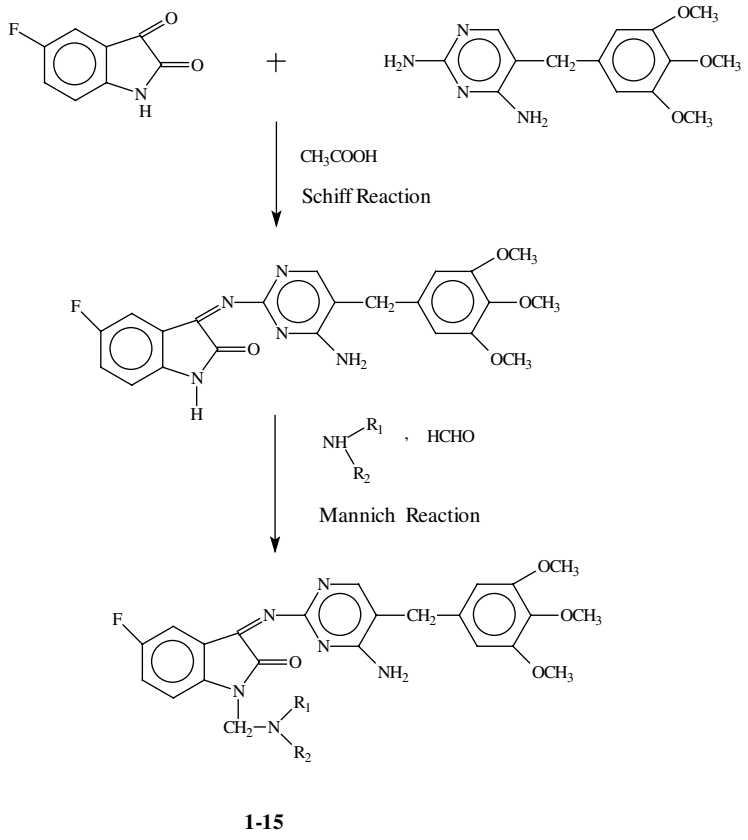
Drugs	AB (in Å)	BC (in Å)	CA (in Å)	Angle BAC
Loviride	4.36–5.19	4.25–6.25	9.4	119°
Trovirdine	4.22–6.67	6.53–7.6	9.44	117.5°
Indole carboxamide	4.20–6.72	4.44–7.08	9.44	118°
Benzothiadiazine-1-oxide	4.25–6.60	4.86–7.02	9.12	114.9°
Range	4.20–6.72	4.25–7.6	9.12–9.44	114.9°–119°
Lead compound	4.23–5.34	4.07–6.63	9.113	116.7°

2.2. Synthesis

The synthesis of various aminopyrimidinimino isatin derivatives was achieved in two steps (Fig. 3).[11] 5-Fluoroisatin was condensed with 5-trimethoxybenzyl-2,4-diaminopyrimidine in the presence of glacial acetic acid to form Schiff's base. The N-Mannich bases of the above Schiff's base were synthesized by condensing acidic imino group of isatin with formaldehyde and various secondary amines. All compounds (Tables 2 and 3) gave satisfactory elemental analysis. IR and ^{1}H NMR spectra were consistent with the assigned structures.

3. 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 4).[12] In the MT-4 cell lines, compound **12** was found to be the most active against replication of HIV-1 with EC_{50} of 12.1 μg/mL and their selectivity index (SI = CC_{50}/EC_{50}) was found to be more than 13 with maximum protection of 96.6%. Other compounds (**4**, **9**, **13**–**15**) showed maximum protection of 54–86% with SI of 2–8. In the T4 lymphocytes (CEM



1-15

Figure 3. Protocol for the synthetic compounds.

cell lines), only compound **14** was found to be active with EC_{50} of 50.8 μg/mL with maximum protection of 63%. Other tested compounds showed marked anti-HIV activity (11–47%) at a concentration below their toxicity threshold. The loss of activity might be due to degeneration/rapid metabolism in the culture conditions used in the screening procedure. Moreover the bioactivity did not have any correlation with the log *P*.

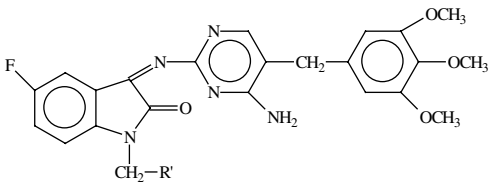
Further, compound **12** was evaluated for the inhibitory effects on HIV-1 RT enzyme[13] and their IC_{50} value was found to be 32.6 ± 6.4 μM. The in vitro IC_{50} values for HIV-1 RT with Poly (νC) oligo (dG) as the template/primer were significantly higher than the corresponding EC_{50} values for 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 the cellular systems.[14]

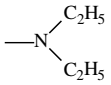
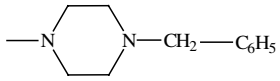
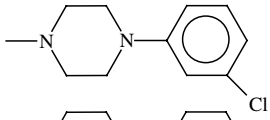
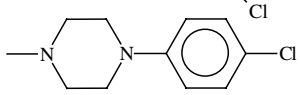
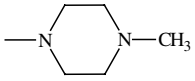
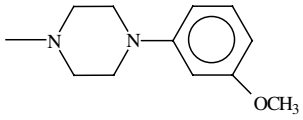
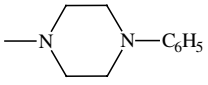
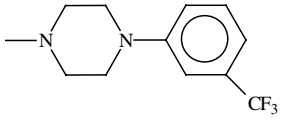
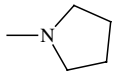
All the synthesized compounds were also evaluated for their inhibition of HCV viral RNA replication in HUH-7 cells at 50 μg/mL,[15] and the results are presented in Table 4. Among these, six compounds (**1**, **3**, **8**, **10**, **11** and **15**) were found to be less toxic to Huh-7 cells (cell growth of >80%) and inhibited HCV viral RNA replication at about 82–100%. Compound **11** was found to be the most active analogue showing 100% inhibition on viral replication and was non-toxic to Huh-7 cells. This paper is first of its kind in which isatin derivatives are reported to possess anti-HCV activity.

The synthesized compounds were also screened against *Mycobacterium tuberculosis* strain $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium at a initial concentration of 6.25 μg/mL (Table 4).[16] Four compounds (**12**–**15**) showed complete inhibition (100%) of *M. tuberculosis* in the primary screening. In the secondary level screening the actual minimum inhibitory concentration (MIC) and cytotoxicity in VERO cells of these three compounds were determined. The MIC's of these compounds were found to be 3.13 μg/mL and they were not cytotoxic upto 62.5 μg/mL to VERO cells.

All the compounds were evaluated for their in vitro anti-bacterial activity against 24 pathogenic bacteria by conventional agar dilution procedures[17] and the results of the assays are summarized in Tables 5 and 6. The data for norfloxacin, ciprofloxacin, lomefloxacin and gatifloxacin were included for comparison. The anti-bacterial activity data revealed that all the test compounds showed mild to moderate activity against tested bacteria. The most sensitive organisms for the tested compounds were *K. ozaenae*, *Vibrio mimicus*, *E. coli* NCTC10439, *P. mirabilis*, *S. Typhimurium* and *S. enteritidis* as these compounds inhibited them at a concentration less than 2.5 μg/mL. Compound **5**, which contain 4-chlorophenyl piperazinomethyl moiety at N-1 position was found to be the most active compound

Table 2. Physical constants of the synthesized compounds **1**–**10**



Compound	R′	Molecular formula	Molecular weight	Yield (%)	Mp (°C)	Log *P*
1		$C_{27}H_{31}N_6O_4F$	522.57	62.08	123	4.00
2		$C_{25}H_{27}N_6O_4F$	494.52	60.10	77	3.23
3		$C_{34}H_{36}N_7O_4F$	625.69	58.65	79	8.30
4		$C_{33}H_{33}N_7O_4ClF$	646.11	65.81	112	8.32
5		$C_{33}H_{33}N_7O_4ClF$	646.11	66.10	114	8.31
6		$C_{28}H_{32}N_7O_4F$	549.60	69.00	111	6.28
7		$C_{34}H_{36}N_7O_5F$	641.70	60.50	132	8.09
8		$C_{33}H_{34}N_7O_4F$	611.67	62.50	60	8.01
9		$C_{34}H_{33}N_7O_4F_4$	679.66	65.27	76	8.89
10		$C_{27}H_{29}N_6O_4F$	520.56	67.91	137	3.58

being more potent than lomefloxacin against 20 tested bacteria. Compound **12** containing norfloxacin moiety at N-1 position was found to be more active than norfloxacin against 18 tested bacteria. Compound **13** containing ciprofloxacin moiety at N-1 position was found to be more active than ciprofloxacin against 13 tested bacteria. When compared to lomefloxacin, compound **14** (lomefloxacin derivative) was found to be more active against 23 tested bacteria. Compound **15** bearing gatifloxacin at N-1 position was found to be more active than gatifloxacin against six tested bacteria. The better antibacterial activity of these compounds might be due to the inhibitory effect of both bacterial dihydrofolate reductase and DNA gyrase enzymes. These data are consistent with our earlier results in which the derivatives of the known fluoroquinolones were potent than the parent drug.[18]

In vivo anti-bacterial activity of some selected compounds against an experimentally induced infection of mice after oral administration[18] is presented in Table 7, along with the in vitro activity against the infecting organism *E. coli* NCTC 10418. Norfloxacin, ciprofloxacin and lomefloxacin were used as reference compounds. Compound **12** was found to be three times more active (ED_{50}: 1.87 mg/kg body weight) than norfloxacin (ED_{50}: 6.0 mg/kg) while

Table 3. Physical constants of the synthesized compounds **11–15**

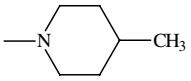
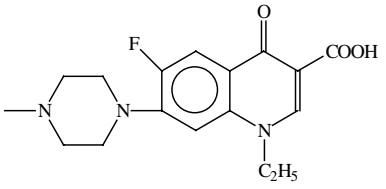
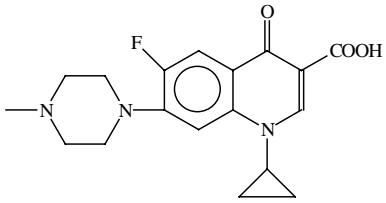
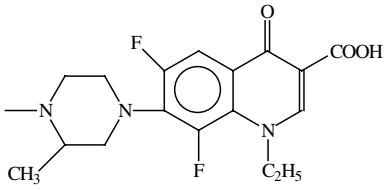
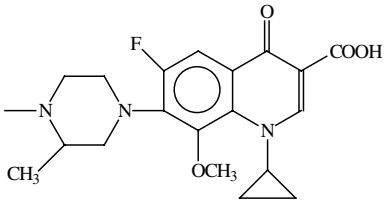
Compound	R′	Molecular formula	Molecular weight	Yield (%)	Mp (°C)	Log *P*
11		$C_{29}H_{33}N_6O_4F$	548.61	65	99	4.59
12		$C_{39}H_{38}N_8O_7F_2$	768.77	63	264	5.33
13		$C_{40}H_{38}N_8O_7F_2$	780.77	64	230	5.29
14		$C_{40}H_{39}N_8O_7F_3$	800.78	68	212	5.71
15		$C_{42}H_{42}N_8O_8F_2$	824.83	69	144	5.86

Table 4. Anti-HIV, anti-HCV and anti-mycobacterial activities

Compound	Anti-HIV activity						Anti-HCV activity at 50 μg/mL		Anti-mycobacterial activity at 6.25 μg/mL
	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	>141.01	141.0	30.2	>116.0	116.0	11.28	90	82	19
2	>62.7	62.7	12.1	NT	NT	NT	55	100	19
3	>139.3	139.3	34.6	>112.0	112.0	11.53	87	97	37
4	>121.6	121.6	46.1	>104.0	104.0	15.53	63	92	20
5	36.2	90.1	59.4	>95.6	95.6	47.47	66	90	45
6	>106.7	106.7	42.6	>115.0	115.0	13.91	68	96	24
7	>81.6	81.6	22.1	NT	NT	NT	10	100	63
8	>84.7	84.7	21.6	NT	NT	NT	91	98	33
9	62.1	136.6	54.2	>127.0	127.0	10.95	71	88	40
10	>129.6	129.6	40.1	>121.0	121.0	14.25	90	88	36
11	>69.6	69.6	23.6	NT	NT	NT	97	100	24
12	12.1	160.2	99.6	50.8	139.0	63.84	48	95	100
13	17.9	141.6	86.2	NT	NT	NT	38	100	100
14	57.1	130.9	61.6	NT	NT	NT	73	89	100
15	25.2	91.6	56.8	NT	NT	NT	89	92	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%.

Table 5. In vitro anti-bacterial activity (MIC's in μg/mL)

Microorganism	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
K. ozaenae	0.31	0.31	0.31	0.31	0.1526	1.22	2.44	1.22	2.44	2.44
K. pneumoniae	2500	1250	1250	625	1.22	2500	2500	2500	1250	2500
S. sonnei	625	625	625	9.765	0.3051	625	625	312.5	625	2500
Plesiomonas	2500	625	625	625	0.6103	625	1250	625	625	1250
S. boydii	2500	625	625	625	0.6103	625	2500	625	625	1250
M. morganii	2500	625	625	625	0.6103	312.5	2500	19.531	625	625
S. aureus	625	312.5	78.125	312.5	0.6103	1250	2500	78.125	625	1250
P. aeroginosa	2500	1250	1250	625	1.22	1.22	1250	1250	1250	2500
V. mimicus	0.31	0.3051	0.31	0.1526	0.3051	0.1526	0.1526	1.22	1.22	2.44
V. fluvialis	2500	1250	625	625	1.22	312.5	2500	0.6103	1250	1250
V. cholerae 0139	1250	1250	625	625	0.3051	0.6103	2500	1250	1250	1250
V. cholerae 01	0.31	0.3051	0.31	0.3051	0.3051	39.06	2500	1250	4.88	1250
V. parahaemolyticus	19.531	78.12	78.12	19.531	2.44	39.06	78.125	0.6103	39.062	39.06
E. coli NCTC10418	0.31	0.3051	0.31	1.22	0.3051	1.22	2.44	0.1526	1.22	1.22
E. tarda	625	625	0.31	625	0.3051	312.5	4.88	312.5	39.062	4.88
P. vulgaris	625	625	0.31	625	4.88	312.5	2.44	312.5	156.25	625
P. mirabilis	0.31	0.31	0.31	0.3051	0.3051	0.6103	2.44	0.6103	0.6103	0.3051
S. typhimurium	0.31	0.31	0.1526	0.3051	0.3051	0.6103	1.22	0.6103	0.6103	0.6103
S. paratyphi A	2500	1250	1250	625	0.3051	625	2500	1.22	1250	78.125
S. typhi	2500	625	0.6103	625	0.1526	625	2500	312.5	1250	625
S. enteritidis	0.31	0.3051	0.31	0.3051	0.3051	1.22	2.44	1.22	2.44	1.22
C. ferundii	625	625	0.61	312.5	0.3051	1.22	1250	1.22	1250	312.5
Enterobacter	9.765	156.25	78.12	312.5	4.88	312.5	1250	312.5	312.5	1250
B. megatherius	625	625	1.22	312.5	0.3051	625	1250	625	2500	1250

Table 6. In vitro anti-bacterial activity (MIC's in μg/mL)

Microorganism	**11**	**12**	**13**	**14**	**15**	Cipro	Lome	Gati	Nor
K. ozaenae	1.22	1.22	0.3051	0.0095	0.3051	0.15	1.22	0.08	1.22
K. pneumoniae	2500	0.3051	0.0190	0.6103	0.15	0.04	2.44	0.08	2.44
S. sonnei	1250	0.1526	0.1526	0.6103	0.0763	0.04	39.06	0.08	9.76
Plesiomonas	2500	0.1526	0.1526	0.6103	0.0381	0.04	1.22	2.44	2.44
S. boydii	2500	1.22	0.0381	0.0095	0.01	0.04	9.77	1.22	1.22
M. morganii	2500	0.1526	0.1526	0.0095	0.0763	0.04	1.22	0.02	9.76
S. aureus	2500	0.3051	0.0763	0.0095	0.1526	0.04	0.61	0.02	39.06
P. aeroginosa	1250	1.22	0.0190	2.441	0.31	0.15	4.88	0.15	19.53
V. mimicus	2.44	1.22	0.1526	0.0095	0.0381	0.04	1.22	0.15	1.22
V. fluvialis	1250	2.44	0.1526	0.0095	0.0763	0.04	1.22	0.02	1.22
V. cholerae 0139	2500	1.22	0.6103	1.22	0.1526	0.04	2.44	0.02	2.44
V. cholerae 01	2500	1.22	0.1526	4.882	1.22	0.04	0.02	0.02	2.44
V. parahaemolyticus	78.125	0.0763	0.0095	0.6103	2.44	0.04	39.06	9.77	2.44
E. coli NCTC10418	1.22	0.0763	0.0095	0.3051	0.15	0.02	0.61	0.02	0.31
E. tarda	312.5	0.1526	0.3051	0.3051	0.0763	0.04	4.88	0.02	9.76
P. vulgaris	625	0.0190	0.0095	0.0095	0.0190	0.04	0.61	0.02	0.61
P. mirabilis	1.22	1.22	0.0095	0.0095	0.0095	0.04	2.44	0.02	1.22
S. typhimurium	1.22	1.22	0.0095	0.0095	0.0381	0.04	4.88	0.02	0.31
S. paratyphi A	2500	0.1526	0.0190	0.3051	0.0763	0.04	0.61	0.02	0.61
S. typhi	2500	0.3051	0.0190	0.0095	0.0381	0.04	9.77	0.02	1.22
S. enteritidis	2.44	1.22	0.0095	0.0095	0.0190	0.04	19.53	0.02	9.76
C. ferundii	1250	1.22	0.0095	0.3051	0.0763	0.04	19.53	0.08	9.76
Enterobacter	1250	0.6103	0.0095	0.0095	0.15	0.04	19.53	0.02	2.44
B. megatherius	1250	0.1526	0.0190	0.1526	0.0190	0.04	19.53	0.08	4.88

Table 7. In vivo anti-bacterial study on *E. coli* NCTC 10419 strain

Compound	In vitro MIC (μg/mL)	In vivo EC_{50} (mg/kg body wt)
12	0.076	1.87
13	0.0095	0.62
14	0.3051	1.25
Norfloxacin	0.30	6.0
Ciprofloxacin	0.0190	1.25
Lomefloxacin	0.6103	1.87

compound **13** was twice more active than ciprofloxacin with ED_{50} of 0.62 mg/kg and compound **14** was slightly more active (ED_{50}: 1.25 mg/kg) than lomefloxacin (ED_{50}: 1.87 mg/kg) against the tested bacteria.

4. Conclusion

Six compounds of the 15 new derivatives developed in this work showed inhibition against replication of

HIV-1 in MT-4 cells with EC_{50} ranging from 12.1 to 62.1 μg/mL. All the compounds were active against HCV RNA replication showing >80% inhibition at 50 μg/mL. Four compounds inhibited *M. tuberculosis* H37Rv with MIC of 3.13 μg/mL. Generally the compounds, which possessed substituted piperazines showed good activity. Especially the quinolone containing compounds (**12**–**15**) had exhibited promising activity profile. Five compounds showed very good activity against various pathogenic bacteria. In this series compound **12** emerged as a more promising broad-spectrum chemotherapeutic agent.

5. Experimental

Melting points were determined in one end open capillary tubes on a Büchi 530 melting point apparatus and are uncorrected. Infrared (IR) and proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded for the compounds on Jasco IR Report 100 (KBr) and Brucker Avance (300 MHz) 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 Perkin–Elmer model 240C analyzer. The homogeneity of the compounds was monitored by ascending thin layer chromatography (TLC) on silica gel-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.).

5.1. Synthesis of (3-{[4′-amino-5(3″,4″,5″-trimethoxybenzyl)pyrimidin-2′-yl]imino}-5-fluoro-1,3-dihydro-2*H*-indol-2-one)

Equimolar quantities (0.01 mol) of 5-flouroisatin 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[19] at 80% intensity with 30 s/cycle for 3 min and set aside. The resultant solid was washed with dilute ethanol dried and recrystallized from ethanol–chloroform mixture. Yield 78.4%; mp: 197 °C; IR (KBr): 3300, 2050, 1665, 1620, 1580 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ (ppm) 3.20 (s, 2H, CH_2), 3.7 (s, 9H, –OCH_3), 5.5 (s, 2H, NH_2), 6.8–7.5 (m, 6H, Ar–H), 10.68 (s, 1H, –NH).

5.2. 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-fluoro-1,3-dihydro-2*H*-indol-2-one (0.02 mol) in ethanol was added appropriate secondary amines (0.02 mol) and 37% formaldehyde (0.5 mL) and irradiated in a microwave oven at an intensity of 80% with 30 s/cycle. The number of cycle in turn depended on the completion of the reaction, which was checked by TLC. The reaction timing varied from 1.5 to 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.

5.2.1. (3-{[4′-Amino-5′-(3″,4″,5″-trimethoxybenzyl)pyrimidin-2′-yl]imino}-5-bromo-1-[(dimethylamino)methyl]-1,3-dihydro-2*H*-indol-2-one) (2). Yield: 60.10%; mp: 77 °C; IR (KBr): 3010, 2850, 2840, 1730, 1616, 1506, 1236, 1129 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ (ppm) 2.5 (s, 6H, –$N(CH_3)$, 3.16 (s, 2H, CH_2 of benzyl), 3.7 (s, 9H, –OCH_3), 5.1 (s, 2H, –NCH_2N–), 5.6 (s, 2H, NH_2), 6.8–7.26 (m, 6H, Ar–H); calculated for $C_{25}H_{27}N_6O_4F$: C, 60.72; H, 5.55; N, 16.99; found: C, 60.13; H, 5.42; N, 17.09.

5.2.2. (3-{[4′-Amino-5′-(3″,4″,5″-trimethoxybenzyl)pyrimidin-2′-yl]imino}-5-bromo-1-[(4-chlorophenyl piperazinyl)methyl]-1,3-dihydro-2*H*-indol-2-one) (5). Yield: 66.10%; mp: 114 °C; IR (KBr): 3010, 2850, 2830, 1730, 1620, 1500, 1240, cm^{-1}; ^{1}H 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 $C_{33}H_{33}N_7O_4ClF$: C, 61.35; H, 5.15; N, 15.17; found: C, 61.60; H, 5.20; N, 15.22.

5.2.3. (3-{[4′-Amino-5′-(3″,4″,5″-trimethoxybenzyl)pyrimidin-2′-yl]imino}-5-bromo-1-[(piperidino)methyl]-1,3-dihydro-2*H*-indol-2-one) (10). Yield: 67.91%; mp: 137 °C; IR (KBr): 3010, 2858, 2842, 1730, 1622, 1506, 1236, 1129 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ (ppm) 2.0 (m, 4H, $2 \times CH_2$), 2.6 (t, 4H, –CH_2N CH_2–), 3.16 (s, 2H, CH_2 of benzyl), 3.72 (s, 9H, –OCH_3), 5.16 (s, 2H, –NCH_2N–), 5.6 (s, 2H, NH_2), 6.82–7.26 (m, 6H, Ar–H); calculated for $C_{27}H_{29}N_6O_4F$: C, 62.3; H, 5.62; N, 16.14; found: C, 62.13; H, 5.58; N, 16.19.

5.2.4. 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[[*N*^{4}-[3′-(4′-amino-5′-trimethoxybenzyl pyrimidin-2′-yl)imino-1′-5-(flouroisatinyl)]methyl]-*N*′-piperazinyl]-3-quinoline carboxylic acid (12). Yield: 63.17%; mp: 264 °C; IR (KBr): 3010, 2850, 2840, 1736, 1620, 1596, 1506, 1236, 1125 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ (ppm) 1.28 (t, 3H, CH_3 of C_2H_5), 3.3 (s, 2H, CH_2 of benzyl), 3.62 (s, 9H, –OCH_3), 3.7–4.1 (m, 8H, –piperazine–H), 4.25 (q, 2H, CH_2 of C_2H_5), 5.1 (s, 2H, –NCH_2N), 5.8 (s, 2H, NH_2), 6.58–8.60 (m, 9H, Ar–H), 8.6 (s, 1H, C_2–H); calculated for $C_{39}H_{38}N_8O_7F_2$: C, 60.93; H, 4.98; N, 14.58; found: C, 60.82; H, 4.91; N, 14.30.

5.2.5. 1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[[*N*^{4}-[3′-(4′-amino-5′-trimethoxybenzyl pyrimidin-2′-yl)imino-1′-5-(flouroisatinyl)]methyl]-*N*′-piperazinyl]-3-quinoline carboxylic acid (13). Yield: 64.5%; mp: 230 °C; IR (KBr): 3010, 2850, 2844, 1740, 1628, 1596, 1506, 1236, 1125 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ (ppm) 0.88–1.1 (m, 4H, cyclopropyl–H), 3.3 (s, 2H, CH_2 of benzyl), 3.5 (m, 1H, cyclopropyl–H), 3.62 (s, 9H, –OCH_3), 3.7–4.1 (m, 8H, –piperazine–H), 5.1 (s, 2H, –NCH_2N), 5.8 (s, 2H, NH_2), 6.58–8.60 (m, 9H, Ar–H), 8.6 (s, 1H, C_2–H); calculated for $C_{40}H_{38}N_8O_7F_2$: C, 61.53; H, 4.91; N, 14.35; found: C, 61.80; H, 4.97; N, 14.33.

5.3. Anti-HIV screening

5.3.1. 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 25 μL 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.

5.3.2. 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 1:200 final dilution of the compound. Uninfected cells with the compound served as a toxicity control, and infected 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 colour 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.

5.3.3. HIV-1 RT assay. The reaction mixture (50 μL) contained 50 mM Tris–HCl (pH 7.8), 5 mM 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(*v*C)·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 the test compounds was determined, fixed concentration of 2.5 M [3H] dGTP was used.

5.4. Anti-viral and cytotoxicity assays against HCV

5.4.1. Cell culture. Huh-7 cells the subgenomic HCV replicon BM4-5 cells were maintained in Dulbecco0s 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.

5.4.2. Cytotoxicity. Huh-7 cells were, respectively, seeded at a density of 3×10^{-4} cells/well in 96-well plates for the cell-viability assay, or at a density of 6×10^{-5} cells/well in six-well plates for the anti-viral assay. Sixteen hours postseeding, cells were treated with the compounds at 50 μg/mL for 3 days. The administration of each drug was renewed each day. Other drugs, including ribavirin (ICN Pharmaceuticals, USA), mycophenolic acid (Sigma, 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).

5.4.3. Anti-viral 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). Hybridization 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.

5.5. Anti-mycobacterial screening

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

5.6. In vitro anti-bacterial activity

Compounds were evaluated for their in vitro anti-bacterial 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.

5.7. In vivo anti-bacterial activity (mouse protection test)

The in vivo anti-bacterial activity of the test compounds was determined in CF-strain male mice (20–25 g bodyweight, 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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19. A domestic microwave oven with the following specifications had been used: Make LG; input 220 V–50 Hz, 980 W, 4.7 A; frequency 2450 MHz.