Aminopyrimidinimino Isatin Analogues: Design and Synthesis of Novel Non- Nucleoside HIV-1 Reverse Transcriptase Inhibitors with Broad-Spectrum Anti-Microbial Properties

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Abstract: HIV is the most significant risk factor for many opportunistic infections like tuberculosis, 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'-chloroben-zylpyrimidin-2'-yl)imino-1'-(5-methylisatinyl)] methyl]N^1-piperazinyl]-3-quinoline carboxylic acid (**10**) emerged as the most potent broad-spectrum chemotherapeutic agent active against HIV-1 replication (EC_{50}: 9.4 μg /ml), *M. tuberculosis* (MIC: 3.13 μg /ml) and various pathogenic bacteria (MIC's: 1.22 μg /ml).

Key Words: Anti-HIV; anti-mycobacterial; anti-bacterial; isatin.

1. INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is caused by the retrovirus, human immuno deficiency virus (HIV) [1]. The 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 infection (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 living with HIV/AIDS. Up to 50% of people with HIV in sub-Saharan Africa develop TB and 1 out of 3 die of TB. TB is one of the leading causes of illness and death among people living with AIDS in developing countries. The development of active TB accelerates the progression of HIV disease towards full-blown AIDS, accompanied by enhanced HIV replication, The majority of people infected with the HIV virus develop TB as the first manifestation of AIDS. 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 inhibiting OIs like TB, and other bacterial infections. Earlier studies in our laboratory have identified various isatinimino derivatives exhibiting broad-spectrum chemo-therapeutic properties [3]. 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.

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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 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 [4]. Based on these premises, more than thirty different classes of NNRTI's could be considered [5]. 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 center ('body) and two hydrophobic outskirts ('wing'). In the present study, the aminopyrimidinimino isatin analogues are designed in accord once with 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 Fig. **1**. 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 [6] 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**).

2.2. Synthesis

The starting material 2,4-diamino-5-(4'chlorobenzyl)-pyrimidine was synthesised according to the literature procedure [7]. 4-Chloro benzaldehyde undergo base-

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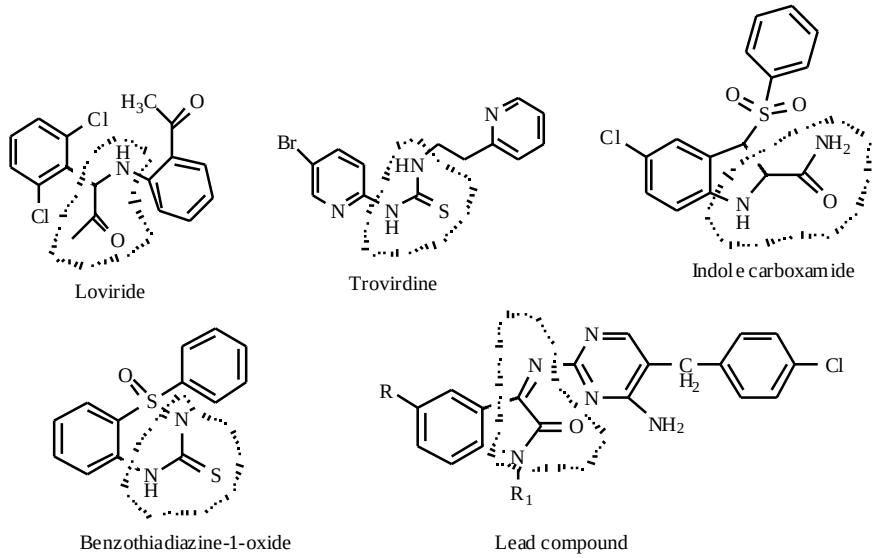


Fig. (1). Existing NNRTI's and Lead Compound.

catalysed condensation with β–ethoxypropinitrile to form β-ethoxy-α-(4-chloro benzylidene) propionitrile, which on reaction with considerable excess of guanidine (2-3 equivalents) undergo cyclisation to form required pyrimidine moiety. The synthesis of various aminopyrimidinimino isatin derivatives was achieved in two steps (Fig. **3**) [8]. 5-Substituted isatin were condensed with 2,4-diamino-5-(4'chlorobenzyl)-pyrimidine, in the presence of glacial acetic acid to form Schiff's base. The N-Mannich bases of the above Schiff's base were synthesised by condensing acidic imino group of isatin with formaldehyde and various secondary amines. All compounds (Table **2** and **3**) gave satisfactory elemental analysis. IR and ^{1}H-NMR spectra were consistent with the assigned structures.

3. BIOLOGICAL INVESTIGATION AND DISCUSSION

The synthesised compounds were evaluated for their inhibitory effect on the replication of HIV-1 in MT-4 and CEM cell lines (Table **4**) [9]. In the MT-4 cell lines, compound **10** was found to be the most active against replication of HIV-1 with EC_{50} of 9.4 μg /ml, and their selectivity index ($SI=CC_{50}/EC_{50}$) was found to be more than 15 with maximum protection of 102%. Other compounds (**1, 6, 7, 14** and **15**) showed maximum protection of 62%-90% with SI of 2-7. In the T4 lymphocytes (CEM cell lines), the compounds showed marked anti-HIV activity (11-43%) 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.

One compound (**10**) was evaluated for the inhibitory effects on HIV-1 RT enzyme [10] and their IC_{50} values were found to be 21.4 ± 3.12 μM. The *in-vitro* IC_{50} values for HIV-1 RT with Poly (_C) 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

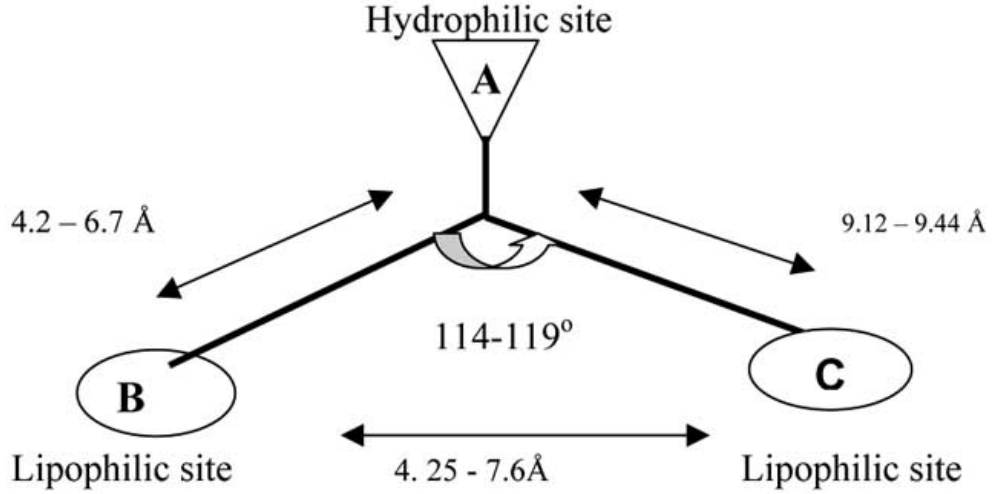


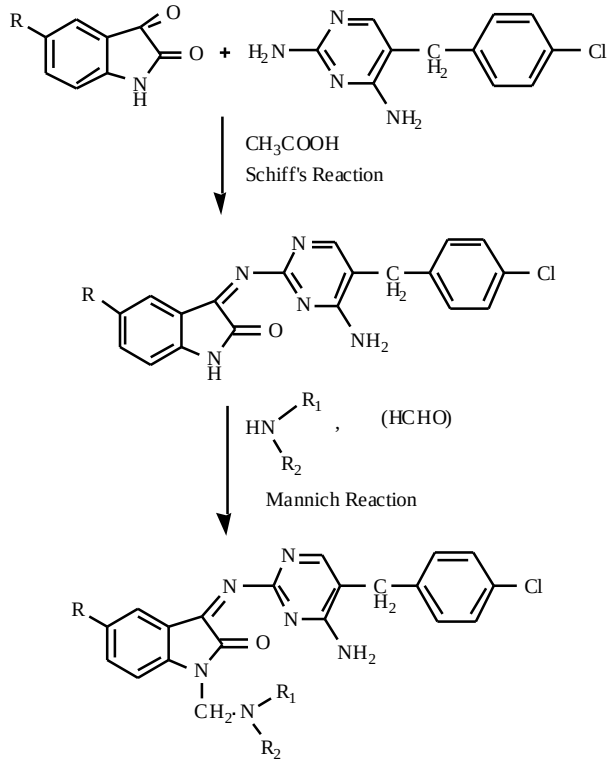
Fig. (2). Schematic representation of a butterfly-like configuration of NNRTI'S and the pharmacophoric distance map.

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°**

may reflect the differences between the *in vitro* HIV-1 RT assay, in which a synthetic template/primer is used, and the cellular systems [11].

The synthesised compounds were also screened against *M. tuberculosis* strain $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium initially at 6.25 μg/ml (Table **4**) [12]. Three compounds (**6**, **10** and **14**) showed complete inhibition (100%) of *M. tuberculosis* in the primary screening. In the secondary level screening (data not shown), 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.



1-15

Fig. (3). Protocol for the synthetic compounds.

All the compounds were evaluated for their *in-vitro* anti-bacterial activity against 24 pathogenic bacteria by conventional agar dilution procedures [13] and the results of these assays are summarized in Table (**5** and **6**). The data for norfloxacin was 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. pneumoniae*, *S. sonnei*, *S. boydii*, *V. cholerae*0139, *E. coli* NCTC 10418, *E. tarda*, *S. typhi* and *S. enteritidis*, as these compounds inhibited them at a concentration less than 10 μg/ml, and all the compounds were more active than norfloxacin. Compounds **6, 10** and **15**, which contain norfloxacin moiety at N-1 position, were found to be the most active compounds which were more potent than norfloxacin against all 24 tested pathogenic bacteriae. These data are consistent with our earlier results [14].

In vivo anti-bacterial activities of some selected compounds against an experimentally induced infection of mice after oral administration [14] are presented in Table **7**, along with the *in-vitro* activity against the infecting organism *E. coli* NCTC 10418. Norfloxacin was used as reference compounds. Compound **10** was found to be most potent compound and it was 5 times more active (ED_{50}: 1.2 mg/kg body weight) than norfloxacin (ED_{50}: 6.0 mg/kg), while compound **6** and **15** were also more active than norfloxacin with ED_{50} of 2.5 and 1.87 mg/kg respectively, against the tested bacteria.

4. CONCLUSION

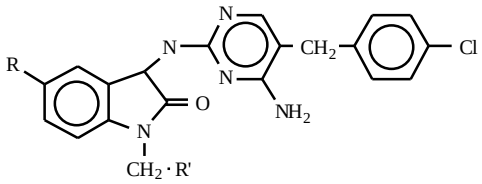
Six compounds of the 15 new derivatives developed in this work showed inhibition against replication of HIV-1 in MT-4 cells, with EC_{50} ranging from 9.4-26.3 μg/ml. Three compounds inhibited *M. tuberculosis* H37Rv with MIC of 3.13 μg/ml. Three compounds showed very good activity against various pathogenic bacteria and were more potent than norfloxacin. Among the synthesised compounds, **6, 10** and **15** emerged as more promising broad-spectrum anti-microbial agents.

5. EXPERIMENTAL PROTOCOLS

5.1. Chemistry

Melting points were determined in one end open capillary tubes on a Büchi 530 melting point apparatus and were

Table 2. Physical Constants of the Synthesized Compounds 1-10



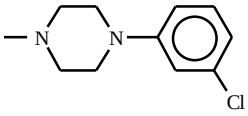
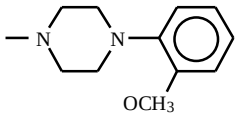
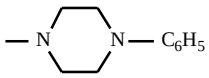
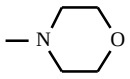
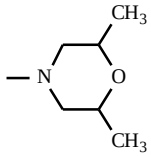
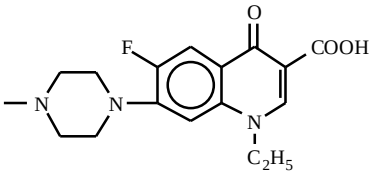
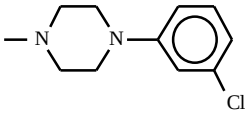
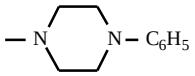
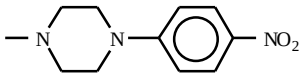
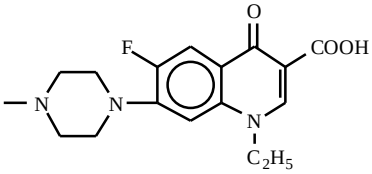
#	R	R'	Molecular Formula	Molecular Weight	Yield (%)	M.P. (°C)	Log P
1	Cl		$C_{30}H_{26}N_7OCl_3$	606.932	69.60	162	8.78
2	Cl		$C_{31}H_{29}N_7O_2Cl_2$	602.513	70.20	144	8.49
3	Cl		$C_{30}H_{27}N_7OCl_2$	572.487	72.50	102	8.50
4	Cl		$C_{24}H_{22}N_6O_2Cl_2$	497.376	71.00	172	7.22
5	Cl		$C_{26}H_{26}N_6O_2Cl_2$	525.429	68.80	138	7.51
6	Cl		$C_{36}H_{31}N_8O_4Cl_2F$	729.587	66.20	177	4.10
7	CH_3		$C_{31}H_{29}N_7OCl_2$	586.514	66.30	121	8.19
8	CH_3		$C_{31}H_{30}N_7OCl$	552.069	68.50	150	7.89
9	CH_3		$C_{31}H_{29}N_8O_3Cl$	597.067	70.10	170	6.95
10	CH_3		$C_{37}H_{34}N_8O_4ClF$	709.169	72.60	184	4.77

Table 3. Physical Constants of the Synthesized Compounds 11-15

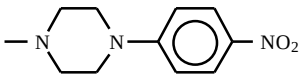
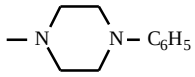
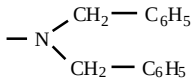
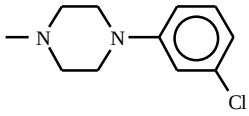
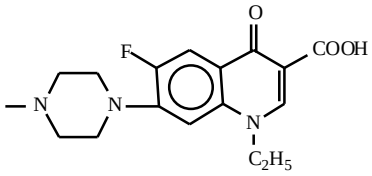
#	R	R'	Molecular Formula	Molecular Weight	Yield (%)	M.P. (°C)	Log P
11	F		$C_{30}H_{26}N_8O_3ClF$	601.031	63.26	167	7.32
12	F		$C_{30}H_{27}N_7OClF$	556.033	68.50	110	8.36
13	F		$C_{34}H_{28}N_6OClF$	591.077	67.90	81	9.44
14	F		$C_{30}H_{26}N_7OCl_2F$	590.478	74.00	141	8.64
15	F		$C_{36}H_{31}N_8O_4ClF_2$	713.132	76.26	211	3.95

Table 4. Anti-HIV, Anti-HCV and Antimycobacterial Activity

	MT-4 cell line			CEM cell line			Antimycobacterial activity at 6.25 μg/ml % inhibition
	EC_{50}[a]	CC_{50}[b]	% Inhibition	EC_{50}[a]	CC_{50}[b]	% Protection	
1	20.02	39.4	62.6	> 25.8	> 25.8	13.78	63
2	> 21.6	21.6	29.12	> 18.7	18.7	22.08	52
3	> 42.1	42.1	36.8	NT	NT	NT	28
4	> 42.8	42.8	18.02	> 34.9	34.9	12.73	34
5	> 46.92	46.92	21.06	> 36.6	36.6	11.75	81
6	11.2	69.8	88.4	> 26.2	26.2	26.61	100
7	26.3	109.26	72.10	> 108.0	108.0	25.4	67
8	> 143.92	143.92	27.62	NT	NT	NT	43
9	> 113.12	113.12	42.10	> 104.0	104.0	42.71	62
10	9.4	143.0	102.6	> 130.0	130.0	11.37	100
11	> 69.12	69.12	32.1	NT	NT	NT	28
12	> 72.72	72.72	8.6	NT	NT	NT	24
13	> 120.92	120.92	21.16	> 116.0	116.0	12.21	79
14	21.6	73.16	69.92	> 65.8	65.8	29.32	83
15	14.9	131.60	90.12	> 123.0	123.0	19.91	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*** **Antibacterial Activity (MIC's in μg/ml)**

Microorganism	1	2	3	4	5	6	7	8	9	10
K. ozaenae	625	625	625	78.13	19.53	0.31	19.53	19.53	156.25	0.15
K. pneumoniae	1.22	1.22	0.62	0.62	0.15	0.018	0.037	0.62	0.62	0.037
S. sonnei	4.88	4.88	2.44	2.44	0.61	0.075	1.22	1.22	2.44	0.009
Plesiomonas	1250	1250	19.53	78.13	19.53	2.44	19.53	9.77	625	2.44
S. boydii	0.61	0.61	0.3	0.61	0.3	0.075	0.15	0.3	0.61	0.018
M. morganii	1250	1250	625	625	625	4.88	39.06	1250	1250	1.22
S. aureus	1250	1250	625	625	625	2.44	39.06	19.53	78.13	1.22
P. aeroginosa	1250	1250	1250	625	625	4.88	9.76	1250	1250	4.88
V. mimicus	78.13	39.06	19.53	0.31	9.76	0.31	4.88	0.31	0.31	0.31
V. fluvialis	78.13	78.12	0.31	0.31	9.76	0.31	0.31	0.31	0.31	0.31
V. cholerae 0139	0.15	0.15	0.15	0.15	0.037	0.037	0.075	0.075	0.15	0.018
V. cholerae 01	39.06	1.22	78.12	39.06	39.06	0.61	0.31	9.77	39.12	0.61
V. parahaemolyticus	78.13	78.12	39.06	78.12	78.12	0.61	0.31	0.31	19.53	0.15
E. Coli NCTC10418	0.15	0.15	0.15	0.15	0.15	0.037	0.15	0.075	0.15	0.018
E. tarda	4.88	4.88	1.22	1.22	2.44	0.6	4.88	4.88	2.44	0.6
P. vulgaris	1250	1250	625	625	625	0.075	9.77	78.13	1250	0.037
P. mirabilis	1250	1250	625.0	625	625	0.61	9.77	2500	1250	0.61
S. typhimurium	1250	1250	625	312.50	625	0.15	312.5	2500	1250	0.15
S. paratyphi A	1250	312.5	625	625	78.12	0.3	4.88	78.13	9.77	0.15
S. typhi	0.61	0.61	0.3	0.61	0.61	0.15	0.31	0.61	0.61	0.075
S. enteritidis	4.88	4.88	2.44	2.44	2.44	0.31	4.88	1.22	0.15	0.75
C. ferundii	0.61	0.61	78.12	78.12	19.53	1.22	9.77	78.13	9.76	0.3
Enterobacter	1.22	1250	19.53	312.5	312.5	0.31	0.31	312.5	156.25	1.22
B. megatherius	625	1250	625	625	312.5	0.31	0.31	9.77	625	0.61

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 (300MHz) instruments, respectively. Chemical shifts were 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 analyser. The homogeneity of the compounds was monitored by ascending thin layer chromatography (TLC) on silicagel-G (Merck) coated aluminium plates, visualised 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.).

5.1.1. General Procedure for the Preparation of Schiff bases

Equimolar quantities (0.01 mole) of 5-substituted isatin and 5-(4'- chlorobenzyl)-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 [15] 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.

5.1.2. (3-{[4'-Amino-5(4'' - chlorobenzyl) pyrimidin-2'-yl]} imino}-5-chloro-1, 3-dihydro-2H-indol-2-one.

Yield 84.2%; m.p.: 189 °C; IR (KBr) : 3300, 2040, 1660, 1620,1580 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).

5.1.3. General Procedure for the Preparation of Mannich Bases

To a suspension of 3-{[4'-amino-5-(4''- chlorobenzyl) pyrimidin-2'-yl]} imino}-5-(sub)-1,3-dihydro-2H-indol-2-

Table 6. ***In Vitro*** **Antibacterial Activity (MIC's in μg/ml)**

Microorganism	11	12	13	14	15	Nor
K. ozaenae	19.53	625	625	1.22	0.01	1.22
K. pneumoniae	0.62	0.62	0.62	0.075	0.018	2.44
S. sonnei	0.31	4.88	4.88	1.22	0.037	9.76
Plesiomonas	625	625	625	0.15	0.61	2.44
S. boydii	0.61	0.61	0.61	0.15	0.018	1.22
M. morganii	1250	625	625	9.76	0.31	9.76
S. aureus	39.06	625	625	39.06	1.22	39.06
P. aeroginosa	2500	78.12	2500	39.06	9.76	19.53
V. mimicus	0.31	0.31	0.31	1.22	0.018	1.22
V. fluvialis	0.31	39.12	78.12	1.22	0.15	1.22
V. cholerae 0139	1.22	2.44	1.22	0.61	0.018	2.44
V. cholerae 01	0.31	0.31	0.31	1.22	0.075	2.44
V. parahaemolyticus	19.53	9.77	19.53	0.15	0.075	2.44
E. Coli NCTC10418	0.15	0.15	0.15	0.075	0.037	0.31
E. tarda	4.88	4.88	4.88	1.22	0.31	9.76
P. vulgaris	625	625	1250	625	0.0018	0.61
P. mirabilis	625	625	1250	312.6	0.0018	1.22
S. typhimurium	625	625	1250	312.5	0.15	0.31
S. paratyphi A	156.25	625	625	156.25	0.3	0.61
S. typhi	0.61	0.3	0.3	0.61	0.075	1.22
S. enteritidis	4.88	2.44	2.44	1.22	0.018	9.76
C. ferundii	19.53	78.13	39.12	1.22	0.31	9.76
Enterobacter	9.76	625	625	9.76	0.15	2.44
B. megatherius	625	625	625	156.25	0.61	4.88

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 in turn 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^oC for 30 min and the resulting precipitate was recrystallized from a mixture of DMF and water.

5.1.3.1. (3-{[4'-Amino-5'-(4''-chlorobenzyl)pyrimidin-2'-yl]} imino}-5-chloro-1-[(3-chlorophenyl piperazinyl) methyl]-1,3-dihydro-2H-indol-2-one) (1)

Yield: 69.60%; m.p.: 162^oC ; IR (KBr) : 3010, 2850, 2830, 1730, 1620, 1500, 1240, cm ; 1H-NMR ($CDCl_3$) δ (ppm): 3.18 (s, 2H, CH_2 of chlorobenzyl), 3.9 -4.26 (m, 8H, piperazine-H), 5.2 (s, 2H, -NCH_2N-), 5.65 (s, 2H, NH_2), 6.67-7.80 (m, 12H, Ar-H) ; Calculated for $C_{30}H_{26}N_7OCl_3$: C, 59.37; H, 4.32; N, 16.15; found: C, 59.17; H, 4.19; N, 16.22

5.1.3.2. 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[[N4-[3'-(4'-amino-5'- chlorobenzyl pyrimidin-2'-yl)imino-1'-5-(methylisatinyl)] methyl] N'-piperazinyl]-3-quinoline Carboxylic Acid (10)

Yield: 72.6%; m.p.: 184^o C ; IR (KBr) : 3016, 2850, 2840, 1736, 1620, 1596, 1506, 1236, 1125 cm ; 1H-NMR ($CDCl_3$) δ (ppm): 1.28 (t, 3H, CH_3 of C_2H_5),1.84(s, 3H, CH_3), 3.3 (s, 2H, CH_2 of benzyl), 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, 11H, Ar-H), 8.6 (s, 1H, C_2-H) ; Calculated for $C_{37}H_{34}N_8O_4FCl$: C, 62.67; H, 4.83; N, 15.8; found: C, 62.82; H, 4.91; N, 15.70.

Table 7. ***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.)
6	0.0190	2.5
10	0.1526	1.20
15	0.0190	1.87
Norfloxacin	0.6103	6.0

5.1.3.3. (3-{[4'-Amino-5'-(4''- chlorobenzyl) pyrimidin-2'-yl]} imino}-5-fluoro-1-[(4-phenyl piperazinyl) methyl]-1,3-dihydro-2H-indol-2-one) (12)

Yield: 68.5; m.p.: 110°C ; IR (KBr) : 3010, 2850, 2830, 1730, 1620, 1500, 1240, cm ; 1H-NMR ($CDCl_3$) δ (ppm): 3.17 (s, 2H, CH_2 of chlorobenzyl), 3.9 -4.1 (m, 8H, piperazine-H), 5.2 (s, 2H, -NCH_2N-), 5.65 (s, 2H, NH_2), 6.67-8.02 (m, 13H, Ar-H) ; Calculated for $C_{30}H_{27}N_7OClF$: C, 64.8; H, 4.89; N, 17.63; found: C, 64.60; H, 4.82; N, 17.70.

5.2. Biology

5.2.1. Anti-HIV Screening

5.2.1.1. 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 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.

5.2.1.2. 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 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

5.2.1.3. 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 MgCl2, 1.25 μg of bovine serum albumine, an appropriate concentration of the radio-labelled substrate [3H] dGTP, 0.1 mM poly(νC)·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 Na4P2O7 (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 analysed for radioactivity. For the experiments in which 50% inhibitory concentration (IC50) of the test compounds was determined, fixed concentration of 2.5 μM [3H] dGTP was used.

5.2.2. 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).

5.2.3. 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.

5.2.4. 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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[15] A domestic microwave oven with the following specifications had been used : Make LG; Input 220V~50 Hz, 980 W, 4.7 A; Frequency 2450 MHz.