Aminopyrimidinimino isatin analogues: Design of novel non-nucleoside HIV-1 reverse transcriptase inhibitors with broad-spectrum chemotherapeutic properties

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Received April 11, 2005; Revised September 22, 2005; Accepted October 19, 2005; Published October 20, 2005

ABSTRACT: Purpose: HIV is the most significant risk factor for many opportunistic infections such as tuberculosis, hepatitis, bacterial infections and others. In this paper, we describe an 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. **Methods:** The synthesis of various aminopyrimidinimino isatin derivatives was achieved in two steps and evaluated for anti-HIV, anti-HCV, antimycobacterial and antibacterial activities. **Results:** Compound 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7[[N^4-[3'-(4'-amino-5'-trimethoxybenzylpyrimidin-2'-yl)imino-1'-isatinyl] 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. Among the synthesized compounds compound **14** and **15** emerged as more promising broad-spectrum chemotherapeutic agents.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is caused by the retrovirus, human immunodeficiency virus (HIV) [1]. The HIV infection, which targets the monocytes expressing surface CD4 receptors, eventually produces profound defects in cell-mediated immunity [2]. Over time 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 co-infection by hepatitis C virus (HCV) and HIV is quite common, mainly because these infections share the same parenteral, sexual and 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 persons. Predisposing liver damage favors a higher rate of hepatotoxicity of antiretroviral drugs, which can limit the benefit of HIV treatment in some individuals [4]. Through logic and orderly thinking, it appears that an ideal drug for HIV/AIDS patients should suppress HIV replication thereby acting as an anti-HIV drug and also should treat OIs like TB, hepatitis and other bacterial infections. Earlier work in our laboratory has identified various isatinimino derivatives exhibiting broad-spectrum chemotherapeutic properties [5]. As a continuation of 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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EXPERIMENTAL

Chemistry

Melting points were determined in one end open capillary tubes on a Büchi 530 melting point apparatus and are uncorrected. 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. Infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR) spectra and ^{13}C NMR were recorded for the compounds on Jasco IR Report 100 (KBr) and Brucker Avance (300MHz) instruments and Varian unity 400 (50MHz) spectrometer 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 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 software (Tripos Co.).

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

Equimolar quantities (0.01 mole) of 5-bromoisatin and 5-(3', 4', 5'-trimethoxybenzyl)-two, 4-diaminopyrimidine (Trimethoprim) were dissolved in warm ethanol containing 1 ml of glacial acetic acid. The reaction mixture was irradiated in an unmodified domestic microwave oven [17] 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.: 185 °C; IR (KBr) : 3300, 2040, 1660, 1620,1580 cm^{-1}; ^{1}H-NMR ($CDCl_3$) 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-bromo-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 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^{o}C for 30 min and the resulting precipitate was recrystallized from a mixture of DMF and water.

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

Yield: 70.26%; m.p.: 68^{o}C ; IR (KBr) : 3010, 2850, 2840, 1730, 1616, 1506, 1236, 1129 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.8 (t, 6H, CH_3 of C_2H_5, J = 15 Hz), 3.16 (s, 2H, CH_2 of benzyl), 3.7 (s, 9H, -OCH_3), 4.2 (q, 4H, CH_2 of C_2H_5, J = 12 Hz), 5.1 (s, 2H, -NCH_2N-), 5.6 (s, 2H, NH_2), 6.8-7.26 (m, 6H, Ar-H) ; ^{13}C NMR (DMSO-d_6) δ (ppm): 13.0 (2C, CH_3s of C_2H_5), 41.3 (CH_2), 46.9 (2C, CH_2s of C_2H_5), 56.1 (2C, OCH_3s at 3- and 5- positions), 56.4 (OCH_3 at 4-position), 70.1 (CH_2), 106.3 (2C at 2- and 6-positions of trimethoxyphenyl), 114.2 (C at 5-position of pyrimidine), 118.8 (C at 5-position of indole), 119.9 (C at 9-position of indole), 123.9 (C at 7-position of indole), 130.5 (C at 1-position of trimethoxyphenyl), 132.9 (C at 4-position of indole), 134.1 (C at 6-position of indole), 136.2 (C at 4-position of trimethoxyphenyl), 146.4 (C at 8-position of indole), 150.7 (2C at 3- and 5-positions of trimethoxyphenyl), 161.1 (C at 4-position of pyrimidine), 163.0 (C at 6-postion of pyrimidine), 163.2 (C at 3-position of indole),163.5 (C at 2-position of indole), 164.1 (C at 2-position of pyrimidine); Calculated for $C_{27}H_{31}N_6O_4Br$: C, 55.58; H, 5.36; N, 14.4; found: C, 55.48; H, 5.39; N, 14.60.

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

Yield: 75.10%; m.p.: 87^{o}C ; IR (KBr) : 3010, 2850, 2840, 1730, 1616, 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), 4.36 (s, 2H, CH_2 of benzylpiperazine), 5.2 (s, 2H, -NCH_2N-), 5.65 (s, 2H, NH_2), 6.67-7.82 (m, 11H, Ar-H) ; ^{13}C NMR (DMSO-d_6) δ (ppm): 41.3 (CH_2), 50.3 (2C of 2- and 6-positions of piperazine), 52.2 (2C of 3- and 5-positions of piperazine), 56.1 (2C of OCH_3s at 3- and 5-positions), 56.4 (C of OCH_3 at 4-position), 60.1 (CH_2) 70.1 (CH_2), 106.3 (C at 2-and 6-positions of trimethoxyphenyl), 114.2 (C at 5- position of pyrimidine), 118.8 (C at 5-position of indole), 119.9 (C at 9-position of indole), 123.9 (C at 7-position of indole), 127.3 (C at 4- position of phenyl), 128.5 (2C at 3- and 5- positions of phenyl), 128.8 (2C at 2- and 6- positions of phenyl), 130.5 (C at 1-position of trimethoxyphenyl), 132.9 (C at 4-position of indole), 134.1 (C at 6-position of indole), 135.5 (C at 1-position of phenyl), 136.2 (C at 4-position of trimethoxyphenyl), 146.4 (C at 8-position of indole), 150.7 (C at 3- and 5-positions of trimethoxyphenyl), 161.1 (C at 4- position of pyrimidine), 163.0 (C at 6-position of pyrimidine), 163.2 (C at 3-position of indole), 163.5 (C at 2-position of indole), 164.1 (C at 2-position of pyrimidine) Calculated for $C_{34}H_{36}N_7O_4Br$: C, 59.48; H, 5.28; N, 14.28; found: C, 59.60; H, 5.20; N, 14.32.

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

Yield: 62.82%; m.p.: 84^{o}C ; IR (KBr) : 3010, 2850,

2830, 1730, 1620, 1500, 1240, cm ; ^{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 $C_{33}H_{33}N_7O_4ClBr$: C, 56.06; H, 4.7; N, 13.87; found: C, 56.12; H, 4.67; N, 13.62

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

Yield: 76%; m.p.: 222^o C ; IR (KBr) : 3010, 2850, 2840, 1736, 1620, 1506, 1236, 1125 cm ; 1H-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) ; ^{13}C NMR (DMSO-d_6) δ (ppm): 5.6 (C at 2- and 3-positions of cyclopropyl), 36.0 (C at 1-position of cyclopropyl), 41.3 (CH_2), 49.6 (2C of 3- and 5-positions of piperazine), 49.9 (2C of 2- and 6-positions of piperazine), 56.3 (2C od OCH_3s at 3- and 5-positions), 56.6 (C of OCH_3 at 4-position), 70.1 (CH_2), 100.0 (C at 8-position of quinoline), 106.3 (2C at 2- and 6-positions of trimethoxyphenyl), 109.3 (C at 3-position of quinoline), 114.2 (C at 5-position of pyrimidine), 116.4 (C at 5-position of quinoline), 118.2 (C at 10-position of quinoline), 118.8 (C at 5-position of indole), 119.9 (C at 9-position of indole), 123.9 (C at 7-position of indole), 130.5 (C at 1-position of trimethoxyphenyl), 132.9 (C at 4-position of indole), 134.1 (C at 6-position of indole), 136.2 (C at 4-position of trimethoxyphenyl), 140.5 (C at 9-position of quinoline), 143.9 (C at 7-position of quinoline), 144.6 (C at 6-position of quinoline), 146.4 (C at 8-position of indole), 148.0 (C at 2-position of quinoline), 150.7 (C at 3- and 5-positions of trimethoxyphenyl), 161.1 (C at 6-position of pyrimidine), 163.0 (C at 4-position of pyrimidine), 163.2 (C at 3-position of indole), 163.5 (C at 2-position of indole), 164.1 (C at 2-position of pyrimidine), 166.2 (COOH) , 177.4 (C at 4-position of quinoline); Calculated for $C_{40}H_{38}N_6O_7FBr$: C, 57.08; H, 4.55; N, 13.31; found: C, 57.12; H, 4.61; N, 13.30.

Anti-HIV activity

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 {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} 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 50mM Tris–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 radiolabeled 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 $Na_4P_2O_7$ (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 2.5µM [^{3}H] dGTP was used.

ANTIVIRAL AND CYTOTOXICITY ASSAYS FOR HCV

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.

Cytotoxicity

Huh-7 cells were respectively seeded at a density of 3 $X10^{-4}$ 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), 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).

Antiviral assay

Total tRNA (transfer RNA) 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 [^{32}P] CTP-labeled 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 activity

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 microdilution assay, the microplate Alamar Blue Assay (MABA) .

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 protocol is approved by Institute Animal Ethical Committee (IAEC/RES/11). 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.

RESULTS AND DISCUSSIONS

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 affecting normal cell viability [6]. Based on these premises, more than thirty different classes of NNRTIs could be considered [7]. Several studies have revealed that although the NNRTIs seemingly belong to a widely diverging classes of compounds, but on closer inspection it has been elucidated that most of them possess some features in common, that is a (thio) carboxamide, (thio) acetamide or (thio) urea entity ('body') which is hydrophilic in nature, surrounded by two hydrophobic, mostly aryl

moieties ('wings'), one of which is quite often substituted by a halogen group. Thus, the overall structure may be considered reminiscent of a butterfly with hydrophilic centre ('body') and two hydrophobic outskirts ('wings'). The 'butterfly-like' conformation has been proven by crystallographic analysis for Nevirapine [8] and TBZ. Based on this hypothesis, a 3D-pharmacophoric distance model was derived utilizing eight well-known NNRTIs, i.e. Nevirapine, Delavirdine, Efavirenz, Trovirdine, Loviride, indole carboxamide, benzothiadiazine-1-oxide and thiocarboxanilide. All the ligands were geometrically optimized based on the internal strain energy calculated by molecular mechanics calculations (MM3 parameterization) in Alchemy Tripos software to ensure uniform sampling of low energy conformers. Then the essential structural components like atoms, centroids of collection of atoms, electron lone pair positions, steric and electrostatic potentials etc were matched in the three-dimensional space of the energetically accessible conformations of the ligands, to arrive at the 3-point pharmacophore model proposed below (Fig 2).

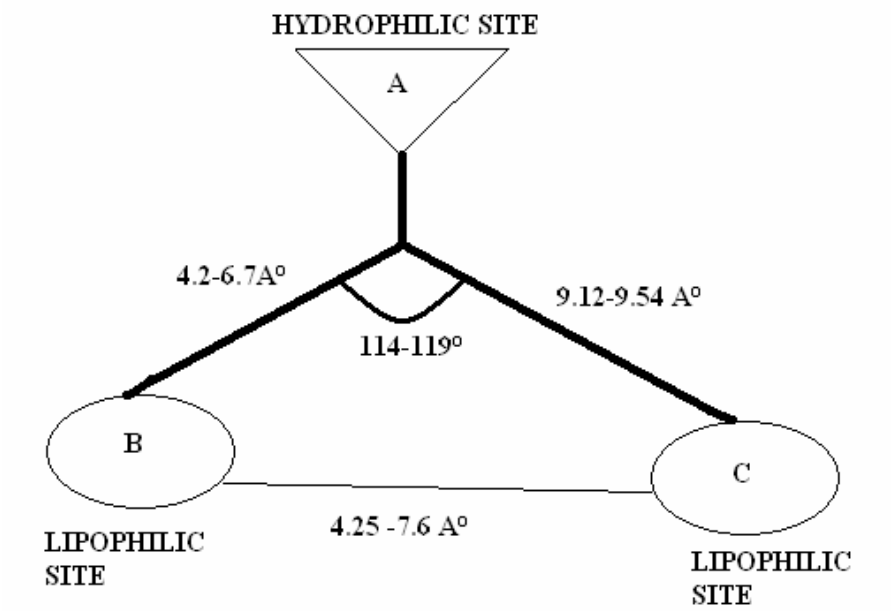
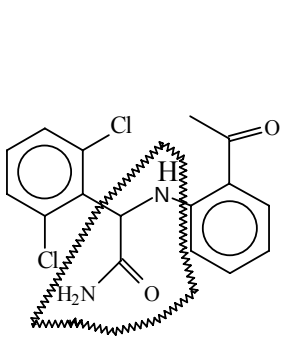


Figure 2: Schematic representation of a butterfly-like configuration of NNRTI'S and the pharmacophoric distance map

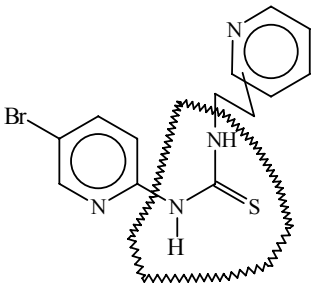
In the present study, the aminopyrimidinimino isatin analogues are designed in accord with this hypothesis. The iminocarbamoyl moiety (-N=C-CO-N-) constitutes the 'body' and the aryl ring of isatin and the pyrimidine derivative constitute the 'wings' as depicted in Fig.1. The crucial structural components included in the proposed model contain a hydrophilic centre (A), which is surrounded by 2 hydrophobic outskirts denoted by B and C. The distance between the 3-pharmacophoric points were calculated for minimum four different conformations and are represented as mean standard deviation. The lead compound was found to comply within the specification of the pharmacophoric distance map (Fig. 2 and Table 1). During the development of this 3D-pharmacophoric model, molecular superposition techniques have also been used to investigate similarities and differences between the selected points in the test molecule (aminopyrimidinimino isatin analogue) and the corresponding points in the reference molecule (Nevirapine, Efavirenz, and Delavirdine) calculated by means of RMS (Root mean square deviation) value. It was deduced from the RMS fit value that the structure fits appreciably with Delavirdine with RMS value of 0.075 (Fig 3)

Synthesis

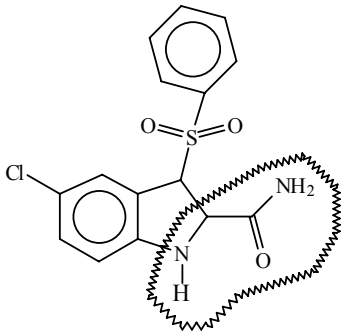
The synthesis of various aminopyrimidinimino isatin derivatives was achieved in two steps (Fig.4) [9]. 5-Bromoisatin was condensed with 5-trimethoxybenzyl-2,4-diamino pyrimidine in the presence of glacial acetic acid to form Schiff's base. The N-Mannich bases of the above Schiff's base were synthesized by condensing acidic imino group of isatin with formaldehyde and various secondary amines. All compounds (Table 2 and 3) gave satisfactory elemental analysis. IR, ^{1}H-NMR and ^{13}C spectra were consistent with the assigned structures.



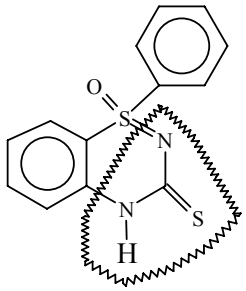
Loviride



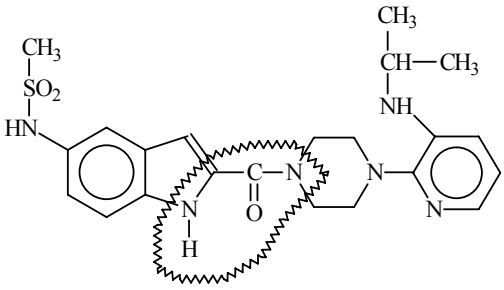
Trovirdine



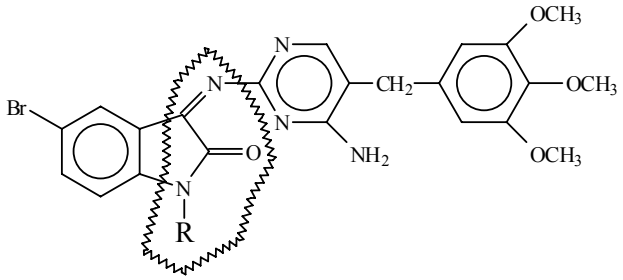
Indole Carboxamide



Benzothiadiazine-1-oxide



Delavirdine



Lead Compound

Figure 1: Existing NNRTIs and Lead Compound

Biological activities

The synthesized compounds were evaluated for their inhibitory effect on the replication of HIV-1 in MT-4 and CEM cell lines (Table 4) [10]. In the MT-4 cell lines, compound **12** and **15** were found to be the most active against replication of HIV-1 with EC_{50} of 5.6 and 7.6 μM respectively and their selectivity index ($SI=CC_{50}/EC_{50}$) was found to be more than 12 with maximum protection of 94-126%. When compared to reference standard Nevirapine (EC_{50} = 0.1 μM) the synthesized compounds were less active. Other compounds (**2**, **3**, **7**, **14**, **and 16**) showed maximum protection of 56%-88% with SI of 2-10. In the T4 lymphocytes (CEM cell lines), the compounds showed marked anti-HIV activity (15-48%) 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. Overall, seven compounds of the 16 new derivatives developed in this work showed inhibition against replication of HIV-1 in MT-4 cells with EC_{50} ranging from 5.6-22.6 μM.

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

COMPOUND	AB (IN Å)		BC (IN Å)		CA (IN Å)	
	LOWER LIMIT	UPPER LIMIT	LOWER LIMIT	UPPER LIMIT	LOWER LIMIT	UPPER LIMIT
DELAVIRDINE	4.328 ± 0.04	6.705 ± 0.15	4.256 ± 0.19	7.542 ± 0.35	9.156± 0.04	9.382± 0.04
TROVIRDINE	4.235 ± 0.01	6.635 ± 0.02	4.289 ± 0.08	7.269 ± 0.16	9.168± 0.04	9.426± 0.04
LOVIRIDE	4.356 ± 0.03	6.709 ± 0.12	4.254 ± 0.06	7.129 ± 0.14	9.132± 0.04	9.368± 0.04
INDOLE CARBOXAMIDE	4.359 ± 0.01	6.705 ± 0.20	4.562 ± 0.14	7.478 ± 0.07	9.125± 0.04	9.434± 0.04
EFAVIRENZ	4.425 ± 0.05	6.689 ± 0.16	4.247 ± 0.23	7.211 ± 0.21	9.145± 0.04	9.440± 0.04
NEVIRAPINE	3.854 ± 0.02	6.538 ± 0.04	4.268 ± 0.31	7.603 ± 0.02	9.215± 0.04	9.421± 0.04
BENZOTHIADIAZINE-1-OXIDE	4.512 ± 0.02	6.459 ± 0.03	4.269 ± 0.14	7.545 ± 0.01	9.129± 0.04	9.406± 0.04
THIOCARBOXANILIDE	4.229 ± 0.04	6.523 ± 0.11	4.223 ± 0.07	7.147 ± 0.39	9.169± 0.04	9.398± 0.04
LEAD COMPOUND	4.235 ± 0.18	6.459 ± 0.12	4.218 ± 0.09	7.547 ± 0.15	9.159± 0.01	9.431± 0.02

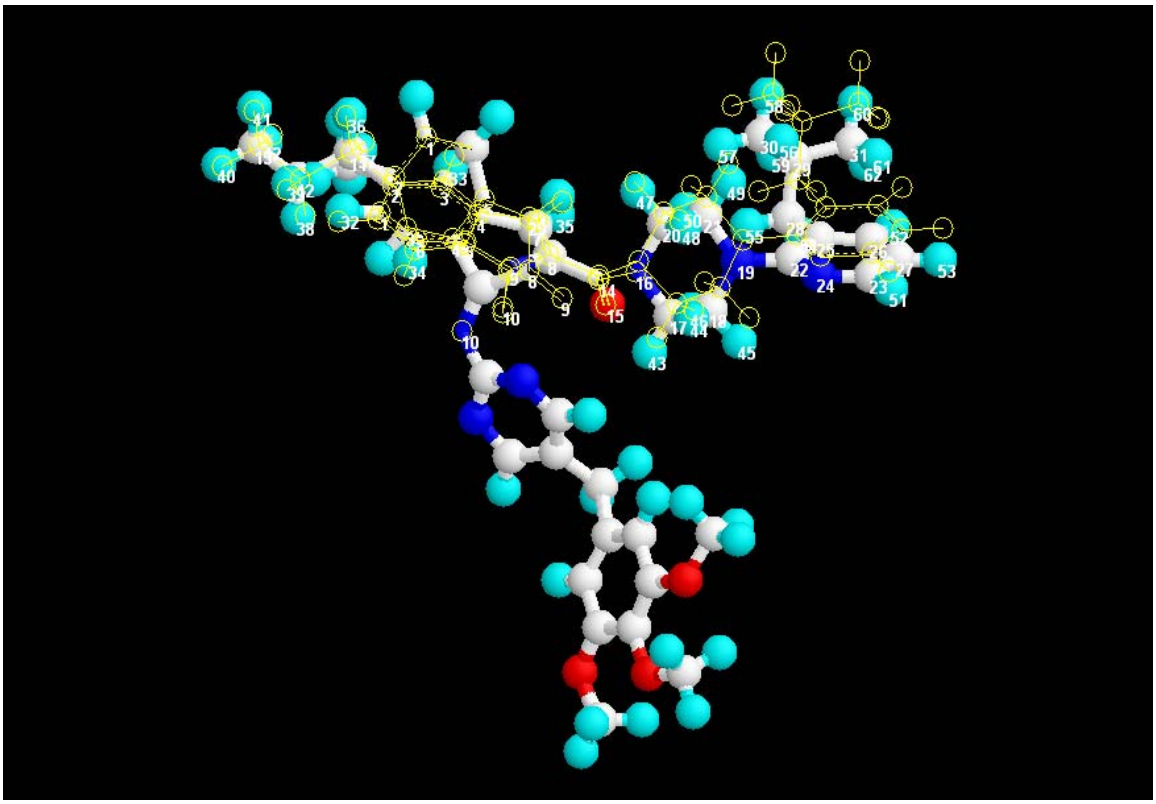
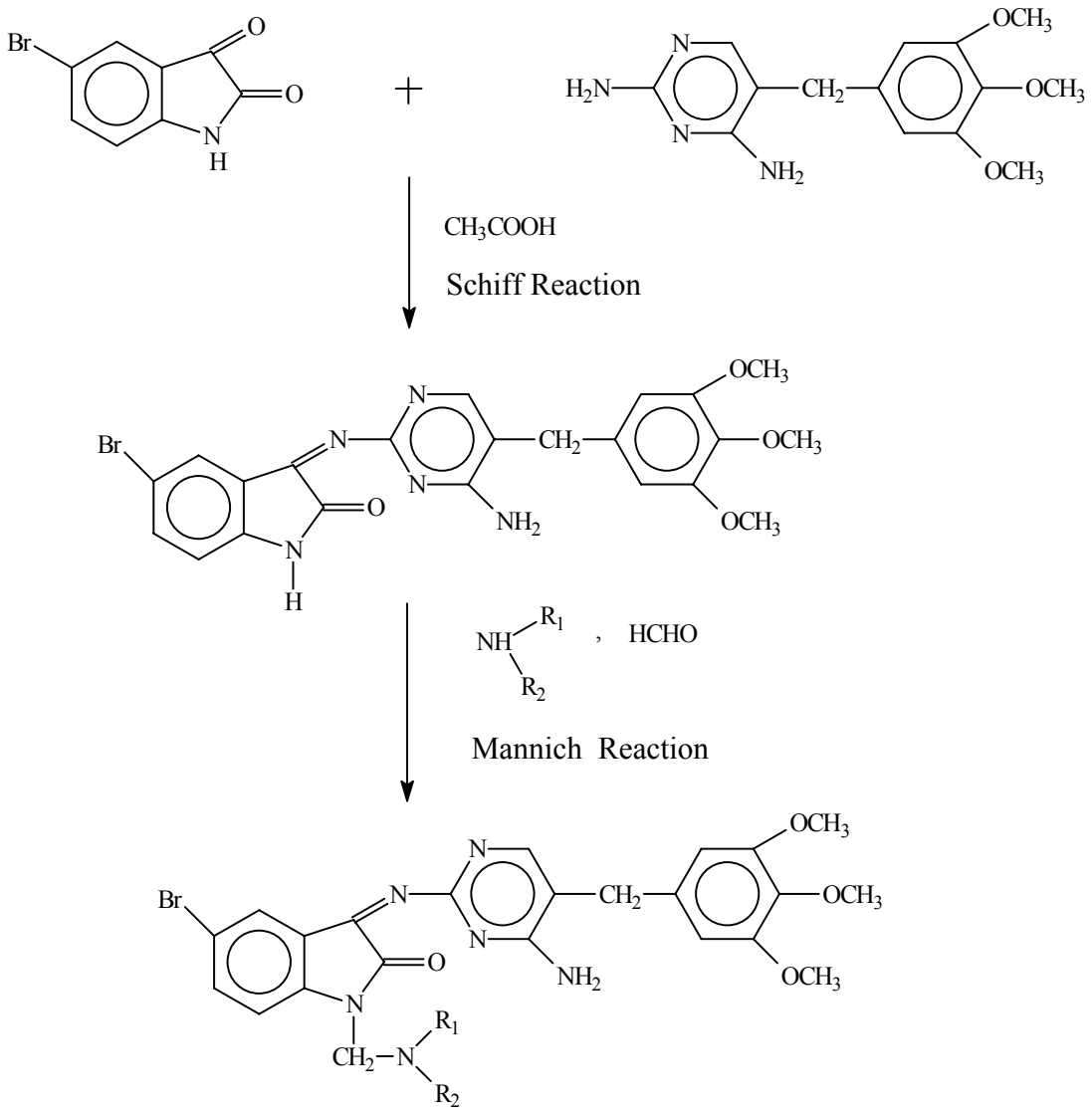


Figure: 3 Superimposition and RMS fit of the proposed lead compound and Delavirdine (RMS value = 0.075)

Two compounds (**14** and **15**) were evaluated for the inhibitory effects on HIV-1 RT enzyme [11] and their IC_{50} values were found to be 28.4 ± 4.4 and 40.2 ± 8.6 µM respectively. 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 [12]. All the synthesized compounds were also evaluated preliminarily for their inhibition of HCV viral RNA replication in Huh-7 cells at 50-µg/ ml [13], and the results are presented in Table 4. Among these, four compounds (**10**, **14**, **15** and **16**) were found to be less toxic to Huh-7 cells (cell growth of > 80%) and inhibited HCV viral RNA replication at about 80-86%. Two compounds (**1** and **13**) inhibited 100% viral replication but they were toxic to Huh-7 cells. Summarizing, twelve compounds were active against HCV RNA replication showing 80% inhibition at 50 µg/ml. This paper is the first of its kind in which isatin derivatives are reported to possess anti- HCV activity.



1-16

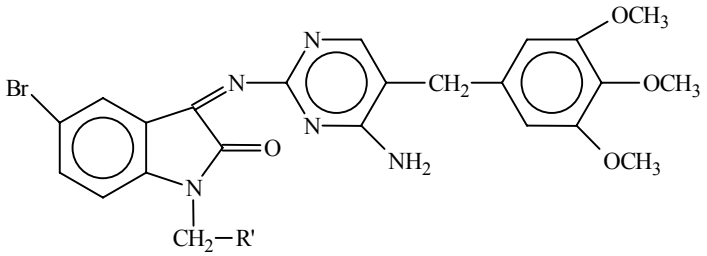
Figure 4. Protocol for the synthetic compounds

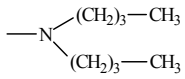
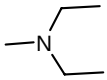
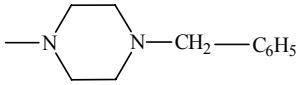
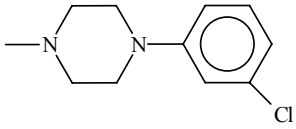
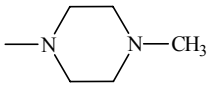
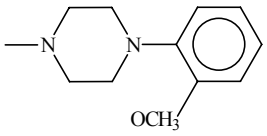
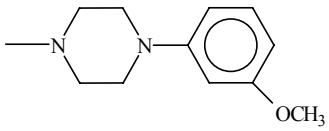
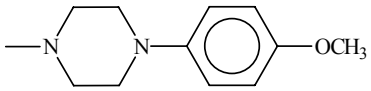
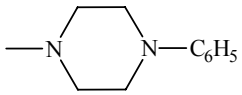
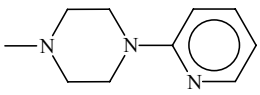
The synthesized compounds were also screened against *M. tuberculosis* strain $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium initially at 6.25 μg/ml (Table 4) [14]. Three compounds (**14**, **15** and **16**) showed complete inhibition (100%) of *M. tuberculosis* in the primary screening. In the secondary 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 up to 62.5 μg/ml to VERO cells.

All the compounds were evaluated for their *in-vitro* antibacterial activity against 24 pathogenic bacteria by conventional agar dilution procedures [15] and the results of these assays are summarized in Table 5 and 6.

The data for Ciprofloxacin, Lomefloxacin and Ggatifloxacin were included for comparison. The antibacterial 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 *S sonnei*, *Vibrio mimicus, V. flurialis, V. cholerae*0139, *V. parahaemolyticus* and *Citrobacter ferundii* as these compounds inhibited them at a concentration less than 50 μM.

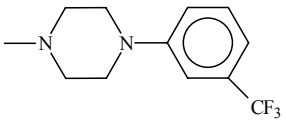
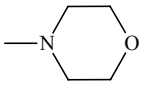
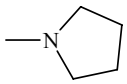
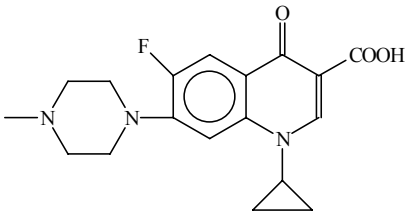
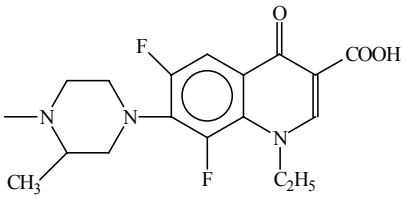
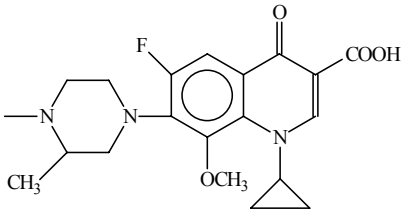
Table 2. Physical constants of the synthesized compounds 1-10



Compound	R'	Molecular Formula	Molecular Weight	Yield (%)	M.P. (°C)
1		$C_{31}H_{39}N_6O_4Br$	639.58	69.10	104
2		$C_{27}H_{31}N_6O_4Br$	583.49	70.26	68
3		$C_{34}H_{36}N_7O_4Br$	686.60	75.10	87
4		$C_{33}H_{33}N_7O_4ClBr$	707.01	62.82	84
5		$C_{28}H_{32}N_7O_4Br$	610.50	65.12	85
6		$C_{34}H_{36}N_7O_5Br$	702.59	69.50	117
7		$C_{34}H_{36}N_7O_5Br$	702.59	72.10	82
8		$C_{34}H_{36}N_7O_5Br$	702.59	70.80	83
9		$C_{33}H_{34}N_7O_4Br$	672.57	71.21	130
10		$C_{32}H_{33}N_8O_4Br$	673.56	71.56	76

Compound **7** which contain 3-methoxyphenyl piperazinomethyl moiety at N-1 position was found to be the most active compound that was more potent than lomefloxacin against *K. ozaenae, S. sonnei, V. flurialis, V. cholerae*0139*, V. parahaemolyticus, E. coli* NCTC 10418*, E tarda, P mirabilis, S. typhi, S. enteritidis, C. ferundii, enterobacter and B. megatherius*. Compound **14**, containing a ciprofloxacin moiety at N-1 position was found to be more active than ciprofloxacin against 17 tested bacteria. When compared to Llomefloxacin, compound **15** (Lomefloxacin derivative) was found to be more active against 23 tested bacteria. Compound **16** bearing Gatifloxacin at N-1 position was found to be more active than Gatifloxacin against 15 tested bacteria. These data are in consistent with our earlier results [16].

Table 3. Physical constants of the synthesized compounds 11-16

Compound	R'	Molecular Formula	Molecular Weight	Yield (%)	M.P. (°C)
11		$C_{34}H_{33}N_7O_4F_3Br$	740.57	66.12	146
12		$C_{27}H_{29}N_6O_5Br$	597.46	65.50	88
13		$C_{27}H_{29}N_6O_4Br$	581.46	64.82	94
14		$C_{40}H_{38}N_8O_7FBr$	841.68	76.15	222
15		$C_{40}H_{39}N_8O_7F_2Br$	861.68	76.50	257
16		$C_{42}H_{42}N_8O_8FBr$	885.73	75.86	137

In-vivo antibacterial activity of some selected compounds against an experimentally induced infection of mice after oral administration [16] are presented in Table 7, 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 3 times more active (ED_{50}: 0.46 mg/kg body weight) than Ciprofloxacin (ED_{50}: 1.25 mg/kg) while compound **15** was equally active as Lomefloxacin with ED_{50} of 1.87 mg/kg against the tested bacteria. Thus, four compounds showed very good activity against various pathogenic bacteria, and among the synthesized compounds, compound **14** and **15** emerged as more promising broad-spectrum chemotherapeutic agents. The more activity of the synthesized compounds might be due to the dual inhibition of bacterial enzymes dihydrofolate reductase (trimethoprim nucleus) and DNA gyrase (fluoroquinoline nucleus).

ACKNOWLEDGEMENTS

The authors acknowledge M/S VenkarChem, Hyderabad (India) and Sun Pharmaceutical, Baroda (India) for providing gift samples of Trimethoprim, Ciprofloxacin, Lomefloxacin and Gatifloxacin. One of the authors Ms. Tanushree Bal deeply acknowledges the Council of Scientific and Industrial Research, INDIA, for providing a Senior Research Fellowship. The authors also thank The authors also thank Dr. G. Nath, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varansi-221005 INDIA, Dr. Robert H. Shoemaker, National Cancer Institute, USA and Dr. Sam Ananthan, TAACF, Southern Research Institute, USA for their support in biological testing.

Table 4. Anti-HIV, anti-HCV and antimycobacterial activity

Compound	Anti-HIV activity (µM)						Anti-HCV activity at 50 µg/ml		Antimycobacterial 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	> 36.1	36.1	29.6	NT	NT	NT	11	100	68
2	19.2	62.6	72.6	> 42.8	42.8	39.93	84	76	46
3	7.8	79.1	88.6	> 74.0	74.0	42.40	62	74	NT
4	> 41.6	41.0	29.1	> 37.2	37.2	29.73	70	80	11
5	> 49.6	49.6	38.1	> 45.3	45.3	38.30	83	76	62
6	> 47.1	47.1	26.2	> 45.3	45.3	38.30	83	45	60
7	22.6	46.4	62.6	> 42.2	42.2	38.39	54	94	57
8	> 59.1	59.1	36.1	> 54.4	54.4	22.28	60	83	NT
9	> 46.2	46.2	31.6	> 36.3	36.2	20.36	62	96	49
10	> 69.7	69.7	26.6	> 55.9	55.9	21.38	88	84	38
11	> 61.6	61.6	20.1	NT	NT	NT	51	98	69
12	5.6	72.6	126	> 55.1	55.1	48.48	62	80	6
13	> 96.2	96.2	39.6	> 123	123.0	22.24	59	100	10
14	12.3	64.6	68.4	NT	NT	NT	88	81	100
15	7.6	92.1	93.6	> 81.9	81.9	35.73	80	80	100
16	19.2	34.6	56.6	NT	NT	NT	86	80	100

NT indicates not tested
a50% Effective concentration, or concentration required to inhibit HIV-1 induced cytopathicity cell lines by 50%.
b50% Cytotoxic concentration, or concentration required to reduce the viability of mock-infected cell lines by 50%.

Table 5. In vitro antibacterial activity (MICs in µM)

Microorganism	1	2	3	4	5	6	7	8	9	10
K. ozaenae	39.00	42.9	18.2	17.7	0.64	17.8	0.009	0.555	4.64	1.16
K. pneumoniae	39.00	42.9	18.2	17.7	5.12	17.8	0.555	17.8	18.6	18.6
S. sonnei	39.00	42.9	18.2	17.7	0.64	1.11	0.278	0.278	0.0091	0.0091
Plesiomonas	39.00	42.9	0.569	2.21	0.64	17.8	0.555	0.278	18.6	18.6
S. boydii	39.00	42.9	18.2	17.7	10.2	17.8	1.11	17.8	37.2	18.6
M. morganii	39.00	42.9	18.2	17.7	20.5	17.8	1.11	35.6	37.2	18.6
S. aureus	39.00	42.9	18.2	17.7	20.5	17.8	1.11	0.555	2.32	1.16
P. aeroginosa	39.00	42.9	36.4	17.7	20.5	35.6	0.278	35.6	37.2	74.2
V. mimicus	2.44	1.34	0.569	0.0086	0.32	0.0086	0.139	0.0086	0.0091	0.0091
V. fluvialis	2.44	2.68	0.009	0.0086	0.32	0.0086	0.0086	0.0086	0.00907	0.0091
V. cholerae 0139	2.44	0.021	0.009	0.0173	0.32	0.0086	0.0086	0.0086	0.00907	0.0091
V. cholerae 01	1.22	0.0048	2.28	1.10	1.28	2.22	0.0086	0.278	1.16	0.145
V. parahaemolyticus	2.44	2.68	1.14	2.21	2.56	1.11	0.0086	0.0086	0.58	1.16
E. Coli NCTC10418	39.00	42.9	18.2	17.7	20.5	17.8	0.0086	35.6	37.2	18.6
E. tarda	39.00	42.9	36.4	17.7	20.5	8.89	0.139	0.139	37.2	18.6
P. vulgaris	39.00	42.9	18.2	17.7	20.5	8.89	0.278	2.22	37.2	18.6
P. mirabilis	39.00	42.9	18.2	17.7	20.5	17.8	0.278	71.1	37.2	18.6
S. typhimurium	39.00	42.9	18.2	8.84	20.5	35.6	8.89	71.1	37.2	18.6
S. paratyphi A	39.00	10.7	18.2	17.7	2.56	2.22	0.139	2.22	0.29	0.58
S. typhi	39.00	42.9	18.2	17.7	20.5	17.8	0.009	71.1	37.2	18.6
S. enteritidis	19.5	42.9	18.2	17.7	20.5	0.0086	0.139	2.22	9.29	4.64
C. ferundii	0.019	0.61	2.28	2.21	0.64	1.11	0.278	2.22	0.29	0.58
Enterobacter	0.0381	42.9	0.569	8.84	10.2	1.11	0.0086	8.89	4.64	4.64
B. megatherius	19.5	42.9	18.2	17.7	10.2	0.0086	0.0086	0.278	18.6	9.28

Table 6. In vitro antibacterial activity (MIC's in μM)

Microorganism	11	12	13	14	15	16	Cipro	Lome	Gati
K. ozaenae	0.527	20.9	21.5	0.0290	0.0002	0.0017	0.0092	0.0629	0.0037
K. pneumoniae	16.9	20.9	21.5	0.0002	0.0035	0.0138	0.0023	0.1259	0.0037
S. sonnei	0.0082	20.9	21.5	0.00003	0.0035	0.0017	0.0023	2.0156	0.0037
Plesiomonas	16.9	20.9	21.5	0.0036	0.0002	0.0035	0.0023	0.0629	0.1182
S. boydii	33.8	20.9	43.0	0.0002	0.0002	0.0008	0.0023	0.5039	0.0590
M. morganii	33.8	20.9	43.0	0.0002	0.0071	0.0008	0.0023	0.0629	0.0009
S. aureus	1.05	20.9	43.0	0.0004	0.0071	0.0008	0.0023	0.0314	0.0009
P. aeroginosa	67.5	2.62	86.0	0.0004	0.0071	0.0278	0.0092	0.2519	0.0074
V. mimicus	0.0082	0.0102	0.0105	0.0290	0.0002	0.0017	0.0023	0.0629	0.0074
V. fluvialis	0.0082	1.31	1.31	0.0004	0.0566	0.0008	0.0023	0.0629	0.0009
V. cholerae 0139	0.0082	0.0102	0.0105	0.0290	0.0002	0.0017	0.0023	0.1259	0.0009
V. cholerae 01	0.0082	0.0102	0.0105	0.0290	0.0566	0.0069	0.0023	0.0009	0.0009
V. parahaemolyticus	0.527	0.327	0.672	0.0004	0.0018	0.0035	0.0023	2.0156	0.4727
E. Coli NCTC10418	33.8	20.9	86.0	0.0004	0.0035	0.0017	0.0011	0.0314	0.0009
E. tarda	16.9	20.9	43.0	0.0004	0.0071	0.0035	0.0023	0.2519	0.0009
P. vulgaris	16.9	20.9	43.0	0.0004	0.0002	0.0008	0.0023	0.0314	0.0009
P. mirabilis	16.9	20.9	43.0	0.0004	0.0002	0.0035	0.0023	0.1259	0.0009
S. typhimurium	16.9	20.9	43.0	0.0002	0.0566	0.0035	0.0023	0.2519	0.0009
S. paratyphi A	4.22	20.9	21.5	0.0036	0.0142	0.0069	0.0023	0.0314	0.0009
S. typhi	16.9	20.9	21.5	0.0002	0.00002	0.0004	0.0023	0.5039	0.0009
S. enteritidis	8.44	0.654	21.5	0.0002	0.00002	0.0008	0.0023	1.0078	0.0009
C. ferundii	0.527	2.62	1.34	0.0290	0.0071	0.0002	0.0023	1.0078	0.0037
Enterobacter	0.264	20.9	21.5	0.0002	0.0035	0.0004	0.0023	1.0078	0.0009
B. megatherius	16.9	20.9	21.5	0.0002	0.00002	0.0004	0.0023	1.0078	0.0037

Table 7. In vitro antibacterial study on *E.coli* NCTC 10419 strain

Compound	*In Vitro* MIC (in μM / ml)	*In Vivo* EC^{50} (in mg / Kg body wt.)
14	0.0004	0.46
15	0.0035	1.87
Ciprofloxacin	0.0011	1.25
Lomefloxacin	0.0314	1.87

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