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Synthesis and Biological Evaluation of N-(4-Fluorophenyl)-6-Methyl-2-Oxo-1, 2, 3, 4-Tetrahydropyrimidine-5-Carboxamides as HIV Integrase Strand Transfer Inhibitors

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ABSTRACT

HIV-1 integrase (IN) catalyzes chromosomal integration of synthesized viral DNA into host DNA by performing two independent reactions, 3'-processing (3'-P) and strand transfer (ST). In the present study, we report synthesis and evaluation of N-(4-fluorophenyl)-6-methyl-2-oxo-4-substituted phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxamides for IN inhibitory activity. All the derivatives were found to inhibit strand transfer reaction in vitro in isolated enzyme assay and most active compound (13e) showed IC50 value of 0.65 µM. Docking studies were also done to justify the IN inhibition and in vitro-in silico correlation was drawn. However, these compounds did not show HIV-1 and HIV-2 inhibition below their cytotoxic concentration in cell culture assay indicating that these compounds cannot be used as lead for anti-HIV activity.

Keywords: HIV-1 integrase, Docking studies, Anti-HIV, Strand transfer inhibitors, Pyrimidine-5-carboxamide.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) is a worldwide health problem with an estimated 39.4 million people infected patients in 2014 [1]. The genome of HIV is encoded as RNA and three enzymes viz. reverse transcriptase (RT), protease (PR) and integrase (IN) play essential role in its replication. The viral life cycle begins with binding of glycoprotein 120 (gp 120) to a CD4 cell receptor on the host cell surface. After attachment, viral RNA is transcribed by RT to synthesize double-stranded DNA (dsDNA). The dsDNA then forms the pre-integration complex (PIC) of which HIV-1 integrase (IN) is a part. IN performs two independent steps: 3′-processing (3′-P) and strand transfer (ST). In 3′-P, IN eliminates a 3′ terminal portion (usually a dinucleotide) at both ends of HIV DNA followed by transportation into nucleus of host cell where insertion and covalent ligation of viral DNA into host genome occurs as a part of strand transfer (ST) reaction. The integrated viral DNA is now referred to as provirus DNA, which works as a template for the formation of new viral RNA *via* transcription process. After transcription, newly formed viral RNA having code for synthesis of viral proteins and enzymes, moves out of the infected cell's nucleus and translated into polypeptide chains, which further form the protein and enzyme components. These newly constructed proteins and enzymes are assembled at plasma membrane to produce complete virions, which are released by cell disintegration [2,3]. To date, nearly 28 anti-retroviral drugs targeting distinct phases of viral replication cycle have been approved for treatment of HIV-1. Most of these target viral fusion, reverse transcription catalyzed by RT and proteolytic maturation step by proteases. To overcome viral resistance, which is a major problem in anti-HIV therapy, triple therapy or highly active antiretroviral therapy (HAART) is the usual treatment for HIV-1 infection. The egressions of multi-drug resistant viral strains, along with toxicity effects, and long-term complicated dosing have rendered the current therapy ineffective [4].

IN has been shown to be a potential target for the management of AIDS, with the entry of raltegravir (MK-0518), elvitegravir (GS-9137) and dolutegravir (GSK- 1349572) in the clinic [5,6]. Moreover, due to absence of HIV-1 IN homologue in human cells, it is an attractive target for development of new anti HIV therapy [7]. It is reported that there are two separate cavities in IN, namely cavity 3P and cavity ST, which are perpendicular to each other. The first cavity ST covers the area of the flexible loop (Phe 139–Ile 151, part of α_4 helix) and pocket is made up of hydrophilic amino acids Gln146, Ser147, Gln148 and hydrophobic amino acids Ile141, Pro142, Tyr143, Pro145, Val150. The cavity 3P is made of amino acids Asp64, Cys65, Thr66, His67, Glu92, Asp116 and Asn120. Here, Asp64 and Asp116 could chelate with Mg^{2+}, which is important for 3′-processing activity [8,9].

Previously, a number of IN inhibitors have been discovered belonging to different chemical classes such as arylamides, aurintricarboxylic acids, catechols, curcumin derivatives, diarylsulfones, depsides, flavones, integrinic acid derivatives, lignanolides, nucleotides analogs, styrylquinoline derivatives, salicylhydrazides, tyrphostins, thiazolothiazepines, triazine derivatives, tetracyclines and naturally extracted derivatives. Although the structures of natural product inhibitors are complicated, small molecule inhibitors have always been in the focus [1,9-11]. Diketo or catechol compounds have been reported to be cytotoxic and hence could not be taken forward [12,13]. There is need to search new leads belonging to new classes of strand transfer inhibitors for the treatment of HIV-1 infection. Several derivatives such as MK-2048, GS9160, PICA, and MK-0536 are in clinical phases (Figure 1) [1,9].

Tetrahydropyrimidines have been extensively evaluated for muscarinic, anti-inflammatory, antiviral, anticancer and acetyl cholinesterase inhibitory activity [14-19]. Recently, antiretroviral potential of tetrahydropyrimidine analogues having 2-chlorophenyl group has been reported [20]; wherein one of the compound *N*-(4-fluorophenyl)-6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxamide (**8**) was found inactive but its bromo analogue ie. *N*-(4-bromophenyl)-6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxamide was found to be active [21]. Previous reports indicate that diketo function imparts cytotoxicity [12,13] and clinically approved as well as some HIV IN inhibitors having potent IN inhibition possess *p*-fluoro phenyl (aromatic ring) feature on a heterocyclic ring along with carboxamide linkage. The *p*-fluoro phenyl is reported to penetrate the active site pocket vacated by 3′ adenine and forms a more favorable π-stacking interaction with the base of nucleotide [22,23]. Compound (**8**) lacked diketo feature and had *p*-fluoro phenyl (aromatic ring) feature separated by carboxamide linkage from tetrahydropyrimidone ring. Therefore, this structural similarity with integrase inhibitors prompted us to explore compound (**8**) further. The 2-tetrahydropyridinone nucleus of compound (**8**) was not changed but modifications were done by changing substitution pattern on 4-phenyl ring and its effect on integrase inhibition was evaluated.

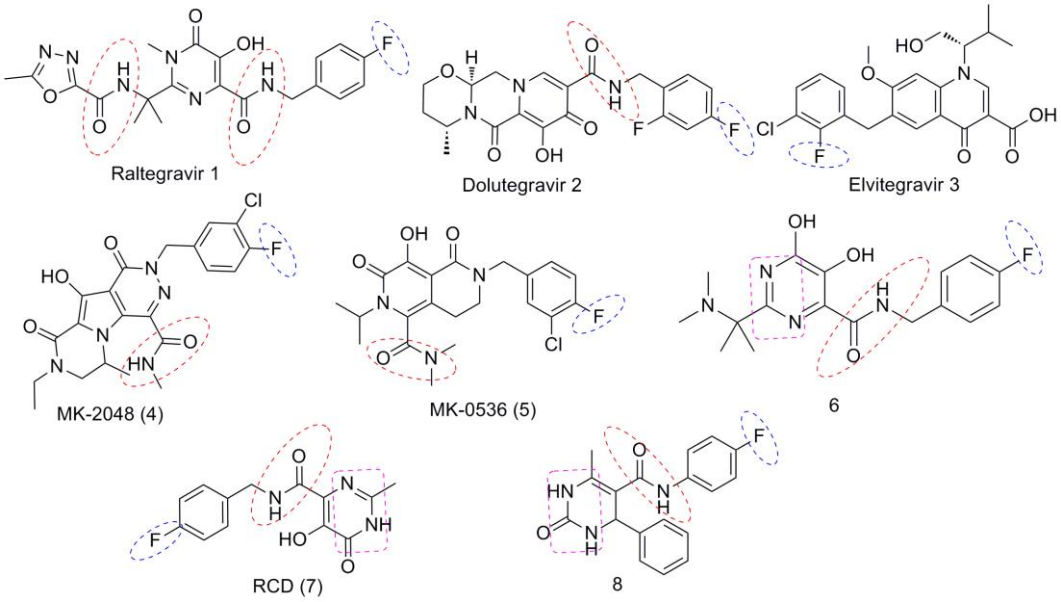


Figure 1: Representative HIV-1 IN inhibitors (1-8).

EXPERIMENTAL PROCEDURE

Chemistry

Chemicals used in synthetic work were purchased from Spectrochem Pvt Ltd, Mumbai, SD Fine Chem Limited, Mumbai and Sigma Aldrich, Mumbai. All the solvents used were of analytical grade without further purification. Analytical TLC was carried out with plates precoated with silicagel 60 F_{254} (0.25 mm thick) by using mobile phase ethyl acetate and hexane in suitable portion. Melting points were determined in open capillary tubes on a Precision Buchi B530 (Flawil, Switzerland) apparatus containing silicon oil. The IR spectra of the synthesized compounds were recorded using FTIR spectrophotometer (Shimadzu IR

Prestige 21, Shimadzu, Mumbai, India). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DPX-400 spectrometer (Bruker India Scientific Pvt. Ltd., Mumbai, India) using tetramethylsilane (TMS) as an internal standard (chemical shifts indicated in δ). ESI-MS were recorded on MICROMASS Quattro-II LCMS system (Waters Corporation, Milford, USA).

Synthesis of N-(4-fluorophenyl)-3-oxobutanamide (11)

To a two-necked 100 ml RBF fitted with condenser, was added ethylacetoacetate 9 (12.75 ml, 0.10 mol) in xylene (30 ml) and pyridine (1 ml) under nitrogen atmosphere. The reaction mass was gently refluxed for 0.5 h, 4-fluoroaniline **10** (10 ml, 0.10 mol) was then added drop wise and further refluxed for 8 h, with removal of distillate through a Dean-Stark tube. The progress of reaction was monitored by TLC. After completion, the mixture was cooled to room temperature (RT) and extracted with 2M solution of NaOH solution (50 ml). The aqueous layer was separated and made weakly acidic with concentrated HCl. The resulting precipitate was collected by filtration, washed with water, and dried to give the desired product as white crystals.

Yield 63%; Mp: 98–100°C; ^{1}H-NMR (DMSO-D_6): δ 2.20 (s, 2H), 3.39 (s, 3H), 7.17 (t, 2H, J = 8.4 Hz), 7.71 (t, 2H), 10.16 (s, 1H); ^{13}C-NMR (DMSO-D_6) 30.65, 52.67, 115.67, 115.89, 135.74, 157.30, 159.68 and 203.40; EI-MS (*m*/*z*): 196.04 (M+H).

Synthesis of N -(4-fluorophenyl)-6-methyl-2-oxo-4-substituted-phenyl-1,2,3,4-tetrahydro pyrimidine-5-carboxamides (8, 13a-13q)

Three necked RBF was charged with *N*-(4-fluoro-phenyl)-3-oxo-butyramide (**11**) dissolved in 30 ml of ethanol. Then substituted aryl aldehydes (**12a-12r**, 0.01 mol), and urea (0.015 mol) were added and the resultant solution was heated under reflux for 7–9 h in presence of a catalytic amount of concentrated HCl. The progress of reaction was monitored by TLC. After the reaction was complete, mixture was kept overnight and the precipitate obtained was filtered. Products were recrystallized from ethanol and purity was assessed using HPLC. For this purpose, column used was Agilent Extend – C18 (250 × 4.6 mm, 5µ), mobile phase: Water: Methanol (50:50), flow rate of 1 mL/min. Detection was done at 254 nm.

N-(4-fluorophenyl)-6-methyl-2-oxo-4-phenyl-1, 2, 3, 4-tetrahydropyrimidine-5-carboxamide (8)

White solid; yield 71%; Mp: 179-182°C; ^{1}H-NMR (DMSO-D_6): δ 2.65 (s, 3H), 5.75 (s, 1H), 7.14– 7.75 (m, 9H), 7.17 (t, 2H, J = 8.4 Hz), 7.71 (t, 2H), 9.56 (s, 1H); ^{13}C-NMR (DMSO-D_6): 18.44, 53.23, 107.17, 126.01, 126.19, 132.66, 143.91, 146.71, 150.32, 162.14 and 163.61; EI-MS (*m*/*z*): 326.06 (M+H).

N-(4-fluorophenyl)-6-methyl-4-(2-nitrophenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13a)

Yellow solid; yield 68%; Mp: 204-206°C, ^{1}H-NMR (DMSO-D_6): δ 2.58 (s, 3H), 5.30 (s, 1H), 7.10– 7.52 (m, 8H), 9.58 (s, 1H); ^{13}C-NMR (DMSO-D_6): 20.36, 51.11, 108.47, 127.24, 127.37, 131.47, 144.01, 152.42, 167.23, 163.22; EI-MS (*m*/*z*): 371.14 (M+H).

N-(4-fluorophenyl)-6-methyl-4-(3-nitrophenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13b)

Yellow solid; yield 75%; Mp: 201-203°C, ^{1}H-NMR (DMSO-D_6): δ 1.98 (s, 3H), 5.71 (s, 1H), 6.30– 7.10 (m, 8H), 8.21 (s, 1H); ^{13}C-NMR (DMSO-D_6): 2.40, 58.30, 104.74, 109.52, 119.52, 119.74,126.72, 126.79, 134.39, 144.64, 151.06, 153.08, 170.27; EI-MS (*m*/*z*): 371.21 (M+H); 369.16 (M-H).

N-(4-fluorophenyl)-6-methyl-4-(4-nitrophenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13c)

Yellow solid; yield 71%; Mp: 203-205°C, ^{1}H-NMR (DMSO-D_6): δ 2.32 (s, 3H), 5.41 (s, 1H), 7.19– 7.56 (m, 8H), 9.41 (s, 1H); ^{13}C-NMR (DMSO-D_6): 19.41, 52.74, 107.20, 126.29, 127.01, 132.52, 143.94, 151.81, 166.14, 170.06; EI-MS (*m*/*z*): 371.10 (M+H).

4-(2-chlorophenyl)-N-(4-fluorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13d)

Yellow creamish solid; yield 73%; Mp: 211-213°C, ^{1}H-NMR (DMSO-D_6): δ 3.36 (s, 3H), 5.32 (s, 1H), 7.06– 8.20 (m, 8H), 10.23 (s, 1H); ^{13}C-NMR (DMSO-D_6): 19.45, 50.74, 108.21, 127.42, 127.53, 131.26, 135.38, 150.42, 165.03, 167.39; EI-MS (*m*/*z*): 360.10 (M+H).

4-(4-chlorophenyl)-N-(4-fluorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13e)

Light yellow solid; yield 72%; Mp: 211-213°C, ^{1}H-NMR (DMSO-D_6): δ 2.51 (s, 3H), 5.45 (s, 1H), 7.30– 7.72 (m, 8H), 10.0 (s, 1H); ^{13}C-NMR (DMSO-D_6): 9.65, 53.04, 108.57, 115.66, 115.72, 126.11, 126.24, 126.34, 126.53, 133.26, 133.38, 141.25, 146.25, 150.72, 162.03,163.42; EI-MS (*m*/*z*): 360.12 (M+H).

4-(2,4-dichlorophenyl)-N-(4-fluorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13f)

Brown solid; yield 70%; Mp: 237-240°C, ^{1}H-NMR (DMSO-D_6): δ 2.38 (s, 3H), 5.49 (s, 1H), 7.24 – 7.51 (m, 7H), 9.60 (s, 1H); ^{13}C-NMR (DMSO-D_6): 21.34, 52.81, 108.04, 116.14, 116.14, 126.44, 126.82, 127.01, 133.17, 133.64, 140.84, 146.24, 150.18, 162.18, 163.54; EI-MS (*m*/*z*): 409.06 (M-H).

N-(4-fluorophenyl)-4-(2-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13g)

Dark brown solid; yield 69%; Mp: 207-210°C, ^{1}H-NMR (DMSO-D_6): δ 3.30 (s, 3H), 5.39 (s, 1H), 7.16– 8.04 (m, 8H), 10.04 (s, 1H); ^{13}C-NMR (DMSO-D_6): 20.15, 52.04, 108.29, 126.18, 127.40, 130.47, 135.66, 150.77, 163.64, 165.07; EI-MS (*m*/*z*): 342.21 (M+H), 340.12 (M-H).

N-(4-fluorophenyl)-4-(4-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13h)

Yellow solid; yield 70%; Mp: 211-214°C, ^{1}H-NMR (DMSO-D_6): δ 3.32 (s, 3H), 5.47 (s, 1H), 7.21– 8.22 (m, 8H), 9.54 (s, 1H); ^{13}C-NMR (DMSO-D_6): 19.45, 53.45, 108.32, 124.77, 128.67, 130.11, 136.01, 150.11, 162.14, 165.07; EI-MS (*m*/*z*): 342.19 (M+H).

N-(4-fluorophenyl)-4-(4-methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13i)

White solid; yield 73%; Mp: 211-214°C, ^{1}H-NMR (DMSO-D_6): δ 3.36 (s, 3H), 3.71 (s, 3H), 5.36 (s, 1H), 6.90– 7.57 (m, 8H), 8.72 (s, 1H), 9.59 (s, 1H); ^{13}C-NMR (DMSO-D_6): 17.50, 54.90, 55.52, 60.23, 114.25, 115.39, 115.61, 121.76, 127.93, 152.95, 158.97, 165.69; EI-MS (*m*/*z*): 356.21 (M+H), 354.11 (M-H).

4-(3,4-dimethoxyphenyl)-N-(4-fluorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13j)

White solid; yield 68%; Mp: 285-287°C, ^{1}H-NMR (DMSO-D_6): δ 2.82 (s, 3H), 3.85-3.87 (s, 6H), 5.46 (s, 1H), 7.17-7.79 (m, 7H), 9.51 (s, 1H); ^{13}C-NMR (DMSO-D_6): 18.09, 53.74, 55.59, 103.81, 104.74, 108.58, 115.04, 115.08, 126.11, 126.58, 131.47, 132.87, 134.58, 141.55, 144.57, 146.58, 160.47, 162.24; EI-MS (*m*/*z*): 386.16 (M+H).

4-(2,5-dimethoxyphenyl)-N-(4-fluorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13k)

Light yellow solid; yield 69%; Mp: 281-283°C, ^{1}H-NMR (DMSO-D_6): δ 2.89 (s, 3H), 3.83-3.85 (s, 6H), 5.67 (s, 1H), 7.20-7.71 (m, 7H), 9.51 (s, 1H); ^{13}C-NMR (DMSO-D_6): 17.62, 54.58, 56.45, 104.55, 105.54, 107.87, 114.54, 115.97, 125.81, 126.23, 130.17, 133.58, 135.71, 140.18, 143.80, 145.88, 161.33, 163.04; EI-MS (*m*/*z*): 386.22 (M+H), 384.12 (M-H).

N-(4-fluorophenyl)-6-methyl-2-oxo-4-(3,4,5-trimethoxyphenyl)-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13l)

Yellowish white solid; yield 69%; Mp:184-187°C, ^{1}H-NMR (DMSO-D_6): δ 2.42 (s, 3H), 3.82-3.84 (s, 9H), 5.61 (s, 1H), 7.14-7.61 (m, 6H), 9.62 (s, 1H); ^{13}C-NMR (DMSO-D_6): 18.04, 56.48, 56.74, 60.47, 104.14, 106.57, 106.05, 113.57, 115.90, 126.01, 131.47, 132.88, 134.27, 138.17, 141.88, 144.87, 160.54, 162.98; EI-MS (*m*/*z*): 416.08 (M+H).

N-(4-fluorophenyl)-4-(4-hydroxy-3,5-dimethoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro pyrimidine-5-carboxamide (13m) Creamish yellow solid; yield 72%; Mp:199-201°C, ^{1}H-NMR (DMSO-D_6): δ 3.32 (s, 3H), 3.84-3.86 (s, 6H), 4.48 (s, 1H), 5.46 (s, 1H), 7.15-7.70 (m, 6H), 9.62 (s, 1H); ^{13}C-NMR (DMSO-D_6): 17.91, 54.24, 56.39, 104.31, 104.64, 109.28, 115.33, 115.64, 125.47, 125.97, 132.44, 133.04, 136.03, 144.25, 146.45, 148.97, 162.41, 163.92; EI-MS (*m*/*z*): 400.22 (M-H).

N-(4-fluorophenyl)-4-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro pyrimidine-5-carboxamide (13n) White solid; yield 68%; Mp: 204-206°C, ^{1}H-NMR (DMSO-D_6): δ 3.32 (s, 3H), 3.84 (s, 3H), 4.56 (s, 1H), 5.40 (s, 1H), 7.04-7.66 (m, 7H), 9.48 (s, 1H); ^{13}C-NMR (DMSO-D_6): 19.30, 55.04, 56.34, 107.23, 112.28, 115.25, 116.64, 126.76, 133.64, 137.93, 145.05, 148.05, 164.20; EI-MS (*m*/*z*): 370.12 (M-H).

4-(3-ethoxy-4-hydroxyphenyl)-N-(4-fluorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro pyrimidine-5-carboxamide (13o) Yellowish brown solid; yield 70%; Mp: 209-211°C, ^{1}H-NMR (DMSO-D_6): δ 0.95 (s, 3H), 2.25 (s, 3H), 2.84 (s, 2H), 4.33 (s, 1H), 5.40 (s, 1H), 6.50-7.50 (m, 7H), 9.50 (s, 1H); ^{13}C-NMR (DMSO-D_6): 15.24, 18.44, 54.34, 63.03, 108.01, 111.28, 115.66, 115.71, 115.92, 125.06, 126.34, 133.97, 136.93, 144.25, 146.05, 146.25, 148.44, 150.65, 162.58, 163.90; EI-MS (*m*/*z*): 386.22 (M+H), 384.17 (M-H).

N-(4-fluorophenyl)-4-(1H-indol-3-yl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13p)

White solid; yield 69%; Mp: 208-210°C, ^{1}H-NMR (DMSO-D_6): δ 2.34 (s, 3H), 5.32 (s, 1H), 7.01-7.83 (m, 8H), 9.27 (s, 1H),10.03 (s, 1H); ^{13}C-NMR (DMSO-D_6):18.71, 55.17, 107.14, 111.37, 111.08, 114.57, 115.33, 118.21, 122.47, 124.58, 126.21, 132.35, 134.01, 143.24, 149.87, 162.57, 163.08; EI-MS (*m*/z): 365.09 (M+H).

N-(4-fluorophenyl)-4-(furan-2-yl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (13q)

Black solid; yield 68%; Mp:178-180°C, ^{1}H-NMR (DMSO-D_6): δ 3.36 (s, 3H), 5.47 (s, 1H), 6.37 (t, 1H), 7.11-7.72 (m, 5H), 8.83 (s, 1H), 9.66 (s, 1H); ^{13}C-NMR (DMSO-D_6): 8.94, 17.63, 45.89, 49.24, 106.03, 110.79, 115.39, 115.61, 121.79, 121.87, 142.93, 150.02, 166.08; EI-MS (*m*/*z*): 316.09 (M+H).

Docking study

The X-ray crystal structure of protein (PDB Code: 1QS4) was retrieved from Protein data bank (PDB) [24]. Target protein was further refined by using protein preparation wizard and 3D energy minimized structures of the designed ligands were prepared using Lig-prep 2.3 module. After Grid generation, the prepared molecules were docked. Validation of docking protocol was done by co-crystallized ligand. The RMSD value obtained by superimposing docked pose over co-crystallized ligand was 0.28, which suggested the reliability of docking protocol. The best-docked poses were visually analyzed for comparing the percentage inhibition values with interactions in the active site.

Biological evaluation

HIV-IN inhibition activity

The HIV-1 IN strand transfer inhibition assay was studied by using kit provided by Xpressbio Life Science Products, USA. Briefly, 100 µL double-stranded HIV-1 LTR U5 donor substrate (DS) DNA was added to the wells of streptavidin-coated 96-well microtiter plates. Following incubation at 37 ºC for 30 min and a wash step, 100 µL purified HIV-1 IN was added onto the pre-processed donor DNA and incubated for 30 min at 37°C. Following a wash step, compounds **8**, **13a–13q** or dolutegravir were titrated into individual wells at a final concentration of 10 µM. The microtiter plates were incubated for 5 min at room temperature, washed and the strand transfer reaction was initiated through the addition of 50 µL double stranded target substrate (TS) DNA. After an incubation period of 30 min at 37°C, the plates were washed and then 100 µL HRP antibody solution per well was added. Finally, the plates were washed and 100 µL TMB peroxidase substrate solution followed by incubation at room temperature for 10 min and 100 µL TMB stop solution was added to allow for detection at 450 nm using a microplate reader. All inhibition values are the average of duplicate experiments [25].

Anti-HIV assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed for evaluating the antiviral activity of the compounds. The principle of MTT assay is based on spectrophotometric measurement of blue-purple formazan produced by reduction of MTT (Acros Organics, Geel, Belgium) by metabolically active cells. The absorbance was read in an eight-channel

computer-controlled photometer (Infinite M1000, Tecan, Mechelen, Belgium), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of three wells.

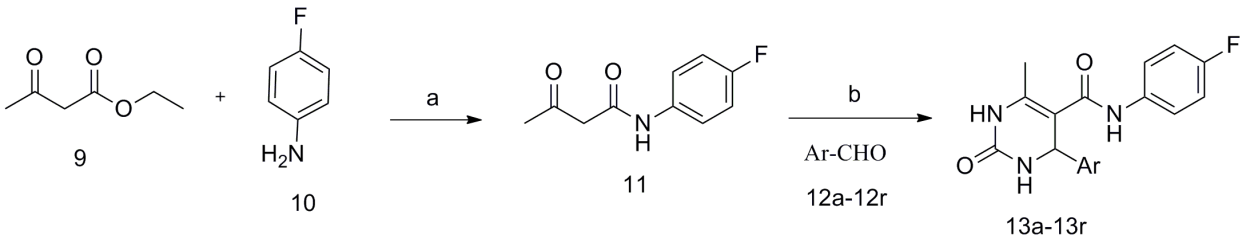
The procedure involved addition of stock solutions (10 x final concentration) of test compounds in 25 µl volumes to two series of triplicate wells for evaluation of their effects on mock- and HIV-infected cells at the start of each experiment. In flat-bottomed 96-well microtiter trays, serial five-fold dilutions of the test compounds were supplemented using Biomek 3000 robot (Beckman instruments, Fullerton, CA). Further, untreated control HIV and mock-infected cell samples followed by HIV-1 (IIIB) or HIV-2 (ROD) stock (50 µl) at 100–300 $CCID_{50}$ (50% cell culture infectious dose) or culture medium were added for each sample. Cytotoxicity effect of test compounds was measured by evaluating mock-infected cells. It involves centrifugation of exponentially growing MT-4 cells for 5 min at 220 g. Then the obtained supernatant was discarded and MT-4 cells were re-suspended at 6 x 10^5 cells/ml and followed by transfer of 50 µl volumes to the microtiter tray wells. After five days of infection, the viability of mock and HIV infected cells were examined spectrophotometrically.

Here, EC_{50} (50% effective antiviral concentration) is defined as the concentration of the tested compound achieving 50% protection from virus-induced cytopathic effect and CC_{50} (50% cytotoxic concentration) was defined as the compound concentration that reduced the viability of mock-infected cells by 50%.

RESULTS AND DISCUSSION

Chemistry

Compounds (**8**, **13a-13q**, Table 1) having different substitution pattern on 4-phenyl ring were synthesized using procedure illustrated in Scheme 1. Ethyl acetoacetate was condensed with 4-fluoro aniline in presence of pyridine to prepare the key intermediate *N*-(4-fluorophenyl)-3-oxobutanamide (compound **11**). Treatment of compound **11** with various substituted aromatic aldehydes (**12a-12r**) and urea in ethanol at 80°C led to the formation of final products **8**, **13a-13q**, in overall yield of 68-75%.



Scheme 1: Reagents and conditions: (a) pyridine, xylene, 8.5 h, reflux (b) urea, hydrochloric acid, 7-9 h, reflux

HIV-1 IN inhibition assay

HIV-1 IN strand transfer inhibition by compounds **8, 13a-13q** was checked by using HIV-1 Integrase Assay kit (Xpressbio Life Science Products, USA) and manufacturer's procedure was followed for the assay. The percentage inhibition of integrase activity of all compounds at 10 µM concentrations and IC_{50} value of few highly active compounds is studied. To substantiate the results obtained, *in silico* docking study was carried out using Glide (maestro version 9.3, Schrödinger suite) in extra precision mode.

The results of *in vitro* evaluation as well as Gscore of designed compounds with dolutegravir as standard are given in Table 1. To extrapolate the results obtained, the synthesized derivatives were further tested for their anti-HIV activity. First, cytotoxicity was studied using MTT based cell viability assay against HeLa cell line and then anti-HIV activity against both HIV-1 (IIIB) and HIV-2 (ROD) at/below their cytotoxic concentration was evaluated. Dolutegravir was used as reference drug. The results of cytoxicity and anti-HIV assays are shown in Table 2.

As mentioned earlier, unsubstituted phenyl ring at 4 position of pyrimidine was used as starting point and effect of various electron donating and electron withdrawing substituents on this ring was checked. Compound **8** having unsubstituted phenyl ring showed hydrogen bonding interactions with Thr 66, Lys 159 (by amidic carbonyl group) and hydrophobic interactions with Asn 155, Lys 156 and Lys 159 were also observed. It did not show interaction with DDE motif and was also least active against IN *in vitro*.

Substitution of electron withdrawing nitro group, either at ortho position (**13a**) or at para position (**13c**), showed hydrophobic interactions with Asp 116, Gln 148 and Glu 152 as well as hydrogen bonding interactions with Thr 66 (*via* carbonyl group of pyridine ring) and Glu 152 (by –NH group of pyridine ring). In addition, **13a** showed hydrogen bonding interactions with Cys 65 (by amidic carbonyl group and *o*-NO_2 group) and His 67 by carbonyl of pyrimidine ring; whereas **13c** displayed hydrogen-bonding interactions Lys 156 and Lys 159 (via *p*-NO_2 group). The% inhibition profile of both compounds was better than **8**, however **13c** was less active than **13a**, which may be attributed to lack of interactions with Cys 65 and His 67. Substitution of nitro group at *meta* position (**13b**) further reduced activity because it did not show interaction with Asp 116, Gln 148 and Glu 152 as well as with Cys 65 and His 67 as compared to *o* & *p* substituted compounds (**13a** and **13c**).

Compounds **13d** and **13e** were substituted with *o*-chloro and *p*-chloro group, respectively and displayed high inhibition; **13d** showed 88.36% and **13e** showed 90.11% inhibition. Analysis of docked poses revealed that amidic carbonyl has hydrogen bonding interaction with Cys 65, carbonyl of pyrimidine ring also showed hydrogen bonding with Thr 66 and His 67; and -NH of pyrimidine formed hydrogen bond with Glu 152. Also, hydrophobic interactions of chlorophenyl ring with Asp 64, Asp 116 and Gln 148 were seen. Moreover, the amidic carbonyl also showed interaction with Mg 1001 and hence these compounds showed very good activity. The *o*-chloro group of **13d** showed hydrophobic interaction with Cys 65 which was not observed in **13e**. But **13e** showed π-π stacking interaction with His 67 which was absent in **13d**. The *p*-chloro group of **13e** appeared to come out of the cavity and hence it showed less docking score. However, **13e** was found to be more active than **13d** *in vitro*. Substitution of chlorine at both ortho and para positions (compound **13f**) showed absence of docking interactions with Asp 64 and Asp 116 (G score -5.21) leading to reduction in Gscore as well as activity as compared to **13d** and **13e**.

Substitution of electron donating hydroxy group at *o* or *p* position (**13g** and **13h**, respectively) showed more than 70% HIV-1 IN inhibition. Compound **13g** showed highest docking score (G Score -7.40) and visual analysis indicated that *o*-hydroxyl group formed hydrogen bonding interactions with Cys 65 and Thr 66 and also showed coordination with Mg1001. The carbonyl of pyrimidine also formed hydrogen bond with His 67 and Lys 159; and amidic carbonyl formed hydrogen bond with Asn 155. Apart from these, hydrophobic interactions were observed with Asp 64, Asp 116, Cys 65, Thr 66, Gln 148, Ile 151, Glu 152, Lys 156 and Lys 159. Substitution of *p*-OH (Compound **13h**) displayed hydrogen-bonding interactions with Cys 65 via –NH of amide and pyrimidine ring and His 67 (via carbonyl group of pyrimidine ring). Hydrophobic interactions were observed with Asp 64, Gln 148 (by *p*-fluoro group), Ile 151, Glu 152, Lys 156 and Lys 159. The *p*-hydroxy group was found to interact with Mg1001.

However, other interactions like **13g** were not seen, which might be reason for less docking score as compare to **13g**. Replacing *p*-OH with *p*-methoxy (**13i**) further reduced the activity as well as docking score because it was observed that the methoxy substituted aryl ring did not enter the active site cavity.

After analyzing the results of **13g**, **13h** and **13i**, next logical step was to check the effect of di- or tri- substitution on activity. Keeping this in mind, compounds **13j** (having 3,4-dimethoxy substitution) and **13k** (having 2,5-dimethoxy substitution) were designed. It was observed that in compound **13j**, there was no improvement in activity although docking score was improved as compared to **13i**. It showed hydrogen bonding interactions with Thr 66 (via 3-methoxy group) and His 67 (by 4-methoxy group) and hydrophobic interactions with Gln 148, Ile 151, Glu 152, Asn 155, Lys 156 and Lys 159. However, compound **13k**, substituted with dimethoxy groups at 2 and 5 positions of aryl ring, displayed similar interactions but the docking score was less and% inhibitory potential was marginally more as compared to **13j**. It displayed hydrogen bonding interactions with Cys 65 (via amidic carbonyl group), Thr 66, His 67 and Glu 152 (via carbonyl and –NH group of pyrimidine ring). Hydrophobic interactions with Asp 64, Glu 92, Gln 148 and Glu 152 were also observed. The π-π stacking between dimethoxy-substituted aryl ring and His 67 were also seen, that might be responsible for better activity as compare to **13j**. When the ring is substituted with 3,4,5-trimethoxy (compound **13l**), it was noted that there was no interaction with DDE motif although hydrogen bonding interactions with Cys 65 (via carbonyl of amide) and Thr 66 (by methoxy) and also hydrophobic interactions with His 67, Asn 155 and Lys 156 were seen. It reduced the inhibitory activity drastically. Since compound **13h**, having 4-hydroxy substitution showed good activity, effect of replacing 4-methoxy with 4-hydroxyl (**13m**) was studied. Indeed, it enhanced the docking score as well as activity but very slightly. It established some interactions with DDE motif, particularly Asp 116 and Glu152, as also 4-hydroxy was found to have interaction with Lys 156.

The observation that 4-hydroxy substitution and 3-methoxy substitution has positive effect on activity, compound **13n** was considered and since methoxy group takes part in hydrophobic interactions, replacing methoxy with ethoxy in compound **13o** was envisaged. Interestingly, it was noted that methoxy substitution decreases the activity and score but ethoxy substitution enhances the interactions and thus potentiates activity. These compounds had similar interactions such as hydrogen bonding with Cys 65 (via *p*-OH); Gln 148 (via amidic carbonyl group); Glu 152 and Lys 156 (via carbonyl and –NH group of pyrimidine ring) as well as hydrophobic interactions with Asp 64, Asp 116, coordinated with Mg 1001. However, **13o** additionally showed hydrophobic interactions with Cys 65, Gln 148 and DDE motif (via ethoxy group) and hydrogen bonding interaction of 4-hydroxy and oxygen of ethoxy with Thr 66, which might be the reason for better activity of **13o**.

Effect of replacing the substituted phenyl ring with fused ring like indole (compound **13p**) or small ring like furan (**13q**) was also thought of. Compound **13p** showed hydrogen bonding interactions with Asp 116 (via -NH of indole ring) and Glu 92 (via –NH- of pyrimidine ring). Hydrophobic interactions with Cys 65, His 67 and Asn 155 were also seen. However, it did not interact with DDE motif and the indole ring was more or less solvent exposed, which might be the reason for less docking score as well as IN inhibitory activity. However, substitution of small heterocyclic furan ring displayed hydrogen bonding interactions with His 67, Thr 66 and Lys 159 (via carbonyl of pyrimidine ring), Asn 155 (via oxygen of furan ring) and Cys 65 (with amidic oxygen); hydrophobic interactions with Asp 64, Asp 116, Thr 66, His 67, Asn 155, Glu 152 and showed coordination with Mg 1001 (with amidic oxygen). Due to these interactions, its docking score was good and showed 84% inhibition of IN.

Overall, it was observed that the HIV-1 IN inhibitory potency of designed compounds altered significantly with variation in nature as well as position of substituent. In general, compounds having *o*-chloro (**13d**) and *p*-chloro substitution (**13e**); electron donating group like 4-hydroxy-3-ethoxy substitution (**13o**) and small furan ring in place of phenyl ring (**13q**) exhibited significant IN inhibitory activity. Since these compounds showed% inhibition more than 80%, these were further tested for calculation of IC_{50} values (given in Table 1). It was observed that compounds **13d** and **13e** with electron withdrawing substituent showed IC_{50} value of 4.01µM and 0.65 µM respectively. Compound **13o** had IC_{50}value of 1.91 µM and furan substituted compound **13q** displayed IC_{50}value of 4.91µM.

Encouraged by the *in silico* and *in vitro* results, all the compounds were tested for anti-HIV activity against HIV-1 (III_B) and HIV-2 (ROD). It was however observed that, all the compounds did not show any anti-HIV activity below their cytotoxic concentration (as shown in Table 2). The compounds showed very good potential *in vitro* however in cell culture assay the activity was not seen. This requires further studies which will eventually be carried out (Figure 2).

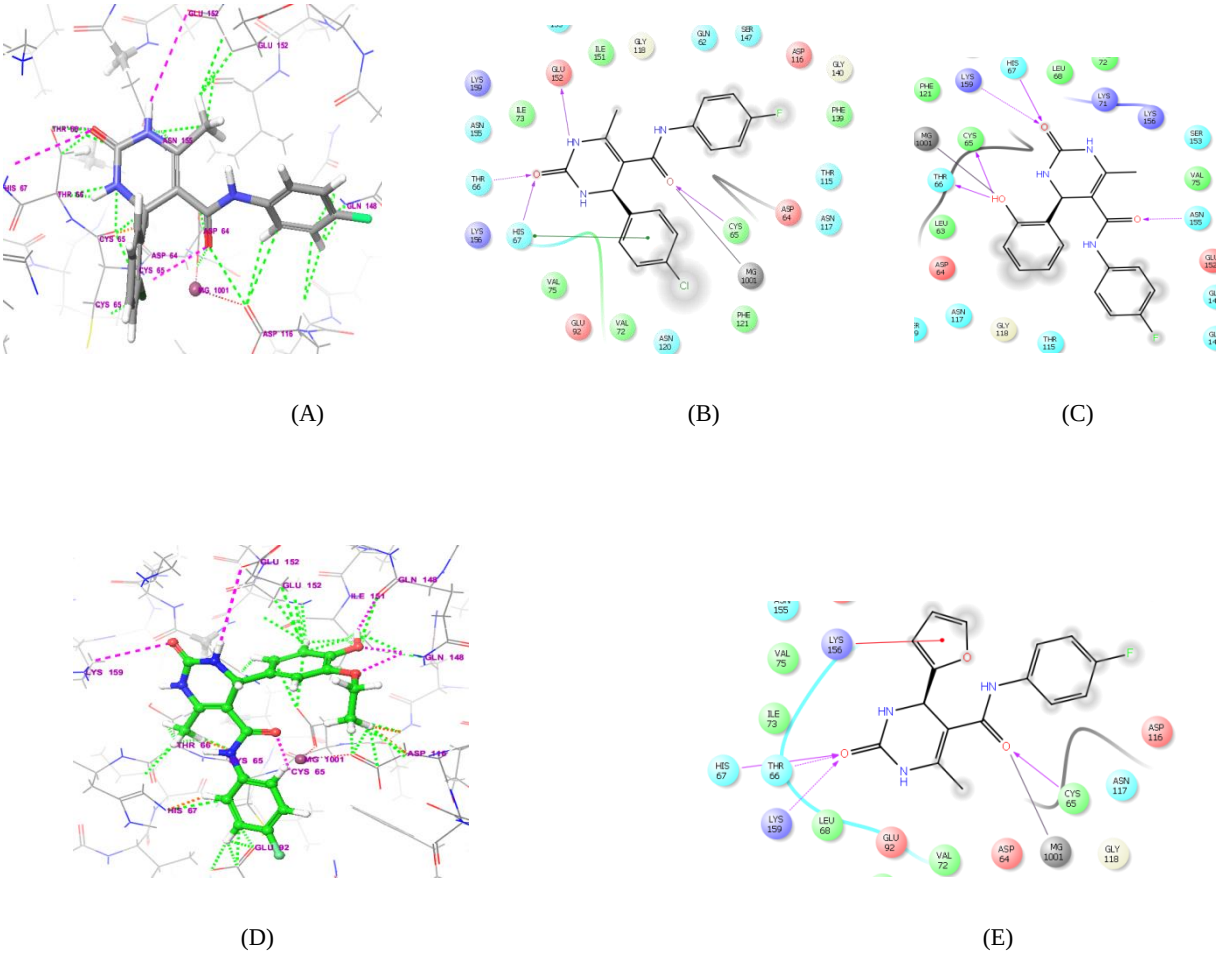


Figure 2: Docking poses of few representative compounds A: Docking pose of 13d; B: 2D interaction plot of 13e; C: 2D interaction plot of 13g; D: 2D interaction plot of 13o; E: 2D interaction plot of 13q.

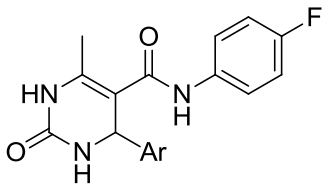


Table 1: Results of *in-vitro* HIV-1 IN inhibition and docking studies.

Compound code	Ar	Glide Score (G score)	% Inhibition*	IC_{50} value**
Dolutegravir	-----	-7.65	95	nd
8	Ph	-4.85	27.35	nd
13a	2-NO_2-Ph	-6.07	45.50	nd
13b	3-NO_2-Ph	-5.82	30.20	nd
13c	4-NO_2-Ph	-5.93	40.82	nd
13d	2-Cl-Ph	-5.95	88.36	4.01
13e	4-Cl-Phs	-5.66	90.11	0.65
13f	2,4-diCl-Ph	-5.21	69.31	nd
13g	2-OH-Ph	-7.40	72.80	nd
13h	4-OH-Ph	-6.66	73.40	nd
13i	4-OMe-Ph	-5.85	65.60	nd
13j	3,4-diOMe-Ph	-6.37	67.20	nd
13k	2,5-diOMe-Ph	-6.01	72.50	nd
13l	3,4,5-triOMe-Ph	-6.16	49.20	nd
13m	4-OH-3,5-diOMe-Ph	-6.69	56.62	nd
13n	4-OH-3-OMe-Ph	-6.70	60.18	nd
13o	4-OH-3-OEt-Ph	-7.03	87.83	1.91
13p	1H-indol-3-yl	-5.81	47.30	nd
13q	furan-2-yl	-6.50	84.13	4.91

Note: *% inhibition at 10µM concentration, values are mean of duplicate experiment performed independently, nd = not done; ** in µM

Table 2: Anti-HIV activity of the synthesized compounds.

Compound code	EC_{50}[1] (µM)		CC_{50}[2] (µM)	HIV-2[3] SI
	HIV-1 (III_B)	HIV-2 (ROD)		
Dolutegravir	0.00082	0.0023	1.44	619
8	>86.13	>86.13	>86.13	<1
13a	>16.65	>16.65	16.65	<1
13b	>61.63	>61.63	61.63	<1
13c	>10.98	>10.98	10.98	<1
13d	>60.18	>60.18	60.18	<1
13e	nd	nd	nd	nd
13f	>4.49	>4.49	4.49	<1
13g	>65.88	>65.88	65.88	<1
13h	>108.90	>108.90	108.90	<1
13i	>59.18	>59.18	59.18	<1
13j	>84.65	>84.65	84.65	<1
13k	>64.08	>64.08	64.08	<1
13l	>125.00	>125.00	125.00	X1
13m	>63.93	>63.93	63.93	<1
13n	>60.95	>60.95	60.95	<1
13o	>60.55	>60.55	60.55	<1
13p	nd	nd	nd	nd
13q	>58.23	>58.23	58.23	<1

[1] EC_{50}: Concentration of compound required to achieve 50% protection of MT-4 cell cultures against HIV induced cytotoxicity, as determined by MTT method.

[2] CC_{50}: Concentration required to reduce the viability of mock-infected cells by 50%, as determined by MTT method.

[3] SI: Selectivity index (CC_{50}/EC_{50} against HIV-2).

CONCLUSION

In summary, eighteen *N*-(4-fluorophenyl)-6-methyl-2-oxo-4-substituted phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxamide derivatives were synthesized and evaluated for anti-HIV activity using isolated enzyme (IN) assay, *in silico* and in cell culture assay against HIV-1 and HIV-2. Four compounds **13d, 13e, 13o,** and **13q** exhibited significant percentage inhibition of HIV-1 IN with IC_{50} value less than 4.91 µM. There was reasonably good correlation between docking simulation and isolated enzyme assay results. However, none of the derivative was active against HIV-1 and HIV-2 below their cytotoxic concentration. This indicates that these type of compounds can be excluded from further exploration for anti-HIV activity. Future course of action could be replacing 6-methyl with hydroxyl or oxo group so that it can bind with the active site magnesium.

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