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Original Article

Design, synthesis and evaluation of cytotoxic, antimicrobial, and anti-HIV-1 activities of new 1,2,3,4-tetrahydropyrimidine derivatives

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Abstract

A series of new 1,2,3,4-tetrahydropyrimidine (THPM) derivatives were designed and synthesized within a one-pot three component Biginelli reaction. The structures of compounds were characterized by FT-IR, ^{1}H-NMR, mass spectroscopy, and elemental analysis. All synthesized derivatives were screened for their cytotoxic, antimicrobial, and anti-HIV activities. Due to significant cytotoxic and antimicrobial effects of 1,2,3,4-THPM scaffold, in this study, cytotoxic and antimicrobial activities of synthesized derivatives were evaluated on two cell lines and four bacterial strains. Compounds **4e** and **4k** showed highest cytotoxic activity against HeLa and MCF-7 cell lines. In addition, **4c** and **4d** were most active against MCF-7 and HeLa cell lines, respectively. Among the compounds, **4e** revealed high antimicrobial activity against four strains. According to the results, **4e** possessing *m*-bromophenyl group at C-4 position of THPM exhibited the highest cytotoxic and antimicrobial effects. Also, all the newly synthesized compounds were evaluated for their anti-HIV-1 assay. Compounds **4l** and **4a** indicated remarkable anti-HIV-1 activity. It is concluded from cytotoxic, antimicrobial, and anti-HIV-1 activities that the 1,2,3,4-tertahydropyrimidines may serve as hit compounds for development of new anticancer small-molecules.

Keywords: Anti-HIV; Antimicrobial; Biginelli; Neoplasm;1,2,3,4-Tetrahydropyrimidine.

INTRODUCTION

Cancer is a kind of multifactorial disease considered the most serious health problem all over the world. It is the second leading cause of death worldwide, accounted for 8.8 million deaths in 2015 and nearly 1 in 6 of all global deaths (1).

The new cancer cases are expected to increase to 15 million per year by 2020, according to the world health organization (WHO), unless further precautionary measures are followed (2). Although great progress in the treatment of this disease has been made with respect to the problems associated with drug resistance, more attempts are essential for discovery of new anticancer agents (3). Multi-drug resistance and acute toxicity are the two major issues with most of the currently available chemotherapeutic agents (4). In a quest for the discovery of more effective anticancer drugs, a large number of structurally diverse synthetic and natural products have been screened for their anticancer potential (5,6).

Bacterial infections are a major cause of complications and death in cancer patients who become granulocytopenic because of intensive myelosuppressive chemotherapy (7).

In addition, infections are recognized as major obstacles impeding the successful management of patients with malignant diseases. The most common organisms causing these infections include *Pseudomonas aeruginosa (P. aeruginosa)* and *Staphylococcus aureus (S. aureus)* (8).

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There are hopes to find a much-needed antibiotic that may exert both antimicrobial and antitumor activity, to be used for prophylaxis as well as for treatment of bacterial superinfections in cancer patients, while being effective in preventing growth of cancer cells (8).

Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus, HIV-1, which is the most common and pathogenic strain of the virus. In the past decades developments of anti-HIV-1 drugs have converted this fatal infection into a chronic disease (9). Morbidity and mortality due to HIV infection have been decreased significantly with the appearance of treatment protocols such as highly active antiretroviral therapy. Problems such as cost, toxicity, and drug resistance limit the efficiency of highly active antiretroviral therapy and other currently existing treatments. New drugs that are better tolerated, active against resistant viruses, and able to completely abolish HIV replication suggest enhancements over current medications (10).

Multicomponent reactions have become popular in organic, medicinal, and combinatorial chemistry because they address both diversity and complexity in organic synthesis. Multicomponent reactions are defined as a process in which three or more different components are combined to yield ideally a single product. Such procedures reduce time and save both energy and starting materials (11,12).

There are two main interdependent reasons for the great increase of interest in 1,2,3,4-tetrahydropyrimidines (THPM) over the last few decades. One is the relative easiness of their preparation and their efficiency in a wide range of derivatizations and the second stems from the recognition of 1,2,3,4-THPM derivatives as valuable pharmacophores exhibiting a wide array of pharmacological activities (13). The current literature reveals that 1,2,3,4-THPM derivatives exhibit remarkable biological activities such as anti-viral (14), antitubercular (15), antimicrobial (16), and anticancer effects (17).

1,2,3,4-THPM comprise of a pyrimidine scaffold having a resemblance with the structures of the nucleic acid bases found in DNA and RNA. Their involvement as bases in nucleic acids has a great significance in drug design. Recent progress in the 1,2,3,4-THPM class of the anticancer agent monastrol, an inhibitor of human kinesin Eg5 (18), has led to the attention for efficient pharmacophore variation of Biginelli THPMs. Human kinesin Eg5 plays a crucial role in bipolar spindle generation during mitosis, inhibition of which leads to mitotic arrest and subsequent apoptotic cell death. It is therefore considered as one of the promising targets in cancer chemotherapy (19).

There are few reports on the anticancer activity of 1,2,3,4-THPM derivatives with benzyl ester at C-5 position of tetrahydropyrimidine ring. Wright, *et al*. have reported N-1 substituted long chain 4-aryl/alkyl-1,2,3,4-tetrahydropyrimidines as a novel class of cytotoxic agents against MCF-7 cell line in 2008 (20). In other works, researchers prepared a series of novel 1,2,3,4-THPMs with different substitutions such as ethyester, methylester, ketones, and amids at C-5 position of tetrahydropyrimidine ring (21). Some of these researches screened anti-cancer activity against MCF-7 and HeLa cell lines (22,23). There is no report for cytotoxic activity against MCF-7 and HeLa cell lines by 1,2,3,4-tetrahydropyrimidines with benzyestre and hydrogen at C-5 and N-1 moieties, respectively. Additionally, several reports of antimicrobial activities of substituted 1,2,3,4-tetrahydropyrimidines have been published in the recent years (16,24,25). These derivatives with different groups at C-2, C-4 and C-5 positions, were screened against diverse Gram negative and Gram positive microorganisms and showed moderate to excellent antimicrobial activities (21). There are some reports on the antibacterial activity of 1,2,3,4-THPM derivatives with ethylester and methylester at C-5 position of tetrahydropyrimidine ring on different strains (21). But there is not any research for 1,2,3,4-tetrahydropyrimidine scaffold with benzyester at C-5 against *S. aureus*, *P. aeruginosa*, *Escherichia coli (E. coli)* , and *Shigella flexneri (S. flexneri) (* strains. In our previous researches, a series of 1,2,3,4-THPM

derivatives were synthesized and evaluated in terms of anti-HIV activity. Then, their molecular docking simulation studies were performed by *in sillico* techniques. These derivatives showed modarete to excellent anti-HIV activity (26,27). We have been the first research group start working on anti-HIV assay of 1,2,3,4-THPM derivatives.

In this study, new 6-methyl-2-oxo-4-aryl-1,2,3,4-THPM-5-benzylester derivatives were designed, synthesized and evaluated for their cytotoxic activity against two human cell lines of MCF-7 and HeLa. Furthermore, all the synthesized compounds were tested for their *in vitro* anti-HIV-1 replication and antimicrobial activities against *S. flexneri*, *S. aureus*, *E. coli,* and *P. aeruginosa* strains.

MATERIALS AND METHODS

Chemistry

All chemicals used in this study were synthesis grade and purchased from Sigma-Aldrich chemical Co. (USA) and Merck Co. (Germany) any and used without further purification. Chemical reactions were monitored by analytical thin-layer chromatography (TLC) using several solvent systems with different polarities on pre-coated silica gel 60 F254 aluminum plates (Merck, Germany).

Fourier-transform infrared (FT-IR) spectra of all the compounds were recorded in the 4000-400 cm^{-1} range with samples in KBr discs using a Perkin-Elmer apparatus (USA). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were scanned on a Bruker Ultrashield 400 MHz spectrometer (Germany) using deuterated dimethyl sulfoxide (DMSO-d_6) as the solvent. Chemical shifts (δ) were reported in ppm relative to internal standard tetramethylsilane (TMS). Mass spectrometry results for all the compounds were recorded on an LC-MS/MS Quattro Micro API micromass Waters 2695 spectrometer (USA).

General procedure for synthesis of benzyl 4-(aryl) -6- methyl - 2-oxo-1,2,3,4- tetrahydro-pyrimidine-5-carboxylate (4a-4l)

A combination of the desirable aldehyde (3 mmol), benzyl 3-oxobutanoate (3.6 mmol), urea (3.9 mmol) and $Co(HSO_4)_2$ (0.6 mmol) in absolute ethanol (4 mL) was heated and stirred under reflux until the end of the reaction. The progress of the reaction was detected by TLC. After the completion of the reaction, the mixture was cooled to room temperature and crushed ice and water were added. Stirring was continued for several minutes till dissolving the catalyst and the excess of urea. Solid products were filtered and washed with water. Pure products were obtained by recrystallization from ethanol.

Biologyical assay

Cell culture

The human cervical cancer cell line (HeLa) and human breast cancer cell line (MCF-7) were provided by the Pasteur Institute of Iran (Tehran, I.R. Iran). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2/air in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA) supplemented with fetal bovine serum (FBS) and penicillin/streptomycin. For plating monolayers of cells, the medium was changed every two days to reach optimal confluence.

Anti-proliferation assay

In vitro cytotoxic activity of all synthesized compounds was evaluated against MCF-7 and HeLa cells using the standard MTT tests (28). MCF-7 and HeLa cells were seeded in 96-well plate at a density of 5×10^4 cell/mL, respectively and incubated for 24 h. Doxorubicin was used as the positive control and the wells containing DMSO (1%; Merck, Germany) and cell suspension was regarded as the negative controls. Then various concentrations of target compounds (1000, 500, 100, 10 μg/mL) were added and plates were incubated for 72 h. After the treatment period, 20 μL of XTT 0.5% (Merck, Germany) was added and further incubated for 3 h. Then supernatant of each well was replaced with 150 μL of DMSO to dissolve the formed formazan crystals. The absorbances were determined at 570 nm with an enzyme-linked immunosorbent assay (ELISA) microplate reader (BioTek, USA). Each assay was carried out three times at three different days, and the results of the experiment were summarized in

Table 1. Percent of cell survival was calculated as follows:

Cell survival (%) = [(absorbance of treated cells - absorbance of culture medium)/(absorbance of untreated cells – absorbance of culture mediun)] × 100

The software Graph pad Prism 6 (GraphPad Software, Inc, CA, USA) was used in the current survey to measure the half maximal inhibitory concentration (IC_{50}) of the drug by applying above mentioned concentrations to draw a graph based on non-linear regression.

Antimicrobial activity

In order to measure IC_{50} of synthetic components on *S. aureus*, *P. aeruginosa*, *E. coli,* and *S. flexneri*, these strains were ordered from Iranian microbial bank (Pasteur Institute of Iran, Tehran, I.R. Iran). (*S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *S. flexneri* ATCC 29508, and *E. coli* ATCC 25922). Subsequently, they were cultured on Mueller-Hinton agar in 37 °C for about 24 h. After that, for gaining the pure culture of each bacteria, they were cultured on especial rich medium. For *S. aureus* and *P. aeruginosa*, blood agar and for *S. flexneri* and *E. coli*, eosin methylene blue agar were applied. After about 24 h in 37 °C, to obtain the number of 1.5×10^8 of bacteria, 0.5 Mc-Farland tube was used. Components were dissolved in DMSO. Compounds 4c and 4f-4k at 1000, 500, 250 and 125 µg/mL and the compounds 4d, 4e, and 4l at 62.5, 125, 250 and 500 µg/mL made and added to microtiter plate. Then, 95 µL of Mueller-Hinton agar and 5µL of bacteria were mixed in each plate. Finally, after 24 h of incubation (37 °C), the optical density of these compounds were read by microtiter reader in 630 nm (29). Gentamycin, imipenem, and cefazole were utilized as positive controls of this test.

Anti-HIV-1 assay

For this purpose, inhibition of the HIV-1 entry by the prepared compounds was investigated. BMS-806 as the control HIV-1 surface glycoprotein inhibitor was used in the experiments (30). Synthesized compounds were dissolved in DMSO at different concentrations. DMSO with the final concentration of 1% v/v (1 mL DMSO in 100 mL H_2O) was considered as the negative solvent control. DMSO plus MT-2 (human lymphocyte) cells and virus were used to normalize the solvent effect (31). BMS-806 (200 mM) was dissolved in DMSO and kept in -20 °C. All tests were performed in triplicate.

Human embryonic kidney (HEK) and MT-2 cells were obtained from Pasteur Institute of Iran, Tehran, I.R. Iran. MT-2 Cells were produced by co-culturing normal human cord leukocytes with leukemic T-cells from a patient with adult T-cell leukemia. To measure the inhibition effect, HEK 293T cells were cultured in RPMI 1640 and DMEM containing 15% L-FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (32). In the step of transfection into HEK cells, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid was added and finally the cells were incubated at 37 °C under 5% CO_2. To produce the SCR HIV-1 virions, pMD2G, pmzNL4-3, and pSPAX2 plasmids were used. These plasmids were co-transfected into HEK 293T cells in 6-well plates (5×10^5 cells/well) in certain proportion using PolyFect reagent (Qiagen, Koria) according to the suggested method by Qiagen. Supernatants of the transfected cells were harvested after 24, 48, and 72 h after infection and fresh medium was added to the culture wells. Mediums containing viruses were mixed together and stored at -70 °C after filtration with 0.22 µm filters (33). Final centrifugation on the filtrate at 60,000 g was performed for 2 h at 4 °C. The supernatant was taken out and the virions pellet was shaken gently overnight in 1/30 volume of RPMI 1640 at 4 °C. SCR HIV-1 virions were produced in 24-well plates to generate viruses for measuring the rate of production and maturation. 70×10^3 HEK 293T cells were used for producing SCR HIV-1 virions by 400 ng of plasmid. Transfection was carried out in the presence of 4 µL of PolyFect reagent. Volume of transfection was 300 µL and the volume of the added DNA complex was 120 µL. When transfection was complete, 800 µL of the complete medium containing the synthesized compounds was added to the cells. The culture medium containing the virus was collected 48 h after transfection and the amount of the purified virus was measured using p24 ELISA assay kit (Cell Biolabs, France) (34).

RESULTS

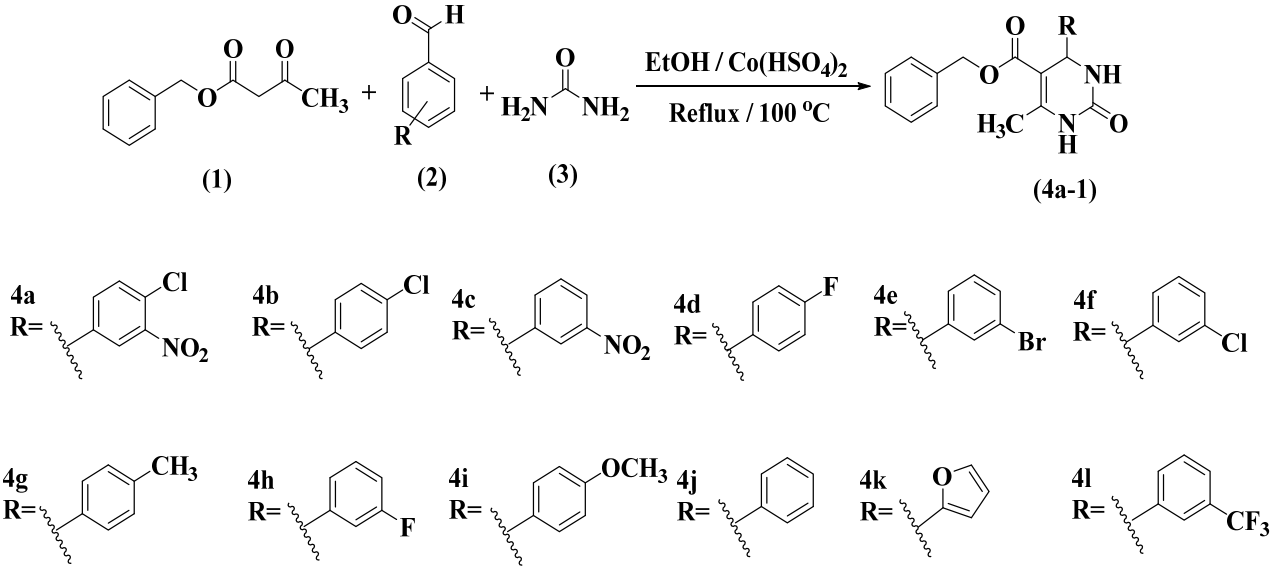
Twelve new 1,2,3,4-THPM derivatives described here were synthesized following the synthetic routes outlined in Scheme 1. The core 1,2,3,4-THPM ring was constructed according to the Biginelli multi-component cyclocondensation reaction.

In brief, a mixture of the different aldehyde, benzyl 3-oxobutanoate, and urea was refluxed in absolute ethanol in the presence of the catalytic amounts of $Co(HSO_4)_2$ to provide benzyl 4-(aryl)-6-methyl-2-oxo-1,2,3,4- THPM -5-carboxylate (**4a-4l**). The structures of all compounds were confirmed by FT-IR, ^{1}H-NMR, mass spectroscopy, and elemental analysis. The details of the spectral data are given in the following.

FT-IR spectra of the final compounds exhibited characteristic absorption peaks for the ureide N-H bonds at 3224-3443 cm^{-1} and 3121-3319 cm^{-1}. ^{1}H-NMR spectra revealed that presence of the THPM ring was confirmed by two peaks at 9.10-9.44 and 7.69-7.93 ppm belonging to ureide N-H bonds and a peak at 5.09-6.09 ppm attributable to the C-4 proton of the THPM ring. Other ^{1}H-NMR spectral signals were in accordance with the proposed structures.

*Benzyl 4-(4-chloro-3-nitrophenyl)-6-methyl-2-oxo - 1,2,3,4 - tetrahydropyrimidine - 5-carboxylate (**4a**)*

White precipitates. yield: 16%. mp: 233-234 °C. R_f = 0.65 (petroleum ether/EtOAc 1:3), R_f = 0.77 (chloroform/methanol 4:0.3). FT-IR (KBr, cm^{-1}): 3443, 3319 (NH, ureide), 3097 (C-H, aromatic), 2919 (C-H, aliphatic), 1652 (C=O, ester), 1605 (C=O, ureide), 1652, 1539, 1479 (C=C, aromatic), 1156 (C-O, ester). ^{1}H-NMR (400 MHz, DMSO-d_6): 9.15 (1H, s, H-$N_{(1)}$: ureide), 7.93 (1H, s, H-$N_{(3)}$: ureide), 7.76 (1H, s, H-$C_{(2)}$, -$C_6H_3ClNO_2$), 7.63 (1H, d, J = 8.28 Hz, H-$C_{(5)}$: -$C_6H_3ClNO_2$), 7.43-7.10 (5H, m, H-C: $CH_2\underline{C_6H_5}$), 6.98 (1H, d, J = 8.16 Hz, H-$C_{(6)}$: -$C_6H_3ClNO_2$), 6.09 (1H, brs, H-$C_{(4)}$: THPM), 5.83 (2H, brs, H-C: $\underline{CH_2}C_5H_6$), 2.30 (3H, s, CH_3-$C_{(6)}$: THPM). ESI-MS (m/z): 400.05. Anal. Calc. for $C_{19}H_{16}ClN_3O_5$ (401.80): C: 56.80, H: 4.01, N: 10.46; found: C: 56.58, H: 3.96, N: 10.31.



Scheme 1. General procedure for the preparation of the 1,2,3,4-tetrahydropyrimidine derivatives (**4a-l**).

*Benzyl 4 - (4-chlorophenyl)- 6- methyl- 2 - oxo- 1,2,3,4- tetrahydropyrimidine-5-carboxylate **(4b)***

Synthesis and the structural features of this compound have been reported previously (26).

*Benzyl 6-methyl-4-(3-nitrophenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate **(4c)***

Synthesis and the structural features of this compound have been reported previously (26).

*Benzyl 4- (4-fluorophenyl) – 6 - methyl - 2 -oxo- 1,2,3,4-tetrahydropyrimidine-5-carboxylate **(4d)***

White crystals yield: 41%. mp: 185-186 °C. R_f = 0.56 (petroleum ether/EtOAc 1:3), R_f = 0.37 (chloroform/methanol 4:0.3). FT-IR (KBr, cm^{-1}): 3355, 3225 (N-H, ureide), 3119 (C-H, aromatic), 2983 (C-H, aliphatic), 1706 (C=O, ester), 1686, 1507, 1498 (C=C, aromatic), 1224 (C-O, ester). ^{1}H-NMR (400 MHz, DMSO-d_6): 9.29 (1H, s, H-$N_{(1)}$: ureide), 7.77 (1H, d, *J*= 3.2 Hz, H-$N_{(3)}$: ureide), 7.30-7.10 (9H, m, H-C: - $CH_2\underline{C_6H_5}$, -$\underline{C_6H_4}$F), 5.18 (1H, brs, H-$C_{(4)}$: THPM), 5.07 (1H, d, *J*= 12.8 Hz, H-$C_{(a)}$: $\underline{CH_2}C_5H_6$), 4.99 (1H, d, *J*= 12.8 Hz, H-$C_{(b)}$: $\underline{CH_2}C_5H_6$), 2.27 (3H, s, CH_3-$C_{(6)}$: THPM), ESI-MS (m/z): 400.05. Anal. Calc. for $C_{19}H_{17}FN_2O_3$ (340.35): C: 67.05, H: 5.03, N: 8.23; found: C: 67.37, H: 4.99, N: 8.06.

*Benzyl 4- (3- bromophenyl)- 6 - methyl- 2- oxo - 1,2,3,4-tetrahydropyrimidine-5-carboxylate **(4e)***

White precipitates yield: 33%. m.p.: 182-183 °C. R_f = 0.67 (petroleum ether/EtOAc 1:3), R_f = 0.61 (chloroform/methanol 4:0.3). FT-IR (KBr, cm^{-1}): 3360, 3219 (NH, ureide), 3109 (C-H, aromatic), 2956 (C-H, aliphatic), 1706 (C=O, ester), 1695, 1572, 1475 (C=C, aromatic), 1251 (C-O, ester), ^{1}H-NMR (400 MHz, DMSO-d_6): 9.43 (1H, s, H-$N_{(1)}$: ureide), 7.89 (1H, s, H-$N_{(3)}$: ureide), 7.55-7.22 (9H, m, H-C: -$CH_2\underline{C_6H_5}$, -$\underline{C_6H_4}$Br), 5.24 (1H, brs, H-$C_{(4)}$: THPM), 5.15 (1H, d, *J*= 12.8 Hz, H-$C_{(a)}$: $\underline{CH_2}C_5H_6$), 5.07 (1H, d, *J*=12.8 Hz, H-$C_{(b)}$: $\underline{CH_2}C_5H_6$), 2.35 (3H, s, CH_3-$C_{(6)}$: THPM). ESI-MS (m/z): 400.05. Anal. Calc. for $C_{19}H_{17}BrN_2O_3$ (401.26): C: 56.87, H: 4.27, N: 6.98; found: C: 57.01, H: 4.51, N: 7.26.

*Benzyl 4- (3-chlorophenyl) -6- methyl-2- oxo- 1,2,3,4-tetrahydropyrimidine-5-carboxylate **(4f)***

White precipitates. yield: 21%. mp: 180-181 °C. R_f = 0.62 (petroleum ether/EtOAc 1:3), R_f = 0.41 (chloroform/methanol 4:0.3), FT-IR (KBr, cm^{-1}): 3359, 3220 (NH, ureide), 3110 (C-H, aromatic), 2957 (C-H, aliphatic), 1706 (C=O, ester), 1697, 1532, 1471 (C=C, aromatic), 1172 (C-O, ester), ^{1}H-NMR (400 MHz, DMSO-d_6): 9.27 (1H, s, H-$N_{(1)}$: ureide), 7.73 (1H, s, H-$N_{(3)}$: ureide), 7.73-7.08 (9H, m, H-C: -$CH_2\underline{C_6H_5}$, -$\underline{C_6H_4}$Cl), 5.09 (1H, brs, H-$C_{(4)}$: THPM), 5.00 (1H, d, *J*= 12.8 Hz, H-$C_{(a)}$: $\underline{CH_2}C_5H_6$), 4.91 (1H, d, *J*=12.8 Hz, H-$C_{(b)}$: $\underline{CH_2}C_5H_6$), 2.20 (3H, s, CH_3-$C_{(6)}$: THPM). ESI-MS (m/z): 355.05. Anal. Calc. for $C_{19}H_{17}ClN_2O_3$ (356.81): C: 63.96, H: 4.80, N: 7.85; found: C: 64.22, H: 4.91, N: 7.59.

*Benzyl 6- methyl- 2 - oxo - 4 -p-tolyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate **(4g)***

Synthesis and the structural features of this compound have been reported previously (26).

*Benzyl 4- (3-fluorophenyl) -6- methyl -2- oxo- 1,2,3,4-tetrahydropyrimidine-5-carboxylate **(4h)***

White precipitates. yield: 39%. mp: 1798-179 °C. R_f = 0.69 (petroleum ether/EtOAc 1:3), R_f = 0.44 (chloroform/methanol 4:0.3). FT-IR (KBr, cm^{-1}): 3366, 3225 (NH, ureide), 3111 (C-H, aromatic), 2969 (C-H, aliphatic), 1705 (C=O, ester), 1692, 1590, 1486 (C=C, aromatic), 1223 (C-O, ester), ^{1}H-NMR (400 MHz, DMSO-d_6): 9.44 (1H, s, H-$N_{(1)}$: ureide), 7.91 (1H, s, H-$N_{(3)}$: ureide), 7.46-7.37 (4H, m, H-C: -$\underline{C_6H_4}$F), 7.25-7.03 (5H, m, H-C: $CH_2\underline{C_6H_5}$), 5.28 (1H, brs, H-$C_{(4)}$: THPM), 5.18 (1H, d, *J* = 12.8 Hz, H-$C_{(a)}$: $\underline{CH_2}C_5H_6$), 5.09 (1H, d, *J* = 12.8 Hz, H-$C_{(b)}$: $\underline{CH_2}C_5H_6$), 2.37 (3H, s, CH_3-$C_{(6)}$: THPM). ESI-MS (m/z): 339.10. Anal. Calc. for $C_{19}H_{17}FN_2O_3$ (340.35): C: 67.05, H: 5.03, N: 8.23; found: C: 66.92, H: 4.98, N: 8.34.

*Benzyl 4- (methoxyphenyl) - 6 -methyl - 2-oxo- 1,2,3,4-tetrahydropyrimidine-5-carboxylate **(4i)***

White precipitates. yield: 57.77%. m.p.: 190-191 °C. R_f = 0.47 (petroleum ether/EtOAc 1:3), R_f = 0.59 (chloroform/methanol 4:0.3). FT-IR (KBr, cm^{-1}): 3358, 3224 (NH, ureide), 3116 (C-H, aromatic), 2952 (C-H, aliphatic), 1706 (C=O, ester), 1685, 1511, 1456 (C=C, aromatic), 1224 (C-O, ester). ^{1}H-NMR (400 MHz, DMSO-d_6): 9.34 (1H, s, H-$N_{(1)}$: ureide), 7.69 (1H, d, *J* = 3.2 Hz, H-$N_{(3)}$: ureide), 7.54-7.41 (5H, m, H-C: -$CH_2\underline{C_6H_5}$),

7.25 (2H, d, *J* = 9.6 Hz, H-$C_{2'}$, H-$C_{6'}$: $\underline{C_6H_5}OCH_3$), 6.78 (2H, d, *J* = 9.6 Hz, H-$C_{3'}$, H-$C_{5'}$: $\underline{C_6H_5}OCH_3$), 5.32 (1H, brs, H-$C_{(4)}$: THPM), 4.95 (1H, d, *J* = 12.8 Hz, H-$C_{(a)}$: $\underline{CH_2}C_5H_6$), 4.81 (1H, d, *J* =12.8 Hz, H-$C_{(b)}$: $CH_2C_5H_6$), 4.12 (3H, s, OCH_3), 2.38 (3H, s, CH_3-$C_{(6)}$: THPM). ESI-MS (m/z): 351.05. Anal. Calc. for $C_{20}H_{20}N_2O_4$ (352.39): C: 68.17, H: 5.72, N: 7.95; found: C: 67.98, H: 5.81, N: 8.20.

Benzyl 6- methyl - 2-oxo- 4- phenyl- 1,2,3,4 - tetrahydropyrimidine-5-carboxylate ***(4j)***

Synthesis and the structural features of this compound have been reported previously (26).

Benzyl 4-(furan-2-yl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate ***(4k)***

White precipitates. yield: 45%. mp: 192-193 °C. R_f = 0.50 (petroleum ether/EtOAc 1:3), R_f = 0.36 (chloroform/methanol 4:0.3). FT-IR (KBr, cm^{-1}): 3241, 3121 (N-H, ureide), 2942 (C-H, aliphatic), 1712 (C=O, ester), 1495, 1454 (C=C, aromatic), 1231 (C-O, ester). ^{1}H-NMR (400 MHz, DMSO-d_6): 9.32 (1H, s, H-$N_{(1)}$: ureide), 7.79 (1H, d, *J* = 3.2 Hz, H-$N_{(3)}$: ureide), 7.56 (1H, brd, *J* = 1.6 Hz, H-$C_{(3)}$: furan), 7.36-7.23 (5H, m, H-C: -$CH_2\underline{C_6H_5}$), 6.36 (1H, dd, *J* = 3.2 Hz, *J* = 2.0 MHz, H-$C_{(4)}$: furan), 6.06 (1H, d, *J* = 3.2 Hz, H-$C_{(5)}$: furan), 5.25 (1H, brs, H-$C_{(4)}$: THPM), 5.10 (1H, d, *J* = 12.8 Hz, H-$C_{(a)}$, -$\underline{CH_2}C_6H_5$), 5.04 (1H, d, *J* = 12.8 Hz, H-$C_{(b)}$: -$\underline{CH_2}C_6H_5$), 2.25 (3H, s, CH_3-$C_{(6)}$: THPM). ESI-MS (m/z): 311.10. Anal. Calc. for $C_{17}H_{16}N_2O_4$ (312.33): C: 65.38, H: 5.16, N: 8.97; found: C: 65.19, H: 5.45, N: 8.63.

Benzyl 6-methyl-2-oxo-4-(3-(trifluoromethyl) phenyl)- 1,2,3,4 - tetrahydropyrimidine – 5 - carboxylate ***(4l)***

White precipitates. yield: 35%. mp: 180-181 °C. R_f = 0.62 (petroleum ether/EtOAc 1:3), R_f = 0.64 (chloroform/methanol 4:0.3). FT-IR (KBr, cm^{-1}): 3355, 3221 (NH, ureide), 3112 (C-H, aromatic), 2964 (C-H, aliphatic), 1705 (C=O, ester), 1495, 1454 (C=C, aromatic), 1217 (C-O, ester), ^{1}H-NMR (400 MHz, DMSO-d_6): 9.10 (1H, s, H-$N_{(1)}$: ureide), 7.70 (1H, d, *J* = 3.2 Hz, H-$N_{(3)}$: ureide), 7.64 (1H, m, H-$C_{(2)}$: -$C_6H_4CF_3$), 7.33-7.31 (3H, m, H-$C_{(4)}$, H-$C_{(5)}$, H-$C_{(6)}$: -$C_6H_4CF_3$), 7.19-7.16 (5H, m, H-C: -$CH_2\underline{C_6H_5}$), 5.34 (1H, brs, H-$C_{(4)}$: THPM), 5.12 (1H, d, *J* = 12.8 Hz, H-$C_{(a)}$: $\underline{CH_2}C_5H_6$), 5.03 (1H, d, *J* =12.8 Hz, H-$C_{(b)}$: $\underline{CH_2}C_5H_6$), 2.36 (3H, s, CH_3-$C_{(6)}$: THPM). ESI-MS (m/z): 389.05. Anal. Calc. for $C_{20}H_{17}F_3N_2O_3$ (390.36): C: 61.54, H: 4.39, N: 7.18; found: C: 61.37, H: 4.43, N: 7.49.

Biological assay

Cytotoxic activity

All the synthesized compounds were tested for cytotoxic activity *vs* MCF-7 and HeLa cell lines by XTT assay. The concentration dependent cytotoxicities against MCF-7 and HeLa cell lines are summarized in Table 1. Results indicated that most compounds exhibited moderate to good activities against MCF-7 and HeLa cell lines. Compounds 4c, 4e, 4j, and 4k indicated IC_{50} values in the range of 45-80 and 15-100 µg/mL against MCF-7 and HeLa cell lines, respectively.

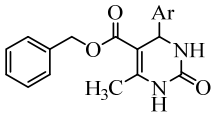
Antimicrobial activity

Compounds were tested for antimicrobial activity against *S. aureus*, *P. aeruginosa*, *E. coli,* and *S. flexneri* strains. The results proved that all of the tested compounds had moderate to good antibacterial activity. **4d**, **4e**, **4j,** and **4l** showed significant growth inhibition at 40.6, 21.7, 37.8, and 19.9 µg/mL concentrations, respectively against *S. aureus* and maximum activity at 34.0, 17.8, 101.4, and 52.4 µg/mL, respectively, against *E. coli*. *In vitro* antibacterial activity of synthesized derivatives are shown in Table 2.

Anti-HIV-1 activity

Anti-HIV activities of all the synthesized derivatives were evaluated in terms of inhibitory activity against HIV-1 replication in HEK cells cultures. HIV-1 replication inhibitory activities at 100 µM from 0 to 72% were recorded for the studied compounds. Among tested compounds, **4a** and **4l** showed the highest anti-HIV-1 activity (61.43 and 71.65%). The anti-HIV-1 results are summarized in Table 3.

Table 1. The IC_{50} (µg/mL) of compounds tested against MCF-7 and HeLa cancer cell lines.



(4a-1)

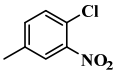
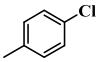
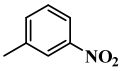
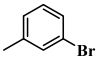
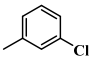
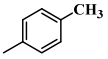
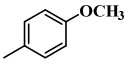
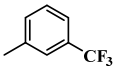
Compounds	Ar	IC_{50} (µg/mL)	
		MCF-7	HeLa
4a		420 ± 2.34	380 ± 3.12
4b		350 ± 2.87	300 ± 2.35
4c		60 ± 2.15	820 ± 2.85
4d		650 ± 3.41	80 ± 3.08
4e		45 ± 2.91	15 ± 2.35
4f		135 ± 2.08	140 ± 2.78
4g		500 ± 3.12	450 ± 3.45
4h		120 ± 3.08	135 ± 3.19
4i		500 ± 2.05	500 ± 2.05
4j		80 ± 2.78	100 ± 4.07
4k		55 ± 3.37	40 ± 2.43
4l		220 ± 3.18	135 ± 3.15
Doxorubicin	-	4 ± 2.34	4 ± 1.92

Table 2. IC_{50} of the synthesized compounds **4a-4l** against Gram-negative and Gram-positive bacteria.

Compounds	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Shigella flexneri*
	IC_{50} (µg/mL)			
4a	249.6 ± 10.4	459.6 ± 9.3	497.5 ± 13.4	282.5 ± 10.6
4b	835.3 ± 26.2	130.5 ± 3.9	322.5 ± 13.4	269.6 ± 5.1
4c	993.0 ± 70.6	71.9 ± 6.6	270.6 ± 18.6	301.1 ± 10.6
4d	40.6 ± 2.1	68.2 ± 3.4	34.0 ± 2.1	96.3 ± 4.3
4e	21.7 ± 1.8	47.1 ± 2.0	17.8 ± 2.5	23.5 ± 0.8
4f	130.3 ± 1.6	138.7 ± 4.1	111.7 ± 5.7	141.3 ± 6.7
4g	508.3 ± 9.0	490.0 ± 22.1	348.7 ± 16.9	294.3 ± 7.6
4h	153.7 ± 9.9	138.3 ± 10.0	107.2 ± 3.1	134.3 ± 10.1
4i	494.3 ± 4.4	752.5 ± 30.5	510.6 ± 40.0	526.3 ± 6.5
4j	37.8 ± 0.9	37.4 ± 1.8	101.4 ± 4.2	20.7 ± 1.3
4k	115.9 ± 9.5	117.0 ± 6.7	109.3 ± 2.4	121.2 ± 9.5
4l	19.9 ± 1.4	48.25 ± 3.2	52.4 ± 7.1	27.3 ± 2.0
Gentamycin	4.9 ± 0.5	6.8 ± 0.4	8.3 ± 1.3	7.2 ± 0.8
Imipenem	3.2 ± 0.1	4.2 ± 1.1	2.1 ± 0.4	7.25 ± 0.25
Cefazole	1.7 ± 0.7	7.3 ± 0.8	3.9 ± 0.8	2.8 ± 0.4

Table 3. Anti-HIV-1 activity of the prepared compounds.

Compounds	100 µM
	Inhibition rate of P_{24} expression (%)
4a	61.43 ± 1.45
4b	47.50 ± 2.50
4c	52.25 ± 1.25
4d	4.75 ± 1.79
4e	12.93 ± 1.31
4f	50.21 ± 2.19
4g	0.00
4h	10.67 ± 2.33
4i	36.28 ± 1.78
4j	23.75 ± 1.25
4k	22.51 ± 2.50
4l	71. 65 ± 1.98
BMS-806	100

DISCUSSION

As it was explained in the introduction, the most relevant compounds which were previously reported as anticancer, antibacterial, and anti-HIV-1 agents were THPM derivatives with diverse amides and esters expect benzylester at C5 of THPM scaffold. In addition, some previously synthesized THPM derivatives were N1 and N3 substituted. They were different from the compounds in the present study in both the chemical structure and some of the biological targets. From the viewpoint of structure activity relationship, we wanted to establish the effect of structural changes on the cytotoxic, anti-bacterial and anti-HIV activities within this group of compounds by comparing the activity of those bearing an aromatic substituent at C4 and C5, a carbonyle moiety at C2 and two hydrogen atoms at N1 and N3 positions. Structure activity relationships of previous researches have shown that presence of aromatic moieties such as substituted phenyls and hetroaromatic rings at C4 position could enhance their cytotoxicity, antimicrobial, and anti-HIV effects compared to aliphatic groups (18-27).

Results of cytotoxic studies indicated that most compounds exhibited moderate to good activities against MCF-7 and HeLa cell lines. Compounds **4c**, **4e**, **4j,** and **4k** showed the highest IC_{50} values among compounds in the range of 45-80 and 15-100 µg/mL against MCF-7 and HeLa cell lines, respectively. These compounds showed higher activity in comparison with previously reported compounds bearing amide groups at C5 position *against* HeLa cell line (21).

Among the assessed compounds, **4e** manifested potent activity against two cell lines with IC_{50} values of 45 and 15 µg/mL *against* MCF-7 and HeLa cells, respectively. It seemed that the presence of bromine atom at *meta* position of C4-phenyl ring provided a good cytotoxic effect. Interestingly, replacement of the unsubstituted phenyl ring (**4j**) with heterocyclic furyl ring (**4k**) at C-4 position significantly enhanced the anticancer activity against both cell lines (Table 1).

The substitution of *meta*-bromo substituent with more electronegative groups such as chloro (**4f**), fluoro (**4h**), and trifluoromethyl (**4l**) resulted in poor activity against both cell lines. However, compound **4c**, with *meta*-nitro substituent retained activity against MCF-7 cell line although it was less active against HeLa cell line. This demonstrated that nitro is a hydrophilic group while halogens are lipophilic groups, so nature of group at *meta* position is important for cytotoxic activity within assessed cell lines. Movement of chlorine or fluorine atom from *meta* to *para* position (compounds **4b** and **4d**) significantly decreased cytotoxic activity against both cell lines for compound **4b** and against MCF-7 cell line for compound **4d**. It was indicated that position of substituents on phenyl ring is a determinant factor in cytotoxic activity.

The presence of chlorine and nitro groups on *para* and *meta* positions of phenyl ring (**4a**) provided poor activity for **4b** and **4c**. These

results exhibited that compounds with two substituents on the phenyl ring exhibited less activity than compounds with one substituent. This structure activity relationship revealed the importance of functional groups such as fluorine and bromine groups at the *meta* positions of C4-phenyl ring.

Compounds **4b** and **4i** showed poor activity against MCF-7 cell line. It should be noted that similar compounds with ethylester moiety at C-5 position were also reported as weak cytotoxic compounds against MCF-7 cell line (34).

Compounds **4l** and **4j** with *m*-(trifluoromethyl) phenyl and phenyl moieties at C4 of THPM ring had potent antibacterial activity against *S. aureus* and *S. flexneri*, respectively (Table 2). This showed that probably the increase of electronegativity at *metha* position is appropriate for activity. Introduction of bromine and chlorine groups at the *meta* or *para* position of phenyl ring resulted in a significant improvement of the antibacterial activity. Compounds **4b** (*para*-chloro), **4c** (*para*-methyl), and **4g** (*meta*-nitro) had the lowest activities against *S. aureus*. In addition, compounds **4g** and **4i** with electron donor substituents (methyl and methoxy groups) exhibited weak activities against *P. aeruginosa.* Interestingly, introducing of OCH_3 group at the *para* position, reduced antibacterial activity against *E. coli* and *S. flexneri*. This might be attributed to the donation of electrons to the benzene ring through inductive effect.

A series of 1,2,3,4-THPM-5-carbohydrazides were synthesized and evaluated against *E. coli* and *Bacillus subtilis* strains by other researchs (35). These derivatives with carbohydrazide moiety at C-5 position were more potent than our compounds with benzyl ester at C-5 position against *E. coli*.

In our previous studies, we synthesized and screened anti-HIV effect of a series 1,2,3,4-THPM-5-benzylcarboxylate derivatives (26). In continuation of our previous exploration, structure activity relationship studies indicated that compounds **4l**, **4a**, and **4c** were more active than the others. The presence of trifluoromethyl group at the *meta* position of phenyl ring provided a potent anti-HIV-1 property to the compound **4l**. Similarly compounds bearing nitro (**4c**), fluorine (**4h**), bromine **(4e),** and chlorine **(4f**) groups at the *meta* position as well as chlorine and nitro groups at *para* and *meta* positions (**4a**) of phenyl were less potent. The presence of fluorine at *meta* position showed better anti-HIV-1 activity than *para* position (**4d**) (10.67 *vs* 4.75% in 100 µM). Moreover, similarity shift of chlorine group from *meta* position (**4f**) to *para* position (**4b**) decreased activity. This revealed that the presence of a group at *meta* position is appropriate for anit-HIV-1 activity when compared to *para* position.

CONCLUSION

In summary, a new series of 1,2,3,4-THPM derivatives were designed and synthesized. All compounds were evaluated for their cytotoxicity against HeLa and MCF-7 cancer cell lines, antibacterial effect aganist *S. aureus*, *P. aeruginosa*, *E. coli,* and *S. flexneri,* and anti-HIV-1 activities. Compounds **4c**, **4e**, **4j,** and **4k** showed the highest cytotoxic activity with IC_{50} values of 60, 45, 55, and 13 µg/mL against MCF-7 cell line and **4d**, **4e**, **4j,** and **4k** exhibited good cytotoxic activity with the IC_{50} values of 80, 15, 100, and 40 µg/mL against HeLa cell line. Substitution at *meta* position of THPM C4-phenyl and incorporating a heteroaromatic moiety instead of phenyl ring at C4 led to the enhanced cytotoxicities. Furthermore, compounds **4d**, **4e**, **4j,** and **4l** revealed the strongest activities against four strains. As evident from antibacterial results, lipophilic and electron-withdrawing groups such as CF_3, F, and Br at *para* and *meta* of phenyl ring exhibited higher antibacterial activities. Interestingly, **4e**, **4d**, and **4j** showed the highest antibacterial and cytotoxic activities. According to the results, **4a**, **4c,** and **4l** efficiently inhibited the rate of P24 expression. The percentage of inhibition values for these compounds were 61.43, 52.25, and 71.65%, respectively. According to anti-HIV-1 results, presence of Cl at *para* position and nitro at *meta* position of phenyl ring as well as CF_3 group resulted in increase of

anti-HIV-1 effect, so it can be concluded that *meta* and *para* substitution pattern is favorable for anti-HIV-1 activity in this group of derivatives.

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