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Evaluation of *N*-arylhydrazone derivatives of orotic acid as stimulators of human mesenchymal stem cell (hMSCs) differentiation.

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Abstract:

Human mesenchymal stem cells (hMSCs) constitute of cells having potential of self-renewal and proliferation and are commonly isolated from bone marrow aspirates of large bones. The osteogenic potential of these stem cells has been extensively exploited by scientists during the last many years for the biological evaluation of synthetic scaffolds with applications in tissue engineering. Current work aimed to synthesize *N*-arylhydrazone derivatives of orotic acid and

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their evaluation as stimulators of human mesenchymal stem cells. Some of the analogs show good to moderate proliferation rate.

Introduction:

Heterocycles and heterocyclic derivatives are continuously serves as a versatile tool to synthesize variety of natural product due to the presence of various chromophores (1-5). Pyrimidine carboxylic acid which is commonly called as orotic acid is a well known compound and found in many naturally occurring products like, milk whey and serves as an intermediate in the biosynthesis of pyrimidine which is an essential component in DNA and RNA synthesis. Moreover, orotic acid is also reported to enhance cardiac output and aid in recovery from heart failure. It also has growth stimulant effects in mammals and may assist in absorption of calcium, magnesium and other essential nutrients. It has also been reported that orotic acid is used to reduce the level of bilirubin in infants and is also useful for the treatment of gout. Many orotic acid analogues have shown promising results against antitumor and antimicrobial activities. Some of them also serve as enzyme inhibitors and gain the attention of chemists and molecular biologists (6-9).

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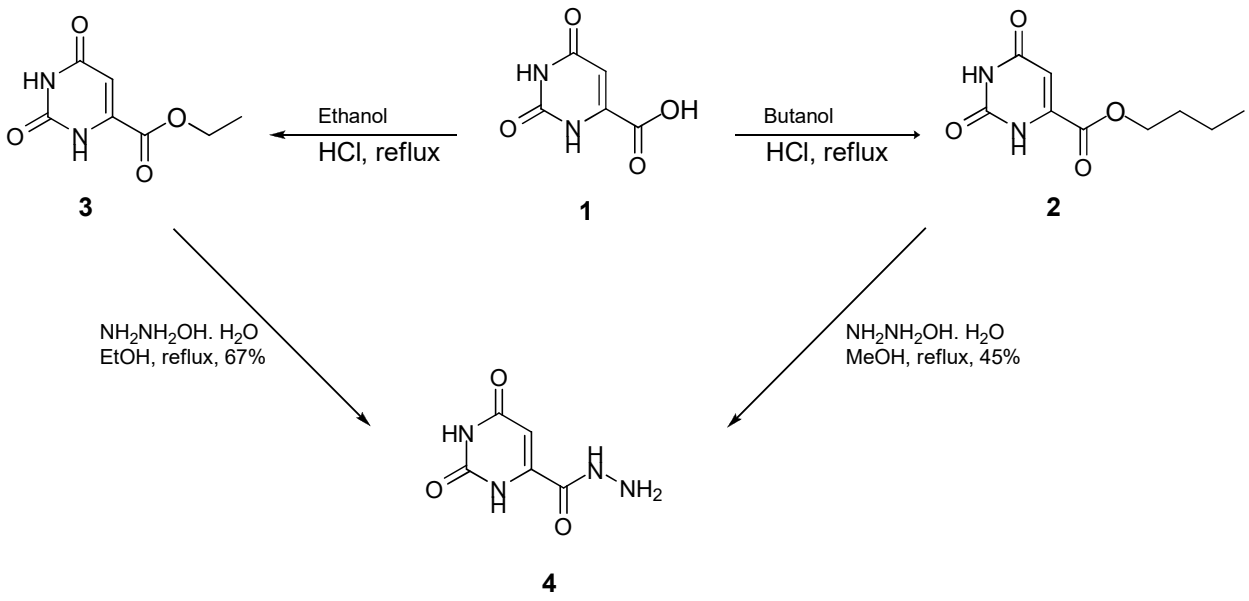

Scheme 1: Reaction scheme for the synthesis of compound **2**,**3** and **4**.

Hydrazones constitutes an important class of compounds in organic synthesis due to azomethine group being part of the molecule. In addition, hydrazones and hydrazides are considered as one of the most useful synthetic intermediate to synthesize variety of molecules and possible drug candidates (10). Since many years, hydrazones derivatives have been the focus of interest for many synthetic chemists and biologists because of the synthetic importance and the biological activity associated with them. The pharmacological profile includes their antimicrobial, antiviral, anticancer and anti inflammatory activities. The bioactivity of the hydrazide-hydrazone analogues is apparently not limited to the core moiety but also depends on the molecules attached to the terminal nitrogen. It is long been known that introduction of aromatic molecules to the heterocyclic system results in more potent molecules (11-13).

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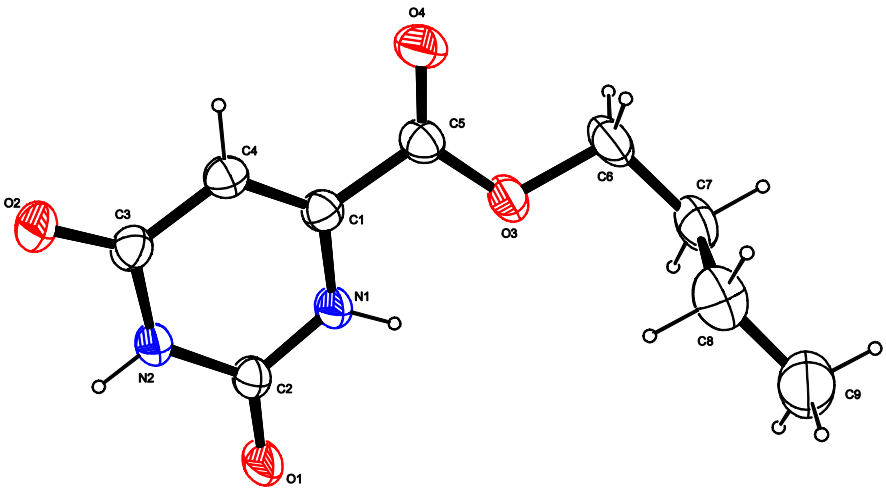

Fig 1. Crystal structure of compound **2**

Human stromal (mesenchymal) stem cells (hMSCs) are multipotent stem cells with ability to differentiate into mesoderm-type cells e.g. osteoblasts and adipocytes and thus they are being introduced into clinical trials for tissue regeneration. Various heterocyclic compounds are having boundless influence in term of stem cell therapy of different organs such as heart, bone marrow transplant. They enhance the stem cell proliferation and differentiation into other mature cell types. Synthetic design of heterocyclic compounds have been aging in substantial numbers of molecular platform including substituted purines pyrimidines, quinazolines, pyrazines, pyrrolopyrimidine, pyrazolopyrimidine, pyridazines, and hydrazones which lend appropriate chemical concern to look into modulate complex cellular mechanism (14-18). However, to the best of our knowledge, an application with pyrimidine carboxylic acid has not yet been reported.

Keeping in mind the biological potential of orotic acid and in continuation to our interest in hydrazone-hydrazide chemistry, the present works describe the preparation of *N*-arylhydrazones starting from orotic acid. Current research work also presents the stem cell proliferation potential of hydrazone derivatives with human stromal stem cells which was never studied before.

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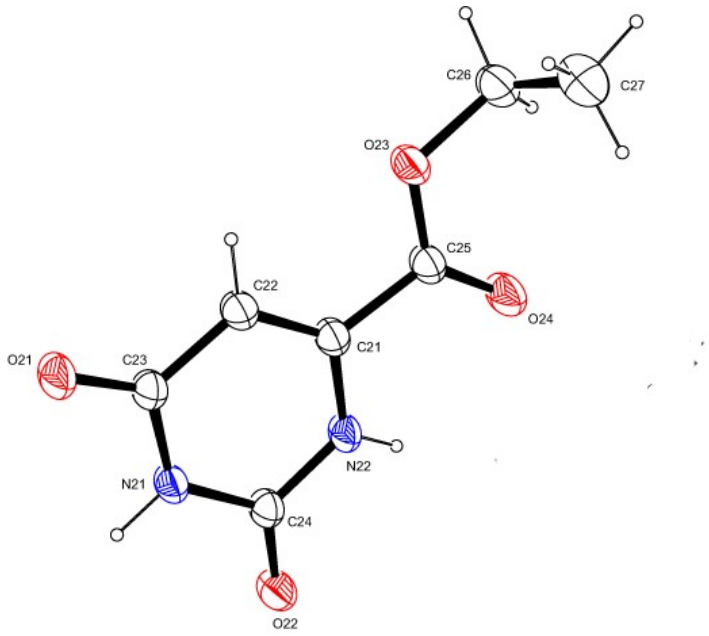

Fig 2.Crystal structure of compound **3**

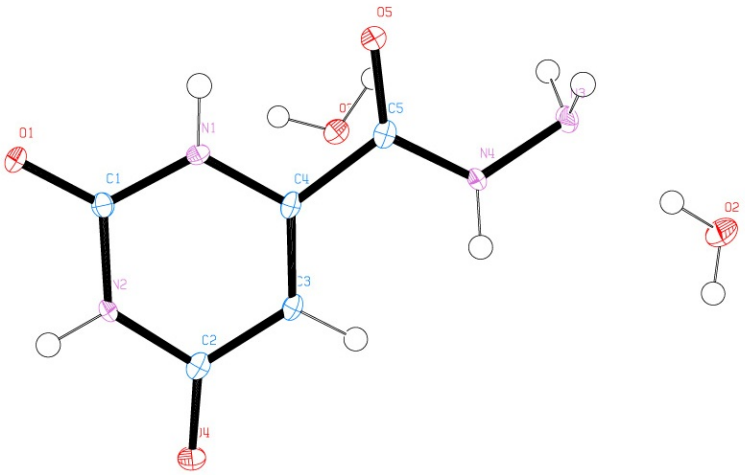

Fig 3. Crystal structure of compound **4**

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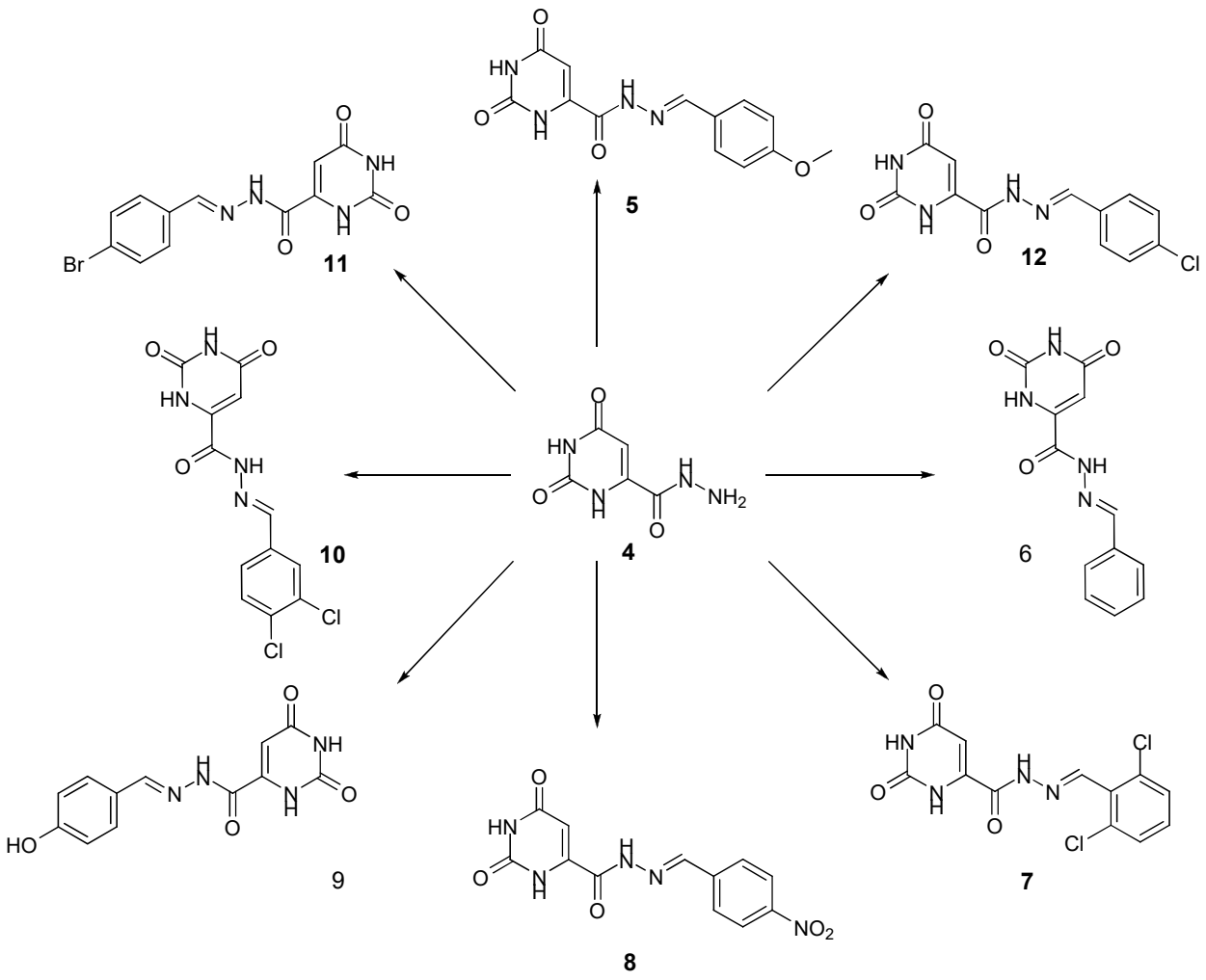

Scheme 2. General synthesis of **5**-**12**. Reagents and conditions: Ethanol, AcOH, reflux 5 h

Results:

Proliferation Method:

In this study we used the already established hTERT-MSC-CL1 (hMSC) cell lines, the cells were used in between passage 24–28. The cells were cultured in T75 culture flask (BD FalconTM, NJ, USA). Cells were monitored with inverted light microscope (Observer A1, Zeiss®, Gottingen, Germany). hMSC were grown in media composed of D-MEM (Gibco, Cat No. 41966052) upplemented with 10% FBS (Gibco, Cat No. 26140087), 1% pen/strep (10,000 units of penicillin

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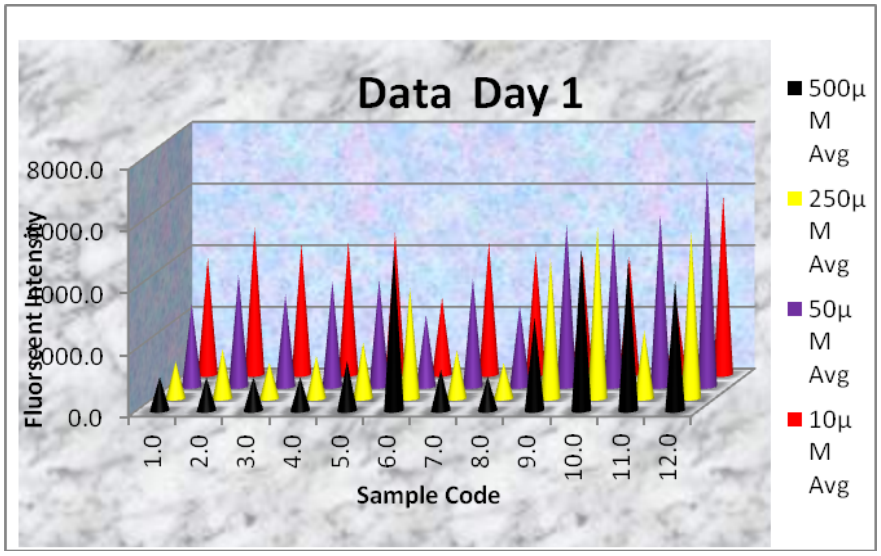

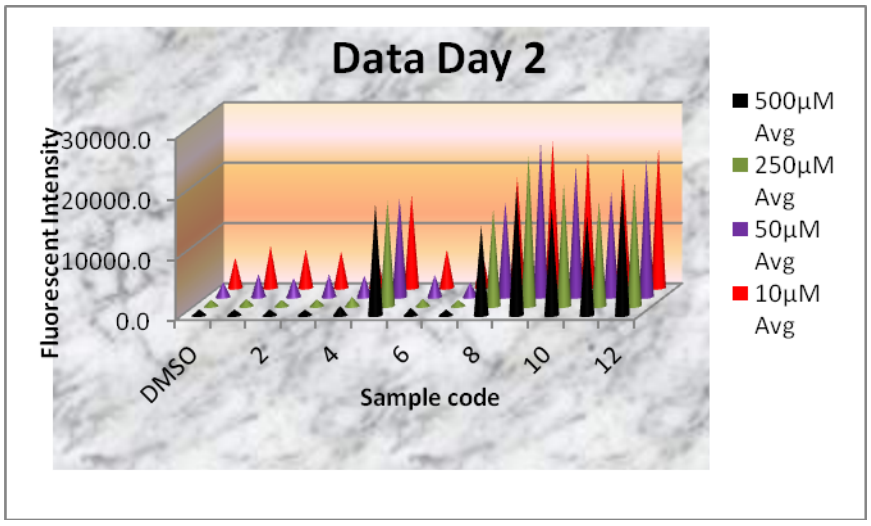

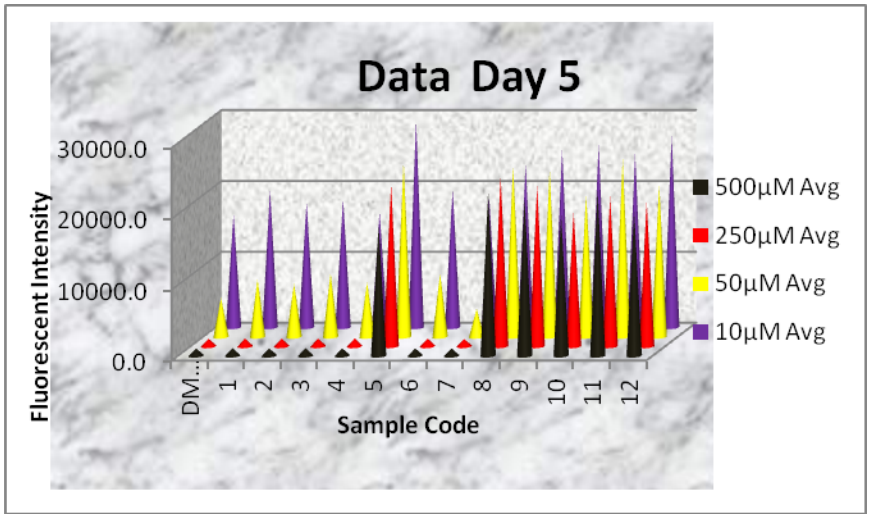

Graph 1: Proliferation data for days **1**, **2** and **5**.

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and 10,000 g of streptomycin/ml; Gibco, Cat No. 15140122) and 1% NEAA (X100; Gibco, Cat No. 11140035). After the cells reached 80–90% confluences in culture flasks, the cells were trypsinized and they were transferred in falcon tubes. Cells were counted using the Neubauer hemocytometer counting chamber (PAUL MARIENFELD GMBH & CO.KG.), the cells were seeded at a density of 0.01×106 cells per well of a 96-well tissue culture plate. Following day the diluted compounds were added to the cells at the desired concentration in triplicate, after additional 2 days the media is changed to normal growth media. The next day is designated as day 1 of proliferation.

Alamar Blue Cell Viability Assay:

Cell viability was measured using alamar blue assay according to the manufacturer's recommendations (AbD Serotec, Raleigh, NC, USA). In brief, we cultured cells in 96-well plates in 100μl of the appropriate medium and at the indicated time point, and 10μl of Alamar Blue substrate was added and plates were incubated in the dark at 37°C for 1h. Reading was subsequently taken using fluorescent mode (Ex 530nm/Em 590nm) using BioTek Synergy II microplate reader (BioTek Inc., Winooski, VT, US.

Chemistry:

All solvents and reagents were purchased from Aldrich Chemical Co and were used as obtained. IR spectra were recorded with a Perkin-Elmer spectrum BX FT-IR spectrometer using KBr pellets. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker instrument (500 and 125 MHz, respectively) in DMSO-d_6, Mass spectra were obtained on a JEOL JMS-700 mass spectrometer, ionization method was EI (70 eV). Melting points were measured with a Thermo Scientific 9100 apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed with fluorescent silica gel HF_{254} plates (Merck) and visualized under UV 254 and charring with the

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EtOH–H_2SO_4 (5:1) system. Merck silica gel 60 (230-400 mesh) was used for column chromatography.

General procedure for the synthesis of compound 2 and 3 (19).

To a solution of orotic acid **1** (2 mmol) in ethanol/butanol (50 mL) was added catalytic amount of HCl. The resulting mixture was refluxed for 10 h with stirring followed by the evaporation of solvent in vacuo. The resulting solid obtained was washed with cold water several times, recrystallized with ethanol-water mixture afforded compounds **2** and **3**.

Ethyl 2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carboxylate (3)

Yield 71 %, m.p. 176. Brown crystals, IR (KBr): ν = cm^{-1}. IR (KBr): 1715, 1730 (C=O), 2990 (NH), 3335 (OH). ^{1}HNMR (500.133 MHz, DMSO-d_6): δ = 1.30 (t, 3H, CH_3), 4.31 (m, 2H, CH_2), 6.05 (s, 1H, CH), 11.14 (br s, 1H, NH), 11.39 (br s, 1H, NH). ^{13}C NMR (125.76 MHz, DMSO-d_6): δ = 15.21, 62.2, 103.50, 142.09, 151.20, 163.05, 164.55. MS (70 eV): *m/z* (I_{rel}, %) 185 (71) $[M+H]^+$, 112 (100): calcd for $C_7H_8N_2O_4$ (184.05): HRMS: 184.0499.

General procedure for the synthesis of arylhydrazones 5, 7, 8, 10, 11 and 12

A mixture containing the orotic hydrazide **4** (1 mmol) and appropriate aromatic aldehyde (1.1 mmol) with catalytic amount of acetic acid was heated under reflux and stirring for 3 h in ethanol. After completion of the reaction, as indicated by TLC, the reaction mixture was poured onto crushed ice and the solid separated was filtered under suction, washed with ice-cold water (50 mL), and subsequently dried to afford pure product.

(*E*)-N-(4-methoxybenzylidene)-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbohydrazide (5)

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Yield 64 %, m.p. 183 ° C. white solid, IR (KBr): ν = cm^{-1}. IR (KBr): 1720, 1723 (C=O), 3019 (NH), 3310 (OH). ^{1}HNMR (500.133 MHz, DMSO-d$_6$): δ = 3.82 (s, 3H, OCH$_3$), 5.94 (s, 1H, CH), 7.70 (d, 2H, *j* = 8.5 Hz, arom), 7.83 (d, 2H, *j* = 9.0 Hz, arom), 8.33 (s, 1H, CH), 10.20 (br s, 1H, NH), 10.77 (br s, 1H, NH), 11.26 (br s, 1H, NH). ^{13}C NMR (125.76 MHz, DMSO-d$_6$): δ = 55.84, 100.79, 114.92, 125.37, 126.67, 129.57, 130.45, 150.38, 151.10, 151.30, 160.96, 164.51. MS (70 eV): *m/z* (I_{rel}, %) 289 (39) [M+H]$^+$, 267 (25), 133 (100): calcd for C$_{13}$H$_{12}$N$_4$O$_4$ (288.09): HRMS: 288.0819.

(*E*)-N'-(2,6-dichlorobenzylidene)-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbohydrazide (7)

Yield 69 %, m.p. 211 ° C. light yellow solid, IR (KBr): ν = cm^{-1}. IR (KBr): 1718, 1723 (C=O), 3029 (NH), 3290 (OH). ^{1}HNMR (500.133 MHz, DMSO-d$_6$): δ = 6.16 (s, 1H, CH), 7.49-7.63 (m, 3H, arom), 7.99 8.62 (s, 1H, CH), 11.03 (br s, 1H, NH), 11.37 (br s, 1H, NH), 12.38 (br s, 1H, NH). ^{13}C NMR (125.76 MHz, DMSO-d$_6$): δ = 101.29, 129.71 (2×), 130.18, 132.78, 133.11, 134.64, 137.63, 148.91, 157.08, 164.46, 167.07. MS (70 eV): *m/z* (I_{rel}, %) 327 (39) [M+H]$^+$, 292 (25), 170 (100): calcd for C$_{12}$H$_8$Cl$_2$N$_4$O$_3$ (326.00): HRMS: 326.0736.

(*E*)-N-(4-nitrobenzylidene)-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbohydrazide (8)

Yield 69 %, m.p. 169 ° C. white solid, IR (KBr): ν = cm^{-1}. IR (KBr): 1715, 1723 (C=O), 3039 (NH), 3290 (OH). ^{1}HNMR (500.133 MHz, DMSO-d$_6$): δ = 5.94 (s, 1H, CH), 7.83 (s, 1H, arom), 8.34 (m, 3H, CH, 2 × arom), 8.74 (s, 1H, arom), 10.12 (br s, 1H, NH), 10.72 (br s, 1H, NH), 11.26 (br s, 1H, NH). ^{13}C NMR (125.76 MHz, DMSO-d$_6$): δ = 103.0, 123.16, 131.16 (2×), 134.91 (2×), 144.20, 149.51, 151.33, 151.90, 164.16, 167.63. MS (70 eV): *m/z* (I_{rel}, %) 304 (41) [M+H]$^+$, 298 (78), 176 (100): calcd for C$_{12}$H$_9$N$_5$O$_5$ (303.06): HRMS: 303.0541.

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(*E*)-N-(3,4-dichlorobenzylidene)-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbohydrazide (10)

Yield 55 %, m.p. 157 ° C. white solid, IR (KBr): ν = cm^{-1}. IR (KBr): 1722, 1723 (C=O), 3031 (NH), 3290 (OH). ^{1}HNMR (500.133 MHz, DMSO-d$_6$): δ = 5.99 (s, 1H, CH), 7.73 (m, 2H, arom), 7.99 (s, 2H, arom), 8.38 (s, 1H, CH), 11.03 (br s, 1H, NH), 11.27 (br s, 1H, NH), 12.33 (br s, 1H, NH). ^{13}C NMR (125.76 MHz, DMSO-d$_6$): δ = 101.17, 127.55, 129.44, 130.60, 132.27, 133.36, 134.74, 135.61, 147.78, 157.67, 164.48, 166.82. MS (70 eV): *m/z* (I_{rel}, %) 289 (39) [M-Cl]$^+$, 267 (25), 133 (100): calcd for $C_{12}H_8Cl_2N_4O_3$ (326.00): HRMS: 326.0317.

(*E*)-N-(4-bromobenzylidene)-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbohydrazide (11)

Yield 59 %, m.p. 177 ° C. white solid, IR (KBr): ν = cm^{-1}. IR (KBr): 1717, 1725 (C=O), 3050 (NH), 3300 (OH). ^{1}HNMR (500.133 MHz, DMSO-d$_6$): δ = 6.11 (s, 1H, CH), 7.65 (d, 2H, *j* = 8.5 Hz, arom), 7.83 (d, 2H, *j* = 8.5 Hz, arom), 8.37 (s, 1H, CH), 8.71 (br s, 1H, NH), 10.25 (br s, 1H, NH), 11.25 (br s, 1H, NH). ^{13}C NMR (125.76 MHz, DMSO-d$_6$): δ = 99.83, 127.41, 129.73, 130.69, 131.86, 132.10 132.50, 145.33, 150.88, 151.20, 164.56, 162.07. MS (70 eV): *m/z* (I_{rel}, %) 337 (30) [M+H]$^+$, 211 (90), 157 (28): calcd for $C_{12}H_9BrN_4O_3$ (335.99): HRMS: 335.9701.

(*E*)-N-(4-chlorobenzylidene)-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbohydrazide (12)

Yield 63 %, m.p. 141 ° C. white solid, IR (KBr): ν = cm^{-1}. IR (KBr): 1715, 1730 (C=O), 2990 (NH), 3335 (OH). ^{1}HNMR (500.133 MHz, DMSO-d$_6$): δ = 6.10 (s, 1H, CH), 7.77 (d, 2H, *j* = 8.5 Hz, arom), 7.90 (d, 2H, *j* = 8.2 Hz, arom), 8.38 (s, 1H, CH), 8.71 (br s, 1H, NH), 10.13 (br s, 1H, NH), 11.26 (br s, 1H, NH). ^{13}C NMR (125.76 MHz, DMSO-d$_6$): δ = 101.04, 128.97 (2x), 129.53 (2x), 130.50, 133.09, 145.04, 149.20, 151.28, 161.05, 164.48. MS (70 eV): *m/z* (I_{rel}, %) 276 (90) [M-OH]$^+$, 247 (45), 110 (100): calcd for $C_{12}H_9ClN_4O_3$ (292.04): HRMS: 292.0390.

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X-ray Crystallographic Study of Compound 2. Crystals of compound **2** were obtained by crystallization from ethanol-water (3:1) by allowing slow solvent evaporation. Crystallographic data for the structure of compound **2** have been deposited at the Cambridge Crystallographic Data Center (deposit CCDC 1483501).

X-ray Crystallographic Study of Compound 3. Brown needles of compound **3** were obtained by crystallization from ethanol-water by allowing slow solvent evaporation. Crystallographic data for the structure of compound **3** have been deposited at the Cambridge Crystallographic Data Center (deposit CCDC 150815).

X-ray Crystallographic Study of Compound 4. Crystals of compound **4** were obtained by crystallization from hot ethanol and by allowing slow solvent evaporation. The compound was crystallized with two molecules of water in crystal lattice. Crystallographic data for the structure of compound **4** have been deposited at the Cambridge Crystallographic Data Center (deposit CCDC 1483500).

Discussion:

Stem cells are characterized by their extensive capability to self-renewal and differentiate into at least one, and usually more, mature cell types in presence of appropriate signals or small molecules which promote stem cells maintenance and specific differentiation [19, 20]. The current paper is focused on the synthesis of heterocyclic molecules specially modulated to enhance cell differentiation in mesenchymal stem cells.

Owing to low solubility in organic solvents, orotic acid has never been a choice as starting material for chemists therefore; very few reports are available on the synthesis of its analogues. To resolve this problem, esterification of orotic acid was carried out in butanol following the

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reported procedure. The product obtained was recrystallized in ethanol-water (3:1) at room temperature to obtain crystals suitable for x-ray crystallography for compound **2** (**fig 1**). However, the cumbersome workup, low yield and toxicity restrict the wide application of this solvent. Considering the limitations of the reported methods, the need for the development of new and efficient method is strongly desirable. Therefore, ethanol is used along with catalytic amount of HCl afforded **3** in good yield. The solid obtained was recrystallized in ethanol-water mixture to afford the crystals suitable for x-ray crystallography for compound **3** (**fig 2**). The ethyl ester was reacted with hydrazine hydrate in refluxing ethanol yielding hydrazide **4**[21] in 67% yields. Comparing the result obtained, the same reaction was carried out with butyl ester of orotic acid but the yield was significantly low with longer reaction time. Yellow crystals of compound **4** (**fig 3**) suitable for X-ray crystallography were obtained by slow evaporation from ethanol at room temperature. Starting material **4** was than subjected through a series of acid catalyzed condensation reaction with various substituted aromatic aldehyde afforded target compounds in excellent yields. All the compounds **2-12** were isolated as (*E*)-isomers which was confirmed by gated-decoupling (GD) measurements. In the ^{1}H NMR spectrum of synthesized molecules, signal of pyrimidine CH appears at the characteristic position, moreover aromatic protons appeared in their respective regions. The IR spectra of all the synthesized compounds showed spectral bands in the regions 3050-3350 (NH), 1710-1723 (C=O) and 1600-1680 (C=O) typical of a uracil ring. The mass and HRMS data are also in accord with the proposed structures of compounds **2-12**.

All the synthesized compounds were evaluated on human bone marrow mesenchymal stem cells (hMSCs), and the results are collected in Table **1**, **2** and **3**. Cells were treated with the test compounds at concentrations ranging from 5.0 to 50 μg/mL in triplicate for 48 h. Moreover, the

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cells were investigated for additional three days to ensure the effect of these compounds on hMSCs. It was clearly observed that after day 1 at high concentration (500 µM) almost every compound including DMSO had slowed their proliferation completely except for sample **1**, **6** and **9**. This effect was more prominent at lower concentrations where the same compounds had significantly higher proliferation rate then the other compounds and DMSO (**Graph 1**). However at lower concentrations other compounds also showed significant up regulation such as, at 250 µM: **1** and **3**, and at 10 µM sample **2** also showed significant difference, it was only **7** which did not have any effect on proliferation at day 1. Same pattern was followed on day 2 also, all the compounds at the highest concentration (500 µM) slowed the proliferation however **1**, **6** and **9** still had significant higher proliferation rate. And at lower concentrations 50 and 10 µM beside **7** all compounds showed significant up regulation of proliferation (**Graph 1**). However, at day 5 only **1** was significantly higher at the highest concentration and **7** showed no change or lower proliferation at 50 µM (**Graph 1**). Other compounds exhibit less significant effect on proliferation which shows that substituent's plays a role in stem cell proliferation.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

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For synthesis of compounds 2, 4, 6 and 9, please check reference 21 and references cited therein.

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