Monastrol derivatives: in silico and in vitro cytotoxicity assessments

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Abstract

Background and purpose: Cancer is the leading cause of death in today's world, therefore the efforts to achieve anticancer drugs with higher potency and fewer side effects have always been conducted by researchers in the field of pharmaceutical chemistry. Monastrol, a cytotoxic small molecule, from dihydropyrimidinone scaffold, is an inhibitor of the kinesin-5 protein. So, efforts to identify more derivatives of this molecule have been of interest.

Experimental approach: Some of monastrol's analogs as Eg5 inhibitors with different substitution patterns were analyzed, synthesized, and their cytotoxic effects were evaluated on MCF-7 and HeLa cancerous cells in vitro using the MTT assay. The structure-activity relationship (SAR) was studied in silico by molecular docking.

Findings / Results: Among all proposed structures, in ducking study, those with hydrophobic moieties on the C2-N3 region, those with a hydroxyl group on the phenyl on C4 position, and those with a carboxylic group on C5 were the best candidates. In vitro studies, on the other side, emphasized that monastrol still was the most potent derivative. Another finding was the more moderate activity of synthesized compounds on the HeLa cell compared to the MCF-7 cell line. During different challenges for substitution at 5-position, some earlier reports around the dihydropyrimidinone reactions were questioned. It seems that the change at the position 5 is not merely accessible, as earlier reports claimed. Also, we could not achieve any better cell cytotoxicity by the larger group in the thiourea region or position 5; nonetheless, it seems that the introduction of a methylene group at this position could be beneficial.

Conclusion and implications: The initial results of this study were valuable in terms of design and synthesis and will be useful for future investigations.

Keywords: Dihydropyrimidinones; Eg5 inhibitor; in vitro cytotoxicity; Molecular ducking; Monastrol.

INTRODUCTION

The biological activity of multi-potential dihydropyrimidinones compounds, their synthesis, and their related reactions have been always an interesting era in chemistry and medicinal chemistry field during the last decades (1-4). After the discovery of monastrol as an efficient inhibitor of kinesin-5 (KIF11 or kinesin Eg5) from this family, it has been the core of attention in several kinds of cancer research (5). Many efforts have been made to design and discover the more potent inhibitors of this family, or to expand the library for high throughput screening in biological projects (6-8).
The most studied mechanism by which monastrol disrupts cell cycle is allosteric inhibition of microtubule-stimulated ADP release from Eg5 (kinesin spindle protein presents in *Xenopus laevis*), followed by apoptotic signaling pathway which leads to efficient cell death. Based on attempts in developing structure-activity relationship (SAR) for Eg5 inhibitors, thione derivatives of dihydropyrimidinones are more potent than oxo ones, and the presence of hydroxyl group in 3’ position, make the molecules more potent than those having none, or with the other substitutes (9,10). Since different parts of a molecule interact differentially with the active site residues, which leads to the optimized binding energies, therefore, the accurate analysis of the enzyme active site enables us to design and synthesis more potent dihydropyrimidinones rather than monastrol. In this work, we tried to evaluate how the structural changes affect the cytotoxicity of some monastrol’s analogs. At first, we studied these changes *in silico*, and then we synthesized the monastrol's analogs to evaluate cytotoxicity profile *in vitro* on MCF-7 and HeLa cell lines.

**MATERIAL AND METHODS**

**Materials**

All of the materials and solvents were supplied commercially and used without further purification. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), hydroxy benzotriazole (HOBt), and anhydrous dimethylformamide (DMF) were purchased from Merck (Germany) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) from Sigma (USA). The RPMI-1640 culture medium was from PAA (Austria). The MCF-7 and HeLa cell lines were supplied by the Pasteur Institute of Iran. Reactions were monitored by thin layer chromatography (TLC) on pre-coated plates of kieselgel 60 F254, which purchased from Merck (Germany), and the spots were detected by exposing the dry plates to UV lamp at 254 nm. We obtained the melting points in open capillaries on a capillary melting point apparatus (Electrothermal 9200 UK), and the data were uncorrected. Proton nuclear magnetic resonance ($^1$H-NMR) and carbon-13 nuclear magnetic resonance ($^{13}$CNMR) spectra were recorded by NMR spectrophotometer (400 MHz, AC-80, Bruker Biospin, Germany) in deuterated dimethyl sulfoxide (DMSO-d6) and CDCl$_3$ as solvents. Chemical shifts $\delta$ were reported in parts per million downfield from tetramethylsilane. Infrared spectra (IR) were recorded on Rayleigh, WQF-510/520, spectrophotometer (China), using KBr disc, and the results were reported in cm$^{-1}$.

**Docking procedure**

Molecular docking of thirteen proposed compounds (Fig. 1) with Eg5 binding pocket was analyzed using protein-ligand docking (11). For this purpose, the crystal structure of the kinesin Eg5 in complex with monastrol (protein data bank (PDB) ID: 1Q0B) was retrieved from the PDB. The co-crystallized monastrol, and all of the irrelevant compartments were removed using Accelrys DS visualizer 3.5 (Studio D, Insight I. Accelrys Software Inc. San Diego, CA 2009; 92121) (12). All missing parts of the active site were merged to their proper places using AutoDock tools (13). For ligand preparation, all structures were drawn and optimized by the MM+ force field and PM3 semi-empirical techniques with the aid of Hyperchem software (Hyperchem, molecular modeling system. Hypercube, Inc., and Auto Desk, Inc.). The partial charges of atoms were calculated using the Gasteiger-Marsili procedure (13). Polar hydrogen atoms of the compounds were added while nonpolar hydrogens were merged. In all dockings, a grid map of $60 \times 60 \times 60$ points with 0.375 Å spacing between grid points was applied. Since the position of the co-crystallized monastrol within the binding site of Eg5 was known, we centered the grid box at the centroid of bounded monastrol in the active site (grid box center coordinate: X: 41.501; Y: 15.727; Z: 48.857), so that roughly encompasses the center of the Eg5 binding pocket. For each ligand, 100 independent docking runs were carried out employing the Lamarckian genetic algorithm (LGA) (11). The factors for LGA were defined as follows: a maximum number of $2.5 \times 106$ energy evaluations; a maximum number of generations of 27000; mutation and crossover rates of 0.02 and 0.8, respectively.
AutoDock Tools were used to generate both grid and docking parameter files. Finally, the free binding energies and inhibition constants (Ki) were calculated. We validated this procedure by the extraction of the monastrol from X-ray complex and redocking it and then superimposing the best-obtained pose with its X-ray counterparts in the 1Q0B structure. The docking poses results were analyzed with AutoDock tools and DS visualizer 3.5.
General chemical procedure for the synthesis of compounds (monastrol-M12)

Synthesis of ethyl 1,2,3,4-tetrahydro-4-(3-hydroxyphenyl)-6-methyl-2-thioxopyrimidine-5-carboxylate (monastrol) and ethyl 1,2,3,4-tetrahydro-4-(3-methoxyphenyl)-6-methyl-2-thioxopyrimidine-5-carboxylate (M1) (4)

A solution of ethyl acetoacetate (60 mmol), aldehyde (40 mmol), and thiourea (60 mmol) in the minimum amount of ethanol (10-15 mL) was prepared. In the presence of the catalytic amount of p-toluenesulfonic acid (PTSA, 6 mmol), the medium was refluxed for 6-8 h. After the completion of the reaction, the mixture was cooled, and 10 mL of cold ethanol was added to it and kept cold overnight. White to yellow precipitates were filtered and rewashed with cold water and ethanol. In the case of monastrol, the product was further purified by recrystallization from ethyl acetate.

Synthesis of ethyl 3,5-dihydro-5-(3-hydroxyphenyl)-7-methyl-3-oxo-2H-thiazolo[3,2-a]pyrimidine-6-carboxylate (M2) and ethyl 3,5-dihydro-5-(3-methoxyphenyl)-7-methyl-3-oxo-2H-thiazolo[3,2-a]pyrimidine-6-carboxylate (M3) (14)

A mixture of compound monastrol or M1 (2 mmol), methyl bromoacetate (2 mmol), and anhydrous sodium acetate (400 mg) was refluxed in anhydrous ethanol for 5 h. The solvent was evaporated under vacuum, and the product was recrystallized from ethyl acetate.

Synthesis of ethyl 1,6-dihydro-6-(3-hydroxyphenyl)-1,4-dimethyl-2-(methylthio) pyrimidine-5-carboxylate (M4) and ethyl 1,6-dihydro-6-(3-methoxyphenyl)-1,4-dimethyl-2-(methylthio) pyrimidine-5-carboxylate (M5) (15)

To a solution of monastrol or M1 (2 mmol) in DMF (5 mL), potassium carbonate (4 mmol) and methyl iodide (4 mmol) was added, and the mixture was stirred for 7-10 h in room temperature. After completion of the reaction, the mixture was poured into cold water, and precipitates were recrystallized from ethanol.

Synthesis of 1,2,3,4-tetrahydro-4-(3-hydroxyphenyl)-6-methyl-2-thioxopyrimidine-5-carboxylic acid (M6) (16)

A solution of NaOH (4 mmol) in 1 mL water was added to a solution of compound monastrol (2 mmol) in 5 mL methanol. The mixture was heated and stirred at 60 to 62 °C for 8 h. After the completion, the solvent was removed under vacuum. The residue was then added to 25 mL ice-cold water and was extracted with chloroform (3 × 10 mL) to remove the unreacted ester. The aqueous layer was acidified to pH 2 using 10% v/v HCl and extracted with ethyl acetate (3 × 15 mL). The organic layer was separated, dried over anhydrous sodium sulfate, and concentrated to give the crude acid. The crude acid was purified by PTLC.

Synthesis of ethyl 3-(4-bromophenyl)-8,8a-dihydro-5-(3-methoxyphenyl)-7-methyl-5H-thiazolo[3,2-a]pyrimidine-6-carboxylate (M7) (17)

A mixture of compound M1 (4 mmol), p-bromo phenacyl bromide (4 mmol), and PTSA (10 mol%) in glacial acetic acid stirred in 100 °C for 4 h, followed by fast reflux at 120 °C about 2 h. After completion of the reaction, the vessel was kept overnight at room temperature. The mixture was added into stirring ice-cold water, and the precipitates were filtered, washed with water and petroleum ether to obtain pure product. Upon the neutralizing of the filtrate (NaHCO3 5%), we got the next portion of precipitates, which was purified the same as before.

Synthesis of (17E)-N’-(4-chlorobenzylidene)-1,2,3,4-tetrahydro-4-(3-hydroxyphenyl)-6-methyl-2-thioxopyrimidine-5-carboxyhydrazide (M8) (18)

4-Chlorobenzaldehyde (5 g) was added to a stirred mixture of hydrazine hydrate (99% in water, 7 mL) and toluene (10 mL) over 0.5 h at 50 °C. After an additional 1 h at 50 °C, the mixture was cooled to room temperature and diluted with water (5 mL). The organic layer was separated and concentrated to dryness in a vacuum. The residue was slurried in heptane (30 mL) and filtered. The dried yellow powder (Hyd1), brought to reaction with activated acid, immediately. Compound M6 (0.38 mmol) was dissolved in anhydrous DMF (10 mL). To this solution was added EDC, HOBt (both 0.42 mmol), Hyd1 (0.38 mmol), and the mixture
stirred at room temperature for 4 h. The medium was concentrated under a vacuum. We added ethyl acetate and hexane mixture to the remaining, and the resulting precipitants were purified by PTLC.

**Synthesis of 1-(1,2,3,4-tetrahydro-4-(3-methoxyphenyl)-6-methyl-2-thioxopyrimidin-5-yl)ethanone (M9)** (19)

A solution of 3-methoxybenzaldehyde (5 mmol), acetylacetone (7.5 mmol), thiourea (6.5 mmol), and two drops of concentrated HCl in absolute ethanol (10 mL) was stirred at 50-55 °C for 20 h. One drop of concentrated HCl was added occasionally. After the completion of the reaction, it was kept at 0 - 4 °C overnight, the precipitate was filtered and recrystallized from ethanol to afford M9 as a yellowish solid.

**Synthesis of (2E)-1-(1, 2, 3, 4-tetrahydro-4-(3-methoxyphenyl)-6-methyl-2-thioxopyrimidin-5-yl)-3-(4-chlorophenyl)prop-2-en-1-one (M10)** (20)

A mixture of M9 (2 mmol) and the 4-chlorobenzaldehyde (2 mmol) in 10% ethanolic sodium hydroxide solution (5 mL) was stirred at room temperature for 24 h following by 1 h reflux. The mixture was cooled, poured onto ice water (20 mL), and neutralized with 37% HCl (0.5 mL). The precipitates were filtered, dried, and recrystallized from aqueous DMF to give the final product as a yellow powder.

**Synthesis of 2-(1,2,3,4-tetrahydro-4-(3-hydroxyphenyl)-6-methyl-2-thioxopyrimidin-5-yl) acetic acid (M11) and 2-(1,2,3,4-tetrahydro-4-(3-methoxyphenyl)-6-methyl-2-thioxopyrimidin-5-yl) acetic acid (M12)** (21)

3-Hydroxy benzaldehyde/ 3-methoxy benzaldehyde (10 mmol), thiourea (10 mmol), and PTSA (1 mmol) were dispersed in 10 mL ethanol, and to this, levulinic acid (10 mmol) was added. The mixture was stirred at 80 °C for 24 h. Ethanol was removed under vacuum, and the remaining sticky product was dissolved in 10 mL water (pH adjusted to 2), and 15 mL ethyl acetate. The organic layer then separated dried over anhydrous sodium sulfate, and removed; the pure product was obtained by recrystallization in glacial acetic acid.

**in vitro cytotoxicity assay**

The cell lines (MCF-7 and HeLa) were cultured in a complete medium (RPMI 1640 medium supplemented with 10% FBS, 1% antibiotic, penicillin/streptomycin). Stock solutions of compounds with a concentration of 10000 µM were prepared in sterile DMSO. Before treatment, the concentrations of 100, 500, 1000, 1500, 2000, and 3000 µM were prepared using the complete medium. Cells were incubated in 96-well plates containing 170 µL of complete medium per well and at a density of 5000 cells/well. After 24 h incubation at standard conditions and complete cell adhesion, 20 µL of each concentration was added to the assigned wells (the maximum concentration of DMSO in each well was justified at 2%). The final concentrations were 10, 50, 100, 150, 200, and 300 µM. Positive control wells (other than monastrol, and for better monitoring of cell line sensitivity) containing 2 mg/mL paclitaxel (Hangzhou, China), prepared in the same manner, along with negative control and blank wells. After 48 h incubation, the supernatant medium of each well, containing soluble chemicals and dead cells, replaced with 100 µL phenol red-free RPMI. Then MTT solution (10 µL, 5 mg/mL in RPMI) was added, and plates were incubated for 3 h, the medium of each well was carefully eliminated, and 100 µL DMSO was added to each well to remove formazon, and the absorbance was measured using a microplate reader at 570 nm. The cell viability was calculated using the following equation:

\[
\text{Cell survival (\%)} = \left( \frac{Tm - Bm}{Cm - Bm} \right) \times 100
\]

where Tm, Bm, and Cm represent mean absorbance of the treatment, blank, and negative control, respectively (22). The above assay was repeated in triplicate, and final mean cell survival used to calculate IC50 of each compound using excel software.

**Statistical analysis**

To compare the statistical differences between groups, one way ANOVA was run in SPSS software, and followed up with post-hoc tests with significant level set at 0.05. Data are presented as mean ± SEM.
RESULTS

Docking studies
The results of this part, including the values for free binding energy ($\Delta G_{\text{bind}}$) and other details for the best-docked positions in the active site of Eg5, are provided in Tables 1 and 2. The molecular interactions of the best redocked pose of monastrol, as well as the best-docked poses of M4 and M11 within the active site, are depicted in Fig. 2.

Chemistry
All of the chemical reactions are shown in Fig. 1, and the proposed mechanism of ring cleavage in the reaction with hydrazine is provided in Fig. 3.

Furthermore, all of spectral details for various derivatives are as follow:
monastrol: Yield: 65%; pale yellow powder; melting point (MP): 185-187 °C (lit. 185-186 °C (24)); C14H16N2O3S; IR $\nu_{\text{max}}$ cm$^{-1}$ 3308 (broad band: O-H and N-H str.), 3184 (C-H str. aromatic), 2981 (C-H str. aliphatic), 1667 (C=O str.), 1574 ( Ar. C=C str.), 1471 (C-H bend.), 1279 (C-N str.), 1191 (C=S str.);

\begin{align*}
\text{1HNMR (DMSO-d6)} \delta \text{ppm:} & \quad 10.39 (s, 1H, N-H), \\
& \quad 9.69 (d, 1H, N-H, J \approx 2.80 \text{ Hz}), \\
& \quad 9.53 (s, 1H, O-H), \\
& \quad 7.19 (m, 1H, H Ar.), \\
& \quad 6.70-6.73 (m, 3H, H Ar.), \\
& \quad 5.17 (d, 1H, J = 2.80 \text{ Hz}), \\
& \quad 4.09 (m, 2H, -OCH2CH3), \\
& \quad 2.35 (s, 3H, Ar-CH3), \\
& \quad 1.20 (t, 3H, -OCH2CH3).
\end{align*}

M1: Yield: 78%; pale yellow powder; MP: 167-168 °C; C12H18N2O3S; IR $\nu_{\text{max}}$ cm$^{-1}$: 3298 (broad band; O-H and N-H str.), 3180, 3102, 2906, 1661 (C=O str.), 1595 (N-H str.), 1571 (C=C aromatic), 1459, 1254, 1189 (C=S str.);

\begin{align*}
\text{1HNMR (DMSO-d6)} \delta \text{ppm:} & \quad 10.35 (s, 1H, N-H), \\
& \quad 9.65 (d, 1H, O-H, J = 2.4 \text{ Hz}), \\
& \quad 7.26-7.30 (m, 1H, H Ar.), \\
& \quad 6.76-6.88 (m, 3H, H Ar.), \\
& \quad 5.16 (d, 1H, H4, J = 2.4 \text{ Hz}), \\
& \quad 4.01-4.06 (m, 2H, -OCH2CH3), \\
& \quad 2.29 (s, 3H, Ar-CH3), \\
& \quad 1.13 (t, 3H, -OCH2CH3), \\
& \quad 2.35 (s, 3H, Ar-CH3), \\
& \quad 1.20 (t, 3H, -OCH2CH3).
\end{align*}

M2: Yield: 78%; white powder; MP: 210-212 °C (lit. 219 °C(25)); C15H18N2O3S; IR $\nu_{\text{max}}$ cm$^{-1}$: 3298 (broad band: O-H and N-H str.), 3180, 3102, 2906, 1661 (C=O str.), 1595 (N-H str.), 1571 (C=C aromatic), 1459, 1254, 1189 (C=S str.);

\begin{align*}
\text{1HNMR (DMSO-d6)} \delta \text{ppm:} & \quad 10.35 (s, 1H, N-H), \\
& \quad 9.65 (d, 1H, O-H, J = 2.4 \text{ Hz}), \\
& \quad 7.26-7.30 (m, 1H, H Ar.), \\
& \quad 6.76-6.88 (m, 3H, H Ar.), \\
& \quad 5.16 (d, 1H, H4, J = 2.4 \text{ Hz}), \\
& \quad 4.01-4.06 (m, 2H, -OCH2CH3), \\
& \quad 2.29 (s, 3H, Ar-CH3), \\
& \quad 1.13 (t, 3H, -OCH2CH3).
\end{align*}

Table 1. Docking results of the proposed compounds and reference ligand (monastrol) into the Eg5 active site (pdb code: 1Q0B).

| Derivatives | $\Delta G_{\text{bind}}$ (kcal/mol) | VHDE (kcal/mol) | EE (kcal/mol) | IE (kcal/mol) | IC (µM) |
|-------------|------------------------------------|----------------|--------------|---------------|--------|
| M1          | -6.34                              | -7.81          | -0.01        | -7.83         | 22.67  |
| M2          | -6.09                              | -7.86          | -0.13        | -7.99         | 34.18  |
| M3          | -5.67                              | -7.06          | 0.19         | -6.87         | 69.55  |
| M4          | -8.01                              | -9.79          | 0.00         | -9.8          | 1.35   |
| M5          | -6.24                              | -7.48          | 0.05         | -7.43         | 26.76  |
| M6          | -7.16                              | -8.47          | -0.18        | -8.65         | 5.63   |
| M6-1        | -6.90                              | -8.34          | -0.06        | -8.40         | 8.70   |
| M7          | -3.05                              | -4.70          | -0.14        | -4.84         | 5810   |
| M8          | -6.51                              | -7.21          | -0.2         | -7.41         | 16.8   |
| M9          | -7.29                              | 8.68           | -0.4         | -9.08         | 4.57   |
| M10         | -5.53                              | -6.66          | 0.24         | -6.43         | 88.22  |
| M11         | -8.63                              | -10.10         | -0.02        | -10.12        | 0.473  |
| M12         | -6.54                              | -8.34          | 0.00         | -8.33         | 16.01  |
| Monastrol   | -6.67                              | -8.06          | -0.1         | -8.16         | 12.95  |

VHDE, Vander Waals H-bond desolvatiation energy; EE, electrostatic energy; IE, intermolecular energy; IC, inhibitory concentration.
Table 2. Details of interaction between the docked compounds and the Eg5 binding site residues (pdb code: 1Q0B).

| Derivatives | Amino acids in hydrophobic interaction | Amino acids in hydrogen bond interaction | Amino acids in cation-π interaction | Amino acids in electrostatic interaction |
|-------------|---------------------------------------|----------------------------------------|-----------------------------------|----------------------------------------|
| M1          | Arg119, Trp127, Ile136, Pro137, Leu160, Tyr211, Leu214, Phe239 | Gly117, Glu116                         | -                                 | Arg221, Glu118, Glu116                 |
| M2          | Thr112, Arg119, Ala133, Ile136, Pro137, Leu160, Tyr211, Leu214, Ala218, Thr213 | Gly117 | Arg221 | Arg221, Glu116, Glu116 |
| M3          | Arg119, Ile136, Leu160, Tyr211, Leu214, Ala218, Phe239 | Arg221 | - | Arg221, Glu116, Glu116 |
| M4          | Thr112, Glu118, Arg119, Ile136, Tyr211, Ala133, Trp127, Pro137, Ile136, Leu160, Leu214, Ala218 | Glu116 | Arg221 | Arg221, Glu116 |
| M5          | Arg119, Trp127, Ile136, Pro137, Leu160, Tyr211, Leu214, Phe239 | - | - | Arg221, Glu118, Glu116 |
| M6          | Met115, Leu132, Ala133, Ile136, Pro137, Tyr211, Leu214, Ala218 | Glu118 | Arg119 | Glu116, Arg119, Glu118 |
| M6-1        | Ile136, Pro137, Leu160, Tyr211, Leu214, Ala218 | Glu116 | Arg221 | Arg119, Glu116, Arg221 |
| M7          | Gly117, Ile136, Leu214, Ala218, Pro136 | Arg221 | - | - |
| M8          | Arg119, Ala133, Ile136, Pro137, Tyr211, Leu214 | Leu214, Gly117 | - | Glu118, Glu116, Arg221 |
| M9          | Leu132, Ala133, Ile136, Pro137, Tyr211, Leu214, Ala218 | - | - | Glu118, Glu116, Arg119 |
| M10         | Ala133, Ile136, Pro137, Tyr211, Leu214, Ala218 | Glu116 | - | Glu118, Glu116, Arg119, Arg221 |
| M11         | Ser120, Trp127, Leu132, Ala133, Gly134, Ile136, Pro137, Tyr211, Leu214, Ala218 | Glu118, Gly117 | Arg119 | Glu118, Glu116, Arg119 |
| M12         | Thr112, Ile136, Leu160, Tyr211, Leu214, Ala218 | Glu116 | Arg221 | Glu116, Arg119, Arg221 |
| Monastrol   | Gly117, Ser120, Trp127, Asp130, Leu132, Ala133, Gly134, Ile136, Pro137, Tyr211, Leu214, Glu215, Ala218 | Glu118, Glu116 | Arg119 | Glu118, Glu116, Arg119 |

M4: Yield: 80%; white fine powder; MP: 153-155 °C (lit. 150-152 °C (26)); C_{16}H_{20}N_{2}O_{3}S; IR v_{max} cm^{-1}: 3312 (O-H str.), 2932 (C-H str. aliphatic), 1654 (C=O str.), 1590 (N-H bend.), 1479, 1275 (C=N str.), 1182 (C=S str.); \textsuperscript{1}HNMR (DMSO-d$_6$) δ ppm: 9.55 (s, 1H, O-H), 7.29-7.33 (m, 1H, H Ar.), 6.82-6.84 (m, 3H, H Ar.), 5.41 (s, 1H, H4), 4.10-4.15 (m, 2H, -OCH$_2$CH$_3$), 3.11 (s, 3H, -N-CH$_3$), 2.66 (s, 3H, Ar-CH$_3$), 1.19 (t, 3H, -OCH$_2$CH$_3$).

M5: Yield: 70%; yellow crystals; MP: 161-162 °C; C_{17}H_{22}N_{2}O_{3}S; IR v_{max} cm^{-1}: 3381 (O-H str.), 3270 (N-H str.), 3172 (N-H str.), 3095, 1610 (C=O, acid), 1470, 1413, 1185 (C=S str.); \textsuperscript{1}HNMR (DMSO-d$_6$) δ ppm: 12.15 (bs, 1H, COOH), 10.39 (s, 1H, N-H), 9.69 (d, 1H, N-H, J = 1.20 Hz), 7.23-7.27 (m, 1H, H Ar.), 6.72-6.89 (m, 3H, H Ar.), 5.19 (d, 1H, H4, J = 1.2 Hz), 3.71 (s, 3H, -OCH$_3$), 2.27 (s, 3H, Ar-CH$_3$).

M6: Yield: 20%; white powder; MP: 168-170 °C (lit. 163-165 °C(16)); C_{12}H_{12}N_{2}O_{3}S; IR v_{max} cm^{-1}: 3381 (O-H str.), 3270 (N-H str.), 3172 (N-H str.), 3095, 1610 (C=O, acid), 1470, 1413, 1185 (C=S str.); \textsuperscript{1}HNMR (DMSO-d$_6$) δ ppm: 12.15 (bs, 1H, COOH), 10.39 (s, 1H, N-H), 9.69 (d, 1H, N-H, J = 1.20 Hz), 7.23-7.27 (m, 1H, H Ar.), 6.72-6.89 (m, 3H, H Ar.), 5.19 (d, 1H, H4, J = 1.2 Hz), 3.71 (s, 3H, -OCH$_3$), 2.27 (s, 3H, Ar-CH$_3$).

M7: Yield: 48%; yellow-brown powder; MP: 228-229 °C; C_{23}H_{23}BrN_{2}O_{3}S; IR v_{max} cm^{-1}: 3107 (N-H str.), 2959, 1706 (C=O str.), 1583 (Ar. C=C), 1530, 1484, 1260 (C=S str.); \textsuperscript{1}HNMR (DMSO-d$_6$) δ ppm: 7.82 (d, 2H, H Ar.), 7.56 (s, 1H, H Ar.), 7.36 (d, 2H, H Ar., J = 8.4 Hz), 7.19-7.23 (m, 1H, H Ar.), 6.87-6.89 (m, 1H, H Ar.), 6.49-6.51 (m, 1H, H Ar.), 6.36 (s, 1H, H Ar.), 6.11 (s, 1H, H4), 4.07-4.20 (m, 2H, -OCH$_2$CH$_3$), 3.63 (s, 3H, -OCH$_3$), 2.54 (s, 3H, Ar-CH$_3$), 1.18 (t, 3H, -OCH$_2$CH$_3$).
\[^{13}\text{C}NMR\ (100\ MHz,\ DMSO-d_6,\ \delta\ (ppm):\ 164.43,\ 161.24,\ 159.26,\ 141.05,\ 139.27,\ 132.29,\ 132.19,\ 130.57,\ 127.07,\ 124.82,\ 119.30,\ 115.01,\ 112.82,\ 112.30,\ 103.22,\ 70.24,\ 61.02,\ 59.41,\ 55.28,\ 18.43,\ 14.36.\]

**Hyd1**: Yield: 75\%; yellow crystal; MP: 65-66 \(^{\circ}\text{C};\ C_7H_7ClN_2;\ \[^1\text{H}\text{NMR\ (CDCl}_3\delta\ (ppm):\ 8.54\ (s, 1H, -CH=N-),\ 7.72\ (d, 2H, H Ar., J = 8.40\ Hz),\ 7.36\ (d, 2H, H Ar., J = 8.40\ Hz),\ 2.11\ (s, 2H, NH2).**

**Fig. 2.** (A1-C1) Best redocked pose of the monastrol, M11, and M4 in the Eg5 active site; (A2-C2) two dimensional diagram of the interaction between the redocked monastrol, M11, and M4 and the critical interacting amino acid residues of binding site. Blue dashed line shows hydrogen bonds and \(\pi\) cationic interaction is represented as an orange line. Figures are prepared using the Accelrys discovery studio visualizer program.
M8: Yield: 13%; yellow powder; MP: 126-128 °C; C_{19}H_{17}ClN_{4}O_{2}S; IR \nu_{\text{max}} \text{ cm}^{-1}: 3383 (broad band; O-H and N-H str.), 3186, 2963, 1657 (C=O str.), 1592 (C=C str. and N-H bend.), 1482, 1262 (C-N str.), 1189 (C=S str.); \textbf{^1}HNMR (DMSO-d_{6}, \delta \text{ ppm}): 10.38 (s, 1H, N-H), 9.69 (s, 1H, N-H, J = 2.40 Hz), 9.53 (s, 1H, O-H), 8.22 (s, 1H, -C=N-NH-CO-), 7.83 (s, 1H, -CH=N-), 7.63 (d, 2H, H Ar., J = 8.40 Hz), 7.51 (d, 2H, H Ar., J = 8.40 Hz), 7.19 (m, 1H, H Ar.), 6.71-6.73 (m, 3H, H Ar.), 5.15 (d, 1H, H4, J = 2.80 Hz), 2.35 (s, 3H, Ar-CH3); \textbf{^{13}}CNMR (100 MHz, DMSO-d_{6}, \delta \text{ ppm}): 178.1, 169.1, 158.2, 150.1, 143.3, 142.1, 130.5, 130.0, 129.4, 129.0, 117.4, 115.1, 113.8, 109.5, 52.9, 16.9.

M9: Yield: 85%; yellow powder; MP: 252-254 °C; C_{14}H_{16}N_{2}O_{2}S; IR \nu_{\text{max}} \text{ cm}^{-1}: 3402 (N-H str.), 3301 (O-H str.), 2981 (C-H str. aliphatic), 1660 (C=O str., 1505 (C-C), 1432 (C-C), 1373 (C-N), 1181 (C=S str.); \textbf{^1}HNMR (DMSO-d_{6}, \delta \text{ ppm}): 10.26 (s, 1H, N-H), 9.72 (d, 1H, N-H, J = 2.40 Hz), 7.11-7.15 (m, 1H, H Ar.), 6.65-6.68 (m, 3H, H Ar.), 5.15 (d, 1H, H4, J = 2.80 Hz), 2.35 (s, 3H, Ar-CH3), 2.14 (s, 3H, CH3-CO-).

M10: Yield: 70%; yellow powder; MP: 171-173 °C; C_{21}H_{19}ClN_{2}O_{2}S; IR \nu_{\text{max}} \text{ cm}^{-1}: 3408 (N-H str.), 3278, 2912, 1715 (C=O str.), 1617 (C-C), 1474, 1425, 1223 (C-S str.); \textbf{^1}HNMR (DMSO-d_{6}, \delta \text{ ppm}): 12.15 (bs, 1H, COOH), 10.35 (s, 1H, N-H), 9.65 (d, 1H, N-H, J = 2.80 Hz), 7.26-7.30 (m, 1H, H Ar.), 6.77-6.87 (m, 3H, H Ar.), 5.15 (d, 1H, H4, J = 2.80 Hz), 3.74 (s, 3H, -OCH3), 2.71 (s, 2H, -OCH2CH3), 2.30 (s, 3H, Ar-CH3), 2.14 (s, 3H, CH3-CO-).

M11: Yield: 27%; off white powder; decomposition point around 200 °C; C_{13}H_{14}N_{2}O_{3}S; IR \nu_{\text{max}} \text{ cm}^{-1}: 3279 (broad bands of O-H and N-H str.), 2985, 1700 (C=O str.), 1679 (C=O str.), 1479, 1455, 1229 (C-S str.); \textbf{^1}HNMR (DMSO-d_{6}, \delta \text{ ppm}): 12.10 (bs, 1H, COOH), 10.25 (s, 1H, N-H), 9.69 (d, 1H, N-H, J = 2.80 Hz), 7.07-7.11 (m, 1H, H Ar.), 6.77-6.79 (m, 3H, H Ar.), 5.19 (d, 1H, H4, J = 2.80 Hz), 2.85 (s, 2H, -CH2-CO-), 2.35 (s, 3H, Ar-CH3).

M12: Yield: 28%; pale yellow powder; MP: 127-129 °C; C_{14}H_{16}N_{2}O_{3}S; IR \nu_{\text{max}} \text{ cm}^{-1}: 3408 (N-H str.), 3278, 2912, 1715 (C=O str.), 1617 (C-C), 1474, 1425, 1223 (C-S str.); \textbf{^1}HNMR (DMSO-d_{6}, \delta \text{ ppm}): 12.15 (bs, 1H, COOH), 10.35 (s, 1H, N-H), 9.65 (d, 1H, N-H, J = 2.80 Hz), 7.26-7.30 (m, 1H, H Ar.), 6.77-6.87 (m, 3H, H Ar.), 5.15 (d, 1H, H4, J = 2.80 Hz), 3.74 (s, 3H, -OCH3), 2.71 (s, 2H, -OCH2CH3), 2.30 (s, 3H, Ar-CH3), 2.14 (s, 3H, CH3-CO-).

Fig. 3. Proposed decomposition mechanism of dihydropyrimidinone by hydrazinolysis, reported by Said et al. (23).
Evaluation of cytotoxic effect

All of the estimated IC50 values on MCF-7 and Hela cells are provided in Table 3.

Table 3. Evaluated IC50 values of monastrol’s derivatives for MCF-7 and HeLa cell lines and the ∆Gbind. The IC50 values are presented as mean ± SEM.

| Derivatives | IC50 values (MCF-7) | IC50 values (HeLa) | ∆G bind (kcal/mol) |
|-------------|---------------------|-------------------|-------------------|
| Monastrol   | 88 ± 23             | 111 ± 25          | -6.67             |
| M1          | 138 ± 6             | 160 ± 6           | -6.34             |
| M2          | 175 ± 20            | 224 ± 20          | -6.09             |
| M3          | 145 ± 3             | 232 ± 3           | -5.67             |
| M4          | 164 ± 21            | 220 ± 21          | -8.01             |
| M7          | 194 ± 30            | 246 ± 30          | -7.16             |
| M10         | 230 ± 63            | > 500             | -6.54             |
| M11         | 167 ± 1             | 270 ± 1           | -3.05             |
| M8          | 187 ± 5             | 225 ± 5           | -6.51             |
| M9          | 168 ± 15            | 228 ± 15          | -7.29             |
| M12         | 205 ± 13            | > 500             | -6.54             |

| Derivatives | IC50 values (MCF-7) | IC50 values (HeLa) | ∆G bind (kcal/mol) |
|-------------|---------------------|-------------------|-------------------|
| M11         | 167 ± 1             | 270 ± 1           | -3.05             |
| M8          | 187 ± 5             | 225 ± 5           | -6.51             |
| M9          | 168 ± 15            | 228 ± 15          | -7.29             |
| M10         | 230 ± 63            | 249 ± 63          | -5.53             |
| M11         | 194 ± 30            | 266 ± 30          | -8.63             |
| M12         | 205 ± 13            | > 500             | -6.54             |

DISCUSSION

In order to better rationalize SAR of the proposed monastrol’s analogs and gain insights into the binding mode of molecules within the motor domain of Eg5, molecular docking studies were conducted. Among available X-ray structures of the Eg5-monastrol complex, the structure (PDB ID: 1Q0B) with a high resolution (1.9 Å) was chosen (6, 27-29). The accuracy of the docking protocol was validated by removing the co-crystallized monastrol from the binding site of Eg5 and redocking into the active site. The results of the redocking indicated that the redocked conformer of monastrol adopted a similar binding mode to that seen in its parent crystal structure. The root mean square deviation (RMSD) value for this compound in comparison with its coordination in the crystal structure was 0.14. Based on Fig. 1A, the redocked monastrol have favorable hydrophobic with a relatively hydrophobic region within the active site surrounded by Trp127, Leu132, Ala133, Ile136, Pro137, Tyr211, Leu214, Glu215, Ala218 amino acid residues. The ligand-binding was further stabilized by favorable hydrogen bonds with crucial residues same as the original cocrystal monastrol. Moreover, for redocking analysis, with the same orientation as we see in the cocrystal structure, the 4-phenyl group was involved in a cation-π interaction with the residue of Arg119. According to these results, the moderate degree of hydrophobicity and the ability to establish hydrogen bonds with Glu118 and Glu116, can be considered as the prime determinants for the binding affinity of inhibitors to the active site. After the validation, as it can be seen from Table 1, all of the proposed derivatives were docked into the same binding site of the Eg5 with negative binding energy values. Previous studies using hydrogen-deuterium exchange and mass spectrometry coupled with directed mutagenesis on the motor domain of human Eg5 pointed out the importance of these interactions at monastrol's binding site (30). As Table 1 shows, the in-silico inhibition trend of proposed derivatives was as following; M11 > M4 > M9 > M6 > D2 > monastrol > M12 > M8 > M1 > M5 > M2 > M3 > M10 > M7.

Among all of derivatives, compounds M4 and M11 were particularly found to possess better binding affinity (ΔGbind: -8.63 and -8.01 Kcal/mol, respectively) than monastrol (ΔGbind: -6.67 Kcal/mol). The best-docked pose of M11 within the active site is depicted in Fig. 1B. As this figure shows, the compound displayed the same pattern of hydrogen bonding interaction with Glu118 as observed for monastrol (Fig. 1A). Moreover, the phenyl ring of the C4 position is situated in such a way that favorably made a π–cationic interaction with a positively charged amino group of the Arg119. Based on the previous reports, hydrophobic and electrostatic interactions of the ligand with Arg119 appear to be crucial for drug binding at the motor domain of human Eg5, therefore the Eg5 motor domain is remarkably tolerant of mutations at Arg119 (31). A comparison of the docking energy values of M11 (ΔGbind: -8.63 Kcal/mol) and M6 (ΔGbind: -7.16 Kcal/mol) indicated that the insertion of an additional methylene unit to the junction of the C5 and carboxylic acid side chain was in favor of binding affinity to the Eg5 active site. This result could be due to the increased flexibility of the C-5 carboxylic acid side chain that enables it to deeper access toward the
hydrophilic residues. For M11, more effective hydrophobic interactions may be achieved with Gly134, Pro137, Ile136, Tyr211, and Leu214 residues. Based on this analysis, the free binding energy can also be improved through N3 and S methylations. In this regard, the N/S-methylated analogs of monastrol, M4, displayed more affinity to interact with the active site ($\Delta G_{\text{bind}}$: -8.01 Kcal/mol). Despite the participation of this compound in the only one hydrogen binding with Glu116, the more hydrophobic interactions of N/S-methyl fragments with the non-polar residues, especially Tyr211, Ala133, Trp127, and Pro137 could promote the affinity (Fig. 1C). Interestingly, the docking models revealed that the replacement of hydroxyl substituent at the meta position of the phenyl ring in M11 by a methoxy group in compound M12 led to a reduction in the binding affinity ($\Delta G_{\text{bind}}$: -6.54 Kcal/mol). Compound M12 adopted a conformation in which the 3-methoxy substituted phenyl ring was pointed to the opposite direction as that seen in M11. Although in this orientation phenyl ring participated in a $\pi$ cationic interaction with Arg221, no meaningful hydrogen bonding could be detected between the methoxy group and critical residues. Similar findings were obtained for M5 ($\Delta G_{\text{bind}}$: -6.24 Kcal/mol) comparing the corresponding 3-hydroxy substituted analog M4 ($\Delta G_{\text{bind}}$: -8.01 Kcal/mol). M5 did not make any vital hydrogen bonding and $\pi$ cationic interactions. As shown in Table 1, M2, M3, and M10 exhibited significantly more reduced affinities compared to monastrol. The $\Delta G_{\text{bind}}$ values of the best docked poses of these compounds were within the range of -6.09 to -5.53 Kcal/mol. Further bulky substitutions at the positions of C2, N3, and C5 of the primary scaffold could hamper the binding affinity to the active site. Limited extension of binding pocket can be attributed to the more accessible accommodation and so better fitting of small groups at the active site compared with bulkier ones. The importance of this issue is more noticeable about bicyclic compound M7. Because of bulky 4-bromophenyl moiety, M7 was not able to entirely insert in the binding pocket $\Delta G_{\text{bind}}$: -3.05 Kcal/mol). Generally, docking results of proposed compounds were in a satisfactory agreement with the published cocrystal structure of monastrol with the Eg5 motor domain (32) as well as the SAR reported for different kinds of monastrol’s derivatives by other research groups (6,29,30).

The synthesis pathways for all derivatives are shown in Fig. 2. Monastrol and M1 were synthesized without any complications, as described in the literature, using PTSA and anhydrous sodium acetate as the primary catalysts, respectively (4,14). We synthesized dimethyl monastrol at S, and N3 (rather than the N1) positions using methyl iodide and potassium carbonate with a slight modification to an earlier report (15). In the next step, the reaction between monastrol and hydrazine hydrate did not proceed, as mentioned in the literature (33), and we could not obtain any pure carboxyhydrazide (compound X). Other efforts with different conditions were studied instead, but we could not succeed in any of them to the synthesis of X. The product of all these attempts was the initial monastrol (confirmed by HNMR) or different products that could not be separated as pure compounds. It seems that in mild conditions (equimolar ratios, and low temperature), hydrazine hydrate is not strong enough to lyse the ester group. Whereas in harsh conditions (excess hydrazine hydrate and high temperature), hydrazine hydrate is not strong enough to lyse the ester group. Whereas in harsh conditions (excess hydrazine hydrate and high temperature), we see the ring decomposition (Fig. 3), as Said et al. observed (23) before. Finally, to make a change in the ring position of 5, we tried to get the carboxylic acid forms of our parent ester. The low efficacy of hydrolysis reaction was the main drawback in this pathway. The leading causes are the $\alpha,\beta$-unsaturated nature of monastrol ring (34), and wide ranges of pKa present in the acid product (predicted values are 9.38, 11.16, and 3.47 for phenolic hydroxyl, N1, and carboxyl group, respectively). Finally, we purified the M6 in low yield and obtained M8 after the reaction of M6 with Hyd1, similar as the reported method (26). Because of the instability of hydrazones, the coupling reaction performed almost in situ in the presence of EDC/HOBt as catalysts (18). We synthesized other derivatives (M2, M3, and M7) without any considerable difficulty. In a direct route for the synthesis of M11 and M12, we used levulinic acid as the starting material.
instead of ethyl acetoacetate. We preferred PTSA catalyst instead of potassium carbonate as reported before (21), to prevent alkaline mediated decarboxylation of the final product. Finally, we synthesized the acetyl derivative of methoxylated monastrol (M9) and chalcone derivative (M10) as the complementary derivatives based on the previous reports (19,20) with minor changes. Among the all synthesized derivatives M5, M7, M8, M11, M12, and M10 are all novel, and their synthesis is reported for the first time. The spectral proofs confirmed the correct structures. All compounds had their special characteristic features in both NMR and IR spectra. All of the dimethylated derivatives (M4 and M5) are N3 methylated, which can be approved by the doublet signal for H4 on the ring. Both M2 and M3 derivatives showed a specific pattern due to the sulfur's effect on the methylene group of the thiazole ring. For M8 and M10, we have a specific pattern of para substituted phenyl group beside the vinylic proton, which can be seen readily in their NMR spectra.

MCF-7 and HeLa cancer cell lines both have assigned for their overexpression of the Eg5 enzyme (35); therefore, we used these two cell lines to study the cell toxicity of the synthesized compounds. Based on estimated IC50 values (Table 2) obtained from MTT assay, monastrol showed more potency than its derivatives (88 and 111 μM for MCF-7 and HeLa, respectively). Furthermore, after monastrol, M1 with a methoxy group instead of hydroxyl, M3 with thiazole moiety at C2-N3 position, and the methylated monastrol, respectively have lower IC50 than others on MCF-7 cell line (Table 2). Guido et al. have reported the same results for various substituted monastrol; in their study, just the derivative of 2-hydroxyl phenyl had better activity than the monastrol (9). Furthermore, Table 2 shows a relatively similar trend on HeLa cell line; again, monastrol itself has a better potency; after that, the M1, M6, M4, and M2, respectively are at the top of the list. Among our novel synthesized derivatives, it seems that M11 can be a potential candidate for further cellular assessments on MCF-7, and M8 has the same condition on the HeLa cell line. Amongst the derivatives with a hydrophobic ligand at thiourea residue, the dimethylated derivative is more potent than the thiazole ones in both cell lines. Another finding from our introductory study was obtained from the HeLa cell line assessment, all of the derivatives even the monastrol itself showed significantly less activity on HeLa cell line compares to the MCF-7. Based on the protein atlas website (www.proteinatlas.org), the expression of Eg5 in terms of TPM (transcripts per million) in the MCF-7 cell line is about 32.5, and in HeLa is 29.8, respectively. Therefore, the lower sensitivity of the HeLa cells may be attributed to the lower expression of the Eg5 enzyme, which should be approved by enzymatic assessment, or to the cell resistance resulting from successive sub-culturing or passaging, which should be checked on the newer source of cells. Furthermore, the study conducted by Tcherniuk et al. demonstrated that mutations in the induced-fit binding pocket of Eg5 could confer drug resistance in cells to inhibitors that are known to bind to this pocket, such as monastrol (31).

CONCLUSION

Eg5 has been a favorite target in anticancer drug designing to extend more potent anticancer agents. Our in-silico studies revealed that all of the proposed derivatives other than the M7 could be suitable candidates for Eg5 inhibition. SAR analysis and molecular modeling studies revealed that the positioning of a hydrogen bond donor/acceptor on the phenyl ring of the monastrol and hydrophobic group on the thiourea moiety play a critical effect in the inhibition of Eg5 enzyme. We synthesized 13 derivatives with different substitution patterns around the monastrol ring. In vitro studies potency results were not in line with those of in silico experiments. The IC50s were in the range of 88 to more than 500 μg/mL against HeLa and MCF-7 cell lines. Although the designed compounds were found to have moderate activity against cancer cell lines compared to the monastrol, the results are expected to contribute toward more profound insight into the SAR. The results could be helpful in further monastrol-based drug discoveries. The reason behind the controversy between the in vitro and in silico results might be that the applied cell-based assays are not appropriate representative of the inhibition of
Eg5 protein. Furthermore, the lower in vitro activity of studied derivatives could be due to the suboptimal physicochemical properties that should further be modified via structural optimization of these compounds in the future. Finally, we should note that in drug development, some parameters, rather than potency, play an essential role, for example, the normal cell cytotoxicity and so on. Furthermore, we found out that some previous reports and claims on the functionalization of the monastrol ring are not reliable and accurate.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest for this study.

AUTHORS’ CONTRIBUTION

The idea was developed by M. Rostami. H. Sirous and M. Rostami designED the molecular docking study. M. Rostami, Gh. Khodarahmi, and F. Hassanzadeh supervised this work. The literature review was done by Z. Bidram, A. Ali Hariri, H. Sirous, and M. Rostami. Data collection and analysis was performed by Z. Bidram, H. Sirous, A.A. Hariri, N. Dana, and M. Rostami. Z. Bidram, M. Rostami, and H. Sirous contributed in manuscript preparation and revision.

REFERENCES

1. Tayeebee R, Amini MM, Ghadamgahi M, Armaghan M. H₃PW₁₀V₂O₄₀/Pip-SBA-15: a novel reusable organic-inorganic hybrid material as potent Lewis acid catalyst for one-pot solvent-free synthesis of 3,4-dihydropyrimidinones. J Mol Catal A: Chem. 2013;366:266-274.
   DOI: 10.1016/j.molcata.2012.10.004.
2. Razzaghi-Asl N, Kamrani-Moghadam M, Farhangi B, Vahabpour R, Zabihollahi R, Sepehri S. Design, synthesis and evaluation of cytotoxic, antimicrobial, and anti-HIV-1 activities of new 1,2,3,4-tetrahydroprymidine derivatives. Res Pharm Sci. 2019;14(2):155-166.
   DOI: 10.4103/1735-5362.253363.
3. Matos LHS, Masson FT, Simeoni LA, Homem-de-Mello M. Biological activity of dihydropyrimidinone (DHPM) derivatives: a systematic review. Eur J Med Chem. 2018;143:1779-1789.
   DOI: 10.1016/j.ejmech.2017.10.073.
4. Jin T, Zhang S, Li T. p-Toluenesulfonic acid-catalyzed efficient synthesis of dihydropyrimidines: improved high yielding protocol for the Biginelli reaction. Synt Comms. 2002;32(12):1847-1851.
   DOI: 10.1081/SCC-120004068.
5. Marques LA, Semprebon SC, Niwa AM, D’Epiro GFR, Sartori D, de Fátima Â, et al. Antiproliferative activity of monastrol in human adenocarcinoma (MCF-7) and non-tumor (HB4a) breast cells. Naunyn-Schmiedebergs Arch Pharmacol. 2016;389(12):1279-1288.
   DOI: 10.1007/s00210-016-1292-9.
6. Tawfik HO, El-Moselhy TF, El-Din NS, El-Hamamsy MH. Design, synthesis, and bioactivity of dihydropyrimidine derivatives as kinesin spindle protein inhibitors. Bioorg Med Chem. 2019;27(23):115126.
   DOI: 10.1016/j.bmc.2019.115126.
7. Russowsky D, Canto RFS, Sanches SAA, D’Oca MGM, de Fátima Â, Pilli RA, et al. Synthesis and differential antiproliferative activity of Biginelli compounds against cancer cell lines: monastrol, oxo-monastrol and oxygenated analogues. Bioorg Chem. 2006;34(4):173-182.
   DOI: 10.1016/j.bioorg.2006.04.003.
8. Reddy S, Suryanarayana CV, Sharmila N, GV Ramana, Anuradha V, Hari Babu B. Synthesis and cytotoxic evaluation for some new dihydropyrimidinone derivatives for anticancer activity. Lett Drug Des Discov. 2013;10(8):699-705.
   DOI: 10.2174/15701808113109990007.
9. Guido BC, Ramos LM, Nolasco DO, Nobrega CC, Andrade BY, Pic-Taylor A, et al. Impact of kinesin Eg5 inhibition by 3,4-dihydropyrimidin-2(1H)-one derivatives on various breast cancer cell features. BMC Cancer. 2015;15:283-297.
   DOI: 10.1186/s12885-015-1274-1.
10. Abnous K, Barati B, Mehr si, Farimani MRM, Alibolandi M, Mohammadpour F, et al. Synth esis and molecular modeling of six novel monastrol analogues: evaluation of cytotoxicity and kinesin inhibitory activity against HeLa cell line. DARU. 2013;21(1):70-77.
   DOI: 10.1186/2008-2231-21-70.
11. Guan B, Zhang C, Ning J. EDGA: a population evolution direction-guided genetic algorithm for protein-ligand docking. J Comput Biol. 2016;23(7):585-596.
   DOI: 10.1089/emb.2015.0190.
12. Studio D, Insight I. Accelrys Software Inc. San Diego, CA. 2009;92121. https://www.3dsbiovia.com/products/collaborative-science/biovia-discovery-studio/visualization-download.php.
13. Morris GM, Huey R, Olson AJ. Using autodock for ligand-receptor docking. Curr Protoc Bioinformatics. 2008;24(1):8-14.
   DOI: 10.1002/0471250953.bi0814s24.
Synthesis and antimicrobial activity of some new 1,3-diphenylpyrazoles bearing pyrimidine, pyrimidinemethine, thiazolopyrimidine, triazolopyrimidine, thio- and alkylthio triazolopyrimidine moieties at the 4-position. Phosphorus Sulfur Silicon Relat Elem. 2006;181(11):2459-2474. DOI: 10.1080/1042650600754695.

Synthesis of some dihydro pyrimidine-based compounds bearing pyrazoline moiety and evaluation of their antiproliferative activity. Eur J Med Chem. 2013;70:273-279. DOI: 10.1016/j.ejmech.2013.10.003.

Design, synthesis, biological evaluation and in silico molecular docking studies of novel benzochromeno[2,3-d]thiazolopyrimidine derivatives. Res Chem Intermediat. 2018;44(3):1833-1846. DOI: 10.1007/s11164-017-0320-3.

Design, synthesis, biological evaluation and in vitro screening, and molecular modeling study. Bioorg Chem. 2018;76:37-52. DOI: 10.1016/j.bioorg.2017.10.021.

A new procedure for preparation of carboxylic acid hydrazides. J Org Chem. 2002;67(2):947-951. DOI: 10.1021/jo012628n.

Design, synthesis, in vitro screening, and molecular modeling study. Bioorg Chem. 2018;76:37-52. DOI: 10.1016/j.bioorg.2017.10.021.

Synthesis and kinases Eg5 inhibition activities of new arene ruthenium complexes of pyrimidine analogs. J Coord Chem. 2017;70(12):2061-2073. DOI: 10.1080/00958972.2017.1334259.

Design, synthesis and bioevaluation of dihydro pyrazolo[3,4-b] pyridine and benzo[4,5]imidazo[1,2-a] pyrimidine compounds as dual KSP and Aurora-A kinase inhibitors for anti-cancer agents. Bioorg Med Chem. 2010;18(22):8035-8043. DOI: 10.1016/j.bmc.2010.09.020.

Design, synthesis and anticanicar activity of new monastrol analogues bearing 1,3,4-oxadiazole moiety. Eur J Med Chem. 2017;138:140-151. DOI: 10.1016/j.ejmech.2017.06.026.

Design and synthesis of novel phenyl-1,4-beta-carboline-hybrid molecules as potential anticancer agents. Eur J Med Chem. 2017;128:123-139. DOI: 10.1016/j.ejmech.2017.01.014.

Mutations in the human kinesin Eg5 that confer resistance to monastrol and S-trityl-L-cysteine in tumor derived cell lines. Biochem Pharmacol. 2010;79(6):864-872. DOI: 10.1016/j.bcp.2009.11.001.

Small-molecule and mutational analysis of allosteric Eg5 inhibition by monastrol. BMC Chem Biol. 2006;6:2-10. DOI: 10.1186/1472-6769-6-2.

Synthesis and anti-inflammatory activity of some 3-(4,6-disubstituted-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-y1) propanoic acid derivatives. Bioorg Med Chem Lett. 2010;20(15):4424-4426. DOI: 10.1016/j.bmc.2010.06.058.

Culture of specific cell types: Wiley Online Library;2005. DOI: 10.1002/0471747599.cae023.

Synthesis, X-ray structure, in vitro HIV and kinesin Eg5 inhibition activities of new arene ruthenium complexes of pyrimidine analogs. J Coord Chem. 2017;70(12):2061-2073. DOI: 10.1080/00958972.2017.1334259.

Design, synthesis and bioevaluation of dihydro pyrazolo[3,4-b] pyridine and benzo[4,5]imidazo[1,2-a] pyrimidine compounds as dual KSP and Aurora-A kinase inhibitors for anti-cancer agents. Bioorg Med Chem. 2010;18(22):8035-8043. DOI: 10.1016/j.bmc.2010.09.020.

Design, synthesis and anticanicar activity of new monastrol analogues bearing 1,3,4-oxadiazole moiety. Eur J Med Chem. 2017;138:140-151. DOI: 10.1016/j.ejmech.2017.06.026.

Design and synthesis of novel phenyl-1,4-beta-carboline-hybrid molecules as potential anticancer agents. Eur J Med Chem. 2017;128:123-139. DOI: 10.1016/j.ejmech.2017.01.014.

Mutations in the human kinesin Eg5 that confer resistance to monastrol and S-trityl-L-cysteine in tumor derived cell lines. Biochem Pharmacol. 2010;79(6):864-872. DOI: 10.1016/j.bcp.2009.11.001.

Small-molecule and mutational analysis of allosteric Eg5 inhibition by monastrol. BMC Chem Biol. 2006;6:2-10. DOI: 10.1186/1472-6769-6-2.

Synthesis and anti-inflammatory activity of some 3-(4,6-disubstituted-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-y1) propanoic acid derivatives. Bioorg Med Chem Lett. 2010;20(15):4424-4426. DOI: 10.1016/j.bmc.2010.06.058.

Culture of specific cell types: Wiley Online Library;2005. DOI: 10.1002/0471747599.cae023.

Synthesis, X-ray structure, in vitro HIV and kinesin Eg5 inhibition activities of new arene ruthenium complexes of pyrimidine analogs. J Coord Chem. 2017;70(12):2061-2073. DOI: 10.1080/00958972.2017.1334259.