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Original Article

Synthesis and cytotoxicity evaluation of novel cyclic/non-cyclic *N*-aryl enamino amides against human cancer cell lines

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Abstract

Background and purpose: Considering the undesirable consequences of prevalent cancer diseases, design and development of potent and selective anticancer chemotherapeutics is a major concern. Several studies have unraveled the potential of dihydropyrimidinone (DHPM) scaffold toward generating anticancer agents.
Experimental approach: In the present work, a series of new dihydropyrimidinethiones (DHPMTs) along with a few acyclic enamino amides were synthesized and evaluated for their cytotoxic activity against human gastric (AGS), liver (Hep-G2), and breast (MCF-7) cancer cell lines.
Findings/Results: Among the assessed compounds, one of the DHPMT derivatives (compound **5**: 4-(3-fluorophenyl)-6-methyl-*N*-phenyl-2-thioxo-1,2,3,4-ttrahydropyrimidine-5-carboxamide) exhibited superior cytotoxicity in all of the target cell lines (AGS, IC_{50} 9.9 µM; MCF-7, IC_{50} 15.2 µM; and Hep-G2, IC_{50} 40.5 µM). Cytotoxicity assessments showed that non-cyclic enamino amides exhibited weaker activities when compared to cyclic analogues (DHPMs).
Conclusion and implications: DHPMTs were better cytotoxic agents than non-cyclic enamino amides. Structure activity relationship studies guided us toward the design of DHPMT derivatives with OH and NH groups particularly on *meta* position of 4-phenyl ring and hydrophobic bulky substituents on carboxamide side chain within the structure. Possible interaction with the hydrophobic site(s) of the cellular target was supposed. The results of this study emphasized the potential role of DHPMTs and their optimized derivatives as privileged medicinal scaffolds to inhibit the growth of gastric, breast, and liver cancer cells.

Keywords: Cancer; Cytotoxicity; Dihydropyrimidinethione; Enamino amide; MTT.

INTRODUCTION

Serious social and economic consequences have been brought as a result of cancer prevalence all over the world. It has been revealed that genetic mutations, heredity, chemicals, and outside factors, obesity, infections, and radiation are principal factors causing cancer (1,2). Regarding the latest cancer incidence statistics, treatment, or control of this disease is a priority (3).

One possible strategy to control the progression of the disease is based on using chemotherapeutic agents against cancer cells which is commonly known as chemotherapy. Many studies have shown that dihydropyrimidinone (DHPM) scaffold has the potential to be developed into anticancer agents. Acyclic N-amino amide derivatives were also demonstrated to kill cancer cells, which have significant cytotoxic effects on prostate PC-3, colon HT-29, and breast MDA-MB-231 cell lines (4).

Monastrol (Fig. 1), a well-known DHPM derivative, is an FDA approved anticancer drug that acts by inhibiting Eg5 enzyme, blocking mitosis, and inducing apoptosis (5,6).

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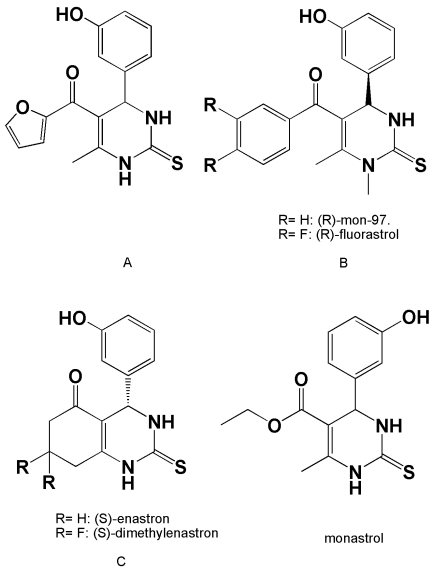


Fig. 1. Chemical structures of some anticancer dihydropyrimidinone (DHPM) derivatives.

The drug possesses a good structural template for further explorations toward more diverse bioactive agents. A furyl derivative i.e. compound A has been proven to be 5 times more active than monastrol in inhibiting Eg5 while compounds B and C could better bind to the allosteric site (6).

A few studies have revealed the cytotoxic effects of 5-carboxamide derivatives on Hep-G2 (7) and kidney (8), and MCF-7 (9) cancer cells. Other researchers pointed to the cytotoxic activity of dihydropyrimidinethiones (DHPMTs) on A431 cancer cells (10). 3,4-DHPMs of Curcuma were synthesized and evaluated for their cytotoxicity against Hep-G2, HCT-116, and QG-5 cancer cells, and results showed a superior effect on HCT-116 cells (11).

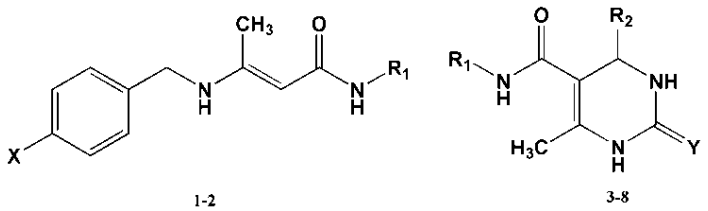
In the light of the above explanations, and in continuation to our previous efforts toward the synthesis of cytotoxic agents (12), a few DHPMT and acyclic enamino amides were prepared and evaluated for their cytotoxic activity against human gastric (AGS), liver (Hep-G2), and breast (MCF-7) cancer cell lines.

MATERIALS AND METHODS

Chemistry

All materials and reagents were purchased from Merck and Aldrich Chemical Companies and applied without further purification. Melting points were determined on an Electro thermal type 9200 melting point apparatus (England) and uncorrected. Fourier-transform infrared (FT-IR) spectra were recorded on a Perkin Elmer-400 FT-IR spectrophotometer (England). Proton nuclear magnetic resonance (^{1}HNMR) spectra were obtained on a Bruker DRX400 spectrometer (400 MHz). Mass (MS) spectra were recorded on an Agilent 7890A spectrometer (USA).

Table 1. Synthetic derivatives of *N*-aryl enamino amide and dihydropyrimidinethiones.



Compounds' No.	R_1	R_2	X	Y	MW	Yield (%)
1	Phenyl	-	H	-	266	54.2
2	Phenyl	-	Cl	-	300	86.5
3	Phenyl	Phenyl	-	S	323	18
4	Phenyl	3-chlorophenyl	-	S	357	58.3
5	Phenyl	3-fluorophenyl	-	S	341	60.7
6	Phenyl	3-bromorophenyl	-	S	309	19.9
7	4-methyl-2-benzothiazolyl	Phenyl	-	S	394	71.7
8	Phenyl	3-phenoxyphenyl	-	S	415	62.9

General procedure for the synthesis of N-phenyl enamino amide derivatives

For the synthesis of enamino amide derivatives (Table 1), 4 mmol *N*-phenyl-3-oxo butane amide was dissolved in isopropyl alcohol and 4.5 mmol of 4-chlorobenzyl amine or benzylamine was added to the mixture. After obtaining a clear solution, the solution was refluxed for 1 h. After achieving room temperature, the precipitate was filtered and washed twice with cold isopropyl alcohol (2 × 3 mL). Obtained products were dried in a desiccator for one day. Structural identification of the synthesized derivatives was performed through melting point, thin-layer chromatography (TLC), ^{1}HNMR, MS, and IR.

General procedure for the synthesis of N-(4-methylbenzo[d]thiazole-2-yl)-3-oxo butane amide

All the synthetic procedure was performed according to the previous report (13,14). For this purpose, 4 mmol of 2-amino-4-methyl benzothiazole was dissolved in 10 mL xylene (140 °C) and 5 mol 2,2,6-trimethyl-1,3-dioxin-4-one was added to the mixture. After 2 h reflux, the mixture was cooled to room temperature. Obtained precipitates were filtered and washed with petroleum ether (3 × 2 mL). Subsequent recrystallization from ethanol afforded the final product.

General procedure for the synthesis of DHPMTs

For the synthesis of DHPMT derivatives (Table 1), 4.8 mmol N-phenyl-3-oxo butane amide, 5.2 mmol thiourea, 1 mL of HCl, and 4 mmol corresponding aldehyde were mixed in 5 mL ethanol and refluxed for 0.5-24 h. Following the reflux, the heater was switched off and after achieving room temperature; some water and ice (deionized water) were added to the flask in order to remove the remaining reactant. After this step, the contents of the flasks were filtered and washed with cold water (2 × 3 mL). Further purification was done by recrystallization in ethanol.

Biological assessment

Reagents and chemicals

Fetal bovine serum (FBS), RPMI 1640, trypsin, and phosphate-buffered saline (PBS) were all obtained from Biosera (Ringmer, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT was purchased from Sigma (Saint Louis, MO, USA), and penicillin/streptomycin was purchased from Invitrogen (San Diego, CA, USA). Cisplatin and dimethyl sulphoxide (DMSO) were obtained from EBEWE Pharma (Unterach, Austria) and Merck (Darmstadt, Germany), respectively.

Cell lines

MCF-7 (human breast adenocarcinoma), AGS (human gastric cancer), and Hep-G2 (human liver cancer) cells were purchased from the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. All cell lines were maintained in RPMI 1640 supplemented with 10% FBS, and 100 units/mL penicillin-G and 100 μg/mL streptomycin. Cells were grown in monolayer cultures.

Cytotoxic assay

Cell viability following exposure to the synthetic compounds was estimated *via* MTT reduction assay (15). Cells were plated in 96-well microplates at a density of 1×10^4 cells per well (200 μL per well). Control wells contained no drugs and blank wells contained only growth medium for background correction. After cell attachment, the medium was removed, and cells were incubated with a serum-free medium containing 1 mg/mL of the synthetic compounds by 1/4 serial dilutions. Compounds were all first dissolved in DMSO and then diluted in medium, therefore, the maximum concentration of DMSO in the wells did not exceed 0.5%. Cells were further incubated for 24 h. At the end of the incubation time, the medium was removed and MTT was added to each well at a final concentration of 0.5 mg/mL, and plates were incubated for another 4 h at 37 °C. Then formazan crystals were solubilized in 200 μL DMSO. The optical density was measured at 570 nm with background correction at 655 nm using a Bio-Rad microplate reader (Model 680, USA). The percentage of inhibition of viability compared to the control wells was calculated for each concentration of the compounds and IC_{50} values were calculated with SigmaPlot version 12.5.

The absorbance of wells containing no cells was subtracted from the sample well absorbance before calculating the percentage of inhibition. Each experiment was carried out in triplicate.

Statistical analysis

Obtained biological data are presented as mean ± standard deviation (SD). Statistically significant differences among groups were determined by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. $P \leq 0.05$ was considered significant.

RESULTS

Chemistry

Spectroscopic results of synthesized compounds are illustrated below.

E-(3-benzylamino)-N-phenyl but-2-enamide (1)

White crystal, yield 54.2%; m.p.: 80-81 °C; IR (KBr) ν_{max} (cm^{-1}): 3274.3, 3240.4, 3040, 2925, 1657.8, 1612.4, 1594.6, 746.2, 696.1; ^{1}HNMR (400 MHz, DMSO-*d6*) δ (ppm) 9.44 (1H, *J* = 6 Hz, NH-enamine), 9.20 (1H, brs, NH-amide), 7.55 (2H, d, *J* = 8.4 Hz, CH-phenyl), 7.26-7.40 (8H, m, CH-phenyl), 4.62 (1H, s, CH-alkene), 4.44 (2H, d, *J* = 6.4 Hz, CH2-benzyl), 1.90 (3H, s, CH3); MS m/z (%): 266 (23) [M+], 174 (95), 147 (16), 91 (100).

E-3-(4-chlorobenzylamino)-N-phenyl but-2-enamide (2)

White crystal, yield 86.5%; m.p.: 140-141 °C; IR (KBr) ν_{max} (cm-1): 3274.3, 3240.4, 3040, 2925, 1657.8, 1612.4, 1594.6, 746.2, 696.1; ^{1}HNMR (400 MHz, DMSO-*d6*) δ (ppm) 9.44 (1H, t, *J* = 6.4 Hz, NH-enamine), 9.22 (1H, brs, NH-amide), 7.55 (2H, d, *J* = 8 Hz, CH-phenyl), 7.44 (2H, d, *J* = 8.4 Hz, CH-phenyl), 7.32 (2H, d, *J* = 8.4 Hz, CH-phenyl), 7.21 (3H, t, *J* = 7.6 Hz, CH- phenyl), 4.63 (1H, s, CH-alkene), 4.43 (2H, d, *J* = 6.4 Hz, CH2-benzyl), 1.87 (3H, s, CH3); MS m/z (%): 300 (27) [M+], 208 (95), 160 (7), 125 (100)

6-methyl -2- N-4-diphenyl-2-thioxo-1,2,3,4-ttrahydropyrimidine-5-carboxamide (3)

White crystal, Yield 18%; M.p.: 165-167 °C; IR (KBr) ν_{max} (cm-1): 3486.3, 3284.1, 3181.4, 3060, 3102.8, 1679.7, 1630.1, 1589.9; 1H-NMR (400 MHz, DMSO-d6) δ (ppm) 10.11 (1H, brs, NH-amide), 9.84 (1H, brs, N1H), 9.55 (1H, brs, N3H), 7.64 (2H, d, J = 8 Hz, CH-phenyl), 7.33-7.46 (8H, m, CH-phenyl), 5.49 (1H, brs, C4H-DHPM), 2.16 (3H, s, CH3-DHPM), MS m/z (%): 323 (74) [M+], 231 (100), 203 (15), 77 (28).

4-(3- chlorophenyl) -6- methyl-N- phenyl-2-thioxo -1,2,3,4- tetrahydropyrimidine -5-carboxamide (4)

White crystal, yield 8.7%; m.p.: 170-171 °C; IR (KBr) ν_{max} (cm^{-1}): 3416.9, 3267.4, 3185.3, 3066.5, 3015.8, 1678.5, 1629.6, 1584.1; ^{1}HNMR (400 MHz, DMSO-*d6*) δ (ppm) 10.24 (1H, brs, NH-amide), 9.90 (1H, brs, N1H), 9.63 (1H, brs, N3H), 7.66 (2H, d, *J* = 7.6 Hz, CH-phenyl), 7.33-7.55 (7H, m, CH-phenyl), 5.51 (1H, brs, C4H-DHPM), 2.20 (3H, s, CH3-DHPM), MS m/z (%): 357 (63) [M+], 281 (24), 265 (100), 246 (86), 77 (25).

4-(3- fluorophenyl)-6- methyl-N- phenyl-2-thioxo- 1,2,3,4 –ttrahydropyrimidine -5-carboxamide (5)

White crystal, yield 60.7%; m.p.: 178-180 °C; IR (KBr) ν_{max} (cm^{-1}): 3406.4, 3174.2, 3108.9, 3064.3, 3016.9, 1678.7, 1629.8, 1571.4; ^{1}HNMR (400 MHz, DMSO-*d6*) δ (ppm) 10.23 (1H, brs, NH-amide), 9.90 (1H, brs, N1H), 9.64 (1H, brs, N3H), 7.67 (2H, d, *J* = 7.6 Hz, CH-phenyl), 7.22-7.57 (7H, m, CH-phenyl), 5.53 (1H, brs, C4H-DHPM), 2.20 (3H, s, CH3-DHPM), MS m/z (%): 341 (66) [M+], 249 (100), 190 (69), 77 (23).

4-(3- bromophenyl)-6- methyl-N- phenyl-2-thioxo- 1,2,3,4- ttrahydropyrimidine -5-carboxamide (6)

White crystal, yield 58.3%; m.p.: 196-197 °C; IR (KBr) ν_{max} (cm^{-1}): 3345.4, 3186.4, 3125.9, 3064.2, 3016.9, 1667.7, 1651.5, 1581.8; ^{1}HNMR (400 MHz, DMSO-*d6*) δ (ppm) 10.24 (1H, brs, NH-amide), 9.90 (1H, brs, N1H), 9.62 (1H, brs, N3H), 7.66 (2H, d, *J* = 7.6 Hz, CH-phenyl), 7.36-7.62 (7H, m, CH-phenyl), 5.51 (1H, brs, C4H-DHPM), 2.20 (3H, s, CH3-DHPM), MS m/z (%): 402 (5) [M+], 371 (26), 309 (13), 292 (100), 77 (28).

Table 2. IC_{50} values of *N*-aryl enamino amides and dihydropyrimidinethiones *vs* assessed cancer cell lines. Data are presented as mean ± SEM, n = 3. [a] and [b] are designated to the compounds which are more potent (lower IC_{50}, $P \leq 0.05$) than cisplatin and monastrol, respectively.

Compound No.	IC_{50} (µM)		
	AGS	**MCF-7**	**Hep-G2**
1	6501.4 ± 21.9	5715.4 ± 8.2	3760.1 ± 11.4
2	395.0 ± 29.9	321.2 ± 3.0	2820.8 ± 14.5
3	467.9 ± 16.4	513.3 ± 11.0	1055.6 ± 14.9
4	796.2 ± 63.3	442.0 ± 14.3	780.3 ± 9.4
5	9.9 ± 0.3[a,b]	15.2 ± 0.4[b]	40.5 ± 1.0[b]
6	26.5 ± 1.1[b]	44.4 ± 1.2[b]	112.6 ± 2.7[b]
7	63.1 ± 5.4[b]	99.9 ± 6.4[b]	272.3 ± 9.8
8	23.8 ± 1.1[b]	25.9 ± 7.2[b]	94.2 ± 7.9[b]
Monastrol	106.0 ± 0.1	133.0 ± 0.9	115.9 ± 4.0
Cisplatin	11.4 ± 2.9	6.0 ± 2.2	23.3 ± 2.8

6-methyl-N-(4-methylbenzo[d]thiazole-2yl)-4-phenyl-2-thioxo-1,2,3,4-ttrahydropyrimidine-5-carboxamide (7)

White crystal, yield 71.7%; m.p.: 253-254 °C; IR (KBr) ν_{max} (cm^{-1}): 3417.8, 3267.4, 3211.3, 3065.8, 3013.8, 1678.5, 1629.6, 1584.1; ^{1}HNMR (400 MHz, DMSO-*d6*) δ (ppm) 12.43 (1H, brs, NH-amide), 10.36 (1H, brs, N1H), 9.79 (1H, brs, N3H), 7.85 (1H, d, *J* = 8 Hz, C7′H-benzothiazole), 7.28-7.41 (7H, m, CH-phenyl), 5.76 (1H, brs, C4H-DHPM), 2.62 (3H, s, CH3-benzothiazole), 2.31 (3H, s, CH3-DHPM), MS m/z (%): 394 (7) [M+], 163 (100), 91 (8), 77 (17).

6- methyl-4-(3- phenoxyphenyl)-N- phenyl-2-thioxo- 1,2,3,4- ttrahydropyrimidine- 5-carboxamide (8)

White crystal, yield 62.9%; m.p.: 235-236 °C; IR (KBr) ν_{max} (cm^{-1}): 3417.8, 3267.4, 3211.3, 3065.8, 3013.8, 1678.5, 1629.6, 1584.1; ^{1}HNMR (400 MHz, DMSO-*d6*) δ (ppm) 10.16 (1H, brs, NH-amide), 9.87 (1H, brs, N1H), 9.59 (1H, brs, N3H), 7.64 (2H, d, *J* = 7.6 Hz, CH-phenyl), 7.49 (1H, t, *J* = 8 Hz, CH-phenyl), 7.37-7.43 (4H, m, CH-phenyl), 7.22 (1H, t, *J* = 7.6 Hz, CH-phenyl), 7.15 (2H, t, *J* = 7.2 Hz, CH-phenyl), 7.07 (2H, t, *J* = 7.6 Hz, CH-phenyl), 7.03 (1H, t, *J* = 7.6 Hz, CH-phenyl), 6.99 (1H, t, *J* = 7.2 Hz, CH-phenyl), 5.51 (1H, brs, C4H-DHPM), 2.62 (3H, s, CH3-benzothiazole), 2.31 (3H, s, CH3-DHPM), MS m/z (%): 415 (10) [M+], 296 (100), 281 (49), 127 (80), 77 (21).

Biological assessment

Prepared *N*-heteroaryl enamino amides and DHPMTs were assessed for their cytotoxic effect against three human cancer cell lines (AGS, MCF-7, and Hep-G2) in terms of IC_{50} values (Table 2).

DISCUSSION

Chemical structures of thesynthesized *N*-heteroaryl enamino amides and DHPMTs (1-8) were confirmed by spectroscopic methods. From a mechanistic aspect of view, the first step of the synthesis involves nucleophilic attack of thiourea/urea nitrogen to aldehyde carbonyl group leading to tautomerism, intramolecular nucleophilic attack, and water elimination to produce imine intermediate. 3-oxo butane amide carbonyl captured proton to produce enol which reacts with imine intermediate to give the second intermediate. The new intermediate was converted to final derivatives *via* cyclization and water removal (Scheme 1).

As could be seen from the obtained results, compound 5 was more cytotoxic than cisplatin *vs* AGS cell line and none of the tested agents were more cytotoxic than cisplatin *vs* Hep-G2 and MCF-7 cell lines. Compound 5 was the most cytotoxic agent against all of the three cell lines (AGS, IC_{50} 9.9 ± 0.3 µM; MCF-7, IC_{50} 15.2 ± 0.4 µM; Hep-G2, IC_{50} 40.5 ± 1.0 µM).

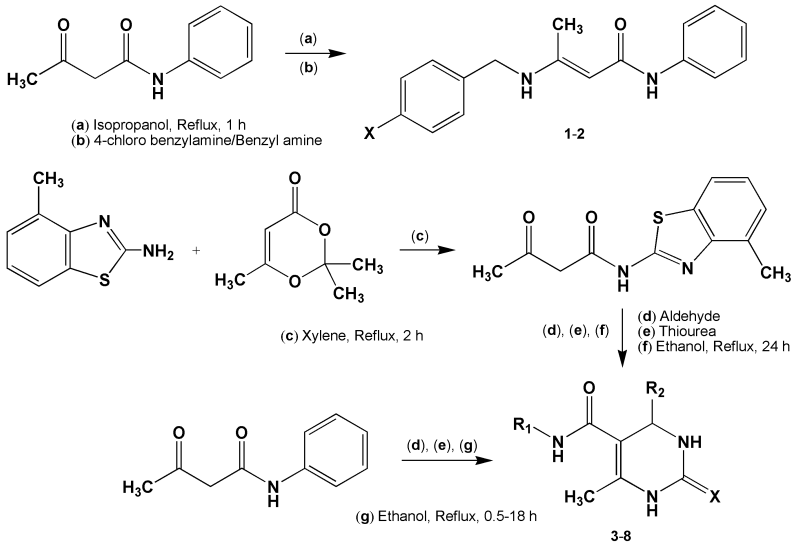
3-fluorophenyl moiety (compound 5) was the superior substitution pattern in all of the assessed cell lines. IC_{50} value in AGS cell proposed compound 5 as an appropriate hit

candidate for further studies. This finding resembled the previous reports on the cytotoxicity of monastrol analogues on Hep-G2 cells (7). Subsequent to compound 5, compound 8 exhibited second-ranked cytotoxicity within all cell lines. A common feature with the previous experience would be the meta position of phenoxy group that confirms the electron-withdrawing effect on the phenyl ring. Lower cytotoxic activity of 8 with regard to 5 might be attributed to the steric hindrance of phenoxy concerning fluoro substituent. The scenario was repeated for compound 6 which contained a bulkier 3-bromophenyl group. Some studies for DHPMs have shown that thioxo analogues possessed higher cytotoxicity than corresponding oxo derivatives due to the antiproliferative effect of sulfur (16). Previous reports revealed that monastrol analogues were good cytotoxic agents due to probably better fitness into Eg5 binding site (17,18). Compound 2 was a significantly better cytotoxic agent than 1 particularly against AGS and MCF-7 cancer cells and such observation could be just attributed to the additional *para*-chloro substituent in compound 2. However, such cases could not be merely interpreted by the occupation of the binding site with additional chlorine group but might be related to the varied conformational orientation of the molecular structure.

Comparison of cytotoxic activities with the control drugs indicated that in AGS and MCF-7 cells, compounds 5-8 were more cytotoxic agents than monastrol while compounds 5 and 8 were more cytotoxic than monastrol in Hep-G2 cells. The results showed that presence of an electron-withdrawing group makes H-bond in the meta position of the phenyl ring determines the cytotoxic activity. In AGS and MCF-7 cells, binding of bulky amide substituent might assist in cytotoxicity. In this regard, other studies have indicated that the incorporation of phenyl ring instead of the ethyl group in monastrol led to the hydrophobic interactions (19).

CONCLUSION

Cytotoxicity assessments indicated that *N*-aryl enamino amides did not exhibit sufficient potency to inhibit the growth of AGS, Hep-G2, and MCF-7 cancer cells and their potential usage in developing further cytotoxic agents needs serious structural modifications. The case for DHPMTs was different since few of them showed much better cell growth inhibitory effects particularly within AGS cells when compared to the *N*-aryl enamino amides. Future trends for boosting the anticancer activities of assessed compounds might be directed toward incorporation of hydrogen bond donating substituents into *meta* position of C4-phenyl ring and hydrophobic groups on carboxamide side chain. This study indicated the scope of DHPMs and their optimized derivatives as privileged medicinal scaffolds to inhibit the growth of gastric, breast, and liver cancer cells.



Scheme 1. Synthetic route toward *N*-aryl enamino amides (1-2) and dihydropyrimidinethiones (3-8).

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST STATEMENT

Authors declared that they have no conflict of interest in this study.

AUTHORS' CONTRIBUTION

S. Bohlooli, S. Sepehri, and N. Razzaghi-Asl contributed to the concept, definition of intellectual content, experimental studies, data analysis, statistical analysis, manuscript editing and manuscript review. N. Nejatkhah and D. Doostkamel did the literature search, experimental studies, data acquisition, data analysis, manuscript preparation.

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