Design, synthesis, anticancer evaluation and docking studies of new pyrimidine derivatives as potent thymidylate synthase inhibitors

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A B S T R A C T

Cancer is a perplexing and challenging problem for researchers. In this study, a series of 6-aryl-5-cyano-pyrimidine derivatives were designed, synthesized and evaluated for their anticancer activity against HepG2, MCF-7 and HCT-116 cell lines. Compounds 2, 3d, 4a-c, 5, 8 and 12 displayed high anticancer activity, comparable to that of 5-fluorouracil. Additionally, compounds with effective anticancer activity were further assessed for their ability to inhibit thymidylate synthase (TS) enzyme. All the tested compounds demonstrated a marked TS inhibitory activity (33.66–74.98%), with IC₅₀ ranging from 3.89 to 15.74 nM. Moreover, apoptosis studies were conducted on the most potent compound 8, to evaluate its proapoptotic potential. Interestingly, compound 8 induced the level of active caspase 3, and elevated the Bax/Bcl2 ratio 44 folds in comparison to the control. Finally, a molecular docking study was conducted to detect the probable interaction between the active compounds and the thymidylate synthase active site.

1. Introduction

Cancer remains one of the most menacing diseases in the world [1]. It is considered the second cause of death after cardiac diseases [2]. Liver cancer is the second most common cancer death causes worldwide [3], while colorectal cancer is the fourth one [4]. Abnormalities in the genetic material are the causes of the various cancer types [5]. One of the important approaches to treat the uncontrolled cell division and growth manifested by the cancer cells is to use drug capable of interfering with the synthesis of the nucleic acids DNA/RNA, and inhibiting their normal function [6,7]. Thymidylate synthase (TS) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2-deoxythymidine-5-monophosphate (dUMP), a DNA building unit, assisted by the cofactor N5,N10-methylenetetrahydrofolate [8–10]. Human TS (hTS) is an essential enzyme for DNA replication, thus it is considered an interesting target for the development of anticancer treatments. Human TS (hTS) exists in the equilibrium of active and inactive conformations [11]. TS plays an important role in the synthesis of key proteins that regulate apoptosis [12]. Inhibition of TS was reported to induce apoptosis.

An example of drugs inhibiting TS enzyme is 5-fluorouracil, the drug of choice for metastatic colorectal cancer with 25% positive patient’s response [13]. 5-Fluorouracil (5-FU) is an analogue of uracil with fluorine atom replacing hydrogen at the C-5 position. It was one of the first rationally designed, synthesized anticancer drugs. 5-FU is converted to 5-fluorodeoxyuridinemonophosphate (FdUMP) which forms a stable ternary complex with thymidylate synthase and the cofactor N5,10-methylenetetrahydrofolate [14–16]. 5-FU remains covalently and irreversibly bound to the active site, misincorporated into RNA, disrupting its function and blocking the synthesis of thymidine leading to DNA damage [1,9,14]. Therefore, targeting TS and its inhibition in TS over expressing cells as tumor cells, have become a major goal in cancer chemotherapy treatment [16]. Herein we provide an approach to explore and expand the anticancer activity of synthesized series of pyrimidine derivatives, via the structural modifications (Fig. 1), of the promising lead compound A which showed a potent anticancer activity against HepG2 cell line with IC₅₀ value of 28 μM [17]. Our rational design is founded on structural diversification of compound A through conserving the cyano-pyrimidine group, while simplification at position 4 and substitution at position 2 with different moieties, to attain an active anticancer agent with an improved activity and selectivity towards cancerous cells. The newly synthesized compounds were evaluated for their anticancer activity against three cell lines; HepG2, HCT-116 and MCF-7. Moreover, the most active compounds were tested for inhibition of thymidylate synthase (TS) enzyme, and their IC₅₀ values were evaluated in addition to the apoptosis induction potential of the compounds. Finally, a molecular docking study was performed to explore the probable interaction of the tested compounds with...
2. Results and discussion

2.1. Chemistry

In connection with our program [18–20], we aim to design and synthesize a novel series of biologically active cyanopyrimidine derivatives to evaluate their anticancer activity. Thus, the sequence of reactions used in the synthesis of the title compounds is shown (Schemes 1–3). The starting key compound 1 was synthesized via one-pot cyclocondensation reaction, in which, equivalent molar quantities of aromatic aldehydes, ethyl cyanoacetate and thiourea were allowed to react in absolute ethanol in presence of anhydrous $K_2CO_3$, then neutralized with acetic acid [21,22]. Treating the thiol derivative 1 with hydrazine hydrate by refluxing in absolute ethanol afforded...
Moreover, we studied the nucleophilicity of NH₂ to form Schiff bases through reaction of 2-hydrazinyl-1,6-dihydro-6-oxo-4-pto1ylpyrimidine-5-carbonitrile 2, with substituted aromatic aldehydes namely benzaldehyde, tolualdehyde, 4-chloroaldehyde, 4-hydroxyl aldehyde, 4-methxyaldehyde and 3,4,5 trimethoxybenzaldehyde. The reaction of the former components in absolute ethanol produced the corresponding target compounds 3a-f, respectively (Scheme 2).

The structure of compounds 3a-f were characterized by spectroscopic tools The I.R showed the presence of bands between 3200 and 3400 and 1680–1685 cm⁻¹ for the amide groups NH and C=O groups, respectively. Moreover, ¹H NMR spectra for this group exhibited a singlet corresponding to the imine proton (CH = N) at the range 8.07–8.17 ppm denoting the formation of the Schiff’s base. Furthermore...
the MS spectrum of a showed the molecular ion peak confirmed the structures (c.f. experimental).

Reflexing equimolar amount of compound 2 and the substituted benzoyl chloride namely, benzoylchloride, 4-chlorobenzoyl chloride and 4-nitrobenzoylchloride in dioxane and TEA as catalyst, afforded the target compounds 4a-c, respectively (Scheme 2). IR spectra for this series were in agreement with the predicted structures as they showed the disappearance of NH2 bands and presence of characteristic absorption bands of carbonyl groups around 1634, 1662 cm\(^{-1}\). In addition, \(^1\)H NMR spectra revealed an increase in the number of aromatic protons. The structure 4a-c is forthcoming from the study of \(^1\)H NMR and mass spectra.

Finally, Compound 2 was condensed with different electrophiles namely, phenyl isothiocyanate, triethylthioformate, acetic anhydride, acetylacetone, ethylacetoacetate, phthalic anhydride and chloroacetyl chloride to afford the title compounds 5–12 respectively (Scheme 3). The suggested mechanisms for formation of the structures of compounds 6 and 7 were in Supplementary Data.

The structures of all newly prepared compounds were confirmed by spectral and elemental analyses, which were in full agreement with the proposed structures.

2.2. Biological evaluation

2.2.1. In vitro cytotoxic activity

The in vitro anticancer activity of the eighteen newly synthesized compounds was evaluated against three cell lines, namely; the liver cancer cell line (HepG2), the colon cancer cell line (HCT-116) and the breast cancer cell line (MCF-7). 5-Flourouracil was used as a reference standard and showed IC\(_{50}\) values of 60.73, 40.74 and 41.51 \(\mu\)M for the three cell lines respectively. The results were expressed as IC\(_{50}\) values and listed in (Table 1). The anticancer results profile suggested that, test compounds showed variable activity compared to the reference drug. The anticancer data revealed that compounds 2, 3d, 4a-c, 5 and 12, possessed a remarkable anticancer activity. Compound 2 displayed 2 and 1.7 folds the activity of 5-Fu against HepG2 (IC\(_{50}\) = 31.08 \(\mu\)M) and HCT-116 cell lines (IC\(_{50}\) = 23.62 \(\mu\)M) respectively, and was equipotent to the reference drug against MCF-7 cell line (IC\(_{50}\) = 38.13 \(\mu\)M), while compound 3d displayed 1.7, 1.3 and 1.2 folds the activity of 5-Fu with IC\(_{50}\) values of 36.19, 31.56 and 35.32 \(\mu\)M against HepG2, HCT-116 and MCF-7 cell lines respectively. Compound 4a was more potent than the reference drug against HepG2 (IC\(_{50}\) = 45.57 \(\mu\)M), MCF-7 (IC\(_{50}\) = 24.90 \(\mu\)M), and equipotent against HCT-116 cell line (IC\(_{50}\) = 40.07 \(\mu\)M) while compound 4b displayed 2.4, 1.7 and 1.1 folds the activity of 5-Fu with IC\(_{50}\) values of 25.03, 23.14 and 36.70 \(\mu\)M against the three cell lines respectively. 4c demonstrated 1.7 fold the activity of 5-Fu, with IC\(_{50}\) values of 32.94, 23.00 and 23.49 \(\mu\)M for the three cell lines respectively. Moreover, Compound 5 exhibited 3, 1.6 and 2.2 folds the activity of 5-Fu with IC\(_{50}\) values of 21.15, 24.97 and 19.12 \(\mu\)M against the three cell lines respectively. Compound 12 was equipotent to the reference drug with IC\(_{50}\) values of 59.48, 41.54 and 38.39 \(\mu\)M against HepG2, HCT-116 and MCF-7 cell lines respectively. Compounds 11 was equipotent to the reference drug against HepG2 cell line, while compounds 8 was equipotent to the reference drug against MCF-7 cell line, but showed good activities against other tested cell lines. Compounds 3a, b and 9 possessed a moderate to weak anticancer activity.

2.2.2. In vitro assay of thymidylate synthase (TS) activity

The most active compounds in cytotoxic assay (2, 3d, 4a-c, 5, 8 and 12) were further evaluated to determine their inhibitory activities against thymidylate synthase (TS) aiming to recognize the probable anticancer activity mechanism of action of these compounds. The activity of TS was measured spectrophotometrically according to reported method [23]. Dasatinib was used as a positive control in this assay [24,25]. The results were reported as enzymatic inhibition percentage (%) and a 50% inhibition concentration value (IC\(_{50}\)) (Table 1).

The tested compounds showed good inhibitory activity with IC\(_{50}\) values ranging from 3.89 to 15.74 \(\mu\)M, which were consistent with that of their in vitro anticancer activities. Dasatinib as a reference compound showed 72.25% TS inhibition. Compounds 4c and 8 showed potent inhibitions more than 74% for the TS activity with IC\(_{50}\) values of 4.21 and 3.89 \(\mu\)M, respectively. Good inhibitions also exhibited by compounds 2, 4b and 5, at low IC\(_{50}\) values of 4.42, 4.72 and 4.63 \(\mu\)M, respectively. Moreover, compounds 3d and 12 displayed moderate inhibitions above 67% with IC\(_{50}\) values of 13.05 and 8.13 \(\mu\)M, respectively.

2.2.3. Apoptosis studies

As previously mentioned, the regulatory role of TS may be implicated in the synthesis of different proteins that regulate apoptosis [11]. As our synthesized compounds successfully inhibited TS enzyme, then expectedly they will induce apoptosis. Their potential to induce apoptosis was determined by monitoring the levels of active caspase 3 and the Bax/Bcl2 ratio that presents proof for induction of apoptosis.

2.2.3.1. Effects on the level of active caspase-3

The investigation of Caspase 3 expression level indicates apoptosis induction. In the present study, HepG2 cells were treated with the most active TS inhibitor 8 with IC\(_{50}\) in the nanomolar range (IC\(_{50}\) = 3.89 \(\mu\)M). Results revealed that compound 8 upregulated the caspase 3 level 7.3 folds as compared to the control, thus proving compound 8 apoptosis induction potential.

2.2.3.2. Effects on mitochondrial apoptosis pathway proteins Bax and Bcl-2

Bcl2 and Bax are members of Bcl-2 family of proteins that are responsible for syncing the mitochondrial apoptotic pathway. Bcl2 and Bax tune this programmed process as Bcl2 suppresses apoptosis whereas Bax induces it. Consequently, the balance between these two proteins is judgmental for the cell potential to undergo apoptosis. In this study, HepG2 cells were treated with compound 8 and its effect on Bcl2 and Bax was quantified as showed in (Table 2, Fig. 2). Results revealed that compound 8 reduced the level of the antiapoptotic protein Bcl2 by 7.5 folds as compared to the control while it elevated the level of the pro-apoptotic protein Bax by 5.9 folds. The ratio between Bax and Bcl2 was also calculated as a more decisive parameter. The Bax/Bcl2 ratio for compound 8 was calculated to be 44 folds as compared to the

<table>
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<tr>
<th>Comp.</th>
<th>IC(_{50}) ((\mu)M)</th>
<th>TS inhibition %</th>
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<tr>
<td>HepG2</td>
<td>HCT-116</td>
<td>MCF-7</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>Dasatinib</td>
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<td>72.25</td>
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</table>

* IC\(_{50}\) values are the mean ± S.D. of three separate experiments.
* NT: Compounds not tested.
control, confirming that the compound shifted the cells towards undergoing apoptosis.

2.3. Molecular docking

To understand the obtained biological data on a structural basis, the most active compounds were evaluated through the molecular docking studies using Molecular Operating Environment (MOE) version 2014, 0901. The enzyme structure was prepared through removal of water molecules and restoration of missing hydrogen atoms.

In the present investigation, the 3D-coordinates in X-ray crystal structure of Human Thymidylate Synthase (PDB code 6QXG) was used as the receptor model in the docking simulation to predict the binding modes, affinities and orientation at the active site of the enzyme (Table 3). The inhibitors are held in the active pocket by combination of various interactions [26]. From the published literatures, Arg50, Ser216, Asn226 and Asp218 are the essential amino acids involved in the interaction with enzyme inhibitors [27,28] (see Table 4).

The proposed binding mode of FdUMP showed an affinity value of −5.39 kcal/mol. The phosphate group was involved in five hydrogen bonding interactions with Arg50 and Arg215. The oxygen atom formed two hydrogen bonds with side chain of His256 and TYR 258. Finally, the two-keto groups formed two hydrogen bonds with Asp218 and Asn226 (Figs. 3 and 4). (Table 3).

Overall, it was found that most of the studied compounds are expected to have the same binding mode of FdUMP (Table 3).

The proposed binding mode of compound 8 showed affinity value of −5.39 kcal/mol. It formed 4 hydrogen bonds, one of them between cyano group at the 5-position of pyrimidine moiety and by two hydrogen bonds interaction with Asp218 and Gly222. The last acetyl group oxygen and Asn226. pyrimidinone oxygen was anchored through two hydrogen bonds with sidechain of His216 (Figs. 5 and 6).

2.3.1. Structure activity relationships (SAR)

The introduction of the hydrazide, 2 or benzohydrazide, 4a-c moieties in position 2 of the pyrimidine scaffold lead to a marked increase in the anticancer activity. However, p-chloro or p-nitro substituted derivatives 4b, c showed higher anticancer activity than its unsubstituted counterpart 4a. This indicates that para substitution with electron withdrawing group might have a positive impact on the activity with respect to the unsubstituted derivative. The incorporation of 4-hydroxy benzylidene 3d to the hydrazide group gave a potent anticancer activity than the other counterparts 3a-c, 3e, but still less than its starting compound which contain the hydrazide moiety 2. The presence of phenylthiosemicarbazide moiety 5 instead of the hydrazide moiety resulted in superior anticancer activity as compared to all newly synthesized compounds.

3. Conclusion

A new series of 6-aryl-5-cyano-pyrimidine derivatives were synthesized and evaluated for their anticancer activity against HePG-2, MCF-7 and HCT-116 cell lines. The thymidylate synthase inhibitory activity of the most active compounds was recorded. The results revealed that compound 8 showed a potent anticancer activity with high TS inhibitory activity, its IC_{50} value was 3.89 nM. The proapoptotic potential of our compounds was evaluated via Apoptosis studies for compound 8. Results showed that compound 8 boosted the level of active caspase 3 by 7.3 folds as compared to the control. Additionally, the Bax/Bcl2 ratio of compound 8 was calculated and was found to be 44 folds in comparison to the control.

4. Experimental section

4.1. Chemistry

4.1.1. General

All melting points were measured on a Gallen Kamp melting point apparatus (Sanyo Gallen Kamp, UK) and were uncorrected. The Microwave reactions were done by Microsynth instrument type MAI43 (Micro wave flux). The IR spectra were recorded on a Pye-Unicam SP-3-300 infrared spectrophotometer (KB dricks) and expressed in wave number (cm\(^{-1}\)). 1H NMR spectra were run at 300 and 400 MHz, on a Varian Mercury VX-300 and Bruker Avance III NMR spectrometer respectively. The mass spectra were recorded on Shimadzu GCMS-QP-
1000EX mass spectrometer at 70 eV. Elemental analyses were performed on CHN analyzer and all compounds were within ± 0.4 of the theoretical values. The reactions were monitored by thin-layer chromatography (TLC) using TLC sheets coated with UV fluorescent silica gel Merck 60 F254 plates and were visualized using UV lamp and different solvents as mobile phases. All reagents and solvents were purified and dried by standard techniques. Compounds \( 1 \) was previously reported.

4.1.2. Synthesis of 2-hydrazinyl-1,6-dihydro-6-oxo-4-p-tolyl pyrimidine-5-carbonitrile (2)

A mixture of \( 1 \) (10 mmol) and hydrazinehydrate 99% (20 mmol) was refluxed in absolute (30 mL) ethanol for 10 h. The formed solid was filtered off, dried and recrystallized from ethanol afforded compound 2. Yield 65%; yellow crystal; m.p. 234–236 °C (EtOH); IR (cm\(^{-1}\)): br 3325, 3289 (NH, NH\(_2\)), 3032 (CH-aromatic), 2947 (CH-aliphatic), 2207 (CN), 1665 (CO) amid; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 2.35 (s, 3H, CH\(_3\)), 7.28 (d, 2H, Ar-H, \( J = 8 \) Hz), 7.66 (d, 2H, Ar-H, \( J = 8 \) Hz), 8.19 (s, 1H, NH\(_2\); D\(_2\)O exchangeable), 10.15 (s, 1H, NH\(_2\); D\(_2\)O exchangeable); MS (m/z): 241.12 (M\(^+\), 100%). Anal. Calcd for C\(_{12}\)H\(_{11}\)N\(_5\)O (371.41): C, 59.74; H, 4.60; N, 29.03; Found: C, 59.74; H, 4.60; N, 29.03%.

4.1.3. General procedure for synthesis of 2-(2-benzylidenehydrazinyl)-6-oxo-4-(p-tolyl)-1,6-dihydropyrimidine-5-carbonitrile (3a-f)

A mixture of compound 2 (10 mmol) and the appropriate aromatic aldehyde (10 mmol) namely, benzaldehyde, tolualdehyde, 4-chlorobenzaldehyde, 4-hydroxybenzaldehyde, 4-methoxybenzaldehyde and 3,4,5-trimethoxybenzaldehyde in ethanol (50 mL) was heated under reflux for about 8–10 h. The solid obtained was filtered off dried and recrystallized from methanol to give the corresponding title compounds (3a-f), respectively.

4.1.3.1. 2-(2-benzylidenehydrazinyl)-6-oxo-4-(p-tolyl)-1,6-dihydropyrimidine-5-carbonitrile (3a).

Yield 73%; yellow crystal; m.p over 300 °C (MeOH); IR (cm\(^{-1}\)): 3143 (NH), 3046 (CH-aromatic), 2921 (CH-aliphatic); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 2.37 (s, 3H, CH\(_3\)), 7.33 (d, 2H, Ar-H), 7.42 (d, 2H, Ar-H), 8.18 (s, 1H, NH\(_2\); D\(_2\)O exchangeable), 10.15 (s, 1H, NH\(_2\); D\(_2\)O exchangeable); MS (m/z): 243.12 (M\(^+\), 100%). Anal. Calcd for C\(_{12}\)H\(_{11}\)N\(_5\)O (371.41): C, 59.74; H, 4.60; N, 29.03; Found: C, 59.74; H, 4.60; N, 29.03%.
7.98–8.01 (m, 3H, Ar-H of phenyl ring), 7.77 (d, 2H, Ar-H of phenyl ring), 8.17 (s, 1H, N=CH), 12.37 (s, 2H, 2NH, D$_2$O exchangeable); MS (m/z) 329.15 (M$^+$, 77.21), 90.06 (100). Anal. Calc'd for C$_{19}$H$_{15}$N$_5$O: C, 69.29; H, 4.59; N, 21.26%; Found: C, 69.29; H, 4.59; N, 21.26%.

4.1.3.2. 2-(2-(4-methylbenzylidene)hydrazinyl)-6-oxo-4-(p-tolyl)-1,6-dihydropyrimidine-5-carbonitrile (3b). Yield 81%; yellow crystal; m.p. over 300 °C (MeOH); IR (cm$^{-1}$): 3194 (NH), 3030 (CH aromatic), 2917 (CH aliphatic), 2208 (CN), 1664 (CO); $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ (ppm): 2.33 (s, 3H, CH$_3$), 2.37 (s, 3H, CH$_3$), 7.23 (d, 2H, Ar-H, $J = 8$ Hz), 7.33 (d, 2H, Ar-H, $J = 8$ Hz), 7.76 (d, 2H, Ar-H, of benzylidene ring, $J = 8$ Hz), 8.13 (s, 1H, N=CH); 12.32 (s, 2H, 2NH, D$_2$O exchangeable); MS (m/z): 329.15 (M$^+$, 42.03), 184.09 (100); Anal. Calc'd for C$_{19}$H$_{15}$N$_5$O (329.36): C, 69.29; H, 4.59; N, 21.26%; Found: C, 69.29; H, 4.59; N, 21.26%.

4.1.3.3. 2-(2-(4-chlorobenzylidene)hydrazinyl)-6-oxo-4-(p-tolyl)-1,6-dihydropyrimidine-5-carbonitrile (3c). Yield 70%; yellow crystal; m.p. over 300 °C (MeOH); IR (cm$^{-1}$): 3229, 3175 (NH), 3048 (CH aromatic), 2204 (CN), 1656 (CO); $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ (ppm): 2.37 (s, 3H, CH$_3$), 7.23 (d, 2H, Ar-H), 7.33 (d, 2H, Ar-H), 7.75 (d, 2H, Ar-H of benzylidene ring), 7.88 (d, 2H, Ar-H of benzylidene ring), 8.13 (s, 1H, N=CH); 12.59 (s, 2H, 2NH, D$_2$O exchangeable); MS (m/z): 365.10 (M$^+$, 42.03), 363.11 (M$^+$, 52.14), 89.08 (100); Anal. Calc'd for C$_{19}$H$_{14}$ClN$_5$O (363.81): C, 62.73; H, 3.88; N, 19.25%; Found: C, 62.73; H, 3.88; N, 19.25%.

4.1.3.4. 2-(2-(4-hydroxybenzylidene)hydrazinyl)-6-oxo-4-(p-tolyl)-1,6-dihydropyrimidine-5-carbonitrile (3d). Yield 89%; yellow crystal; m.p. over 300 °C (MeOH); IR (cm$^{-1}$): 3478 (OH), 3394, 3160 (2NH), 3062 (CH aromatic), 2206 (CN), 1673 (CO); $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ (ppm): 2.37 (s, 3H, CH$_3$), 6.79 (d, 2H, Ar-H of benzylidene ring), 7.32 (d, 2H, Ar-H, of benzylidene ring, $J = 8$ Hz), 7.76 (d, 2H, Ar-H), 8.07 (s, 1H, N=CH); 9.87 (s, 1H, OH, D$_2$O exchangeable); 12.13 (s, 2H, 2NH, D$_2$O exchangeable); MS: m/z(%) 345.12 (M$^+$, 39.75%), 40.17 (100); Anal. Calc'd for C$_{19}$H$_{15}$N$_5$O$_2$ (345.36): C, 66.08; H, 4.38; N, 20.40%.

Fig. 5. Predicted binding mode of compound 8 at the binding site of TS.

Fig. 6. Mapping surface showing compound 8 occupying the active pocket of TS.
20.28; Found: C, 66.08; H, 4.38; N, 20.28%.

4.1.3.5. 2-(2-(4-methoxybenzylidene)hydrazinyl)-6-oxo-4-(p-tolyl)-1,6-dihydropyrimidine-5-carbonitrile (3e). Yield 83%; yellow crystal; m.p. 335–337 °C (MeOH); IR (cm⁻¹): 3209, 3160 (NH), 3041 (CH-aromatic), 2925 (CH-aliyclic), 2213 (CN), 1662 (CO); ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 2.37 (3H, CH₃), 3.79 (3H, OCH₃), 6.97 (2H, d, Ar-H, J = 8 Hz), 7.32 (2H, d, Ar-H, J = 8 Hz), 7.75 (2H, d, Ar-H, J = 8 Hz), 7.94 (d, 2H, Ar-H, J = 8 Hz), 7.97 (s, 1H, NH, D₂O exchangeable), 7.24 (1H, NH, D₂O exchangeable), 10.15 (2H, 2H, NH, D₂O exchangeable); MS (m/z): 359.14 (M⁺, 52.20%), 198.12 (100%). Anal. Calcd for: C₁₉H₁₄N₆O₄ (390.32 M⁺) C: 58.46, H: 3.61, N: 21.53; Found: C, 58.73; H, 3.67; N, 21.80%.

4.1.5. Synthesis of 1-(5-cyano-1,6-dihydro-6-oxo-4-p-tolylpyrimidin-2-yl)-4-phenylthiosemicarbazide (5)

A mixture of compound 2 (2.4 g, 10 mmol), phenyl isothiocyanate (1.2 g, 10 mmol) was refluxed in pyridine for 6h. After cooling, the reaction mixture was poured on ice and neutralized with HCl. The solid separated was filtered, washed several times with cold water and re-crystallized from ethylacetate to give compound 5.

Yield 90%; yellow crystal; m.p.130–132 °C (Ethylacetate); IR (KBr) (cm⁻¹): 3205 (NH), 2210 (CN), 1669 (CO). ¹H NMR (DMSO-d₆-D₂O) δ (ppm): 2.35 (3H, CH₃), 6.89–7.32 (5H, 5H, Ar-H), 7.46 (d, 2H, 4-CH₃-ph-H₃,J = 8 Hz), 7.52 (d, 2H, 4-CH₂-ph-H₆,J = 8 Hz), 9.73 (s, 3H, 3NH; exchangeable with D₂O), 9.80 (s, 1H, NH; exchangeable with D₂O); MS (m/z): 376.11 (M⁺, 2.61%), 268.10 (100%); Anal. Calcd for: C₁₉H₁₄N₆O₄ (376.43) C: 60.62, H: 4.28, N: 22.33; Found: C, 60.94; H, 4.39; N, 22.61%.

4.1.6. Synthesis of ethyl-N-(5-cyano-6-oxo-4-(p-tolyl)-1,6-dihydropyrimidin-2-yl)formamidehydrazone (6)

A mixture of 2 (2.4 g, 10 mmole), triethylthioformate (5mL) and acetic anhydride (5mL) was refluxed for 10h, and allowed to cool and poured on ice. The obtained precipitate was filtered off, washed with H₂O, dried, and re-crystallized from ethanol to give compound 6.

Yield 76%; yellow crystal; m.p. 242–244 °C (EtOH); IR (KBr) (cm⁻¹): 3117 (NH), 2218 (CN), 1681 (CO); ¹H NMR (DMSO-d₆-D₂O) δ (ppm): 1.39–1.42 (t, 3H, OCH₂CH₃), 2.39 (3H, CH₃), 4.30–4.32 (q, 2H, OCH₂), 7.37 (d, 2H, 4-CH₂-ph-H₃,J = 8 Hz), 7.80 (d, 2H, 4-CH₃-ph-H₆,J = 8 Hz), 8.36 (s, 1H, NH = CH); MS (m/z): 345.00 (100%); Anal. Calcd for: C₁₉H₁₅N₅O₄ (345.32 M⁺) C: 60.87, H: 5.16, N: 23.38%.

4.1.7. Synthesis of N',N'-diacetyl-N-(5-cyano-4-ethoxy-6-p-tolylpyrimidin-2-yl) acetohydrazide (7)

Anequimolaramountofcompound2andtriethylthioformate,was refluxed in acetic anhydride (10mL) for 3h. After standing, the crystals formed were filtered, and washed with ethanol to give compound 7. X-ray crystallography was performed for the resultant crystals.

Yield 69%; yellow crystal; m.p. 137–139 °C (EtOH); IR (KBr) (cm⁻¹): 2218 (CN), 1733, 1710 (CO); ¹H NMR (DMSO-d₆-D₂O) δ (ppm): 1.34–1.38 (t, 3H, OCH₂CH₃), 2.30 (3H, CH₂), 2.39 (3H, CH₃), 2.75 (6H, 2CH₃CO), 4.38–4.54 (q, 2H, CH₂), 7.40 (d, 2H, 4-CH₂-ph-H₆,J = 8 Hz), 7.84 (d, 2H, 4-CH₂-ph-H₆,J = 8 Hz); MS (m/z): 395.16 (M⁺, 6.97%), 311.15 (100%); Anal. Calcd for: C₁₉H₁₅N₆O₂ (395.41) C: 60.75, H: 5.35, N: 17.71; Found: C, 60.89; H, 5.44; N, 18.06%.

4.1.8. Synthesis of N',N'-diacetyl-N-(5-cyano-1,6-dihydro-6-oxo-4-p-tolylpyrimidin-2-yl) uctohydrazide (8)

An equimolar amount of compound 2 and triethylthioformate, was refluxed in acetic anhydride (10mL) for 3h. After standing, the crystals formed were filtered, and washed with ethanol to give compound 8. X-ray crystallography was performed for the resultant crystals.

Yield 81%; yellow crystal; m.p. 172–174 °C (EtOH); IR (KBr) (cm⁻¹): 3135 (NH), 2223 (CN), 1734, 1669 (CO). ¹H NMR (DMSO-d₆-D₂O) δ (ppm): 2.31 (3s, 3H, COCH₃), 2.38 (3s, 3H, CH₂), 2.64 (6H, 2COCH₃), 3.64 (s, 1H, NH; exchangeable with D₂O), 7.37 (d, 2H, 4-CH₂-ph-H₃,J = 8 Hz), 7.79 (d, 2H, 4-CH₂-ph-H₆,J = 8 Hz); MS (m/z): 367.13 (M⁺, 5.14%), 283.10 (100%); Anal. Calcd for: C₁₉H₁₄N₆O₄ (367.36) C: 58.85, H: 4.66, N: 19.06; Found: C, 59.16; H, 4.70; N, 19.31%.

4.1.9. Synthesis of 1,6-dihydro-2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-oxo-4-p-tolylpyrimidine-5-carbonitrile (9)

A mixture of compound 2 (10mmol) and acetylacetone (10mmol) was refluxed in glacial acetic acid for 20h. The reaction mixture was
cooled and poured on ice cold water. The formed precipitate was filtered, dried and recrystallized from ethanol afforded compound 9.

Yield 56%; yellow crystal; m.p. 271–273 °C (EtOH); IR (KBr) (cm\(^{-1}\)): 3332 (NH), 2218 (CN), 1666 (CO). \(^1\)H NMR (DMSO-d\(_6\)-D\(_2\)O) δ (ppm): 2.22 (s, 3H, CH\(_3\)), 2.38 (s, 3H, CH\(_3\)), 2.61 (s, 3H, CH\(_3\)), 6.29 (s, 1H, CH, pyrazole), 7.05 (d, 2H, 4-CH\(_3\)-ph-H\(_{5,6}\), J = 8 Hz), 7.19 (d, 2H, 4-CH\(_3\)-ph-H\(_{5,6}\), J = 8 Hz); MS (m/z): 305.17 (M\(^+\), 100%); Analy. Calcld for: C\(_{17}\)H\(_{15}\)N\(_5\)O\(_2\) (305.33), C: 66.87, H: 4.95, N: 22.94; Found: C, 66.72; H, 4.96; N, 23.28%.

4.1.10. Synthesis of 1,6-dihydro-2-(3-methyl-5-oxo-2H-pyrazol-1(5H)-yl)-6-oxo-4-p-tolylpyrimidin-5-carbonitrile (10)

A mixture of compound 2 (10 mmol), ethyl acetoacetate (10 mmol) in glacial acetic acid was refluxed for 12 h. The reaction mixture was cooled and poured on ice- water. The formed precipitate was filtered off, dried and recrystallized from ethanol to give compound 10.

Yield: 68%; yellow crystal; m.p. 353–355 °C (EtOH); IR (KBr) (cm\(^{-1}\)): 3324 (NH), 2221 (CN), 1746, 1665 (CO). \(^1\)H NMR (DMSO-d\(_6\)-D\(_2\)O) δ (ppm): 2.30 (s, 3H, CH\(_3\)), 2.48 (s, 3H, CH\(_3\)), 4.31 (s, 1H, CH), 7.40 (d, 2H, 4-CH\(_3\)-ph-H\(_{5,6}\), J = 8.4 Hz), 8.03 (d, 2H, 4-CH\(_3\)-ph-H\(_{5,6}\), J = 8.4 Hz), 11.79 (s, 1H, NH; exchangeable with D\(_2\)O), 12.05 (s, 1H, NH; exchangeable with D\(_2\)O); MS (m/z): 307.00 (M\(^+\), 27.35%), 76.98 (100%); Analy. Calcld for: C\(_{16}\)H\(_{13}\)N\(_5\)O\(_2\) (307.31), Calcld for C: 62.53, H: 4.37, N: 23.12%.

4.1.11. Synthesis of 2-(1,3-dioxoisoindolin-2-ylamino)-1,6-dihydro-6-oxo-4-p-tolylpyrimidin-5-carbonitrile (11)

A mixture of compound 2 (10 mmol), and phthalic anhydride (10 mmol), was refluxed in glacial acetic acid (20 mL) for 12 h. The reaction mixture was cooled and poured on ice water. The formed precipitate was filtered, dried and recrystallized from ethanol.

Yield 73%; yellow crystal; m.p. 293–295 °C (EtOH); IR (KBr) (cm\(^{-1}\)): 3459 (NH), 2220 (CN), 1798, 1745, 1676 (CO); \(^1\)H NMR (DMSO-d\(_6\)-D\(_2\)O) δ (ppm): 2.39 (s, 3H, CH\(_3\)), 7.36 (d, 2H, 4-CH\(_3\)-ph-H\(_{5,6}\), J = 8 Hz), 7.54–7.68 (m, 2H, isoindolin-H), 7.74 (s, 1H, NH, D\(_2\)O exchangeable), 7.62 (s, 2H, 4-CH\(_3\)-ph-H\(_{5,6}\), J = 8 Hz), 7.92–8.00 (m, 2H, isoindolin-H); MS (m/z): 371.04 (M\(^+\) 3.18%), 77.02 (100%); Analy. Calcld for: C\(_{15}\)H\(_{12}\)ClN\(_5\)O\(_2\) (371.35), C: 64.69, H: 3.53, N: 22.79; Found: C, 62.79; H: 3.47, N: 23.12%.

4.2. Biological evaluation

4.2.1. In vitro anticancer activity

Three human cancer cell lines, namely hepatocellular carcinoma (HePG-2), mammary gland (MCF-7) and colorectal carcinoma (HCT-116), are used to determine in vitro the anticancer activity of the synthesized compounds. The tested cell lines were supplied from the US National Cancer Institute. The reported standard procedure \cite{35} was utilized as follows. The tested cells were plated in 96-well microplates; the total volume per well was adjusted at 100 μL. Then, incubation of cells was performed at 37 °C, 5% CO\(_2\), 95% air and 100% relative humidity for 24 h before addition of synthesized compounds. After 24 h, only two plates of each cell line were selected and fixed in situ with TCA, in order to exemplify a measurement of the cell population for each cell line during drug application. The title compounds and fluorouracil, the reference drug, were dissolved in DMSO at 400-fold the desired final maximum test concentration and stored at freezing point prior to use. During addition of drug, the frozen concentrate was dissolved and diluted to twice the desired final maximum test concentration with gemtacimicin solution (50 mg/mL). To reach the desired final drug concentrations, different tested compound dilutions (100 μL) were added to the appropriate microtiter wells containing 100 μL of medium. The tested compounds as well as 5-fluorouracil as reference drug were added. Then, the plates were incubated for an additional 48 h at 37 °C, 5% CO\(_2\), 95% air and 100% relative humidity. The assay was terminated by addition of cold TCA for adherent cells followed by incubation for 60 min at 4 °C. The supernatant was removed, and the plates were washed five times with excessive water and dried. A solution of 0.4% w/v sulforhodamine B (100 μL) in 1% acetic acid was added to each well, followed by incubation at room temperature for 10 min. After staining, the plates were washed with 1% acetic acid to remove unbound dye and air-dried. Bound stain was dissolved with 10 mM Trizma base. Spectrophotometric assay of the optical density (OD) of each well was determined at 564 nm with an ELISA microplate reader (ChroMate-4300, FL, USA). Boltzmann sigmoidal concentration response curves was used to calculate the IC\(_{50}\) values through the non-linear regression fitting models (GraphPad Prism, version 5). The means of three separate experiments was reported as final result. ANOVA test was used to analyze the statistical differences, wherein the differences were considered to be significant at p < 0.05.

4.2.2. In vitro assay of thymidylate synthase (TS) activity

The activity of TS was measured spectrophotometrically at pH 7.4 and 30 °C in a mixture containing 0.1 M 2-mercaptoethanol, 0.0003 M (6R,S)-tetrahydrofolate, 0.02 M MgCl\(_2\), 0.012 M formaldehyde, 0.04 M TrisHCl, 0.001 M dUMP and 0.00075 M Na EDTA according to the reported procedure \cite{23}. In general, to initiate the reaction, the enzyme was added in the absence of inhibitor producing a change in absorbance at 340 nm of 0.016/min. Then, four inhibitor concentrations were used to determine the percent inhibition. Next, concentration-inhibition response curve for the test compounds was generated to determine median inhibitory concentration (IC\(_{50}\)). The obtained data were compared with 5-fluorouracil as a standard TS inhibitor. The standard deviations for determination of the 50% points were within ± 10% of the values given.

HTS enzyme used to perform the kinetic assays was obtained from Sigma Co., St. Louis, USA, via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt.

4.2.3. Determination of the active caspase-3

To determine the effect of the synthesized compounds on apoptosis, the active caspase-3 level was measured by using Quantikine-Human active Caspase-3 Immunoassay (R&D Systems, Inc. Minneapolis, USA).
according to the manufacturer protocol. Briefly, after washing the cells with PBS, the cells were collected and lysed by adding it to the extraction buffer containing protease inhibitors (1 mL per 1–107 cells.) then the lysate was diluted immediately prior to the assay. At the end of the assay the optical density of each well was determined within 30 min using a microplate reader set at 450 nm.

4.2.4. RNA extraction, real time PCR analysis and quantification of gene expression

The gene expression of Bax and Bcl-2 was assessed by total RNA extraction from cells using RNeasy Mini Kit® (Qiagen Inc. Valencia, CA, USA). cDNA library was constructed from different treatments using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The archived cDNA libraries were then subjected to quantitative real time PCR reactions [36] using cyber green fluorophore (Fermentas Inc., Glen Burnie, MD, USA).

4.3. Molecular docking

The docking simulation studies were performed using Molecular Operating Environment (MOE®) version 2014, 0901 Chemical Computing Group Inc., Montreal, Canada. The X-ray crystallographic structure hTS (code 6QXG) was obtained from the Protein Data Bank through the internet. The enzyme was prepared for docking studies by: The ligand and water molecules were removed from hTS active site. Hydrogen atoms were added to the system with their standard geometry. The atoms connection and type were checked for any errors with automatic correction. Selection of the receptor and its atoms potential were fixed. MOE Alpha Site Finder was used for the active site search in the enzyme structure using all default items. Dummy atoms were created from the obtained alpha Spheres. The following methodology was applied: The enzyme active site file was loaded and the Dock tool was initiated. The program specifications were adjusted to: Dummy atoms as the docking site. Triangle matcher as the placement methodology to be used. London dG as Scoring methodology to be used and was adjusted to its default values. The MDB file of the ligand to be docked was loaded and Dock calculations were run automatically. The obtained poses were studied and the poses showed best ligand-enzyme interactions were selected and stored for energy calculations. The docking scores were expressed in negative energy terms; the lower the binding free energy, the better the binding affinity. The structures of ligands were drawn in Chem Draw Ultra 8.0 (Cheminformatics Software company based in Cambridge, Massachusetts, USA) and saved as mol. The two-dimensional structure of the selected compounds was converted into their three-dimensional form and energy minimized using the MMFF94x force field until a root-mean-square deviation of atomic position gradient of 0.01 Kcal/mol/E was reached.

Declaration of Competing Interest

The authors declare no conflict of interest, financial or otherwise.

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Appendix A. Supplementary material

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