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RESEARCH ARTICLE

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Design, synthesis and molecular docking of novel diarylcyclohexenone and diarylindazole derivatives as tubulin polymerization inhibitors

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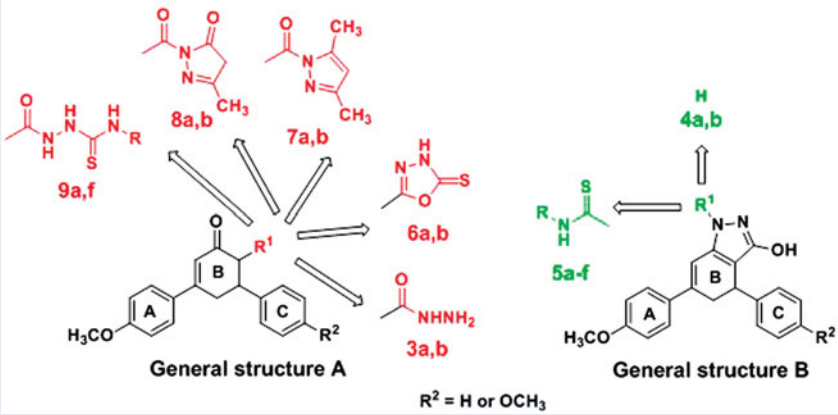
ABSTRACT

New target compounds were designed as inhibitors of tubulin polymerization relying on using two types of ring B models (cyclohexenone and indazole) to replace the central ring in colchicine. Different functional groups (R^1) were attached to manipulate their physicochemical properties and/or their biological activity. The designed compounds were assessed for their antitumor activity on HCT-116 and MCF-7 cancer cell lines. Compounds **4b**, **5e** and **5f** exhibited comparable or higher potency than colchicine against colon HCT-116 and MCF-7 tumor cells. The mechanism of the antitumor activity was investigated through evaluating the tubulin inhibition potential of the active compounds. Compounds **4b**, **5e** and **5f** showed percentage inhibition of tubulin in both cell line homogenates ranging from 79.72% to 89.31%. Cell cycle analysis of compounds **4b**, **5e** and **5f** revealed cell cycle arrest at G_2/M phase. Molecular docking revealed the binding mode of these new compounds into the colchicine binding site of tubulin.



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Introduction

Drugs that disrupt microtubule/tubulin dynamics are widely used in cancer chemotherapy. The vast majority of these molecules act by binding to the protein tubulin, an α,β-heterodimer that forms the core of the microtubules which play a crucial role in the maintenance of cell shape, signal transduction and chromosome segregation during mitosis. Inhibitors of microtubules engage the cell cycle surveillance mechanisms to arrest cell division in mitosis. Microtubules targeting agents, also called antimitotic agents, perturb not only mitosis, but also arrest cells during interphase[1,2].

Microtubules targeting agents are known to interact with tubulin through at least three binding sites: the paclitaxel domain, vinca site and the colchicine domain. So far, tubulin binding agents can be classified into two types based on their site of action as microtubule destabilizing drugs (vinca site and the colchicine site) and microtubule-stabilizing drugs (taxane site). Out of the three binding domains, colchicine binds with high affinity to β-tubulin and forms entangled tubulin dimer, which inhibits the microtubule assembly[3]. Literature revealed many colchicine site inhibitors being evaluated under clinical investigation, and even more in preclinical studies[4]. Colchicine **I** is a rigid molecule whose rigidity is imparted by the B-ring which anchored rings A and C. Rings A and C are aimed to fit with hydrophobic pockets in the colchicine binding site. In addition, an H-bond acceptor (OCH_3) group on ring A is a key feature of these inhibitors. Therefore, in the course of developing new more flexible colchicine analogs, many trials were made to modify the bridge between rings A and C[5,6]. More flexible derivatives involved the replacement of ring B with an olefinic bridge as in combretastatin A-4 **II**[7,8], insertion of a carbonyl function[9–11] or a variety of heterocyclic rings[12–14] handling rings A and C, as exemplified by the pyrazole derivative **III**[15].

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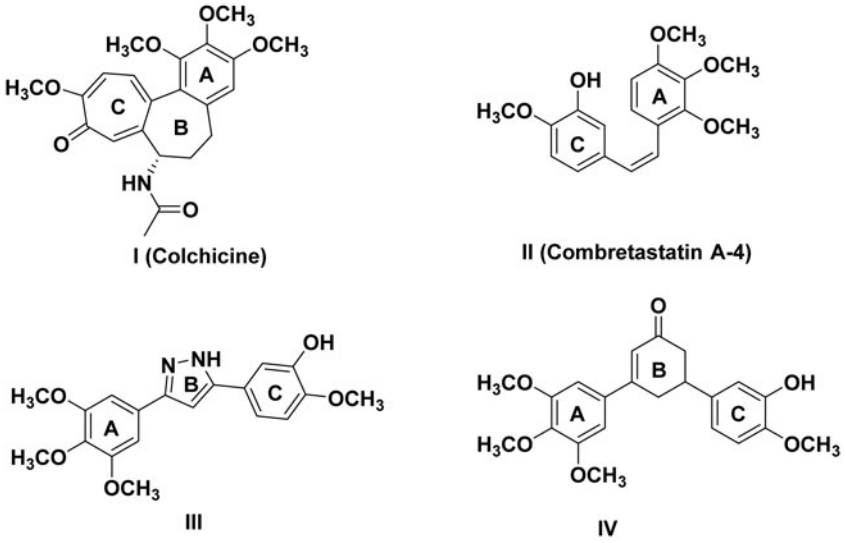


Figure 1. Examples of colchicine binding site inhibitors.

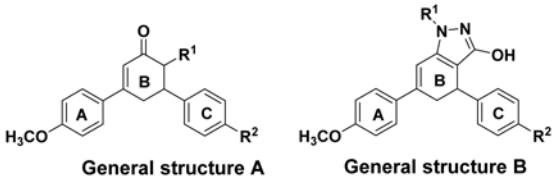


Figure 2. General structures of target compounds.

Alternatively, an alicyclic ring was used as demonstrated by the cyclohexenone derivative **IV**[16] (Figure 1).

With the goal of producing new antitumor agents targeting the microtubules at the colchicine binding site, and based on the aforementioned facts, the design of the new target compounds relied on using two types of ring B models. The first involved the cyclohexenone ring (General structure A) and the other involved the indazole ring (General structure B) as linker moieties between the two hydrophobic rings A and C. Different functional groups (R^1) were attached to ring B to manipulate their physicochemical properties and/or their biological activity. While retaining the H-bond acceptor methoxy group pendent on ring A, another methoxy anchor group (R^2) was introduced in ring C for comparative reasons (Figure 2).

The designed compounds were assessed for their antitumor activity through *in vitro* cytotoxicity study on selected human cancer cell lines. The mechanism of the antitumor activity was investigated through evaluating the tubulin inhibition potential of the active compounds. Finally, a molecular docking study was carried out.

Materials and methods

Chemistry

Melting points were uncorrected and were detected by open capillary tube using Electrothermal 9100 melting point apparatus (Bibby Scientific Limited, Stone, UK). Thin layer chromatography was performed using silica gel cards DC-Alufolien-Kiesel gel with fluorescent indicator UV254 using chloroform or hexane–ethyl acetate 8.5:1.5 as the eluting system and the spots were visualized using Vilber Lourmet ultraviolet lamp at $\lambda = 254$ nm. Elemental microanalyses were performed at the Regional Center for Mycology and Biotechnology, Al-Azhar University. NMR spectra were recorded at the Microanalytical unit, Faculty of pharmacy, Cairo University on Bruker Avance III spectrometer (Zurich, Switzerland) at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shift values (δ) were given downfield from TMS. Samples were dissolved in DMSO-d_6, addition of D_2O was used to confirm the exchangeable protons. Compounds **1a,b** were prepared according to the previously reported procedure[17].

General procedure for the preparation of 2a,b

A solution of ethyl acetoacetate (1.56 ml, 12 mmol) in sodium ethoxide solution (0.3 g sodium metal in 140 ml absolute ethanol) was stirred at room temperature for 1 h. The propenone **1a,b** (12 mmol) was added to the above solution with stirring. The reaction mixture was heated under reflux for 12 h and poured onto cold hydrochloric acid. The obtained solid was filtered off, washed with water, dried and crystallized from methanol.

Ethyl 6-phenyl-4-(4-methoxyphenyl)-2-oxocyclohex-3-enecarboxylate (2a)

Compound **2a** was prepared from compound **1a** and ethylacetoacetate. 61% yield, mp 70–73 °C. ^{1}H NMR δ 0.96 (t, 3H, $J = 7.2$ Hz, CH_3 ethyl), 2.98 (dd, 1H, $J = 4.6$ Hz, $J = 17.8$ Hz, $H5_{cyclohex.ax.}$), 3.08 (ddd, 1H, $J = 2.1$ Hz, $J = 11.2$ Hz, $J = 17.68$ Hz, $H5'_{cyclohex.eq.}$), 3.64 (m, 1H, $H6_{cyclohex}$), 3.40 (s, 3H, OCH_3), 3.93 (q, 2H, $J = 7.2$, CH_2 ethyl), 4.13 (d, 1H, $J = 3.9$, $H1_{cyclohex}$), 6.56 (d, 1H, $J = 1.9$ Hz, $H3_{cyclohex}$), 7.40–7.73 (m, 9H, Ar–Hs). Anal. calcd. for $C_{22}H_{24}O_4$ (350.41): C, 75.41; H, 6.33. Found: C, 75.64; H, 6.42.

Ethyl 4,6-(4-methoxyphenyl)-2-oxocyclohex-3-enecarboxylate (2b)

Compound **2b** was prepared from compound **1b** and ethylacetoacetate. 64% yield, mp 78–81 °C. ^{1}H NMR δ 0.95 (t, 3H, $J = 7.2$ Hz, CH_3 ethyl), 3.02 (m, 2H, $H5_{cyclohex.ax.}$, $H5'c_{yclohex.eq.}$), 3.66 (m, 1H, $H6_{cyclohex}$), 3.80 (s, 6H, $2OCH_3$), 3.94 (q, 2H, $J = 7.2$ Hz, CH_2 ethyl), 4.11 (d, 1H, $J = 3.8$ Hz, $H1_{cyclohex}$), 6.52 (s, 1H, $H3_{cyclohex}$), 6.98–7.72 (m, 8H, Ar–Hs). Anal. Calcd. for $C_{23}H_{24}O_5$ (380.43): C, 72.61; H, 6.36. Found: C, 72.88; H, 6.41.

General procedure for the preparation of 3a,b

To a solution of the ester **2a,b** (10 mmol) in absolute ethanol (30 ml), 98% hydrazine hydrate (0.64 ml, 20 mmol) was added. The reaction mixture was stirred for 24 h. The precipitated solid was filtered off and recrystallized from absolute ethanol.

2-Hydroxy-4-(4-methoxyphenyl)-6-phenylcyclohexa-1,3-diene carbohydrazide (3a)

Compound **3b** was prepared from compound **2b** and 98% hydrazine hydrate by stirring at RT 67% yield, mp 124–127 °C. ^{1}H NMR δ 2.69 (dd, 1H, $J = 4.7$, $J = 17.6$, $H5c_{cyclohex.ax.}$), 2.79 (ddd, 1H, $J = 2.1$ Hz, $J = 11.2$ Hz, $J = 17.6$ Hz, $H5'_{cyclohex.eq.}$), 3.72 (m, 1H, $H6_{cyclohex}$), 3.87 (s, 3H, OCH_3), 6.30 (brs, 3H, $NHNH_2$, D_2O exchange), 6.87 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 6.98–7.50 (m, 9H, Ar–Hs), 10.80 (brs, 1H, OH, D_2O exchange). ^{13}C NMR δ 29.8 ($C6_{cyclohex.}$), 34.8 ($C5_{cyclohex.}$), 55.5 (OCH_3), 114.2, 114.3, 123.7, 127.6, 128.6, 131.2, 133.0, 137.1, 138.6, 142.5 (Ar–Cs), 160.4 (C=O). MS (EI): *m/z* (%): 336.21 (14.91). Anal. calcd. For $C_{20}H_{20}N_2O_3$ (336.38): C, 71.41; H, 5.99; N, 8.33. Found: C, 71.78; H, 6.07; N, 8.51.

2-Hydroxy-4,6-bis(4-methoxyphenyl) cyclohexa-1,3-diene carbohydrazide (3b)

Compound **3b** was prepared from compound **2b** and 98% hydrazine hydrate. 73% yield, mp 144–146 °C. ^{1}H NMR δ 2.75 (dd, 1H, $J = 4.7$ Hz, $J = 17.8$ Hz, $H5_{cyclohex.ax.}$), 2.97 (ddd, 1H, $J = 2.2$ Hz, $J = 11.2$ Hz, $J = 17.7$ Hz, $H5'_{cyclohex.eq.}$), 3.72 (m, 1H, $H6_{cyclohex}$), 3.87 (s, 6H, $2OCH_3$), 6.26 (brs, 3H, $NHNH_2$, D_2O exchange), 6.81 (d, 1H, $J = 1.9$ Hz, $H3_{cyclohex}$), 6.91–7.45 (m, 9H, Ar–Hs and OH). ^{13}C NMR δ 30.0 ($C6_{cyclohex.}$), 35.1 ($C5_{cyclohex.}$), 55.5, 55.6 ($2OCH_3$), 114.2, 114.3, 123.7, 128.5, 133.1, 137.1, 137.9, 146.4 (Ar–Cs), 159.0 (C=O). MS (EI): *m/z* (%): 336.48 (1.10). Anal. calcd For $C_{21}H_{22}N_2O_4$ (336.41): C, 68.84; H, 6.05; N, 7.65. Found: C, 68.97; H, 6.13; N, 7.69.

General procedure for the preparation of 4a,b

A mixture of **2a** or **2b** (10 mmol) and hydrazine hydrate (0.32 ml, 10 mmol) in ethanol (20 ml) was heated under reflux for 8 h. The reaction mixture was evaporated under reduced pressure. After cooling, the reaction mixture was poured onto crushed ice and the solid thus obtained was filtered off, washed with water and crystallized from ethanol to give **4a** and **4b**, respectively.

4-Phenyl-6-(4-methoxyphenyl)-4,5-dihydro-1H-indazol-3-ol (4a)

Compound **4a** was prepared from compound **2a** and 98% hydrazine hydrate under reflux. 76% yield, mp 107–110 °C. ^{1}H NMR δ 2.86, 2.90 (dd, 1H, $J = 3.1$ Hz, $J = 16.7$ Hz, $H5_{indazol.eq.}$), 3.12 (ddd, 1H, $J = 1.7$ Hz, $J = 8.4$ Hz, $J = 16.68$ Hz, $H5'_{indazol.ax.}$), 3.79 (s, 3H, OCH_3), 4.15 (dd, 1H, $J = 3.0$ Hz, $J = 8.2$ Hz, $H4_{indazol}$), 6.27 (s, 1H, NH, D_2O exchange), 6.66 (d, 1H, $J = 3.0$ Hz, $H7_{indazol}$), 7.00–7.70 (m, 9H, Ar–Hs), 10.80 (brs, 1H, OH, D_2O exchange). ^{13}C NMR δ 29.8 ($C4_{indazol}$), 34.8 ($C5_{indazol}$), 55.5 (OCH_3), 114.4, 114.6, 126.4, 127.7, 128.5, 128.8, 129.6, 133.0 (Ar–Cs), 137.1 ($C7_{indazol}$), 145.9, 146.3 (C4 of the 2 phenyl rings), 159.1 ($C3_{indazol}$). MS (EI): *m/z* (%): 318.09 (12.02). Anal. calcd. For $C_{20}H_{18}N_2O_2$ (318.37): C, 75.45; H, 5.70; N, 8.80. Found: C, 75.49; H, 5.76; N, 8.94.

4,6-Bis(4-methoxyphenyl)-4,5-dihydro-1H-indazol-3-ol (4b)

Compound **4b** was prepared from compound **2b** and 98% hydrazine hydrate under reflux. 78% yield, mp 86–89 °C. ^{1}H NMR δ 2.84, 2.88 (dd, 1H, $J = 3.1$ Hz, $J = 16.8$ Hz, $H5_{indazol.eq.}$), 3.14 (ddd, 1H, $J = 1.7$ Hz, $J = 8.4$ Hz, $J = 16.69$ Hz, $H5'_{indazol.ax.}$), 3.80 (s, 6H, $2OCH_3$), 4.16,4.18 (dd, 1H, $J = 3.0$ Hz, $J = 8.3$ Hz, $H4_{indazol}$), 6.20 (s, 1H, NH, D_2O exchange), 6.89 (s, 1H, $H3_{cyclohex}$), 7.00–7.70 (m, 8H, Ar–Hs), 10.79 (brs, 1H, OH, D_2O exchange). ^{13}C NMR δ 33.4 ($C4_{indazol}$), 35.0 ($C5_{indazol}$), 55.3, 55.6 ($2OCH_3$), 114.2, 114.4, 128.2, 128.5, 129.6, 132.7 (Ar–Cs), 137.5 ($C7_{indazol}$), 157.9. 158.1 (C4 of the two phenyl rings), 160.4 ($C3_{indazol}$). MS (EI): *m/z* (%): 348.36 (2.81). Anal. calcd. for $C_{21}H_{20}N_2O_3$ (348.40): C, 72.40; H, 5.79; N, 8.04. Found: C, 72.53; H, 5.84; N, 8.17.

General procedure for the preparation of 5a–f

A mixture of the corresponding hydrazide **4a,b** (10 mmol) and the appropriate isothiocyanate derivative (10 mmol) in ethanol (20 ml) was heated under reflux for 3 h. The formed solid was filtered off, washed with ethanol and crystallized from ethanol.

3-Hydroxy-6-(4-methoxyphenyl)-4-phenyl-N-methyl-4,5-dihydroindazole-1-carbothioamide (5a)

Compound **5a** was prepared from compound **4a** and methyl isothiocyanate. 88% yield, mp 220–224 °C. ^{1}H NMR δ 2.80, 2.84 (dd, 1H, $J = 3.2$ Hz, $J = 16.8$ Hz, $H5_{indazol.eq}$), 3.00 (s, 3H, CH_3), 3.11 (ddd, 1H, $J = 1.8$, $J = 8.3$ Hz, $J = 16.7$ Hz, $H5'_{indazol.ax.}$), 3.79 (s, 3H, OCH_3), 4.10, 4.14 (dd, 1H, $J = 3.0$ Hz, $J = 8.1$ Hz, $H4_{indazol}$) 6.61 (d, 1H, $J = 1.8$ Hz, $H7_{indazol}$), 7.10–8.30 (m, 9H, Ar–Hs), 10.36 (s, 1H, NH, D_2O exchange), 11.05 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 31.1 ($NHCH_3$), 34.7 ($C4_{indazol}$), 36.3 ($C5_{indazol}$), 55.6 (OCH_3), 114.3, 114.6, 122.1, 126.9, 127.6, 128.6, 131.9, 132.1 (Ar–Cs), 137.1 ($C7a_{indazol}$), 144.9 ($C6_{indazol}$), 147.1, 147.7 (C4 of the 2 phenyl rings), 160.5 ($C3_{indazol}$), 178.6 (C=S). MS (EI): *m/z* (%): 391.24 (1.80). Anal. calcd. for $C_{22}H_{21}N_3O_2S$ (391.49): C, 67.50; H, 5.41; N, 10.73. Found: C, 67.84; H, 5.44; N, 10.49.

3-Hydroxy-4,6-bis(4-methoxyphenyl)-N-methyl-4,5-dihydroindazole-1-carbothioamide (5b)

Compound **5b** was prepared from compound **4b** and methyl isothiocyanate. 90% yield, mp 170–173 °C. ^{1}H NMR δ 2.80, 2.85 (dd, 1H, $J = 3.2$ Hz, $J = 16.8$ Hz, $H5_{indazol.eq}$), 2.99 (ddd, 1H, $J = 1.8$, $J = 8.3$ Hz, $J = 16.7$ Hz, $H5'_{indazol.ax.}$), 3.08 (s, 3H, CH_3), 3.79 (s, 3H, OCH_3), 4.10, 4.14 (dd, 1H, $J = 3.0$ Hz, $J = 8.1$ Hz, $H4_{indazol}$) 6.61 (d, 1H, $J = 1.8$ Hz, $H7_{indazol}$), 7.10–8.30 (m, 9H, Ar–Hs), 10.30 (s, 1H, NH, D_2O exchange), 11.01 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 31.2 ($NHCH_3$), 34.9 ($C4_{indazol}$), 36.0 ($C5_{indazol}$), 55.4, 55.7 ($2OCH_3$), 114.2, 114.4, 122.0, 126.9, 128.4, 131.9, 132.1 (Ar–Cs), 137.1 ($C7a_{indazol}$), 144.9 ($C6_{indazol}$), 158.3, 159.9 (C4 of the two phenyl rings), 160.4 ($C3_{indazol}$), 178.5 (C=S). MS (EI): *m/z* (%): 421.48 (3.61). Anal. calcd. for $C_{23}H_{23}N_3O_3S$ (421.51): C, 65.54; H, 5.50; N, 9.97. Found: C, 65.81; H, 5.57; N, 10.04.

3-Hydroxy-6-(4-methoxyphenyl)-4-phenyl-N-ethyl-4,5-dihydroindazole-1-carbothioamide (5c)

Compound **5c** was prepared from compound **4a** and ethyl isothiocyanate. 83% yield, mp 202–205 °C. ^{1}H NMR δ 1.01 (t, 3H, $J = 7.2$, $CH_{3\ ethyl}$), 2.72, 2.74 (dd, 1H, $J = 3.2$ Hz, $J = 16.8$ Hz, $H5_{indazol.eq}$), 2.83 (ddd, 1H, $J = 1.8$ Hz, $J = 8.3$ Hz, $J = 16.7$ Hz, $H5'_{indazol.ax.}$), 3.50 (q, 2H, $J = 7.2$, $CH_{2\ ethyl}$), 3.78 (s, 3H, OCH_3), 4.11,4.14 (dd, 1H, $J = 3.0$ Hz, $J = 8.1$ Hz, $H4_{indazol}$), 6.60 (d, 1H, $J = 1.8$ Hz, $H7_{indazol}$), 6.95–8.37 (m, 9H, Ar–Hs), 10.27 (s, 1H, NH, D_2O exchange), 10.99 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 15.0 ($CH_{3\ ethyl}$), 34.6 ($C4_{indazol}$), 35.8 ($CH_{2\ ethyl}$), 38.1 ($C5_{indazol}$), 55.6 (OCH_3), 114.2, 122.1, 127.0, 127.4, 128.1, 128.6, 128.9, 131.9, 132.1 (Ar–Cs), 137.2 ($C7a_{indazol}$), 144.9 ($C6_{indazol}$), 159.9 (C4 of the two phenyl rings), 160.5 ($C3_{indazol}$), 177.6 (C=S). MS (EI): *m/z* (%): 405.17 (1.90). Anal. calcd. for $C_{23}H_{23}N_3O_2S$ (405.51): C, 68.12; H, 5.72; N, 10.36. Found: C, 68.35; H, 5.81; N, 10.49.

3-Hydroxy-4,6-bis(4-methoxyphenyl)-N-ethyl-4,5-dihydroindazole-1-carbothioamide (5d)

Compound **5d** was prepared from compound **4b** and ethyl isothiocyanate. 86% yield, mp 210–213 °C. ^{1}H NMR δ 1.11 (t, 3H, $J = 7.2$, $CH_{3\ ethyl}$), 2.71, 2.73 (dd, 1H, $J = 3.2$ Hz, $J = 16.8$ Hz,

$H5_{indazol.eq}$), 2.85 (ddd, 1H, $J = 1.8$ Hz, $J = 8.3$ Hz, $J = 16.7$ Hz, $H5'_{indazol.ax.}$), 3.50 (q, 2H, $J = 7.2$, $CH_{2\ ethyl}$), 3.78 (s, 6H, $2OCH_3$), 4.12, 4.15 (dd, 1H, $J = 3.0$ Hz, $J = 8.1$ Hz, $H4_{indazol}$), 6.60 (d, 1H, $J = 1.8$ Hz, $H7_{indazol}$), 6.90–7.70 (m, 8H, Ar–Hs), 10.28 (s, 1H, NH, D_2O exchange), 10.99 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 15.7 ($NHCH_2\underline{C}H_3$), 31.7 ($NH\underline{C}H_2CH_3$), 34.7 ($C4_{indazol}$), 36.1 ($C5_{indazol}$), 55.4, 55.6 ($2OCH_3$), 114.2, 114.3, 114.5, 122.1, 126.8, 127.6, 128.6, 131.9, 132.1, 133.0 (Ar–Cs), 137.1 ($C7a_{indazol}$), 145.0 ($C6_{indazol}$), 157.8, 158.3 (C4 of the 2 phenyl rings), 160.5 ($C3_{indazol}$), 177.6 (C=S). MS (EI): *m/z* (%): 435.20 (1.27). Anal. calcd. for $C_{24}H_{25}N_3O_3S$ (435.54): C, 66.18; H, 5.79; N, 9.65. Found: C, 66.35; H, 5.82; N, 9.78.

3-Hydroxy-6-(4-methoxyphenyl)-4-phenyl-N-phenyl-4,5-dihydroindazole-1-carbothioamide (5e)

Compound **5e** was prepared from compound **4a** and phenyl isothiocyanate. 76% yield, mp 138–140 °C. ^1H NMR δ 2.75, 2.78 (dd, 1H, $J = 3.2$ Hz, $J = 16.8$ Hz, $H5_{indazol.eq}$), 2.87 (ddd, 1H, $J = 1.8$ Hz, $J = 8.3$ Hz, $J = 16.7$ Hz, $H5'_{indazol.ax.}$), 3.80 (s, 3H, OCH_3), 4.11, 4.15 (dd, 1H, $J = 3.0$ Hz, $J = 8.1$ Hz, $H4_{indazol}$), 6.70 (d, 1H, $J = 1.8$ Hz, $H7_{indazol}$), 7.00–7.80 (m, 14H, Ar–Hs), 10.06 (s, 1H, NH, D_2O exchange), 10.77 (s, 1H, OH, D_2O exchange). MS (EI): *m/z* (%): 453.11 (0.81). Anal. calcd. for $C_{27}H_{23}N_3O_2S$ (453.56): C, 71.40; H, 5.11; N, 9.26. Found: C, 71.63; H, 5.14; N, 9.38.

3-Hydroxy-4,6-bis(4-methoxyphenyl)-N-phenyl-4,5-dihydroindazole-1-carbothioamide (5f)

Compound **5f** was prepared from compound **4b** and phenyl isothiocyanate. 78% yield, mp 155–159 °C. ^1H NMR δ 2.78, 2.81 (dd, 1H, $J = 3.2$ Hz, $J = 16.8$ Hz, $H5_{indazol.eq}$), 2.88 (ddd, 1H, $J = 1.8$ Hz, $J = 8.3$ Hz, $J = 16.7$ Hz, $H5'_{indazol.ax.}$), 3.80 (s, 6H, $2OCH_3$), 4.11, 4.14 (dd, 1H, $J = 3.0$ Hz, $J = 8.1$ Hz, $H4_{indazol}$), 6.70 (d, 1H, $J = 1.8$ Hz, $H7_{indazol}$), 7.10–7.80 (m, 13H, Ar–Hs), 10.06 (s, 1H, NH, D_2O exchange), 10.76 (s, 1H, OH, D_2O exchange). MS (EI): *m/z* (%): 483.20 (2.22). Anal. calcd. for $C_{28}H_{25}N_3O_3S$ (483.58): C, 69.54; H, 5.21; N, 8.69. Found: C, 69.78; H, 5.29; N, 8.94.

General procedure for the preparation of 6a,b

To a solution of the corresponding hydrazide **3a,b** (10 mmol) and potassium hydroxide (0.56 g, 10 mmol) in absolute ethanol (5 ml), carbon disulfide (0.95 ml, 15 mmol) was added. The reaction mixture was heated under reflux for 5 h till the release of hydrogen sulfide gas ceased. After dilution with water, the reaction mixture was filtered. The filtrate was acidified with 1 N hydrochloric acid. The precipitated solid was filtered off, washed with water and crystallized from ethanol.

5-[2-Hydroxy-4-(4-methoxyphenyl)-6-phenyl-cyclohexa-1,3-dienyl]-1,3,4 oxadiazole-2-(3H)-thione (6a)

Compound **6a** was prepared from compound **3a** and carbon disulfide. 86% yield, mp 282–284 °C. ^1H NMR δ 2.73, 2.75 (dd, 1H, $J = 4.7$, $J = 17.6$, $H5_{cyclohex.ax.}$), 2.82 (ddd, 1H, $J = 2.1$ Hz, $J = 11.2$ Hz, $J = 17.6$ Hz, $H5'_{cyclohex.eq.}$), 3.77 (m, 1H, $H6_{cyclohex}$), 3.84 (s, 3H, OCH_3), 6.95 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 7.00–7.90 (m, 9H, Ar–Hs), 13.09 (s, 1H, NH, D_2O exchange), 14.05 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 30.8 ($C6_{cyclohex.}$), 39.4 ($C5_{cyclohex.}$), 55.8 (OCH_3), 114.3, 114.4, 114.9, 123.9, 127.1, 127.4, 128.1, 128.6, 129.3, 134.5, 136.0 (Ar–Cs), 159.6 (C=O), 161.1 ($C2_{cyclohex.}$), 180.7 (C=S). MS (EI): *m/z* (%): 378.19 (2.61). Anal. calcd. for $C_{21}H_{18}N_2O_3S$ (378.44): C, 66.65; H, 4.79; N, 7.40. Found: C, 66.82; H, 4.846; N, 7.53.

5-[2-Hydroxy-4,6-bis(4-methoxyphenyl) cyclohexa-1,3-dienyl]-1,3,4 oxadiazole-2-(3H)-thione (6b)

Compound **6b** was prepared from compound **3b** and carbon disulfide. 89% yield, mp 278–281 °C. ^1H NMR δ 2.73, 2.75 (dd, 1H, $J = 4.7$, $J = 17.6$, $H5_{cyclohex.ax.}$), 2.82 (ddd, 1H, $J = 2.1$ Hz, $J = 11.3$ Hz, $J = 17.8$ Hz, $H5'_{cyclohex.eq.}$), 3.74 (m, 1H, $H6_{cyclohex}$), 3.79 (s, 6H, $2OCH_3$), 6.97 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 7.00–7.60 (m, 8H, Ar–Hs), 10.09 (s, 1H, NH, D_2O exchange), 11.47 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 33.7 ($C6_{cyclohex.}$), 40.6 ($C5_{cyclohex.}$), 55.5, 55.7 ($2OCH_3$), 113.9, 114.2, 114.7, 127.1, 127.4, 128.3, 128.5, 129.1, 134.5, 135.7 (Ar–Cs), 159.0 (C=O), 163.9 ($C2_{cyclohex.}$), 198.7 (C=S). MS (EI): *m/z* (%): 408.24 (0.29). Anal. calcd. for $C_{22}H_{20}N_2O_4S$ (408.47): C, 64.69; H, 4.94; N, 6.86. Found: C, 64.82; H, 4.98; N, 6.95.

General procedure for the preparation of 7a,b

A mixture of compound **3a,b** (10 mmol) and acetylacetone (1 ml, 10 mmol) in a mixture of ethanol–acetic acid (100:10 v/v) was heated under reflux for 10 h. The reaction mixture was cooled and the precipitated solid was filtered off, washed with water, dried and crystallized from ethanol.

(3,5-Dimethyl-1H-pyrazol-1-yl) [2-hydroxy-4-(4-methoxyphenyl)-6-phenylcyclohexa-1,3-dienyl] methanone (7a)

Compound **7a** was prepared from compound **3a** and acetylacetone. 45% yield, mp 277–279 °C. ^1H NMR δ 2.40 (s, 3H, CH_3 at $C3_{pyrazole}$), 2.42 (s, 3H, CH_3 at $C5_{pyrazole}$), δ 2.73, 2.77 (dd, 1H, $J = 4.7$, $J = 17.6$, $H5_{cyclohex.ax.}$), 2.98 (ddd, 1H, $J = 2.3$ Hz, $J = 11.3$ Hz, $J = 17.8$ Hz, $H5'_{cyclohex.eq.}$), 3.75 (m, 1H, $H6_{cyclohex}$), 3.83 (s, 3H, OCH_3), 6.43 (s, 1H, $C4_{pyrazole}$), 6.71 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 7.10–8.30 (m, 9H, Ar–Hs), 10.82 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 12.8, 13.7 (2 CH3), 35.8 ($C6_{cyclohex.}$), 44.3 ($C5_{cyclohex.}$), 55.5, 55.7 ($2OCH_3$), 114.3, 114.8, 122.8, 128.4, 131.0, 132.1, 136.3, 139.9, 141.8, 148.4 (Ar–Cs), 159.7 (C=O), 161.3 ($C2_{cyclohex.}$). MS (EI): *m/z* (%): 400.23 (1.00). Anal. calcd. for $C_{25}H_{24}N_2O_3$ (400.47): C, 74.98; H, 6.04; N, 7.00. Found: C, 75.12; H, 6.13; N, 7.24.

(3,5-Dimethyl-1H-pyrazol-1-yl) [2-hydroxy-4,6-bis(4-methoxyphenyl)cyclohexa-1,3-dienyl] methanone (7b)

Compound **7b** was prepared from compound **3b** and acetylacetone. 48% yield, mp 268–270 °C. ^1H NMR δ 2.39 (s, 3H, CH_3 at $C3_{pyrazole}$), 2.45 (s, 3H, CH_3 at $C5_{pyrazole}$), δ 2.72, 2.75 (dd, 1H, $J = 4.8$, $J = 17.7$, $H5_{cyclohex.ax.}$), 2.93 (ddd, 1H, $J = 2.3$ Hz, $J = 11.3$ Hz, $J = 17.8$ Hz, $H5'_{cyclohex.eq.}$), 3.73 (m, 1H, $H6_{cyclohex}$), 3.79 (s, 6H, $2OCH_3$), 6.41 (s, 1H, $C4_{pyrazole}$), 6.89 (d, 1H, $J = 1.6$ Hz, $H3_{cyclohex}$), 7.00–7.80 (m, 8H, Ar–Hs), 10.55 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 12.8,13.7 (2 CH_3), 35.8 ($C6_{cyclohex.}$), 44.3 ($C5_{cyclohex.}$), 55.5, 55.7 ($2OCH_3$), 114.3, 114.8, 122.8, 128.4, 131.0, 132.1, 136.3, 139.9, 141.8, 148.4 (Ar–Cs), 159.7 (C=O), 161.3 ($C2_{cyclohex.}$). MS (EI): *m/z* (%): 430.14 (1.08). Anal. calcd. for $C_{26}H_{26}N_2O_4$ (430.50): C, 72.54; H, 6.09; N, 6.51. Found: C, 72.69; H, 6.08; N, 6.57.

General procedure for the preparation of 8a,b

A mixture of compound **3a,b** (1 mmol), ethyl acetoacetate (0.13 ml, 1 mmol) and anhydrous potassium carbonate (0.21 g, 1.5 mmol) in ethanol (15 ml) was heated under reflux for 10 h. The reaction mixture was poured on water and the precipitated solid was filtrated off, washed with water, dried and crystallized from ethanol.

1-[2-Hydroxy-4-(4-methoxyphenyl)-6-phenyl cyclohexa-1,3-dienecarbonyl]-3-methyl-1H-pyrazole-5(4H)-one (8a)

Compound **8a** was prepared from compound **3a** and ethyl acetoacetate. 51% yield, mp 249–252 °C. ^{1}H NMR δ 1.66 (s, 3H, CH_3 at $C3_{pyrazole}$), 1.84 (s, 2H, $CH_{2pyrazolone}$), 2.81, 2.88 (dd, 1H, $J = 4.6$ Hz, $J = 17.6$ Hz, $H5_{cyclohex.ax.}$), 2.92 (ddd, 1H, $J = 2.1$ Hz, $J = 11.2$ Hz, $J = 17.6$ Hz, $H5'_{cyclohex.eq.}$), 3.72 (m, 1H, $H6_{cyclohex}$), 3.81 (s, 3H, OCH_3), 6.90 (s, 1H, $H3_{cyclohex}$), 7.00–7.90 (m, 9H, Ar–Hs), 10.30 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 25.1 (CH_3), 35.2 ($C6_{cyclohex.}$), 39.3 ($C5_{cyclohex.}$), 40.5 ($CH_{2pyrazoline}$), 55.6 (OCH_3), 69.7 ($C3_{pyrazoline}$), 114.1, 114.7, 126.9, 127.4, 128.5, 128.9, 129.0, 132.1, 136.3, 139.9, 141.8, 144.5, 148.4 (Ar–Cs), 158.6 (C=O), 175.7 ($C2_{cyclohex.}$). MS (EI): *m/z* (%): 402.17 (10.39). Anal. calcd. for $C_{24}H_{22}N_2O_4$ (402.44): C, 71.63; H, 5.51; N, 6.96. Found: C, 71.81; H, 5.58; N, 7.11.

1-[2-Hydroxy-4,6-bis(4-methoxyphenyl)cyclohexa-1,3-dienecarbonyl]-3-methyl-1H-pyrazole-5(4H)-one (8b)

Compound **8b** was prepared from compound **3b** and ethyl acetoacetate. 53% yield, mp 261–263 °C. ^{1}H NMR δ 1.68 (s, 3H, CH_3 at $C3_{pyrazole}$), 1.85 (s, 2H, $CH_{2pyrazolone}$), 2.75, 2.79 (dd, 1H, $J = 4.7$ Hz, $J = 17.7$ Hz, $H5_{cyclohex.ax.}$), 2.94 (ddd, 1H, $J = 2.2$ Hz, $J = 11.3$ Hz, $J = 17.7$ Hz, $H5'_{cyclohex.eq.}$), 3.72 (m, 1H, $H6_{cyclohex}$), 3.81 (s, 6H, $2OCH_3$), 6.82 (s, 1H, $H3_{cyclohex}$), 7.00–7.69 (m, 8H, Ar–Hs), 10.50 (s, 1H, OH, D_2O exchange). MS (EI): *m/z* (%): 432.19 (5.24). Anal. calcd. for $C_{25}H_{24}N_2O_5$ (432.47): C, 69.43; H, 5.59; N, 6.48. Found: C, 69.54; H, 5.63; N, 6.61.

General procedure for the preparation of 9a–f

A mixture of the corresponding hydrazide **3a,b** (10 mmol) and the appropriate isothiocyanate derivative (10 mmol) in ethanol (20 ml) was heated under reflux for 5 h. The formed precipitate was filtered off, washed with ethanol and crystallized from ethanol.

1-[2-Hydroxy-4-(4-methoxyphenyl)-6-phenylcyclohexa-1,3-dienecarbonyl]-4-N- methyl thiosemicarbazide (9a)

Compound **9a** was prepared from compound **3a** and methyl isothiocyanate. 83% yield, mp 136–139 °C. ^{1}H NMR δ 2.86 (dd, 1H, $J = 4.7$, $J = 17.6$, $H5_{cyclohex.ax.}$), 3.10 (s, 3H, CH_3), 3.18 (ddd, 1H, $J = 2.1$ Hz, $J = 11.2$ Hz, $J = 17.6$ Hz, $H5'_{cyclohex.eq.}$), 3.60 (m, 1H, $H6_{cyclohex}$), 3.79 (s, 3H, OCH_3), 6.80 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 7.10–8.3 (m, 9H, Ar–Hs), 10.80 (brs, 4H, 3NHs + OH, D_2O exchange). ^{13}C NMR δ 31.0 (CH_3), 34.9 ($C6_{cyclohex.}$), 38.3 ($C5_{cyclohex.}$), 55.7 (OCH_3), 114.3, 114.6, 127.5, 127.6, 128.6, 128.7, 131.2, 138.7 (Ar–Cs), 145.6 ($C4_{cyclohex.}$), 158.9 ($C4_{methoxyphenyl}$), 159.9 (C=O), 160.4 ($C2_{cyclohex.}$), 175.2 (C=S). MS (EI): *m/z* (%): 409.32 (1.28). Anal. calcd. for $C_{22}H_{23}N_3O_3S$ (409.50): C, 64.53; H, 5.66; N, 10.26. Found: C, 64.70; H, 5.72; N, 10.47.

1-[2-Hydroxy-4,6-bis(4-methoxyphenyl)-6-phenyl cyclohexa-1,3-dienecarbonyl]-4-N-methyl thiosemicarbazide (9b)

Compound **9b** was prepared from compound **3b** and methyl isothiocyanate. 80% yield, mp 147–150 °C. ^{1}H NMR δ 2.69 (dd, 1H, $J = 4.6$, $J = 17.5$, $H5_{cyclohex.ax.}$), 2.80 (ddd, 1H, $J = 2.2$ Hz, $J = 11.3$ Hz, $J = 17.8$ Hz, $H5'_{cyclohex.eq.}$), 3.10 (s, 3H, CH_3), 3.30 (m, 1H, $H6_{cyclohex}$), 3.78 (s, 6H, $2OCH_3$), 6.60 (d, 1H, $J = 1.7$ Hz, $H3_{cyclohex}$), 6.90–8.30 (m, 8H, Ar–Hs), 10.30 (s, 3H, 3NHs, D_2O exchange), 11.01 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 31.1 (CH_3), 34.9 ($C6_{cyclohex.}$), 36.1 ($C5_{cyclohex.}$), 55.5, 55.7 ($2OCH_3$), 112.9, 114.2, 114.6, 122.1, 126.9, 128.2, 128.4, 128.7, 132.2, 137.1 (Ar–Cs), 144.9 ($C4_{cyclohex.}$), 158.3 ($C4_{methoxyphenyl}$), 159.9 (C=O), 160.5 ($C2_{cyclohex.}$), 178.7 (C=S). MS (EI): *m/z* (%): 439.25 (3.11). Anal. calcd. for $C_{23}H_{25}N_3O_4S$ (439.50): C, 62.85; H, 5.73; N, 9.56. Found: C, 63.04; H, 5.76; N, 9.68.

1-[2-Hydroxy-4-(4-methoxyphenyl)-6-phenylcyclohexa-1,3-dienecarbonyl]-4-N-ethyl thiosemicarbazide (9c)

Compound **9c** was prepared from compound **3a** and ethyl isothiocyanate. 86% yield, mp 140–143 °C. ^{1}H NMR δ 0.91 (t, 3H, $J = 7.2$ Hz, $CH_{3\ ethyl}$), 2.62 (dd, 1H, $J = 4.7$ Hz, $J = 17.7$ Hz, $H5_{cyclohex.ax.}$), 3.13 (ddd, 1H, $J = 2.1$ Hz, $J = 11.2$ Hz, $J = 17.7$ Hz, $H5'_{cyclohex.eq.}$), 3.40 (m, 1H, $H6_{cyclohex}$), 3.50 (q, 2H, $J = 7.3$ Hz, $CH_{2\ ethyl}$), 3.76 (s, 3H, OCH_3), 6.60 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 6.90–7.40 (m, 9H, Ar–Hs), 9.80 (s, 3H, 3NHs, D_2O exchange), 11.96 (s, 1H, OH, D_2O exchange). Anal. calcd. for $C_{23}H_{25}N_3O_3S$ (423.53): C, 65.23; H, 5.95; N, 9.92. Found: C, 65.41; H, 6.02; N, 9.98.

1-[2-Hydroxy-4,6-bis(4-methoxyphenyl) cyclohexa-1,3-dienecarbonyl]-4-N-ethyl thiosemicarbazide (9d)

Compound **9d** was prepared from compound **3b** and ethyl isothiocyanate. 87% yield, mp 155–157 °C. ^{1}H NMR δ 1.10 (t, 3H, $J = 7.22$, $CH_{3\ ethyl}$), 2.69 (dd, 1H, $J = 4.7$, $J = 17.6$, $H5_{cyclohex.ax.}$), 2.83 (ddd, 1H, $J = 2.1$ Hz, $J = 11.4$ Hz, $J = 17.7$ Hz, $H5'_{cyclohex.eq.}$), 3.40 (m, 1H, $H6_{cyclohex}$), 3.60 (q, 2H, $J = 7.23$, $CH_{2\ ethyl}$), 3.79 (s, 6H, $2OCH_3$), 6.60 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 6.90–7.50 (m, 8H, Ar–Hs), 10.20 (s, 3H, 3NHs, D_2O exchange), 11.00 (s, 1H, OH, D_2O exchange). ^{13}C-NMR δ 15.0 ($CH_{3\ ethyl}$), 34.9 ($C6_{cyclohex.}$), 36.0 (CH_{2ethyl}), 38.3 ($C5_{cyclohex.}$), 55.4, 56.5 ($2OCH_3$), 112.8, 114.2,

114.5, 122.0, 127.0, 128.3, 128.5, 131.9, 132.1 (Ar–Cs), 144.9 ($C4_{cyclohex.}$), 158.3 ($C4_{methoxyphenyl}$), 159.9 (C=O), 160.4 ($C2_{cyclohex.}$), 177.4 (C=S). MS (EI): *m/z* (%): 453.37 (0.45). Anal. calcd. for $C_{24}H_{27}N_3O_4S$ (453.55): C, 63.56; H, 6.00; N, 9.26. Found: C, 63.62; H, 6.09; N, 9.43.

1-[2-Hydroxy-4-(4-methoxyphenyl)-6-phenyl cyclohexa-1,3-dienecarbonyl]-4-N-phenyl thiosemicarbazide (9e)

Compound **9d** was prepared from compound **3a** and phenyl isothiocyanate. 67% yield, mp 188–190 °C. ^{1}H NMR δ 2.94 (dd, 1H, $J = 4.8$, $J = 17.6$, $H5_{cyclohex.ax.}$), 3.16 (ddd, 1H, $J = 2.3$ Hz, $J = 11.2$ Hz, $J = 17.8$ Hz, $H5'_{cyclohex.eq.}$), 3.74 (m, 1H, $H6_{cyclohex}$), 3.80 (s, 3H, OCH_3), 6.80 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 7.00–7.80 (m, 14H, Ar–Hs), 10.98 (s, 3H, 3NHs, D_2O exchange), 11.00 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 34.7 ($C6_{cyclohex.}$), 39.3 ($C5_{cyclohex.}$), 55.7 (OCH_3), 114.2, 114.6, 117.3, 121.5, 123.3, 124.9, 125.1, 127.5, 127.6, 128.4, 128.8, 129.4, 131.9, 138.9 (Ar–Cs), 145.4 ($C4_{cyclohex.}$), 156.2 ($C4_{methoxyphenyl}$), 158.7 (C=O), 160.1 ($C2_{cyclohex.}$), 180.6 (C=S). MS (EI): *m/z* (%): 471.18 (0.71). Anal. calcd. for $C_{27}H_{25}N_3O_3S$ (471.57): C, 68.77; H, 5.34; N, 8.91. Found: C, 68.94; H, 5.38; N, 9.02.

1-[2-Hydroxy-4,6-bis(4-methoxyphenyl) cyclohexa-1,3-dienecarbonyl]-4-N-phenyl thiosemicarbazide (9f)

Compound **9d** was prepared from compound **3b** and phenyl isothiocyanate. 61% yield, mp 196–198 °C. ^{1}H NMR δ 2.68 (dd, 1H, $J = 4.7$, $J = 17.6$, $H5_{cyclohex.ax.}$), 2.73 (ddd, 1H, $J = 2.2$ Hz, $J = 11.1$ Hz, $J = 17.6$ Hz, $H5'_{cyclohex.eq.}$), 3.71 (m, 1H, $H6_{cyclohex}$), 3.84 (s, 6H, $2OCH_3$), 6.70 (d, 1H, $J = 1.8$ Hz, $H3_{cyclohex}$), 6.90–7.60 (m, 13H, Ar–Hs), 9.80 (s, 3H, 3NHs, D_2O exchange), 10.80 (s, 1H, OH, D_2O exchange). ^{13}C NMR δ 35.7 ($C6_{cyclohex.}$), 38.6 ($C5_{cyclohex.}$), 55.5, 55.8 ($2OCH_3$), 114.2, 114.5, 117.9, 122.0, 125.0, 125.1, 126.8,

127.8, 128.5, 129.1, 129.6, 132.0, 136.8 (Ar–Cs), 142.7 ($C4_{cyclohex.}$), 156.2 ($C4_{methoxyphenyl}$), 159.2 (C=O), 161.7 ($C2_{cyclohex.}$), 181.7 (C=S). MS (EI): *m/z* (%): 501.67 (1.39). Anal. calcd. for $C_{28}H_{27}N_3O_4S$ (501.60): C, 67.05; H, 5.43; N, 8.38. Found: C, 67.13; H, 5.48; N, 8.49.

In vitro *antitumor evaluation by MTT assay*

Antiproliferative activity of the target compounds was determined in cells treated with the different concentrations of the tested compounds in comparison with untreated control using MTT assay as following:

1. Cells were grown as monolayer in media supplemented with 10% inactivated fetal bovine serum.
2. The monolayers of 10 000 cells were plated (104 cells/well) in a 96-well tissue culture plate and incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2 before treatment with the compounds to allow attachment of cell to the plate except blank wells without cells.
3. Different concentrations of 100, 10, 1.0, 0.1 and 0.01 μM of each tested compound and positive control drug were tested for cytotoxicity. Tetraplicate wells were prepared for each concentration in addition to cell control (cell only without compounds).
4. Cells were incubated with the tested compounds for 48 h into CO_2 incubator at 37 °C and 5% CO_2.
5. Culture media containing different concentration of tested compounds and dead cells were decanted leaving only viable attached cells into the tissue culture plate.
6. The plate was washed twice with pre-warmed phosphate buffered saline (PBS).
7. MTT reagent (40 μl) was added to each well including blank and negative control wells.
8. After addition of MTT reagent the plates were incubated in dark for 4 h for the reduction of MTT into formazan (purple needle color) by dehydrogenase activity in mitochondria of viable cells.
9. DMSO (150 μl) was added to each well to solubilize the purple crystals of formazan.
10. Absorbance was measured at 570 nm with microplate reader (ROBONIK TM P2000 Eliza plate reader; Robonik India Pvt. Ltd, Maharashtra, India).
11. The percentage of cell survival was calculated by the following equation:

$$Survival\ rate\,\% = \frac{(A_s - A_b)}{(A_c - A_b)} \times 100,$$

12. where A_s is the absorbance of sample, A_b is the absorbance of blank and A_c is the absorbance of control.
The inhibitory concentration 50 (IC50) was calculated from the equation of the plot between molar concentration of the tested compounds against survival rate percent.

Tubulin polymerization assay

Standard curve construction

Seven different dilution of standard such as 2000, 1000, 500, 250, 125, 62.5, 31.2 pg/mL, and the last tubes with the blank 0 pg/mL concentration were prepared, while test drugs were taken at their IC50 concentration. The duplicate readings for each standard, control and samples were averaged and subtracted from the average zero standard optical density. A standard curve was constructed by plotting the mean OD and concentration for each standard. A best fit curve was drawn through the points on the graph, with concentration on the *y*-axis and absorbance on the *x*-axis. In order to make the calculation easier, the OD values of the standard (*x*-axis) were plotted against the known concentrations of the standard (*y*-axis), although concentration is the independent variable and OD value is the dependent variable.

Sample preparation

The cell lysates obtained after incubation of MCF-7 and HCT-116 cells with the tested compounds at their IC50 concentration were prepared according to the following:

1. Adherent cells should be detached with trypsin and then collected by centrifugation (suspension cells can be collected by centrifugation directly).
2. Cells were washed three times in cold PBS.
3. Cells were resuspended in PBS (1×) and the cells was subjected to ultrasonication for four times (or freeze cells at ≤ −20 °C. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle for three times.)
4. Centrifugation was done at 1500*g* for 10 min at 2–8 °C to remove cellular debris.

Calculation of results

From the curve OD of each sample is converted to tubulin concentration, then percentage inhibition of tubulin polymerization can be calculated by the following equation:

$$\%\,\text{inhibition} = \left(\frac{\text{conc. of control} - \text{conc. of test}}{\text{conc. of control}}\right) \times 100.$$

Cell cycle analysis by fluorescence-activated cell sorting analysis

Fluorescence-activated cell sorting analysis following cell staining with propidium iodide (PI) was used according to the following protocol:

1. Approximately 10^6 cells (HCT-116 or MCF-7) were suspended in 0.5 ml of PBS. The suspension was gently vortexed (5 s) or gently aspirated several times with a Pasteur pipette to obtain a mono-dispersed cell suspension, with minimal cell aggregation.
2. Cells were fixed by transferring this suspension, with a Pasteur pipette, into centrifuge tubes containing 4.5 ml of 70% ethanol, on ice. Cells were kept in ethanol for at least 2 h at 4 °C. Cells may be stored in 70% ethanol at 4 °C for weeks.
3. The ethanol-suspended cells were centrifuged for 5 min at 300*g*. Ethanol was decanted thoroughly.
4. The cell pellet was suspended in 5 ml of PBS, and after about 30 s it was centrifuged at 300*g* for 5 min.
5. The cell pellet was suspended in 1 ml of PI staining solution and kept in the dark at room temperature for 30 min, or at 37 °C for 10 min.
6. The sample was transferred to the flow cytometer, Becton Dickinson Immunocytometry Systems and cell fluorescence was measured. Maximum excitation of PI bound to DNA is at 536 nm, and emission is at 617 nm.

7. Phoenix Flow Systems software (Phoenix Flow systems, Inc., San Diego, CA) was used to deconvolute the DNA content frequency histograms and to estimate the proportions of cells in the respective phases of the cycle.
8. The cell cycle progression was analyzed at a 10 μM concentration for 72 h.

Molecular docking procedure

X-ray crystal structure of tubulin in complex with DAMA-colchicine and the stathmin-like domain (SLD) at 3.5 Å resolution (PDB: 1SA0) was downloaded from protein data bank[7]. All molecular modeling calculations and docking studies were carried out using Discovery Studio software v4.0.0.13259[18] running on a Windows7 PC.

Binding site sphere determination

The protein–ligand complex obtained from the protein data bank was prepared for docking as follows: Deletion of chains A, B and E of the protein together with co-crystallized water molecules was performed. Automatic protein preparation module was used applying CHARMm forcefield. The binding site sphere has been defined automatically by the software.

Preparation of target compounds for docking

The docked compounds were prepared for docking by applying the following protocol: 2D structures of the docked ligands were built using Marvin Sketch and copied to Discovery Studio 4. Ligands were prepared using "Prepare Ligands" protocol in Discovery Studio where hydrogen atoms were added at their standard geometry, optical isomers and 3D conformations were automatically generated.

Running docking

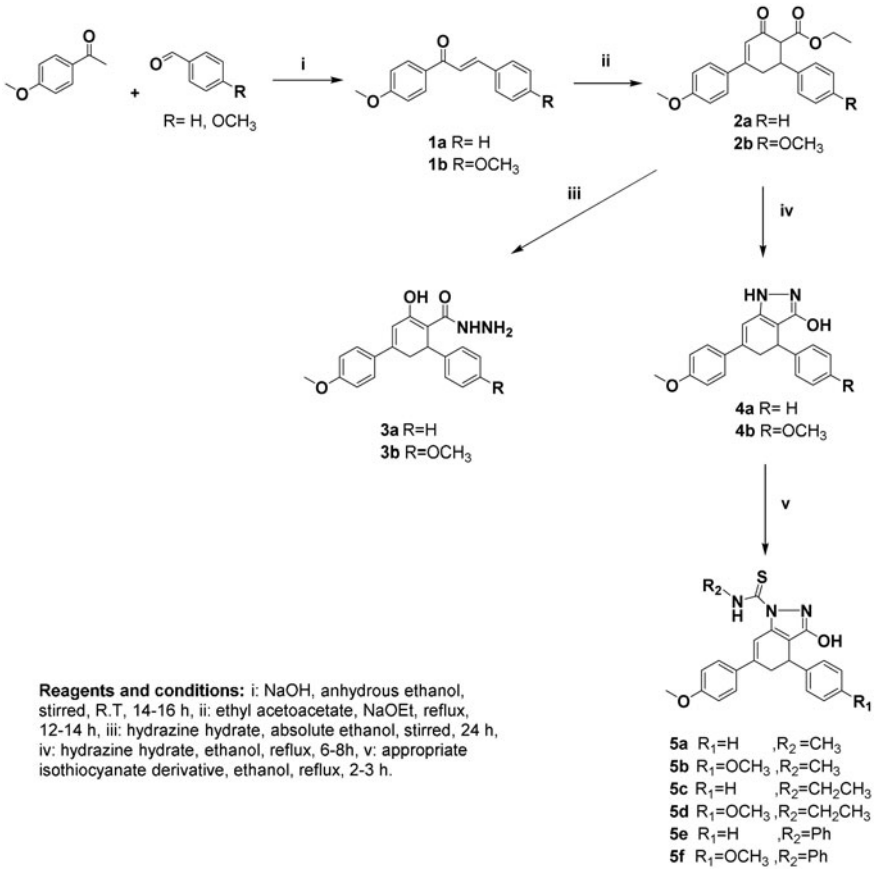
Docking was performed using CDOCKER protocol in Discovery Studio keeping the parameters at default. The best scoring pose of the docked compounds was recognized. Receptor–ligand interactions of the complexes were examined in 2D and 3D styles.

Results and discussion

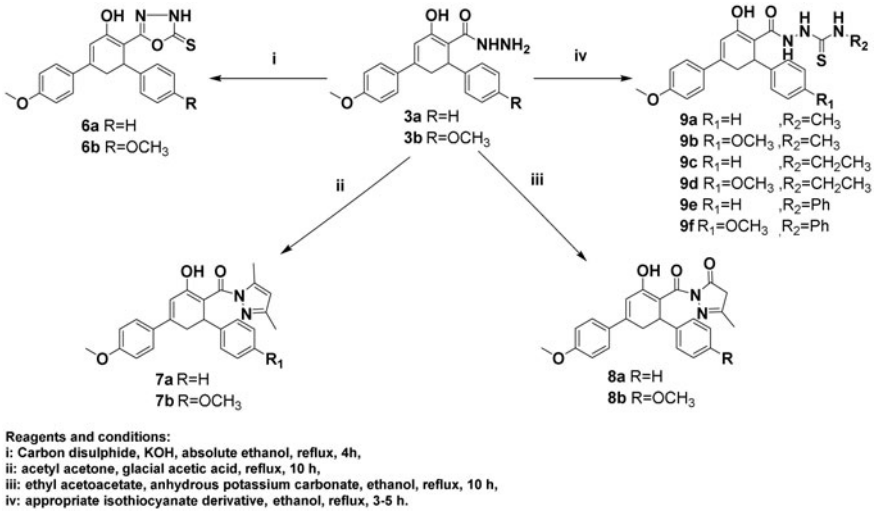
Chemistry

The designed compounds were synthesized adopting the chemical pathways outlined in Schemes 1 and 2.

In the present work, the synthesis of the propenones **1a,b** was achieved by reacting benzaldehyde or 4-methoxybenzaldheyde with 4-methoxy acetophenone in ethanol using aqueous NaOH as a catalyst[17]. The cyclohexenone intermediates **2a,b** were prepared via Michael addition through a cyclo-condensation reaction between the propenones **1a,b** and the β-keto ester, ethyl acetoacetate using sodium ethoxide as a catalyst. As the explored reaction was not stereo selective, two chiral centers (C_1 and C_6) in the structure of the cyclohexenones **2a,b** were generated, which would result in a mixture of diastereomers. No attempt to separate the diastereomeric cyclohexenones was undertaken, and the cyclo-condensation products were characterized in the form of the mixture originated from the synthesis. The characteristic triplet-quartet



Scheme 1. Synthesis of compounds **3a,b**, **4a,b** and **5a–f**.



Scheme 2. Synthesis of compounds **6a,b**, **7a,b**, **8a,b** and **9a–f**.

pattern confirmed the presence of the ethyl group of the ester. The characteristic signal in the ^{1}H NMR spectrum of **2a** was, however, the two protons at C-5, being magnetically nonequivalent, appeared as two different signals, the axial proton appeared as double of doublet of doublets at around δ 2.98 ppm showing geminal coupling, vicinal coupling with H-6 and long range coupling with vinyl proton H-3. The equatorial proton at C-4 appeared as doublet of doublets at around δ 3.08 ppm showing both germinal and vicinal coupling. The vinyl proton, H-3, appeared as doublet at δ 6.56 ppm. Proton at C-6 appeared as multiplet due to vicinal coupling to H-5 and H-1. H-1 proton appeared as doublet being coupled to H-6. Reaction of the cyclohexenones **2a**,**b** with 98% hydrazine hydrate in ethanol at room temperature afforded derivatives **3a,b**, respectively. Proceeding the reaction under reflux condition resulted in cyclization with formation of indazole derivatives **4a,b**. The appearance of the OH stretching band confirmed the presence of the enol tautomer, which resulted in the loss of one of the two chiral centers. According to a previous report on the tautomeric forms of indazole, the obtained compounds **4a,b** could be present in three tautomeric forms A, B and C (Figure 3). The absence of carbonyl bands in the IR spectra of the products ruled out lactam structures **A** and **B**[19]. The ^{1}H NMR spectra of the indazole derivatives exhibited three protons in the sp^3 shift range (H-5$_{eq}$, H-5$_{ax}$ and H-4). H-5$_{eq}$ and H-4 appeared as doublet of doublets at around δ 2.90 and 4.18 ppm, respectively. While the signal for H-5$_{ax}$ appeared as doublet of doublet of doublets at δ 3.12 ppm showing geminal coupling with H-5$_{eq}$, vicinal coupling with H-4 and long range proton coupling with H-7. The vinylic proton at H-7 appeared as doublet at δ 6.89 ppm with J = 1.7 Hz.

Target compounds **5a–f** were prepared by reacting compounds **4a,b** with the appropriate substituted alkyl/aryl isothiocyanate in absolute ethanol under reflux condition.

Substituted 1,3,4-oxadiazole-2(3*H*)-thione derivatives **6a,b** were synthesized by reacting the hydrazide derivatives **3a,b** with carbon disulfide in absolute ethanol in the presence of potassium hydroxide. Pyrazole derivatives **7a,b** were synthesized via cyclo-condensation of acetyl acetone with the hydrazide derivatives **3a,b** in a mixture of ethanol and glacial acetic acid. The yield was found to be solvent-dependent as cyclocondensation in a 10:1 (v/v) mixture of ethanol–acetic acid afforded the corresponding pyrazole derivatives in high yield, while the yield decreased upon using a mixture of ethanol and triethyl amine. Using ethyl acetoacetate as a β-diketone, cyclocondensation reaction with the appropriate hydrazides **3a,b** afforded the 3-methyl-1*H*-pyrazole-5-(4*H*)-ones **8a,b**, respectively. Finally, reaction of isothiocyanates with hydrazides **3a,b** in absolute ethanol under reflux furnished the corresponding thiosemicarbazides **9a–f**.

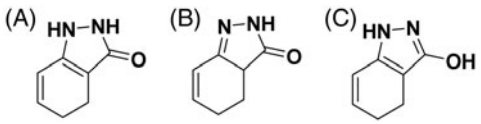


Figure 3. Tautomeric forms of 1*H*-indazol-3-ol.

Table 1. Antiproliferative activity against HCT-116 cell line and MCF-7.

Compound number	R_1	R_2	IC_{50} (μM) (HCT-116)	IC_{50} (μM) (MCF-7)
3a	H	–	55.35	63.39
3b	OCH_3	–	28.04	42.07
4a	H	–	19.21	26.70
4b	OCH_3	–	6.78	11.40
5a	H	CH_3	>100	>100
5b	OCH_3	CH_3	>100	60.90
5c	H	CH_2CH_3	>100	>100
5d	OCH_3	CH_2CH_3	>100	>100
5e	H	C_6H_5	6.71	5.50
5f	OCH_3	C_6H_5	11.05	11.55
6a	H	–	58.46	44.70
6b	OCH_3	–	59.80	>100
7a	H	–	30.25	42.75
7b	OCH_3	–	88.83	>100
8a	H	–	85.07	58.45
8b	OCH_3	–	27.31	60.41
9a	H	CH_3	60.60	>100
9b	OCH_3	CH_3	>100	29.30
9c	H	CH_2CH_3	>100	>100
9d	OCH_3	CH_2CH_3	34.40	>100
9e	H	C_6H_5	>100	>100
9f	OCH_3	C_6H_5	78.20	>100
Colchicine	–	–	12.13	9.41

The structures of all the synthesized compounds were confirmed using the EI MS, FT-IR, ^{1}H and ^{13}C NMR spectral analyses.

Biological screening

In vitro *antitumor evaluation by MTT assay*

The antiproliferative activity of the target compounds against colon cancer HCT-116 and breast cancer MCF-7 cell lines was

measured at Vacsera, Egypt. The MTT method of assay was adopted and the IC_{50} values are listed in Table 1.

An overview of the results of MTT assay revealed that few compounds exhibited IC_{50} values lower than or slightly higher than colchicine. Concerning the antitumor activity against colon HCT-116 tumor cell line, the obtained results showed that compounds **4b, 5e** and **5f** exhibited higher potency than colchicine. Furthermore, compound **5f** revealed comparable activity to doxorubicin, while compounds **3b**, **8b** and **9d** exerted moderate activity. Regarding antitumor activity against MCF-7 breast tumor cell line, it can be revealed that compound **5e** demonstrated higher potency than colchicine. Meanwhile, compounds **4b** and **5f** were less active than colchicine. The cyclohexenols **3a,b** displayed IC_{50} of 55.35–63.39 μM, respectively. Regarding the effect of substitution on the phenyl ring (R^1) the 4-methoxy derivative **3b** showed higher activity than the unsubstituted derivative **3a**. Interestingly, structure rigidification of **3a,b** into the indazole derivatives **4a,b** resulted in increase in the antitumor activity especially for the 4-methoxyphenyl derivative **4b** (IC_{50}= 6.78 and 11.40 μM against HCT-116 and MCF-7 cells, respectively). Structure extension of the indazole derivatives **4a,b** with *N*-substituted carbothioamide moiety was successful only with the *N*-phenyl derivatives **5e,f** (IC_{50} ranging from 5.50 to 11.55 μM). The *N*-methyl (**5a,b**) and *N*-ethyl derivatives (**5c,d**) were inactive. Unfortunately, introduction of heterocylic rings at position 1 of the cyclohexanol nucleus as in compounds **6a,b**, **7a,b**, **8a,b** did not reveal any advantage toward the activity of the compounds, compared to their precursor less bulky hydrazides **3a,b**. In conclusion, it could be revealed that the bicylic indazole derivatives **4a,b** and **5e,f** were the most potent of all derivatives. The hydrazides **3a,b** and the azacyclic related derivatives **6a,b**, **7a,b**, **8a,b** showed only moderate activity. The thiosemicarbazides **9a–f** and *N*-methyl **5a,b** and *N*-ethyl **5c,d** indazole-1-carbothioamide derivatives were the least potent.

Table 2. Percentage inhibition of tubulin polymerization.

	% Inhibition of tubulin polymerization	
Compound	HCT-116	MCF-7
4b	86.96	84.53
5e	89.31	79.72
5f	84.33	86.30
DAMA-colchicine	82.24	86.84

Table 3. Results of cell cycle analysis in HCT-116 and MCF-7 for compounds **4b**, **5e** and **5f**.

	% of cells in each phase HCT-116			% of cells in each phase MCF-7		
	G_0-G_1	S	G_2-M	G_0-G_1	S	G_2-M
Control	67.8	24	8.2	71.3	19.1	9.6
4b	32.8	42	25.2	47.7	24.1	28.2
5e	27.1	35.5	37.4	34.2	27.3	38.5
5f	42.4	24.5	33.1	44.8	21.3	33.9

Tubulin polymerization inhibition assay

Further investigation to assess the mechanism of action of the most active compounds in the MTT assay as potential tubulin polymerization inhibitors was carried out using tubulin polymerization assay. The percentage inhibition of tubulin polymerization following sandwich enzyme immunoassay by ELISA method using Enzyme-linked Immunosorbent Assay Kit was performed. Results are summarized in Table 2. Percentage inhibition of tubulin polymerization was performed on the compounds with the highest activity profile in the MTT assay, namely, **4b**, **5e** and **5f**. The tested compounds showed percentage inhibition of tubulin in both cell line homogenates ranging from 79.72% to 89.31%. Compound **5e** was the most active on HCT-116 and **5f** was the most active on MCF-7 cells. It is noteworthy that activities of the tested

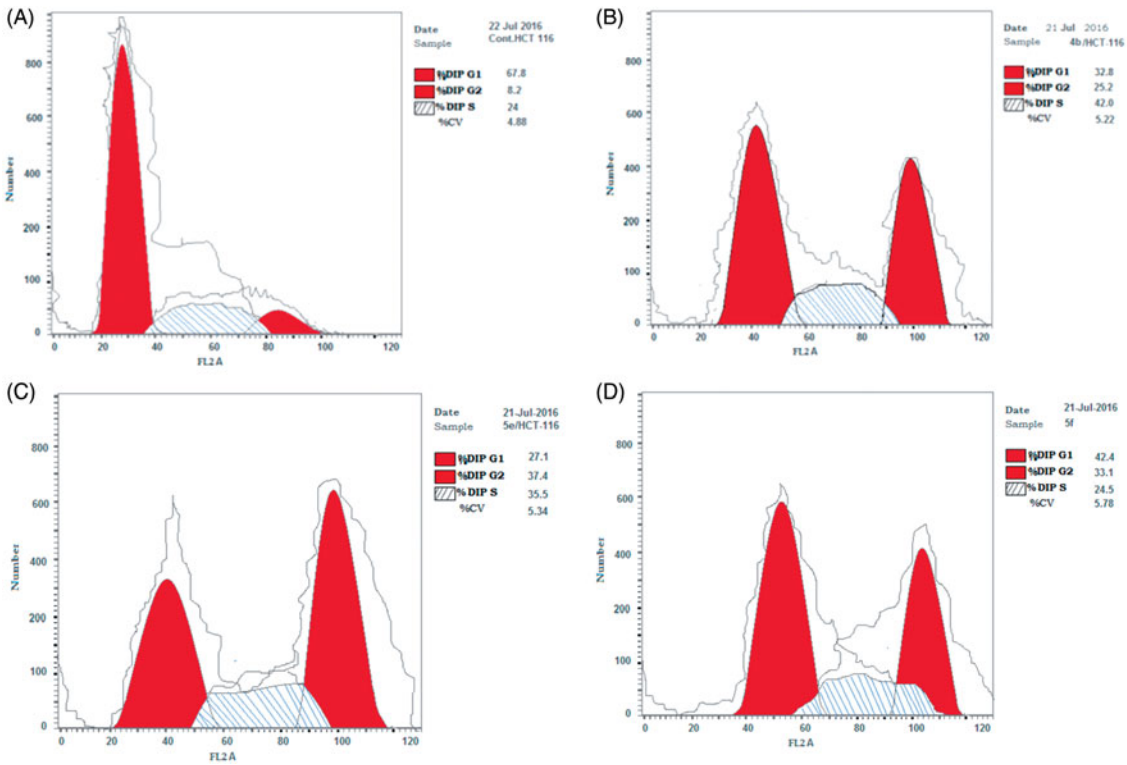


Figure 4. Cell cycle analysis histograms for HCT-116 cells. (A) Control, (B) **4b**, (C) **5e** and (D) **5f**.

compounds were comparable to that of colchicine or even higher especially on HCT-116 cells homogenate.

Cell cycle analysis

It was hypothesized that the mechanism of action of compounds **4b, 5e** and **5f** involved arresting the process of mitosis. Accordingly, cell cycle analysis was performed on HCT-116 and MCF-7 cells after treatment with these compounds. Upon exposure of the cells to the tested compounds, the percentages of cells in the G_0/G_1 phase of the cell cycle in both cell lines, were markedly decreased, especially with compound **5e,** while the percentages in the G_2/M phase of the cell cycle increased. Compound **5e** had the highest effect on G_2/M phase in both cell lines (Table 3, Figures 4 and 5). Compared with the untreated control, tested compounds disturbed the cell cycle strongly at G_2/M phase, which was in agreement with the proposed mechanism of action.

Molecular modeling

Based on the results of the tubulin polymerization assay, docking of the most active compounds **4b, 5e** and **5f** was performed at X-ray crystal structure of tubulin in complex with (*N*-deacetyl-*N*-(2-mercaptoacetyl)colchicine) (DAMA-colchicine) and the SLD at 3.5 Å resolution (PDB: 1SA0)[7] using Discovery Studio 4 software package[18] to shed light on their potential binding modes and investigate their similarity to the native ligand. Since the synthesized

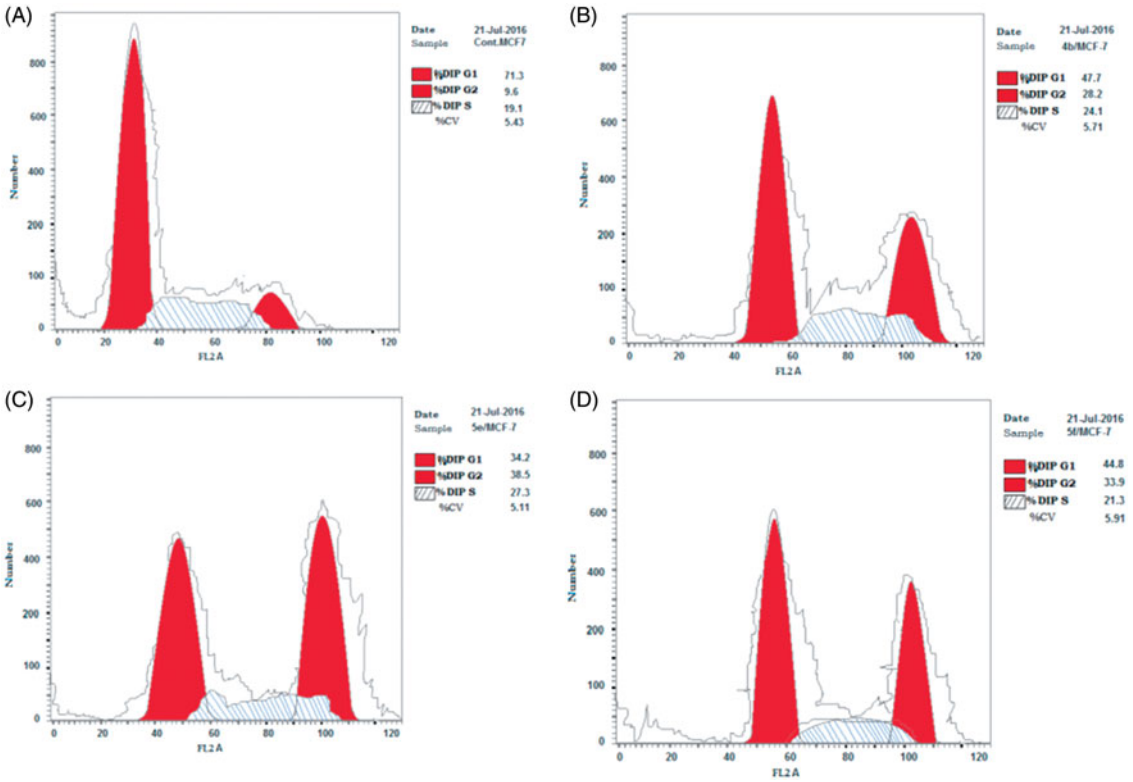


Figure 5. Cell cycle analysis histograms for MCF-7 cells. (A) Control, (B) **4b**, (C) **5e** and (D) **5f**.

Table 4. Results of the molecular docking study.

Compound	CDOCKER interaction energy	Type of interaction	Distance	Interacting moiety in the drug	Amino acid involved
DAMA–colchicine	−55.6986	H-Bonding	2.1	S**H**	C:THR179
		H-Bonding	2.3	**C**arbonyl of tropone	C:VAL181
		H-Bonding	1.9	**O**CH_3	D:CYS241
(*R*)-**4b**	−47.1318	H-Bonding	2.4	O**H**	C:SER178
		Sigma-Pi	2.8	Pyrazole ring	D:LYS352
		H-Bonding	2.5	N**H** Pyrazole ring	D:THR353
(*S*)-**4b**	−40.6168	H-Bonding	2.3	O**H**	C:SER178
(*R*)-**5e**	−45.1529	H-Bonding	2.1	O**H**	C:THR179
		Cation-Pi	6.3	Pyrazole ring	D:LYS352
(*S*)-**5e**	−48.6425	H-Bonding	2.3	**O**H	D:ALA250
		Sigma-Pi	2.8	Pyrazole ring	C:LEU248
(*R*)-**5f**	−51.0859	H-Bonding	2.1	O**H**	C:THR179
		H-Bonding	2.7	**O**CH_3	D:CYS241
		Cation-Pi	4.9	Phenyl ring	D:LYS254
		Cation-Pi	6.3	Pyrazole ring	D:LYS352
(*S*)-**5f**	−52.3539	H-Bonding	2.3	**O**CH_3	D:CYS241
		Sigma-Pi	2.7	Pyrazole ring	C:LEU248
		H-Bonding	2.2	**O**H	D:ALA250

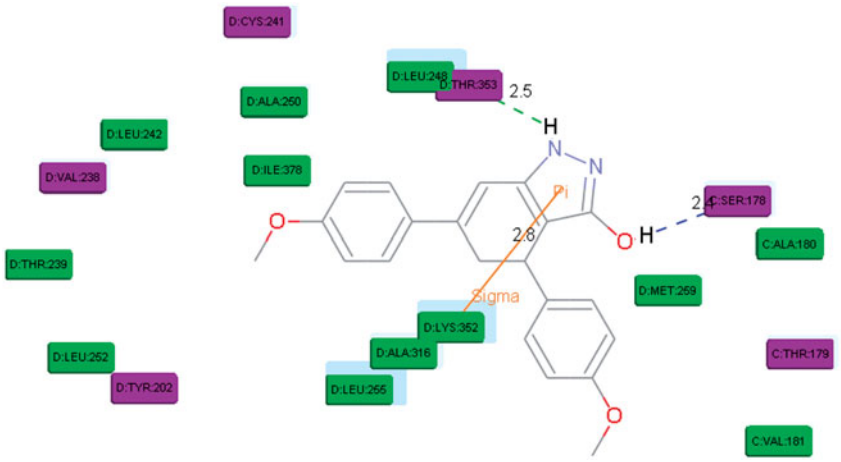


Figure 6. 2D interaction diagram of the top docking pose of the R isomer of compound **4b**.

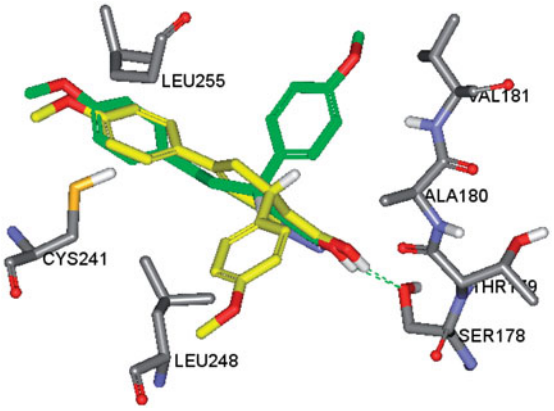


Figure 7. Overlay of the top docking poses of R (green) and S (yellow) isomers of **4b** in the active site of tubulin (PDB: 1SA0).

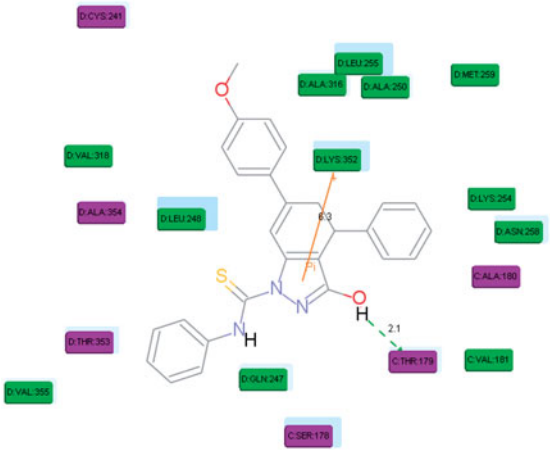


Figure 8. 2D interaction diagram of the top docking pose of the R isomer of compound **5e**.

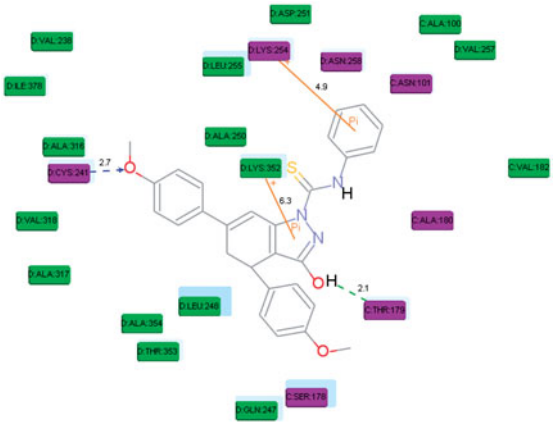


Figure 9. 2D interaction diagram of the top docking pose of the R isomer of compound **5f**.

compounds are chiral, both isomers were enrolled in the docking study. Table 4 illustrates the bonding and the nonbonding interactions of the docked compounds with amino acids of the active site. To ensure the validity of the docking protocol, re-docking of the co-crystallized DAMA-colchicine into the active site of tublin was performed. The coordinates of the best scoring docking pose of DAMA-colchicine was compared with its coordinates in the co-crystallized PDB file based on the binding mode and root mean square deviation (rmsd). The docking validation showed a near perfect alignment with the original ligand as obtained from the X-ray resolved pdb file. The re-docked ligand showed an rmsd of 0.6995 Å with CDOCKER interaction energy of −55.6986 and the same binding interactions. The binding site of DAMA-colchicine is composed of two hydrophobic cavities accommodated by the phenyl ring and tropone ring of DAMA-colchicine. Essential hydrogen bond of CYS241 with a methoxy group in DAMA-colchicine was reported. Thiol and the carbonyl of tropone ring were engaged in two hydrogen bonds with THR179 and VAL181, respectively.

Analysis of the docking results revealed that the docked compounds showed comparable CDOCKER energy to the reference ligand and they interacted with variable amino acids previously reported in molecular modeling studies of CSIs[20]. A properly positioned OH group at pyrazole ring of both isomers of compound **4b** (Figures 6 and 7) and R isomers of compounds **5e** and **5f** was engaged in hydrogen bond interaction with SER178 or THR179, respectively (Figures 8 and 9), this hydrogen bond was reported to increase the activity[21]. The S isomers of compounds **5e** and **5f** were flipped in such a way that their OH group was forming a hydrogen bond with Ala250 (Figures 10 and 11). Additional hydrogen bond interaction was observed at the methoxy group of compound **5f** with CYS241 which was reported to be crucial for CBSIs[20]. It is worth mentioning that

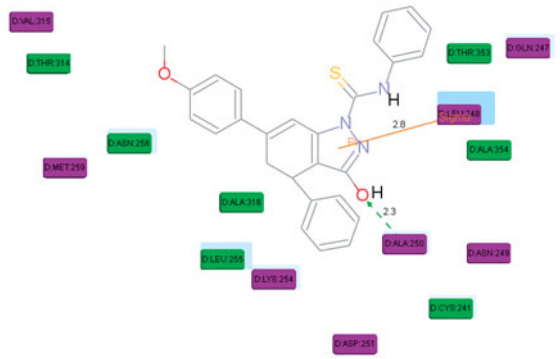


Figure 10. 2D interaction diagram of the top docking pose of the S isomer of compound **5e**.

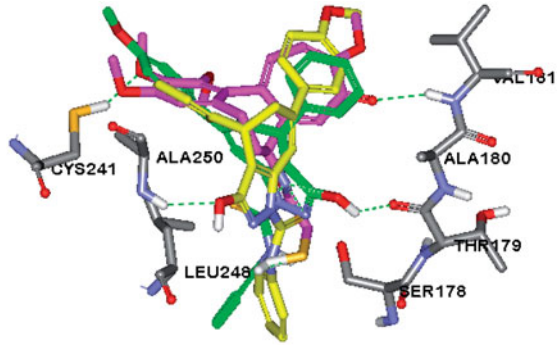


Figure 11. Overlay of the top docking poses of R (green), S (yellow) isomers of **5e** and DAMA-colchicine (magenta) in the active site of tubulin (PDB: 1SA0).

although the hydrogen bonding to CYS241 was not reported in the docking poses of compounds **4b** and **5e**, a methoxy group in these compounds was in the vicinity of this amino acid (Figures 7 and 11). Moreover, pyrazole ring shows Pi interaction with LEU 248 in S isomer of **5e** and **5f** or with LYS254 or LYS352 in their R isomer, in addition to other valuable hydrophobic interactions. These results suggested that the new compounds had the potential to exhibit antitumor activity through inhibition of tubulin polymerization.

Conclusion

Twenty two new target compounds were designed as inhibitors of tubulin polymerization relying on using two types of ring B models (cyclohexenone and indazole) to replace the central ring in colchicine. The designed compounds were assessed for their antitumor activity through *in vitro* cytotoxicity study on HCT-116 and MCF-7 cancer cell lines. Few compounds exhibited IC_{50} values lower than or slightly higher than colchicine. The bicylic indazole derivatives **4a,b** and **5e,f** were the most potent of all derivatives. Derivatives **4b** and **5e** exhibited higher potency than colchicine against colon HCT-116 tumor cell. Compound **5f** revealed comparable activity to colchicine. Compound **5e** demonstrated higher potency than colchicine against MCF-7 breast tumor cell line. The mechanism of the antitumor activity of the most active compounds **4b**, **5e** and **5f** was investigated through evaluating the tubulin inhibition potential of the active compounds. These indazole derivatives **4b**, **5e** and **5f** showed percentage inhibition of tubulin in both cell line homogenates ranging from 79.72% to 89.31%. The effects of **4b**, **5e** and **5f** on cell cycle in HCT-116 and MCF-7 cell lines were analyzed revealing an increase of cell percentage at G_2/M phase. Molecular docking was performed to reveal the interaction of the active compounds into the colchicine binding site of tubulin. Thereby, it could be claimed that the indazole derivatives represented a promising starting point for further study.

Disclosure statement

The authors report that that they have no conflicts of interest.

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