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Research Article

# Newly Designed and Synthesized Curcumin Analogs with *in vitro* Cytotoxicity and Tubulin Polymerization Activity

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**Novel curcumin analogs with 4-piperidone ring were designed, synthesized, and evaluated for their cytotoxic activities against five different cancer cell lines. 3,5-bis(4-Hydroxy-3-methoxybenzylidene)-4-oxo-*N*-phenylpiperidine-1-carbothioamide (XIIe) exhibited considerable cytotoxic activity with $IC_{50}$ values in 1–2.5 $\mu$M range. *In silico* and *in vitro,* studies were also performed to predict the binding affinity of the target compounds to the $\beta$-chain of tubulin receptor (PDB code 1SA1) and their abilities to affect microtubules polymerization cycle. 3,5-bis(3-Iodo-5-methoxy-4-propoxybenzylidene)-*N*-acetylpiperidin-4-one (VIIa) was found to exert 93.3% inhibition of tubulin and destabilization of microtubules *in vitro* compared to vincristine while, 3,5-bis(3,4,5-trimethoxybenzylidene)-*N*-benzoylpiperidin-4-one (XIIc) showed high potency in a different way where it exerted 94.8% stabilization of microtubules *in vitro* compared to positive control paclitaxel.**

**Key words:** curcumin analogs, cytotoxicity, molecular modeling, tubulin polymerization assay

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Cancer is a life-threatening disease and a leading cause of death. Although a range of therapies based on chemotherapy, surgery, and radiotherapy are available, yet they are of limited efficacy. Moreover, current anticancer regimens are associated with significant levels of toxicity and serious adverse side-effects. Hence, many research projects have been focused on developing new chemotherapies with minimal side-effects on mammalian cells. Natural products have been found to be a source of novel and potent bioactive compounds with minimal side-effects *in vivo* (1).

Extensive research conducted within the past years revealed that curcumin (**1**) [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione] (Figure S1) (2) extracted from the rhizome of the plant Curcuma longa modulates and interacts with a diverse range of molecular targets, and hence, it possess antiproliferative activities against tumor cells *in vitro*, anti-inflammatory, antibacterial, antiviral, antihepatotoxic, hypotensive, and anticholesterolemic activities (2–8). As cancer is a result of the dysregulation of multiple cell signaling pathways, curcumin's multitargeting ability may be the key to its therapeutic potential against cancer. Reported structural activity relationship studies performed on curcumin analogs highlighted the critical sites for developing novel analogs where 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (**2**) was found to be very important in activity of the compound as this group reacts with cellular constituents at a specific binding site (primary binding site A) (Figure S2) (9).

This binding may be influenced by the nature of the group attached to the heterocyclic nitrogen atom where charged molecules were found to be unable to penetrate cell membranes and exert a cytotoxic effect, and hence, N-acylation was considered a route to render the nitrogen atom in a non-basic form (9,10). Recent research also revealed that chalcones (**3**) (Figure S3), curcumin, and their derivatives possess cytotoxic activity associated with tubulin inhibition and interference with microtubule formation, which is essential in cellular processes such as mitosis and cell replication. It was found that curcumin exerted inhibition to tubulin inside *Plasmodium falciparum* cells with $IC_{50}$ = 5 $\mu$M (11). Also benzylidene curcumin derivative (**4**) (Figure S4) was found to be more effective than curcumin in inhibiting tubulin self-assembly (12), while chalcone derivative (Figure S5A) exerted high tubulin inhibition activity compared to podophyllotoxin (Figure S5B) (13).

According to all the previous findings in the literature, several modifications were applied to curcumin analogs to develop novel curcumin analogs with cytotoxic activity

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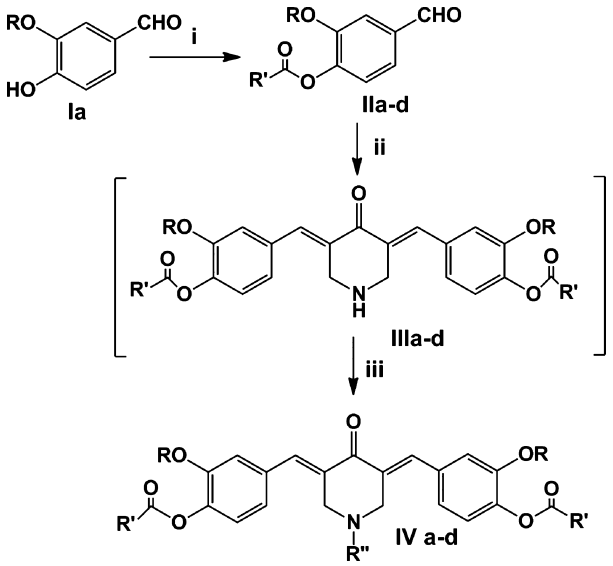
through targeting tubulin and inhibiting microtubules polymerization (13) where esterification and etherification of the aromatic hydroxyl groups took place to increase the hydrophobicity of the target compounds. Also, it was noticed that methylene group in the curcumin structure is unstable in acidic medium (14), so cyclization through the use of 4-piperidone ring was applied. Besides, it was found in the literature that oxygen atom in 4-piperidone ring forms a bond with N-H terminal in protein sequence, and so it was kept in the structure of target compounds. N-terminal in 4-piperidone ring was acylated to render targets non-basic, and hence, ease of penetration of cellular membranes also some targets were synthesized as thiourea derivatives as it was found in the literature that the phenyl isothiocyanate group is responsible for thiocarbomylation of thiol group of glutathione inside the cells and hence leads to depletion of glutathione from cancerous cells that leads to apoptosis (15,16). Conjugated characteristics were kept in the structure of the target compounds or elongated as it was found to be very essential in activity (17).

## Chemistry

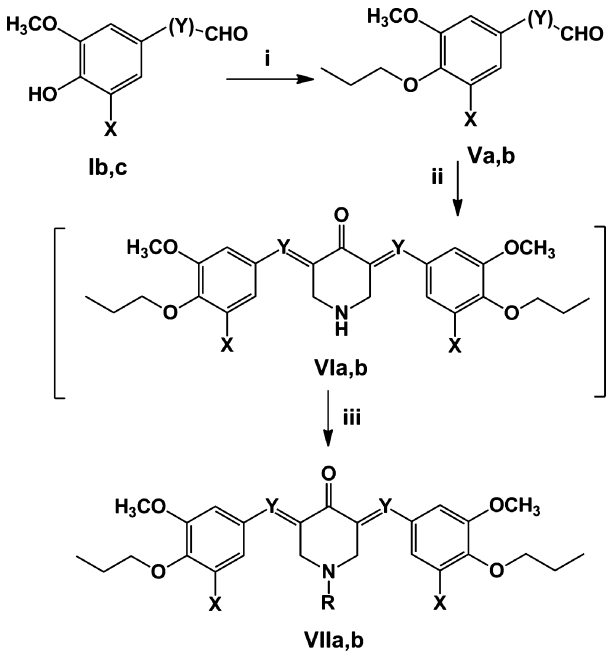
Analogs **IVa–d**, **VIa and VIIa,b**, **IXa and Xa–c**, and **XIIa–e** which represent four different series of compounds were designed and synthesized with 1,5-diaryl-3-oxo-1,4-pentadienyl core moiety. Reported intermediates **IIa–d** of series 1, **Va,b** of series 2, and **XIa,c,d** of series 4 were prepared according to reported procedures (18–25). The syntheses of the target compounds **IVa–d** of series 1 follow two-step procedure. First, condensation of the intermediates **IIa–d** with 4-piperidone.HCl in acidic medium (26) yielded **IIIa–d** that are pure enough to enter the final acylation step, which was carried out using acetyl chloride or benzoyl chloride (10) to give the corresponding target compounds **IVa–d** as in (Scheme 1).

As for series 2, the target compounds **VIa** and **VIIa,b** were synthesized by applying Claisen–Schmidt condensation reaction procedure (26) where the intermediates **Va,b** were condensed with 4-piperidone.HCl in acidic medium. **VIa** was obtained after neutralization and crystallization. Then both **VIa** and the pure mixture **VIb** were acetylated in a reaction catalyzed with triethylamine to obtain the final compounds **VIIa,b** (Scheme 2).

To synthesize the final target compounds of series 3 (**IXa** and **Xa-c**), thiation reaction was applied on the starting materials **Id,e** in the presence of strong catalyst, then the pure prepared mixture **VIIIa,b** entered the Claisen–Schmidt condensation reaction with 4-piperidone.HCl to obtain the target **IXa** and the mixture **IXb** which were pure enough to be acetylated in the presence of triethylamine yielding the final target compounds **Xa,b**. As for the target **Xc,** it was prepared by reacting **IXa** with phenyl isothiocyanate in triethylamine under reflux (Scheme 3).




**Scheme 1:** Synthesis of 3,5-bis(4-Acyloxy-3-methoxybenzylidene)-N-acylpiperidin-4-one (IVa-d). [1] ***Reagents and conditions:*** i: acyl chloride + triethylamine (5 °C), ii: 4-piperidone.HCl + conc. HCl, iii: acyl chloride + triethylamine (5 °C). [R = -$CH_3$].




**Scheme 2:** Synthesis of 3,5-bis(3-Methoxy-5-non-substituted/iodo-4-propoxybenzylidene/allylidene) piperidin-4-one and 3,5-bis(3-Methoxy-5-non-substituted/iodo-4-propoxybenzylidene/allylidene)-*N*-acetylpiperidin-4-one (**VIa and VIIa,b**). [2] ***Reagents and conditions:*** i: Na metal/reflux + alkyl chloride in ethanol/reflux, ii:4-piperidone.HCl + conc. HCl, iii: acyl chloride + triethylamine (5 °C). [R = -$CH_3$].

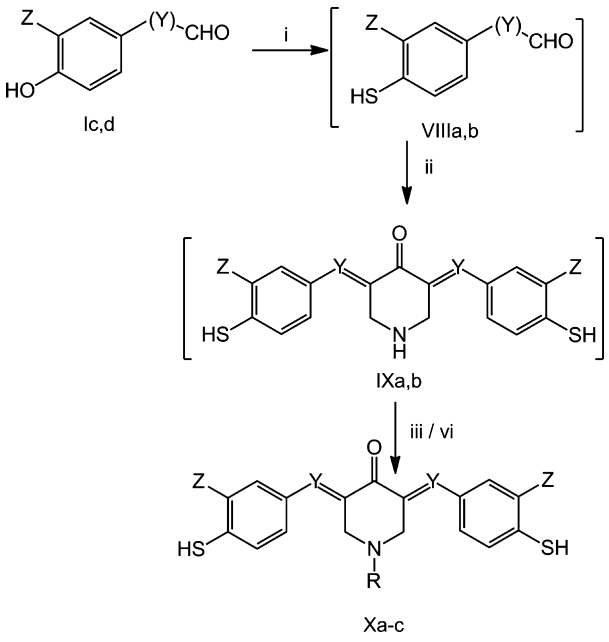
Compounds **XIIa–d** of series 4 were prepared via reacting the intermediates **XIa–d** with acetyl chloride or benzoyl chloride in triethylamine to yield the corresponding final

compounds **XIIa–d**. Target **XIIe** of series 4 was prepared through different pathway where 4-piperidone.HCl was reacted with phenyl isothiocyanate in triethylamine under reflux followed by condensation of the product with **Ia** in the presence of acidic medium to obtain **XIIe** (Scheme 4).

## Experimental

### *Materials and instrumentation*

Starting materials and reagents were purchased from Sigma-Aldrich (Stockholm, Sweden). Melting points were recorded on Stuart Scientific apparatus. $^{1}$H-NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer (International Equipment Trading Ltd, Vernon Hills, IL, USA). $^{13}$C-NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. MS spectra mass were recorded on Shimadzu GCMS-QP 5050A gas chromatograph mass spectrometer (70 eV). Elemental analyses were performed at The Regional Center for Mycology and Biotechnology Al-Azhar University, Cairo, Egypt. *In vitro* antiproliferative assay and tubulin polymerization assay were performed at Uppsala University Hospital, division of Clinical Pharmacology, department of Medical Sciences, Uppsala, Sweden. One compound **(XIIb)** was tested for its cytotoxic activity by the American National Cancer Institute.




**Scheme 3:** Synthesis of 3,5-bis(3-Substituted-4-mercaptobenzylidene/allylidene) piperidin-4-one and 3,5-bis(3-Substituted-4-mercaptobenzylidene/allylidene)-N-substituted piperidin-4-one (**IXa and Xa-c**). [3]***Reagents and conditions:*** i: $P_2S_5$, $CS_2$, conc. HCl, ii: 4-piperidone.HCl, conc. HCl, iii: acyl chloride, triethylamine (5 °C), iv; Phenylisothiocyanate, triethylamine (reflux).

### *Synthesis of reported intermediates*

#### 4-Acyloxy-3-methoxybenzaldehyde (IIa–d) General procedure

0.03 Moles of the appropriate acyl chloride and (0.06 moles, 6 mL) of triethylamine were dissolved in 10 mL chloroform and added dropwise at 5 °C to solution of (0.02 moles, 3 g) of vanillin '**Ia**' in 5 mL chloroform then stirred for 2 h at room temperature.

**4-Formyl-2-methoxyphenyl butyrate (IIa). Yield:** 87%; **melting point** (104–105 °C) (18).

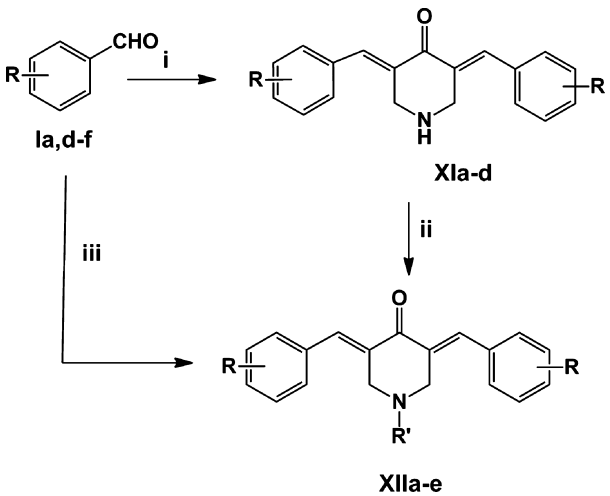
**4-Formyl-2-methoxyphenyl propionate (IIb). Yield:** 66%; **melting point** (114–115 °C) (18, 19).

**Tricyclo[3.3.1.1$^{3,7}$]decane-1-carboxylic acid, 4-formyl-2-methoxyphenyl ester (IIc). Yield:** 71%; **melting point** (118–119 °C) (20).

**4-Formyl-2-methoxyphenyl heptanoate (IId). Yield:** 75%; **melting point** (163–164 °C) (21).

#### Methoxy-5-(unsubstituted/iodo)-4-propoxybenzaldehyde/cinnmaldehyde (Va,b) General procedure

0.02 Moles of the appropriate available start **Ib,c** was refluxed with (0.02 moles, 46 mg) sodium metal in (15 mL) ethanol for one hour. The product was washed with water and filtered and then alkylation reaction was performed by the addition of chloroform solution of propyl chloride (0.02 moles, 1.5 mL) to 0.02 moles of the activated sodium salt of **Ib,c**. The whole reaction mixture was refluxed for further one hour to obtain the corresponding intermediate **Va,b**.




**Scheme 4:** Synthesis of 3,5-bis(Substituted benzylidene)-N-substituted-piperidin-4-one (**XIIa-e**). [4]***Reagents and conditions:*** i: 4-piperidone.HCl, conc. HCl, ii: acyl chloride, triethylamine (5 °C), iii: N-phenylisothiocyanate-4-piperidone, conc. HCl.

**1-Iodo-5-methoxy-4-propoxybenzaldehyde (Va). Yield:** 71%; **melting point** (148 °C) (22).

**3-Methoxy-4-propoxycinnamaldehyde (Vb). Yield:** 36%; **mp** (164 °C) (23).

### 3,5-bis(Substituted benzylidene)-piperidin-4-one (XIa-d) General procedure

Condensation of 0.02 moles of **Ia,d–f** dissolved in 6 mL chloroform with (0.01 mole, 1.5 g) of 4-piperidone.HCl was carried out in presence of 2 mL conc. HCl, and the mixture was stirred for 2 h at room temperature, then left for 2 days. The reaction mixture was neutralized and the chloroform layer was extracted with chloroform (3 × 10 mL) and evaporated under vacuum.

**3,5-bis(4-Hydroxy-3-methoxybenzylidene) piperidin-4-one (XIa). Yield:** 65%; **melting point** (254–256 °C) (24).

**3,5-bis(3,4,5-Trimethoxybenzylidene) piperidin-4-one (XIc). Yield:** 91%; **melting point** (251 °C) (24).

**3,5-bis(3,4-Dihydroxybenzylidene) piperidin-4-one (XId). Yield:** 65%; **melting point** (>300 °C) (25).

### *Synthesis of unreported analogs*

### 3,5-bis(4-Acyloxy-3-methoxybenzylidene)-*N*-acylpiperidin-4-one (IVa–d) General procedure

First, **IIIa–d** intermediates were prepared according to a reported condensation reaction (26) where 0.02 moles of **IIa–d** were dissolved in 5 mL chloroform and condensed with 0.01 mole, 1.5 g of 4-piperidone. HCl in the presence of 2 mL conc. HCl and then stirred for 2 h at room temperature, then left to stand for 2 days. The reaction mixture was neutralized with a suitable base as (10% sodium hydroxide, 10% sodium carbonate, 10% ammonium hydroxide, or triethylamine). The above neutralized solution was then extracted with $CHCl_3$ (3 × 10 mL). The chloroform extract was evaporated under vacuum to afford **IIIa–d** as a sticky mass, which is pure enough to the next step. Acylation of **IIIa–d** was carried out according to a similar reported reaction (10) where 0.01 mole of the appropriate acyl chloride dissolved in chloroform (10 mL) was mixed with (0.02 moles, 2 mL) triethylamine and added dropwise to 0.006 mole of **IIIa–d** at 5 °C then stirred for 2 h at room temperature. The reaction mixture was then recrystallized from appropriate solvent to afford target compounds **IVa–d**.

**3,5-bis(4-Butyryloxy-3-methoxybenzylidene)-*N*-acetylpiperidin-4-one (IVa).** The titled compound was separated as dark brown oily liquid, **yield:** 66.24%, **boiling point:** 125–127 °C. **FT-IR** ($\acute{\upsilon}$ max, $cm^{-1}$): 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1735 (ester C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 7.61 (d, 2H, $J$ = 6.5 Hz, 2 [-CH=C]), 7.59 (d, 2H, $J$ = 7 Hz, ArH), 7.56 (d, 2H, $J$ = 7 Hz, ArH), 7.31–7.36 (m, 2H, ArH), 5.05(q, 4H,-$CH_2NCH_2$), 3.85 (s, 6H, 2[$OCH_3$]), 2.57 (t, 4H, $J$ = 7.5 Hz, 2[CO-**$CH_2$**-$CH_2$-$CH_3$]), 2.29 (s, 3H, $COCH_3$), 1.74–1.64 (m, 4H, $J$ = 6 Hz, 2[CO-$CH_2$-**$CH_2$**-$CH_3$]), 0.99 (t, 6H, J = 7.5 Hz,2[CO-$CH_2$-$CH_2$-**$CH_3$**]). **Ms:** (MW: 549.00): *m/z* 549 ($M^+$, 8.64%), 350.25 (100%). **Anal. Calc. for $C_{31}H_{35}NO_8$:** C, 67.74; H, 6.42; N, 2.55; O, 23.29, **Found:** C, 67.81; H, 6.48; N, 2.62.

**3,5-bis(3-Methoxy-4-propyloloxybenzylidene)-*N*-benzoylpiperidin-4-one (IVb).** The titled compound was separated as yellow oily liquid, **yield:** 65.78%, **boiling point:** 199–204 °C. **FT-IR** ($\acute{\upsilon}$ max, $cm^{-1}$): 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1735 (ester C=O), 1700 (Ar C=C-), 1680 (C=C-) and 1679 (NH-C=O). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 7.98 (d, $J$ = 6 Hz, 2H, ArH), 7.95 (d, 2H, 2[-CH=C]), 7.69 (t, 1H, ArH), 7.66 (t, 1H, ArH), 7.64 (t, 1H, ArH), 7.56–7.55 (m, 2H, ArH), 7.53–7.52 (m, 2H, ArH), 7.51–7.50 (m, 2H, ArH), 4.33 (d, 2H, $J$ = 6 Hz, -$CH_2NCH_2$), 4.31 (d, 2H, $J$ = 6 Hz, -$CH_2NCH_2$), 3.86 (s, 6H, 2[$OCH_3$]), 1.36 (q, 4H, $J$ = 4 Hz, 2[CO-$CH_2$-]), 1.33 (t, 6H, $J$ = 6 Hz, 2[-$CH_3$]). **Ms:** (MW: 583.00): *m/z* 582 ($M^{+-1}$, 0.42%), 75.85 (100%). **Anal. Calc. for $C_{34}H_{33}NO_8$:** C, 69.97; H, 5.70; N, 2.40; O, 21.93, **Found:** C, 70.04; H, 5.74; N, 2.49.

**3,5-bis(4-Adamantoyloxy-3-methoxybenzylidene)-*N*-acetylpiperidin-4-one (IVc):.** The product was crystallized from ethanol/benzene and separated as yellow crystals, **yield:** 48.57%, **melting point:** 122–125 °C. **FT-IR** ($\acute{\upsilon}$ max, $cm^{-1}$): 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1735 (ester C=O), 1700 (Ar C=C-), 1680 (C=C-) and 1679 (NH-C=O). **$^1$H NMR (**300 MHz, **DMSO-*d6*):** $\delta$ 7.59 (d, 2H, 2[-CH=C]), 7.56 (d, 2H, ArH), 7.36–7.23 (m, 2H, ArH), 7.29 (d, 2H, ArH), 3.91 (s, 2H, -$CH_2NCH_2$), 3.84 (s, 6H, 2[$OCH_3$]), 3.78 (s, 2H,-$CH_2NCH_2$), 2.29 (s, 3H, $COCH_3$), 2.06–1.66 (m, 30H, -$CH_2$-, -CH-). **Ms:** (MW: 733.00): *m/z* 733 ($M^+$, 55.41%), 76 (100%). **Anal. Calc. for $C_{45}H_{51}NO_8$:** C, 73.65; H, 7.00; N, 1.91; O, 17.44, **Found:** C, 73.72; H, 6.98; N, 1.97.

**3,5-bis(4-Heptanoyloxy-3-methoxybenzylidene)-*N*-acetylpiperidin-4-one (IVd):.** The titled compound was separated as orange oily liquid, **yield:** 37.5%, **boiling point:** 177–178 °C. **FT-IR** ($\acute{\upsilon}$ max, $cm^{-1}$): 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1735 (ester C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR** (300 MHz, DMSO-*d6*): $\delta$ 7.61 (d, 2H, 2[-CH=C]), 7.59 (d, 2H, ArH), 7.57 (d, 2H, ArH), 7.33 (d, 2H, ArH), 3.87 (d, 2H, -$CH_2NCH_2$), 3.86 (s, 6H, 2[$OCH_3$]), 3.84 (d, 2H, -$CH_2NCH_2$), 2.59 (t, 4H, $J$ = 6 Hz, 2[CO-**$CH_2$**-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_3$]), 2.3 (s, 3H, $COCH_3$), 1.70–1.60 (m, 4H, $J$ = 7.5 Hz, 2 CO-$CH_2$-**$CH_2$**-$CH_2$-$CH_2$-$CH_2$-$CH_3$]), 1.41–1.34 (m, 4H, 2 [CO-$CH_2$-$CH_2$-**$CH_2$**-$CH_2$-$CH_2$-$CH_3$]), 1.32–1.24 (m, 8H, 4 [CO-$CH_2$-$CH_2$-$CH_2$-**$CH_2$-$CH_2$**-$CH_3$]), 1.06 (t, 6H, $J$ = 7.5 Hz, 2[CO-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-**$CH_3$**]).

**Ms:** (MW: 633.00): *m/z* 633 ($M^+$, 3.24%), 151.6 (100%). **Anal. Calc. for $C_{37}H_{47}NO_8$:** C, 70.12; H, 7.47; N, 2.21; O, 20.20, **Found:** C, 70.22; H, 7.53; N, 2.28.

### 3,5-bis[3-Methoxy-5-(non-substittuted/iodo)-4-propoxybenzylidene/allylidene] piperidin-4-one (VIa,b) General procedure

**VIa,b** were prepared by condensation reaction procedure applied in the preparation of compounds **IIIa–d**. Compound **VIb** gave sticky mass pure enough to enter next step.

**3,5-bis(3-Iodo-5-methoxy-4-propoxybenzylidene) piperidin-4-one (VIa).** The above titled compound was separated as yellow buff powder, **yield:** 41.42%, **melting point:** 149–155 °C. **FT-IR** ($\acute{\upsilon}$ max, $cm^{-1}$): 3450 (NH), 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), and 1680 (C=C-). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 9.7 (s, 1H, NH), 7.88 (d, 2H, 2[-CH=C]), 7.47 (s, 2H, ArH), 7.42 (d, 2H, ArH), 3.89 (s, 6H, 2 [$OCH_3$]), 3.38 (t, 4H, $J$ = 6 Hz, 2[O**$CH_2$**-$CH_2$-$CH_3$]), 3.06 (q, 4H, $J$ = 8 Hz, -$CH_2NCH_2$), 2.08 (s, 1H, NH), 1.79–1.75 (m, 4H, 2[$OCH_2$-**$CH_2$**-$CH_3$]), 1.21 (t, 6H, 2[$OCH_2$-$CH_2$-**$CH_3$**]). **Ms:** (MW: 703.00): *m/z* 703 ($M^+$, 0.04%), 277.55 (100%)**. Anal. Calc. for $C_{27}H_{31}I_2NO_5$:** C, 46.11; H, 4.44; I, 36.09; N, 1.99; O, 11.37, **Found:** C, 46.18; H, 4.47; N, 2.06.

### 3,5-bis(3-Methoxy-5-non-substituted/iodo-4-propoxybenzylidene/allylidene)-*N*-acetylpiperidin-4-one (VIIa,b) General procedure

0.03 Mole of acetyl chloride dissolved in chloroform (4 mL) was mixed with (0.06 moles, 6 mL) triethylamine and added dropwise to 0.02 mole of **VIa,b** at 5 °C then stirred for 1 h at room temperature. The reaction mixture was then recrystallized from appropriate solvent to afford target compounds **VIIa,b**.

**3,5-bis(3-Iodo-5-methoxy-4-propoxybenzylidene)-*N*-acetylpiperidin-4-one (VIIa).** Recrystallization was applied for the above titled product from ethanol/benzene and separated as brown crystals, **yield:** 46.41%, **melting point:** 102–107 °C. **FT-IR** ($\acute{\upsilon}$ max, $cm^{-1}$): 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR** (300 MHz, DMSO-*d6*): $\delta$ 7.99 (d, 2H, 2[-CH=C]), 7.87 (s, 2H, ArH), 7.60 (d, 2H, ArH), 3.84 (t, 4H, 2[O**$CH_2$**-$CH_2$-$CH_3$]), 3.05 (q, 4H, $J$ = 6 Hz, -$CH_2NCH_2$), 2.36 (s, 6H, 2[$OCH_3$]), 2.29 (s, 3H, $COCH_3$), 2.09–1.91 (m, 4H, 2[$OCH_2$-**$CH_2$**-$CH_3$]), 1.18 (t, 6H, 2[$OCH_2$-$CH_2$-**$CH_3$**]). **Ms:** (MW: 745.00): *m/z* 747 ($M^{++2}$, 0.06%), 50.90 (100%)**. Anal. Calc. for $C_{29}H_{33}I_2NO_6$:** C, 46.73; H, 4.46; I, 34.05; N, 1.88; O, 12.88**, Found:** C, 46.79; H, 4.52; N, 1.93.

**3,5-bis[(3-Methoxy-4-propoxyphenyl) allylidene]-*N*-acetylpiperidin-4-one (VIIb).** Recrystallization from ethanol/benzene was applied for the above titled product and separated as reddish brown powder, **yield:** 72.34%, **melting point:** 114–116 °C. FT-IR ($\acute{\upsilon}$ max, $cm^{-1}$): 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 7.59 (d, 2H,2[-CH=C]), 7.36–7.27 (m, 2H, 2[CH=CH-CH]), 7.24 (d, 2H, ArH), 7.17 (d, 2H, ArH), 6.91 (d, 2H, 2 [=CH-C]), 6.74 (q, 2H, $J$ = 8 Hz, ArH), 3.83 (t, 4H, 2[O**$CH_2$**-$CH_2$-$CH_3$]), 2.58 (q, 4H, $J$ = 6 Hz, -$CH_2NCH_2$), 2.5 (s, 6H, 2 [$OCH_3$]), 2.30–2.10 (m, 4H, $J$ = 6 Hz, 2[$OCH_2$-**$CH_2$**-$CH_3$]), 1.88 (s, 3H, $COCH_3$), 1.01 (t, 6H, 2[$OCH_2$-$CH_2$-**$CH_3$**]). **Ms:** (MW: 545.00): *m/z* 550 ($M^{++5}$, 0.16%), 76 (100%)**. Anal. Calc. for $C_{33}H_{39}NO_6$:** C, 72.64; H, 7.20; N, 2.57; O, 17.59**, Found:** C, 72.75; H, 7.27; N, 2.69.

### 3,5-bis(3-Substituted-4-mercaptobenzylidene/allylidene) piperidin-4-one (IXa,b) General procedure

An equimolar mixture of '4-hydroxy-3-methoxy cinnamaldehyde' **Ic** (0.01 mole, 1.78 g)**/**'3, 4 dihydroxybenzaldehyde' **Id** (0.01 mole, 1.38 g) and phosphorus pentasulfide (0.01 mole, 1.78 g) were mixed and stirred together at room temperature with the addition of (12 mL) of carbon disulfide dropwise and (6 mL) of conc. HCl then afterward continue stirring with gentle heating at (40–50 °C) for 1 h. The product was then washed with water and filtered to afford a sticky mass **VIIIa,b** pure enough for next step. The same condensation reaction applied in the preparation of compounds **IIIa–d** was carried out to prepare the new intermediates **IXa,b,** whereas compound **IXb** gave sticky mass pure enough to enter next step.

**3,5-bis[(4-Mercapto-3-methoxyphenyl) allylidene] piperidin-4-one (IXa).** The titled compound was separated as violet powder, **yield:** 16.9%, **melting point:** 234–246 °C. **FT-IR** ($\acute{\upsilon}$ max, $cm^{-1}$): 3450 (NH), 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), and 1680 (C=C-). **$^1$H NMR** (300 MHz, DMSO-*d6*): $\delta$ 7.25 (d, 2H, 2[-CH=C]), 7.23 (d, 2H, ArH), 7.18 (d, 2H, 2[CH-CH=C]), 7.16 (d, 2H, ArH), 6.74 (m, 4H, =CH-C, ArH), 3.81–3.80 (s, 6H, 2[$OCH_3$]), 3.21 (s, 2H, 2[SH]), 3.02 (q, 4H, -$CH_2NCH_2$), 1.91 (s, 1H, NH). **Ms:** (MW: 451.00): *m/z* 451 ($M^+$, 9.38%), 51 (100%)**. Anal. Calc. for $C_{25}H_{25}NO_3S_2$:** C, 66.49; H, 5.58; N, 3.10; O, 10.63; S, 14.20, **Found:** C, 66.52; H, 5.64; N, 3.08; S, 13.14.

### 3,5-bis(3-Substituted-4-mercapto-benzylidene/allylidene)-*N*-substituted piperidin-4-one (Xa–c). General procedure

Compounds **Xa,b** were obtained via acetylation reaction according to a similar reported reaction (10). (0.03 moles, 1 mL) of acetyl chloride dissolved in chloroform (5 mL) mixed with (0.06 moles, 6 mL) triethylamine and added dropwise to 0.002 moles of **IXa,b** at 5 °C then stirred for 2 h at room temperature. The reaction mixture was

then recrystallized from appropriate solvent to afford target compounds **Xa,b**. The target compound **Xc** was obtained by reacting a solution of (0.25 g) **IXa** in chloroform (5 mL) with (3 mL) phenyl isothiocyanate in equimolar quantity and then refluxed for 2 h with stirring. Dropwise addition of (1–3 mL) of triethylamine was applied along the reflux. The product **Xc** was then washed with acetone, filtered, and crystallized from toluene.

**3, 5-bis ([4-Mercapto-3-methoxyphenyl] allylidene)-*N*-acetylpiperidin-4-one (Xa).** The above titled compound was crystallized from ethanol/benzene and separated as brown powder, **yield:** 81.21%, **melting point:** 240–247 °C. **FT-IR (ύ max, cm$^{-1}$):** 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 8.96 (s, 2H, 2[SH]), 7.24 (d, 2H, $J$ = 6 Hz, 2[-CH=C]), 7.19–7.14 (m, 4H, ArH), 7.17 (d, 2H, $J$ = 6 Hz, 2[CH=CH-CH]), 6.90–6.60 (dd, 4H,2[=CH-C]), 3.79 (q, 4H, -$CH_2NCH_2$), 3.30 (s, 6H, 2[$OCH_3$]), 2.43 (s, 2H, 2[SH]), 2.30 (s, 3H, $COCH_3$). **Ms:** (MW: 493.00): *m/z* 494 ($M^{++1}$, 2.86%), 49.90 (100%). **Anal. Calc. for $C_{27}H_{27}NO_4S_2$:** C, 65.69; H, 5.51; N, 2.84; O, 12.96; S, 12.99**, Found:** C, 65.73; H, 5.57; N, 2.93; S, 13.05.

**3,5-bis(3, 4 Dimercaptobenzylidene)-*N*-acetylpiperidin-4-one (Xb).** The above titled compound was crystallized from ethanol/benzene and separated as dark blue powder, **yield:** 93.02%, **melting point:** 238–243 °C. **FT-IR (ύ max, cm$^{-1}$):** 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 9.70 (s, 4H, 4 [SH]), 6.82 (d, 2H, 2[-CH=C]), 6.80–6.72 (m, 2H, ArH), 6.69 (d, 2H, ArH), 5.53 (d, 2H, ArH), 3.45 (q, 4H, $J$ = 6 Hz, -$CH_2NCH_2$), 2.85 (s, 4H, 2[SH]), 1.10 (s, 3H, $COCH_3$). **$^{13}$C-NMR (300 MHz, DMSO-*d6*):** $\delta$ 186.89 (1C, C=O), 161.89 (1C, N-C=O), 146.00 (2C, -CH=), 145.47 (2C, C-CO), 131.13 (4C, SH), 116.002 (4C, ArC), 114.71 (4C, ArC), 45.61 (2C, -$CH_2NCH_2$), 38.64 (1C, $CH_3$). **Ms:** (MW: 445.00): *m/z* 445 ($M^+$, 0.40%), 67.90 (100%). **Anal. Calc. for $C_{21}H_{19}NO_2S_4$:** C, 56.60; H, 4.30; N, 3.14; O, 7.18; S, 28.78, **Found:** C, 56.68; H, 4.32; N, 3.19; S, 28.86.

**3,5-bis[(4-Mercapto-3-methoxyphenyl)allylidene]-4-oxo-*N*-phenylpiperidine-1-carbothioamide (Xc).** The above titled compound was crystallized from acetone/toluene and separated as brown powder, **yield:** 24.6%, **melting point:** 216–219 °C. **FT-IR (ύ max, cm$^{-1}$):** 3450 (NH), 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1500 (C=S). **$^1$H NMR** (300 MHz, DMSO-*d6*): $\delta$ 7.51 (d, 2H, 2[-CH=C]), 7.32–7.30 (m, 2H, ArH), 7.28 (t, 2H, ArH), 6.80–6.62 (m, 11H, =CH-CH=C, ArH, =CH-C, ArH), 5.31–5.29 (m, 4H, -$CH_2NCH_2$), 5.14 (s, 1H, NH), 3.75–3.67 (s, 6H, 2[$OCH_3$]), 2.75–.72 (s, 2H, 2 [SH]). **Ms:** (MW: 586.00): m/z 580 ($M^{+-6}$, 0.74%), 85.80 (100%). **Anal. Calc. for $C_{32}H_{30}N_2O_3S_3$:** C, 65.50; H, 5.15; N, 4.77; O, 8.18; S, 16.39, **Found:** C, 65.58; H, 5.19; N, 4.82; S, 16.42.

## 3,5-bis(Substituted benzylidene)-*N*-acylpiperidin-4-one (XIIa-d) General procedure

The target compounds **XIIa–d** were prepared by acylation reaction of 0.01 mole of acetyl chloride (1 mL)/0.01 mole of benzoyl chloride (2 mL) dissolved in chloroform (6 mL) mixed with (0.02 moles, 2 mL) triethylamine and added dropwise to 0.006 mole of the previously prepared **XIa–d** at 5 °C then stirred for 2 h at room temperature. Recrystallization from the appropriate solvent was then carried out.

**3,5-bis(4-Hydroxy-3-methoxybenzylidene)-*N*-acetylpiperidin-4-one (XIIa).** The titled compound was subjected to recrystallization from ethanol/benzene and separated as dark yellow powder, **yield:** 86.81%, **melting point:** 94–102 °C. FT-IR (ύ max, cm$^{-1}$): 3500 (Ar-OH), 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 9.97 (s, 2H, 2[OH]),7.61–7.58 (dd, 2H, 2[-CH=C]), 7.57 (d, 2H, ArH), 7.35 (d, 2H, ArH), 6.97 (d, 2H, $J$ = 6 Hz, ArH), 3.875 (s, 2H, 2[OH]), 3.02 (q, 4H, -$CH_2NCH_2$), 2.30 (s, 6H, 2[$OCH_3$]), 1.89 (s, 3H, $COCH_3$). **Ms:** (MW: 409.00): *m/z* 409 ($M^+$, 40.39%), 350.20 (100%). **Anal. Calc. for $C_{23}H_{23}NO_6$:** C, 67.47; H, 5.66; N, 3.42; O, 23.45, **Found:** C, 67.56; H, 5.69; N, 3.53.

**3,5-bis(3,4-Dioxymethylene-6-methylbenzylidene)-*N*-acetylpiperidin-4-one (XIIb).** 0.02 moles of **Ie** was dissolved in 6 ml chloroform and condensed with (0.01 mole, 1.5 g) of 4-piperidone.HCl in the presence of 2 mL conc. HCl and stirred for 2 h at room temperature, then left for 2 days. The reaction mixture was neutralized and the chloroform layer was extracted and evaporated to give **XIb** pure enough intermediate to be acetylated according to the general procedure explained in **6.3.3** to yield the final target **XIIb,** which was crystallized from ethanol/benzene.

The above titled product was separated as yellow powder, **yield:** 42.55%, **melting point:** 126–129 °C. **FT-IR** (ύ max, cm$^{-1}$): 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 7.29 (s, 2H, 2[-CH=C]), 6.91 (d, 2H, ArH), 6.11 (s, 2H, ArH), 6.11 (s, 2H, $OCH_2O$), 3.29 (s, 4H, -$CH_2NCH_2$), 2.56 (s, 6H, 2[Ar-$CH_3$]), 2.50 (s, 3H, $COCH_3$). **Ms:** (MW: 433.00): *m/z* 433 ($M^+$, 21.68%), 76 (100%). **Anal. Calc. for $C_{25}H_{23}NO_6$:** C, 69.27; H, 5.35; N, 3.23; O, 22.15, **Found:** C, 69.33; H, 5.39; N, 3.31.

**3,5-bis(3, 4, 5-Trimethoxybenzylidene)-*N*-benzoylpiperidin-4-one (XIIc).** The above titled compound was crystallized from ethanol/benzene and separated as yellowish brown powder, **yield:** 89.34%, **melting point:** 115–122 °C. **FT-IR (ύ max, cm$^{-1}$):** 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-),

and 1679 (NH-C=O). **$^1$H NMR** (300 MHz, DMSO-*d6*): $\delta$ 7.94 (d, 2H, ArH), 7.61 (t, 1H, ArH), 7.48 (t, 2H, $J$ = 6 Hz, ArH), 7.27 (d, 2H, $J$ = 6 Hz, 2[-CH=C]), 6.69 (d, 4H, ArH), 3.06 (q, 4H, J = 6 Hz, -$CH_2NCH_2$), 3.86–3.47 (m, 18H, 6 [$OCH_3$]). **Ms:** (MW: 559.00): m/z 559 ($M^+$, 0.33%), 103.95 (100%)**. Anal. Calc. for $C_{32}H_{33}NO_8$:** C, 68.68; H, 5.94; N, 2.50; O, 22.87, **Found:** C, 68.77; H, 5.98; N, 2.61.

**3,5-bis(3, 4-Dihydroxybenzylidene)-*N*-benzoylpiperidin-4-one (XIId).** The above compound was crystallized from ethanol/benzene and separated as reddish brown powder, **yield:** 56.15%, **melting point:** 160–162 °C. **FT-IR ($\acute{\upsilon}$ max, cm$^{-1}$):** 3500 (Ar-OH), 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1679 (NH-C=O). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 8.15 (d, 2H, ArH), 7.93 (d, 2H, $J$ = 6 Hz, 2[-CH=C]), 7.77 (t, 1H, ArH), 7.65 (t, 2H, ArH), 7.49 (d, 2H, ArH), 7.33 (d, 2H, ArH), 6.90 (d, 2H, ArH), 5.10–4.90 (s, 4H, 4[OH]), 2.63 (d, 4H, -$CH_2NCH_2$). **$^{13}$C-NMR (300 MHz, DMSO-*d6*):** $\delta$ 180.91 (1C, C=O), 168.42 (1C, N-C=O), 147.23 (2C, ArC-OH), 145.31 (2C, ArC-OH), 134.43 (2C, -CH=), 132.31 (2C, C-CO), 131.52 (1C, ArC-C=O), 129.60 (1C, ArC), 129.27 (2C, ArC-CH=), 128.90 (2C, ArC), 128.06 (2C, ArC), 125.36 (2C, ArC), 117.89 (2C, ArC), 115.75 (2C, ArC), 45.30 (2C, -$CH_2NCH_2$). **Ms:** (MW: 443.00): *m/z* 443 ($M^+$, 0.36%), 50.10 (100%)**. Anal. Calc. for $C_{26}H_{21}NO_6$:** C, 70.42; H, 4.77; N, 3.16; O, 21.65, **Found:** C, 70.51; H, 4.82; N, 3.25.

### Synthesis of target 3,5-bis(4-Hydroxy-3-methoxybenzylidene)-4-oxo-*N*-phenylpiperidine-1-carbothioamide (XIIe)

The final target compound **XIIe** was obtained through a reverse pathway by mixing 4-piperidone.HCl (1.5 g) dissolved in 4 mL chloroform with phenyl isothiocyanate (9 mL) in equimolar amount and then refluxing for 2 h with stirring while dropwise addition of (1–3 mL) of triethylamine along the reflux. The product '4-oxo-*N*-phenylpiperidine-1-carbothioamide' was then washed with acetone and filtered. (1 mole, 1.8 g) of that product was then reacted with (2 moles, 2.4 g) of the available vanillin **Ia** through condensation procedure mentioned previously under the preparation of **IIIa–d**. The product **XIIe** was then washed again with acetone and petroleum ether and subjected to recrystallization from methanol/benzene.

The titled compound was separated as orange powder, **yield:** 18.05%, **melting point:** 236–245 °C. **FT-IR ($\acute{\upsilon}$ max, cm$^{-1}$):** 3500 (Ar-OH), 3450 (NH), 3010 (Ar C-H), 2890 (C-H), 1750 (ketonic C=O), 1700 (Ar C=C-), 1680 (C=C-), and 1500 (C=S). **$^1$H NMR (300 MHz, DMSO-*d6*):** $\delta$ 9.85–9.84 (s, 2H, 2[OH]), 9.47 (s, 1H, NH), 7.81 (s, 2H,2[-CH=C]), 7.13 (d, 4H, ArH), 7.01–6.92 (m, 7H, ArH), 4.51 (s, 4H, -$CH_2NCH_2$), 4.39 (s, 1H, NH), 3.84–3.81 (s, 6H, 2[$OCH_3$]), 3.17 (s, 2H, 2[OH]). **$^{13}$C-NMR (300 MHz, DMSO-*d6*):** $\delta$ 181.94 (1C, C=O), 181.84 (1C, C=S), 150.00 (2C, ArC-O), 149.18 (2C, -CH=), 147.69 (2C, ArC-OH), 147.60 (2C, C-CO), 139.58 (1C, ArC-NH), 139.34 (2C, ArC), 125.16 (2C, ArC-CH), 124.71 (2C, ArC), 124.46 (1C, ArC), 115.99 (2C, ArC), 115.64 (2C, ArC), 115.41 (2C, ArC), 55.86 (2C, $OCH_3$), 55.58 (2C, -$CH_2NCH_2$). **Ms:** (MW: 502.00): m/z 502 ($M^+$, 0.57%), 161.10 (100%)**. Anal. Calc. for $C_{28}H_{26}N_2O_5S$:** C, 66.91; H, 5.21; N, 5.57; O, 15.92; S, 6.38, **Found:** C, 66.98; H, 5.27; N, 5.62; S, 6.43.

## *Biology*

### Cell lines

The *in vitro* analysis were carried out in a panel of cancer cell lines, including A2780 (ECACC Salisbury, UK), ACHN, Hct-116 and PC-3 (all American Type Culture Collection, LGC Standards, Borås, Sweden), and U937-GTB (kind gift from Kennet Nilsson, Department of pathology, Uppsala University). The different cell lines were selected as representatives of various kinds of cancer types, including ovarian cancer (A2780), renal adenocarcinoma (ACHN), prostate cancer (PC-3), colorectal cancer (Hct-116), and a leukemic monocyte lymphoma (U937-GTB). Cell growth medium RPMI 1640 (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FCS; Sigma-Aldrich), 2 mmol/L L-glutamine, 100 $\mu$g/mL streptomycin, and 100 U/mL penicillin (Sigma-Aldrich), was used.

Target **XIIb** was the only compound tested by the *American National Cancer Institute* (NCI) on 60 different cell lines for different types of cancer as (leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, prostate cancer, renal cancer, and breast cancer).

### Cytotoxic study

A semi-automated fluorometric microculture cytotoxicity assay (FMCA) was used to assess drug sensitivity (27,28). The method was based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes. Using the pipetting robot BioMek 2000 (Beckman Coulter, Fullerton, CA, USA), 384-well microplates (NUNC) were prepared with 5 $\mu$L drug solution in 10 times the final drug concentration. The plates were then stored at −70 °C until further use. Tumor cells from cell lines (5000 cells/well) were seeded in the drug-prepared 384-well plates using the pipetting robot Precision 2000 (Bio-Tek Instruments, Winooski, VT, USA). Three columns without drugs served as controls and one column with medium only served as a blank. The plates were incubated at 37 °C for 72 h and were then analyzed using the FMCA. Cell survival, expressed as survival index (SI), is defined as fluorescence in test wells divided by fluorescence of control wells, with blank values subtracted, ×100. Quality criteria for a successful assay included a mean coefficient of variation of <30% in the control and a fluorescence signal in control wells of more than

five times the blank. From the mean SI% curves, the half maximal inhibitory concentration ($IC_{50}$) was determined using nonlinear regression analysis in Prism 5 Software Package (Graph Pad, San Diego, CA, USA).

Compound **XIIb** was selected and tested by the NCI on 60 different cell lines using concentration of 1–5 M and both mean growth percent and growth percent of the cancer cell lines were calculated.

### Tubulin polymerization assay

All compounds have been tested at 10 $\mu$M. The substances' effect on tubulin polymerization were investigated using the Tubulin Polymerization Assay Kit (porcine tubulin and fluorescence based), which utilize fluorescent reporter enhancement (Cytoskeleton Inc. Denver, CO, USA). Fluorescence was measured using a FLUOstar OPTIMA instrument. Paclitaxel was used as a positive control for microtubule-stabilizing agent and vincristine was used as a second positive control for microtubule destabilization. The positive controls were obtained from the Swedish Pharmacy and diluted with PBS to a final concentration of 3 $\mu$M.

### Molecular modeling

All molecular modeling studies were performed using Accelrys Discovery Studio 2.5 operating system (Accelrys Inc., San Diego, CA, USA), at the Faculty of Pharmacy, Ain Shams University; Cairo, Egypt. Molecules were built within DS, and conformational models for each compound were generated automatically. This emphasizes representative coverage over a 20 Kcal/mol energy range above the estimated global energy minimum, and the best quality generation technique was chosen. Docking study involved the following steps: the docking analysis was carried out on $\beta$-chain of tubulin protein. The 3D protein structure of tubulin cocrystallized with podophyllotoxin (code; 1SA1) was downloaded from the Protein Data Bank of the Research Collaboration for Structural Bioinformatics (RCSB) Web site [www.rcsb.org]. The colchicine binding pocket of the $\beta$-chain of tubulin was docked with podophyllotoxin and the test analogs **IVa–d**, **VIa and VIIa,b**, **IXa and Xa-c**, and **XIIa-e,** after deleting the water structure and $\alpha$, $\gamma$ chains of tubulin protein, cleaning the protein, adding the missing hydrogens and side chains, and energy minimization according to DS protocol. The binding pocket of the complexed compound (podophyllotoxin) with the connected amino acid molecules at sphere of radius = 14 □ was identified and then docked with test analogs using CDocker-CHARMm-based technique. After that the docking scores (-CDOCKER interaction energy) of the best-fitted conformation of each of the docked molecules as well as the total number of hydrogen bonds and Pi-bonds with the amino acids at the colchicine binding pocket were recorded.

## Results and Discussion

### *Pharmacology*

### Antiproliferative activity

The newly synthesized compounds **IVa,c and d**, **VIa and VII a,b**, **IXa and Xa–c**, and **XIIa–e** were screened for their antiproliferative activities against: ovarian cancer (A2780), renal adenocarcinoma (ACHN), prostate cancer (PC-3), colorectal cancer (Hct-116), and a leukemic monocyte lymphoma (U937-GTB) utilizing the fluorometric microculture cytotoxicity assay FMCA method.

From the observed results (Table S1), it was noticed that most of the synthesized compounds revealed mild to moderate cytotoxic properties. However, compounds **XIIe** and **IVc** were found to be extremely potent, as both exhibited considerable antiproliferative activity against the five cancer cell lines used in the assay with ($IC_{50}$, concentration required to produce 50% inhibition of cell growth compared to control experimental) = 1–2.5 $\mu$M and 11.4–23.2 $\mu$M, respectively. Compound **XIId** showed moderate cytotoxicity with $IC_{50}$ = 18.9–52.7 $\mu$M, while rest of compounds exhibited mild to moderate activity according to the type of cancer cell line with $IC_{50}$ ranging from 36 to 269 $\mu$M. Compound **XIIb** was tested on 60 different cancer cell lines by The American National Cancer Institute showing mild cytotoxic activity among them, with mean value 101.15 and range of 35.20 of the growth percents of the cancer cell lines. The assay was inapplicable on compound **IVb** due to its oily nature and poor solubility.

### Tubulin polymerization assay

The effect of 10 $\mu$M of the compounds **IVa,c and d**, **VIa and VIIa,b**, **IXa and Xa–c**, and **XIIa–e** on tubulin polymerization were investigated using the Tubulin Polymerization Assay Kit (porcine tubulin- and fluorescence based), which utilize fluorescent reporter enhancement (Cytoskeleton Inc.). The results are summarized in (Tables S2 and S3) where inhibition % of the newly synthesized compounds were calculated using (equation 1) 'Inhibition % = [((Test sample) – (Vehicle control))/((Control ligand) – (Vehicle control))] × 100', and stabilization % were also calculated using (equation 2) 'Stabilization % = {1-[((Test sample) – (Vehicle control))/((Control ligand) – (Vehicle control))] × 100}'.

The *in vitro* assay revealed that only few compounds inhibited microtubule assembly. Compound **VIIa** displayed the highest activity in destabilizing microtubules compared to the positive control drug, 'vincristine' as a microtubule destabilizing agent (Figure S6).

On the other hand, compound **XIIc** was found to be the most potent one in stabilizing microtubules compared to the other positive control drug, 'paclitaxel' as an example of microtubule-stabilizing agent (Figure S7).

Compound **XIIc** acted by different mechanism where stabilizing microtubules causes chromosomes to become unable to achieve a metaphase spindle configuration, which blocks progression of mitosis, also prolonged activation of the mitotic checkpoint triggers apoptosis or reversion to the G-phase of the cell cycle without cell division.

### *Molecular modeling*

The molecular docking study was carried out using Discovery Studio 2.5 software. The study was started by determining the binding mode of bioactive conformation of podophyllotoxin cocrystallized with $\beta$-chain of tubulin having the code 1SA1 obtained from the protein data bank without change in its conformation to investigate the detailed intermolecular interactions between the ligand and the target protein then to validate this study, it was matched with another peer article (27) where the study of the binding mode of Podophyllotoxin and chalcone derivatives-structures which mimic our compounds- was carried out with the same procedure.

Interactive docking using CDOCKER protocol was then applied between the designed analogs **IVa–d**, **VIa and VIIa,b**, **IXa and Xa–c**, and **XIIa–e,** and the binding site of the prepared $\beta$-chain of tubulin protein. Each tested molecule gave 10 possible docked poses. The ideal pose of each molecule was selected according to the similarity of its binding mode in the binding site to that of podophyllotoxin. The corresponding CDOCKER interaction energy (kcal/mole) was considered in our study to prioritize their virtual affinity to the binding site, in comparison to the ideal pose of the podophyllotoxin, curcumin, and other reported analogs.

Results revealed that these compounds have the ability to interact with the deep two hydrophobic centers of colchicine binding site region in the $\beta$-chain of tubulin similar to podophyllotoxin. One hydrophobic center is surrounded by Met 259, Ala 316, and Lys 352 (occupied by the benzodioxole fragment in podophyllotoxin), and the other one is surrounded by Leu 242, Ala 250, and Leu 255 (occupied by the trimethoxyphenyl moiety) (29) (Figure S8).

Compound **VIIa,** which showed the highest destabilizing activity on microtubules *in silico* as well as in the *in vitro* tubulin polymerization assay, interacted in the same binding mode as podophyllotoxin forming an extra hydrogen bond with Lys 352 (Figure S9).

Also compound **XIIc** with the highest stabilizing activity on microtubules interacted similarly in the same binding mode as podophyllotoxin (Figure S10), where it was ranked within the compounds which showed high interaction energy *in silico* while *in vitro* assay revealed its high stabilization activity mechanism on microtubules.

All these findings proved that the *in silico* study results correlated with the *in vitro* tubulin polymerization assay. A validation of the ideal pose was also performed by alignment of the X-ray bioactive conformers of **(VIIa and XIIc)**, with the best-fitted pose of podophyllotoxin. The alignment showed good coincidence between them with RMSD = 0.401 □, indicating the validity of the selected poses for both analogs (Figures S11 and S12).

Compound **IVc** having adamantyl bulky group failed to attain a pose, and the docking operation did not work out.

## Structure–Activity Relationship (SAR)

Based on the *in vitro* studies, it was concluded that acylation of heterocyclic nitrogen of 4-piperidone ring is very essential for enhancing cytotoxic activity. This was supported by the structure–activity relationship study performed previously on curcumin analogs (9), which stated the importance of such acylation to render nitrogen atom in non-basic form to be able to penetrate cell membrane and hence exerting efficient cytotoxic effect. Also, introduction of thiourea moiety produced potent cytotoxic compounds which coincides with reports describing that phenylisothiocyanate group is responsible for thiocarbomylation of thiol group of glutathione inside cancerous cells which leads to its depletion and hence apoptosis (15,16). Additionally, introduction of iodo group at the aromatic rings could enhance the tubulin destabilization activity of compounds as the size of iodine atom just perfectly fitted into the binding pocket of tubulin; this was concluded from the *in silico* docking study performed to iodine containing compound compared with other halide containing compounds and validated by the results of the *in vitro* assay.

## Conclusion

Esterification/etherification or sulfurization of vanillin and its derivatives yielded products that could be condensed successfully with 4-piperidone ring, where its heterocyclic N-atom was then acylated, benzoylated, or reacted with phenylisothiocyanate to give the targeted compounds **IVa–d**, **VIa and VIIa,b**, **IXa and Xa–c**, and **XIIa–e** in high yield.

The target compounds that were tested *in vitro* for their cytotoxicity on five different cancer cell lines and for their activity on tubulin polymerization proved to possess high to moderate cytotoxic activity. Also, *in silico* study results were consistent with that obtained from *in vitro* tubulin polymerization assay. Based on the previous results, it was concluded that the thiourea derivative **XIIe** was the most potent cytotoxic compound with $IC_{50}$ values in 1–2.5 $\mu$M range, while it showed moderate effect on tubulin polymerization.

Compound **IVc** displayed lower cytotoxicity with $IC_{50}$ values in 11.4–23.2 $\mu$M range and 79.8% stabilization of

microtubules in the tubulin polymerization assay, although it showed failure of docking operation in the *in silico* study, whereas compound **VIIa** destabilized microtubules with 93.3% of inhibition of tubulin and showed cytotoxicity with $IC_{50}$ = 36–102.6 $\mu$M. On the other hand, compound **XIIc** stabilized microtubules with 94.8% and showed cytotoxicity with $IC_{50}$ = 54.4–187.4 $\mu$M. Hence, we concluded that the synthesized curcumin analogs provide a nucleus for further optimization, which could lead to the development of new active chemical entities against cancer via tubulin targeting.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Curcumin structure (2).

**Figure S2.** Primary binding site shown on curcumin analog (9).

**Figure S3.** Chalcones Scaffold (11).

**Figure 4.** Benzylidene curcumin derivative (12).

**Figure S5.** (A) Podophyllotoxin docked into the colchicine binding site of $\beta$-tubulin (13). (B) The most potent chalcone derivative inhibiting tubulin polymerization docked into the same colchicine binding site of $\beta$-tubulin as podophyllotoxin (13).

**Figure S6.** Inhibition % of target compounds towards tubulin with Vincristine as control ligand.

**Figure S7.** Stabilization % of target compounds towards tubulin with Paclitaxel as control ligand.

**Figure S8.** Binding mode of Podophyllotoxin inside colchicine binding site of $\beta$-tubulin.

**Figure S9.** Binding mode of analog **VIIa** inside colchicine binding site of $\beta$-tubulin.

**Figure S10.** Binding mode of analog **XIIc** inside colchicine binding site of $\beta$-tubulin.

**Figure S11.** The alignment of analog **VIIa** with Podophyllotoxin.

**Figure S12.** The alignment of analog **XIIc** with Podophyllotoxin.

**Table S1.** Results of the cytotoxicity assay of the new curcumin analogs expressed as $IC_{50}$ in $\mu$M.

**Table S2.** Inhibition % of target compounds towards tubulin with vincristine as control ligand.

**Table S3.** Stabilization % of target compounds towards tubulin with paclitaxel as control ligand.