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Design, synthesis, and mechanistic evaluation of novel pyrazole/thiazole chalcone hybrids as dual tubulin polymerization and COX-2 inhibitors with potent antiproliferative activity

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A novel series of pyrazole/thiazole chalcone hybrids (**9a–o**) was designed, synthesized, and evaluated as dual tubulin/COX-2 inhibitors with anticancer activity. The synthesized compounds were screened for antiproliferative activity against MDA-MB-231, HCA-7, and A549 cancer cell lines. Several derivatives exhibited promising activity, with **9m** being the most potent against MDA-MB-231 cells ($IC_{50} = 1.96 \pm 0.10 \mu\text{M}$), while **9l** emerged as the most balanced lead compound, showing strong antiproliferative activity against HCA-7, MDA-MB-231, and A549 cells with IC_{50} values of 2.18 ± 0.11 , 2.92 ± 0.15 , and $4.86 \pm 0.25 \mu\text{M}$, respectively. Mechanistic studies revealed that the anticancer activity of this series is mediated through a dual mechanism involving tubulin polymerization inhibition and selective COX-2 inhibition. In particular, compound **9l** inhibited tubulin polymerization with an IC_{50} of $4.21 \pm 0.25 \mu\text{M}$ and showed potent COX-2 inhibition ($IC_{50} = 0.10 \pm 0.01 \mu\text{M}$) with high selectivity over COX-1 ($IC_{50} = 10.92 \pm 0.78 \mu\text{M}$; selectivity index = 109.20). Further investigation in HCA-7 cells demonstrated that **9l** significantly increased Bax level to $438.64 \pm 15.72 \text{ pg mL}^{-1}$ and reduced Bcl-2 to $6.74 \pm 0.19 \text{ pg mL}^{-1}$, while markedly elevating caspase-3 and caspase-9 levels to $496.80 \pm 14.90 \text{ pg mL}^{-1}$ and $47.86 \pm 1.18 \text{ ng mL}^{-1}$, respectively. Moreover, **9l** strongly suppressed PGE-2 production to $0.56 \pm 0.04 \text{ ng mL}^{-1}$, corresponding to 89.2% inhibition, and induced G2/M cell-cycle arrest. It also showed promising anti-migratory activity in the wound-healing assay, favorable microsomal stability, and acceptable *in silico* ADMET properties. Molecular docking further supported its favorable binding within the active sites of both tubulin and COX-2. Collectively, these findings identify **9l** as a promising dual tubulin/COX-2-targeting anticancer candidate.

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1. Introduction

Cancer remains one of the leading causes of death worldwide and continues to impose a major therapeutic burden despite substantial advances in surgery, radiotherapy, and systemic treatment.¹ Its lethality is driven not only by uncontrolled

proliferation but also by invasion, metastasis, and the progressive acquisition of resistance to therapy.^{2,3} According to the World Health Organization, cancer accounted for nearly 10 million deaths in 2020,⁴ and roughly one-third of cancer deaths are linked to modifiable risk factors such as tobacco use, alcohol consumption, obesity, unhealthy diet, and physical inactivity. These realities continue to justify the search for mechanistically innovative small molecules capable of interfering with multiple cancer-relevant pathways simultaneously.^{5–8}

Among validated anticancer strategies, disruption of microtubule dynamics remains one of the most successful approaches in medicinal chemistry.⁹ Microtubules are dynamic polymers of α/β -tubulin heterodimers that are essential for cell architecture, intracellular transport, and, most importantly, chromosome segregation during mitosis.¹⁰ Agents that inhibit tubulin polymerization suppress spindle formation, arrest cells at the G2/M phase, and ultimately promote apoptotic death.¹¹

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Within this class, ligands that target the colchicine-binding site are especially attractive because they prevent tubulin from adopting the conformation required for microtubule assembly, and combretastatin A-4 (CA-4) remains one of the best-known prototypes.¹² However, the therapeutic exploitation of CA-4 has been limited by drawbacks associated with its *cis*-stilbene system, especially its tendency to lose the active geometry and related pharmaceutical limitations,¹³ which has encouraged the development of structurally constrained mimics and heterocyclic replacements.

In this context, both thiazoles and chalcones have emerged as highly valuable motifs in the design of tubulin polymerization inhibitors.¹⁴ Thiazole-containing scaffolds are well-established in anticancer drug discovery, where they serve as rigid heteroaromatic frameworks suitable for colchicine-site binding.^{15–17} Likewise, chalcones offer a privileged aryl–enone scaffold capable of occupying hydrophobic regions within the tubulin pocket and promoting antiproliferative activities.^{18,19} The [2,1-*b*]thiazole–chalcone conjugate **I** illustrates this, exhibiting broad cytotoxicity alongside microtubule disruption, G2/M arrest, and induction of apoptosis.²⁰ The benzo[*d*]imidazo[2,1-*b*]thiazole–chalcone hybrid **II** similarly demonstrated effective inhibition of tubulin assembly with consequent mitotic arrest.²¹ In addition, the thiazole-based chalcone **III** highlighted the intrinsic potential of this hybrid framework as a tubulin-targeting pharmacophore.²² This strategy was further substantiated by compounds **IV** and **V**, which combined tubulin interference with carbonic anhydrase IX inhibition.^{23,24} In another study, the bisthiazole chalcone **VI** exhibited pronounced anti-tubulin activity, with clear cell-cycle arrest and apoptotic effects in MCF-7 cells.²⁵ Similarly, the thiazole-2-acetamide derivative **VII** displayed potent tubulin-associated antiproliferative activity accompanied by apoptosis induction.²⁶ The structures of these representative compounds are shown in Fig. 1. Collectively, these findings reinforce the versatility of thiazole–chalcone hybrids as antimetabolic agents.

A second pathway highly relevant to malignant progression is the cyclooxygenase pathway, particularly COX-2.²⁷ COX-1 is constitutively expressed and is involved in homeostatic functions such as gastric mucosal protection and platelet

physiology, whereas COX-2 is an inducible isoform that becomes markedly upregulated during inflammation and in many tumors.²⁸ Beyond its role in inflammation, COX-2 contributes to several hallmarks of cancer by promoting prostaglandin-dependent proliferation, angiogenesis, migration, invasion, immune evasion, and resistance to apoptosis.²⁹ These findings explain the continued interest in selective COX-2 inhibitors as anticancer agents.^{29,30} Classical coxibs such as celecoxib, rofecoxib, valdecoxib, and etoricoxib were developed to exploit structural differences between COX-1 and COX-2, particularly the larger secondary side pocket in COX-2. However, safety concerns have limited the use of some members of this class.^{31,32} Even so, the COX-2/PGE₂ axis remains an attractive target for hybrid anticancer design.

The pyrazole ring is regarded as a privileged scaffold in medicinal chemistry because of its broad and well-documented spectrum of pharmacological activities, including analgesic, anti-inflammatory, anticancer, antimicrobial, and CNS effects.^{33,34} In particular, pyrazole-based compounds are well-established in NSAID chemistry and have been widely exploited in the design of COX-2-directed agents.³⁵ Several marketed NSAID-related drugs also contain a pyrazole core, such as celecoxib, tepoxalin, lonazolac, and difenamizole.³⁶ This importance is further underscored by several reports on pyrazole-based selective COX-2 inhibitors (Fig. 2). For example, pyrazole-1,3,4-oxadiazole hybrid **VIII** exhibited selective COX-2 inhibition, along with anti-inflammatory, analgesic, and gastro-sparing properties.³⁷ Similarly, pyrazole–chrysin hybrid **IX** combined selective COX-2 inhibition with antiproliferative activity.³⁸ Likewise, pyrazole–thiadiazole hybrid **X** emerged as a highly selective COX-2 inhibitor with a favorable drug-likeness profile,³⁹ whereas more recent difenamizole-inspired pyrazole **XI** bearing a bulkier *N*-substituent showed improved COX-2 selectivity together with EGFR inhibition.⁴⁰

1.1 Rational design

The simultaneous targeting of tubulin polymerization and COX-2 is biologically and pharmacologically justified. Tubulin inhibition disrupts mitotic spindle formation and promotes

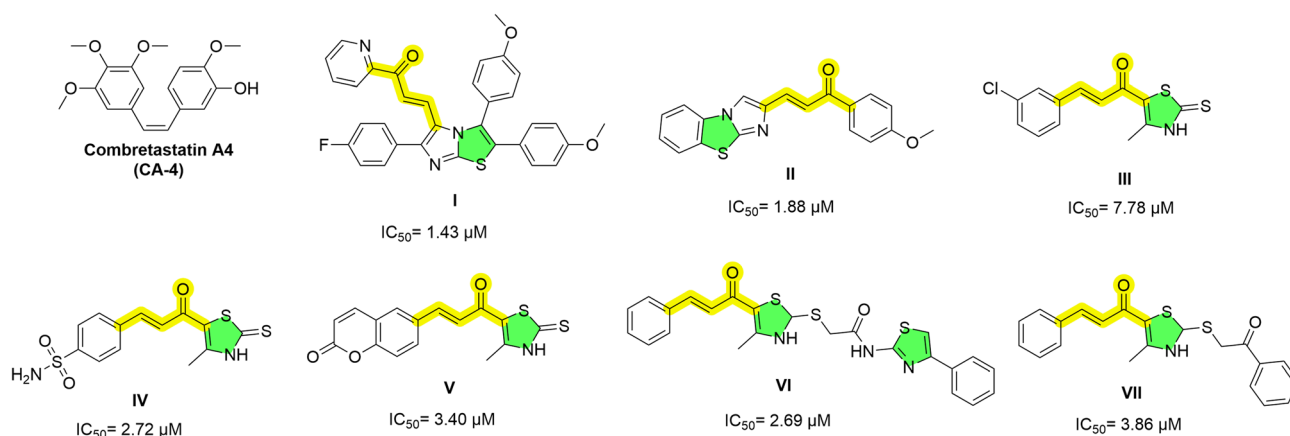


Fig. 1 Selected thiazole/chalcone-based tubulin polymerization inhibitors (I–VII) and combretastatin A-4.

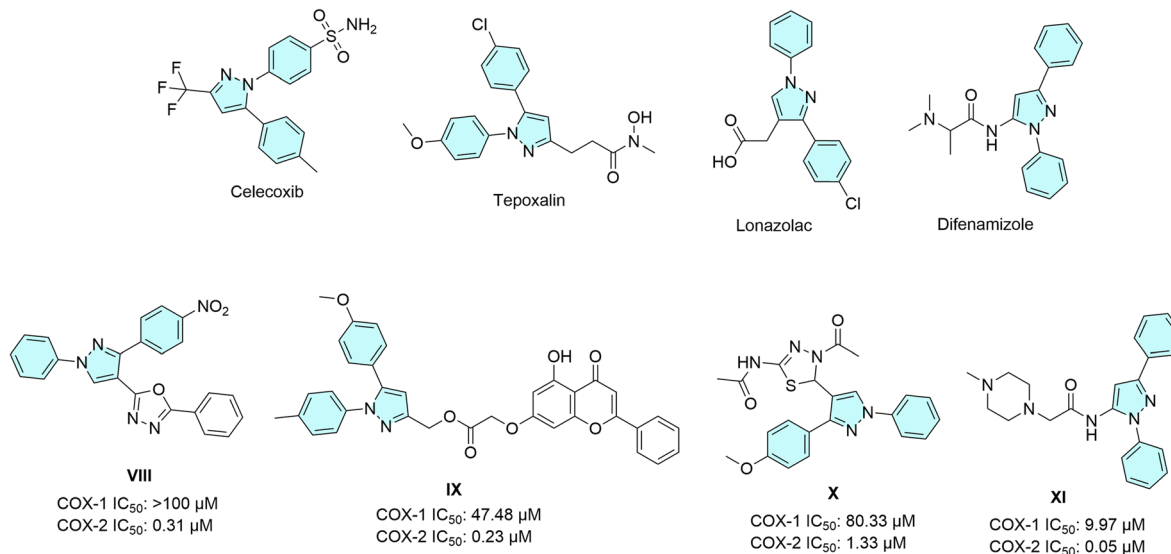


Fig. 2 Selected pyrazole-containing drugs and pyrazole-based selective COX-2 inhibitors (VIII–XI).

apoptotic cell death, whereas COX-2 inhibition is expected to suppress prostaglandin-driven signaling involved in tumor growth, angiogenesis, invasion, and resistance to apoptosis. This concept is supported by both preclinical and clinical evidence, including studies of celecoxib combined with taxane-based regimens.^{41–43} Moreover, the feasibility of integrating both activities within a single scaffold has already been demonstrated through molecular hybridization; notably, the rofecoxib/combretastatin hybrid KSS19 was designed to unite

COX-2 inhibition with microtubule-disrupting activity while avoiding the *cis-trans* isomerization liability of CA-4, and later coxib–combretastatin hybrids likewise confirmed the potential of combining antiproliferative and anti-inflammatory properties within one molecule.

Guided by these considerations, the present design was developed to integrate tubulin polymerization inhibition and COX-2 targeting within a single hybrid molecule (Fig. 3). From the tubulin perspective, it was guided by the classical ring A-

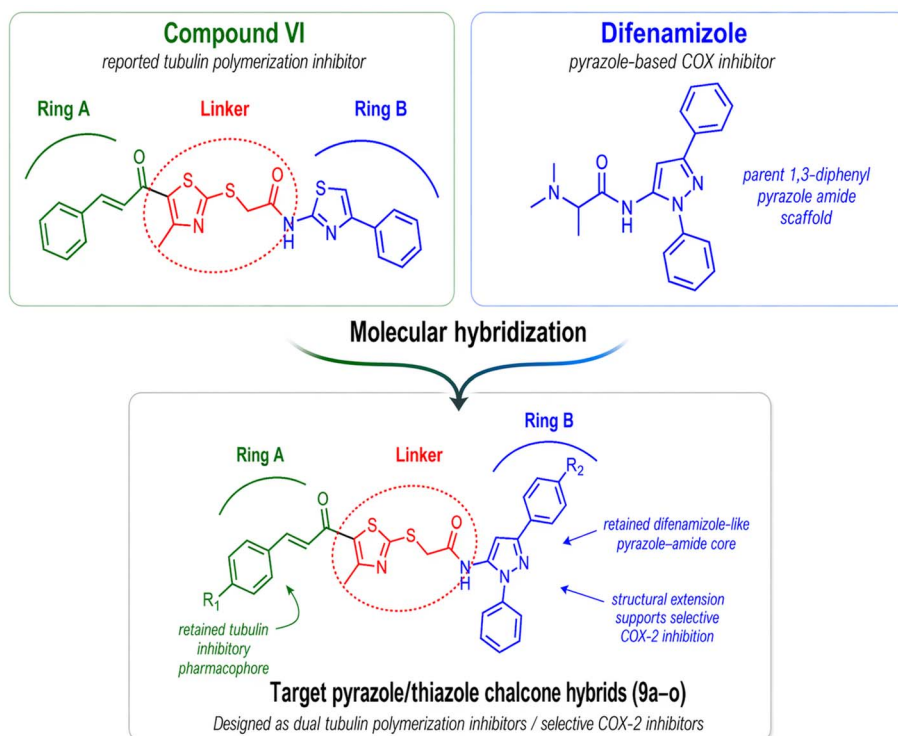


Fig. 3 Rational design of the target hybrids 9a–o.



linker-ring B organization of CA-4-inspired colchicine-site inhibitors. In the current series, ring A consisted of a thiazole–chalcone fragment, selected as an extended hydrophobic pharmacophore capable of occupying the lipophilic region of the colchicine-binding pocket while also acting as a conformationally restricted replacement for the labile *cis*-stilbene system of CA-4. The linker incorporated a thioacetamide spacer, whereas ring B was introduced as a 1,3-diphenylpyrazole unit, chosen as a heteroaromatic moiety expected to establish favorable hydrophobic interactions within adjacent regions of the colchicine-binding site while also providing a COX-2-relevant structural element within the same framework.

More specifically, ring B was selected as a 1,3-diphenylpyrazole unit because the pyrazole nucleus represents the key heterocyclic motif of difenamizole, whereas the diphenyl substitution was expected to provide the steric expansion required for preferential accommodation within the larger COX-2 secondary pocket relative to COX-1. Applying this principle, the current design retained the 1,3-diphenylpyrazole–amide core while replacing the smaller dimethylamino substituent in difenamizole with a more extended thiazole–chalcone moiety. This modification was expected not only to favor binding to COX-2 but also to introduce a tubulin-directed pharmacophore. In addition, variation at the chalcone aryl ring and the 3-phenyl substituent of the pyrazole nucleus was

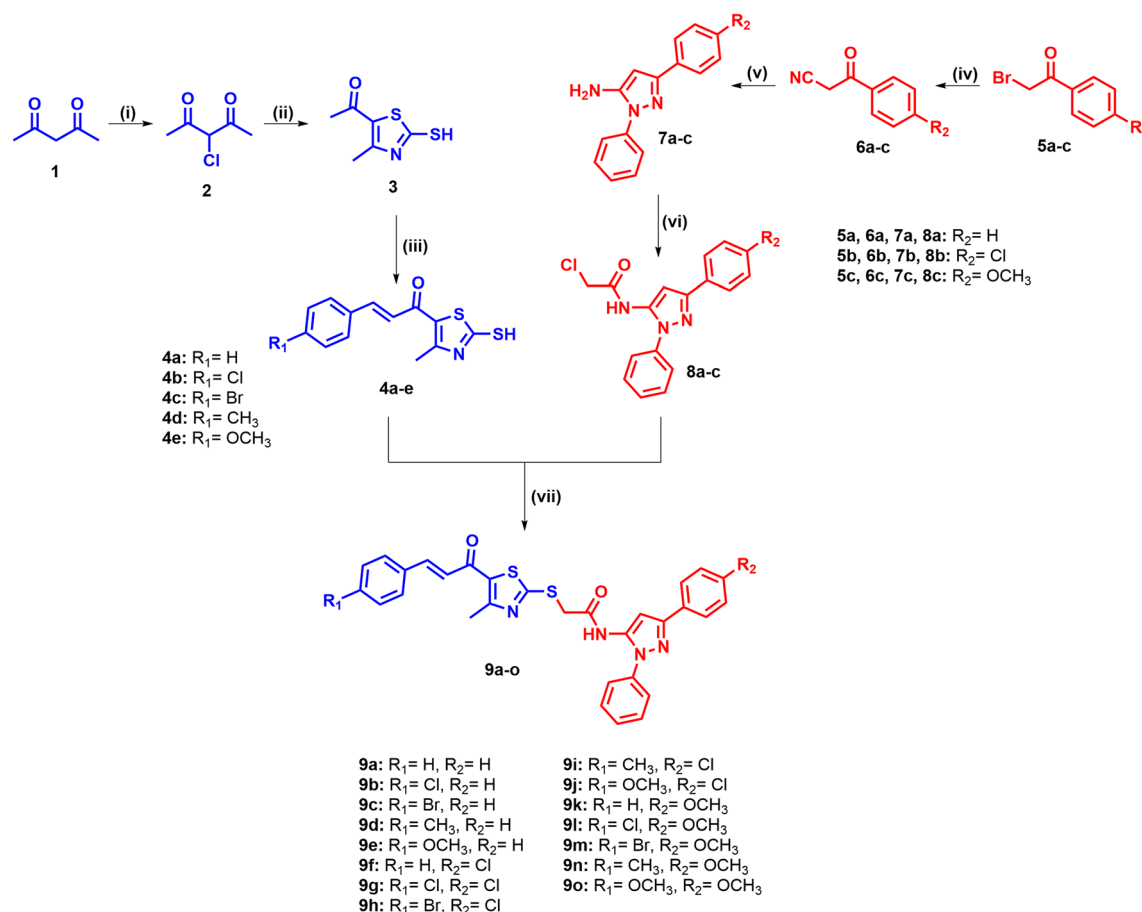
intended to fine-tune hydrophobic and physicochemical features across both targets, thereby supporting the development of dual-acting anticancer candidates.

2. Results and discussion

2.1 Chemistry

The target compounds **9a–o** were synthesized as outlined in Scheme 1. Pentane-2,4-dione (**1**) underwent regioselective chlorination at the activated methylene position upon treatment with suluryl chloride in toluene at 0 °C for 12 h, affording 3-chloropentane-2,4-dione (**2**). Subsequent reaction of **2** with ammonia and carbon disulfide in ethanol at room temperature for 6 h generated the dithiocarbamate *in situ*, which cyclized to afford the 2-mercaptothiazole intermediate (**3**). Claisen–Schmidt condensation of **3** with the appropriate *para*-substituted benzaldehydes in ethanolic 60% NaOH at 0 °C for 18 h, then yielded the chalcone derivatives **4a–e** bearing the free mercapto group at the thiazole C2 position.

In parallel, the pyrazole fragment was prepared from the corresponding phenacyl bromides **5a–c**. Nucleophilic substitution of **5a–c** with potassium cyanide in ethanol at 50 °C for 2 h afforded the β -ketonitriles **6a–c**, which then underwent cyclocondensation with phenylhydrazine hydrochloride under reflux for 8 h to yield the amino pyrazoles **7a–c**. *N*-acylation of the



Scheme 1 Synthesis of the thiazole chalcone/pyrazole hybrids **9a–o**.



amino group in **7a–c** was achieved with chloroacetyl chloride in dichloromethane at 0 °C for 12 h, giving the key chloroacetamido pyrazoles **8a–c**. The final step involved *S*-alkylation of the mercaptochalcones **4a–e** with the α -chloroacetamides **8a–c** in acetonitrile containing triethylamine at room temperature for 6 h, affording the target compounds **9a–o**.

2.2 Reagents and conditions

(i) SO₂Cl₂, toluene, 0 °C, 12 h; (ii) NH₃, CS₂, EtOH, RT, 6 h; (iii) appropriate benzaldehyde derivative, 60% NaOH, EtOH, 0 °C, 18 h; (iv) KCN/ethanol, 50 °C, 2 h; (v) phenylhydrazine/HCl, reflux, 8 h; (vi) chloroacetyl chloride, DCM, 0 °C, 12 h; (vii) acetonitrile, TEA, RT, 6 h.

The synthesized compounds **9a–o** were characterized by ¹H NMR, ¹³C NMR, and elemental analysis. The ¹H NMR spectra, recorded in DMSO-*d*₆, exhibited characteristic signals consistent with the proposed structures. A downfield broad singlet for the amide NH proton was observed at δ 10.45–10.56 ppm. The pyrazole C4-H resonated as a singlet between δ 6.82 and 6.94 ppm. The aromatic protons of the three phenyl rings appeared in the region δ 6.95–7.86 ppm. The *trans* chalcone olefinic protons were observed as two doublets at δ 7.62–7.68 ppm and δ 7.17–7.35 ppm, confirming the (*E*)-geometry of the enone system. The thioacetamide methylene protons appeared as a sharp singlet at δ 4.25–4.26 ppm, while the thiazole methyl group gave a singlet at δ 2.62–2.63 ppm. In derivatives containing a *p*-methyl substituent, an additional singlet was observed at δ 2.32 ppm. Methoxy groups on the aromatic rings (when present) appeared as singlets in the range of δ 3.75–3.79 ppm.

The ¹³C NMR spectra further supported the assigned structures, revealing two distinct carbonyl resonances: the chalcone ketone carbonyl at δ 181.71–181.97 ppm and the amide carbonyl at δ 168.21–168.98 ppm. The pyrazole C4 carbon appeared characteristically upfield at δ 99.31–99.99 ppm. The remaining aromatic carbons of the three phenyl rings and thiazole, along with the two olefinic chalcone carbons, resonated in the region δ 114.63–166.45 ppm. The thioacetamide methylene carbon was observed at δ 37.60–37.69 ppm, while the thiazole methyl carbon appeared at δ 18.72–18.84 ppm. When present, the *p*-methyl carbon resonated at δ 21.64–21.65 ppm and the methoxy carbons at δ 55.61–55.96 ppm. These spectral data collectively confirmed the successful formation of the target thiazole–pyrazole hybrids.

2.3 Biological evaluation

2.3.1. Antiproliferative activity. The antiproliferative activity of the synthesized hybrids **9a–o** was evaluated against three human cancer cell lines: MDA-MB-231 (triple-negative breast), HCA-7 (colon), and A549 (lung) carcinoma cell lines, with doxorubicin and combretastatin A-4 included as reference compounds (Table 1).

The parent compound **9a**, featuring unsubstituted phenyl rings at both positions (R₁ = H, R₂ = H), displayed the lowest activity within the series, exhibiting IC₅₀ values of 11.92 ± 0.62, 15.84 ± 0.82, and 12.34 ± 0.64 μM against MDA-MB-231, HCA-7,

Table 1 Antiproliferative activity of compounds **9a–o** against MDA-MB-231, HCA-7, and A549 cancer cell lines after 48 h treatment, expressed as IC₅₀ values (μM) ± SEM, with doxorubicin and combretastatin A-4 as reference compounds

Compound	Antiproliferative activity IC ₅₀ ± SEM (μM)		
	MDA-MB-231	HCA-7	A549
9a	11.92 ± 0.62	15.84 ± 0.82	12.34 ± 0.64
9b	6.38 ± 0.33	8.76 ± 0.45	6.92 ± 0.36
9c	5.21 ± 0.27	7.42 ± 0.38	5.36 ± 0.28
9d	8.42 ± 0.43	10.48 ± 0.53	7.24 ± 0.37
9e	4.86 ± 0.25	3.28 ± 0.17	6.94 ± 0.35
9f	8.08 ± 0.41	6.54 ± 0.33	10.62 ± 0.54
9g	3.88 ± 0.20	4.92 ± 0.25	5.64 ± 0.29
9h	3.41 ± 0.17	4.36 ± 0.22	2.84 ± 0.14
9i	6.91 ± 0.35	5.86 ± 0.30	8.42 ± 0.43
9j	4.11 ± 0.21	3.14 ± 0.16	5.92 ± 0.30
9k	6.84 ± 0.35	5.12 ± 0.26	8.76 ± 0.44
9l	2.92 ± 0.15	2.18 ± 0.11	4.86 ± 0.25
9m	1.96 ± 0.10	2.57 ± 0.13	4.18 ± 0.21
9n	5.42 ± 0.27	4.26 ± 0.21	6.48 ± 0.33
9o	4.26 ± 0.21	3.02 ± 0.15	5.48 ± 0.28
Doxorubicin	1.62 ± 0.18	3.54 ± 0.36	4.96 ± 0.22
Combretastatin A-4	2.08 ± 0.27	1.91 ± 0.22	2.64 ± 0.24

and A549 cell lines, respectively. All substituted analogs showed improved activity relative to **9a**, confirming that modifications at both aromatic positions enhance the anticancer activity. Among monosubstituted compounds (R₂ = H), the potency order against MDA-MB-231 cells followed Br (**9c**, 5.21 ± 0.27 μM) > OCH₃ (**9e**, 4.86 ± 0.25 μM) > Cl (**9b**, 6.38 ± 0.33 μM) > CH₃ (**9d**, 8.42 ± 0.43 μM) > H (**9a**, 11.92 ± 0.62 μM). Statistical analysis of mean IC₅₀ values by R₁ substituent confirmed that bromo substitution yielded the lowest average IC₅₀ (3.53 ± 0.18 μM against MDA-MB-231), followed by chloro (4.39 ± 0.23 μM) and methoxy (4.41 ± 0.24 μM), whereas unsubstituted (8.95 ± 0.48 μM) and methyl (6.92 ± 0.36 μM) derivatives exhibited reduced potency. Notably, compound **9e** (R₁ = OCH₃, R₂ = H) demonstrated activity against HCA-7 cells (3.28 ± 0.17 μM) that surpassed doxorubicin (3.54 ± 0.36 μM) against this cell line.

Methoxy substitution at R₂ yielded the most potent compounds across all three cell lines, with disubstituted analogs showing superior activity to those bearing R₂ = Cl or single substitutions. Notably, compounds with R₂ = OCH₃ exhibited the lowest mean IC₅₀ against MDA-MB-231 (4.28 ± 0.23 μM), compared to R₂ = Cl (5.28 ± 0.28 μM) and monosubstituted analogs (7.36 ± 0.40 μM). Compound **9m** (R₁ = Br, R₂ = OCH₃) emerged as the most potent compound overall in the series, displaying IC₅₀ values of 1.96 ± 0.10, 2.57 ± 0.13, and 4.18 ± 0.21 μM against MDA-MB-231, HCA-7, and A549 cells, respectively. The activity of **9m** against MDA-MB-231 cells represented an 83.6% improvement over the parent compound **9a** and showed potency comparable to doxorubicin (1.62 ± 0.18 μM) and combretastatin A-4 (2.08 ± 0.27 μM). Compound **9l** (R₁ = Cl, R₂ = OCH₃) ranked as the second most potent analog overall, demonstrating IC₅₀ values of 2.92 ± 0.15, 2.18 ± 0.11, and 4.86 ± 0.25 μM against MDA-MB-231, HCA-7, and A549,



respectively, with activity against HCA-7 cells exceeding that of doxorubicin ($3.54 \pm 0.36 \mu\text{M}$).

Compound **9h** ($R_1 = \text{Br}$, $R_2 = \text{Cl}$) showed exceptional activity against A549 lung carcinoma cells ($2.84 \pm 0.14 \mu\text{M}$) that exceeded its activity against the other two cell lines, indicating cell line-specific responses within the disubstituted series containing $R_2 = \text{Cl}$. MDA-MB-231 triple-negative breast cancer cells generally demonstrated the highest sensitivity to the compound series, exhibiting the lowest mean IC_{50} values across most substitution patterns. HCA-7 colon cancer cells showed intermediate sensitivity, while A549 lung carcinoma cells displayed the most variable responses, with certain compounds (particularly **9h**) showing exceptional activity while others demonstrated reduced potency.

2.3.2. Cytotoxicity against normal MCF-10A breast epithelial cells. The cytotoxicity of the target compounds (**9a–o**) against normal MCF-10A breast epithelial cells was evaluated to assess selectivity toward cancer cells. Selectivity indices (SI) were calculated as the ratio of IC_{50} values for MCF-10A cells to those for each cancer cell line, with doxorubicin and combretastatin A-4 included as reference compounds (Table 2).

The target compounds exhibited substantially lower cytotoxicity against MCF-10A normal cells than against cancer cell lines, with IC_{50} values ranging from $12.76 \pm 0.46 \mu\text{M}$ (**9m**) to $24.24 \pm 1.06 \mu\text{M}$ (**9a**). Doxorubicin showed an IC_{50} of $2.80 \pm 0.25 \mu\text{M}$ against MCF-10A cells, indicating considerable toxicity toward normal cells. The selectivity indices for compounds **9a–9o** against MDA-MB-231 cells ranged from 2.03 (**9a**) to 6.51 (**9m**), with most analogs exceeding an SI value of 3.0. Doxorubicin demonstrated an SI of 1.73 against MDA-MB-231, while combretastatin A-4 showed an SI of 2.31. Against HCA-7 cells, doxorubicin exhibited an SI below 1 (0.79), indicating greater toxicity toward normal cells than this cancer cell line. All target

compounds maintained SI values above 1.53, with compounds **9l** (6.84) and **9e** (5.77) showing favorable selectivity.

Compounds **9m** and **9l**, which demonstrated the highest antiproliferative potency against cancer cells, also exhibited the most favorable selectivity profiles. Compound **9m** showed SI values of 6.51, 4.96, and 3.05 against MDA-MB-231, HCA-7, and A549 cells, respectively, while compound **9l** displayed SI values of 5.11, 6.84, and 3.07 against the same cell lines. These values exceed those of doxorubicin across all three cancer cell lines and surpass those of combretastatin A-4 in MDA-MB-231 and A549 cells. Compound **9h** ($R_1 = \text{Br}$, $R_2 = \text{Cl}$) also demonstrated strong selectivity, with an SI of 4.85 against A549 cells, consistent with its activity against this cell line observed in the antiproliferative assay.

The selectivity indices against A549 lung carcinoma cells were generally lower than those observed for MDA-MB-231 and HCA-7 cells across the compound series, with values ranging from 1.96 (**9a**) to 4.85 (**9h**) compared to combretastatin A-4 (1.82) and doxorubicin (0.56). Compounds **9m** and **9l** achieved SI values above 3.0 against A549 cells, indicating acceptable selectivity margins for this cell line.

2.3.3. In vitro tubulin polymerization inhibitory assay. The effects of the synthesized hybrids **9a–o** on tubulin polymerization, using combretastatin A-4 (CA-4) as the reference inhibitor, are illustrated in Fig. 4. Compounds **9a–o** exhibited clear inhibitory activity against tubulin polymerization, with IC_{50} values ranging from 3.97 to 12.18 μM , compared to CA-4 ($\text{IC}_{50} = 3.84 \pm 0.28 \mu\text{M}$). Among the tested derivatives, compounds **9m**, **9l**, **9h**, and **9g** were the most active, with IC_{50} values of 3.97 ± 0.24 , 4.21 ± 0.25 , 4.43 ± 0.27 , and $4.96 \pm 0.31 \mu\text{M}$, respectively. In particular, **9m** exhibited the highest tubulin polymerization-inhibitory activity in the series and was only 1.03-fold less potent than CA-4, whereas **9l** and **9h** were 1.10-fold and 1.15-fold less potent than the reference compound, respectively.

Table 2 Cytotoxicity of compounds **9a–o** against normal MCF-10A breast epithelial cells after 48 h treatment, expressed as IC_{50} values (μM) \pm SEM, together with their selectivity indices relative to MDA-MB-231, HCA-7, and A549 cancer cell lines; doxorubicin and combretastatin A-4 were included as reference compounds

Compound	MCF-10A $\text{IC}_{50} \pm \text{SEM}$ (μM)	Selectivity index (SI)		
		MDA-MB-231	HCA-7	A549
9a	24.24 ± 1.06	2.03	1.53	1.96
9b	19.31 ± 0.80	3.03	2.20	2.79
9c	16.94 ± 0.67	3.25	2.28	3.16
9d	20.34 ± 0.85	2.42	1.94	2.81
9e	18.92 ± 0.78	3.89	5.77	2.73
9f	21.86 ± 0.93	2.71	3.34	2.06
9g	15.43 ± 0.60	3.98	3.14	2.74
9h	13.78 ± 0.51	4.04	3.16	4.85
9i	17.94 ± 0.73	2.60	3.06	2.13
9j	16.74 ± 0.66	4.07	5.33	2.83
9k	20.88 ± 0.88	3.05	4.08	2.38
9l	14.92 ± 0.57	5.11	6.84	3.07
9m	12.76 ± 0.46	6.51	4.96	3.05
9n	18.08 ± 0.73	3.34	4.24	2.79
9o	16.02 ± 0.63	3.76	5.30	2.92
Doxorubicin	2.80 ± 0.25	1.73	0.79	0.56
Combretastatin A-4	4.80 ± 0.40	2.31	2.51	1.82



Tubulin Polymerization Inhibition

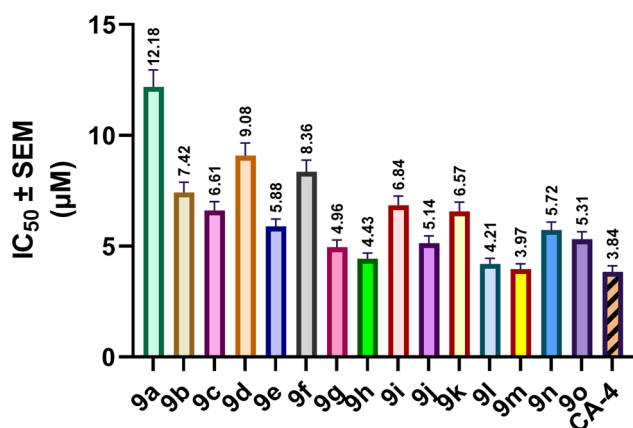


Fig. 4 Tubulin polymerization inhibitory activity of compounds **9a–o** and the reference inhibitor CA-4, expressed as IC₅₀ values (μM), as determined from a fluorescence-based polymerization assay monitored kinetically for 60 min at 37 °C. Data are presented as mean ± SEM.

The anti-tubulin activity of compounds **9a–o** was markedly affected by the substitution pattern at both R₁ and R₂. When R₂ was kept unsubstituted, modification of the chalcone phenyl ring (R₁) produced a substantial enhancement in activity relative to the parent derivative **9a** (IC₅₀ = 12.18 ± 0.76 μM). Within this subset, compound **9e** (R₁ = OCH₃, R₂ = H) was the most active analogue, with an IC₅₀ value of 5.88 ± 0.34 μM, followed by **9c** (R₁ = Br, R₂ = H; 6.61 ± 0.39 μM), **9b** (R₁ = Cl, R₂ = H; 7.42 ± 0.46 μM), and **9d** (R₁ = CH₃, R₂ = H; 9.08 ± 0.57 μM). Thus, when the pyrazole-linked phenyl ring remained unsubstituted, activity increased in the order H < CH₃ < Cl < Br < OCH₃.

A different but related trend was observed upon substitution at R₂ with chlorine. Compounds **9f–j**, bearing R₂ = Cl, showed improved activity relative to their corresponding R₂ = H analogs. For example, the introduction of a chlorine substituent reduced the IC₅₀ value from 12.18 to 8.36 μM for **9a/9f**, from 7.42 to 4.96 μM for **9b/9g**, from 6.61 to 4.43 μM for **9c/9h**, from 9.08 to 6.84 μM for **9d/9i**, and from 5.88 to 5.14 μM for **9e/9j**. Within this subgroup, activity followed the order H < CH₃ < OCH₃ < Cl < Br, with **9h** (R₁ = Br) and **9g** (R₁ = Cl) representing the most active derivatives.

The most favorable profile was obtained when R₂ carried a methoxy group. Compounds **9k–o**, bearing R₂ = OCH₃, constituted the most active subgroup overall, with IC₅₀ values ranging from 3.97 to 6.57 μM. Within this subset, compound **9m** (R₁ = Br) was the most potent derivative in the entire series, followed by **9l** (R₁ = Cl), **9o** (R₁ = OCH₃), **9n** (R₁ = CH₃), and **9k** (R₁ = H). Direct comparison with the corresponding R₂ = H analogs further demonstrated the favorable contribution of methoxy substitution at R₂. Thus, **9k** was 1.85-fold more potent than **9a**, **9l** was 1.76-fold more potent than **9b**, **9m** was 1.67-fold more potent than **9c**, **9n** was 1.59-fold more potent than **9d**, and **9o** was slightly more active than **9e**.

The tubulin polymerization results were also in good agreement with the antiproliferative data obtained against MDA-MB-231, HCA-7, and A549 cells. Compounds **9m** and **9l**, which displayed the highest tubulin polymerization inhibitory activity, were likewise the most potent antiproliferative derivatives in the cellular assay. In contrast, the weaker tubulin polymerization inhibitor **9a** displayed the lowest antiproliferative activity within the series. This parallel behavior supports the conclusion that inhibition of tubulin polymerization is a major contributor to the cytotoxic activity of these hybrids.

2.3.4. In vitro COX inhibition assay. The five most promising compounds, namely **9h**, **9j**, **9l**, **9m**, and **9o**, were selected for further evaluation of their inhibitory activity against COX-1 and COX-2, based on their superior antiproliferative activity and potent inhibition of tubulin polymerization. The obtained COX-1/COX-2 inhibitory profiles are illustrated in Fig. 5, while the corresponding selectivity indices are presented in Table 3. All the tested hybrids demonstrated preferential inhibition of COX-2 over COX-1, with COX-2 IC₅₀ values spanning a sub-micromolar range (0.10–0.86 μM) and selectivity indices ranging from 9.79 to 109.20. In contrast, indomethacin exhibited a COX-2 SI of only 0.19, reflecting its well-established preferential inhibition of the constitutive COX-1 isoform.

Among the tested derivatives, compound **9l** (R₁ = Cl, R₂ = OCH₃) exhibited the most remarkable COX-2 inhibitory profile, with an IC₅₀ value of 0.10 ± 0.01 μM that was comparable to that of the clinically approved selective COX-2 inhibitor celecoxib (IC₅₀ = 0.08 ± 0.01 μM), being only 1.25-fold less potent than the reference drug. Furthermore, **9l** displayed a COX-2 selectivity index of 109.20, which represents approximately 61% of celecoxib's SI (178.00) and is notably exceptional for a non-coxib scaffold devoid of the sulfonamide or methylsulfonyl pharmacophore that is classically considered essential for high COX-2 selectivity. This observation suggests that the chloro substituent on the chalcone phenyl ring, in conjunction with the methoxy group on the pyrazole-linked phenyl ring, provides

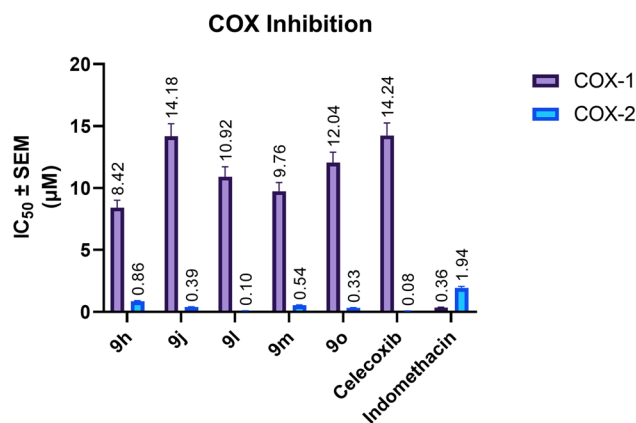


Fig. 5 COX-1 and COX-2 inhibitory activities of compounds **9h**, **9j**, **9l**, **9m**, and **9o**, compared with celecoxib and indomethacin, expressed as IC₅₀ values (μM), obtained after 10 min compound/enzyme pre-incubation at 37 °C, 2 min arachidonic-acid reaction, and 15 min post-SnCl₂ incubation. Data are presented as mean ± SEM.



Table 3 COX-2 selectivity indices of selected compounds (**9h**, **9j**, **9l**, **9m**, and **9o**) compared with reference drugs (celecoxib and indomethacin), calculated from IC₅₀ values obtained after 10 min compound/enzyme preincubation at 37 °C, 2 min arachidonic-acid reaction, and 15 min post-SnCl₂ incubation

Compound	COX-2 selectivity index
9h	9.79
9j	36.36
9l	109.20
9m	18.07
9o	36.48
Celecoxib	178.00
Indomethacin	0.19

a highly favorable substitution pattern for selective COX-2 inhibition. The relatively high COX-1 IC₅₀ of **9l** (10.92 ± 0.78 μM) further contributes to its pronounced selectivity profile.

The remaining derivatives also demonstrated appreciable COX-2 inhibitory activity, with IC₅₀ values of 0.33 ± 0.03, 0.39 ± 0.03, 0.54 ± 0.04, and 0.86 ± 0.06 μM for compounds **9o**, **9j**, **9m**, and **9h**, respectively. The overall COX-2 potency within the evaluated subset followed the order **9l** > **9o** > **9j** > **9m** > **9h**, while the corresponding selectivity indices decreased in the same order: 109.20, 36.48, 36.36, 18.07, and 9.79. All five compounds surpassed the COX-2 potency of indomethacin (IC₅₀ = 1.94 ± 0.14 μM) by margins ranging from 2.3-fold (**9h**) to 19.4-fold (**9l**), and all exhibited substantially superior selectivity profiles relative to the non-selective reference.

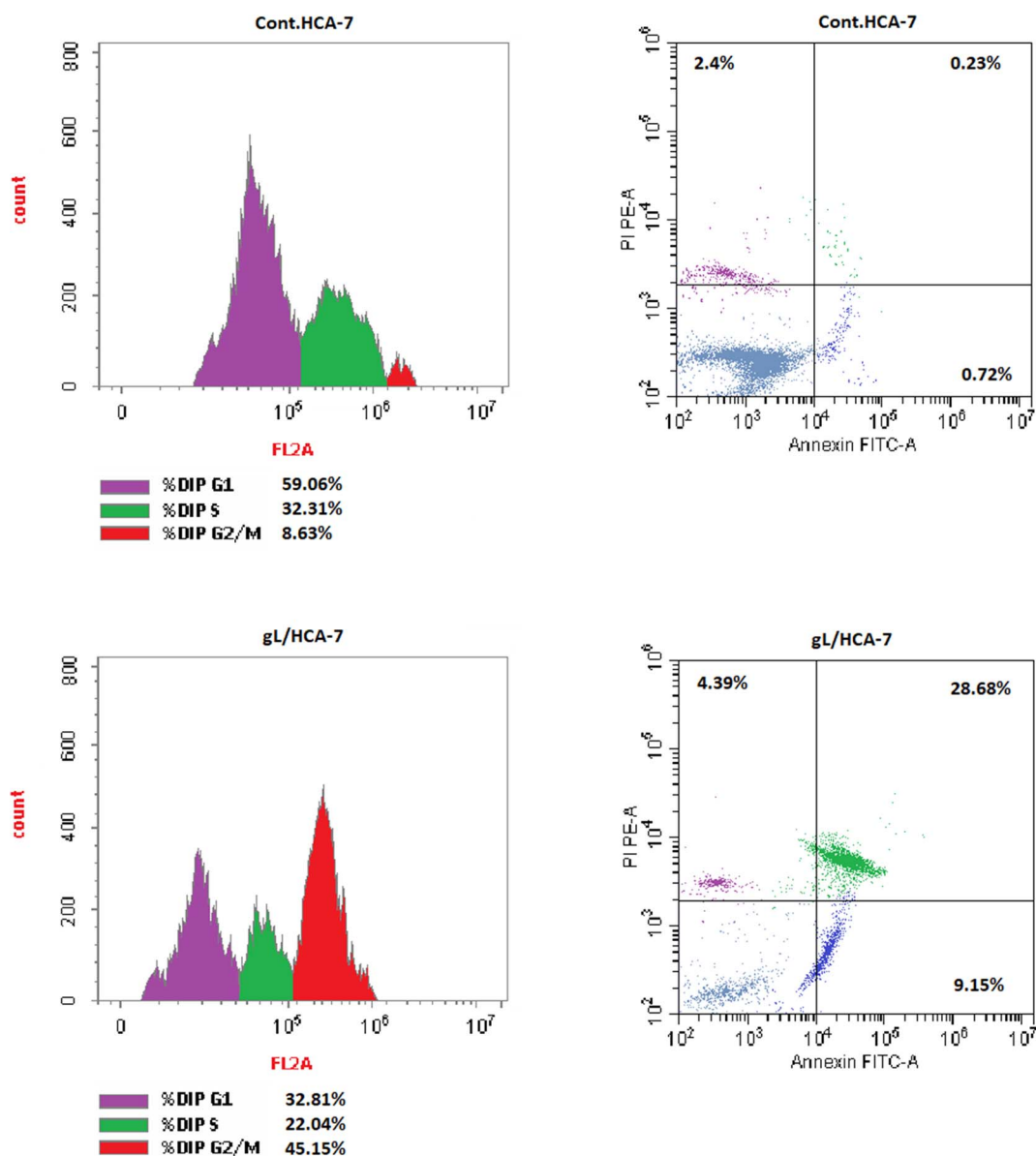


Fig. 6 Effect of compound **9l** on cell-cycle progression and apoptosis in HCA-7 cells after 48 h treatment at its IC₅₀ concentration. Left panels show PI-based cell-cycle distribution, while right panels represent Annexin V-FITC/PI analysis of apoptotic populations.



2.3.5. Cell cycle analysis and apoptosis. Flow-cytometric analysis of PI-stained HCA-7 cells showed that treatment with **9I** markedly altered cell-cycle distribution. The cell-cycle data are illustrated in Fig. 6 and summarized in Table 4. Compared with the untreated control, **9I** reduced the G0/G1 population from 59.06% to 32.81% and the S-phase fraction from 32.31% to 22.04%, while markedly increasing the G2/M population from 8.63% to 45.15%. This pronounced accumulation of cells at the G2/M phase indicates that **9I** induced cell-cycle arrest at this stage. Such a profile is consistent with the anti-mitotic behavior expected for tubulin-targeting agents. It also supports the tubulin polymerization data, indicating that disruption of mitotic progression is a major contributor to the anti-proliferative effect of **9I** in HCA-7 cells.

Annexin V-FITC/PI double staining further demonstrated that the growth-inhibitory effect of **9I** was primarily mediated through induction of apoptotic cell death. The apoptotic distributions of treated and untreated cells are shown in Fig. 6 and summarized in Table 5. Treatment with **9I** increased the early and late apoptotic populations to 9.15% and 28.68%, respectively, whereas the necrotic population remained limited at 4.39%. In contrast, the untreated control showed only 0.72% early apoptosis, 0.23% late apoptosis, and 2.40% necrosis. The marked elevation in the apoptotic fractions, together with the predominance of late apoptosis over early apoptosis, indicates efficient progression of HCA-7 cells toward irreversible apoptosis rather than nonspecific cytotoxic injury.

Table 4 Effect of compound **9I** on cell-cycle distribution in HCA-7 cells after 48 h treatment at its IC₅₀ concentration, expressed as percentages of cells in G0/G1, S, and G2/M phases

Compound no.	DNA content		
	% G0-G1	% S	% G2/M
Compound 9I	32.81	22.04	45.15
DMSO (control)	59.06	32.31	8.63

Table 5 Effect of compound **9I** on apoptosis in HCA-7 cells after 48 h treatment at its IC₅₀ concentration, expressed as percentages of total apoptotic, early apoptotic, late apoptotic, and necrotic cell populations

Compound no.	Apoptosis			Necrosis
	Total	Early	Late	
Compound 9I	42.22	9.15	28.68	4.39
DMSO (control)	3.35	0.72	0.23	2.4

Table 6 Effect of compound **9I** on Bax and Bcl-2 protein expression levels in HCA-7 cells after 48 h treatment at its IC₅₀ concentration, relative to the DMSO-treated control

Compound	Bax (pg μL^{-1}) \pm SEM	Fold change	Bcl-2 (pg μL^{-1}) \pm SEM	Fold change
Compound 9I	438.64 \pm 15.72	5.63	6.74 \pm 0.19	0.332
DMSO (control)	77.94 \pm 2.08	1.00	20.32 \pm 0.46	1.00

2.3.6. Effect of compound 9I on Bax and Bcl-2 protein expression. The effect of compound **9I** on the expression levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 was assessed in HCA-7 cells (Table 6). Bax and Bcl-2 are key regulators of apoptosis, with Bax promoting and Bcl-2 inhibiting cell death. Accordingly, the Bax/Bcl-2 ratio represents a critical determinant of cellular fate, particularly in cancer cells where apoptotic pathways are frequently dysregulated.

The results indicated a significant increase in Bax protein levels following treatment with compound **9I**, with a concentration of 438.64 \pm 15.72 pg mL⁻¹, representing a 5.63-fold increase compared to the DMSO control. This finding aligns with Bax's role as a pro-apoptotic factor that triggers the intrinsic apoptosis pathway by promoting mitochondrial cytochrome c release, ultimately leading to caspase activation and cell death. The upregulation of Bax suggests that compound **9I** may induce apoptosis *via* mitochondrial pathways, a desirable feature for anticancer agents aiming to selectively eliminate tumor cells.

Conversely, compound **9I** caused a marked reduction in Bcl-2 levels, with a concentration of 6.74 \pm 0.19 pg mL⁻¹, indicating a 0.332-fold change relative to the control. Bcl-2 is known to prevent apoptosis by inhibiting the release of cytochrome c from mitochondria and blocking caspase activation. The observed downregulation of Bcl-2 by compound **9I** could further enhance the pro-apoptotic environment in treated cells, as decreased Bcl-2 levels would diminish its antagonistic effect on Bax, thereby facilitating apoptosis.

2.3.7. Effect of compound 9I on caspase-3 and caspase-9 levels. The impact of compound **9I** on apoptotic signaling was assessed through caspase-3 and caspase-9 assays, providing further evidence of its potential as a dual tubulin/COX-2 inhibitor with pronounced pro-apoptotic activity (Table 7). Caspase-9 acts as an initiator caspase, while caspase-3 functions as an executioner within the apoptotic cascade, and the marked elevation of both enzymes reflects activation of the intrinsic apoptotic pathway.

In HCA-7 cells, treatment with compound **9I** resulted in a substantial increase in caspase-3 levels to 496.80 \pm 14.90 pg mL⁻¹, corresponding to an 8.54-fold rise relative to the DMSO control. This elevation confirms progression toward the execution phase of apoptosis and highlights the compound's ability to promote programmed cell death in cancer cells.

Consistently, caspase-9 levels increased to 47.86 \pm 1.18 ng mL⁻¹ (9.08-fold vs. control), indicating activation of the mitochondrial apoptotic pathway. This upregulation is associated with mitochondrial perturbation, leading to cytochrome c release, apoptosome formation, and subsequent activation of



Table 7 Effect of compound **9l** on caspase-3 and caspase-9 levels in HCA-7 cells after 48 h treatment at its IC₅₀ concentration, relative to the DMSO-treated control

Compound	Caspase-3 (pg mL ⁻¹) ± SEM	Fold change	Caspase-9 (ng mL ⁻¹) ± SEM	Fold change
Compound 9l	496.80 ± 14.90	8.54	47.86 ± 1.18	9.08
DMSO (control)	58.16 ± 1.62	1.00	5.27 ± 0.23	1.00

Table 8 Effect of compound **9l** on extracellular PGE-2 production in HCA-7 cells after 24 h treatment with compound **9l** (1.0 μM), relative to the untreated control

Compound	PGE-2 level (ng mL ⁻¹)	Fold change	Relative PGE-2 production	Inhibition of PGE-2 release (%)
Untreated control	5.16 ± 0.18	1.00	100.0	0.0
Compound 9l	0.56 ± 0.04	0.11	10.8	89.2

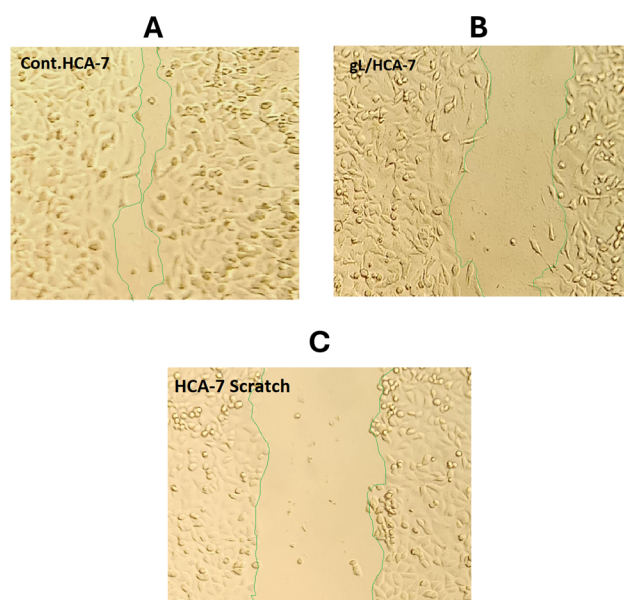
the caspase cascade. Collectively, these findings confirm that compound **9l** effectively induces apoptosis *via* the intrinsic mitochondrial pathway.

2.3.8. Effect of compound 9l on PGE-2 production. The effect of compound **9l** on prostaglandin E2 (PGE-2) production was evaluated by quantifying its extracellular levels in HCA-7 cell culture supernatants using a competitive ELISA assay. PGE-2 is a key downstream mediator of cyclooxygenase-2 activity and plays a central role in tumor progression through its involvement in proliferation, angiogenesis, and resistance to apoptosis. The corresponding results are presented in Table 8.

Treatment with compound **9l** at 1.0 μM resulted in a pronounced suppression of PGE-2 release relative to the untreated control. In HCA-7 cells, **9l** reduced extracellular PGE-2 levels to 0.56 ± 0.04 ng mL⁻¹, corresponding to a 0.11-fold change and an 89.2% decrease compared to the control (5.16 ± 0.18 ng mL⁻¹). This substantial reduction demonstrates the ability of **9l** to effectively attenuate prostaglandin biosynthesis in intact cancer cells. This pronounced suppression is consistent with the previously established selective COX-2 inhibitory profile of **9l** and indicates effective inhibition of COX-2-mediated signaling in a cellular context. Moreover, the marked reduction in PGE-2 complements the observed anti-proliferative and pro-apoptotic effects, further supporting the multitarget anticancer potential of compound **9l**.

2.3.9. Effect of compound 9l on cell migration. The migratory response of HCA-7 cells to **9l** was further examined using the CytoSelect™ wound-healing assay, in which removal of the insert generates a defined 0.9 mm wound field that enables direct monitoring of cell migration into the gap over time. This assay is particularly relevant because reduced wound closure reflects impaired collective cell migration, a process closely linked to tumor invasion and metastatic dissemination.

As shown in Fig. 7, the results further demonstrated that **9l** significantly impaired the migratory capacity of HCA-7 cells. Compared with the untreated control, which showed almost complete wound closure after 72 h (97.04 ± 3.15%), treatment with **9l** reduced wound closure to 58.52 ± 1.88% (Fig. 8). This reduction was also reflected in the migrated cell area, which

**Fig. 7** Wound-healing images of HCA-7 cells after 72 h: (A) control, (B) **9l**-treated, and (C) initial wound field (0 h). Green lines indicate wound edges.

decreased from 0.786 in the control to 0.474 in the treated group, together with a clear reduction in migration length ($\Delta L = 0.4367$ mm for control *vs.* 0.2633 mm for **9l**-treated cells). These findings indicate that **9l** markedly suppresses HCA-7 cell migration, further extending its anticancer profile beyond growth inhibition and apoptosis induction.

2.3.10. Metabolic stability of compound 9l in human liver microsomes. To assess its metabolic liability, the microsomal stability of compound **9l** was evaluated using pooled human liver microsomes under NADPH-supported oxidative conditions at substrate concentrations of 0.8, 4.0, and 20.0 μM. The obtained data are summarized in Table 9.

The results revealed a clear concentration-dependent improvement in apparent stability across the tested range. Increasing the starting concentration from 0.8 to 4.0 and 20.0



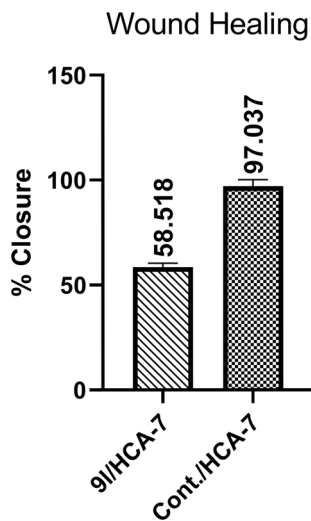


Fig. 8 Wound closure (%) of HCA-7 cells after 72 h treatment with **9l** versus control.

Table 9 Microsomal stability parameters of compound **9l** in pooled human liver microsomes at different starting concentrations, expressed as $t_{1/2}$ (h), intrinsic clearance (CL_{int}; $\mu\text{L min}^{-1} \text{mg}^{-1}$), and percentage of parent compound remaining at 60 min

Start conc. (μM)	$t_{1/2}$ (h)	CL _{int} ($\mu\text{L min}^{-1} \text{mg}^{-1}$)	% Remaining at 60 min
0.8	0.542 ± 0.032	42.69 ± 2.55	28.0 ± 0.7
4.0	0.873 ± 0.013	26.46 ± 0.40	50.3 ± 1.2
20.0	1.321 ± 0.013	17.49 ± 0.18	61.0 ± 0.2

μM was associated with progressive prolongation of the half-life from 0.542 ± 0.032 h (32.5 ± 1.9 min) to 0.873 ± 0.013 h (52.4 ± 0.8 min) and 1.321 ± 0.013 h (79.3 ± 0.8 min), respectively. In parallel, the apparent intrinsic clearance decreased from 42.69 ± 2.55 to 26.46 ± 0.40 and 17.49 ± 0.18 $\mu\text{L min}^{-1} \text{mg}^{-1}$, while the percentage of parent compound remaining at 60 min increased from $28.0 \pm 0.7\%$ to $50.3 \pm 1.2\%$ and $61.0 \pm 0.2\%$. This moderate-to-good profile, which improves at higher substrate levels consistent with possible enzyme saturation, falls within acceptable drug-like ranges and supports its suitability as a promising anticancer lead with acceptable metabolic stability under the tested *in vitro* conditions.

2.4 Computational studies

2.4.1. Molecular docking

2.4.1.1. Docking into the colchicine-binding site of tubulin. To clarify the molecular basis of the potent anti-tubulin activity of compound **9l**, docking studies were performed within the colchicine-binding site of tubulin (PDB ID: 4O2B).⁴⁴ The docking protocol was first validated by redocking the co-crystallized ligand colchicine into its native binding pocket. Superimposition of the redocked and crystallographic poses gave an RMSD value of 0.7508 \AA , confirming the reliability of the adopted docking procedure. The predicted binding affinity of colchicine was $-8.4 \text{ kcal mol}^{-1}$. The validation result is shown in Fig. 9.

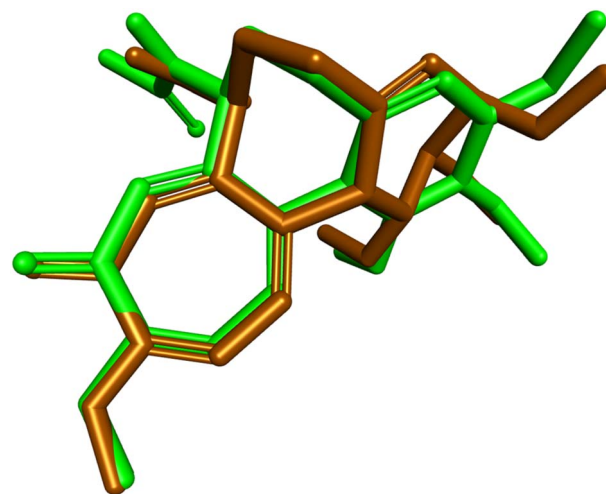


Fig. 9 Superimposition of the co-crystallized (green) and redocked (brown) colchicine poses within the colchicine-binding site of tubulin (PDB ID: 4O2B).

Docking of compound **9l** into the same binding cavity afforded a predicted binding affinity of $-9.6 \text{ kcal mol}^{-1}$, indicating a theoretically more favorable interaction than colchicine. Examination of the best-ranked pose revealed that **9l** was well accommodated within the colchicine pocket and formed multiple stabilizing interactions, predominantly hydrophobic in nature. The pyrazole ring established π -sigma interaction with Leu255, π -alkyl interaction with Leu242, and π -lone pair interaction with Val238. The two pyrazole-linked phenyl rings further reinforced binding through several hydrophobic contacts with Ala250, Cys241, Leu242, Leu255, Val238, Thr239, and Leu252, indicating efficient occupation of the hydrophobic region of the binding site.

Additional stabilization was provided by the methoxy substituent on the pyrazole-linked phenyl ring, which formed hydrophobic contacts with Leu242, Leu252, and Ile4, together with carbon-hydrogen bond interactions with Tyr52 and Gln136. Notably, the amidic NH formed a classical hydrogen bond with Val238, which may play an important role in anchoring the ligand within the pocket. The thiazole ring also contributed to binding through hydrophobic interactions with Ala316, Ala354, and Lys352, while the chalcone phenyl ring interacted with Lys352, Met259, and Val181. Moreover, the chloro substituent on the chalcone phenyl ring formed additional lipophilic contacts with Phe404, Ile347, and Val181. These results suggest that **9l** binds favorably within the colchicine site, consistent with its potent tubulin polymerization inhibitory and antiproliferative activities. The detailed binding interactions are shown in Fig. 10.

2.4.1.2. Docking into COX-2 enzyme. To investigate the second intended pharmacological target, compound **9l** was docked into the active site of COX-2 (PDB ID: 1CX2).³¹ The docking protocol was validated by redocking the co-crystallized ligand SC-558 into its native binding site. Superimposition of the redocked and crystallographic poses gave an RMSD value of 0.8631 \AA , confirming the validity of the protocol for this target.



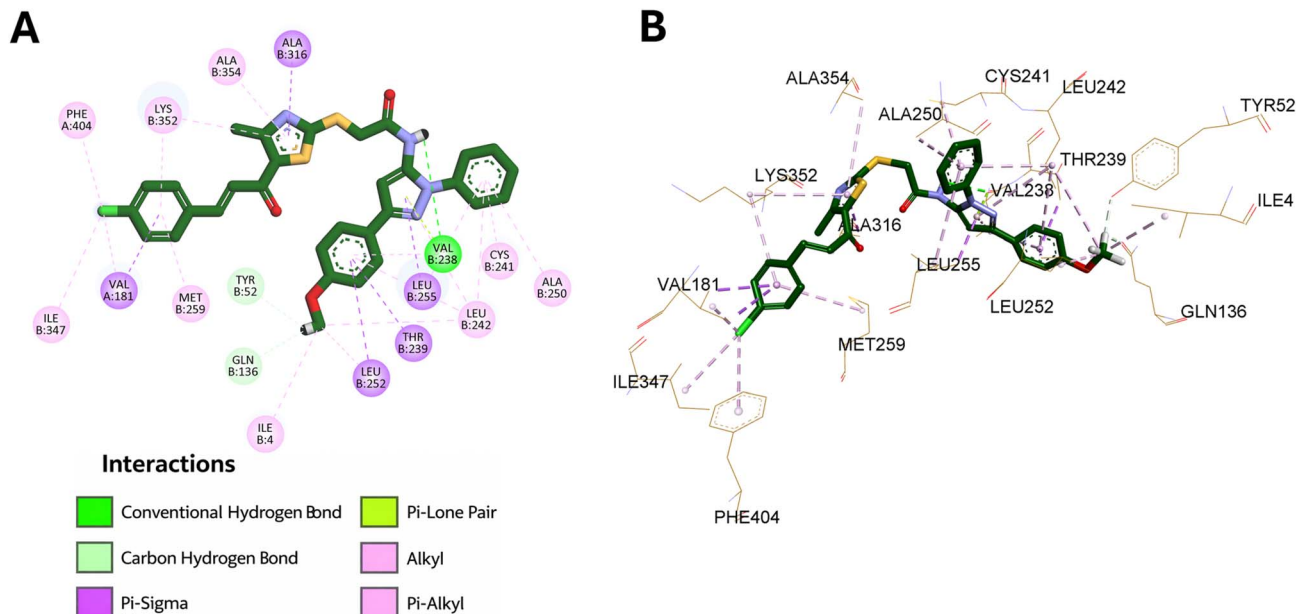


Fig. 10 Binding interactions of compound **9I** within the colchicine-binding site of tubulin (PDB ID: 4O2B): (A) 2D interaction diagram and (B) 3D binding mode.

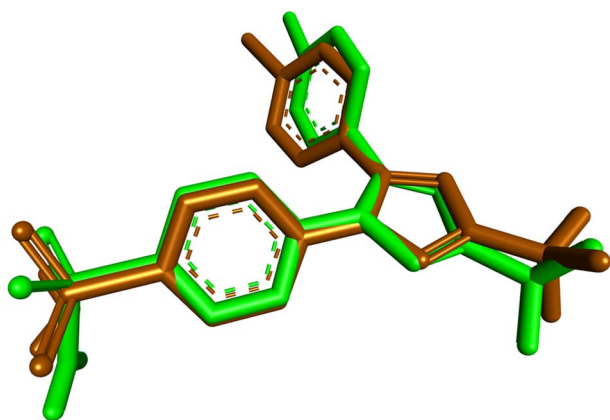


Fig. 11 Superimposition of the co-crystallized (green) and redocked (brown) SC-558 poses within the active site of COX-2 (PDB ID: 1CX2).

The predicted binding affinity of SC-558 was $-10.3 \text{ kcal mol}^{-1}$. The superimposition result is presented in Fig. 11.

Compound **9I** exhibited a predicted binding affinity of $-11.0 \text{ kcal mol}^{-1}$, suggesting a theoretically favorable interaction within the COX-2 active site. Analysis of the docked pose revealed that the pyrazole ring formed π -sigma interaction with Val349 and π -alkyl interaction with Ala527, while the two pyrazole-linked phenyl rings established multiple hydrophobic contacts with Val523, Ala527, Val349, Val116, Leu359, and Leu531. Additional stabilization arose from an amide- π stacked interaction with Gly526 and a π -donor non-classical hydrogen bond with Arg120.

The methoxy substituent on the pyrazole-linked phenyl ring further enhanced binding through hydrophobic interactions with Leu384, Tyr385, Phe381, and Trp387. In addition, the

amidic carbonyl formed a classical hydrogen bond with Tyr355, which likely contributes to stabilization of the docked complex. The sulfur atom of the linker showed a sulfur-X interaction with Ser353, whereas the thiazole ring and its methyl substituent participated in several hydrophobic interactions with Ala516, Val523, Ile517, and Phe518. Moreover, the thiazole sulfur formed a π -sulfur interaction with His90. At the distal end, the chalcone phenyl ring interacted with Pro514, while its chloro substituent formed additional hydrophobic contacts with His95 and Pro514. These findings indicate that **9I** is well accommodated within the COX-2 active site, in agreement with its strong and selective COX-2 inhibitory activity. The interaction pattern of **9I** in the COX-2 active site is presented in Fig. 12.

2.4.1.3. In Silico ADMET prediction. The pharmacokinetic and safety-related properties of compound **9I** were preliminarily evaluated using the ADMETlab 3.0 platform.⁴⁵ The predicted profile revealed several favorable features supporting its potential as a bioactive lead. Compound **9I** showed a molecular weight of 599.11, TPSA of 74.08 \AA^2 , 6 hydrogen-bond acceptors, no hydrogen-bond donors, and 11 rotatable bonds, indicating acceptable polarity and flexibility. Although its lipophilicity was relatively high ($\log P = 6.03$) and aqueous solubility was low ($\log S = -7.38$), these features are consistent with the highly aromatic scaffold and may favor hydrophobic target binding.

This trend was also reflected in the ADMET radar plot (Fig. 13), where most properties were positioned within or close to the recommended physicochemical space, with the main deviations related to lipophilicity and distribution coefficient. Compound **9I** also showed no PAINS, BMS, or chelator alerts, together with very low probabilities of reactivity (0.02) and promiscuity (0.006), and remained synthetically accessible (Synth = 2).



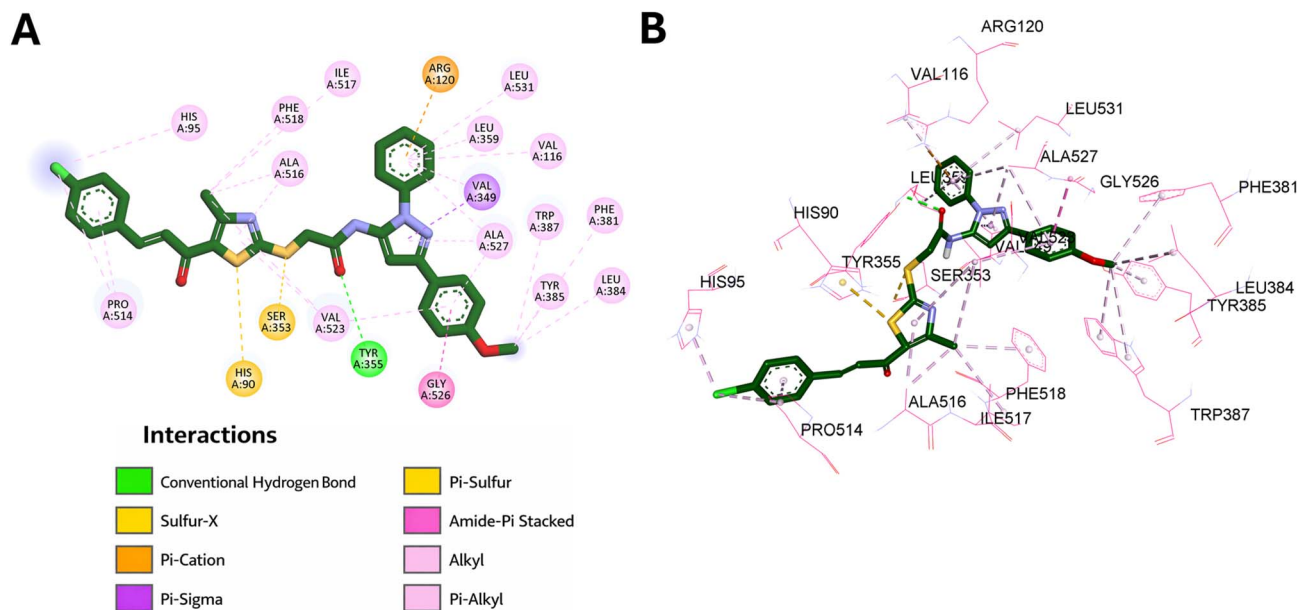


Fig. 12 Binding interactions of compound **9l** within the active site of COX-2 (PDB ID: 1CX2): (A) 2D interaction diagram and (B) 3D binding mode.

Absorption-related prediction was encouraging, with a low probability for poor intestinal absorption ($HIA = 0.00$), favorable oral bioavailability-related outputs ($F20\% = 0.037$, and $F30\% = 0.064$, and $F50\% = 0.064$), and non-substrate behavior toward *P*-glycoprotein (*P*-gp substrate = 0.00), although compound **9l** was predicted to act as a *P*-gp inhibitor (1.00). In addition, compound **9l** showed very low predicted BBB penetration (0.067), acceptable VDss (0.174 L kg⁻¹), a relatively high unbound fraction ($F_u = 0.438$), low probability of microsomal

instability (0.256), and low predicted plasma clearance (2.571 mL min⁻¹ kg⁻¹), despite a short predicted half-life (0.914 h).

On the toxicity side, several favorable predictions were obtained, including low probabilities for Ames mutagenicity (0.215), rat oral acute toxicity (0.163), eye corrosion (0.00), eye irritation (0.031), hematotoxicity (0.187), RPMI-8226 immunotoxicity (0.029), and A549 cytotoxicity (0.154). Although alerts were predicted for hERG liability and hepatotoxicity, these findings require experimental confirmation. Overall, the ADMETlab 3.0 results support compound **9l** as a synthetically feasible and pharmacologically promising lead.

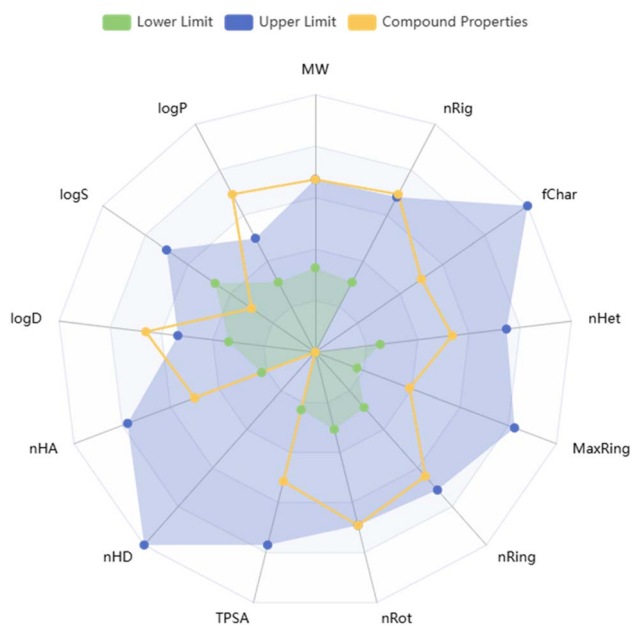


Fig. 13 ADMET radar plot of compound **9l** generated using ADMETlab 3.0, showing key physicochemical properties relative to the optimal drug-likeness space.

3. Conclusion

In conclusion, a new series of thiazole-chalcone/pyrazole hybrids (**9a-o**) was successfully designed, synthesized, and biologically evaluated as potential dual-acting anticancer agents targeting tubulin polymerization and COX-2. Several derivatives exhibited promising antiproliferative activity against MDA-MB-231, HCA-7, and A549 cancer cell lines, with improved selectivity over normal MCF-10A cells. Compounds **9m** and **9l** emerged as the most active members of the series. Among them, compound **9l** showed the most balanced biological profile, displaying potent tubulin polymerization inhibition, strong and selective COX-2 inhibition, marked suppression of PGE-2 production, induction of G2/M cell-cycle arrest, and pronounced apoptotic effects associated with Bax upregulation, Bcl-2 downregulation, and activation of caspase-3 and caspase-9. In addition, **9l** significantly inhibited HCA-7 cell migration, further supporting its anticancer potential. These experimental findings were consistent with the docking results, which demonstrated favorable accommodation of **9l** within both the colchicine-binding site of tubulin and the active site of COX-2. At the same time, ADMET prediction and human liver microsomal



stability studies suggested an acceptable drug-like and metabolic profile. Overall, compound **9l** represents a promising lead for further optimization as a multitarget anticancer agent.

4. Experimental

4.1 Chemistry

4.1.1 General details. Refer to SI.

3-Chloroacetylacetone (**2**),⁴⁶ 1-(2-mercapto-4-methylthiazol-5-yl)ethan-1-one (**3**) and chalcones (**4a–e**),²² amino pyrazoles (**7a–c**) and chloroacetamides (**8a–c**)⁴⁰ were prepared according to reported methods.

4.1.2 General procedures for the synthesis of compounds 9a–o. To a well-stirred solution of the appropriate chalcones (**4a–e**) (1 mmol) and triethylamine (1.5 mmol, 0.152 mg) in acetonitrile, the pyrazole derivative (**8a–c**) (1 mmol) was added. The resulting clear mixture was stirred at room temperature for 6 hours. The formed precipitate was filtered off, washed with cold acetonitrile and distilled water, and dried. The crude product was recrystallized from acetonitrile/water.

4.1.3 2-((5-Cinnamoyl-4-methylthiazol-2-yl)thio)-N-(1,3-diphenyl-1H-pyrazol-5-yl)acetamide (9a). Yellow powder (396 mg, 74% yield), m.p. 219–221 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.51 (s, 1H), 7.85 (d, *J* = 8.5 Hz, 2H), 7.79–7.74 (m, 2H), 7.67 (d, *J* = 15.5 Hz, 1H), 7.56 (d, *J* = 7.6 Hz, 2H), 7.49–7.41 (m, 7H), 7.40–7.31 (m, 3H), 6.94 (s, 1H), 4.26 (s, 2H), 2.63 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.97, 168.77, 166.43, 158.48, 149.66, 144.23, 138.77, 137.48, 134.66, 133.14, 132.57, 132.10, 131.43, 129.75, 129.55, 129.31, 128.39, 128.32, 127.42, 124.86, 124.41, 99.91, 37.61, 18.79. Anal. Calcd. For C₃₂H₂₈N₄O₄S₂: C, 64.41; H, 4.73; N, 9.39. Found: C, 64.58; H, 4.59; N, 9.58. MS (ESI) calcd for C₃₂H₂₈N₄O₄S₂: 536.13, found [M]⁺: 536.38.

4.1.4 (E)-2-((5-(3-(4-chlorophenyl)acryloyl)-4-methylthiazol-2-yl)thio)-N-(1,3-diphenyl-1H-pyrazol-5-yl)acetamide (9b). Yellow powder (445 mg, 78% yield), m.p. 227–230 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.56 (s, 1H), 7.85 (d, *J* = 7.1 Hz, 2H), 7.81 (d, *J* = 6.9 Hz, 2H), 7.65 (d, *J* = 15.7 Hz, 1H), 7.56 (d, *J* = 6.9 Hz, 2H), 7.52–7.42 (m, 6H), 7.40–7.31 (m, 3H), 6.93 (s, 1H), 4.26 (s, 2H), 2.62 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.85, 168.91, 166.45, 158.62, 149.65, 142.75, 138.77, 137.47, 135.92, 133.61, 133.13, 132.49, 132.09, 131.00, 129.74, 129.57, 129.29, 128.30, 127.42, 125.55, 124.38, 99.95, 37.61, 18.79. Anal. Calcd. for C₃₀H₂₃ClN₄O₂S₂: C, 63.09; H, 4.06; N, 9.81. Found: C, 62.95; H, 4.17; N, 9.63. MS (ESI) calcd for C₃₀H₂₃ClN₄O₂S₂: 570.10, found [M]⁺: 571.07.

4.1.5 (E)-2-((5-(3-(4-bromophenyl)acryloyl)-4-methylthiazol-2-yl)thio)-N-(1,3-diphenyl-1H-pyrazol-5-yl)acetamide (9c). Yellow powder (473 mg, 77% yield), m.p. 226–228 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.54 (s, 1H, NH), 7.86 (d, *J* = 8.4 Hz, 2H), 7.74 (d, *J* = 7.9 Hz, 2H), 7.67–7.60 (m, 4H), 7.56 (d, *J* = 7.7 Hz, 2H), 7.49–7.43 (m, 4H), 7.40–7.34 (m, 2H), 6.94 (s, 1H), 4.26 (s, 2H), 2.63 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.90, 168.97, 166.40, 158.63, 149.62, 142.87, 138.77, 137.47, 133.98, 133.11, 132.52, 132.10, 131.25, 129.76, 129.30, 128.31, 127.43, 125.77, 125.62, 124.83, 124.40, 99.94, 37.60, 18.84. Anal. Calcd. for C₃₀H₂₃BrN₄O₂S₂: C, 58.54; H, 3.77; N, 9.10. Found: C,

58.72; H, 3.65; N, 9.01. MS (ESI) calcd for C₃₀H₂₃BrN₄O₂S₂: 614.04, found [M]⁺: 614.30.

4.1.6 (E)-N-(1,3-diphenyl-1H-pyrazol-5-yl)-2-((4-methyl-5-(*p*-tolyl)acryloyl)thiazol-2-yl)thioacetamide (9d). Yellow powder (462 mg, 84% yield), m.p. 241–243 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.50 (s, 1H), 7.85 (d, *J* = 8.3 Hz, 2H), 7.66 (d, *J* = 7.5 Hz, 3H), 7.64–7.53 (m, 3H), 7.51–7.43 (m, 4H), 7.40–7.34 (m, 1H), 7.31–7.21 (m, 3H), 6.94 (s, 1H), 4.26 (s, 2H), 2.62 (s, 3H), 2.31 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.86, 168.52, 166.40, 158.30, 149.66, 144.32, 141.59, 138.81, 137.50, 133.13, 132.62, 132.12, 131.94, 130.16, 129.75, 129.35, 129.27, 128.29, 127.42, 124.42, 123.76, 99.85, 37.68, 21.65, 18.76. Anal. Calcd. for C₃₁H₂₆N₄O₂S₂: C, 67.61; H, 4.76; N, 10.17. Found: C, 67.45; H, 4.89; N, 10.37. MS (ESI) calcd for C₃₁H₂₆N₄O₂S₂: 550.15, found [M]⁺: 550.69.

4.1.7 (E)-N-(1,3-diphenyl-1H-pyrazol-5-yl)-2-((5-(3-(4-methoxyphenyl)acryloyl)-4-methylthiazol-2-yl)thio)acetamide (9e). Yellow powder (475 mg, 84% yield), m.p. 236–238 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.50 (s, 1H), 7.85 (d, *J* = 5.9 Hz, 2H), 7.74 (d, *J* = 5.5 Hz, 2H), 7.64 (d, *J* = 16.0 Hz, 1H), 7.55 (d, *J* = 6.2 Hz, 2H), 7.49–7.34 (m, 6H), 7.19 (d, *J* = 14.4 Hz, 1H), 7.01–6.91 (m, 3H), 4.25 (s, 2H), 3.79 (s, 3H), 2.62 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.77, 168.21, 166.42, 162.14, 158.03, 149.66, 144.30, 138.82, 137.51, 133.14, 132.74, 132.12, 131.28, 129.74, 129.26, 128.28, 127.41, 127.27, 124.41, 122.26, 115.03, 99.82, 55.89, 37.67, 18.73. Anal. Calcd. for C₃₁H₂₆N₄O₃S₂: C, 65.70; H, 4.62; N, 9.89. Found: C, 65.81; H, 4.45; N, 10.03. MS (ESI) calcd for C₃₁H₂₆N₄O₃S₂: 566.14, found [M]⁺: 566.56.

4.1.8 N-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-5-yl)-2-((5-cinnamoyl-4-methylthiazol-2-yl)thio)acetamide (9f). Yellow powder (450 mg, 79% yield), m.p. 231–233 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.50 (s, 1H), 7.85 (d, *J* = 8.5 Hz, 2H), 7.80–7.75 (m, 2H), 7.67 (d, *J* = 15.6 Hz, 1H), 7.56 (d, *J* = 7.6 Hz, 2H), 7.48–7.41 (m, 6H), 7.39–7.31 (m, 3H), 6.94 (s, 1H), 4.26 (s, 2H), 2.63 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.82, 168.76, 166.38, 158.45, 149.66, 144.26, 138.81, 137.82, 134.69, 133.23, 133.12, 132.11, 131.47, 129.74, 129.55, 129.32, 128.30, 127.71, 127.44, 124.94, 124.43, 99.91, 37.64, 18.76. Anal. Calcd. for C₃₀H₂₃ClN₄O₂S₂: C, 63.09; H, 4.06; N, 9.81. Found: C, 62.88; H, 4.15; N, 9.68. MS (ESI) calcd for C₃₀H₂₃ClN₄O₂S₂: 572.10, found [M+2H]⁺: 572.29.

4.1.9 (E)-N-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-5-yl)-2-((5-(3-(4-chlorophenyl)acryloyl)-4-methylthiazol-2-yl)thio)acetamide (9g). Brown powder (466 mg, 77% yield), m.p. 205–207 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.56 (s, 1H), 7.85 (d, *J* = 8.4 Hz, 2H), 7.81 (d, *J* = 8.3 Hz, 2H), 7.65 (d, *J* = 15.5 Hz, 1H), 7.56 (d, *J* = 7.7 Hz, 2H), 7.50–7.43 (m, 6H), 7.38–7.33 (m, 2H), 6.93 (s, 1H), 4.26 (s, 2H), 2.63 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.88, 168.91, 166.47, 158.64, 149.62, 142.75, 138.78, 137.72, 137.51, 135.92, 131.08, 130.10, 129.74, 129.55, 129.27, 128.31, 128.27, 127.45, 125.60, 124.59, 124.39, 100.03, 37.67, 18.87. Anal. Calcd. for C₃₀H₂₂Cl₂N₄O₂S₂: C, 59.50; H, 3.66; N, 9.25. Found: C, 59.65; H, 3.56; N, 9.47. MS (ESI) calcd for C₃₀H₂₂Cl₂N₄O₂S₂: 604.06, found [M]⁺: 604.44.

4.1.10 (E)-2-((5-(3-(4-bromophenyl)acryloyl)-4-methylthiazol-2-yl)thio)-N-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-5-



yl)acetamide (**9h**). Brown powder (500 mg, 77% yield), m.p. 216–219 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.50 (s, 1H), 7.86 (d, *J* = 6.9 Hz, 2H), 7.74 (d, *J* = 6.9 Hz, 2H), 7.68–7.59 (m, 3H), 7.56 (d, *J* = 7.0 Hz, 2H), 7.50–7.43 (m, 4H), 7.36 (d, *J* = 11.8 Hz, 2H), 6.94 (s, 1H), 4.26 (s, 2H), 2.63 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.83, 168.92, 166.42, 158.65, 149.65, 142.84, 138.76, 137.47, 133.93, 133.13, 132.50, 132.07, 131.19, 129.75, 129.29, 128.31, 127.41, 125.57, 124.82, 124.39, 124.11, 99.87, 37.61, 18.77. Anal. Calcd. for C₃₀H₂₂BrClN₄O₂S₂: C, 55.43; H, 3.41; N, 8.62. Found: C, 55.31; H, 3.60; N, 8.51. MS (ESI) calcd for C₃₀H₂₂BrClN₄O₂S₂: 651.01, found [M+3H]⁺: 651.58.

4.1.11 (*E*)-*N*-(3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazol-5-yl)-2-((4-methyl-5-(3-(*p*-tolyl)acryloyl)thiazol-2-yl)thio)acetamide (**9i**). Brown powder (520 mg, 89% yield), m.p. 196–199 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.50 (s, 1H), 7.85 (d, *J* = 8.5 Hz, 2H), 7.65 (t, *J* = 11.9 Hz, 3H), 7.55 (d, *J* = 7.5 Hz, 2H), 7.48–7.42 (m, 4H), 7.39–7.34 (m, 1H), 7.28 (d, *J* = 15.5 Hz, 1H), 7.24 (d, *J* = 7.8 Hz, 2H), 6.94 (s, 1H), 4.26 (s, 2H), 2.63 (s, 3H), 2.32 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.95, 168.52, 166.41, 158.28, 149.65, 144.36, 141.62, 138.80, 137.49, 133.14, 132.64, 132.13, 131.97, 130.19, 129.76, 129.38, 129.30, 128.31, 127.44, 124.41, 123.86, 99.99, 37.69, 21.65, 18.78. Anal. Calcd. for C₃₁H₂₅ClN₄O₂S₂: C, 63.63; H, 4.31; N, 9.58. Found: C, 63.83; H, 4.18; N, 9.67. MS (ESI) calcd for C₃₁H₂₅ClN₄O₂S₂: 584.11, found [M]⁺: 584.47.

4.1.12 (*E*)-*N*-(3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazol-5-yl)-2-((5-(3-(4-methoxyphenyl)acryloyl)-4-methylthiazol-2-yl)thio)acetamide (**9j**). Yellow powder (444 mg, 74% yield), m.p. 216–219 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.50 (s, 1H), 7.85 (d, *J* = 8.5 Hz, 2H), 7.74 (d, *J* = 8.6 Hz, 2H), 7.64 (d, *J* = 15.4 Hz, 1H), 7.55 (d, *J* = 7.6 Hz, 2H), 7.50–7.41 (m, 4H), 7.39–7.34 (m, 1H), 7.19 (d, *J* = 15.4 Hz, 1H), 7.00–6.92 (m, 3H), 4.25 (s, 2H), 3.79 (s, 3H), 2.62 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.83, 168.26, 166.44, 162.15, 158.02, 149.64, 144.33, 138.79, 137.49, 133.12, 132.75, 132.11, 131.32, 129.76, 129.29, 128.30, 127.42, 127.27, 124.40, 122.30, 115.06, 99.92, 55.96, 37.63, 18.75. Anal. Calcd. for C₃₁H₂₅ClN₄O₃S₂: C, 61.94; H, 4.19; N, 9.32. Found: C, 61.75; H, 4.35; N, 9.10. MS (ESI) calcd for C₃₁H₂₅ClN₄O₃S₂: 601.11, found [M + H]⁺: 601.50.

4.1.13 2-((5-Cinnamoyl-4-methylthiazol-2-yl)thio)-*N*-(3-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)acetamide (**9k**). Yellow powder (453 mg, 80% yield), m.p. 190–191 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.46 (s, 1H), 7.81–7.73 (m, 4H), 7.67 (d, *J* = 15.4 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 2H), 7.49–7.41 (m, 5H), 7.38–7.30 (m, 2H), 6.95 (d, *J* = 8.4 Hz, 2H), 6.82 (s, 1H), 4.26 (s, 2H), 3.75 (s, 3H), 2.64 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.90, 168.80, 166.38, 159.77, 158.51, 150.71, 144.21, 138.97, 137.13, 134.65, 132.55, 131.41, 129.70, 129.54, 129.30, 128.00, 127.06, 125.86, 124.81, 124.27, 114.63, 99.32, 55.61, 37.66, 18.78. Anal. Calcd. for C₃₁H₂₆N₄O₃S₂: C, 65.70; H, 4.62; N, 9.89. Found: C, 65.80; H, 4.41; N, 10.05. MS (ESI) calcd for C₃₁H₂₆N₄O₃S₂: 566.14, found [M]⁺: 566.29.

4.1.14 (*E*)-2-((5-(3-(4-chlorophenyl)acryloyl)-4-methylthiazol-2-yl)thio)-*N*-(3-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)acetamide (**9l**). Yellow powder (456 mg, 76% yield), m.p. 214–216 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.51 (s,

1H), 7.81 (d, *J* = 7.9 Hz, 2H), 7.75 (d, *J* = 7.8 Hz, 2H), 7.65 (d, *J* = 15.5 Hz, 1H), 7.55 (d, *J* = 7.6 Hz, 2H), 7.50–7.42 (m, 4H), 7.39–7.30 (m, 2H), 6.95 (d, *J* = 7.8 Hz, 2H), 6.82 (s, 1H), 4.26 (s, 2H), 3.75 (s, 3H), 2.63 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.81, 168.92, 166.41, 159.78, 158.64, 150.69, 142.72, 138.97, 137.12, 135.92, 133.61, 132.45, 130.98, 129.69, 129.56, 127.99, 127.05, 125.87, 125.54, 124.24, 114.64, 99.45, 55.67, 37.67, 18.79. Anal. Calcd. for C₃₁H₂₅ClN₄O₃S₂: C, 61.94; H, 4.19; N, 9.32. Found: C, 61.81; H, 4.31; N, 9.15. MS (ESI) calcd for C₃₁H₂₅ClN₄O₃S₂: 600.11, found [M]⁺: 600.81.

4.1.15 (*E*)-2-((5-(3-(4-bromophenyl)acryloyl)-4-methylthiazol-2-yl)thio)-*N*-(3-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)acetamide (**9m**). Yellow powder (477 mg, 74% yield), m.p. 206–208 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.46 (s, 1H), 7.77–7.71 (m, 4H), 7.66–7.60 (m, 3H), 7.55 (d, *J* = 7.6 Hz, 2H), 7.49–7.42 (m, 2H), 7.39–7.32 (m, 2H), 6.95 (d, *J* = 8.5 Hz, 2H), 6.82 (s, 1H), 4.26 (s, 2H), 3.76 (s, 3H), 2.63 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.71, 168.90, 166.36, 159.77, 158.68, 150.71, 143.11, 142.79, 138.96, 137.13, 133.89, 132.47, 131.12, 129.70, 128.00, 127.05, 125.86, 125.49, 124.82, 124.26, 114.63, 99.31, 55.61, 37.70, 18.79. Anal. Calcd. for C₃₁H₂₅BrN₄O₃S₂: C, 57.67; H, 3.90; N, 8.68. Found: C, 57.89; H, 3.81; N, 8.81. MS (ESI) calcd for C₃₁H₂₅BrN₄O₃S₂: 644.06, found [M]⁺: 643.88.

4.1.16 (*E*)-*N*-(3-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)-2-((4-methyl-5-(3-(*p*-tolyl)acryloyl)thiazol-2-yl)thio)acetamide (**9n**). Brown powder (435 mg, 75% yield), m.p. 225–228 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.45 (s, 1H), 7.75 (d, *J* = 8.7 Hz, 2H), 7.65 (t, *J* = 11.9 Hz, 3H), 7.54 (d, *J* = 7.6 Hz, 2H), 7.45 (t, *J* = 7.7 Hz, 2H), 7.37–7.32 (m, 1H), 7.28 (d, *J* = 15.5 Hz, 1H), 7.24 (d, *J* = 7.8 Hz, 2H), 6.95 (d, *J* = 8.7 Hz, 2H), 6.82 (s, 1H), 4.25 (s, 2H), 3.76 (s, 3H), 2.63 (s, 3H), 2.32 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.92, 168.59, 166.41, 159.77, 158.34, 150.69, 144.36, 141.65, 138.94, 137.11, 132.61, 131.92, 130.19, 129.71, 129.37, 128.02, 127.06, 125.84, 124.25, 123.75, 114.64, 99.37, 55.62, 37.62, 21.64, 18.76. Anal. Calcd. for C₃₂H₂₈N₄O₃S₂: C, 66.19; H, 4.86; N, 9.65. Found: C, 66.08; H, 5.09; N, 9.50. MS (ESI) calcd for C₃₂H₂₈N₄O₃S₂: 580.16, found [M]⁺: 580.44.

4.1.17 (*E*)-*N*-(3-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)-2-((5-(3-(4-methoxyphenyl)acryloyl)-4-methylthiazol-2-yl)thio)acetamide (**9o**). Yellow powder (477 mg, 80% yield), m.p. 220–221 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.46 (s, 1H), 7.78–7.71 (m, 4H), 7.64 (d, *J* = 15.4 Hz, 1H), 7.55 (d, *J* = 7.8 Hz, 2H), 7.49–7.41 (m, 2H), 7.38–7.31 (m, 1H), 7.19 (d, *J* = 15.4 Hz, 1H), 6.97 (dd, *J* = 11.6, 8.7 Hz, 4H), 6.82 (s, 1H), 4.25 (s, 2H), 3.78 (s, 3H), 3.75 (s, 3H), 2.62 (s, 3H); ¹³C NMR (120 MHz, DMSO-*d*₆) δ (ppm): 181.81, 168.25, 166.40, 162.15, 159.79, 158.04, 150.70, 144.30, 138.98, 137.14, 132.74, 131.29, 129.70, 128.00, 127.27, 127.06, 125.89, 124.26, 122.30, 115.05, 114.64, 99.36, 55.95, 55.67, 37.66, 18.72. Anal. Calcd. for C₃₂H₂₈N₄O₄S₂: C, 64.41; H, 4.73; N, 9.39. Found: C, 64.58; H, 4.59; N, 9.58. MS (ESI) calcd for C₃₂H₂₈N₄O₄S₂: 596.16, found [M]⁺: 596.99.

4.2 Biology

4.2.1. Antiproliferative activity. The antiproliferative activities of compounds **9a–o** were evaluated against MDA-MB-231, HCA-7, and A549 cancer cell lines, as well as MCF-10A normal



mammary epithelial cells, using the MTT assay (Sigma-Aldrich *In Vitro* Toxicology Assay Kit, MTT-based, TOX-1), with combretastatin A-4 and doxorubicin as reference drugs. IC₅₀ values were determined from the corresponding dose–response curves. Further experimental details are provided in the SI.

4.2.2. Tubulin polymerization inhibitory assay. The effects of compounds **9a–o** on tubulin polymerization were assessed using a fluorescence-based tubulin polymerization assay (Cytoskeleton, Inc., Cat. No. BK011P), with combretastatin A-4 as the reference inhibitor. The inhibitory activities were expressed as IC₅₀ values. Additional assay details are provided in the SI.

4.2.3. COX inhibition assay. The inhibitory activities of compounds **9h**, **9j**, **9l**, **9m**, and **9o** against COX-1 and COX-2 were evaluated using the Cayman COX (ovine/human) Inhibitor Screening Assay Kit (Item No. 560131). The inhibitory activities were expressed as IC₅₀ values, and the selectivity index (SI) was calculated as IC₅₀(COX-1)/IC₅₀(COX-2). Further methodological details are given in the SI.

4.2.4. Cell cycle analysis and apoptosis. The effects of compound **9l** on cell-cycle progression and apoptosis in HCA-7 cells were analyzed by flow cytometry using propidium iodide staining and Annexin V-FITC/PI dual staining, respectively. The detailed experimental procedures are described in the SI.

4.2.5. Effect on Bax and Bcl-2 protein expression. The effect of compound **9l** on Bax and Bcl-2 protein expression in HCA-7 cells was evaluated using ELISA kits (DRG Human BAX ELISA Kit, Cat. No. EIA-4487; Zymed Bcl-2 ELISA Kit, Cat. no. 99-0042). Full assay details are provided in the SI.

4.2.6. Effect on Caspase-3 and Caspase-9 levels. The effects of compound **9l** on caspase-3 and caspase-9 levels in HCA-7 cells were determined using ELISA-based assays (Invitrogen Human Active Caspase-3 ELISA Kit, Cat. No. KHO1091; Thermo Fisher Human Caspase-9 ELISA Kit, Cat. No. BMS2025). Additional methodological details are given in the SI.

4.2.7. Effect on PGE-2 production. The effect of compound **9l** on PGE-2 production in HCA-7 cells was assessed using a competitive ELISA assay (R&D Systems Parameter™ PGE-2 Immunoassay, Cat. No. KGE004B). Further experimental details are provided in the SI.

4.2.8. Wound healing assay. The effect of compound **9l** on the migratory behavior of HCA-7 cells was evaluated using the CytoSelect™ 24-Well Wound Healing Assay (Cell Biolabs, Inc., Cat. No. CBA-120). The detailed experimental procedure is described in the SI.

4.2.9. Metabolic stability studies. The *in vitro* metabolic stability of compound **9l** was evaluated using pooled human liver microsomes under NADPH-supported oxidative conditions. Detailed incubation conditions, sample processing, and data analysis are provided in the SI.

4.3 Computational studies

The computational studies of compound **9l** comprised molecular docking and ADMET prediction. Docking against tubulin and COX-2 was performed using AutoDock Vina, with interaction analysis performed in Discovery Studio Visualizer, while

ADMETlab 3.0 was used for *in silico* ADMET assessment. Full details are given in the SI.

Author contributions

conceptualization, B. A. A. S., K. S. A. and S. B.; methodology, B. A. A. S., A. A. Q., A. R. S., G. A. and A. A.; validation, K. S. A. and S. B.; formal analysis, A. A. Q., A. R. S., M. A.-Z., G. A. and A. A.; investigation, B. A. A. S., A. R. S., M. A.-Z., G. A. and A. A.; resources, A. A. Q., M. A.-Z. and S. B.; data curation, B. A. A. S. and M. A.-Z.; writing—original draft, B. A. A. S. and A. R. S.; writing—review and editing, K. S. A. and S. B.; supervision, K. S. A. and S. B.; project administration, A. A. Q.; funding acquisition, A. A. Q. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The original contributions presented in this study are included in the article; further inquiries can be directed to the corresponding author.

Supplementary information (SI) is available. See DOI: <https://doi.org/10.1039/d6ra03557d>.

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