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Synthesis and tubulin-binding properties of non-symmetrical click C5-curcuminoids

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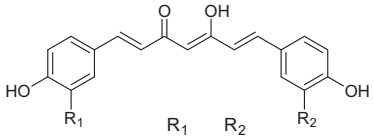
ABSTRACT

A click-type entry into shortened curcuminoids of the diarylpentanoid type has been developed. The reaction is ideally suited to generate non-symmetrical analogues of curcumin, a class of natural products difficult to access but of growing biomedical relevance and special mechanistic interest to investigate the unique binding mode of curcumin to tubulin. Investigation of a series of click diarylpentane curcuminoids and their pyrazole adducts in various cellular tubulin functional assays validated this class of compounds as a novel type of anti-mitotic agents, evidencing structure–activity relationships, and identifying the pyrazole adduct **4k** as a promising lead.

1. Introduction

No plant better than turmeric (*Curcuma longa* L.) exemplifies the versatility of spices in human nutrition and health, flavoring all dishes of the cuisine of India and, allegedly, also curing all diseases in its folk medicine.[1] Unlike other panaceas of traditional medicine, preclinical investigations have confirmed the potential of curcumin (**1a**), the major phytochemical of turmeric, in the management of a multitude of conditions that include some of the hottest areas of current pharmaceutical research (cancer,[2] inflammation,[3] infectious disease[4]). Unsurprisingly given its cure-all status, the hallmark of the biological profile of curcumin is the redundancy of targets, but, from a mechanistic standpoint, there is a general agreement that curcumin is mainly an epigenetic agent, acting on key transcription factors (NF-kB,[5] Nrf2,[6] STAT-3,[7] PPAR-γ[8]) involved in anti-inflammatory/anti-oxidant responses and in cancer development and progression. Curcumin has, indeed, been used with promising results in a phase II clinical study for pancreatic cancer[6] and in some small anti-inflammatory clinical trials.[9] However, its clinical potential remains largely untapped, and megadoses (>5 g/day) are required because of a combination of moderate potency and very poor oral bioavailability[1,10] Significant clinical progress has recently been obtained with a lecithin formulation of curcumin as a sparing agent for anti-inflammatory drugs in various chronic diseases.[11] From a medicinal chemistry standpoint, the pleiotropic bioactivity and substantial lack of toxicity, make it unlikely that any synthetic analogue will displace natural curcumin in this broad-spectrum, but essentially supportive, medical use.

On the other hand, with over 100 different distinct biological targets,[12] curcumin is also a most versatile privileged structure for the discovery of bioactivity, directly interfering with the function of a host of enzymes and receptors. In this context, the activity on tubulin has received considerable attention, since curcumin can affect microtubule function in cancer cells in a way substantially different from all known antitubulin agents,[13] acting at a binding site located between the α and β-subunits of two α, β-dimers, and clearly distinct from those of colchicine, taxoids and Vinca alkaloid. Despite compelling evidence of anti-mitotic activity, curcumin shows an extraordinary level of tolerance in humans, possibly because the functional translation of its anti-tubulin activity involves process(es) differently regulated in normal and in cancer cells. Taken together, these observations qualify curcumin as an interesting anticancer lead, worth exploration in terms of structure–activity relationships.[14]



	R_1	R_2
1a	OMe	OMe
1b	OMe	H

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Many tubulin ligands have a dimeric structure. Thus, Vinca alkaloids are heterodimeric structures, and tubulin ligands like combretastatins and podophyllotoxin share a non-symmetrical dibenzyl dimeric structure. Conversely, curcumin is a symmetric feruloyl dimer, and it was therefore interesting to investigate the tubulin binding properties of non-symmetrical curcuminoids. Owing to difficulties in the synthesis of these compounds, most structure–activity studies on this class of compounds have focused on symmetrical analogues of the natural product, despite the increased potency of demethoxycurcumin (**1b**)[9] in several anti-inflammatory and biochemical assays. To address this issue and extend the structure–activity relationships of curcuminoids as tubulin ligands, we have developed a click-type entry C5-curcuminoids of the benzoyl-feruloylmethane type, capitalizing on the reaction of the borate complex of a series of 1-aryl-2,4-butandiones with various aromatic aldehydes. The stepwise carbonyl chemistry involved in their synthesis makes it possible to easily access curcuminoids with a non-symmetrical aryl substitution pattern. Compared to the C5 curcuminoids of the monocarbonyl-type, these abridged curcuminoids retain the 1,3-dicarbonyl system and its associated rich chemistry, including the conversion to the corresponding pyrazole derivatives, a validated maneuver to improve the potency of curcuminoids in many biological assays.[15,16]

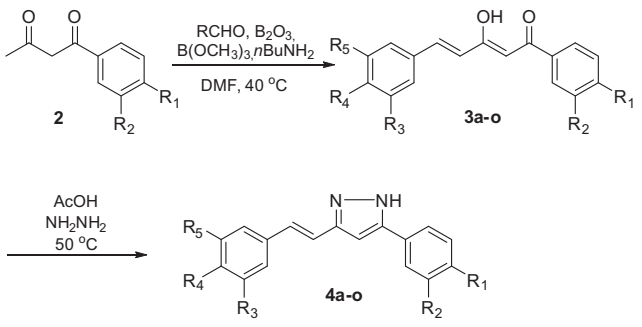
2. Results

2.1. Chemistry

The synthesis of the C5-curcuminoids was carried out by condensing a series of aromatic aldehydes with the boric complex of various 1-aryl-1,3-butanediones,[17] in turn obtained from the reaction of the corresponding acetophenones with ethyl acetate (Scheme 1). By stabilizing its enol tautomer, formation of a boric complex prevents alkylation of the central methylene of phenylbutanedione, quenching the methylene-centered Knoevenagel reactivity at the expenses of the methyl-centered aldol-type reactivity.[18] The reaction was carried out in a click fashion, and the resulting α,β-unsaturated diketones (**3a–o**) were simply obtained by precipitation with acetic acid and washing with water. Purity was evaluated by HPLC, and, when unsatisfactory (<95%), was improved by re-crystallization to reach the threshold set of purity, set at least 95%.

The pyrazole analogues were prepared by overnight treatment with hydrazine hydrate in acetic acid at 50 °C. An aqueous work-up and crystallization of the crude products afforded pure compounds without any further purification.

The non-symmetrical dicarbonyl nature of compounds **3a–o** could bias their tautomeric equilibration, favoring one tautomeric form over the other possible one. All compounds showed only one single set of signals in their NMR spectra, with two well-differentiated 'carbonyl' resonances around δ 190 and 180. In curcumin, there is one single carbonyl resonance at 180 ppm, and, in the absence of any bias, two close values could be expected. The larger separation between the 'carbonyl' resonances, suggests the prevalence of one tautomer, and considerations of calculated chemical shifts suggest the prevalence of the tautomeric form A compared to B.



Scheme 1. Synthesis of mono-α,β-unsaturated β-diketones (**3a–o**) and mono-α,β-unsaturated pyrazoles (**4a–o**).

2.2. Biological results

Cytotoxicity in a human melanoma cell line (MeWo) was used as preliminary screening, treating cells with curcumin or its non-symmetrical analogues **3a–o** and **4a–o** at a fixed concentration of 10 μM for 48 h (Table 1). In accordance with its low potency, **1a** was unable to significantly affect viability at this concentration, while four analogues of the pyrazole series (**4i**, **4k**, **4n**, **4p**) reduce cell viability below 50% of the control sample that was taken as a threshold value for cytotoxicity. For these hits, a concentration–response curve with built, again using **1a** as a positive reference. Curcumin (**1a**) showed an IC_{50} of 35.5 ± 4.7 μM, with **4p** being the most potent (3.6 ± 0.7 μM; Fig. 1). The activity of the lead compounds, as well as of curcumin, was then investigated, building a full concentration–response curve, in a neuroblastoma cell line (SH-SY5Y) exquisitely sensitive to antitubulin agents.[19] The cytotoxic activity was confirmed, but the rank of potency was slightly modified, with only **4k** significantly more cytotoxic than curcumin.

Table 1
Summarization of synthesized compounds and relative cytotoxicity in Mewo cells, after 48 h administration at 10 μM

Compound	R_1	R_2	R_3	R_4	R_5	Viability (%)
1a						90 ± 1
3a	H	H	OCH_3	OH	H	90 ± 11
3b	H	H	H	OH	H	90 ± 11
3c	H	H	H	OCH_3	H	90 ± 4
3d	H	H	OCH_3	H	OCH_3	92 ± 6
3e	H	H	OCH_3	OCH_3	OCH_3	93 ± 2
3f	OH	H	OCH_3	OH	H	89 ± 5
3g	OH	H	H	OH	H	77 ± 9
3h	OH	H	H	OCH3	H	93 ± 4
3i	OH	H	OCH_3	H	OCH_3	90 ± 2
3j	OH	H	OCH_3	OCH_3	OCH_3	85 ± 1
3k	OH	OCH_3	OCH_3	OH	H	97 ± 4
3l	OH	OCH_3	H	OH	H	98 ± 2
3m	OH	OCH_3	H	OCH_3	H	84 ± 4
3n	OH	OCH_3	OCH_3	H	OCH_3	88 ± 5
3o	OH	OCH_3	OCH_3	OCH_3	OCH_3	89 ± 2
4a	H	H	OCH_3	OH	H	51 ± 2
4b	H	H	H	OH	H	98 ± 2
4c	H	H	H	OCH_3	H	92 ± 4
4d	H	H	OCH_3	H	OCH_3	90 ± 4
4e	H	H	OCH_3	OCH_3	OCH_3	89 ± 4
4f	OH	H	OCH_3	OH	H	69 ± 2
4g	OH	H	H	OH	H	87 ± 8
4h	OH	H	H	OCH_3	H	81 ± 7
4i	OH	H	OCH_3	H	OCH_3	38 ± 10
4j	OH	H	OCH_3	OCH_3	OCH_3	85 ± 2
4k	OH	OCH_3	OCH_3	OH	H	36 ± 3
4l	OH	OCH_3	H	OH	H	95 ± 7
4m	OH	OCH_3	H	OCH_3	H	92 ± 5
4n	OH	OCH_3	OCH_3	H	OCH_3	43 ± 1
4o	OH	OCH_3	OCH_3	OCH_3	OCH_3	75 ± 2
4p	OH	OCH_3	OCH_3	H	H	27 ± 1

Results relative to the vehicle (DMSO) and representative of 8–12 different data points AV ± S.D.

The anti-tubulinic activity of the hit compounds was next investigated by FACS analysis in a cell cycle progression assay (Fig. 2). Blockade in the G2/M phase is a phenotypic signature of antitubulinic agents, since impairment of microtubules does not allow cell cycle progression. The incubation of **1a** or compound **4i**, **4k**, **4n**, **4p** at a concentration 3-fold higher than the IC_{50} induced indeed G2/M phase block of the cell cycle. Combretastatin CA-4 (CA-4) was used as a positive control in this assay.

The involvement of tubulin in the cytotoxicity of **4i**, **4k**, **4n**, **4p** was confirmed by direct investigation of the cellular tubulin state by immunofluorescence. Thus, MeWo cells were treated with curcumin or **4i**, **4k**, **4n**, **4p**, for 16 h and next fixed, staining the tubulin cytoskeleton with a specific antibody. The microtubule network was clearly observable in control cells but disappeared in all treated samples (Fig. 3).

Finally, tubulin polymerization was investigated in an in cellular assay. Briefly, cells were treated with the desired compound for 16 h, and proteins were next extracted in presence of paclitaxel to prevent subsequent microtubule re-arrangement (Fig. 3). Western blotting of the pelletable fraction and the soluble fraction made then possible to distinguish between polymerized and free tubulin. As expected, in the control cells tubulin was present in similar amounts in the polymerized (pellet) and free form (supernatant), while the equilibrium was significantly shifted toward the free form when cells were treated with curcumin or the hit compounds **4i**, **4k**, **4n** and **4p**. CA-4 served also in this case as positive control for its well-characterized antitubulin action. Taken together, these experiments provide conclusive evidence that tubulin polymerization represents the primary mechanism of action of these cytotoxic compounds.

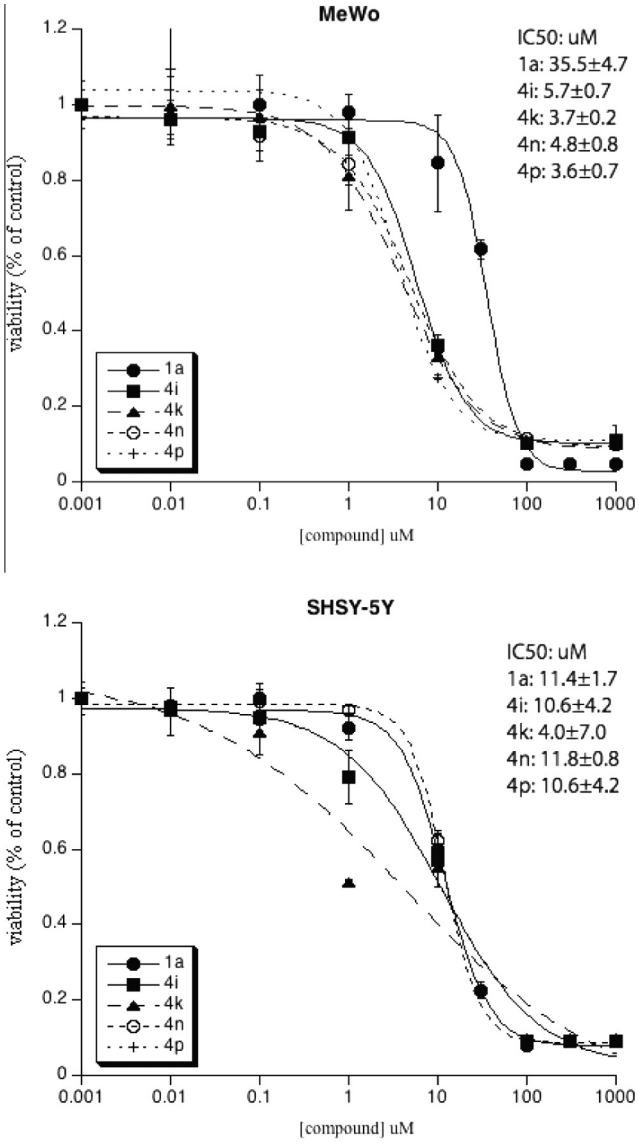


Figure 1. Concentration–response curves of curcumin and the most potent compounds in MeWo and SH-SY5Y cells lines after 48 h treatment. Values are mean + S.D. of 8–16 replicates from 4 separate experiments.

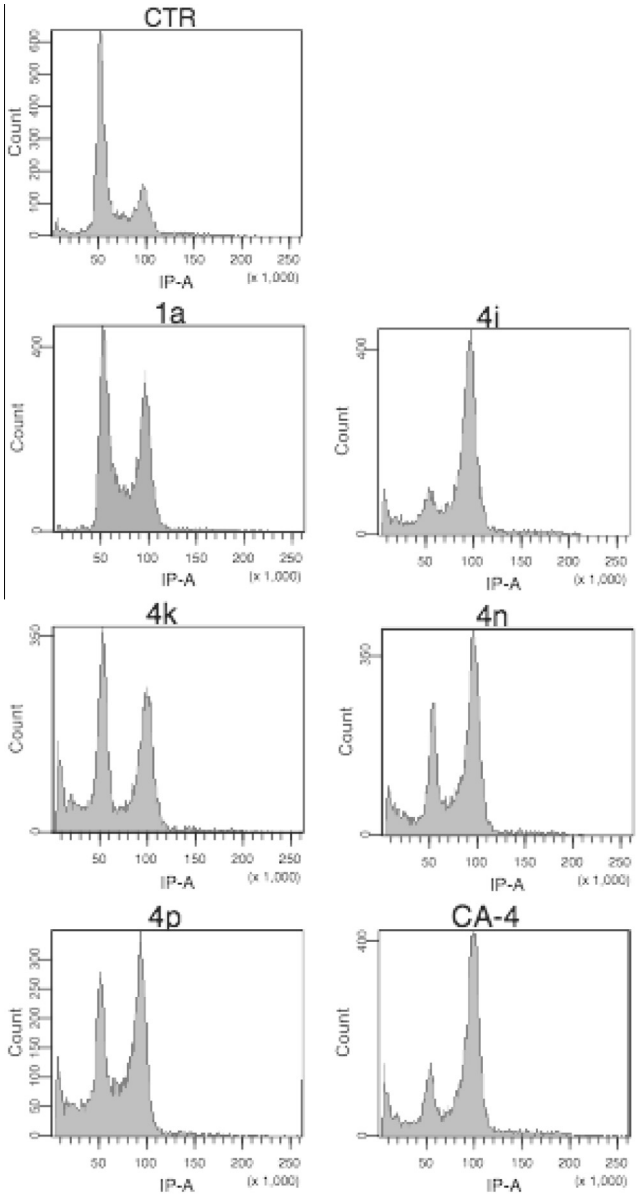


Figure 2. Curcumin and the most potent synthesized analogues induced cell cycle arrest. Cell cycle analysis of Mewo cells treated for 16 h with vehicle (CTR) or 3 X IC_{50} values of indicated compounds. Data are representative of three separate experiments. The *Y*-axis represents cell number, and the *X*-axis represents fluorescence on a linear scale.

3. Discussion

Curcumin, the bioactive ingredient of turmeric, has been used for multiple medical purpose for thousand of years. Modern research has shown that curcumin can modulate a host of cellular

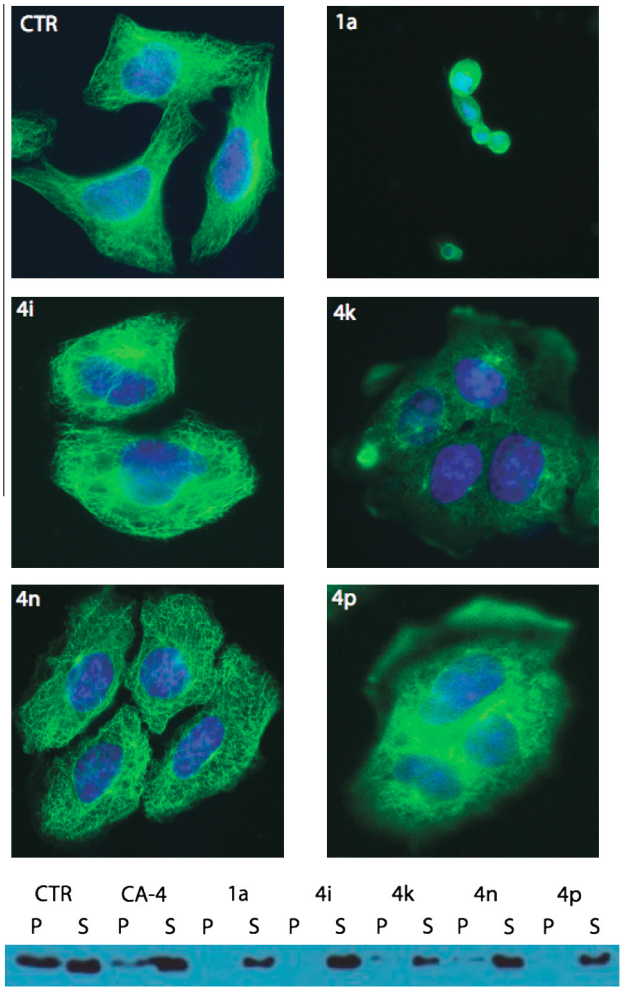


Figure 3. Curcumin and the most active compounds affect tubulin polymerization. Immunofluorescence using an anti α-tubulin antibody (green) and DRAQ5 (blue) for nuclear staining of control cells (CTR) or cells treated for 16 h with 3 × IC_{50} value of the indicated compounds. Images have been obtained with a 63× oil immersion objective (upper pannel). Western blot of α-tubulin extracted in the presence of paclitaxel from Mewo cells treated with the indicated compounds at 3 × IC_{50} value (lower panel). Results are representative of three separate experiments. P = pelletable fraction. S = soluble fraction.

processes, probably because of its extended and oxygenated conjugated system endows it with Michael acceptor, an antioxidant,[20] and a metal chelation properties. Most structure- activity studies on curcumin have focused on the modification of the diacryloylmethane moiety, with simplification to C5-curcuminoids, that is curcuminoids of the dienone-type, being expecially popular and often providing more potent analogueds. Replacement of the β-diketone system with a pyrazole has also been validated as a potency increasing maneuver,[21] The classic C5 curcuminoid lack the enolized β-dicarbonyl moiety of the natural product, and might behave as more potent analogue in assays strongly dependant on, since they can outperform curcumin in assays of thio trapping capacity.[22,23] Competitive experiments were carried out by reacting equimolecular binary mixtures of curcumin and C5 monocarbonyl-curcumin with substoichiometric amount of cysteamine. Under those conditions C5 curcumin showed a higher reactivity than curcumin (unpublished results). We have developed an alternative class of C5 curcuminoids that retain the hallmark of the curcumin pharmacophore, namely the presence of an extensively conjugated enolized β-dicarbonyl system, while differing for a shortened π-conjugation between the peripheral aromatic rings. Compounds of this type are available in a click fashion from a stepwise carbonyl chemistry that make it possible to easily access analogues with different substitution at the terminal aryl moieties, something that is difficult to achieve in curcuminoids of the diarylheptanoid type. Furthermore, these analogues retain the rich chemistry of curcuminoids associated to the presence of the enolized β-dicarbonyl system, including the capacity to afford pyrazole derivative, an important potency-inreasing maneuver in curcuminoids.

Cytotoxicity at a concentration where curcumin shows only marginal activity was used as a rapid comparative initial screening. Most C5 curcuminoids outperformed the natural product, with significant cytotoxicity being observed in the four pyrazoles **4i**, **4k**, **4n**, **4p**, all having non-symmetrically substituted terminal aryl moieties. These findings are in line with the general potency trend observed in curcuminoids of the natural C7-type, and these hits were then investigated for their capacity to disrupt tubulin, one intriguing property of curcumin. In accordance with the cytotoxicity data on the human melanoma cell line MeWo, all four compounds outperformed curcumin. While the data-set should be increased to draw extensive structure–activity relationship within this novel class of C5-curcuminoids, some considerations are evident, mirroring those observed in curcuminoids regarding the replacement of the enolized β-dicarbonyl system with a pyrazole and the non-identical substitution pattern of the peripheral rings. Formation of pyrazole derivatives impose a conformational constraint that is beneficial for tubulin activity, that is seemingly increased by rigidification.[15] Regarding the substitution pattern at the peripheral phenyls, oxygenated functions are important for activity, with a particularly beneficial role for a *p*-hydroxyl in benzoyl-type phenyl, and a *m*-methoxyl in the stiryl-type phenyl.

4. Conclusions

A novel class of non-symmetrical C5-curcuminoids has been identified, with cytotoxicity being especially remarkable in their pyrazole analogues. Despite the shortening of the conjugated system, these compounds show similar, or even better, bioactivity compared with curcumin, with a series of four pyrazole analogues qualifying as lead compounds to develop curcuminoid-based antimitotic agents potentially selective for cancer cells. Due to an increased potency, these compounds also qualify as probe to investigate the molecular basis of the selectivity of action of curcuminoids.

5. Experimental section

5.1. Chemistry

IR spectra were taken on a FT-IR Thermo Nicolet equipment. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were measured on a JEOL Eclipse 300 instrument. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: $\delta_H = 7.26$, $\delta_C = 77.0$, CD_3OD: $\delta_H = 3.34$, $\delta_C = 49.0$). Low- and high-resolution ESIMS were obtained on a LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. Silica gel 60 (70–230 mesh) and RP-18 used for gravity column chromatography was purchased from Macherey-Nagel. Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, that were visualized by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. Organic phases were dried with Na_2SO_4 before evaporation.

5.1.1. General procedure for the synthesis of mono-a,β-unsaturated β-diketones (3a–o)

To a stirred solution of vanillin (1 equiv mol) in dry DMF (2 mL/mmol), 1-phenyl-1,3-butanedione[17] (1 equiv mol), B_2O_3 (1,6 equiv

mol), $B(OCH_3)_3$ (0,73 equiv mol) and butyl amine (0,08 equiv mol) were sequentially added. The reaction was stirred at 40 °C overnight and quenched with a 5% AcOH (2.5 mL/mmol). A precipitate was formed, and after filtration and washing with water **3a** was obtained as a yellow solid (55%) without further purification. Mp: 163 °C; IR (KBr) cm^{-1}: 3438, 3422, 2354, 1866, 1538, 1504, 1470, 1427, 1285,1271, 837, 764, 689, 421, 402; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.94 (d, 2H, J = 7.02), 7.62 (d, 1H, J = 15.6), 7.50 (m, 3H), 7.13 (dd, 1H, J_1 = 1.3, J_2 = 7.9), 7.06 (d, 1H, J = 1.4), 6.94 (dd, 1H, J_1 = 0.8, J_2 = 8.1), 6.32 (s, 1H), 3.95 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 188.22, 181.35, 149.30, 148.01, 140.71, 136.29, 132.61, 128.79, 128.63, 127.36, 127.25, 123.10, 120.69, 115.48, 110.69, 96.83; CI-EIMS: m/z [M^+] 295; $C_{18}H_{16}O_4$ (296.31): calcd C 72.96, H 5.44; found: C 72.99, H 5.29.

5.1.2. (*E*)-5-(4-Hydroxyphenyl)-1-phenylpent-4-ene-1,3-dione (3b)

43% as a yellow powder. Mp: 179 °C; IR (KBr) cm^{-1}: 3468, 3417, 2961, 2733, 2589, 1618, 1525, 1453, 1364, 1166, 769, 688, 418, 407; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.94 (d, 2H, J = 8.5), 7.62 (d, 1H, J = 15.7), 7.47 (m, 5H), 6.86 (d, 2H, J = 8.8), 6.52 (d, 1H, J = 15.6), 6.31 (s, 1H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 190.24, 157.61, 132.91, 130.00, 128.70, 128.57, 127.90, 127.65, 125.38, 115.68, 114.51, 99.38; CI-EIMS: m/z [M^+] 265; $C_{17}H_{14}O_3$ (266.29): calcd C 76.68, H 5.30; found: C 76.70, H 5.31.

5.1.3. (*E*)-5-(4-Methoxyphenyl)-1-phenylpent-4-ene-1,3-dione (3c)

49% as an orange powder. Mp: 130 °C; IR (KBr) cm^{-1}: 3954, 3800, 3428, 2858, 2589, 1903, 1715, 1505, 1398, 769, 395, 438; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.94 (d, 2H, J = 8.2), 7.66 (d, 1H, J = 15.9), 7.51 (m, 5H), 6.92 (d, 2H, J = 8.8), 6.53 (d, 1H, J = 15.6), 6.31 (s, 1H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 188.42, 161.66, 139.77, 136.36, 132.65, 129.93, 128.80, 127.90, 127.31, 121.10, 114.54, 96.98, 54.99; CI-EIMS: m/z [M^+] 279; $C_{18}H_{16}O_3$ (280.31): calcd C 77.12, H 5.75; found: C 77.15, H 5.77.

5.1.4. (*E*)-5-(3,5-Dimethoxyphenyl)-1-phenylpent-4-ene-1,3-dione (3d)

53% as a yellow powder. Mp: 126 °C; IR (KBr) cm^{-1}: 3898, 3867, 3805, 3750, 3342, 2837, 2335, 1734, 1518, 1501, 1471, 1323, 437, 418; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.95 (d, 2H, J = 8.2), 7.61 (d, 1H, J = 15.9), 7.50 (m, 3H), 6.70 (d, 2H, J = 2.1), 6.60 (d, 1H, J = 15.6), 6.49 (t, 1H, J = 2.1), 6.33 (s, 1H), 3.82 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ:189.55, 179.24, 161.14, 140.07, 137.04, 136.31, 132.69, 128.73, 127.45, 123.91, 107.21, 106.03, 102.43, 97.83,55.49; CI-EIMS: m/z [M^+] 309; $C_{19}H_{18}O_4$ (310.34): calcd C 73.53, H 5.85; found: C 73.55, H 5.88.

5.1.5. (*E*)-1-Phenyl-5-(3,4,5-trimethoxyphenyl)pent-4-ene-1,3-dione (3e)

50% as a yellow powder. Mp: 117 °C; IR (KBr) cm^{-1}: 3900, 3868, 3829, 3800, 3768, 3710, 3422, 2839, 2365, 1908, 1715, 1538, 1505, 1455, 1418, 1346, 431, 421, 414; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.94 (d, 2H, J = 8.2), 7.59 (d, 1H, J = 15.9), 7.50 (m, 3H), 6.79 (s, 2H), 6.55 (d, 1H, J = 15.9), 6.34 (s, 1H), 3.89 (m, 9H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 189.03, 153.86, 140.43, 140.23, 136.28, 132.78, 130.75, 128.82, 127.33, 122.76, 105.80, 97.28, 59.89, 55.71; CI-EIMS: m/z [M^+] 339; $C_{20}H_{20}O_5$ (340.13): calcd C 70.57, H 5.92; found: C 70.58, H 5.95.

5.1.6. (*E*)-5-(4-Hydroxy-3-methoxyphenyl)-1-(4-hydroxy phenyl)pent-4-ene-1,3-dione (3f)

40% as a yellow oil purified on silica gel (PE/EtOAc 6:4 as eluant). IR (KBr) cm^{-1}: 3799, 3748, 3710, 2923, 2852, 2362, 1866, 1747, 1731, 1567, 1538, 1455, 421, 410; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.94 (d, 2H, J = 8.5), 7.59 (d, 1H, J = 15.6), 7.30 (d, 1H, J = 1.8), 7.16 (dd, 1H, J_1 = 1.8, J_2 = 8.3), 6.95 (d, 2H, J = 8.8), 6.88 (d, 1H, J = 7.9), 6.81 (d, 1H, J = 15.6), 6.49 (s, 1H), 3.91 (s, 9H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 188.84, 179.16, 161.87, 148.99, 147.97, 139.52, 129.76, 128.01, 127.52, 122.78, 120.74, 115.51, 115.42, 110.55, 96.22, 55.49 ; CI-EIMS: m/z [M^{2+}] 310; $C_{18}H_{16}O_5$ (312.10): calcd C 69.22, H 5.16; found: C 69.21, H 5.17.

5.1.7. (*E*)-1,5-Bis(4-hydroxyphenyl)pent-4-ene-1,3-dione (3g)

43% as a dark yellow solid. Mp: 194 °C; IR (KBr) cm^{-1}: 3820, 33730, 3710, 2718, 2507, 2363, 1907, 1728, 1557, 1514, 1435, 1372, 1168, 963, 837, 487, 419, 409; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.94 (d, 2H, J = 8.5), 7.60 (d, 1H, J = 15.87), 7.54 (d, 2H, J = 8.8), 6.94 (d, 2H, J = 8.5) 6.89 (d, 2H, J = 8.5), 6.68 (d, 1H, J = 15.6), 6.50 (s, 1H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 188.79, 179.21, 161.87, 159.52, 139.17, 129.94, 129.75, 128.00, 127.06, 120.55, 115.98, 115.52, 96.19; CI-EIMS: m/z [M^{2+}] 280; $C_{17}H_{14}O_4$ (282.09): calcd C 72.33, H 5.00; found: C 72.34, H 5.01.

5.1.8. (*E*)-1-(4-Hydroxyphenyl)-5-(4-methoxyphenyl)pent-4-ene-1,3-dione (3h)

47% as a yellow solid. Mp: 165 °C; IR (KBr) cm^{-1}: 3852, 3800, 3627, 2856, 1914, 1716, 1557, 1460, 1211, 845, 682, 545; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.93 (d, 2H, J = 8.5), 7.61 (m, 3H), 6.98 (d, 2H, J = 8.8) 6.52 (d, 2H, J = 8.8), 6.73 (d, 1H, J = 15.6), 6.52 (s, 1H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 189.03, 178.83, 161.91, 161.46, 138.74, 129.79, 129.71, 128.05, 121.26, 115.51, 114.49, 96.35, 54.95; CI-EIMS: m/z [M^+] 295; $C_{18}H_{16}O_4$ (296.10): calcd C 72.96, H 5.44; found: C 72.95, H 5.43.

5.1.9. (*E*)-5-(3,5-Dimethoxyphenyl)-1-(4-hydroxyphenyl)pent-4-ene-1,3-dione (3i)

51% as a yellow solid. Mp: 139 °C; IR (KBr) cm^{-1}: 3900, 3852, 3742, 3648, 3496, 3418, 2839, 2364, 1887, 1539, 1455, 1350, 964, 823, 419; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.95 (d, 2H, J = 8.2), 7.57 (d, 1H, J = 15.9), 6.96 (d, 2H, J = 8.2) 6.93 (d, 1H, J = 10.4), 7.85 (d, 2H, J = 2.1), 6.57 (s, 1H), 6.52 (brt, 1H,) 3.84 (s, 6H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 189.82, 177.57, 162.14, 161.40, 138.78, 137.40, 130.71, 127.97, 124.19, 115.58, 105.82, 102.10, 97.04, 54.96; CI-EIMS: m/z [M^+] 325; $C_{19}H_{18}O_5$ (326.11): calcd C 69.93, H 5.56; found: C 69.90, H 5.55.

5.1.10. (*E*)-1-(4-hydroxyphenyl)-5-(3,4,5-trimethoxyphenyl) pent-4-ene-1,3-dione (3j)

49% as a yellow solid. Mp: 129 °C; IR (KBr) cm^{-1}: 3880, 3549, 2847, 2325, 1903, 1683, 1652, 1524, 1348, 1211, 1189, 934, 780, 682; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.89 (d, 2H, J = 8.5), 7.57 (d, 1H, J = 15.6), 6.89 (d, 2H, J = 8.5) 6.77 (s, 2H), 6.88 (d, 1H, J = 2.1), 6.52 (d, 1H, J = 15.6), 6.27 (s, 1H), 3.90 (s, 6H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 189.14, 178.12, 160.01, 153.53, 139.89, 139.52, 130.85, 129.87, 129.14, 122.78, 115.63, 105.21, 97.16, 61.10, 56.25; CI-EIMS: m/z [M^+] 355; $C_{20}H_{20}O_6$ (356.36): calcd C 67.41, H 5.66; found: C 67.43, H 5.65.

5.1.11. (*E*)-1,5-Bis(4-hydroxy-3-methoxyphenyl)pent-4-ene-1,3-dione (3k)

56% as a pale orange solid. Mp: 172 °C; IR (KBr) cm^{-1}: 3890, 3831, 3658, 3410, 3387, 3004, 2927, 2843, 2659, 1887, 1715, 1516, 1469, 1310, 1263, 1034, 960, 790, 421; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 8.55 (–OH), 8.20 (–OH), 7.61 (s, 2H), 7.58 (d, 1H, J = 7.3), 7.31 (d, 1H, J = 1.8), 7.16 (dd, 1H, J_1 = 1.8, J_2 = 8.2), 6.94 (d, 1H, J = 8.5), 6.88 (d, 1H, J = 8.2), 6.72 (d, 1H, J = 15.9), 6.53 (s, 1H), 3.91 (s, 3H), 3.89 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 189.06, 178.72, 151.42, 149.01, 147.98, 147.70, 139.49, 128.46, 127.53, 122.80, 122.09, 120.68, 115.43, 114.98, 110.54, 110.38, 96.41, 55.55, 55.51; CI-EIMS: m/z [M^{2+}] 340; $C_{19}H_{18}O_6$ (342.11): calcd C 66.66, H 5.30; found: C 66.68, H 5.33.

5.1.12. (*E*)-1-(4-Hydroxy-3-methoxyphenyl)-5-(4-hydroxy phenyl)pent-4-ene-1,3-dione (3l)

49% as a orange solid. Mp: 124 °C; IR (KBr) cm^{-1}: 3780, 3651, 3411, 2999, 2867, 2833, 1890, 1746, 1539, 1475, 1215, 1127, 983, 879, 775, 685; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.61 (s, 2H), 7.58 (d, 1H, J = 6.7), 7.53 (d, 2H, J = 8.5), 6.94 (d, 1H, J = 8.5), 6.90 (d, 2H, J = 8.5), 6.67 (d, 1H, J = 15.9), 6.54 (s, 1H), 3.90 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 189.05, 178.75, 159.50, 151.42, 147.70, 139.16, 129.96, 128.46, 127.09, 122.13, 120.48, 116.01, 115.00, 110.40, 96.45, 55.56; CI-EIMS: m/z [M^{2+}] 310; $C_{18}H_{16}O_5$ (312.10): calcd C 69.22, H 5.16; found: C 69.20, H 5.17.

5.1.13. (*E*)-1-(4-Hydroxy-3-methoxyphenyl)-5-(4-methoxy phenyl)pent-4-ene-1,3-dione (3m)

45% as a yellow solid. Mp: 157 °C; IR (KBr) cm^{-1}: 3780, 3334, 3005, 2967, 2845, 1587, 1643, 1557, 1475, 1310, 1230, 1171, 695; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.62 (m, 5H), 6.98 (d, 2H, J = 8.8), 6.93 (d, 1H, J = 8.8), 6.72 (d, 1H, J = 15.9), 6.56 (s, 1H), 3.91 (s, 3H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 189.28, 178.38, 161.46, 151.49, 147.72, 138.69, 129.70, 128.42, 128.06, 122.14, 121.18, 114.97, 114.50, 110.41, 96.57, 55.53, 54.96; CI-EIMS: m/z [M^+] 325; $C_{19}H_{18}O_5$ (326.11): calcd C 69.93, H 5.56; found: C 69.91, H 5.55.

5.1.14. (*E*)-5-(3,5-Dimethoxyphenyl)-1-(4-hydroxy-3-methoxy phenyl pent-4-ene-1,3-dione (3n)

47% as a yellow solid. Mp: 141 °C; IR (KBr) cm^{-1}: 3890, 3831, 3760, 3288, 2941, 2842, 1635, 1519, 1471, 1438, 1349, 1287, 1204, 1066, 960, 791; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.57 (m, 3H), 6.94 (d, 1H, J = 7.9), 6.84 (m, 3H), 6.56 (s, 1H), 6.50 (s, 1H), 3.91 (s, 3H), 3.81 (s, 6H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 190.01, 177.15, 161.37, 151.63, 147.72, 138.80, 137.39, 128.45, 124.10, 122.39, 115.01, 110.45, 105.84, 102.10, 97.31, 55.56, 54.97; CI-EIMS: m/z [M^+] 355; $C_{20}H_{20}O_6$ (356.12): calcd C 67.41, H 5.66; found: C 67.43, H 5.69.

5.1.15. (E)-1-(4-Hydroxy-3-methoxyphenyl)-5-(3,4,5-tri methoxyphenyl)pent-4-ene-1,3-dione (3o)

48% as a yellow solid. Mp: 160 °C; IR (KBr) cm^{-1}: 3800, 3788, 3309, 3099, 3004, 1975, 1715, 1567, 1473, 1315, 1128, 554; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.57 (m, 3H), 6.96 (m, 3H), 6.81 (d, 1H, J = 15.9), 6.53 (s, 1H), 3.90 (s, 3H), 3.86 (s, 6H), 3.77 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 189.47, 177.41, 153.56, 150.28, 146.88, 140.08, 139.34, 130.83, 129.10, 122.65, 122.27, 114.29, 109.69, 105.35, 105.13, 7.17, 61.02, 56.26, 56.15; CI-EIMS: m/z [M^+] 385; $C_{18}H_{16}N_2O_3$ (308.11): calcd C 70.12, H 5.23, N 9.09; found: C 70.09, H 5.17, N 9.07.

5.1.16. General procedure for the synthesis of pyrazole (4a–o)

To a stirred solution of 3a (1 equiv mol) in AcOH (2.5 mL/1 mmol.) hydrazine monohydrate (2.3 equiv mol) was added. The reaction was stirred at 50 °C overnight and quenched with the addition of brine and extraction with EtOAc, to give **6a** as a yellow solid (80%) without further purification. Mp: 158 °C; IR (KBr) cm^{-1}: 3821, 3801, 3711, 2839, 2364, 1898, 1733, 1513, 1460, 1368, 1029, 961, 764, 420; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.85 (dd, 2H, J_1 = 1.23, J_2 = 7.3), 7.41 (t, 2H, J = 7.6), 7.30 (t, 1H, J = 7.3), 7.21 (d, 1H, J = 2.1), 7.14 (s, 1H), 7.05 (s, 1H), 7.01 (d, 1H, J=), 6.87 (s, 1H), 6.83 (d, 1H, J = 7.9), 3.90 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 147.86, 147.03, 132.89, 130.18, 129.22, 128.69, 128.55, 127.62, 125.33, 120.79, 120.45, 115.20, 114.90, 109.10, 99.35, 55.44; CI-EIMS: m/z [M^+] 291; $C_{18}H_{16}N_2O_2$ (292.12): calcd C 73.95, H 5.52, N 9.58; found: C 73.92, H 5.55, N 9.60.

5.1.17. (*E*)-4-(2-(5-Phenyl-1*H*-pyrazol-3-yl)vinyl)phenol (4b)

79% as a yellow solid. Mp: 160 °C; IR (KBr) cm^{-1}: 3004, 2601, 1897, 1684, 1512, 1235, 1169, 964, 700, 532; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.85 (brdd, 2H, J = 7.3), 7.39 (m, 5H), 7.18 (d, 1H, J = 16.4), 6.99 (d, 1H, J = 16.5), 6.85 (m, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 157.64, 149.21, 149.95, 132.81, 132.60, 130.11, 128.91, 127.70, 127.26, 125.40, 115.71, 114.46, 99.40 ; CI-EIMS: m/z [M^+] 261; $C_{17}H_{14}N_2O$ (262.11): calcd C 77.84, H 5.38, N 10.68; found: C 77.84, H 5.40, N 10.69.

5.1.18. (*E*)-3-(4-Methoxystyryl)-5-phenyl-1*H*-pyrazole (4c)

84% as a pale yellow solid. Mp: 148 °C; IR (KBr) cm^{-1}: 3649, 3336, 2943, 2379, 2356, 1987, 1670, 1620, 1416, 832, 700, 551, 533; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.72 (brdd, 2H, J = 8.2), 7.38 (m, 5H), 7.05 (d, 1H, J = 16.5), 6.88 (m, 3H), 6.71 (m, 1H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 159.82, 149.23, 146.07, 132.81, 129.71, 128.78, 127.82, 127.74, 125.45, 115.37, 114.28, 99.61, 54.84; CI-EIMS: m/z [M^+] 275; $C_{18}H_{16}N_2O$ (276.12): calcd C 78.24, H 5.84, N 10.14; found: C 78.26, H 5.85, N 10.17.

5.1.19. (*E*)-3-(3,5-Dimethoxystyryl)-5-phenyl-1*H*-pyrazole (4d)

77% as an amorphous solid. IR (KBr) cm^{-1}: 3587, 3466, 3400, 2965, 2476, 2455, 1740, 1665, 1489, 1402, 1256, 1213, 783, 582, 320; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.71 (brdd, 2H, J = 7.9), 7.41 (m, 3H), 7.03 (brd, 2H), 6.88 (brs, 1H), 6.63 (brd, 2H), 6.41 (brt, 1H), 3.81 (s, 6H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 161.37, 148.89, 146.23, 139.17, 132.39, 130.16, 128.85, 127.90, 125.49, 118.37, 104.52, 100.18, 100.14, 54.89; CI-EIMS: m/z [M^+] 305; $C_{19}H_{18}N_2O_2$ (306.13): calcd C 74.49, H 5.92, N 9.14; found: C 74.48, H 5.90, N 9.15.

5.1.20. (*E*)-5-Phenyl-3-(3,4,5-trimethoxystyryl)-1*H*-pyrazole (4e)

75% as a pale yellow solid. Mp: 146 °C; IR (KBr) cm^{-1}: 3566, 3487, 3451, 3418, 2838, 2362, 1662, 1506, 1458, 1335, 763, 692, 421; ^1H NMR (300 MHz, $CDCl_3$) δ: 7.71 (brdd, 2H, J = 7.9), 7.44 (brdd, 2H, J = 7.2), 7.36 (brdd, 1H, J = 7.4), 7.02 (m, 2H), 6.74 (m, 3H), 3.91 (s, 6H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 153.50, 148.75, 147.27, 138.36, 132.39, 131.29, 130.84, 129.00, 128.45, 125.69, 117.29, 103.71, 100.20, 61.06, 56.18; CI-EIMS: m/z [M^+] 335; $C_{20}H_{20}N_2O_3$ (336.14): calcd C 71.41, H 5.99, N 8.33; found: C 71.42, H 5.98, N 8.34.

5.1.21. (*E*)-4-(2-(5-(4-Hydroxyphenyl)-1*H*-pyrazol-3-yl)vinyl)-2-methoxyphenol (4f)

81% as a pale yellow solid. Mp: 219 °C; IR (KBr) cm^{-1}: 3875, 3813, 3710, 2938, 2701, 2363, 1887, 1715, 1519, 1455, 1269, 963, 943, 787; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.65 (d, 2H, J = 8.8), 7.19 (d, 1H, J = 1.8), 7.11 (s, 1H), 7.04 (s, 1H), 6.98 (dd, 1H, J_1 = 1.8, J_2 = 6.3), 6.88 (d, 2H, J = 8.5), 6.82 (d, 1H, J = 8.1), 6.75 (s, 1H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 157.32, 148.47, 147.83, 146.89, 146.65, 129.79, 129.36, 126.74, 124.13, 120.35, 115.68, 115.51, 115.16, 109.04, 98.44, 55.43; CI-EIMS: m/z [M^+] 307; $C_{18}H_{16}N_2O_3$ (308.11): calcd C 70.12, H 5.23, N 9.09; found: C 70.15, H 5.24, N 9.10.

5.1.22. (*E*)-4-(2-(5-(4-Hydroxyphenyl)-1*H*-pyrazol-3-yl) vinyl) phenol (4g)

70% as an amorphous solid. IR (KBr) cm^{-1}: 3710, 3648, 3417, 2572, 2358, 1867, 1731, 1557, 1455, 1048, 733, 700, 461; ^1H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.61 (d, 2H, J = 8.5), 7.39 (d, 2H, J = 8.5), 7.15 (d, 1H, J = 16.5), 7.01 (d, 1H, J = 16.5), 6.88 (d, H, J = 8.5), 6.81 (d, 2H, J = 8.5), 6.78 (s, 1H); ^{13}C NMR (75 MHz, $(CD_3)_2$-CO) δ: 157.52, 157.33, 149.55, 146.98, 129.55, 128.82, 127.81, 126.75, 124.16, 115.65, 115.49, 115.28, 98.45; CI-EIMS: m/z [M^+] 277; $C_{17}H_{14}N_2O_2$ (278.10): calcd C 73.37, H 5.07, N 10.07; found: C 73.40, H 5.08, N 10.09.

5.1.23. (*E*)-4-(3-(4-Methoxystyryl)-1*H*-pyrazol-5-yl)phenol (4h)

82% as pale yellow solid. Mp: 187 °C; IR (KBr) cm^{-1}: 3833, 3800, 3675, 2987, 2620, 1950, 1716, 1683, 1558, 1530, 1414, 1389, 1151, 1066, 1012, 957, 841, 535; ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.68 (d, 2H, J = 8.5), 7.49 (d, 2H, J = 8.8), 7.09 (q, 2H, J = 16.5), 6.93 (d, H, J = 8.5), 6.89 (d, 2H, J = 8.5), 6.75 (s, 1H), 3.80 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 159.73, 157.36, 148.55, 146.57, 142.36, 129.90, 129.17, 128.34, 127.67, 126.77, 124.10, 116.13. 115.53, 114.22, 98.56, 54.79 ; CI-EIMS: m/z [M^+] 291; $C_{18}H_{16}N_2O2$ (292.12): calcd C 73.95, H 5.52, N 9.58; found: C 73.93, H 5.49, N 9.55.

5.1.24. (E)-4-(3-(3,5-dimethoxystyryl)-1H-pyrazol-5-yl)phenol (4i)

78% as a pale yellow solid. Mp: 294 °C; IR (KBr) cm^{-1}: 3820, 3800, 3710, 2925, 2840, 2601, 2362, 1907, 1586, 1512, 1457, 1343, 1204, 1151, 1066, 826, 784; ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.68 (d, 2H, J = 8.5), 7.12 (d, 2H, J = 2.1), 6.89 (d, 2H, J = 8.8), 6.80 (s, 1H), 6.72 (d, 2H, J = 2.1), 6.41 (brt, 1H), 3.80 (s, 6H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 161.35, 157.42, 148.01, 146.99, 139.33, 129.49, 126.78, 123.99, 119.17, 115.56, 104.32, 99.98, 98.95, 54.82; CI-EIMS: m/z [M^+] 321; $C_{19}H_{18}N_2O_3$ (322.13): calcd C 70.79, H 5.63, N 8.69; found: C 70.84, H 5.65, N 8.70.

5.1.25. (E)-4-(3-(3,4,5-trimethoxystyryl)-1H-pyrazol-5-yl) phenol (4j)

81% as a pale yellow solid. Mp: 246 °C; IR (KBr) cm^{-1}: 3870, 3821, 3765, 3749, 1549, 1533, 1430, 1415, 1394, 1200, 1172, 1151, 892, 450; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.67 (d, 2H, J = 8.58), 7.12 (d, 2H, J = 1.1), 6.89 (d, 2H, J = 8.5), 6.86 (s, 2H), 6.77 (s, 1H) 3.86 (s, 6H), 3.73 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 157.46, 153.79, 148.13, 146.35, 138.46, 132.95, 129.66, 126.78, 123.73, 118.05, 115.60, 103.90, 98.73, 59.79, 55.60; CI-EIMS: m/z [M^+] 351; $C_{20}H_{20}N_2O_4$ (352.14): calcd C 68.17, H 5.72, N 7.95; found: C 68.15, H 5.75, N 7.94.

5.1.26. (E)-4-(2-(5-(4-hydroxy-3-methoxyphenyl)-1H-pyrazol-3-yl)vinyl)-2-methoxyphenol (4k)

83% as a yellow solid. Mp: 97 °C; IR (KBr) cm^{-1}: 3846, 3458, 3400, 2924, 2853, 2358, 1715, 1515, 1455, 1276, 814, 421; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.22 (d, 1H, J = 1.6), 7.18 (dd, 1H, J_1 = 1.8, J_2 = 8.1), 6.92 (m, 6H), 6.59 (s, 1H) 3.86 (s, 6H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 148.46, 148.17, 147.84, 147.75, 146.91, 146.83, 146.63, 129.84, 129.34, 124.35, 120.33, 118.43, 115.74, 115.17, 109.04, 108.97, 98.57, 55.48, 55.43; CI-EIMS: m/z [M^+] 337; $C_{19}H_{18}N_2O_4$ (338.12): calcd C 67.44, H 5.36, N 8.28; found: C 67.45, H 5.37, N 8.27.

5.1.27. (E)-4-(3-(4-hydroxystyryl)-1H-pyrazol-5-yl)-2-methoxy phenol (4l)

83% as an amorphous solid. IR (KBr) cm^{-1}: 3780, 3761, 3317, 2498, 2458, 1961, 1745, 1312, 1168, 993, 567, 490, 461; ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.46 (d, 1H, J = 2.1), 7.40 (d, 2H, J = 8.5), 7.30 (dd, 1H, J_1 = 1.83, J_2 = 7.9), 7.15 (d, 1H, J = 16.5), 6.99 (d, 1H, J = 16.5), 6.88 (d, 1H, J = 7.9), 6.85 (d, 2H, J = 8.5), 6.80 (s, 1H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 157.61, 148.77, 147.78, 146.81, 146.69, 129.79, 128.75, 127.84, 124.30, 118.52, 115.67, 115.28, 115.21, 108.95, 98.67, 55.45; CI-EIMS: m/z [M^+] 307; $C_{18}H_{16}N_2O_3$ (308.11): calcd C 70.12, H 5.23, N 9.09; found: C 70.14, H 5.21, N 9.06.

5.1.28. (E)-2-methoxy-4-(3-(4-methoxystyryl)-1H-pyrazol-5-yl) phenol (4m)

74% as a brown solid. Mp: 164 °C; IR (KBr) cm^{-1}: 4001, 3765, 3456, 2980, 2748, 2567, 1589, 1503, 1457, 1345, 987, 937, 435; ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.49 (d, 1H, J = 2.1), 7.46 (d, 2H, J = 8.4), 7.33 (dd, 1H, J_1 = 2.1, J_2 = 8.2), 7.21 (d, 1H, J = 16.6), 7.03 (d, 1H, J = 16.4), 6.91 (d, 1H, J = 8.2), 6.90 (d, 2H, J = 8.9), 6.86 (s, 1H), 3.86 (s, 3H), 3.78 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 159.74, 148.57, 147.76, 146.66, 145.14, 129.89, 129.20, 127.67, 124.36, 119.92, 118.45, 116.19, 115.35, 115.22, 114.23, 109.00, 98.70, 55.49, 54.79; CI-EIMS: m/z [M^+] 321; $C_{19}H_{18}N_2O_3$ (322.13): calcd C 70.79, H 5.63, N 8.69; found: C 70.75, H 5.63, N 8.70.

5.1.29. (E)-4-(3-(3,5-dimethoxystyryl)-1H-pyrazol-5-yl)-2-methoxyphenol (4n)

82% as a pale yellow solid. Mp: 184 °C; IR (KBr) cm^{-1}: 3890, 3801, 3743, 2926, 2835, 2601, 1584, 1415, 1355, 949, 817, 420; ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.41 (brd, 1H), 7.30 (brdd, 1H, J = 7.9), 7.23 (d, 1H, J = 16.4), 7.15 (d, 1H, J = 16.5), 6.90 (d, 1H, J = 8.22), 6.87 (s, 1H), 6.71 (brd, 2H), 6.41 (s, 1H), 3.88 (s, 3H), 3.79 (s, 6H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 161.35, 148.21, 147.81, 146.90, 146.76, 139.30, 129.64, 123.93, 119.19, 118.52, 115.31, 109.02, 104.35, 100.02, 99.16, 55.49, 54.83; CI-EIMS: m/z [M^+] 351; $C_{20}H_{20}N_2O_4$ (352.14): calcd C 68.17, H 5.72, N 7.95; found: C 65.20, H 5.70, N 7.96.

5.1.30. (E)-2-methoxy-4-(3-(3,4,5-trimethoxystyryl)-1H-pyrazol-5-yl)phenol (4o)

83% as a pale yellow solid. Mp: 191 °C; IR (KBr) cm^{-1}: 3910, 3855, 3821, 3799, 2843, 1598, 1512, 1445, 1434, 1003, 957, 419; ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.46 (d, 1H, J = 1.8), 7.31 (dd, 1H, J_1 = 1.9, J_2 = 8.2), 7.15 (s, 2H), 6.90 (d, 1H, J = 7.9), 6.85 (s, 3H), 3.87 (s, 3H), 3.84 (s, 6H), 3.73 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 154.83, 147.83, 146.78, 132.94, 129.71, 124.09, 118.54, 118.08, 115.29, 109.13, 104.09, 98.91, 59.81, 55.61, 55.49; CI-EIMS: m/z [M^+] 381; $C_{21}H_{22}N_2O_4$ (382.15): calcd C 65.96, H 5.80, N 7.33; found: C 65.98, H 5.79, N 7.35.

5.1.31. (E)-2-methoxy-4-(3-(3-methoxystyryl)-1H-pyrazol-5-yl)phenol (4p)

83% as a brown oil. IR (KBr) cm^{-1}: 3981, 3812, 3334, 2758, 2697, 1635, 1601, 1457, 1278, 1003, 988, 565, 461; ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ: 7.49 (d, 1H, J = 1.83), 7.32 (dd, 1H, J_1 = 1.8, J_2 = 8.2), 7.26 (d, 1H, J = 7.9), 7.18 (m, 5H), 6.92 (d, 1H, J = 7.9), 6.85 (brdd, 1H), 3.87 (s, 3H), 3.84 (s, 6H), 3.79 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 172.05, 160.26, 147.91, 146.89, 138.67, 129.88, 129.81, 123.86, 119.04, 118.76, 118.71, 115.46, 113.62, 111.61, 109.16, 55.54, 54.76; CI-EIMS: m/z [M^+] 321; $C_{19}H_{18}N_2O_3$ (322.12): calcd C 70.79, H 5.63, N 8.69; found: C 70.81, H 5.64, N 8.66.

5.2. Biology

5.2.1. Cell Culture and Cytotoxicity Assay

The SH-SY5Y human neuroblastoma cell line was obtained from ATCC (LGC Promochem Teddington, UK) and cultured in 50% DMEM and 50% F-12 supplemented with 10% foetal bovine serum, 2 mM l-glutamine, penicillin (100 μg/mL), and streptomycin (100 μg/mL). The MeWo Human melanoma cell line was obtained from ATCC (LGC Promochem Teddington, UK) and cultured in DMEM supplemented with 10% foetal bovine serum, 2 mM l-glutamine, penicillin (100 μg/mL), and streptomycin (100 μg/mL). For cytotoxicity assays, cells were plated on 24-well plates and grown for 48 h in the presence or absence of Curcumin or the synthesized compounds. On the experimental day, cells were washed twice in Locke's solution (134 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 10 mM HEPES [pH 7.6], 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM sucrose) and incubated for 90 min with MTT (250 μg/mL in Locke's solution) at 37 °C. Reactions were then stopped and the crystals solubilized in isopropyl alcohol/HCl before being read at 570 nm in a spectrophotometer. To determine $_{IC50}$ values, data were plotted and fitted using the Kaleidagraph software (Synergy software, Reading, PA).

5.2.2. Immunocytochemistry

MeWo were plated on glass coverslips and grown to subconfluency in the presence or absence of drugs for 24 h. Cover slips were then fixed in paraformaldehyde (3.7%) for 20 min at RT. Cells were then washed once in phosphate buffered saline (PBS) and permeabilized in ice cold 80% MeOH for 10 min in ice. Cells were then washed twice in PBS and blocked with 0,2% cold fish gelatine. Anti-tubulin (mouse) primary antibody (Sigma-Aldrich; 1:1000) was incubated for 20' at RT, and after three further washes, coverslips were incubated with a goat anti-mouse Alexa fluo 488 (Molecular Probes, 1:2000) and DAPI (Cell Signaling 1:10.000). Slides were then visualized in a Leica DMI6000B wide field microscope.

5.2.3. Tubulin Polymerization Assay

To measure the degree of tubulin polymerization, we used an adaptation of the methods described by Minotti et al.[15] In brief, cells were grown in 75 cm^2 flasks in the presence or absence of drugs (3 x IC_{50}) for 16 h. Cells were then tripsinised and centrifuged at 600*g* for 3 min, washed once by PBS at room temperature. Cells were then re-suspended in 70 μL of hypotonic buffer (20 mM Tris-Hcl pH 6.8, 1 mM MgCl2, 2 mM EGTA, protease inhibitors, 0.5% NP-40) containing 4 μg/mL paclitaxel. Lysates were incubated for 10 min at room temperature. Lysates were then corrected for protein amounts (Bradford assay, Sigma-Aldrich) and 50 μg (corresponding to 50 μls) was centrifuged at 13000 rpm for 15 min at room temperature. Supernatant and pellet were then re-suspended in equal volumes of SDS-loading buffer and run on a 10% SDS−PAGE polyacrylamide gel. After transfer of proteins to nitrocellulose (blocked in 5% milk), tubulin was identified with an anti-tubulin primary antibody (1:1000, Sigma-Aldrich) and anti mouse secondary antibody peroxidase-conjugated (1:8000, Amersham Bioscience) and visualized by chemiluminescence (Supersignal WestPico, Pierce).

5.2.4. Flow-Cytometric Analysis of Cell-Cycle Status

MeWo grown in the presence or absence of compounds for 16 h were washed once in PBS and re-suspended in 1 mL of 30:70 ice cold PBS/EtOH and stored at −20 °C. Cells were then washed twice in PBS and re-suspended in PBS containing RNAse (100 μg/mL) for 30' at r.t. DNA was then stained with a PBS solution containing 5 mM EDTA and 100 μg/mL propidium iodide for 30 min at RT in the dark. Cell cycle analysis was determined with a FACSVantage SE DiVa (Becton Dickinson).

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