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Synthesis and in vitro antitumor activity of new 4,5-dihydropyrazole derivatives

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ABSTRACT

A series of 3,5-diaryl-4,5-dihydropyrazole regioisomers, and their 1-acetylated derivatives, bearing a 3,4,5-trimethoxyphenyl moiety combined with a variety of substituted phenyl rings, was synthesized and evaluated for antitumor activity. Results of the in vitro assay against a non-small cell lung carcinoma cell line (NCI-H460) showed several compounds to be endowed with cytotoxicity in micromolar to submicromolar range, depending on substitution pattern and position of aryl rings on 4,5-dihydropyrazole core. Potent and selective activity was also observed in the NCI 60 human cancer cell line panel. 5-(3,4,5-Trimethoxyphenyl)pyrazolines **31** and **39** were found to possess potent antiproliferative activity against SR and MDA-MB-435, with GI_{50} inhibitory values in nanomolar range. Structure–activity relationships revealed that introduction of a (hydroxy)acetyl group at N-1 of inactive 5-(3,4,5-trimethoxyphenyl)pyrazolines, results in a clear in vitro activating effect. Compound **31** (IC_{50} = 5.16 μM) showed inhibition of tubulin polymerization comparable to that of CA-4 (IC_{50} = 4.92 μM).

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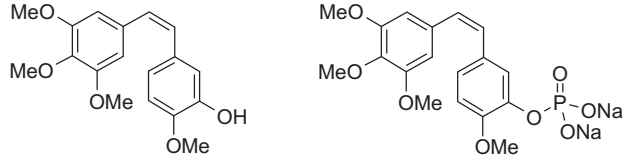
1. Introduction

Combretastatins are a group of antimitotic agents derived from the African Willow tree (*Combretum caffrum*) and they are powerful reversible inhibitors of tubulin polymerization. Among natural combretastatins, Combretastatin A-4 (3,4,5-trimethoxy-3′-hydroxy-4′-methoxy-(*Z*)-stilbene, CA-4, **1**) (Fig. 1) was found to be the most potent as antiproliferative agent against a broad range of cancer cells including multi-drug-resistant (MDR) cell lines.[1] CA-4 inhibits mitosis and microtubule assembly and is a competitive inhibitor of colchicine binding to tubulin.[2] In the recent literature it has been reported that drugs binding to the colchicine domain are intensively investigated as vascular-disrupting agents for cancer therapy.[3] CA-4 itself is poorly water-soluble and several attempts have been made to produce an active soluble derivative.[4] A disodium phosphate derivative Combretastatin A-4 phosphate (CA-4P, **2**, Zybrestat) (Fig. 1) has shown promising results in phase I/II cancer clinical trials.[5]

As microtubule inhibitor, CA-4P act as a vascular-disrupting agent that rapidly depolymerizes microtubules of newly formed vasculatures and subsequently shut down the blood supply to tumors.[6] Studies on CA-4 have established that *cis*-orientation of ethenyl bridge is essential for strong cytotoxicity. However, during storage and administration, *cis*-olephine double bond is prone to isomerize into *trans*-form, resulting in dramatic reduction in both antitubulin activity and cytotoxicity. With the aim of developing *cis*-restricted biologically active analogs, a number of compounds containing different 1,2-substituted heterocyclic rings as replacement for the stilbene core of CA-4 have been reported.[7] On the other hand, there is a limited number of analogs where the stilbene core of CA-4 has been replaced with 1,3-substituted heterocycles. Examples of heterocyclic bridges include isoxazolines,[8] oxadiazolines,[9] and pyrazoles.[10] In our efforts to discover new potential anti-cancer CA-4 based chemotherapeutic agents,[11] we decided to explore series of 3,5-diaryl-4,5-dihydropyrazole compounds bearing a 3,4,5-trimethoxyphenyl moiety as the prerequisite for potent cytotoxicity,[6d] combined with a variety of substituted phenyl rings, while the ethenyl bridge in the CA-4 skeleton was replaced with a structurally flexible non-aromatic nitrogen heterocycle which would augment biological solubility. Recently, Johnson et al. developed a series of pyrazolines and a range of N-acetylated derivatives bearing in particular a 3,4,5-trimethoxyphenyl group in 3-position of the heterocyclic ring.[12] Among these, a non-acetylated pyrazoline with the same substituents as CA-4 was the most active compound as both in vitro antitumor and



1: Combretastatin A-4 (**CA-4**)

2: **CA-4P**

Figure 1.

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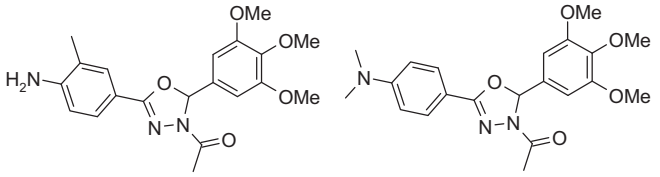


Figure 2.

microtubules disrupting agent; in contrast, the N-acetylated derivative of the same compound showed poor activity. Moreover, earlier reported studies on the similar acetylated oxadiazoline series[9a] demonstrated compounds **3**[13] and **4**[14] (Fig. 2), bearing the acetyl moiety in position adjacent to a 3,4,5-trimethoxyphenyl group, to be endowed with promising in vitro and in vivo antineoplastic properties due to their ability to inhibit a variety of cancer cell lines, including multi-drug resistant cells, and microtubules polymerization.

In view of these findings, substitution on N-1 nitrogen atom of the 5-(3,4,5-trimethoxyphenyl)pyrazolines should be informative to evaluate the effect on the activity of these series of compound. Therefore, a new series of 3-(3,4,5-trimethoxyphenyl)-4,5-dihydropyrazole **A**, and their 1-acetyl derivatives **B** were designed along with **C** and **D** analogs, respectively, bearing a 3,4,5-trimethoxyphenyl ring in 5-position, for SARs studies on series of 4,5-dihydropyrazole regioisomers (Fig. 3).

Moreover, the 1-acetyl moiety in representative **D** analogs was further functionalized, that is, introduction of hydroxy or acethoxy groups (**E** analogs), for further SARs studies (Fig. 3). Here we report the synthesis of these novel 4,5-dihydropyrazole-based CA-4 analogs and results of their in vitro biological activities as both antitumor agents and tubulin polymerization inhibitors.

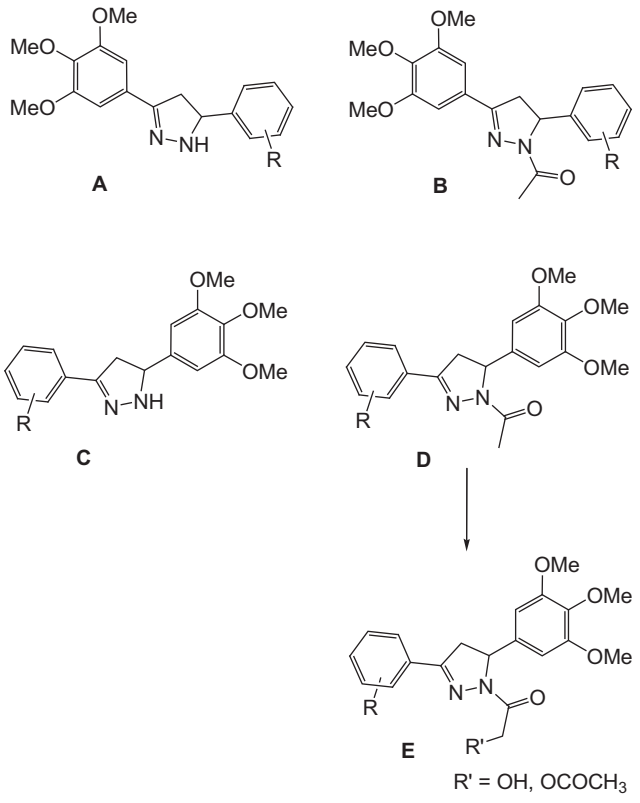


Figure 3. Pyrazoline-based CA-4 analogs.

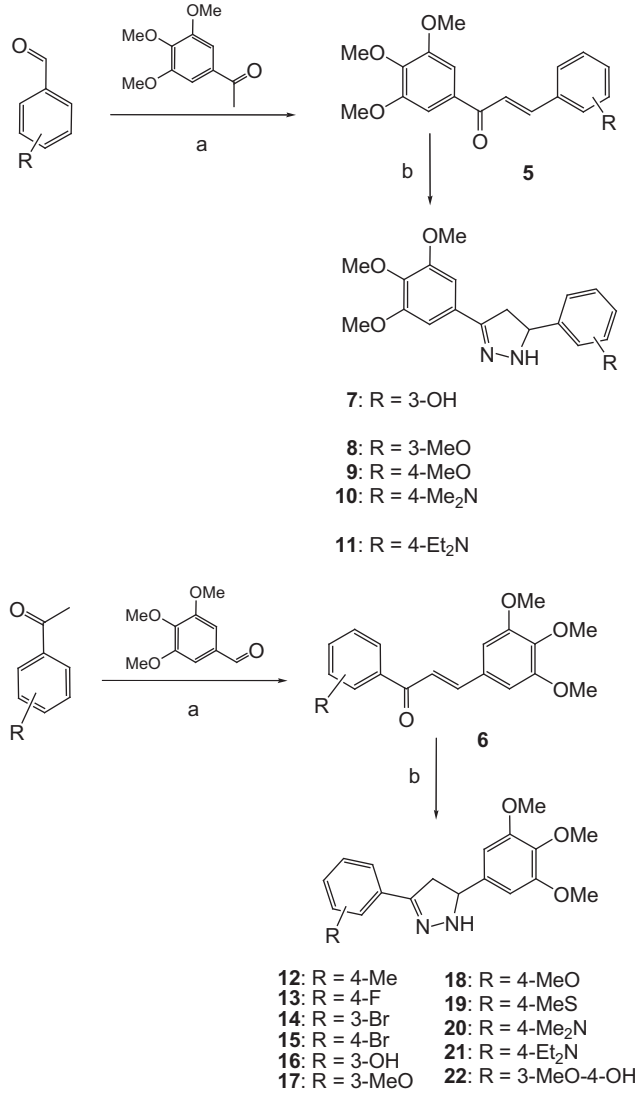
2. Results and discussion

2.1. Chemistry

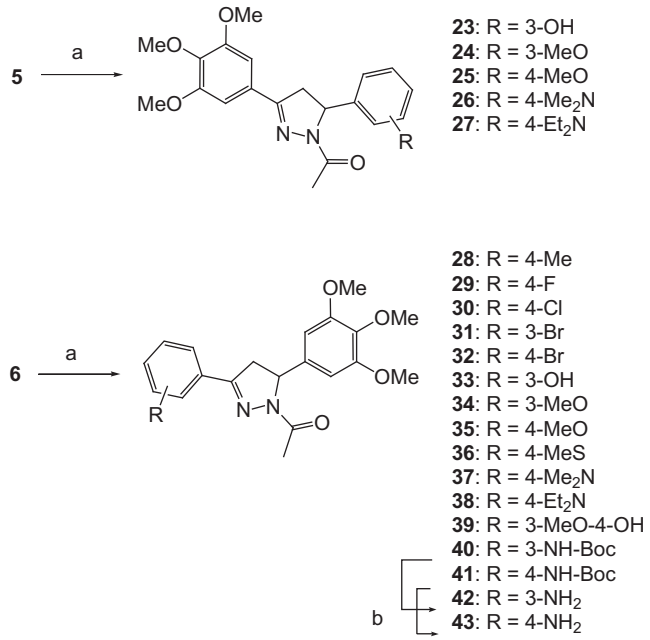
3,5-Diaryl-4,5-dihydropyrazole derivatives were easily prepared by the reaction sequence showed in Schemes 1–3. The starting materials for the synthesis of compounds **A** and **B** were chalcones **5**, prepared through the well-established Claisen–Schmidt condensation[15] of 3,4,5-trimethoxyacetophenone with substituted benzaldehydes in methanol solution in presence of NaOH (Scheme 1).

Similarly, chalcones **6**, which serve as the precursors for compounds **C** and **D**, were prepared by the condensation of 3,4,5-trimethoxybenzaldehyde with substituted acetophenones. Chalcones **5** and **6** were then reacted with hydrazine hydrate in boiling ethanol[16] to effect the assembly of pyrazolines **7**–**11** in 62–92% yields, and **12**–**22** in 69–88% yields, respectively (Scheme 1).

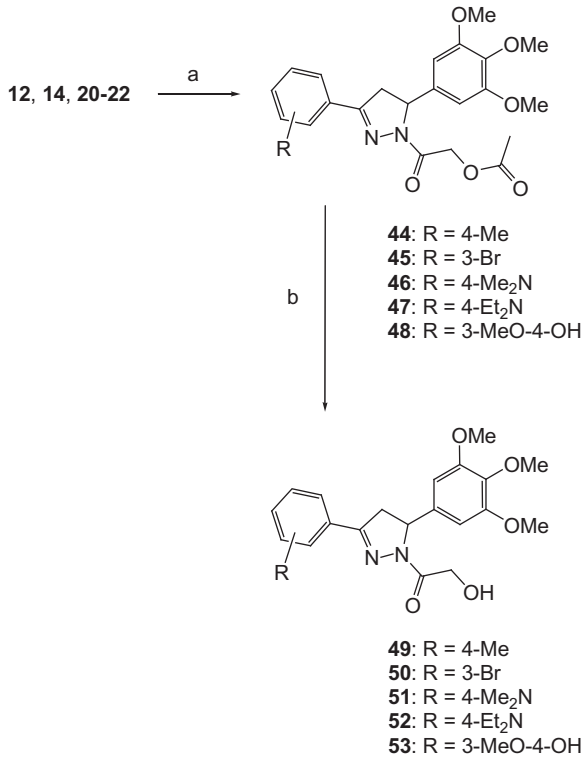
In order to evaluate the effect of substitution on the N-1 nitrogen atom, 1-acetylated derivatives were prepared. Firstly, upon treatment with hydrazine hydrate in boiling acetic acid[17] chalcones **5** and **6** were converted into 1-acetyl-3,5-diaryl-4,5-dihydropyrazoles **23–27** in 56–90% yields, and **28–41** in 60–95% yields, respectively (Scheme 2). Boc-deprotection of **40** and **41** with triflu-



Scheme 1. Reagents and conditions: (a) MeOH, NaOH pellets, rt; (b) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux, 2 h.



Scheme 2. Reagents and conditions: (a) $NH_2NH_2{\cdot}H_2O$, AcOH, reflux, 3 h; (b) TFA, CH_2Cl_2, rt, 16 h.



Scheme 3. Reagents and conditions: (a) (i) HOBt, EDC, chloroacetic acid, MeCN, 24 h, rt; (ii) potassium acetate, DMSO, 12 h, rt; (b) K_2CO_3, MeOH/H_2O 10:1, 6 h, rt.

oroacetic acid in dichloromethane gave **42** (86%) and **43** (77%), respectively.

Compounds **44–53**, with a further functionalized acetyl moiety at the 1-position of the pyrazoline ring, were synthesized starting from 3-aryl-5-(3,4,5-trimethoxyphenyl)-4,5-dihydropyrazoles, as shown in Scheme 3. Upon coupling pyrazolines **12, 14, 20–22** with chloroacetic acid in the presence of 1-(3-dimethylaminopropyl)-3-ethylcardodiimide hydrochloride (EDC) and hydroxybenzotriazole (HOBt), in dry MeCN, followed by treatment with potassium acetate in DMSO, 1-(acethoxyacetyl)pyrazolines **44–48** were obtained in 72–89% overall yields. Hydrolysis of **44–48** using potassium carbonate in aqueous methanol provided 1-(hydroxyacetyl)pyrazolines **49–53** in almost quantitative yields (Scheme 3).

2.2. Pharmacology

In a primary assay, synthesized pyrazolines and their 1-acetylated derivatives were screened for their ability to inhibit cell growth of a non-small cell lung carcinoma (NSLC) NCI-H460 cell line determining the number of surviving cells with sulforhodamine B test.[18] Results, expressed as concentrations that inhibit 50% of cell growth (IC_{50}), were calculated by the ALLFIT program, and are shown in Table 1.

Among non-acetylated pyrazolines, analogs **8–11**, bearing the 3,4,5-trimethoxyphenyl moiety in 3-position showed antiproliferative effect, being derivative **11** the most potent agent with IC_{50} value of 0.30 μM; in contrast, pyrazoline regioisomers **12–22** were inactive. Within 1-acetylated derivatives **23–27**, only compound **27** exhibited a weak 13.8 μM IC_{50} value, whereas regioisomers **28–33**, **36–39**, **42**, and **43**, bearing a 3,4,5-trimethoxyphenyl moiety in 5-position of the 4,5-dihydropyrazole core showed growth inhibition in micromolar to sub-micromolar range. Derivatives **31** and **39** were the most potent agents with IC_{50} values of 0.35 and 0.21 μM, respectively. Across the series of derivatives, a 4-(diethylamino)phenyl moiety favorably affects the inhibitory activity against the NCI-H460 cell line. Therefore, initial SAR study was focused on modifications of this substituent. Among non-acetylated pyrazolines **7–11**, replacement of 4-(diethylamino) group of **11** results in a progressive decrease in potency, from **10** to **7**. In the series of 1-acetylpyrazolines **28–43**, introduction of a 4-methoxyphenyl group leads to inactive compound **35**; introduction at the same position of phenyl ring of either halogen atoms (compounds **29**, **30**, and **32**), or methylthio (**36**) substituents results in weakly active analogs. On the other hand, 4-amino- (**43**), 4-(dimethylamino)- (**37**), and 4-methylphenyl (**28**) analogs exhibit increasing potency in the low micromolar range. While the 3-bromo- (**31**) and 3-aminophenyl (**42**) derivatives show sub-micromolar inhibitory potency, the 3-hydroxy analog (**33**) is moderately active, and the 3-methoxy analog (**34**) is devoid of activity. Interestingly, introduction of a 4-hydroxy substituent in this last compound results in the most active analog (**39**) in the whole series. This result can be rationalized by comparing molecular modeling of **39** and **1** (Fig. 4). Initially, compounds were constructed with standard bond and angles, and their geometry were optimized using Spartan'08 software. The optimal structure and conformation of **39** and **1** were further optimized using standard Hartree–Fock models. The resulting conformation of pyrazoline **39** is shown in Figure 4A. It is evident that the 4,5-dihydropyrazole derivative adopts a twisted conformation similar to **1** (Fig. 4B). In particular, the phenyl ring bearing the hydroxy and methoxy groups of **39** adopts a orientation comparable to **1**, resulting in its improved ability to interact with the biological target, although the hydroxy and methoxy substituents are in inverted position respect to CA-4.

The effect of the functionalization of the 1-acetyl group was evaluated in compounds **44–53**. Compounds **44–48** show a substantial decrease in inhibition ability as compared to the parental 1-acetylated analogs, likely because of steric hindrance imposed by the acetoxy group; however, hydrolysis of **44–48** to give 1-hydroxyacetyl derivatives **49–53** restores inhibitory efficacy, as a result of both enhanced hydrophilicity and decreased steric hindrance.

Comprehensive examination of cytotoxicity data shows that introduction of a (hydroxy)acetyl group at the N-1 of inactive 3-aryl-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-pyrazoles **12–22** results in clear activating effect on their ability to inhibit cell

Table 1
Inhibitory activity of pyrazoline **7**–**53** against a non-small cell lung carcinoma NCI-H460 cell line[a]

Compd	IC_{50} ± SE (μM)
7	>20
8	11.6 ± 0.1
9	3.8 ± 0.1
10	1.1 ± 0.05
11	0.30 ± 0.01
12	>20
13	>20
14	>20
15	>20
16	>20
17	>20
18	>20
19	>20
20	>20
21	>20
22	>20
23	>20
24	>20
25	>20
26	>20
27	13.8 ± 0.1
28	1.3 ± 0.05
29	12.0 ± 0.9
30	11.9 ± 1.6
31	0.35 ± 0.01
32	6.0 ± 0.09
33	2.3 ± 0.06
34	>20
35	>20
36	8.0 ± 0.1
37	1.8 ± 0.06
38	0.98 ± 0.03
39	0.21 ± 0.005
40	Not tested
41	Not tested
42	0.74 ± 0.03
43	2.6 ± 0.1
44	3.3 ± 0.1
45	>20
46	>20
47	5.0 ± 0.2
48	4.5 ± 0.1
49	0.69 ± 0.02
50	1.1 ± 0.02
51	1.3 ± 0.01
52	0.95 ± 0.05
53	1.85 ± 0.07

[a] Inhibitory activity was assayed by exposure for 72 h to substances and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are expressed as mean ± SE from the dose–response curves of at least three independent experiments.

growth. This result well correlate with those of the above mentioned acetylated oxadiazoline series.[9a] Likely, the acetyl moiety in position adjacent to a 3,4,5-trimethoxyphenyl group of a 4,5-dihydropyrazole core, as in compounds **31**, **38**, and **39**, allows the molecule to adopt a conformation that would activate their ability to interact with mitotic microtubules, leading to cytotoxic effect. In contrast, the same substituents in the 1–3 positions, as in compounds **23**–**27**, results in opposite effect, accordingly to the previous reported result.[12]

Selected pyrazolines were submitted to the US National Cancer Institute (NCI; Bethesda, MD), for the more extensive in vitro testing against a panel of approximately 60 tumor cell lines, derived from nine different cancer types: leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast.[19] The compounds were tested at five concentrations at 10-fold dilution. A 48 h continuous drug exposure protocol was used and sulforhodamine B (SRB) protein assay was used to estimate cell growth.[20] The antitumor activity of tested compounds is given by three parameters for each cell line: pGI_{50} value (−log of the molar concentration of the compound that inhibits 50% net cell growth), pTGI value (−log of the molar concentration of the compound leading to total inhibition), and pLC_{50} value (−log of the molar concentration of the compound leading to 50% net cell death). Furthermore, a mean graph midpoint (MG-MID) is calculated for each of the mentioned parameters, giving an averaged activity parameter over all cell lines. For the calculation of the MG-MID, insensitive cell lines are included with the highest concentration tested.

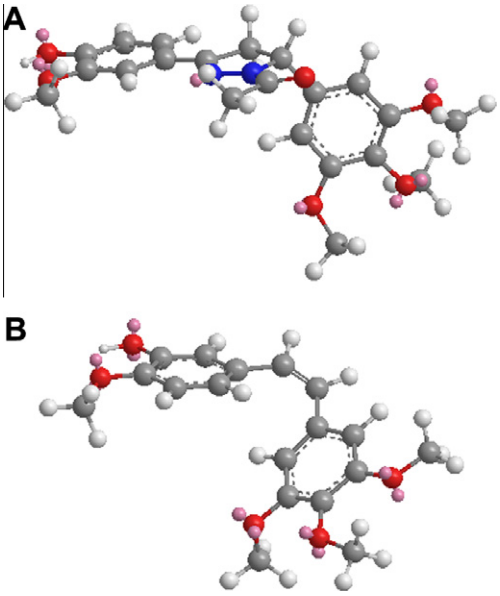


Figure 4. Molecular ball models of pyrazoline **39** (A) and **1** (B). Gray represents carbon, white hydrogen, blue nitrogen, and red oxygen.

Screening data against the NCI in vitro 60 human cancer cell panel corroborated the results described above. Representative growth inhibitory properties of tested compounds compared to CA-4 (**1**) as the reference compound are reported in Table 2. The pGI_{50} MG-MID values across the series of compounds are in the 5.1–6.4 range, being **11**, **31, 38**, and **39** the most potent agents, with a variety of significant cell selectivity. As compared with **1**, all tested compounds exhibit better inhibitory efficacy against the ovarian SK-OV-3 and breast T-74D cell lines, and better or comparable effect against the colon COLO 205 and HT29, and CNS SNB-75 cell lines, at both pGI_{50} and pTGI levels.

Selectively, compounds **31**, **33**, **38**, and **39** show better inhibitory potency than **1** against the leukemic SR cell line, with GI_{50} values in nanomolar range. Compounds **31** and **39** also inhibit the MDA-MB-435 cell line (pGI_{50} 7.3) with potency comparable to **1** (pGI_{50} 7.5). Significantly, all tested compounds exhibit antiproliferative effect against ovarian NCI/ADR-RES cell line, with pGI_{50} values in the 5.2–6.7 range. This finding is important because NCI/ADR-RES, an adriamycin-resistant cell line, expresses high levels of MDR-1 and P-glycoprotein, and is a useful model for new drug development, given its utility in identifying compounds subject to multi-drug resistance.

To investigate a possible mechanism of action responsible for the observed antiproliferative effect, active agents, and inactive or less active congeners, were evaluated for their ability to affect tubulin polymerization. The tubulin polymerization, performed

Table 2
Overview of growth inhibitory properties of compounds **9**–**11**, **31**, **33**, **37**–**39**, and **1** against the NCI human cancer cell lines, and the average pGI_{50} value in the full panel cell lines screening[a]

Compound	**9**		**10**		**11**		**31**		**33**		**37**		**38**		**39**		**1**[b]	
Panel/cell line	pGI_{50}[c]	pTGI[d]	pGI_{50}	pTGI	pGI_{50}	pTGI	pGI_{50}	pTGI	pGI_{50}	pTGI	pGI_{50}	pTGI	pGI_{50}	pTGI	pGI_{50}	pTGI	pGI_{50}	pTGI
Leukemia																		
CCRF-CEM	5.2	4.0	5.8	5.2	6.7	6.2	6.5	5.7	6.0	5.3	5.6	4.0	6.5	6.0	6.5	6.0	7.0	5.3
HL-60(TB)	5.7	4.9	5.8	5.3	6.6	5.7	6.6	6.3	5.8	5.3	6.0	5.2	6.9	6.3	6.8	6.3	7.5	7.2
K-562	5.6	4.0	6.4	4.1	6.4	4.9	6.9	4.9	6.2	4.2	5.8	4.0	6.2	4.0	6.4	4.0	7.5	5.7
MOLT-4	5.3	4.6	5.5	4.9	5.9	5.3	6.5	5.8	5.3	4.0	5.5	4.0	6.6	5.5	6.4	4.0	7.0	5.4
RPMI-8226	5.5	4.7	5.8	4.9	6.4	5.4	6.8	6.2	6.0	5.3	5.5	4.8	6.8	6.3	6.7	6.1	6.8	5.2
SR	6.0	5.4	6.6	5.1	6.6	6.0	7.4	4.0	7.0	4.0	6.2	4.0	7.2	6.2	7.3	6.3	6.9	4.1
NSCL[e]																		
EKVX	5.0	4.0	5.7	5.2	6.4	6.1	6.2	4.0	5.2	4.0	5.3	4.0	6.4	5.6	6.1	4.0	6.4	4.1
HOP-62	4.5	4.0	5.9	4.0	6.5	5.4	6.0	4.1	6.9	5.7	6.1	4.0	5.8	4.6	6.3	4.0	6.7	5.6
NCI-H322M	4.1	4.0	4.9	4.0	4.0	4.0	6.3	4.0	5.4	4.0	5.4	4.2	6.3	4.9	6.3	4.0	7.1	4.1
NCI-H460	5.5	4.52	5.9	5.2	6.5	4.9	6.5	4.9	5.7	5.0	6.2	4.0	6.4	5.7	6.4	4.8	7.5	4.2
NCI-H522	5.5	4.44	5.7	5.2	6.6	5.7	6.4	4.7	5.8	4.4	6.2	5.1	6.4	6.2	6.5	4.0	7.5	5.4
Colon																		
COLO 205	5.4	4.5	5.6	4.9	6.4	5.8	6.7	6.2	5.4	5.0	5.5	4.8	6.5	5.9	6.6	6.1	5.6	4.3
HCC-2998	5.0	4.4	5.8	5.3	6.2	5.1	5.8	4.6	5.7	4.8	5.9	4.7	6.1	5.1	nt[f]	nt	7.3	5.9
HCT-116	5.4	4.0	5.7	4.7	6.6	6.0	6.3	4.0	5.6	4.0	6.3	4.7	6.5	4.9	6.4	4.3	7.5	6.7
HCT-15	5.5	4.0	5.6	4.0	6.2	4.0	6.5	4.5	6.1	4.0	6.2	4.0	6.4	4.9	6.4	4.0	7.5	5.0
HT29	5.4	4.0	6.2	4.0	6.5	4.0	nt	nt	5.5	4.0	6.4	4.8	6.4	5.8	6.5	4.0	6.2	5.2
KM12	5.4	4.0	6.3	4.9	6.4	4.0	6.5	4.7	6.0	4.0	6.1	4.5	6.5	4.9	6.6	4.0	7.3	6.1
SW-620	5.3	4.0	6.4	4.0	6.4	4.0	6.5	4.0	6.1	4.0	6.3	4.0	6.5	4.8	6.5	4.0	7.5	4.1
CNS																		
SF-268	4.7	4.0	5.7	4.0	6.1	nt	6.0	4.1	5.1	4.0	5.6	4.0	6.1	4.7	6.2	4.0	6.9	4.3
SF-295	5.3	4.0	6.6	6.1	7.0	6.5	6.7	4.9	6.5	5.3	6.8	6.2	6.7	6.2	6.7	nt	7.2	5.9
SF-539	5.2	4.4	6.3	5.5	6.5	5.9	6.5	5.0	5.7	5.2	6.0	5.3	6.5	6.2	6.5	6.0	7.4	6.7
SNB-75	5.4	nt	6.0	4.0	6.7	6.2	6.4	4.0	6.4	nt	6.1	4.0	6.6	6.2	6.6	4.0	6.1	4.6
U251	5.4	4.0	6.2	4.0	6.4	4.0	6.3	4.5	5.6	4.0	6.2	4.0	6.4	4.9	6.4	4.0	7.3	4.9
Melanoma																		
MALME-3M	4.6	4.0	5.1	4.0	4.7	4.0	nt	4.3	5.6	4.0	4.0	4.0	6.7	4.6	nt	4.0	6.3	4.1
M14	4.9	4.0	5.6	nt	nt	nt	6.2	4.2	6.1	4.0	6.3	5.6	6.4	5.5	6.4	4.0	6.8	6.6
SK-MEL-2	nt	nt	5.5	4.5	5.8	4.0	4.7	4.1	5.2	4.0	5.1	4.0	6.3	4.4	6.3	4.0	5.1	4.1
MDA-MB-435	5.5	4.7	6.0	nt	6.8	6.5	7.3	6.6	6.6	6.1	6.7	6.2	6.8	6.4	7.3	6.7	7.5	7.1
SK-MEL-28	4.4	4.0	4.8	4.0	nt	nt	6.1	4.2	5.3	4.0	4.8	4.0	6.1	4.7	6.3	4.0	5.5	4.3
SK-MEL-5	5.4	4.6	5.7	5.2	6.1	5.5	6.6	4.9	6.2	5.3	6.2	4.9	6.4	5.4	7.2	6.3	8.0	8.0
UACC-62	5.1	4.0	5.4	4.0	6.4	4.3	6.7	4.7	5.6	4.0	6.2	4.0	6.5	4.9	6.5	4.0	8.0	4.2
Ovarian																		
IGROV1	5.2	4.0	6.3	5.5	6.5	5.2	5.7	4.0	5.4	4.0	5.6	nt	6.1	4.5	6.3	4.0	7.2	4.2
OVCAR-3	5.4	4.3	5.2	4.0	6.4	4.0	nt	nt	5.9	5.4	6.6	6.0	6.4	5.0	6.6	6.2	7.4	6.8
OVCAR-4	4.9	4.0	4.8	4.0	6.0	4.0	6.2	4.0	5.2	4.0	5.4	4.0	6.2	4.6	6.4	4.0	6.6	4.1
OVCAR-8	4.6	4.0	5.4	4.0	6.4	5.1	6.2	4.0	5.3	4.0	5.3	4.0	6.4	5.3	6.2	4.0	7.4	4.4
NCI/ADR-RES	5.2	4.0	5.7	4.0	6.3	4.0	6.4	4.0	5.9	5.4	6.5	5.9	6.7	6.3	6.6	4.0	7.2	6.4
SK-OV-3	4.9	4.0	5.4	4.0	6.3	4.0	6.3	4.2	5.6	4.0	5.4	4.0	6.4	4.8	6.5	4.0	4.5	4.0
Renal																		
786-0	4.7	4.0	5.7	4.0	6.8	6.3	6.1	4.0	5.9	5.4	5.9	4.2	6.3	4.9	6.4	4.0	7.7	4.6
A498	5.2	4.3	5.5	5.1	6.4	6.1	6.4	5.4	5.6	5.0	5.6	4.7	6.5	5.6	6.6	5.4	7.3	6.1
CAKI-1	5.1	4.2	5.6	4.0	6.7	6.1	6.6	4.4	5.6	4.0	6.3	4.0	6.9	4.7	6.5	4.0	7.4	4.8
RXF 393	5.0	4.0	5.9	5.3	nt	nt	6.6	6.0	5.4	4.0	5.8	5.3	6.4	5.6	6.9	nt	6.0	4.1
TK-10	4.4	4.0	5.0	4.0	4.4	4.0	4.9	4.2	5.1	4.0	4.9	4.0	6.4	4.7	6.2	4.0	6.2	5.4
UO-31	5.3	4.0	5.3	4.3	6.1	4.0	4.9	4.0	5.0	4.0	5.6	4.0	6.4	4.8	6.4	4.0	6.7	4.3
Prostate																		
PC-3	5.2	4.0	6.1	4.0	6.5	4.2	6.7	4.5	5.7	4.0	6.3	4.0	6.5	4.9	6.6	4.0	7.4	4.0
DU-145	4.5	4.0	5.7	5.3	6.4	5.8	6.4	4.9	5.5	nt	5.7	4.9	6.4	5.7	6.5	4.9	8.0	4.0
Breast																		
MCF7	5.6	4.6	6.4	4.0	6.8	5.8	6.8	4.8	6.3	5.0	6.4	4.0	6.5	5.0	6.6	4.0	7.4	4.1
HS 578T	4.8	4.0	5.6	5.2	6.6	6.2	nt	nt	6.1	5.1	6.4	5.5	6.5	4.6	6.7	6.2	6.8	5.1
BT-549	5.1	4.0	6.1	5.0	6.4	5.3	nt	4.4	5.3	4.0	5.6	4.0	6.2	5.3	6.2	4.0	8.0	5.7
T-47D	5.4	4.0	4.7	4.0	nt	4.0	nt	4.4	5.5	4.0	5.2	4.0	6.7	4.7	nt	nt	4.3	4.0
MDA-MB-468	5.4	4.2	nt	nt	nt	nt	6.7	4.0	6.3	5.0	5.8	4.0	6.6	6.2	6.6	6.1		
MG-MID[g]	5.1		5.6		6.2		6.2		5.5		5.6		6.4		6.3		7.0	

[a] Data obtained from the NCI's in vitro disease-oriented human tumor cells screen.
[b] http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp.
[c] pGI_{50} is the −log of the molar concentration of the compound that causes 50% inhibition of net cell growth.
[d] pTGI is the −log of the molar concentration of the compound leading to total inhibition of cell growth.
[e] Non-small cell lung cancer.
[f] Not tested.
[g] MG-MID = mean graph midpoint = arithmetical mean value for all tested cell lines.

Table 3
In vitro cytotoxic effect against NCI-H460 cell line and tubulin polymerization inhibition (TPI) of selected compounds and CA-4 (**1**)

Compd	IC_{50} ± SE (μM)	
	NCI-H460	TPI[a]
11	0.30 ± 0.01	>10
12	>20	>10
21	>20	>10
28	1.3 ± 0.05	>10
31	0.35 ± 0.01	5.16 ± 0.17
38	0.98 ± 0.03	8.09 ± 0.14
39	0.21 ± 0.005	8.56 ± 0.09
42	0.74 ± 0.03	>10
43	2.6 ± 0.1	10 ± 0.2
49	0.69 ± 0.02	7.72 ± 0.17
52	0.95 ± 0.05	6.14 ± 0.11
1	0.032 ± 0.002	4.92 ± 0.2

[a] Concentration of compound that inhibited tubulin polymerization by 50% (IC_{50}) versus 100% polymerization (maximal attainable polymerization) of the untreated control.

by CytoDINAMIX ScreenTM (Cytoskeleton Inc., Denver, CO), was detected by measuring the absorbance of the solution (340 nm) for 60 min. The results show pyrazolines **31**, **38**, **39**, **49**, and **52** to affect tubulin polymerization with inhibition potency in the same magnitude order of that of **1** (Table 3), being compound **31** (IC_{50} = 5.16 μM) as potent as CA-4 (**1**) (IC_{50} = 4.92 μM). The tubulin assembly assay well correlates with cytotoxicity screening results, suggesting that these new derivatives could exert their antiproliferative activity through tubulin polymerization inhibition. The lower cytotoxicity of these compounds than CA-4 indicates that the latter compound may exert its cytotoxic effects through additional targets besides tubulin.[21] Unexpectedly, the sub-micromolar cytotoxic compounds **11** and **42** did not inhibit microtubule assembly up to 10 μM, suggesting that their cytotoxicity may be due to a mechanism other than antitubulin activity.

In conclusion, we have synthesized and evaluated in vitro a series of novel 4,5-dihydropyrazole-based CA-4 analogs as potential antitumor agents. Results from the anticancer screening showed compounds **31** and **39** to possess potent antiproliferative activity against SR and MDA-MB-435 of the NCI 60 human cancer cell line panel, with GI_{50} inhibitory values in nanomolar range. Compounds **31**, **38**, **39**, **49**, and **52** exhibited inhibition potency on tubulin polymerization in the same order of magnitude as CA-4. From the current investigation, structure–activity relationships revealed that a 4-(diethylamino)phenyl moiety favorably affects the inhibitory activity within the series of derivatives. Most importantly, introduction of a (hydroxy)acetyl group at N-1 of inactive 5-(3,4,5-trimethoxyphenyl)pyrazolines, resulted in clear activating effect on their ability to inhibit both cell growth and tubulin polymerization.

Based on easy synthetic approach and biological results, 3,5-diaryl-4,5-dihydropyrazole structure constitutes a valid template for access to potential anticancer chemotherapeutic CA-4 analogs. In order to investigate observed structure–activity relationships and corroborate our findings, further studies are on the way, and the results will be reported elsewhere.

3. Experimental

3.1. Chemistry

Unless otherwise noted, all solvents, including anhydrous solvents and chemicals, were purchased from Aldrich Co. and/or Alfa Aesar, and used without further purification. Melting points were recorded on a Stuart Scientific melting point SMP1 apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Unity 300 spectrometer (300 MHz) in DMSO-d_6 as the solvent. Chemical shifts are expressed in ppm relative to tetramethylsilane. Splitting patterns are designated as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Infrared spectra were run on Bruker Vector 22 spectrophotometer. Silica gel thin-layer chromatography (TLC) sheets from Fluka (silica gel precoated aluminum sheets with fluorescent indicator at 254 nm) were used for TLC. Developed plates were visualized by a Spectroline ENF 260C/F UV apparatus. Concentration and evaporation of the solvent after reaction or extraction were carried out on a rotary evaporator (Büchi Rotavapor) operating at reduced pressure. Elemental analyses were carried out with a Carlo Erba model 1106 elemental analyzer, and all values were within 0.4% of the calculated values, which indicates >95% purity of the tested compounds.

3.2. General procedure for the preparation of 3,5-diaryl-4,5-dihydro-1*H*-pyrazoles 7–22

To a solution of chalcone derivative **3**, **4** (1 mmol) in ethanol (3 mL) hydrazine hydrate (0.3 mL, 6 mmol) was added. The mixture was refluxed under stirring for 2 h, and then water (1 mL) was added. The mixture was stored at 4–5 °C for 24 h, and the formed precipitate was filtered off, washed with cold water, and allowed to air dry to give pyrazolines **7–22**.

3.2.1. 5-(3-Hydroxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (7)

Yield 80%, mp 118–120 °C. ^{1}H NMR: δ 2.95 (dd, *J* = 15.8, 10.2 Hz, 1H), 3.60 (m, 1H) 3.78 (s, 3H), 3.89 (s, 6H), 4.89 (t, *J* = 10.2 Hz, 1H), 6.76–6.90 (m, 3H), 7.02 (s, 2H), 7.24 (m, 1H), 7.38 (m, 1H), 7.56 (br s, 1H), 9.50 (s, 1H, OH); IR (Nujol) 3453, 3283, 1590, 1566 cm^{-1}. Anal. Calcd for $C_{18}H_{20}N_2O_4$: C, 65.84; H, 6.14; N, 8.53. Found: C, 65.80; H, 6.16; N, 8.49.

3.2.2. 5-(3-Methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (8)

Yield 68%, mp 106–108 °C. ^{1}H NMR: δ 2.97 (dd, *J* = 16.4, 10.4 Hz, 1H), 3.54 (dd, *J* = 16.4, 10.4 Hz, 1H) 3.78 (s, 3H), 3.86 (s, 3H), 3.91 (s, 6H), 4.91 (t, *J* = 10.4 Hz, 1H), 6.95 (m, 1H), 7.02 (s, 2H), 7.06 (m, 1H), 7.38 (m, 1H), 7.61 (br s, 1H); IR (Nujol) 3288, 1594, 1566 cm^{-1}. Anal. Calcd for $C_{19}H_{22}N_2O_4$: C, 66.65; H, 6.48; N, 8.18. Found: C, 66.61; H, 6.45; N, 8.20.

3.2.3. 5-(4-Methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (9)

Yield 78%, mp 122–124 °C. ^{1}H NMR: δ 2.95 (dd, *J* = 16.3, 10.4 Hz, 1H), 3.46 (dd, *J* = 16.3, 10.4 Hz, 1H) 3.79 (s, 3H), 3.85 (s, 3H), 3.91 (s, 6H), 4.91 (t, *J* = 10.4 Hz, 1H), 6.73 (d, *J* = 8.4 Hz, 2H), 7.02 (m, 4H), 7.41 (d, *J* = 8.8 Hz, 2H), 7.50 (br s, 1H); IR (Nujol) 3335, 1591, 1565 cm^{-1}. Anal. Calcd for $C_{19}H_{22}N_2O_4$: C, 66.65; H, 6.48; N, 8.18. Found: C, 66.60; H, 6.47; N, 8.14.

3.2.4. 5-[4-(Dimethylamino)phenyl]-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (10)

Yield 92%, mp 142–144 °C. ^{1}H NMR: δ 2.98 (m 7H), 3.79 (s, 3H), 3.91 (m, 7H), 4.84 (t, *J* = 10.2 Hz, 1H), 6.82 (d, *J* = 8.4 Hz, 2H), 7.02 (s, 2H), 7.29 (d, *J* = 8.4 Hz, 2H), 7.45 (br s, 1H); IR (Nujol) 3290, 1589, 1565 cm^{-1}. Anal. Calcd for $C_{20}H_{25}N_3O_3$: C, 67.58; H, 7.09; N, 11.82. Found: C, 67.54; H, 7.12; N, 11.83.

3.2.5. 5-[4-(Diethylamino)phenyl]-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (11)

Yield 62%, mp 130–132 °C. ^{1}H NMR: δ 1.17 (t, *J* = 6.5 Hz, 6H), 2.95 (dd, *J* = 16.5, 10.4 Hz, 1H), 3.41 (q, *J* = 6.5 Hz, 4H) 3.78 (s, 3H), 3.89 (s, 6H), 4.81 (t, *J* = 10.4 Hz, 1H), 6.73 (d, *J* = 8.4 Hz, 2H), 7.02 (s, 2H), 7.25 (d, *J* = 8.4 Hz, 2H), 7.40 (br s, 1H); IR (Nujol)

3222, 1592, 1564 cm^{-1}. Anal. Calcd for $C_{22}H_{29}N_3O_3$: C, 68.90; H, 7.62; N, 10.96. Found: C, 68.92; H, 7.60; N, 10.91.

3.2.6. 3-(4-Methylphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (12)

Yield 82%, mp 116–118 °C. ^{1}H NMR: δ 2.43 (s, 3H), 2.94 (dd, J = 16.2, 11.2 Hz, 1H), 3.75 (s, 3H), 3.87 (s, 6H), 3.94 (m, 1H), 4.88 (t, J = 11.2 Hz, 1H), 6.82 (s, 2H), 7.30 (d, J = 8.0 Hz, 2H), 7.63 (d, J = 8.0 Hz, 2H); IR (Nujol) 3264, 1590 cm^{-1}. Anal. Calcd for $C_{19}H_{22}N_2O_3$: C, 69.92; H, 6.79; N, 8.58. Found: C, 69.90; H, 6.81; N, 8.55.

3.2.7. 3-(4-Fluorophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (13)

Yield 74%, mp 128–130 °C. ^{1}H NMR: δ 3.00 (dd, J = 16.2, 10.6 Hz, 1H), 3.75 (s, 3H), 3.86 (s, 6H), 3.94 (m, 1H), 4.88 (t, J = 10.6 Hz, 1H), 6.87 (s, 2H), 7.40 (m, 2H), 7.92 (m, 2H); IR (Nujol) 3274, 1593 cm^{-1}. Anal. Calcd for $C_{18}H_{19}FN_2O_3$: C, 65.44; H, 5.80; N, 8.48. Found: C, 65.41; H, 5.81; N, 8.51.

3.2.8. 3-(3-Bromophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (14)

Yield 77%, mp 142–144 °C. ^{1}H NMR: δ 2.98 (dd, J = 16.8, 10.8 Hz, 1H), 3.75 (s, 3H), 3.88 (s, 6H), 3.95 (m, 1H), 4.90 (t, J = 10.8 Hz, 1H), 6.88 (s, 2H), 7.48–7.88 (m, 3H), 8.02 (s, 1H); IR (Nujol) 3354, 1593 cm^{-1}. Anal. Calcd for $C_{18}H_{19}BrN_2O_3$: C, 55.26; H, 4.89; N, 7.16. Found: C, 55.27; H, 4.90; N, 7.14.

3.2.9. 3-(4-Bromophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (15)

Yield 86%, mp 138–140 °C. ^{1}H NMR: δ 2.98 (dd, J = 16.4, 11.0 Hz, 1H), 3.76 (s, 3H), 3.85 (s, 6H), 3.96 (m, 1H), 4.90 (t, J = 11.0 Hz, 1H), 6.88 (s, 2H), 7.48 (d, J = 8.2 Hz, 2H), 7.86 (d, J = 8.2 Hz, 2H); IR (Nujol) 3336, 1591 cm^{-1}. Anal. Calcd for $C_{18}H_{19}BrN_2O_3$: C, 55.26; H, 4.89; N, 7.16. Found: C, 55.25; H, 4.87; N, 7.15.

3.2.10. 3-(3-Hydroxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (16)

Yield 80%, mp 127–129 °C. ^{1}H NMR: δ 3.00 (dd, J = 16.4, 10.6 Hz, 1H), 3.76 (s, 3H), 3.86 (s, 6H), 3.94 (m, 1H), 4.88 (t, J = 10.6 Hz, 1H), 6.85 (s, 2H), 7.04 (s, 1H), 7.12–7.36 (m, 3H), 7.48 (br s, 1H), 9.82 (s, 1H); IR (Nujol) 3368, 3311, 1592 cm^{-1}. Anal. Calcd for $C_{18}H_{20}N_2O_4$: C, 65.84; H, 6.14; N, 8.53. Found: C, 65.81; H, 6.15; N, 8.50.

3.2.11. 3-(3-Methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (17)

Yield 68%, mp 124–126 °C. ^{1}H NMR: δ 2.98 (dd, J = 16.5, 10.8 Hz, 1H), 3.76 (s, 3H), 3.85 (s, 3H), 3.90 (s, 6H), 3.95 (m, 1H), 4.88 (t, J = 10.8 Hz, 1H), 6.86 (s, 2H), 7.04 (s, 1H), 7.10–7.40 (m, 3H), 7.50 (br s, 1H); IR (Nujol) 3330, 1591 cm^{-1}. Anal. Calcd for $C_{19}H_{22}N_2O_4$: C, 66.65; H, 6.48; N, 8.18. Found: C, 66.60; H, 6.46; N, 8.13.

3.2.12. 3-(4-Methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (18)

Yield 82%, mp 120–122 °C. ^{1}H NMR: δ 2.96 (dd, J = 16.5, 10.8 Hz, 1H), 3.74 (s, 3H), 3.88 (s, 6H), 3.90 (s, 3H), 3.95 (m, 1H), 4.90 (t, J = 10.8 Hz, 1H), 6.84 (s, 2H), 7.26 (d, J = 7.8 Hz, 2H), 7.50 (br s, 1H), 7.78 (d, J = 7.8 Hz, 2H); IR (Nujol) 3336, 1592 cm^{-1}. Anal. Calcd for $C_{19}H_{22}N_2O_4$: C, 66.65; H, 6.48; N, 8.18. Found: C, 66.61; H, 6.45; N, 8.15.

3.2.13. 3-(4-Methylthiophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (19)

Yield 88%, mp 145–147 °C. ^{1}H NMR: δ 2.95 (dd, J = 16.4, 10.4 Hz, 1H), 3.45 (s, 3H), 3.76 (s, 3H), 3.88 (s, 6H), 3.92 (m, 1H), 4.90 (t, J = 10.4 Hz, 1H), 6.83 (s, 2H), 7.38 (d, J = 8.4 Hz, 2H), 7.67 (d, J = 8.4 Hz, 2H); IR (Nujol) 3331, 1591 cm^{-1}. Anal. Calcd for $C_{19}H_{22}N_2O_3S$: C, 63.66; H, 6.19; N, 7.81. Found: C, 63.62; H, 6.21; N, 7.80.

3.2.14. 3-[4-(Dimethylamino)phenyl]-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (20)

Yield 81%, mp 140–142 °C. ^{1}H NMR: δ 2.88 (dd, J = 16.6, 10.2 Hz, 1H), 3.04 (s, 6H), 3.76 (s, 3H), 3.85 (s, 6H), 3.94 (m, 1H), 4.84 (t, J = 10.2 Hz, 1H), 6.74 (d, J = 8.2 Hz, 2H), 6.82 (s, 2H), 7.50 (d, J = 8.2 Hz, 2H); IR (Nujol) 3291, 1610, 1591 cm^{-1}. Anal. Calcd for $C_{20}H_{25}N_3O_3$: C, 67.58; H, 7.09; N, 11.82. Found: C, 67.60; H, 7.12; N, 11.77.

3.2.15. 3-[4-(Diethylamino)phenyl]-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (21)

Yield 76%, mp 138–140 °C. ^{1}H NMR: δ 1.19 (t, J = 7.0 Hz, 6H), 2.84 (m, 1H), 3.44 (d, J = 7.0 Hz, 4H), 3.74 (s, 3H), 3.86 (s, 7H), 4.81 (t, J = 9.8 Hz, 1H), 6.75 (d, J = 8.4 Hz, 2H), 6.81 (s, 2H), 7.53 (d, J = 8.4 Hz, 2H); IR (Nujol) 3315, 1610, 1590 cm^{-1}. Anal. Calcd for $C_{22}H_{29}N_3O_3$: C, 68.90; H, 7.62; N, 10.96. Found: C, 68.87; H, 7.60; N, 10.98.

3.2.16. 3-(4-Hydroxy-3-methoxy-phenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (22)

Yield 84%, mp 146–148 °C. ^{1}H NMR: δ 2.90 (m, 1H), 3.75 (s, 3H), 3.86 (s, 6H), 3.94 (s, 3H), 4.86 (t, J = 10.0 Hz, 1H), 6.74 (d, J = 8.2 Hz, 2H), 6.82 (s, 2H), 6.90–7.36 (m, 2H), 7.45 (s, 1H), 9.80 (s, 1H); IR (Nujol) 3346, 3328, 1592 cm^{-1}. Anal. Calcd for $C_{19}H_{22}N_2O_5$: C, 63.68; H, 6.19; N, 7.82. Found: C, 63.65; H, 6.16; N, 7.80.

3.3. General procedure for the preparation of 1-acetyl-3,5-diaryl-4,5-dihydro-1*H*-pyrazoles 23-41

To a solution of chalcone derivative **3**, **4** (1 mmol) in acetic acid (3 mL) hydrazine hydrate (0.3 mL, 6 mmol) was added. The mixture was refluxed under stirring for 3 h, and then poured onto crushed ice. The precipitate was filtered off, washed with cold water, and crystallized from methanol to give pyrazolines **23–41**.

3.3.1. 1-Acetyl-5-(3-hydroxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (23)

Yield 65%, mp 116–118 °C. ^{1}H NMR: δ 2.42 (s, 3H), 2.96 (m, 1H), 3.76 (s, 3H), 3.85 (s, 6H), 3.93 (s, 3H), 5.58 (m, 1H), 6.66–7.00 (m, 3H), 7.18 (s, 2H), 7.36 (s, 1H), 9.58 (br s, 1H); IR (Nujol) 3421, 1660, 1588 cm^{-1}. Anal. Calcd for $C_{20}H_{22}N_2O_5$: C, 64.85; H, 5.99; N, 7.56. Found: C, 64.81; H, 6.03; N, 7.55.

3.3.2. 1-Acetyl-5-(3-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (24)

Yield 63%, mp 120–122 °C. ^{1}H NMR: δ 2.43 (s, 3H), 3.02 (m, 1H), 3.75 (s, 3H), 3.86 (s, 6H), 3.90 (s, 3H), 3.93 (dd, J = 10.4, 17.0 Hz, 1H), 5.58 (dd, J = 4.8, 10.4 Hz, 1H), 6.80–7.06 (m, 3H), 7.18 (s, 2H), 7.96 (s, 1H); IR (Nujol) cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.61; H, 6.29; N, 7.29. Found: C, 65.64; H, 6.30; N, 7.31.

3.3.3. 1-Acetyl-5-(4-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (25)

Yield 56%, mp 134–136 °C. ^{1}H NMR: δ 2.42 (s, 3H, Me), 3.00 (dd, J = 4.2, 17.0 Hz, 1H), 3.82 (s, 3H), 3.84 (s, 6H), 3.90 (s, 3H), 5.55 (dd, J = 4.2, 11.0 Hz, 1H), 7.18 (s, 2H), 7.26 (d, J = 7.6 Hz, 2H), 7.78 (d, J = 7.6 Hz, 2H); IR (Nujol) 1660, 1620, 1589 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.61; H, 6.29; N, 7.29. Found: C, 65.60; H, 6.27; N, 7.26.

3.3.4. 1-Acetyl-5-[4-(dimethylamino)phenyl]-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (26)

Yield 90%, mp 148–150 °C. ^{1}H NMR: δ 2.40 (s, 3H), 2.96 (s, 6H), 3.30 (m, 1H), 3.82 (s, 3H), 3.90 (s, 3H) 3.94 (s, 6H), 5.56 (m, 1H), 6.78 (d, J = 8.0 Hz, 2H), 7.14 (d, J = 8.0 Hz, 2H), 7.17 (s, 2H); IR (Nujol) 1661, 1620, 1572 cm^{-1}. Anal. Calcd for $C_{22}H_{27}N_3O_4$: C, 66.48; H, 6.85; N, 10.57. Found: C, 66.45; H, 6.86; N, 10.55.

3.3.5. 1-Acetyl-5-[4-(diethylamino)phenyl]-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (27)

Yield 88%, mp 143–145 °C. ^{1}H NMR: δ 1.16 (t, J = 6.9 Hz, 6H), 2.40 (s, 3H), 3.30 (dd, J = 4.0, 18.0 Hz, 1H), 3.40 (d, J = 6.9 Hz, 4H), 3.82 (s, 3H), 3.88 (dd, J = 11.5, 18.0 Hz, 1H), 3.94 (s, 6H), 5.52 (dd, J = 4.0, 11.5 Hz, 1H), 6.70 (d, J = 8.4 Hz, 2H), 7.07 (d, J = 8.4 Hz, 2H), 7.17 (s, 2H); IR (Nujol) 1657, 1612, 1571 cm^{-1}. Anal. Calcd for $C_{24}H_{31}N_3O_4$: C, 67.74; H, 7.34; N, 9.87. Found: C, 67.71; H, 7.35; N, 9.84.

3.3.6. 1-Acetyl-3-(4-methylphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (28)

Yield 71%, mp 92–94 °C. ^{1}H NMR: δ 2.43 (s, 3H), 2.45 (s, 3H), 3.24 (dd, J = 4.6, 17.0 Hz, 1H), 3.73 (s, 3H), 3.83 (s, 6H), 3.88 (dd, J = 10.8, 17.0 Hz, 1H), 5.58 (dd, J = 4.6, 10.8 Hz, 1H), 6.56 (s, 2H), 7.38 (d, J = 6.0 Hz, 2H), 7.78 (d, J = 6.0 Hz, 2H); IR (Nujol) 1660, 1593 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_2O_4$: C, 68.46; H, 6.57; N, 7.60. Found: C, 68.42; H, 6.55; N, 7.61.

3.3.7. 1-Acetyl-3-(4-fluorophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (29)

Yield 78%, mp 135–137 °C. ^{1}H NMR: δ 2.44 (s, 3H), 3.31 (dd, J = 5.0, 17.0 Hz, 1H), 3.73 (s, 3H), 3.89 (s, 6H), 3.94 (dd, J = 10.6, 17.0 Hz, 1H), 5.60 (dd, J = 5.0, 10.6 Hz, 1H), 6.57 (s, 2H), 7.42 (m, 2H), 7.95 (m, 2H); IR (Nujol) 1650, 1594 cm^{-1}. Anal. Calcd for $C_{20}H_{21}FN_2O_4$: C, 64.51; H, 5.68; N, 7.52. Found: C, 64.50; H, 5.66; N, 7.50.

3.3.8. 1-Acetyl-3-(4-chlorophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (30)

Yield 95%, mp 147–149 °C. ^{1}H NMR: δ 2.44 (s, 3H), 3.28 (dd, J = 4.8, 16.8 Hz, 1H), 3.74 (s, 3H), 3.84 (s, 6H), 3.94 (dd, J = 10.8, 16.8 Hz, 1H), 5.59 (dd, J = 4.8, 10.8 Hz, 1H), 6.58 (s, 2H), 7.65 (d, J = 7.7 Hz, 2H), 7.91 (d, J = 7.7 Hz, 2H); IR (Nujol) 1667, 1592 cm^{-1}. Anal. Calcd for $C_{20}H_{21}ClN_2O_4$: C, 61.78; H, 5.44; N, 7.20. Found: C, 61.75; H, 5.41; N, 7.24.

3.3.9. 1-Acetyl-3-(3-bromophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (31)

Yield 64%, mp 152–154 °C. ^{1}H NMR: δ 2.45 (s, 3H), 3.31 (dd, J = 5.2, 17.2 Hz, 1H), 3.74 (s, 3H), 3.85 (s, 6H), 3.93 (dd, J = 10.2, 17.6 Hz, 1H), 5.60 (dd, J = 5.0, 10.2 Hz, 1H), 6.58 (s, 2H), 7.52–7.91 (m, 3H), 8.06 (s, 1H); IR (Nujol) 1656, 1593 cm^{-1}. Anal. Calcd for $C_{20}H_{21}BrN_2O_4$: C, 55.44; H, 4.89; N, 6.47. Found: C, 55.46; H, 4.87; N, 6.44.

3.3.10. 1-Acetyl-3-(4-bromophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (32)

Yield 91%, mp 138–140 °C. ^{1}H NMR: δ 2.44 (s, 3H), 3.27 (dd, J = 4.6, 16.8 Hz, 1H), 3.74 (s, 3H), 3.84 (s, 6H), 3.93 (dd, J = 10.6, 16.8 Hz, 1H), 5.60 (dd, J = 4.6, 10.6 Hz, 1H), 6.57 (s, 2H), 7.78 (d, J = 6.0 Hz, 2H), 7.84 (d, J = 6.0 Hz, 2H); IR (Nujol) 1667, 1592 cm^{-1}. Anal. Calcd for $C_{20}H_{21}BrN_2O_4$: C, 55.44; H, 4.89; N, 6.47. Found: C, 55.43; H, 4.90; N, 6.43.

3.3.11. 1-Acetyl-3-(3-hydroxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (33)

Yield 76%, mp 141–143 °C. ^{1}H NMR: δ 2.43 (s, 3H), 3.20 (dd, J = 4.6, 16.8 Hz, 1H), 3.73 (s, 3H), 3.84 (s, 6H), 3.90 (m, 1H), 5.58 (m, 1H), 6.56 (s, 2H), 6.97 (m, 1H), 7.27–7.40 (m, 3H), 9.84 (s, 1H); IR (Nujol) 3245, 1632, 1593 cm^{-1}. Anal. Calcd for $C_{20}H_{22}N_2O_5$: C, 64.85; H, 5.99; N, 7.56. Found: C, 64.81; H, 5.96; N, 7.58.

3.3.12. 1-Acetyl-3-(3-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (34)

Yield 71%, mp 115–117 °C. ^{1}H NMR: δ 2.45 (s, 3H), 3.20 (dd, J = 4.8, 17.0 Hz, 1H), 3.74 (s, 3H), 3.84 (m, 7H), 3.98 (s, 3H), 5.56 (m, 1H), 6.54 (s, 2H), 7.00 (m, 1H), 7.21–7.38 (m, 3H); IR (Nujol) 1663, 1589 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.61; H, 6.29; N, 7.29. Found: C, 65.60; H, 6.31; N, 7.26.

3.3.13. 1-Acetyl-3-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (35)

Yield 60%, mp 102–104 °C. ^{1}H NMR: δ 2.43 (s, 3H, Me), 3.22 (dd, J = 5.0, 17.0 Hz, 1H), 3.73 (s, 3H), 3.83 (s, 6H), 3.92 (dd, J = 11.2, 17.0 Hz, 1H), 5.58 (dd, J = 5.0, 11.2 Hz, 1H), 6.57 (s, 2H), 7.46 (d, J = 6.4 Hz, 2H), 7.80 (d, J = 6.1 Hz, 2H); IR (Nujol) 1660, 1590 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.61; H, 6.29; N, 7.29. Found: C, 65.63; H, 6.28; N, 7.31.

3.3.14. 1-Acetyl-3-(4-methylthiophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (36)

Yield 77%, mp 149–151 °C. ^{1}H NMR: δ 2.43 (s, 3H), 3.24 (dd, J = 4.8, 16.8 Hz, 1H), 3.73 (s, 3H), 3.83 (s, 6H), 3.90 (dd, J = 10.8, 16.8 Hz, 1H), 5.58 (dd, J = 4.8, 10.8 Hz, 1H), 6.56 (s, 2H), 7.42 (d, J = 6.1 Hz, 2H), 7.81 (d, J = 6.1 Hz, 2H); IR (Nujol) 1654, 1592 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_2O_4S$: C, 62.98; H, 6.04; N, 6.99. Found: C, 62.98; H, 6.04; N, 7.03.

3.3.15. 1-Acetyl-3-[4-(dimethylamino)phenyl]-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (37)

Yield 88%, mp 143–145 °C. ^{1}H NMR: δ 2.45 (s, 3H), 3.08 (s, 6H), 3.21 (dd, J = 4.8, 17.4 Hz, 1H), 3.74 (s, 3H), 3.83 (s, 6H), 3.90 (m, 1H), 5.54 (dd, J = 4.8, 10.4 Hz, 1H), 6.55 (s, 2H), 6.85 (d, J = 6.4 Hz, 2H), 7.70 (d, J = 6.4 Hz, 2H); IR (Nujol) 1642, 1606 cm^{-1}. Anal. Calcd for $C_{22}H_{27}N_3O_4$: C, 66.48; H, 6.85; N, 10.57. Found: C, 66.44; H, 6.87; N, 10.55.

3.3.16. 1-Acety-3-[4-(diethylamino)phenyl]-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (38)

Yield 82%, mp 138–140 °C. ^{1}H NMR: δ 1.21 (t, J = 6.5 Hz, 6H), 2.45 (s, 3H), 3.17 (dd, J = 5.0, 17.6 Hz, 1H), 3.48 (d, J = 6.5 Hz, 4H), 3.74 (s, 3H), 3.84 (s, 6H), 5.53 (dd, J = 5.0, 11.0 Hz, 1H), 6.55 (s, 2H), 6.80 (d, J = 8.8 Hz, 2H), 7.67 (d, J = 8.8 Hz, 2H); IR (Nujol) 1655, 1607 cm^{-1}. Anal. Calcd for $C_{24}H_{31}N_3O_4$: C, 67.74; H, 7.34; N, 9.87. Found: C, 67.70; H, 7.33; N, 9.84.

3.3.17. 1-Acetyl-3-(4-hydroxy-3-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (39)

Yield 84%, mp 164–166 °C. ^{1}H NMR: δ 2.43 (s, 3H), 3.24 (dd, J = 5.0, 17.2 Hz, 1H), 3.73 (s, 3H), 3.84 (s, 6H), 3.93 (s, 3H), 5.58 (dd, J = 5.0, 11.2 Hz, 1H), 6.56 (s, 2H), 6.94 (d, J = 8.4 Hz, 1H), 7.39 (m, 1H), 7.43 (s, 1H), 9.79 (br s, 1H); IR (Nujol) 3082, 1629, 1595 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 63.03; H, 6.02; N, 7.01.

3.3.18. 1-Acetyl-3-[3-[(*tert*-buthoxycarbonyl)amino]phenyl]-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (40)

Yield 82%, mp 126–128 °C. ^{1}H NMR: δ 1.57 (s, 9H), 2.44 (s, 3H), 3.29 (m, 1H), 3.73 (s, 3H), 3.84 (s, 6H), 3.93 (m, 1H), 5.65 (m, 1H), 6.56 (s, 2H), 6.80–7.10 (m, 2H), 7.20 (s, 1H), 7.29 (m, 1H), 9.74 (s, 1H); IR (Nujol) 1728, 1660, 1589 cm^{-1}. Anal. Calcd for $C_{25}H_{31}N_3O_6$: C, 63.95; H, 6.65; N, 8.95. Found: C, 63.92; H, 6.66; N, 8.99.

3.3.19. 1-Acetyl-3-[4-[(*tert*-buthoxycarbonyl)amino]phenyl]-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (41)

Yield 73%, mp 115–117 °C. ^{1}H NMR: δ 1.59 (s, 9H), 2.43 (s, 3H), 3.25 (m, 1H), 3.73 (s, 3H), 3.83 (s, 6H), 3.92 (m, 1H), 5.65 (m, 1H), 6.55 (s, 2H), 7.67 (d, J = 8.4 Hz, 2H), 7.79 (d, J = 8.4 Hz, 2H), 9.71 (s, 1H); IR (Nujol) 3360, 1724, 1658, 1592 cm^{-1}. Anal. Calcd for $C_{25}H_{31}N_3O_6$: C, 63.95; H, 6.65; N, 8.95. Found: C, 63.96; H, 6.62; N, 8.98.

3.4. General procedure for Boc-deprotection of 40 and 41

To a solution of pyrazoline **40**, **41** (0.6 mmol) in dichloromethane (5 mL) trifluoroacetic acid (2 mL) was added. The mixture was stirred at room temperature for 16 h, and then evaporated to dryness in vacuo. To the residue water (15 mL) was added and the solution treated with 28% ammonia solution to pH 9–10. The formed solid was filtered off, air died, and crystallized from *iso*-propyl ether-methanol 1:1 to give **42**, **43**.

3.4.1. 1-Acetyl-3-(3-aminophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (42)

Yield 84%; mp 122–124 °C. ^{1}H NMR: δ 2.42 (s, 3H), 3.14 (dd, J = 5.0, 17.4 Hz, 1H), 3.74 (s, 3H), 3.84 (s, 6H), 3.90 (m, 1H), 5.56 (dd, J = 5.0, 10.8 Hz, 1H), 5.35 (s, 2H), 6.55 (s, 2H), 6.77 (m, 1H), 6.98 (m, 1H), 7.17 (s, 1H), 7.20 (m, 1H); IR (Nujol) 3432, 3349, 1644, 1594 cm^{-1}. Anal. Calcd for $C_{20}H_{23}N_3O_4$: C, 65.03; H, 6.28; N, 11.37. Found: C, 65.00; H, 6.29; N, 11.40.

3.4.2. 1-Acetyl-3-(4-aminophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (43)

Yield 76%; mp 108–110 °C. ^{1}H NMR: δ 2.39 (s, 3H), 3.14 (dd, J = 4.8, 17.0 Hz, 1H), 3.73 (s, 3H), 3.83 (s, 6H), 3.90 (m, 1H), 5.52 (dd, J = 4.8, 11.0 Hz, 1H), 5.76 (s, 2H), 6.54 (s, 2H), 6.70 (d, J = 8.0 Hz, 2H), 7.56 (d, J = 6.4 Hz, 2H); IR (Nujol) 3433, 3353, 1631, 1607 cm^{-1}. Anal. Calcd for $C_{20}H_{23}N_3O_4$: C, 65.03; H, 6.28; N, 11.37. Found: C, 65.06; H, 6.32; N, 11.41.

3.5. General procedure for the preparation of 1-(Acetoxyacetyl)-4,5-dihydro-1*H*-pyrazoles 44–48

3.5.1. 1-(Acetoxyacetyl)-3-(4-methylphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (44)

To a solution of chloroacetic acid (0.19 g, 2 mmol) in 5 mL of anhydrous MeCN, HOBt (0.27 g, 2 mmol) and EDC (0.43 g, 2.2 mmol) were added. The mixture was stirred for 30 min at room temperature, then pyrazoline **12** (0.65 g, 2 mmol) was added, and the stirring continued for additional 18 h. After evaporation of the solvent in vacuo, 0.1 M aqueous hydrochloric acid (30 mL) was added and the mixture extracted with ethyl acetate (3 × 5 mL). The combined ethyl acetate layers were back-extracted with saturated sodium bicarbonate (1 × 5 mL) and brine (1 × 5 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was dissolved in 5 mL of DMSO, then potassium acetate (0.20 g, 2 mmol) was added, and the mixture stirred at room temperature for 12 h. The reaction mixture was poured into 50 mL of ice-water and the formed solid filtered off, air dried, and crystallized from *iso*-propyl ether-methanol 1:1 to give **44**. Yield 80%; mp 156–158 °C. ^{1}H NMR: δ 2.20 (s, 3H), 2.46 (s, 3H), 3.31 (dd, J = 4.8, 16.8 Hz, 1H), 3.74 (s, 3H), 3.86 (s, 6H), 3.90 (m, 1H), 5.10 (d, J = 15.8 Hz, 1H), 5.37 (d, J = 15.8 Hz, 1H), 5.61 (dd, J = 4.8, 10.8 Hz, 1H), 6.60 (s, 2H), 7.40 (d, J = 8.0 Hz, 2H), 7.82 (d, J = 8.0 Hz, 2H); IR (Nujol) 1743, 1678, 1593 cm^{-1}. Anal. Calcd for $C_{23}H_{26}N_2O_6$: C, 64.78; H, 6.15; N, 6.57. Found: C, 64.86; H, 6.10; N, 6.60.

3.5.2. 1-(Acetoxyacetyl)-3-(3-bromophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (45)

Following the general procedure, the title compound was obtained from **14**. Yield 78%; mp 146–148 °C (from methanol). ^{1}H NMR: δ 2.42 (s, 3H), 3.26 (dd, J = 5.2, 17.4 Hz, 1H), 3.73 (s, 3H), 3.83 (s, 6H), 3.94 (dd, J = 10.2, 17.4 Hz, 1H), 5.04 (d, J = 13.0 Hz, 1H), 5.10 (d, J = 13.0 Hz, 1H), 5.58 (dd, J = 5.2, 10.2 Hz, 1H), 6.56 (s, 2H), 7.40–7.82 (m, 3H), 8.08 (s, 1H); IR (Nujol) 1747, 1681, 1593 cm^{-1}. Anal. Calcd for $C_{22}H_{23}BrN_2O_6$: C, 53.78; H, 4.72; N, 5.70. Found: C, 53.74; H, 4.75; N, 5.68.

3.5.3. 1-(Acetoxyacetyl)-3-[4-(dimethylamino)phenyl]-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (46)

Following the general procedure, the title compound was obtained from **20**. Yield 89%; mp 132–134 °C (from methanol). ^{1}H NMR: δ 2.19 (s, 3H), 3.08 (s, 6H), 3.19 (dd, J = 4.4, 16.4 Hz, 1H), 3.74 (s, 3H), 3.86 (s, 6H), 3.90 (m, 1H), 5.07 (d, J = 12.4 Hz, 1H), 5.35 (d, J = 12.4 Hz, 1H), 5.54 (dd, J = 4.4, 11.0 Hz, 1H), 6.58 (s, 2H), 6.85 (d, J = 9.0 Hz, 2H), 7.71 (d, J = 9.0 Hz, 2H); IR (Nujol) 1740, 1676, 1610 cm^{-1}. Anal. Calcd for $C_{24}H_{29}N_3O_6$: C, 63.28; H, 6.42; N, 9.22. Found: C, 63.24; H, 6.46; N, 9.26.

3.5.4. 1-(Acetoxyacetyl)-3-[4-(diethylamino)phenyl]- 5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (47)

Following the general procedure, the title compound was obtained from **21**. Yield 83%; mp 122–124 °C (from ethanol). ^{1}H NMR: δ 1.20 (t, J = 6.8 Hz, 6H), 2.42 (s, 3H), 3.21 (dd, J = 5.0, 17.0 Hz, 1H), 3.46 (d, J = 6.8 Hz, 2H), 3.74 (s, 3H), 3.85 (s, 6H), 3.90 (m, 1H), 5.04 (d, J = 12.8 Hz, 1H), 5.32 (d, J = 12.8 Hz, 1H), 5.55 (dd, J = 5.0, 10.8 Hz, 1H), 6.56 (s, 2H), 6.78 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 8.4 Hz, 2H); IR (Nujol) 1747, 1666, 1607 cm^{-1}. Anal. Calcd for $C_{26}H_{33}N_3O_6$: C, 64.58; H, 6.88; N, 8.69. Found: C, 64.54; H, 6.85; N, 8.73.

3.5.5. 1-(Acetoxyacetyl)-3-(4-hydroxy-3-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (48)

Following the general procedure, the title compound was obtained from **22**. Yield 72%; mp 158–160 °C (from MeCN). ^{1}H NMR: δ 2.20 (s, 3H), 3.08 (s, 6H), 3.28 (m, 1H), 3.73 (s, 3H), 3.90 (m, 7H), 5.06 (d, J = 16.0 Hz, 1H), 5.36 (d, J = 16.0 Hz, 1H), 5.58 (m, 1H), 6.57 (s, 2H), 6.88–7.36 (m, 3H), 7.43 (s, 1H), 9.80 (br s, 1H, OH); IR (Nujol) 3108, 1748, 1681, 1594 cm^{-1}. Anal. Calcd for $C_{23}H_{26}N_3O_8$: C, 60.26; H, 5.72; N, 6.11. Found: C, 60.23; H, 5.74; N, 6.09.

3.6. General procedure for the preparation of 1-(hydroxy acetyl)-4,5-dihydro-1*H*-pyrazoles 49–53

To a solution of **44–48** (0.5 mmol) in methanol (10 mL), a solution of potassium carbonate (0.35 g, 2.5 mmol) in water (1 mL) was added. The mixture was stirred at room temperature for 4 h, then diluted with water (30 mL), and extracted with dichloromethane (3 × 10 mL). The combined organic layers were back-extracted with brine (1 × 10 mL), dried over MgSO4, filtered, and concentrated in vacuo. Crystallization from MeCN gave **49–53**.

3.6.1. 1-(Hydroxyacetyl)-3-(4-methylphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (49)

Following the general procedure, the title compound was obtained from **44**. Yield 94%; mp 106–108 °C. ^{1}H NMR: δ 2.44 (s, 3H), 3.21 (m, 1H), 3.73 (s, 3H), 3.84 (s, 6H), 3.93 (m, 1H), 4.57 (m, 2H), 5.05 (m, 1H), 5.59 (dd, J = 4.8, 11.2 Hz, 1H), 6.57 (s, 2H), 7.37 (d, J = 7.2 Hz, 2H), 7.76 (d, J = 7.2 Hz, 2H); IR (Nujol) 3480, 1652, 1594 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.61; H, 6.29; N, 7.29. Found: C, 65.60; H, 6.25; N, 7.34.

3.6.2. 3-(3-Bromophenyl)-1-(hydroxyacetyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (50)

Following the general procedure, the title compound was obtained from **45**. Yield 93%; mp 134–136 °C. ^{1}H NMR: δ 3.28 (m, 1H), 3.74 (s, 3H), 3.85 (s, 6H), 3.94 (m, 1H), 4.61 (m, 1H), 5.05 (br s, 1H), 5.65 (m, 1H), 6.60 (s, 2H), 7.45–7.78 (m, 3H), 8.06 (s, 1H); IR (Nujol) 3445, 1661, 1592 cm^{-1}. Anal. Calcd for $C_{20}H_{21}BrN_2O_5$: C, 53.47; H, 4.71; N, 6.23. Found: C, 53.44; H, 4.70; N, 6.18.

3.6.3. 3-[4-(Dimethylamino)phenyl]-1-(hydroxyacetyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (51)

Following the general procedure, the title compound was obtained from **46**. Yield 95%; mp 118–120 °C. ^{1}H NMR: δ 3.08 (s, 6H), 3.20 (dd, J = 4.8, 17.2 Hz, 1H), 3.74 (s, 3H), 3.84 (s, 6H), 3.92 (dd, J = 10.8, 17.2 Hz, 1H), 4.56 (d q, J = 6.3, 15.4 Hz, 2H), 4.87 (t, J = 6.3 Hz, 1H), 5.55 (dd, J = 4.8, 10.8 Hz, 1H), 6.58 (s, 2H), 6.84 (d, J = 8.8 Hz, 2H), 7.70 (d, J = 8.8 Hz, 2H); IR (Nujol) 3431, 1645, 1607 cm^{-1}. Anal. Calcd for $C_{22}H_{27}N_3O_5$: C, 63.91; H, 6.58; N, 10.16. Found: C, 63.93; H, 6.54; N, 10.12.

3.6.4. 3-[4-(Diethylamino)phenyl]-1-(hydroxyacetyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (52)

Following the general procedure, the title compound was obtained from **47**. Yield 92%; mp 110–112 °C. ^{1}H NMR: δ Anal. Calcd for ($C_{24}H_{31}N_3O_5$ 441.53) C, H, N. 1.21 (t, J = 6.8 Hz, 6H), 3.20 (dd, J = 4.6, 17.0 Hz, 1H), 3.58 (d, J = 6.8 Hz, 2H), 3.74 (s, 3H), 3.88 (m, 7H), 4.56 (d q, J = 6.2, 16.3 Hz, 2H), 4.87 (t, J = 6.2 Hz, 1H), 5.53 (dd, J = 4.6, 10.8 Hz, 1H), 6.58 (s, 2H), 6.79 (d, J = 8.7 Hz, 2H), 7.67 (d, J = 8.7 Hz, 2H); IR (Nujol) 3440, 1648, 1600 cm^{-1}. Anal. Calcd for $C_{24}H_{31}N_3O_5$: C, 65.29; H, 7.05; N, 9.52. Found: C, 65.32; H, 7.02; N, 9.47.

3.6.5. 1-(Hydroxyacetyl)-3-(4-hydroxy-3-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1*H*-pyrazole (53)

Following the general procedure, the title compound was obtained from **48**. Yield 93%; mp 128–130 °C. ^{1}H NMR: δ 3.24 (m, 1H), 3.58 (d, J = 6.8 Hz, 2H), 3.74 (s, 3H), 3.85 (s, 3H), 3.94 (m, 4H), 4.58 (m, 1H), 4.87 (m, 1H), 5.53 (m, 1H), 6.58 (s, 2H), 6.90–7.34 (m, 3H), 7.42 (s, 1H), 9.84 (br s, 1H); IR (Nujol) 3446, 3144, 1650, 1594 cm^{-1}. Anal. Calcd for $C_{21}H_{24}N_3O_7$: C, 60.57; H, 5.80; N, 6.73. Found: C, 60.55; H, 5.77; N, 6.70.

3.6.6. Cell culture and cytotoxicity assay

NCI-H460 human non-small cell lung carcinoma cell line was purchased from ATCC and cultured according to manufacturer's instructions. To test the effect of the drugs on cell growth, the cell line was resuspended at different concentrations in 200 μL of appropriate growth medium and seeded in 96-well plates. Twenty-four hours after plating, scalar concentrations of each drug were then added to the cells. Upon 72 h of treatment, the number of surviving cells was then determined by staining with sulforhodamine B test.[18] Optical density was measured at 570 nm. Results, expressed as concentrations that inhibit 50% of cell growth (IC_{50}), were calculated by the ALLFIT program.

3.6.7. Tubulin polymerization assay

The tubulin polymerization test was performed by CytoDINAMIX ScreenTM (Cytoskeleton Inc., Denver, CO). Pipetted into wells of a 96-well plate were 100 μL of 3 mg/mL HTS tubulin in G-PEM buffer plus 5% glycerol at 4 °C, incubated at 37 °C in presence or absence of single compounds. Tubulin polymerization was detected by measuring the absorbance of the solution (340 nm) for 60 min. Because the amount of polymerized tubulin was directly proportional to the AUC (area under the curve), we used AUC to determine the concentration that inhibited tubulin polymerization by 50% (IC_{50}). The AUC of the untreated control was set to 100% polymerization (maximal attainable polymerization), and the IC_{50} was calculated by non-linear regression.

3.6.8. Determination of GI_{50}, TGI, and LC_{50} values

A total of 60 human tumor cell lines, derived from nine cancer types (leukemia, lung, colon, brain, melanoma, ovarian, renal, prostate, and breast) formed the basis of this test. The tumor cells were cultured in RPMI1640 medium supplemented with 5% fetal calf serum and 2 mM L-glutamine. The tumor cells are inoculated over a series of standard 96-well microtrite plates in 100 mL of medium.[22,23] Density of inoculum depends on the type of tumor cell and from its growth characteristics. These cells are then preincubated on the microtrite plate for 24 h before adding the compounds. These were tested in DMSO solution at five different concentrations (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M). After an incubation of the chemical agent for 48 h with the tumor cell lines, a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The cytotoxic effects are evaluated and the assay results and dose–response parameters were calculated as previously described.[24]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.037. These data include MOL files and InChiKeys of the most important compounds described in this article.

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