Synthesis of New N\(^1\)-Substituted-5-aryl-3-(3,4,5-trimethoxyphenyl)-2-pyrazoline Derivatives as Antitumor Agents Targeting the Colchicine Site on Tubulin

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A series of pyrazoline derivatives 2a–e, 3a–e and 4a–e structurally related to combretastatin A4 (CA-4) were synthesized and characterized by spectroscopic means and elemental analyses. In these compounds, the cis double bond of CA-4 was replaced with the pyrazoline ring aiming to enhance the cytotoxic effects displayed by CA-4 and to prevent the cis/trans isomerization that is associated with inactivation of CA-4. The cytotoxic activity of all new compounds was investigated in vitro against MCF-7 and HCT-116 cell lines. The inhibition of tubulin polymerization by the most active compounds 3d, 4a and e was evaluated. The cytotoxicity of 4e was correlated with induction of apoptosis and caspase-3 activation in vitro thus indicating the apoptotic pathway of anticancer effect of these compounds. Furthermore, in vivo evaluation of the synthesized compounds was carried out against Ehrlich's ascites carcinoma (EAC) solid tumor grown in mice. Compounds 2c, 3a and e showed significant reduction in tumor weight, and about 2–4 fold increase in caspase-3 expression.

Key words  tubulin; combretastatin A4; 2-pyrazoline; cytotoxic activity; caspase-3

Microtubules are attractive targets for cancer chemotherapy. They are composed of \(\alpha\) and \(\beta\) tubulin heterodimers that are involved in numerous cellular functions including motility, division and intracellular transport.\(^1–6\) A number of small molecules bind tubulin interfering with microtubule polymerization and depolymerization, thereby inducing cell cycle arrest leading to apoptosis. It is known that the antitumor efficacy of many chemotherapeutic agents is correlated to their ability to promote apoptosis in cancer cells.\(^7\) One of the important pathways for apoptosis induction is the activation of caspase-3, which belongs to a family of cysteine protease proteins that are crucial mediators of apoptosis. Therefore, compounds that induce apoptosis in cancer cells is an attractive approach in cancer treatment.\(^8,9\)

The majority of microtubule targeting agents are derived from natural sources. Colchicine is a natural tubulin depolymerizing agent however, its therapeutic value against cancer is restricted due to poor bioavailability, high toxicity and low therapeutic index.\(^10\) Its structure is formed of three rings; a trimethoxy phenyl ring (A), a seven membered ring with an acetamide group at C\(_7\) (B) and a methoxy tropone ring (C). Rings A and C comprise the main pharmacophoric scaffold important for tubulin binding, whereas the central ring B was found to be not essential for tubulin interaction\(^11\) (Fig. 1). Lately, new tubulin targeting agents have been intensively investigated and developed. Combretastatin A4 acts as both cytotoxic and vascular disrupting agent acting through inhibition of endothelial cell proliferation and vascular tube formation important for angiogenesis.\(^12–15\) It is structurally related to colchicine where the ring B in colchicine was replaced by a cis-double bond, which allows placement of two benzene rings at right distance and dihedral angle to get maximum interaction with the colchicine binding site.\(^16\) Although CA-4 exhibits its high cytotoxic and vascular disrupting activities in vitro, it does not show efficacy in vivo because of its poor aqueous solubility as well as isomerization of its cis-double bond into the inactive trans-isomer.\(^17\) CA-4 phosphate (Zybrestat) is a water soluble produg that is currently in phase III clinical trials.\(^18–20\) One of the strategies to prevent in vivo cis/trans isomerization associated with the inactivation of CA-4 was the replacement of the olefinic bridge in CA-4 with an appropriate heterocyclic ring aiming to rigidify the structure and restrict its rotation.\(^21,22\)

In the present study, the design of the new compounds was based upon the potent cytotoxic activity displayed by the oxadiazoline derivative A-105972 that interacts with the microtubules and induces apoptosis.\(^23\) Herein, we performed the synthesis of certain CA-4 analogues containing pyrazoline core (2a–e, 3a–e and 4a–e) (Fig. 1). These compounds possess a two ring scaffold; one of them contains a trimethoxyphenyl group for anchorage, which was reported to efficiently inhibit tubulin polymerization.\(^16,21\) Moreover, due to limited water solubility of CA-4, the cis-double bond was replaced with the more polar pyrazoline ring. Furthermore, incorporation of formyl, carboxamide or carbothioamide residues at \(N^1\) of pyrazoline moiety is supposed to provide additional hydrogen binding interactions with the colchicine binding site.

RESULTS AND DISCUSSION

Chemistry As shown in Chart 1, the key intermediate chalcones 1a–e have been synthesized by Claisen–Schmidt condensation of 3,4,5-trimethoxyacetophenone and different substituted benzaldehydes in ethanol containing potassium hydroxide as a catalyst.\(^24\) Cyclization of 1a–e with hydrazine hydrate in formic acid afforded the corresponding \(N\)-formyl

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pyrazolines 2a–e. On the other hand, cyclization of 1a–e with semicarbazide hydrogen chloride (HCl) or thiosemicarbazide in alkaline medium afforded 3a–e and 4a–e, respectively.

The structures of the new compounds 2a–e, 3a–e and 4a–e were confirmed by elemental analyses and spectral data. IR spectra of the carboxaldehyde derivatives 2a–e and the carboxamide derivatives 3a–e showed a prominent band in the range of 1683–1670 cm$^{-1}$ corresponding to the carbonyl function. Further, the IR spectra of 3a–e and 4a–e revealed a forked band in the region of 3473–3253 cm$^{-1}$ indicating NH$_2$ group. In addition, compounds 4a–e showed a characteristic band at 1367–1357 cm$^{-1}$ corresponding to C=S. All compounds displayed bands corresponding to C=N due to ring closure.

Moreover, the formation of 2-pyrazoline ring in 2a–e, 3a–e and 4a–e was confirmed by the appearance of ABX system in their $^1$H-NMR spectra as a result of geminal-vicinal coupling. The pyrazoline ring system displayed three signals; a doublet of doublets in the range of 3.07–3.28 ppm (Ha), a multiplet in the range of 3.71–3.94 ppm (Hb) and a doublet of doublets...
downfield in the range of 5.28–5.89 ppm (H₂).

The protons belonging to the aromatic system and phenyl substituents were observed at the expected chemical shifts and integral values. The aldehyde proton in compounds 2a–e was displayed at 8.86–8.93 ppm. D₂O exchangeable signals corresponding to NH₂ protons in 3a–e and 4a–e were observed in the range of 6.48–6.56 and 7.96–8.10 ppm, respectively.

Mass spectra of the compounds 2a, 3a and 4a showed the characteristic M and M+2 peaks confirming the presence of Br. In addition, compounds 3a–e showed a characteristic fragmentation peak corresponding to M–CONH₂, however the mass spectra of the compounds 4a–e displayed a peak at m/z 60 corresponding to CSNH₂. The synthetic route for the preparation of the new compounds 2a–e, 3a–e and 4a–e is outlined in Chart 1.

**Biological Study**

**In Vitro Screening**

**Cytotoxic Activity**

All the synthesized compounds 2a–e, 3a–e and 4a–e were evaluated for cytotoxic activity against breast cancer cell line...
MCF-7 and colon cancer cell line HCT-116 using sulforhodamine B stain (SRB) colorimetric assay. The results were summarized and represented graphically (Table 1, Fig. 2).

Compounds 3a, d, 4a and e displayed the highest inhibitory activity (IC$_{50}$ = 6.88–12.43 $\mu$M) against both breast and colon cancer cell lines. These compounds showed reasonable cytotoxic activity against MCF-7 cell line and a moderate activity against HCT-116 cell line in comparison to colchicine as a reference compound. Moreover, compounds 2c and 4d revealed moderate cytotoxic activity against MCF-7 with IC$_{50}$ of 16.73 and 19.50 $\mu$M respectively.

Among the pyrazoline derivatives 2a–e, compound 2c containing 3,4-dimethoxy substitution on the ring B showed the highest cytotoxic activity against both tested cell lines, whereas compounds 3a and d with the 4-bromo and 3,4,5-trimethoxy substitution respectively were the most active in 3a–e series against MCF-7 cell line. Furthermore, compounds 4a and e containing the 4-bromo and 4-dimethylamino substitution respectively showed comparable cytotoxic activity against both tested cancer cell lines.

Data in Table 1 revealed that the carboxamide derivatives 3a–e and the carbothioamide derivatives 4a–e were more active than the carboxaldehyde derivatives 2a–e against both tested cancer cell lines. This may be attributed to presence of NH$_2$ group that could provide additional hydrogen bonding interaction with the colchicine binding site.

Immunohistochemistry

Three of the most active compounds in the SRB assay (3d, 4a and e) were subjected for immunohistochemistry evaluation to establish the effect of these compounds on cellular microtubules. MCF-7 cells were treated with 30% of the IC$_{50}$ of the tested compounds for 48 h, fixed and immunostained for microtubules, then compared to the untreated cells (negative control). The resulted microscope images are presented in Fig. 3.

Tubulin analysis in the untreated MCF-7 cells (A) showed living cells with no signs of toxicity as well as distinct acridine stained nucleus with homogenous and normal tubulin expression pattern and normal cytoplasm/nucleus proportion. The cell–cell microtubules mesh was intact and healthy active with a homogenous intracellular distribution. On the other hand, cells treated with the tested compounds 3d, 4a and e showed moderate to high malformed cellular structure with shrunk nuclei, abnormal reduced cytoplasm/nucleus proportion, abnormal tubulin expression pattern and reduction in the cell–cell microtubules mesh. The photo micrographs illustrated that the cytotoxicity of these compounds may be attributed to interference with the microtubule assembly.

Tubulin Polymerization Inhibition Assay

Compounds 3d, 4a and e were further investigated for inhibition of tubulin polymerization following enzyme linked immunosorbent assay (ELISA) using human $\beta$-tubulin assay kit SEB870Hu (Cloud-Clone Corp., U.S.A.). Colchicine was used as a positive control and the results are summarized in Table 2. The results revealed that the tested compounds produced significant tubulin suppression compared to the reference antimitotic agent colchicine. Therefore, cytotoxic activity of the tested compounds may be referred to their tubulin inhibitory activity.

Caspase-3 Activation Assay

Activation of caspases plays a crucial role in induction of apoptosis. Caspase-3 is one of the key effector caspases that is essential for certain biochemical events and cell morphological changes associated with the execution of programmed cell death. Compound 4e was selected to correlate its cytotoxic activity with the induction of apoptosis through activation of caspase-3. The amount of activated caspase-3 was investigated following ELISA using human active caspase-3 Invitrogen EIA kit, Catalogue No. KHO1091 (Camarillo, CA, U.S.A.). Colchicine was used as a reference and the results are summarized in Table 3 and Fig. 4. The results showed that 4e produced about 5 fold increases in cleaved caspase-3.

In Vivo Evaluation of Antitumor Activity

All newly synthesized compounds 2a–e, 3a–e and 4a–e were tested for in vivo antitumor activity against solid tumors.

Table 2. Percentage Inhibition of Tubulin Polymerization at IC$_{50}$ Concentration for the Compounds 3d, 4a and e

| Compound No. | IC$_{50}$ ($\mu$g/mL) | % Inhibition of tubulin polymerization (MCF-7) |
|-------------|-----------------------|--------------------------------------------|
| 3d          | 3.60                  | 75.70                                      |
| 4a          | 3.70                  | 84.40                                      |
| 4e          | 2.84                  | 81.60                                      |
| Colchicine  | 4.92                  | 74.30                                      |

Table 3. Caspase-3 Concentration of 4e, Colchicine and Control MCF-7 Cells

| Compound No. | Concentration of active caspase-3 in ng/mL |
|--------------|-------------------------------------------|
| 4e           | 0.645                                     |
| Colchicine   | 0.483                                     |
| Control      | 0.134                                     |
Ehrlich’s ascites tumors (EAT) grown in mice and compared to colchicine as the reference compound. The results showed that colchicine produced marginally non-significant decrease in tumor mass compared to EAT control group, which may be attributed to its poor bioavailability. On the other hand, all tested compounds except 4c, produced significant reduction in tumor masses in comparison to the control (Fig. 5). Some selected compounds that showed significant reduction in tumor masses; 2c, 3a and e were further investigated for their proapoptotic potential via induction of the expression of caspase-3.

Microscopic examination of solid tumor sections stained with hematoxylin and eosin (H&E) revealed that colchicine did not significantly reduce the total histological score compared to EAT control group. However, the three selected highly-active compounds 2c, 3a and e showed reduction in the total histological score compared to both EAT control group and colchicine-treated group as well ($p<0.05$, Table 4, Fig. 6).

Immunostaining for caspase-3 activity demonstrated low degree of staining in both EAT control group and the colchicine group (Fig. 7a). On the other hand, treatment with the selected new compounds 2c, 3a and e resulted in significant increase in tumoral expression of caspase-3, leading to stronger antitumor activity compared to EAT control group and colchicine-treated group (Fig. 7b).

**Molecular Modelling**

Docking simulation was performed to predict the probable binding mode of the most active compounds 3d, 4a and e with the crystal structure of tubulin at the colchicine binding site (PDB ID: 3E22) using discovery studio software version 2.55. The co-crystallized ligand (colchicine) revealed H-bond interaction of the 4-methoxy group in ring A with $\beta$Cyst241 (distance 2.15 Å). To gain better understanding on the important

**Fig. 4.** Effect of 4e and Colchicine on Caspase-3 Activity

MCF-7 cells were treated with the tested compounds at IC$_{50}$ concentration values for 24h.

**Fig. 5.** Effect of Colchicine and the Tested Compounds 2a–e, 3a–e and 4a–e on Tumor Mass in Ehrlich’s Tumor Bearing Mice

Mice were inoculated with Ehrlich’s tumor at day 1 while treatment with different drugs was initiated at day 8. Data were presented as the mean±S.E.M. and analyzed using one-way ANOVA followed by post-hoc analysis at $p<0.05$ employing SPSS program. * Compared to EAT control. $ Compared to colchicine.

**Table 4.** Scoring for Sections from Solid EAT Grown in Mice Treated with Colchicine or the Tested Compounds

| Groups       | Necrosis area | Giant cell | Mitotic picture | Total score |
|--------------|---------------|------------|-----------------|-------------|
| EAT control  | 3±0           | 3±0        | 3±0             | 9±0         |
| Colchicine   | 2.7±0.2       | 2.2±0.2    | 2.3±0.2         | 7.2±0.5     |
| 2c           | 1.8±0.2*$^*$  | 0±0*$^*$   | 1.83±0.2*       | 3.7±0.2*$^*$|
| 3a           | 1.8±0.2*$^*$  | 0±0*$^*$   | 1.83±0.2*       | 3.7±0.2*$^*$|
| 3e           | 1.8±0.2*$^*$  | 0±0*$^*$   | 1.83±0.2*       | 3.7±0.2*$^*$|

EAT: Ehrlich’s ascites tumor. Scoring for H&E stained tumors was done as (0) absent, (1) low or weak, (2) mild to moderate and (3) high or frequent and the total score was calculated. Results were presented as the mean±S.E.M. and analyzed using one-way ANOVA followed by Bonferroni’s post-hoc test at $p<0.05$. *Significantly different from EAT control. $^*$Significantly different from colchicine group.
interactions with the colchicine binding site, we proceeded to examine CA-4 and A-105972 binding modes. CA-4 was docked into the active site of the enzyme and showed three H-bond interactions with two amino acid residues βCyst241 and αAsn101. SH of βCyst241 interacted with oxygen atom of 4-methoxy group in ring A through one H-bond (distance 2.18 Å). In addition, αAsn101 NH2 and C=O groups interacted with oxygen atom of 3-methoxy group and hydrogen atom of OH group in ring B respectively (distance 2.80, 2.05 Å, respectively) through two H-bonds with an estimated binding energy score = −15.50 kcal/mol (Fig. 8).

Both lead compounds, CA-4 and A-105972 showed comparable H-bond interactions and binding energy scores. Docking simulation of A-105972 revealed two H-bond interactions between oxygen atom of 3- and 4-methoxy groups in ring A and SH of βCyst241 (distance 2.28, 2.79 Å, respectively), a H-bond between oxygen of 3-methoxy group and hydrogen atom of OH group in ring B respectively (distance 2.80, 2.05 Å, respectively) through two H-bonds with an estimated binding energy score = −16.07 kcal/mol (Fig. 9).

Similarly, all docked compounds were capable of occupying the colchicine binding site while maintaining the essential H-bond interactions with the same amino acid residue (βCyst241). The trimethoxy phenyl (ring A) in compounds 3d, 4a and e was superimposed with that of colchicine. Compound 3d was bound to tubulin at the colchicine binding site through five H-bonds with three amino acid residues; βCyst241, βAsn258 and αAsn101 with an estimated binding energy score = −11.68 kcal/mol. βCyst241 forms two SH…O H-bonds (distance 2.97, 2.24 Å) with oxygens of 3- and 4-methoxy groups of ring A respectively, while βAsn258 forms two H-bonds (distance 2.47, 2.86 Å) with the NH2 hydrogens. Furthermore, αAsn101 was bound to 3-methoxy group of ring B through one H-bond (distance 2.13 Å). In addition, βLys352 was involved in π–cation interaction with the ring B (trimethoxyphenyl) (Fig. 10). Although 3d was involved in several H-bonding interactions with the colchicine binding site, it showed lower binding energy score compared to 4a and e. This may be referred to steric hindrance associated with the presence of trimethoxy group on ring B leading to twisting of the compound 3d to an unfavourable orientation.

Compounds 4a and e showed comparable both cytotoxic activity and free binding energy scores. Compound 4a was bound appropriately through four H-bonds with βCyst241, αThr179 and βLys254 with an estimated interaction energy = −15.27 kcal/mol. βCyst241 forms a H-bond with the oxygen of 4-methoxy group in ring A (distance 2.34 Å). It is worth to mention that 4a takes a different orientation so that carbothioamide moiety is in proximity to αThr179 forming two H-bonds with hydrogens of the NH2 group (distance 2.13, 2.63 Å) (Fig. 9). H-Bonding with αThr179 amino acid residue at the colchicine binding site was reported to increase the cytotoxic activity. βLys254 was bound to the bromine atom in ring B through a H-bond (distance 2.71 Å). It was also involved in π–cation interaction with 4-bromophenyl ring (Fig. 11).

Binding model of 4e revealed three H-bond interactions with βCyst241 and βAsn258 with an estimated binding free energy score = −16.28 kcal/mol. SH of βCyst241 forms one H-bond (distance 2.35 Å) with the oxygen of 4-methoxy group in ring A, while βAsn258 forms two H-Bonds; one with the
hydrogen atom of NH₂ group (distance 2.18 Å) and the other with the sulphur atom of C=S (distance 2.87 Å) (Fig. 12). H-Bonding with the polar amide amino acid residue βAsn258 increases stability of the ligand–colchicine binding site complex.27,28) The binding modes of 3d, 4a and e were similar to that of CA-4 and A-105972, they interacted with similar amino acid residues including βCys241, αAsn101 and βLys254 and showed comparable binding energy scores=−11.68 to −16.28 kcal/mol. The length of all H-bond interactions was less than 3 Å. It is clear that these compounds fit well in the colchicine binding site showing good interactions and binding free energy scores compared to colchicine.

**CONCLUSION**

In this study, modifications of prior lead compound A-105972 resulted in the synthesis of three series of 2-pyrazoline derivatives as a novel class of tubulin polymerization inhibitors that bind to the colchicine site on tubulin. Four compounds; 3a, d, 4a and e showed reasonable cytotoxic activity compared to colchicine against MCF-7 cell line with IC₅₀ (6.85–8.51 µM) range, and a moderate effect against HCT-116 cell line with IC₅₀ (6.88–12.43 µM) range. Three most active
compounds 3d, 4a and e showed percentage suppression of tubulin comparable to that of colchicine in breast cancer cells (MCF-7) (75.7, 84.4, 81.6%, respectively). Immunohistochemistry assay showed that the cytotoxic effect exerted by these compounds may be referred to the interference with the microtubule assembly.

In vivo studies against Ehrlich’s ascites (EAT) solid tumor grown in mice revealed that significant reduction in tumor weight has been shown by the compounds 2c, 3a and e. These compounds demonstrated 2−4 fold increases in tumoral expression of caspase-3 compared to control.

Aiming to rationalize the biological results, molecular docking was further performed. After analysis of the binding modes of the compounds 3d, 4a and e with the colchicine site of tubulin and comparing the results with that of CA-4 and A-105972, it was obvious that these compounds could appropriately bind to the colchicine binding site of α- and β-tubulin through H-bond interactions with βCys241, which may play a crucial role in its antitubulin polymerization and antiproliferative activities. In addition, the NH2 group in the pyrazoline derivatives 3a−e and 4a−e also interacted with βAsn258 or αThr179. Therefore, tubulin could be considered a good target for pyrazoline derivatives containing a trimethoxyphenyl scaffold.

MATERIALS AND METHODS

Chemistry The starting materials 1a−e were prepared as reported. Other chemicals and reagents were obtained from Aldrich, Fluka or Merck and were used without further purification. Progress of the reactions was monitored using TLC sheets precoated with UV fluorescent silica gel Merck 60F 254. The solvent system was benzene−chloroform−methanol (5:9:1) and spots were visualized using UV lamp. IR spectra were determined on Shimadzu FT-IR 8400s spectrophotometer (KBr, cm−1). 1H-NMR spectra were carried out using a Mercury 300-BB 300 MHz and Bruker 400 MHz spectrophotometers using tetramethylsilane (TMS) as internal standard. 13C-NMR spectra were carried out using a Mercury 300-BB 75 MHz and Bruker 100 MHz using TMS as internal standard. Chemical shifts (δ) are recorded in ppm on δ scale. Mass spectra were performed on Shimadzu QP-2010 plus mass spectrophotometer at 70 eV. Elemental analysis was carried out at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt. Melting points were determined on Stuart apparatus and the values given are uncorrected.

General Procedure for the Preparation of 2a−e To a mixture of the appropriate aldehyde 1a−e (0.001 mol) and formic acid (5 mL), hydrazine hydrate 99% (0.001 mol, 0.05 g) was added. The reaction mixture was heated under reflux for 8−12 h, then cooled and poured onto ice-cold water. The solid product formed was collected and crystallized from ethanol.

5-(4-Bromophenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbaldehyde (2a)

Yield 90%; mp 147−148°C; 1H-NMR (DMSO-d6, 300 MHz) δ (ppm): 3.26 (1H, dd, Jαδ=5Hz, Jαβ=18Hz), 3.71 (3H, s), 3.82 (6H, s), 3.88−3.94 (1H, m), 5.51 (1H, dd, Jαδ=5Hz, Jαβ=12Hz), 7.06 (2H, s), 7.19 (2H, d, J=7.5Hz), 7.54 (2H, d, J=7.5Hz), 8.90 (1H, s); IR (KBr) cm−1: 3074, 2968, 2918, 2798, 1670; electron ionization (EI)-MS m/z (% rel. int.): 419.80 (M+2), 418.85 (M+H), 417.80 (M+), 234.95, 208.95, 193.95. Anal. Calc. for C19H16BrN2O4: C, 45.68; H, 3.32; N, 7.51. Found: C, 45.62; H, 3.34; N, 7.50.

5-(4-Dimethylaminophenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbaldehyde (2b)

Yield 69%; mp 129−130°C; 1H-NMR (DMSO-d6, 300 MHz) δ (ppm): 3.23 (1H, dd, Jαδ=4Hz, Jαβ=18Hz), 3.69 (3H, s), 3.72 (3H, s), 3.82 (6H, s), 3.86−3.90 (1H, m), 5.46 (1H, dd, Jαδ=5Hz, Jαβ=12Hz), 6.89 (2H, d, J=7Hz), 7.08 (2H, s), 7.16 (2H, d, J=7Hz), 8.88 (1H, s); IR (KBr) cm−1: 3062, 2931, 2868, 2829, 1674; EI-MS m/z (% rel. int.): 370.85 (M+H), 369.95 (M+), 340.90, 193.95. Anal. Calc. for C22H25N2O4: C, 64.85; H, 5.99; N, 7.56. Found: C, 65.52; H, 6.42; N, 8.13.

5-(3,4-Dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbaldehyde (2c)

Yield 81%; mp 156−157°C; 1H-NMR (DMSO-d6, 400 MHz) δ (ppm): 3.25 (1H, dd, Jαδ=4.8Hz, Jαβ=18Hz), 3.64 (3H, s), 3.73 (3H, s), 3.74 (3H, s), 3.83 (6H, s), 3.84−3.90 (1H, m), 5.46 (1H, dd, Jαδ=5Hz, Jαβ=12Hz), 6.70 (1H, d, J=8Hz), 6.84 (1H, s), 6.90 (1H, d, J=8Hz), 7.09 (2H, s), 8.90 (1H, s); IR (KBr) cm−1: 3001, 2941, 2877, 2829, 1674; EI-MS m/z (% rel. int.): 410.01 (M+H), 400.00 (M+), 371, 234.95, 208.95, 91.00. Anal. Calc. for C20H22N2O5: C, 62.99; H, 6.04; N, 7.63. Found: C, 63.17; H, 6.08; N, 7.08.

5-(3,4,5-Trimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbaldehyde (2d)

Yield 90%; mp 149−150°C; 1H-NMR (DMSO-d6, 400 MHz) δ (ppm): 3.28 (1H, dd, Jαδ=4Hz, Jαβ=18Hz), 3.64 (3H, s), 3.70 (3H, s), 3.75 (6H, s), 3.83−3.87 (1H, m), 5.46 (1H, dd, Jαδ=4Hz, Jαβ=12Hz), 6.51 (2H, s), 7.06 (2H, s), 8.93 (1H, s); 13C-NMR (DMSO-d6) ppm: 43.09, 56.47, 59.23, 60.60, 103.08, 104.65, 126.65, 137.13, 137.64, 140.00, 153.61, 156.57, 159.45, 160.20; IR (KBr) cm−1: 3074, 2937, 2887, 2825, 1670; EI-MS m/z (% rel. int.): 430.95 (M+H), 430.00 (M), 429.05 (M−H), 401.23, 245.94, 200.00. Anal. Calc. for C2H2N2O5: C, 61.39; H, 6.09; N, 6.51. Found: C, 61.54; H, 6.13; N, 6.62.

5-(4-Dimethylaminophenyl)-3-(3,4,5-Trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbaldehyde (2e)

Yield 71%; mp 144−145°C; 1H-NMR (DMSO-d6, 400 MHz) δ (ppm): 2.85 (6H, s), 3.22 (1H, dd, Jαδ=4Hz, Jαβ=18Hz), 3.71 (3H, s), 3.79 (6H, s), 3.83−3.86 (1H, m), 5.40 (1H, dd, Jαδ=4Hz, Jαβ=11.5Hz), 6.67 (2H, d, J=8.5Hz), 7.02 (2H, d, J=8.5Hz), 7.06 (2H, s), 8.86 (1H, s); IR (KBr) cm−1: 3009,
δ (2H, d, 3H, s), 3.73 (3H, s), 3.74–3.77 (1H, m), 3.82 (6H, s), 5.33 (1H, m). Rel. Int.: 446.00 (M+H), 344.80, 234.90, 103, 90.95; Anal. Cacld for C19H20BrN3O3S: C, 50.67; H, 4.48; N, 9.33. Found: C, 50.81; H, 4.53; N, 9.41.

5-(4-Methylphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide (4a)

Yield 65%; mp 199–200°C; 1H-NMR (DMSO-d6, 400 MHz) δ (ppm): 3.20 (1H, dd, δax=3Hz, δax=11Hz), 3.69 (3H, s), 3.83 (6H, s), 3.85–3.89 (1H, m), 5.89 (1H, dd, δax=3Hz, δax=11Hz), 7.13–7.19 (6H, m), 8.04 (2H, O=CH, O=CH) (KBr) cm−1: 3439, 3273, 3074, 2995, 2933, 1367; EI-MS m/z (% rel. int.): 400.80 (M+), 206.90, 192.90, 59.90; Anal. Cacld for C22H27N3O7: C, 59.83; H, 5.77; N, 10.47. Found: C, 59.99; H, 5.83; N, 10.65.

5-(3,4-Dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide (4d)

Yield 75%; mp 177–178°C; 1H-NMR (DMSO-d6, 400 MHz) δ (ppm): 3.21 (1H, dd, δax=3Hz, δax=18Hz), 3.69 (3H, s), 3.71 (3H, s), 3.72–3.82 (1H, m), 3.83 (6H, s), 5.85 (1H, dd, δax=3Hz, δax=11Hz), 6.57 (1H, d, δ=8.5Hz), 7.03 (2H, d, δ=8.5Hz), 7.18 (2H, s), 8.04 (2H, O=CH, O=CH) (KBr) cm−1: 3446, 3321, 3061, 2933, 2833, 1367; EI-MS m/z (% rel. int.): 432.00 (M+H), 431.00 (M+), 430.05 (M+H), 371.00, 222.95, 163, 91.00, 59.95; Anal. Cacld for C22H27N3O7S: C, 58.45; H, 5.84; N, 9.74. Found: C, 58.62; H, 5.88; N, 9.89.

5-(3,4,5-Trifluoromethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide (4e)

Yield 79%; mp 189–190°C; 1H-NMR (DMSO-d6, 400 MHz) δ (ppm): 3.25 (1H, dd, δax=3.5Hz, δax=18Hz), 3.64 (3H, s), 3.70 (3H, s), 3.73 (6H, s), 3.74–3.83 (1H, m), 3.84 (6H, s), 5.86 (1H, dd, δax=3.5Hz, δax=11Hz), 6.43 (2H, s), 7.19 (2H, s), 8.10 (2H, O=CH, O=CH) (KBr) cm−1: 3446, 3331, 3074, 2937, 2840, 167; EI-MS m/z (% rel. int.): 461.95 (M+H), 461.00 (M+), 460.00 (M–H), 410.00, 267.95, 266.95, 234.95, 191.00, 59.95; Anal. Cacld for C22H17F3N3O7S: C, 57.25; H, 5.90; N, 9.10. Found: C, 57.49; H,
5.98; N, 9.23.

5-(4-Dimethylaminophenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (4e)

Yield 96%; mp 169–170°C. 1H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.83 (6H, s), 3.20 (1H, dd, J=α=3 Hz, J=β=18 Hz), 3.68 (3H, s), 3.82 (6H, s), 3.84–3.92 (1H, m), 5.89 (1H, dd, J=α=3 Hz, J=β=11 Hz), 6.66–7.58 (6H, m), 7.96 (2H, s, DIO exchangeable); IR (KBr) cm\(^{-1}\): 3410, 3253, 3061, 2993, 2931, 1361; EI-MS m/z (% rel. int.): 413.90 (M\(^+\)), 219.90, 146.00, 59.95; Anal. Calcd for C\(_{21}\)H\(_{26}\)N\(_4\)O\(_3\)S: C, 60.85; H, 6.32; N, 13.52. Found: C, 61.04; H, 6.41; N, 13.74.

Biological Study

In Vitro Screening

Growth Inhibition Assay

The response of two different carcinoma cell lines; colon cancer cell line (HCT-116) and breast cancer cell line (MCF-7) to the newly synthesized compounds was evaluated by the determination of cell survival using SRB assay following the method of Skehan et al.\(^{29}\) Colchicine was used as a positive control. Cells from different cell lines were cultured in 96-well plate (104 cells/well) for 24 h before treatment with the compounds, to allow cell attachment. Different concentrations of each compound (0, 1, 2.5, 5, 10 \(\mu\)g/mL) were added to the cell monolayer triplicate wells, which were prepared for each individual dose. Cells were incubated with the compounds for 48 h at 37°C and in atmosphere of 5% CO\(_2\), then they were collected, washed, and stained with sulphorhodamine B stain. Excess stain was washed with acetic acid and the attached stain was recovered with tris ethylenediaminetetraacetic acid (EDTA) buffer. Color intensity was measured using an ELISA reader. The relation between surviving fraction and drug concentration was plotted to get the survival curve of each tumor cell line. The IC\(_{50}\) value was calculated using sigmoidal dose response curve-fitting models (GraphPad, Prizm Software Incorporated).

Immunohistochemistry

Human breast carcinoma cells were cultured onto multiwell confocal adhesive slides until confluence. The cells were treated with 30% of the IC\(_{50}\) of the tested compounds for 48 h then fixed on slides by glacial acetic acid–methanol mixture. Antigen retrieval was performed, then the slides were incubated with the primary antibody (1:1000) [Abcam, U.S.A.]. After washing, slides were soaked in conjugated fluorescein isothiocyanate (FITC) goat immunoglobulin G (IgG) antibodies (1:2000) [Abcam] then in acridine orange solution (100 \(\mu\)g/mL in phosphate buffered saline (PBS)) for 10 min, and washed by PBS. Fluorescent images were visualized using Apotome fluorescence microscope (Axioskop Plus, Zeiss, Göttingen, Germany) equipped with image analyzer and digital camera (PowerShot A20, Canon, U.S.A.).

In Vitro Tubulin Polymerization Assay

MCF-7 cells were obtained from American Type Culture Collection, and cultured using Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen/Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 10 \(\mu\)g/mL of insulin (Sigma-Aldrich, MO, U.S.A.), and 1% penicillin–streptomycin. All other chemicals and reagents were purchased from Sigma, or Invitrogen. MCF-7 cells were cultured in 96 well plate (10000 cells/well, cells density 1.2–1.8). The cells were incubated with the tested compounds at IC\(_{50}\) concentration of each compound (Table 2) 24 h before enzyme assay. Tubulin polymerization inhibitory activity was determined using human \(\beta\)-tubulin SEB870HU assay kit (Cloud-Clone Corp., U.S.A.). The procedure of the used kit was performed according to the manufacturer’s instructions.

In Vitro Caspase-3 Activation Assay

MCF-7 cells were purchased from American Type Culture Collection. Cells were grown in Roswell Park Memorial Institute medium (RPMI) 1640 containing 10% foetal bovine serum at 37°C, stimulated with the compounds to be tested for caspase-3 at their IC\(_{50}\) concentration and lysed with cell extraction buffer. This lysate was diluted in standard diluent buffer and measured for human active caspase-3 content. The assay was performed using human active caspase-3 Invitrogen EIA kit, Catalogue No. KHO1091 (Camarillo, CA, U.S.A.). The procedure of the used kit was done according to the manufacturer’s instructions.

In Vivo Evaluation of Antitumor Activity

Induction of Solid EAT in Mice

In this study, adult female Swiss albino mice were used under fixed housing conditions under normal dark/light cycle. Mice were housed in clean polyethylene cages with food and water provided ad libitum. The study protocol was approved by the Research Ethics Committee at the Faculty of Pharmacy, Suez Canal University (license number 201412A2). A mouse carrying EAT cell line was purchased from the Department of Tumor Biology at the National Cancer Institute (Cairo, Egypt). First, Trypan blue exclusion test was employed to detect the viability of EAT cells according to the method reported previously.\(^{30}\) Second, a suspension of EAT cells was prepared in sterile saline to obtain a concentration equals 2.5 million of EAT cells/0.1 mL. At the beginning of the experiment, mice were shaved at their back and inoculated subcutaneously with 0.1 mL of the EAT suspension in their back.

Experimental Design

EAT is commonly employed as a solid form to test antitumor activity of drugs and natural compounds.\(^ {32,33}\) Seven days after inoculation with the tumor cells, mice were randomly divided into 17 groups. The therapeutic regimens (or vehicle) were initiated at day 8 for 14 d as follows, Group 1: mice treated with DMSO (5 mL/kg, intraperitoneally (i.p.)) daily, Group 2: mice treated with the reference compound, colchicine (1 mg/kg, i.p.) daily.\(^ {34}\) Groups 3–7: mice were treated with compounds \(2a, b, c, d\) and \(e\) (1 mg/kg, i.p.) daily. Groups 8–12: mice were treated with compounds \(3a, b, c, d\) and \(e\) (1 mg/kg, i.p.) daily. Groups 13–17: mice were treated with compounds \(4a, b, c, d\) and \(e\) (1 mg/kg, i.p.) daily. All therapies were continued for 14 d.

Measurement of Tumor Mass and Histopathological Examination

At day 22, mice were anesthetized by ether, sacrificed by cervical dislocation and the skin on the back was cut to dissect the implanted solid tumors. The tumors were weighed and immersed in 10% formalin solution then embedded in paraffin and sectioned at 4 \(\mu\)m for routine staining with H&E. Histopathological examination for the tumor sections determined the extension of necrotic area, the presence of neoplastic giant cells and demonstrated the typical mitotic picture under the light microscope. It was done blindly and each of these findings was scored according to their frequency in the microscopic fields as: (0) absent, (1) weak or low, (2) mild to...
moderate and (3) high or frequent. The total of these scores was calculated and processed for statistical comparison.23

Immunohistochemical Staining for Caspase-3 and Image Analysis

Immunohistochemical staining for caspase-3 was done on another 4-μm tissue section. Primary antibodies against caspase-3 [ab4051], supplied by Abcam Company (Cambridge Science Park, Cambridge CB4 0FL, U.K.), were added to each section and incubated at 4°C. The biotinylated secondary antibodies were added followed by the enzyme conjugate and 3,3-diaminobenzidine, which was used as a chromogen. Images from different areas covering the surface of the cut section were captured under a light microscope and processed for analysis using the Image J 1.45 system (National Institute of Health, U.S.A.). The percent of the area of caspase-3 immunostaining was determined and compared.

Statistical Analysis

Data of the experiment were collected and presented as the mean±standard error of the mean (S.E.M.). One-way ANOVA was employed for statistical analysis and was followed by Bonferroni’s post-hoc analysis at p value <0.05. Statistical tests were performed using the statistical package for social science (Chicago, U.S.A.), version 16.

Molecular Docking

The X-ray crystal structure of DAMA–colchicine–tubulin complex (PDB code 3E22) was downloaded from http://www.rcsb.org/pdb. All molecular modelling calculations and docking studies were carried out using Discovery Studio software (version 2.55). Automatic protein preparation was done using CHARMM forcefield. 3D structures of target compounds were built using ChemBioDraw Ultra 11.0. Our ligands were prepared using Accelry’s discovery studio prepare ligands protocol. The validation results showed the same binding interactions of the co-crystallized and the re- docked ligand with rmsd of 0.50 Å and docking score of −6.66 kcal/mol.

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Conflict of Interest

The authors declare no conflict of interest.

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