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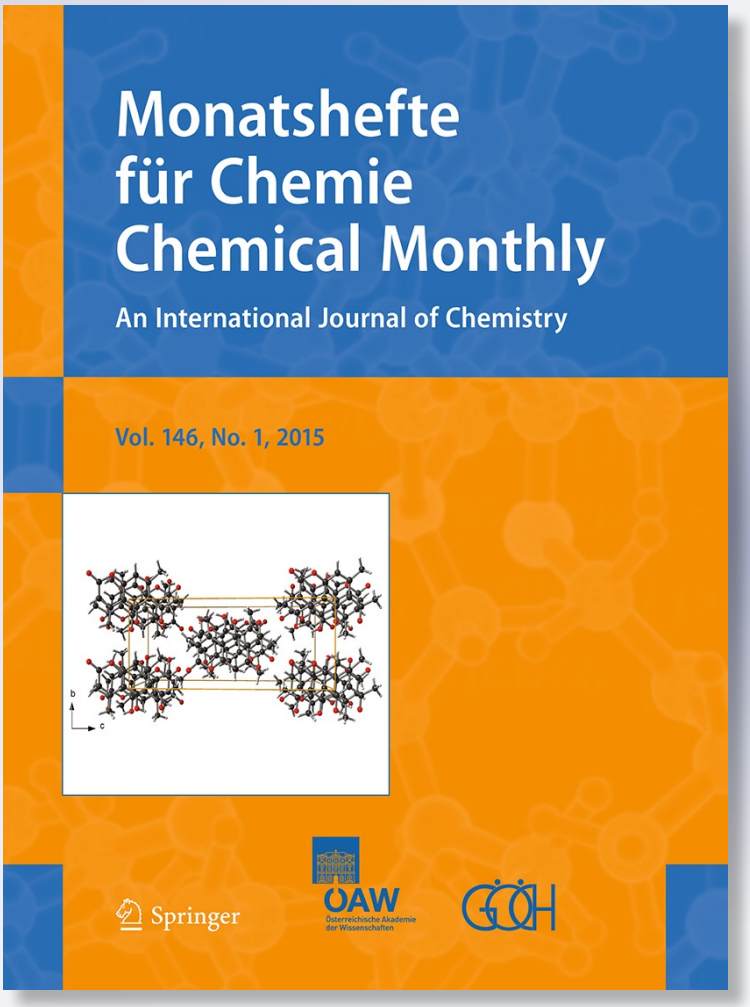

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ORIGINAL PAPER

Synthesis, characterization, and pharmacological evaluation of some novel thiadiazoles and thiazoles incorporating pyrazole moiety as anticancer agents

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Abstract Two series of novel 2-[[1-(5-methyl-1-phenyl-5-substituted-1*H*-pyrazol-4-yl)ethylidene]hydrazono]-3-phenyl-2,3-dihydro-1,3,4-thiadiazole derivatives and 2-[5-(4-chlorophenyl)-5′-methyl-1′-phenyl-3,4-dihydro-1′*H*,2*H*-[3,4′-bipyrazol]-2-yl]-4-substituted-5-(phenyldiazenyl)thiazole derivatives were prepared from reaction of hydrazonoyl halides with methyl 2-[1-(5-methyl-1-phenyl-1*H*-pyrazol-4-yl)ethylidene]hydrazine-carbodithioate and thiosemicarbazide derivative, respectively. The newly synthesized derivatives were elucidated by elemental analysis, spectral data, and alternative synthetic routes, whenever possible. The anti-cancer activity of the selected products against the breast carcinoma cell line MCF-7 was determined by WST-1 assay indicating concentration-dependent cellular growth inhibitory effect especially for three compounds with dose response curves indicating IC_{50} values of 21.3 ± 0.72, 21.3 ± 0.72, and 23.56 ± 0.81 μg cm^{-3}, respectively. Confocal laser scanning imaging of the treated cells stained by rhodamin 123 and acridine orange dyes confirms that the selected compounds inhibit the mitochondrial lactate dehydrogenase enzymes. The obtained results revealed promising anticancer activity.

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Keywords 1,3,4,-Thiadiazoles · Thiazole · Antitumor agents · Hydrazonoyl halides · Bioorganic chemistry · Reaction mechanisms

Introduction

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells, which can lead to death if left untreated. Despite the considerable progress made over the last few decades in oncology research and treatment, cancer remains as one of the foremost causes of morbidity and mortality worldwide, with 12.7 million new cases and 7.6 million deaths in 2008. Therefore, it is imperative to find novel drugs and treatments to overcome this predicted situation [1–6]. 1,3,4-Thiadiazole derivatives have attracted considerable interest owing to their wide spectra of biological activities such as antibacterial, antifungal, antituberculosis, antihepatitis B viral, antileishmanial, anti-inflammatory, analgesic, CNS depressant, anticancer, antioxidant, antidiabetic, molluscicidal, antihypertensive, diuretic, analgesic, antimicrobial, antitubercular, and anticonvulsant activities [7–24]. Thiazole derivatives are reported to exhibit diverse biological activities as antimicrobial, antioxidant, antitubercular, anticonvulsant, anticancer, and anti-inflammatory agents [25–31].

Literature survey showed that many derivatives of thiazole and 1,3,4-thiadiazole have antitumor activity with excellent IG_{50} and IC_{50} as depicted in Fig. 1 [32–35]. In view of these facts, we report herein the synthesis of a new series of substituted thiadiazoles and thiazoles for the examination of their antitumor activity against the breast carcinoma cell line MCF-7.

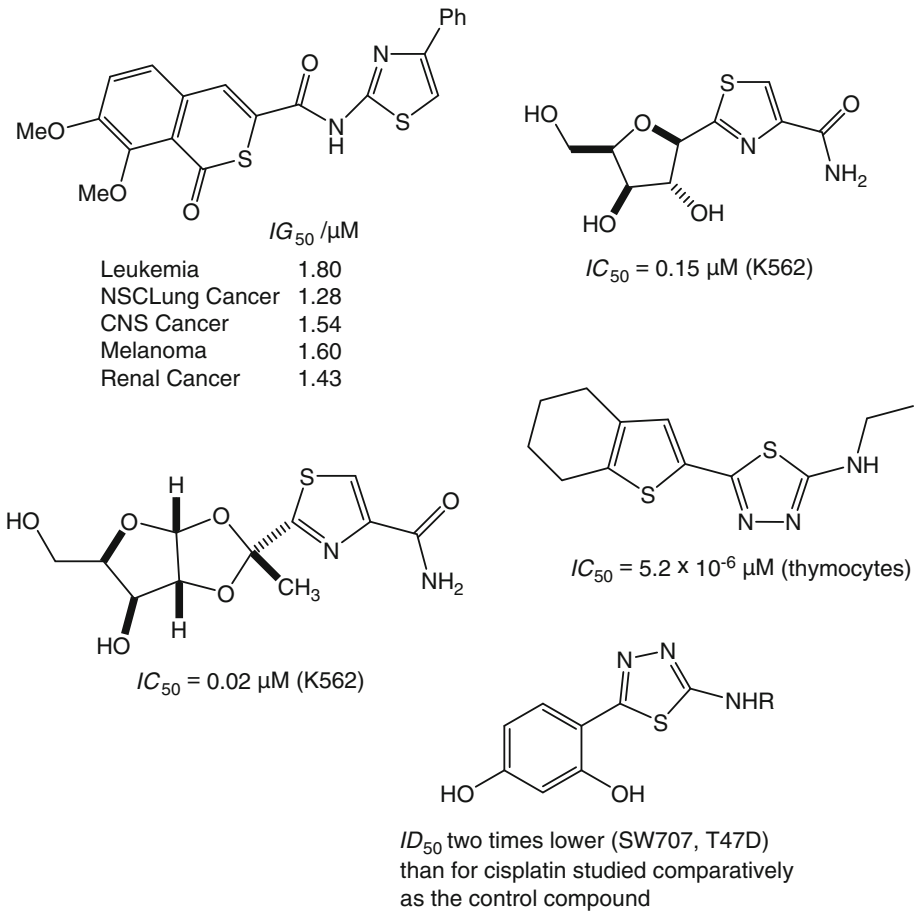


Fig. 1 Antitumor activity of thiazoles and 1,3,4-thiadiazoles

Results and discussion

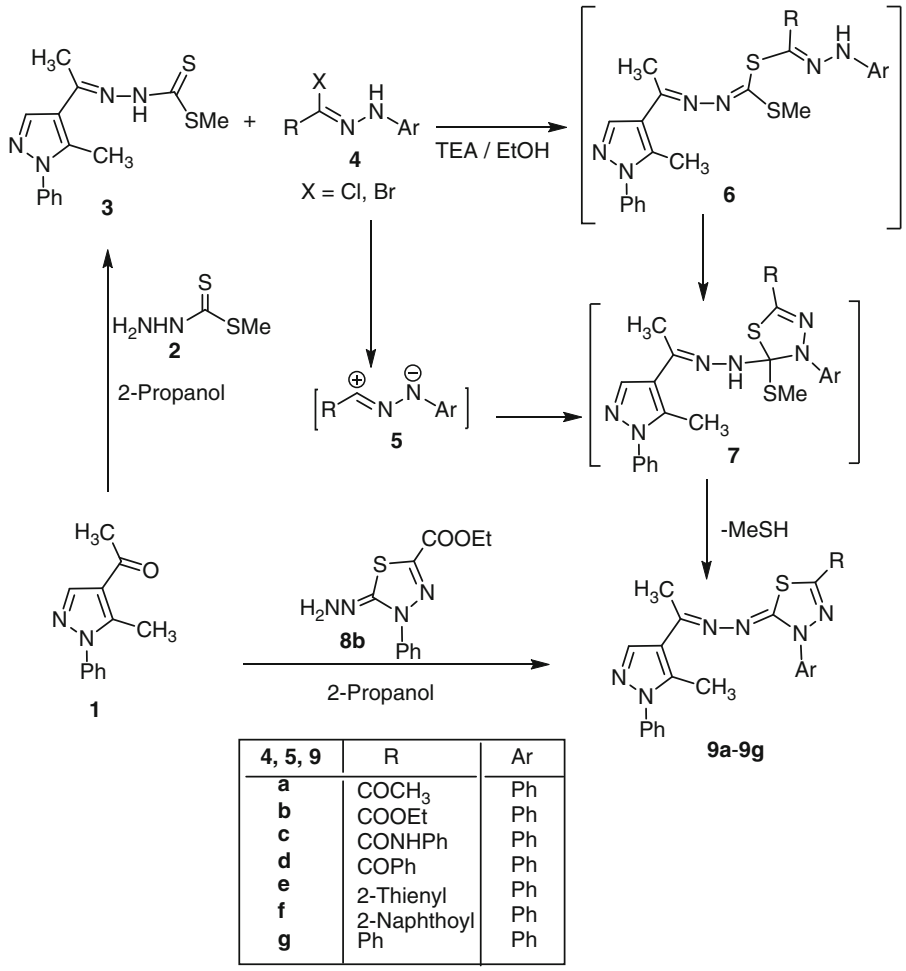
Treatment of 1-(5-methyl-1-phenyl-1*H*-pyrazol-4-yl)ethanone (**1**) with methyl hydrazinecarbodithioate (**2**) in 2-propanol gave methyl 2-[1-(5-methyl-1-phenyl-1*H*-pyrazol-4-yl)ethylidene]hydrazinecarbodithioate (**3**) (Scheme 1). Structure **3** was confirmed by elemental analysis, spectral data, and chemical transformations. Thus, *C*-ethoxycarbonyl-*N*-phenylhydrazonoyl chloride (**4b**) reacted with methyl carbodithioate **3** in ethanol containing triethylamine to afford ethyl 5-[[1-(5-methyl-1-phenyl-1*H*-pyrazol-4-yl)ethylidene]hydrazono]-4-phenyl-4,5-dihydro-1,3,4-thiadiazole-2-carboxylate (**9b**). Structure **9b** was established by elemental analyses, spectral data, and alternative syntheses. Thus, ethyl 2-hydrazono-3-phenyl-1,3,4-thiadiazoline-5-carboxylate (**8b**) [36] reacted with **1** in 2-propanol to give a product identical in all aspects (m.p., mixed m.p., and spectra) with **9b** (Scheme 1).

In the light of the foregoing results, the mechanism outlined in Scheme 1 seems to be the most plausible pathway for the formation of **9b** from the reaction of **2** with **4b**. The reaction involves initial formation of thiohydrazonate **6**, which undergoes intermolecular cyclization as soon as it is formed to yield the intermediate **7** or via 1,3-dipolar cycloaddition of nitrileimine **5b** (prepared in situ from **4b** with triethylamine) to the C=S double bond of **2**. The formation of **6** and **7** are similar to the reaction of hydrazonoyl chloride with 1-phenyl-1,4-dihydrotetrazole-5-thione [37] and 5-phenyl-1,3,4-thiadiazole-2(3*H*)-thione [38]. Compound **9** was produced from **7** by elimination of methyl mercaptane. Analogously, the appropriate **4a** and **4c**–**4g** reacted with **3** in ethanolic triethylamine to afford 2,3-dihydro-1,3,4-thiadiazoles **9a** and **9c**–**9g**, respectively.

Next, 3-(4-chlorophenyl)-1-(5-methyl-1-phenyl-1*H*-pyrazol-4-yl)prop-2-en-1-one (**10**) [39] was reacted with thiosemicarbazide to afford 5-(4-chlorophenyl)-5′-methyl-1′-phenyl-4,5-dihydro-1*H*,1′*H*-[3,4′-bipyrazole]-1-carbothioamide (**11**). Compound **11** reacted with the appropriate hydrazonoyl halides **4a**, **4e**, and **4h**–**4k** in boiling ethanol containing triethylamine to give 5-(aryldiazenyl)-2-[5-(4-chlorophenyl)-5′-methyl-1′-substituted 3,4-dihydro-1′*H*,2*H*-[3,4′-bipyrazol]-2-yl]-4-phenylthiazole **12a**, **12e**, and **12h**–**12k**, respectively (Scheme 2).

Structures **12** were confirmed by elemental analyses, spectral data, and alternative synthetic routes. Thus, a-renediazonium chloride reacted with 2-[5-(4-

Scheme 1



4, 5, 9	R	Ar
a	$COCH_3$	Ph
b	COOEt	Ph
c	CONHPh	Ph
d	COPh	Ph
e	2-Thienyl	Ph
f	2-Naphthoyl	Ph
g	Ph	Ph

chlorophenyl)-5′-methyl-1′-phenyl-3,4-dihydro-1′*H*,2*H*-[3,4′-bipyrazol]-2-yl]-4-substituted-thiazole **14** (prepared via reaction of **11** with chloroacetone) in pyridine to give a product identical in all aspects (m.p., mixed m.p., and IR spectra) with compound **12a** (Scheme 2). On other hand, reaction of 2-hydrazinyl-4-methyl-5-(phenyldiazenyl)thiazole (**13**) [40] with compound **10** afforded a product identical in all aspects (m.p., mixed m.p., and IR spectra) with **12a** (Scheme 2).

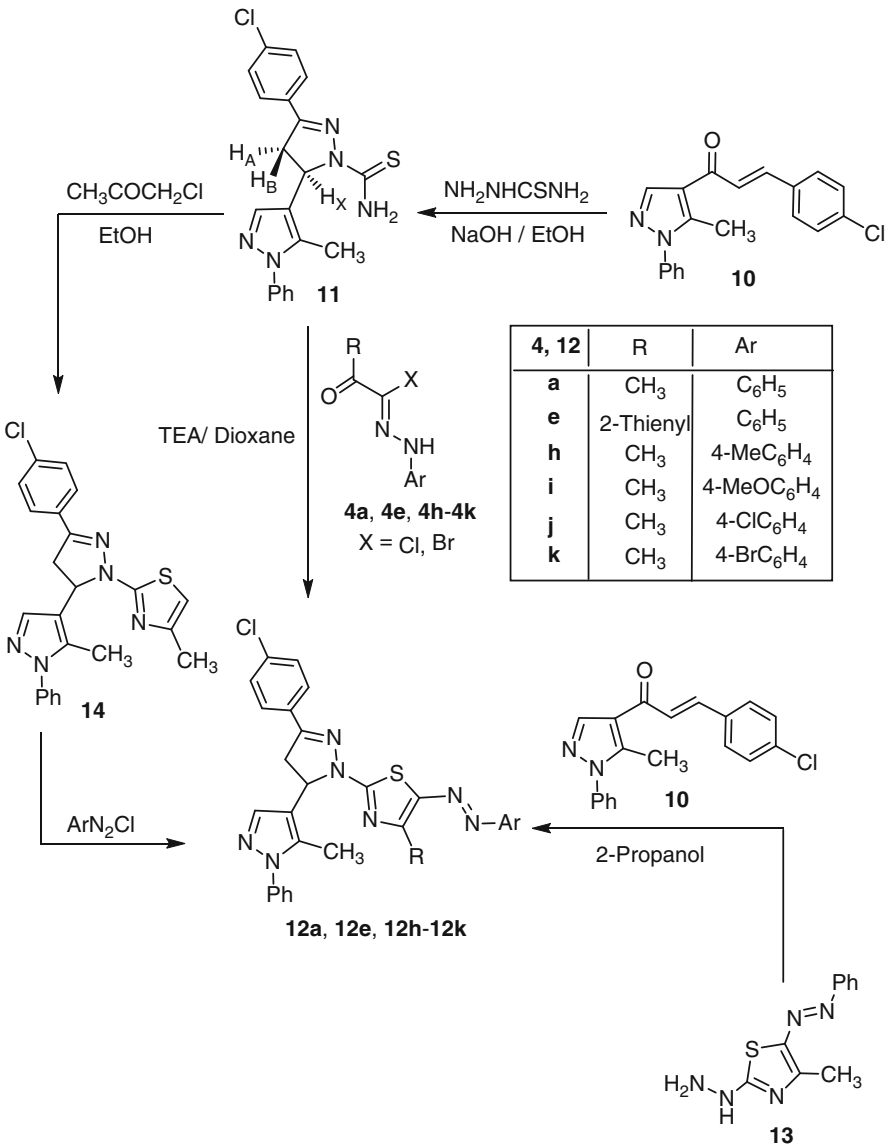
Pharmacology

Anti-cancer activity against MCF-7 cell line

The anti-tumor activity of synthesized products group 1 (**9a**–**g**, **3**, **9e**, **f**) and group 2 (**12a**, **i**, **j**, **12h**) were evaluated against human breast cancer cell line MCF-7 using the WST-1cell proliferation assay as a fast and sensitive quantification of cell proliferation and viability. The assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. Expansion in the number of viable cells results in an increase in the overall activity of the mitochondrial dehydrogenases in the sample. The augmentation in enzyme activity leads to the increase in the amount of formazan dye formed. The formazan dye produced by viable cells can be quantified by a multiwell spectrophotometer (microplate reader) by measuring the absorbance of the dye solution at 450 nm. Tables 1 and 2 represent the viability of the tested compounds in response to their concentration as illustrated in Figs. 2 and 3 [data generated were used to plot a dose response curve of which the concentration of test compounds required to kill 50 % of cell population (IC_{50}) was estimated exponentially]. Cytotoxic activity was expressed as the mean IC_{50} of three independent experiments. The results are represented in Table 3 and Fig. 4.

The results revealed that compounds **9a**, **c**, **g**, and **d** (IC_{50} were 21.3, 22.65, 23.56, and 42.05 μg cm^{-3}, respectively) has promising antitumor activity against

Scheme 2



breast carcinoma cell line MCF-7, while **9b**, **3**, **9e**, and **f** have moderate activity (IC_{50} were 63.16, 73.89, 78.73, and 82.89 μg cm^{-3}). On the other hand, **12a**, **i**, **j**, and **h** have poor inhibitory activity against MCF-7 (IC_{50} = 112.57, 188.23, 245.69, and 303.84 μg cm^{-3}, respectively). The small values of IC_{50} for the selected compounds indicate that for more anticancer effect higher concentrations can be used.

The WST-1 assay results revealed a significant decrease in the growth rate of the tumor cells but did not explain the mode of action of the compounds. Confocal laser scanning microscopic (CLSM) imaging of breast carcinoma cell line MCF-7 stained with acridine orange laser dye for nucleic acids (green stain) and rhodamine 123 (orange stain) for inner mitochondrial membrane reflects the activity of mitochondrial lactate dehydrogenase enzyme activity. It was obvious that the activity of lactate dehydrogenase significantly decreased with cells treated with **9a**, **c**, **g**, and **d**, while moderate decrease for **9b**, **3**, **9e**, and **f** (Fig. 5) and lower decrease for the rest of the compounds. The more amount of orange color reflects the higher activity of mitochondrial dehydrogenase enzyme which means higher viability and vice versa compared to the untreated control group (Fig. 6).

Conclusion

We have developed a simple and convenient method for the synthesis of 1,3,4-thiadiazolines and thiazoles incorporating pyrazole moiety. The synthesized compounds

Table 1 Viability values of tested group 1 against MCF-7

Sample conc./μg cm^{-3}	Viability/%							
	3	**9a**	**9b**	**9c**	**9d**	**9e**	**9f**	**9g**
0	100	100	100	100	100	100	100	100
10	100	80.654	93.334	90.321	95.337	100	99.447	92.871
20	95.291	62.895	82.089	81.898	86.727	94.433	93.983	66.774
40	80.094	20.456	68.9	20.234	47.605	82.46	80.417	24.766
60	62.376	11.654	51.7	8.985	31.741	56.574	59.789	11.75
80	46.761	9.324	33.45	5.273	24.671	48.612	51.975	7.403
100	25.293	4.654	24.142	3.097	19.952	39.167	40.008	3.546

Table 2 Viability values of tested group 2 against MCF-7

Sample conc./μg cm^{-3}	Viability/%			
	12a	**12h**	**12i**	**12j**
0	100	100	100	100
10	100	100	100	100
20	95.843	100	97.832	100
40	86.898	100	90.227	98.1
60	77.228	94.819	85.089	91.895
80	61.966	90.543	80.876	84.761
100	56.252	81.338	73.405	79.484

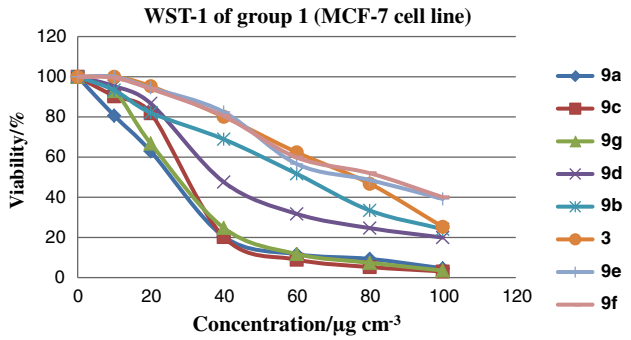


Fig. 2 Viability chart of tested group 1 against MCF-7 cell line

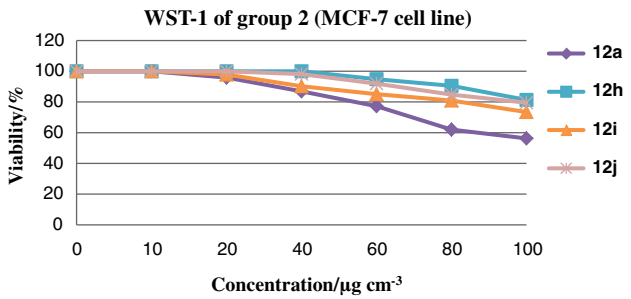


Fig. 3 Viability chart of tested group 2 against MCF-7 cell line

Table 3 IC_{50} values of tested compounds ± standard deviation against MCF-7

Compound	IC_{50}/μg cm^{-3}	Compound	IC_{50}/μg cm^{-3}
3	73.89 ± 1.93	**9f**	82.89 ± 1.97
9a	21.3 ± 0.72	**9g**	23.56 ± 0.81
9b	63.16 ± 1.78	**12a**	112.57 ± 2.22
9c	22.65 ± 0.79	**12h**	303.84 ± 7.62
9d	42.05 ± 1.02	**12i**	188.23 ± 4.12
9e	78.73 ± 1.82	**12j**	245.69 ± 5.76

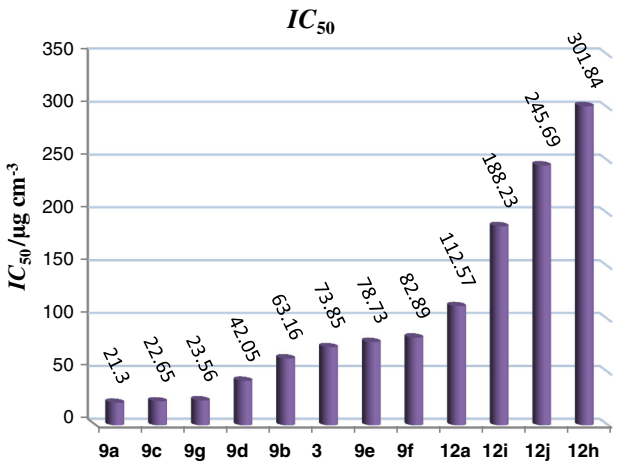


Fig. 4 IC_{50} values of tested compounds against MCF7

were tested for in vitro antitumor activity against HCF-7 and could therefore serve as lead chemical entities for further modification to render them clinically useful drug agents.

Experimental

Melting points were measured on an Electrothermal IA 9000 series digital melting point apparatus. IR spectra were

recorded in potassium bromide discs on Pye Unicam SP 3300 and Shimadzu FTIR 8101 PC infrared spectrophotometers. NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer operating at 300 MHz (^{1}H NMR) and run in deuterated dimethylsulfoxide (DMSO-d_6). Chemical shifts were related to that of the solvent. ^{13}C NMR was recorded on a Bruker spectrometer at 75 MHz. Mass spectra were recorded on a Shimadzu GCMS-QP1000 EX mass spectrometer at 70 eV. Elemental analyses were measured by using a German made Elementar vario LIII CHNS analyzer. Antitumor activity was evaluated by the Nanotechnology and Advanced materials central lab, Agricultural Research Center, Giza, Egypt. 4-Acetyl-5-methyl-1-phenyl-1*H*-pyrazole (**1**) [41] and hydrazonoyl halides **4** [42, 43] were prepared as reported in the respective literature.

Methyl 2-[1-(5-methyl-1-phenyl-1H-pyrazol-4-yl)-ethylidene]hydrazinecarbodithioate (**3**, $C_{14}H_{16}N_4S_2$)
To a solution of 2.00 g 4-acetyl-5-methyl-1-phenyl-1*H*-pyrazole **1** (10 mmol) in 20 cm^3 2-propanol, 1.22 g methyl hydrazinecarbodithioate **2** (10 mmol) was added. The mixture was stirred at room temperature for 2 h. The solid product was filtered off, recrystallized from ethanol to afford **3** as yellow solid in 82 %. M.p.: 212–214 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.34 (3H, s, CH_3), 2.49 (3H, s, CH_3), 2.69 (3H, s, CH_3), 3.30 (1H, s, NH or SH), 7.45–7.94 (5H, m, Ar–H), 8.06 (1H, s, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 14.9 (CH_3), 17.9 (CH_3), 21.0 (CH_3), 116.4, 125.8, 129.6, 129.8, 132.4, 133.1, 134.7, 164.6 (Ar–C), 191.3 (C=S) ppm; IR (KBr): $\bar{\nu}$ = 3,142 (NH), 3,054, 2,920 (CH), 1,576 (C=N) cm^{-1}; MS: *m/z* (%) = 304 (M$^+$, 7), 257 (100), 201 (32), 129 (39), 79 (62).

General procedure for synthesis of 2-[[1-(5-methyl-1-phenyl-5-substituted-1H-pyrazol-4-yl)-ethylidene]hydrazono]-3-phenyl-2,3-dihydro-1,3,4-thiadiazoles ***9a–g***

To a mixture of alkyl carbodithioate **3** (1 mmol) and the appropriate hydrazonoyl halides **4a–g** (1 mmol) in 20 cm^3 ethanol, 0.5 cm^3 triethylamine was added, the mixture was stirred at room temperature for 2 h. The resulting solid was collected and recrystallized from dimethylformamide to give the corresponding 1,3,4-thiadiazolines **9a–g**.

1-[5-[[1-(5-Methyl-1-phenyl-1H-pyrazol-4-yl)-ethylidene]hydrazono]-4-phenyl-4,5-dihydro-1,3,4-thiadiazol-2-yl]ethanone (**9a**, $C_{22}H_{20}N_6OS$)
Orange solid; 76 % yield; m.p.: 166–168 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.37 (3H, s, CH_3), 2.49 (3H, s, CH_3), 2.63 (3H, s, CH_3), 7.10–7.61 (10H, m, Ar–H), 8.09 (1H, s, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 11.3 (CH_3), 14.7 (CH_3), 17.5 (CH_3), 112.4, 116.1, 116.3, 127.9, 129.1, 129.4, 129.6, 129.7, 129.8, 130.3, 133.1, 134.7, 138.5, 163.1 (Ar–C), 192.1 (C=O) ppm; IR (KBr): $\bar{\nu}$ = 3,054, 2,920 (CH), 1,678 (C=O), 1,603 (C=N) cm^{-1}; MS: *m/z* (%) = 416 (M$^+$, 43), 344 (19), 183 (100), 142 (32), 79 (50).

Ethyl 5-[[1-(5-methyl-1-phenyl-1H-pyrazol-4-yl)-ethylidene]hydrazono]-4-phenyl-4,5-dihydro-1,3,4-thiadiazole-2-carboxylate (**9b**, $C_{23}H_{22}N_6O_2S$)
Orange solid; 74 % yield; m.p.: 212–214 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.37 (3H, t, CH_2CH_3), 2.30 (3H, s, CH_3), 2.63 (3H, s, CH_3), 3.91 (2H, q, CH_2CH_3), 7.46–7.95 (10H, m, Ar–H), 8.04 (1H, s, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 11.3 (CH_3), 14.4 (CH_3), 17.1 (CH_3), 64.3 (CH_2), 112.4, 114.4, 116.1, 116.3, 125.8, 127.9, 129.1, 129.6, 131.6, 133.1, 134.7, 134.8, 138.5, 164.3 (Ar–C), 177.9 (C=O) ppm; IR (KBr): $\bar{\nu}$ = 3,054, 2,919 (CH), 1,708 (C=O), 1,605 (C=N) cm^{-1}; MS: *m/z* (%) = 446 (M$^+$, 20), 344 (52), 329 (29), 197 (70), 183 (100), 118 (59), 78 (90).

5-[[1-(5-Methyl-1-phenyl-1H-pyrazol-4-yl)ethylidene]-hydrazono]-N,4-diphenyl-4,5-dihydro-1,3,4-thiadiazole-2-carboxamide (**9c**, $C_{27}H_{23}N_7OS$)
Orange solid; 72 % yield; m.p.: 200–201 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.50 (3H, s, CH_3), 2.63 (3H, s, CH_3), 7.12–8.09 (15H, m, Ar–H), 8.10 (1H, s, pyrazole-H3), 11.43 (1H, s, NH) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 12.0 (CH_3), 17.3 (CH_3), 112.4, 116.1, 116.3, 125.8, 127.9, 129.1, 129.4, 129.6, 129.7, 129.8, 130.3, 133.1, 134.7, 134.8, 138.5, 145.4, 148.5, 160.6 (Ar–C), 163.1 (C=O) ppm; IR (KBr): $\bar{\nu}$ = 3,367 (NH), 3,053, 2,920 (CH), 1,647 (C=O), 1,575 (C=N) cm^{-1}; MS: *m/z* (%) = 493 (M$^+$, 3), 344 (99), 198 (100), 183 (84), 118 (55), 78 (48), 65 (27).

[5-[[1-(5-Methyl-1-phenyl-1H-pyrazol-4-yl)ethylidene]-hydrazono]-4-phenyl-4,5-dihydro-1,3,4-thiadiazol-2-yl](phenyl)methanone (**9d**, $C_{27}H_{22}N_6OS$)
Orange solid; 72 % yield; m.p.: 152–154 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.36 (3H, s, CH_3), 2.63 (3H, s, CH_3), 7.02–7.86 (15H, m, Ar–H), 8.22 (1H, s, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 12.9 (CH_3), 17.6 (CH_3), 112.9, 115.9, 116.1, 119.1, 119.4, 126.4, 128.1, 128.7, 129.6, 129.2, 129.4, 130.9, 132.2, 133.6, 135.6, 135.7, 136.1, 162.8 (Ar–C), 189.1 (C=O) ppm; IR (KBr): $\bar{\nu}$ = 3,059, 2,916 (CH), 1,643 (C=O), 1,599 (C=N) cm^{-1}; MS: *m/z* (%) = 478 (M$^+$, 44), 183 (35), 142 (27), 106 (52), 78 (100), 65 (56).

[5-[[1-(5-Methyl-1-phenyl-1H-pyrazol-4-yl)ethylidene]-hydrazono]-4-phenyl-4,5-dihydro-1,3,4-thiadiazol-2-yl](thiophen-2-yl)methanone (**9e**, $C_{25}H_{20}N_6OS_2$)
Orange solid; 76 % yield; m.p.: 188–190 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.35 (3H, s, CH_3), 2.61 (3H, s, CH_3), 6.99–7.78 (14H, m, Ar–H, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 12.0 (CH_3), 17.5 (CH_3),

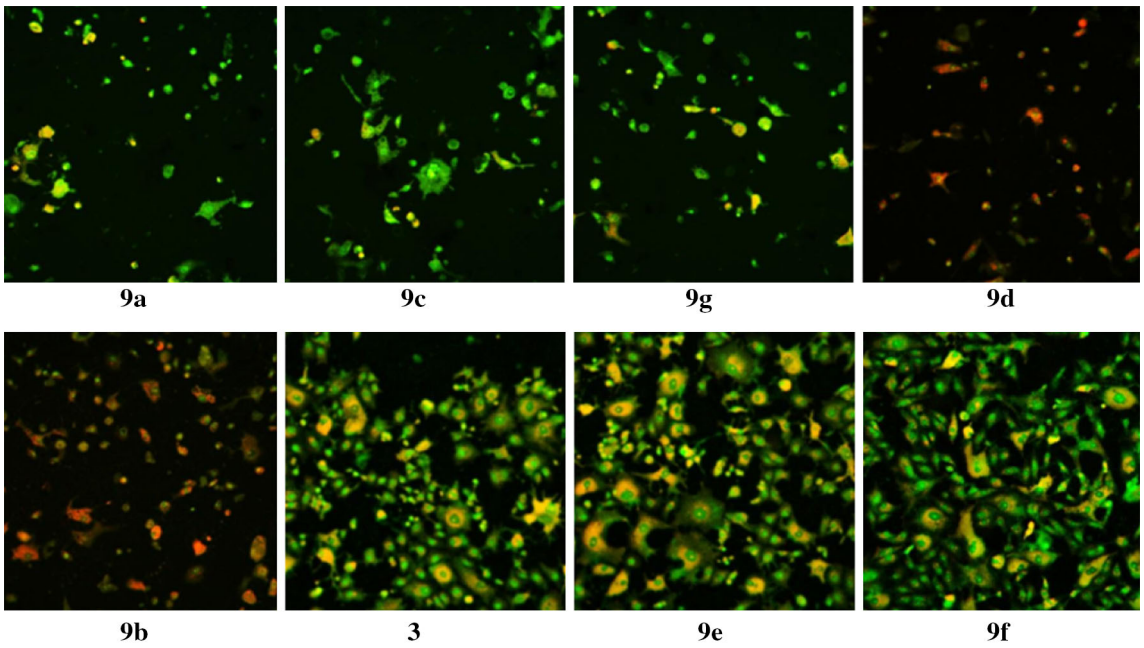


Fig. 5 CLSM image of MCF-7 cell line treated with IC_{50} concentration of group 1 tested compounds. Stained by acridine orange (*green*) and rhodamine 123 (*orange*) (color figure online)

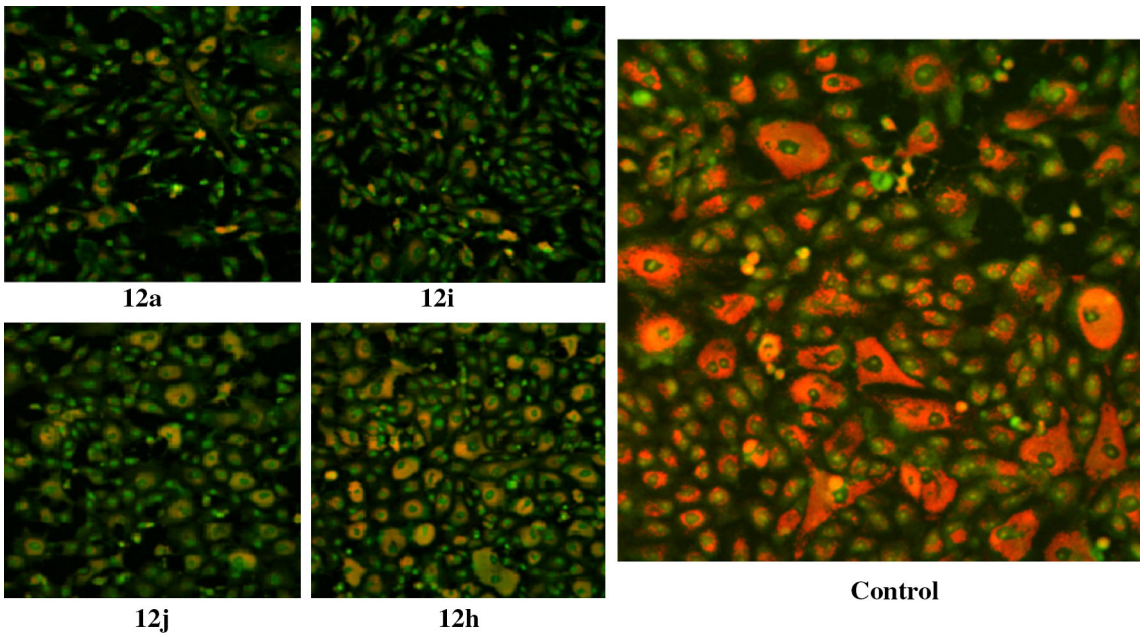


Fig. 6 CLSM image of MCF-7 cell line treated with IC_{50} concentration of group 2 tested compounds compared to control one. Stained by acridine orange (*green*) and rhodamine 123 (*orange*) (color figure online)

112.4, 116.2, 116.4, 125.8, 127.9, 129.1, 129.6, 129.7, 129.8, 132.3, 132.4, 133.1, 134.6, 134.7, 138.6, 148.5, 149.9, 160.7 (Ar–C), 188.3 (C=O) ppm; IR (KBr): $\bar{\nu}$ = 3,059, 2,919 (CH), 1,636 (C=O), 1,570 (C=N) cm^{-1}; MS: *m/z* (%) = 485 ($[M+1]^+$, 3), 484 (M^+, 16), 344 (6), 197 (27), 183 (73), 112 (51), 78 (100).

[5-[[1-(5-Methyl-1-phenyl-1H-pyrazol-4-yl)ethylidene]-hydrazono]-4-phenyl-4,5-dihydro -1,3,4-thiadiazol-2-yl](naphthalen-2-yl)methanone (**9f**, $C_{31}H_{24}N_6OS$)
Orange solid; 78 % yield; m.p.: 264–266 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.36 (3H, s, CH_3), 2.63 (3H, s, CH_3), 7.00–7.82 (17H, m, Ar–H, pyrazole-H3), 8.20 (s, 1H,

naphthalene-H1) ppm; IR (KBr): $\bar{\nu}$ = 3,054, 2,923 (CH), 1,630 (C=O), 1,599 (C=N) cm^{-1}; MS: *m/z* (%) = 529 ($[M+1]^+$, 2), 528 (M^+, 9), 198 (9), 183 (45), 155 (47), 128 (56), 78 (100), 65 (46).

2-[[1-(5-Methyl-1-phenyl-1H-pyrazol-4-yl)ethylidene]-hydrazono]-3,5-diphenyl-2,3-dihydro-1,3,4-thiadiazole (**9g**, $C_{26}H_{22}N_6S$)

Orange solid; 76 % yield; m.p.: 206–208 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.33 (3H, s, CH_3), 2.63 (3H, s, CH_3), 6.84–7.72 (15H, m, Ar–H), 8.12 (1H, s, pyrazole-H3) ppm; IR (KBr): $\bar{\nu}$ = 3,053, 2,923 (CH), 1,598 (C=N) cm^{-1}; MS: *m/z* (%) = 450 (M^+, 46), 433 (77), 198 (87), 183 (91), 118 (55), 78 (100).

Alternate synthesis of **9b**

To a solution of 0.2 g 4-acetyl-5-methyl-1-phenyl-1*H*-pyrazole (**1**, 1 mmol) in 10 cm^3 2-propanol, 0.264 g ethyl 5-hydrazono-4-phenyl-4,5-dihydro-1,3,4-thiadiazole-2-carboxylate (**8b**, 1 mmol) was added. The mixture was refluxed for 2 h then cooled to room temperature. The solid precipitated was filtered off, washed with water, dried, and recrystallized from dimethylformamide to give the corresponding product **9b**, which was identical in all aspects (m.p., mixed m.p., and IR spectra) with those obtained from reaction of **3** with **4b** but in 67 % yield.

5-(4-Chlorophenyl)-5′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole-2-carbothioamide (**11**, $C_{20}H_{18}ClN_5S$)

A mixture of 3.22 g chalcone **10** (10 mmol), 0.92 g thiosemicarbazide (10 mmol), and 0.3 g sodium hydroxide in 20 cm^3 ethanol was heated under reflux for 6 h. The resulting solid was collected, washed with ethanol, and recrystallized from acetic acid to give pure product of compound **11** as white solid (83 %). M.p.: 158–160 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.58 (s, 3H, CH_3), 3.18 (dd, 1H, H_A, J = 17.6, 6.1 Hz), 3.94 (dd, 1H, H_B, J = 17.6, 12.2 Hz), 5.88 (dd, 1H, H_X, J = 12.4, 6.1 Hz), 7.16–8.04 (m, 9H, Ar–H, pyrazole-H3), 8.23 (s, 1H, pyrazole-H3), 11.46 (s, br, 2H, NH_2) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 12.0 (CH_3), 52.6 (CH_2), 72.0 (CH), 111.5, 117.5, 118.0, 118.2, 122.2, 122.6, 129.0, 133.7, 139.9, 153.6, 154.0, 163.4 (Ar–C), 180.5 (C=S) ppm; IR (KBr): $\bar{\nu}$ = 3,432, 3,276 (NH_2), 1,593 (C=N) cm^{-1}; MS: *m/z* (%) = 395 (M^+, 48), 361 (62), 241 (42), 198 (100), 138 (90), 117 (79), 65 (43).

Method A

*Synthesis of 2-[5-(4-chlorophenyl)-5′-methyl-1′-phenyl-3,4-dihydro-1′H, 2H-[3,4′-bipyrazol]-2-yl]-4-substituted-5-(phenyldiazenyl)thiazoles **12a**, **12e**, and **12h–k***

Amixture of 0.395 g 5-(4-chlorophenyl)-5′-methyl-1′-phenyl-3,4-dihydro-1′*H*,2*H*-[3,4′-bipyrazole]-2-carbothioamide (**11**, 1 mmol) and the appropriate hydrazonoyl halides **4a**, **e**, **4h–k** (1 mmol) in 20 cm^3 dioxane containing 0.5 cm^3 TEA was refluxed for 4 h (monitored by TLC), allowed to cool, and the solid formed was filtered off, washed with ethanol, dried, and recrystallized from dimethylformamide to give **12a**, **e**, and **12h–k**.

5-(4-Chlorophenyl)-5′-methyl-2-[4-methyl-5-[(E)-phenyldiazenyl]-1,3-thiazol-2-yl]-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**12a**, $C_{29}H_{24}ClN_7S$)

White solid; 78 % yield; m.p.: 106–108 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.44 (s, 3H, CH_3), 2.59 (s, 3H, CH_3), 3.26 (dd, 1H, H_A, J = 17.6, 6.1 Hz), 4.18 (dd, 1H, H_B, J = 17.6, 12.2 Hz), 5.86 (dd, 1H, H_X, J = 12.4, 6.1 Hz), 7.31–8.03 (m, 14H, Ar–H), 8.69 (s, 1H, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 12.6 (CH_3), 13.1 (CH_3), 54.6 (CH_2), 71.5 (CH), 112.8, 113.2, 115.9, 116.2, 118.9, 126.1, 128.5, 128.6, 129.0, 129.1, 129.3, 129.5, 129.6, 129.7, 130.8, 130.9, 135.5, 160.4, 162.8 (Ar–C) ppm; IR (KBr): $\bar{\nu}$ = 2,922 (CH), 1,603 (C=N) cm^{-1}; MS: *m/z* (%) = 539 ($[M+2]^+$, 2), 539 ($[M+1]^+$, 5), 538 (M^+, 6), 402 (22), 355 (13), 183 (11), 118 (15), 77 (100).

5-(4-Chlorophenyl)-5′-methyl-2-[4-(thiophen-2-yl)-5-[(E)-phenyldiazenyl]-1,3-thiazol-2-yl]-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**12e**, $C_{32}H_{24}ClN_7S_2$)

White solid; 72 % yield; m.p.: 182–184 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.44 (s, 3H, CH_3), 3.14 (dd, 1H, H_A, J = 17.6, 6.1 Hz), 3.48 (dd, 1H, H_B, J = 17.6, 12.2 Hz), 5.84 (dd, 1H, H_X, J = 12.4, 6.1 Hz), 7.05–8.29 (m, 12H), 8.44 (s, 1H, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 11.9 (CH_3), 53.7 (CH_2), 71.5 (CH), 116.0, 116.2, 125.8, 126.1, 128.5, 128.6, 128.9, 129.5, 129.6, 130.8, 130.9, 131.1, 132.1, 132.4, 135.3, 135.4, 143.8, 160.4, 162.8 (Ar–C) ppm; IR (KBr): $\bar{\nu}$ = 2,957 (CH), 1,599 (C=N) cm^{-1}; MS: *m/z* (%) = 618 ($[M+2]^+$, 7), 617 ($[M+1]^+$, 15), 616 (M^+, 15), 552 (15), 402 (18), 235 (15), 198 (21), 77 (100), 67 (35).

5-(4-Chlorophenyl)-5′-methyl-2-[4-methyl-5-[(E)-p-tolyldiazenyl]-1,3-thiazol-2-yl]-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**12h**, $C_{30}H_{26}ClN_7S$)

White solid; 74 % yield; m.p.: 128–130 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.17 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 3.14 (dd, 1H, H_A, J = 17.6, 6.1 Hz), 3.48 (dd, 1H, H_B, J = 17.6, 12.2 Hz), 5.87 (dd, 1H, H_X, J = 12.4, 6.1 Hz), 7.23–7.97 (m, 13H, Ar–H), 8.63 (s, 1H, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 12.5 (CH_3), 13.9 (CH_3), 18.4 (CH_3), 55.3 (CH_2), 71.3 (CH), 112.4, 114.4, 116.1, 116.3, 125.6, 125.8, 127.9, 129.1, 129.6, 129.7, 129.8, 131.6, 133.1, 134.7,

134.8, 138.4, 148.4, 163.6, 164.3 (Ar–C) ppm; IR (KBr): $\bar{\nu}$ = 2,927 (CH), 1,600 (C=N) cm^{-1}; MS: *m/z* (%) = 554 ($[M+2]^+$, 1), 553 ($[M+1]^+$, 4), 552 (M^+, 4), 470 (78), 325 (13), 198 (45), 184 (54), 92 (63), 65 (100).

5-(4-Chlorophenyl)-5′-methyl-2-[4-methyl-5-[(E)-4-methoxyphenyldiazenyl]-1,3-thiazol-2-yl]-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**12i**, $C_{30}H_{26}ClN_7OS$)
White solid; 72 % yield; m.p.: 218–220 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.32 (s, 3H, CH_3), 2.60 (s, 3H, CH_3), 3.18 (dd, 1H, H_A, J = 17.6, 6.1 Hz), 3.46 (dd, 1H, H_B, J = 17.6, 12.2 Hz), 3.67 (s, 3H, OCH_3), 5.82 (dd, 1H, H_X, J = 12.4, 6.1 Hz), 7.19–7.44 (m, 13H, Ar–H), 8.53 (s, 1H, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 11.2 (CH_3), 16.0 (CH_3), 45.6 (CH_2), 53.2 (CH_3), 71.5 (CH), 112.8, 113.1, 115.8, 116.1, 125.8, 126.4, 128.1, 128.7, 129.0, 129.1, 129.6, 130.7, 135.8, 135.9, 136.2, 136.3, 144.8, 144.9, 162.8 (Ar–C) ppm; IR (KBr): $\bar{\nu}$ = 2,917 (CH), 1,598 (C=N) cm^{-1}; MS: *m/z* (%) = 570 ($[M+2]^+$, 4), 569 ($[M+1]^+$, 12), 568 (M^+, 13), 402 (45), 335 (10), 118 (33), 77 (100), 65 (21).

5-(4-Chlorophenyl)-5′-methyl-2-[4-methyl-5-[(E)-4-chlorohenyl diazenyl]-1,3-thiazol-2-yl]-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**12j**, $C_{29}H_{23}Cl_2N_7S$)
Yellow solid; 72 % yield; m.p.: 114–116 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.42 (s, 3H, CH_3), 2.63 (s, 3H, CH_3), 3.14 (dd, 1H, H_A, J = 17.6, 6.1 Hz), 3.50 (dd, 1H, H_B, J = 17.6, 12.2 Hz), 5.86 (dd, 1H, H_X, J = 12.4, 6.1 Hz), 7.36–7.89 (m, 13H, Ar–H), 8.61 (s, 1H, pyrazole-H3) ppm; IR (KBr): $\bar{\nu}$ = 2,959 (CH), 1,600 (C=N) cm^{-1}; MS: *m/z* (%) = 574 ($[M+2]^+$, 1), 573 ($[M+1]^+$, 1), 572 (M^+, 1), 451 (4), 402 (100), 183 (18), 111 (21), 77 (98), 51 (49).

5-(4-Chlorophenyl)-5′-methyl-2-[4-methyl-5-[(E)-4-bromophenyldiazenyl]-1,3-thiazol-2-yl]-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**12k**, $C_{29}H_{23}BrClN_7S$)
White solid; 86 % yield; m.p.: 144–146 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.41 (s, 3H, CH_3), 2.71 (s, 3H, CH_3), 3.14 (dd, 1H, H_A, J = 17.6, 6.1 Hz), 3.62 (dd, 1H, H_B, J = 17.6, 12.2 Hz), 5.58 (dd, 1H, H_X, J = 12.4, 6.1 Hz), 7.19–8.27 (m, 13H, Ar–H), 8.62 (s, 1H, pyrazole-H3) ppm; IR (KBr): $\bar{\nu}$ = 2,919 (CH), 1,602 (C=N) cm^{-1}; MS: *m/z* (%) = 618 ($[M+2]^+$, 7), 617 ($[M+1]^+$, 15), 616 (M^+, 15), 552 (15), 402 (18), 235 (15), 198 (21), 77 (100), 67(35).

Method B

2-[5-(4-Chlorophenyl)-5′-methyl-1′-phenyl-3,4-dihydro-1′H, 2H-[3,4′-bipyrazol]-2-yl]-4-methylthiazole (**14**, $C_{23}H_{20}ClN_5S$)
A mixture of 2.025 g **11** (5 mmol) and 0.460 g chloroacetone (1 mmol) in 30 cm^3 absolute ethanol was refluxed for 4 h. The product started to separate out during the course of reaction. The crystalline solid was filtered, washed with water, dried, and recrystallized from ethanol to give pure thiazole **14** as yellow crystals in 70 % yield. M.p.: 166–168 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.48 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 3.11 (dd, 1H, H_A, J = 17.6, 6.1 Hz), 4.23 (dd, 1H, H_B, J = 17.6, 12.2 Hz), 5.89 (dd, 1H, H_X, J = 12.4, 6.1 Hz), 6.88 (s, 1H, thiazole-H5), 7.34–8.06 (m, 9H, Ar–H), 8.63 (s, 1H, pyrazole-H3) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 12.9 (CH_3), 16.6 (CH_3), 53.6 (CH_2), 71.4 (CH), 112.0, 123.8, 125.6, 128.2, 129.3, 129.8, 132.5, 132.6, 132.7, 139.0, 140.4, 140.5, 141.2, 153.4, 160.1 (Ar–C) ppm; IR (KBr): $\bar{\nu}$ = 2,926 (CH), 1,603 (C=N) cm^{-1}; MS: *m/z* (%) = 435 ($[M+2]^+$, 6), 433 (M^+, 21), 355 (43), 183 (53), 118 (47), 77 (100).

Coupling of thiazole **14** *with arenediazonium chloride*
To a solution of 0.433 g **14** (1 mmol) in 20 cm^3 ethanol was added 0.138 g sodium acetate trihydrate (1 mmol), and the mixture was cooled to 0–5 °C in an ice bath. To the resulting cold solution was added portionwise a cold solution of arenediazonium chloride [prepared by diazotizing aniline derivatives (1 mmol) dissolved in 1 cm^3 hydrochloric acid (6 M) with a solution of 0.07 g sodium nitrite (1 mmol) in 2 cm^3 water]. After complete addition of the diazonium salt, the reaction mixture was stirred for a further 30 min in an ice bath. The solid that separated was filtered off, washed with water, and finally recrystallized from dimethylformamide to give product proved identical in all respects (m.p., mixed m.p., and IR spectra) with compounds **12a** and **12h–k** obtained from method A.

Alternate synthesis of **12a**
Equimolar amounts of 0.322 g chalcone **10** (l mmol) and 0.233 g 2-hydrazinyl-4-methyl-5-(phenyldiazenyl)thiazole (**13**, 1 mmol) in 10 cm^3 2-propanol were refluxed for 2 h then cooled to room temperature. The solid precipitated was filtered off, washed with water, dried, and recrystallized from dimethylformamide to give the corresponding product **12a**, which were identical in all aspects (m.p., mixed m.p., and IR spectra) with those obtained from reaction of **11** with **4a** but in 72 % yield.

Cytotoxic activity
WST-1 assay The human breast carcinoma cell lines MCF-7 were cultured and tested at Nanotechnology and Advanced materials central lab, Cairo, Egypt. The culture was maintained in DMEM with 10 % FBS at 37 °C humidified with 5 % CO_2. Various concentrations of the compound under test (0, 10, 20, 40, 60, 80, and 100 μg cm^{-3}) were added to the cell monolayer in

triplicate wells individual dose and its cytotoxicity was tested using a standard WST-1(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate) assay, in a 96-well microtiter plate for 24 h and measured at 450 nm [44].

Confocal laser scanning microscopy

The mode of potential cytotoxicity action was evaluated using confocal laser scanning microscopic (Carrl Zeiss CLSM 710) imaging of MCF-7-treated cell lines at IC_{50} concentration of the tested compounds. Cells were plated in 96-multiwall plates (about 10^4 cells/well) for 24 h before treatment with the tested compound to allow attachment of cell to the wall of the plate. Selected concentrations of the compounds under test were added to the cell monolayer in triplicate wells individual dose, monolayer cells were incubated with the compounds for 24 h at 37 °C and in atmosphere of 5 % CO_2. After 24 h, cells were stained by rhodamine 123 and acridine orange stains (Sigma-Aldrich), wait for 5 min and microscopic examination was done using excitation laser lines at 588 and 633 nm by two channel detection.

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