Full Paper

Synthesis and Structure-activity Relationship Studies of Pyrazole-based Heterocycles as Antitumor Agents

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Several 4-cyano-1,5-diphenylpyrazoles attached to different heterocyclic ring systems at position 3 were synthesized starting from ethyl 4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carboxylate **1**. The newly synthesized compounds were tested *in vivo* for their anti-estrogenic effects and evaluated *in vitro* for their cytotoxic properties against estrogen-dependent tumors. 3-(5-Mercapto-1,3,4-oxadiazole-2-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **13** revealed the highest cytotoxic activity with a GI_{50} value equal to 40 nM against the IGROVI ovarian tumor cell line. It also showed an anti-estrogen activity 1.6 more effective than the reference drug, in addition to a high tolerable dose. 3-(5-(Methylthio)-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **7** was found to have the highest anti-estrogenic activity, while 1,5-diphenyl-3-[5-(phenylamino)-1,3,4-thiadiazol-2-yl]-1*H*-pyrazole-4-carbonitrile **11** showed the lowest activity. The oral LD_{50} values revealed that most of the tested compounds are relatively nontoxic.

Keywords: Anti-estrogenic activity / 1,5-Diphenylpyrazoles / 1,3,4-Oxadiazoles / 1,3,4-Thiadiazole / 1,2,4-Triazoles

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Introduction

Estrogens control the development and maintenance of the female sex organs, secondary sex characteristics, and mammary glands, as well as certain functions of the uterus and its accessory organs. They are also formed in the placenta in late pregnancy, which increases the spontaneous activity of the uterine muscle and its response to oxytocic drugs. Estradiol is the most active of the estrogens formed from androgen precursors in the ovarian follicles of premenopausal women. In men and postmenopausal women, estrogens are also formed in adipose tissue from adrenal androgens.

The class of anti-estrogens is used therapeutically in several diseases such as malignant neoplasms of the breast [1], gynaecomastia [2], mastalgia [3], and reduction of pelvic pain scores in women with refractory endometriosis [4]. This class includes nonsteroidal derivatives which have both estrogenic and anti-estrogenic properties such as clomifene [5, 6], cyclofenil, and the more selective nonsteroidal anti-estrogens ormeloxifene [7] and raloxifene [8, 9].

Since some types of breast cancer and other cancers were found to be estrogen-dependent, in which binding of estrogen with its receptors activates transcription of its target genes, the latter are responsible for cancer cell proliferation in estrogen-dependent breast tumor [10]. Therefore, treatment of these conditions focuses on decreasing estrogen, either by oophorectomy or by hormonal therapy [11–13]. The anti-estrogens, used in the hormonal treatment of breast cancer, include the estrogen receptor antagonists tamoxifen [14–16], toremifene [17, 18], and various aromatase cytochrome P450 (CYP19) inhibitors, which catalyze the conversion of androstenedione and testostron to estradiol [19, 20], such as formestane [21], anastrozole [22, 23], vorozole [24, 25], fadrozole [26], testolactone [27], and letrozole [28, 29].

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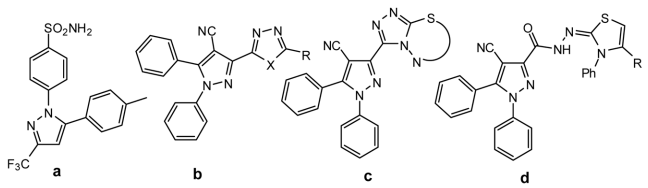


Figure 1. a) Celecoxib; b–d) general formulae of the synthesized compounds.

The importance of 1,5-diphenylpyrazoles as anti-estrogens, recently became important after discovering the effect of cyclooxygenase-2 (COX-2) inhibitors such as celecoxib (Fig. 1a) and meloxicam [30] on many types of malignant tumors, especially breast cancer [31–37]. It is proved that 1,5-diphenylpyrazoles exert this action via inhibition of the aromatase enzyme [38].

In continuation of our recent work aiming at the synthesis of heterocyclic systems with remarkable biological importance [39–52], we report here on the synthesis of some new 1,5-diphenylpyrazole derivatives with different substituted triazole and triazole bioisosteres at position 3 (Fig. 1b) as an important counterpart to fit with the required enzyme (CYP19) [28]. The present study also involves the anti-estrogenic effects of the synthesized compounds *in vivo* and evaluating their cytotoxic properties *in vitro* against different breast and ovarian tumor cell lines.

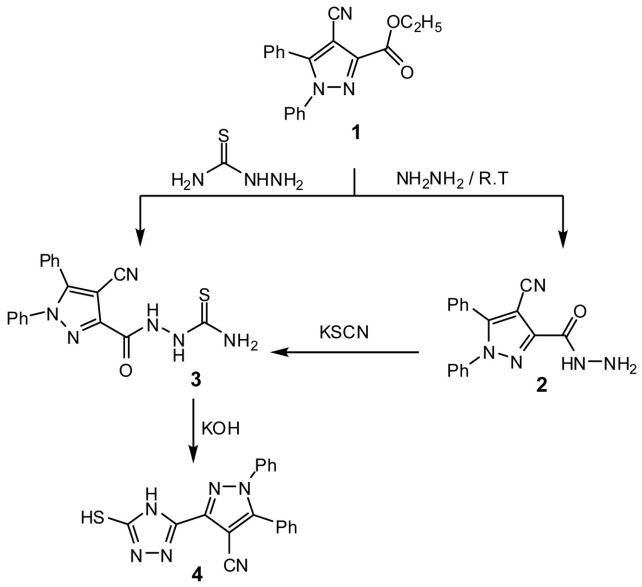
The structure-activity relationship (SAR) study of this new promising group was carried out in order to get new agents that could be optimized as potent antitumor drugs. In a second line of chemical optimization, several 1,5-diphenylpyrazole derivatives were synthesized with different fused triazole ring systems at position 3 (Fig. 1c). To achieve this goal, we utilized several intermediates such as carboxylic acid hydrazide **2** and thiosemicarbazide derivatives **3** and **5**. Since the acid hydrazide **2** showed a promising anti-estrogenic activity as well as a significant cytotoxic activity against certain types of breast tumor cell lines, we decided to optimize its structure (Fig. 1d) in order to increase its selectivity towards ovarian tumors.

All of the synthesized compounds were designed to contain a nitrile group as it is common in different aromatase inhibitors such as anastrozole [22], fadrozole [24], and letrozole [28].

Results and discussion

Chemistry

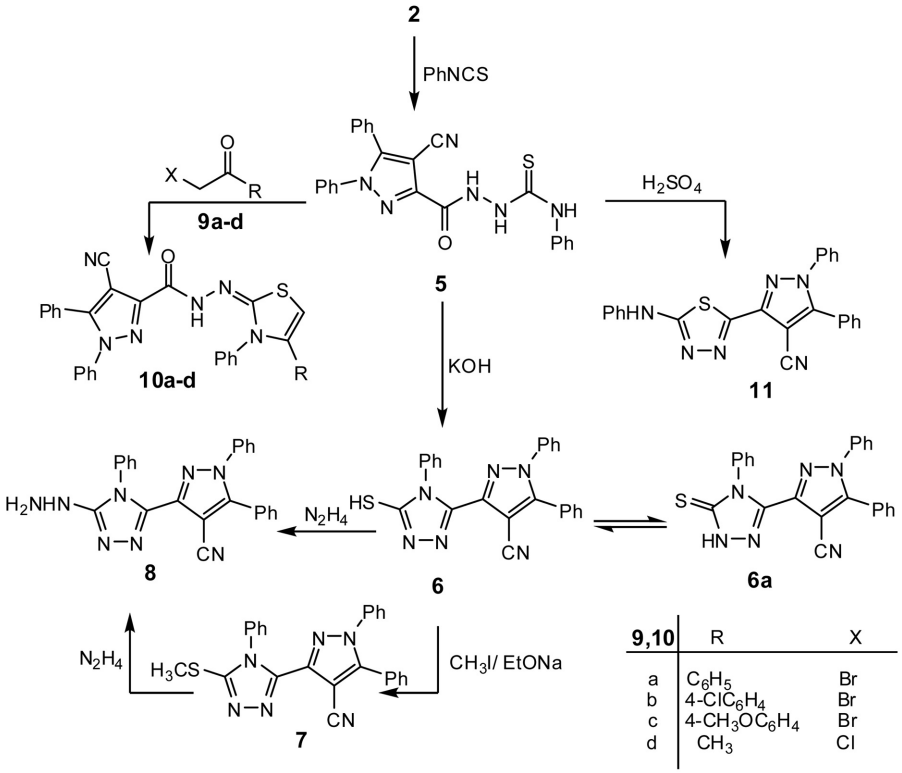
In the course of our investigation, we have found that ethyl 4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carboxylate **1**



Scheme 1. Synthetic pathway for the formation of compounds **2**, **3**, and **4**.

[53] is an excellent building block for the synthesis of several heterocyclic ring systems. Thus, when the latter compound was treated with hydrazine hydrate in ethanol, it afforded the corresponding 4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carboxylic acid hydrazide **2** in high yield (Scheme 1). The structure of product **2** was established on the basis of its elemental analysis and spectral data. For example, its IR spectrum revealed absorption bands at 3309, 3160, 3111, 2237, and 1674 cm^{-1} corresponding to NH, NH_2, nitrile, and amide carbonyl groups, respectively. Its ^{1}H-NMR spectrum showed broad D_2O-exchangeble signals at δ = 2.78 and 8.50 ppm corresponding to NH_2 and NH protons, respectively, in addition to a multiplet at δ = 7.21–7.43 characteristic for aromatic protons. The mass spectrum of the same product showed a peak at *m*/*z*: 303 corresponding to its molecular ion.

Treatment of the acid hydrazide **2** with potassium thiocyanate and HCl, under reflux condition, afforded 1-(4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carbonyl)thiosemicarbazide **3**. The ^{1}H-NMR spectrum of the latter product displayed broad D_2O-exchangeable signals at δ = 11.30, 7.85, and 4.34 ppm characteristic for two NH and NH_2 protons, respectively. Its mass spectrum showed a peak at *m*/*z*: 362 corresponding to its molecular ion. The structure of compound **3** was further confirmed by its alternate synthesis from the reaction of pyrazole-3-carboxylic acid ethyl ester **1** with thiosemicarbazide in refluxing dioxan (Scheme 1). When the pyrazole thiosemicarbazide **3** was treated with potassium hydroxide in refluxing ethanol, it afforded 3-(5-mercapto-4*H*-1,2,4-triazol-3-yl)-1,5-di-



Scheme 2. Synthetic pathway for the formation of compounds **5**, **6**, **7**, **8**, **9**, and **10**.

phenyl-1*H*-pyrazole-4-carbonitrile **4** (Scheme 1). The ^{1}H-NMR spectrum of the latter product revealed two D_2O-exchangeable signals at δ = 10.5 and 14.1 ppm characteristic for NH and SH protons, respectively. Its mass spectrum showed a peak at *m*/*z*: 344 corresponding to its molecular ion.

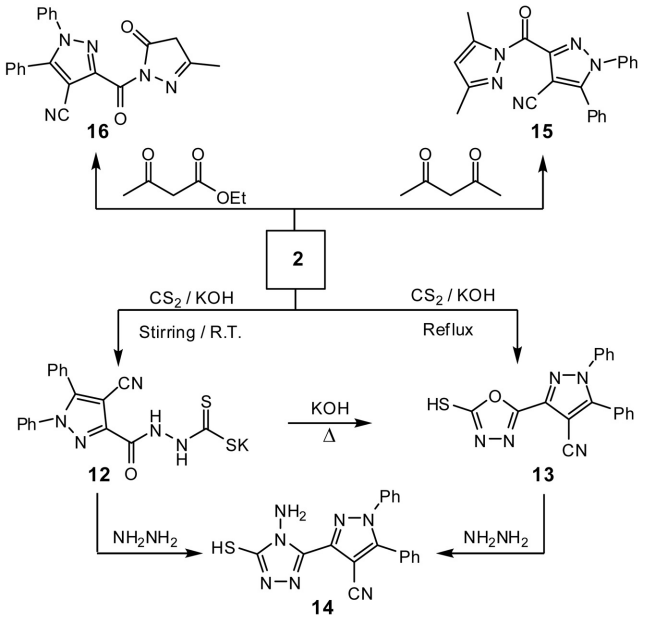
Treatment of the acid hydrazide **2** with phenyl isothiocynate, in ethanol under reflux, afforded a single product identified as 1-(4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carbonyl)-4-phenylthiosemicarbazide **5** (Scheme 2). The ^{1}H-NMR spectrum of the product **5** displayed three D_2O-exchangeble signals at δ = 7.93, 8.74, and 11.34 ppm characteristic for three NH protons. Its mass spectrum showed a peak corresponding to its molecular ion at *m*/*z*: 438.

Heating of the thiosemicarbazide derivative **5** in potassium hydroxide solution afforded a product identified as 3-(5-mercapto-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **6** (Scheme 2). The IR spectrum of the latter product showed two characteristic bands at 3265 and 2228 cm^{-1} corresponding to a SH group and a nitrile function, respectively. Its ^{1}H-NMR spectrum displayed broad D_2O-exchangeable signals at δ = 11.2 and 14.16 ppm corresponding to NH and SH protons, respectively, in addition to a multiplet at δ = 6.84–7.47 ppm characteristic for fifteen aromatic protons. The mass spectrum of the same product showed a peak corresponding to its molecular ion peak at *m*/*z*: 420 (see Experimental, section 4).

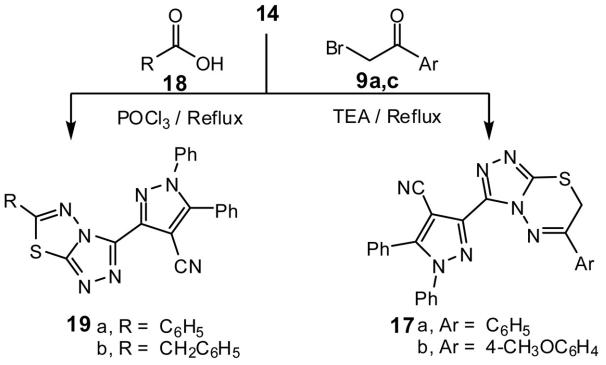
Treatment of compound **6** with an ethanolic solution of sodium ethoxide followed by the addition of an equimolar amount of methyl iodide, afforded a single product identified as 3-(5-(methylthio)-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **7** (Scheme 2). The ^{1}H-NMR spectrum of the latter product displayed a singlet signal at δ = 3.45 ppm characteristic for S-CH_3 protons. Its mass spectrum showed a peak corresponding to its molecular ion at *m*/*z*: 434. Treatment of a suspension of compound **7** with hydrazine hydrate under reflux condition afforded 3-(5-hydrazino-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **8**. The mass spectrum of the latter product revealed a peak corresponding to its molecular ion at *m*/*z*: 418.

The structure of compound **8** was further confirmed by its alternate synthesis from the reaction of **6** with hydrazine hydrate in refluxing ethanol (Scheme 2).

Treatment of compound **5** with phenacyl bromide derivatives **9a–c**, in the presence of a catalytic amount of triethylamine, afford the corresponding 4-cyano-1,5-diphenyl-1*H*-pyrazole-[4-aryl-3-phenyl-3*H*-thiazol-2-ylidene]-



Scheme 3. Synthetic pathway for the formation of compounds **12**, **13**, **14**, **15**, and **16**.



Scheme 4. Synthetic pathway for the formation of compounds **17** and **19**.

3-carboxylic acid hydrazide derivatives **10a–c** (Scheme 2). The IR spectra of the isolated products **10a–c** showed, in each case, one carbonyl absorption band in the region 1680 to 1690 cm^{-1}. The ^{1}H-NMR spectrum of compound **10c**, taken as a typical example of the prepared series, displayed signals at δ = 3.68, 7.74, 6.51 ppm characteristic for OCH_3, NH, and CH of thiazole protons, respectively, in addition to a multiplet at δ = 7.15–7.37 ppm characteristic for aromatic protons. Its mass spectrum showed a peak corresponding to its molecular ion at *m*/*z*: 568 (see Experimental, section 4).

Similarly, the thiosemicarbazid derivative **5** reacts with chloroacetone **9d** in the presence of a catalytic amount of triethylamine to afford the corresponding 4-cyano-1,5-diphenyl-1*H*-pyrazole-[4-methyl-3-phenyl-3*H*-thiazol-2-ylidene]-3-carboxylic acid hydrazide **10d** (Scheme 2).

When the thiosemicarbazide **5** was treated with sulphuric acid, it afforded 1,5-diphenyl-3-(5-(phenylamino)-1,3,4-thiadiazol-2-yl)-1*H*-pyrazole-4-carbonitrile **11** (Scheme 2). The mass spectrum of the latter product revealed a peak at *m*/*z*: 420 corresponding to its molecular ion. Its IR spectrum revealed an absorption band at 3190 cm^{-1} due to the NH group. The ^{1}H-NMR spectrum of the same product displayed a broad D_2O-exchangable singlet signal at δ = 7.75 ppm characteristic for the NH proton.

When the acid hydrazide **2** was treated with carbon disulphide and potassium hydroxide in ethanol at room temperature, it afforded the corresponding potassium salt **12** (Scheme 3). Heating of the potassium salt intermediate **12** in potassium hydroxide solution, afforded 3-(5-mercapto-1,3,4-oxadiazole-2-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **13** (Scheme 3).

Treatment of the potassium salt **12** with hydrazine hydrate under reflux condition, afforded a single product identified as 3-(5-mercapto-4-amino-4*H*-1,2,4-triazole-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitile **14** (Scheme 3). The IR spectrum of the latter product revealed absorption bands at 3294, 3120, and 2585 cm^{-1} characteristic for NH_2 and SH groups, respectively. The ^{1}H-NMR spectrum of the same product displayed signals at δ = 5.56 and 14.1 ppm (D_2O-exchangable) characteristic for NH_2 and SH protons, respectively. Its mass spectrum revealed a peak at *m*/*z*: 359 corresponding to its molecular. The same product was also obtained from the reaction of oxadiazole derivative **13** with hydrazine hydrate (Scheme 3; see Experimental, section 4).

The pyrazole carboxylic acid hydrazide **2** reacts with acetylacetone or with ethyl acetoacetate, in ethanol solution under reflux condition, to afford products **15** and **16**, respectively (Scheme 4).

The ^{1}H-NMR spectrum of compound **15** reveled two-singlet signal at δ = 1.95 and 2.06 ppm corresponding to two methyl groups and a singlet signal at 5.32 ppm corresponding to pyrazole-4H, whereas its mass spectrum showed a peak at *m*/*z*: 367 corresponding to its molecular ion. The IR spectrum of compound **16** exhibited two bands at 1665 and 1651 cm^{-1} characteristic for two carbonyl groups.

Treatment of the mecaptotriazole **14** with phenacyl bromide derivatives **9a**, **c** in ethanol, in the presence of a catalytic amount of triethylamine, at reflux temperature, afford the corresponding 3-(4-cyano-1,5-diphenyl-1*H*-pyrazole-3-yl)-6-aryl-7*H*-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazine derivatives **17a**, **b** (Scheme 4). The IR spectrum of compound **17b**, taken as a typical example, revealed a

band at 2230 cm^{-1} characteristic for a nitrile function. Its ^{1}H-NMR spectrum displayed a singlet signal at δ = 3.86 ppm corresponding to OCH_3 protons and a singlet signal at δ = 4.71 ppm corresponding to the CH_2 protons of thiadiazine, in addition to a multiplet at δ = 7.15–7.37 ppm due to aromatic protons.

The mecaptotriazole **14** reacts also with benzoic acid and with phenylacetic acid, in the presence of $POCl_3$, to afford 6-phenyl-3-(4-cyano-1,5-diphenyl–1*H*-pyrazole-3-yl)-1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazole **19a** and 6-benzyle-3-(4-cyano-1,5-diphenyl-1*H*-pyrazole-3-yl)-1,2,4-triazolo-[3,4-*b*]-1,3,4-thiadiazole **19b**, respectively (Scheme 4). The IR spectrum of compound **19b**, taken as a typical example, revealed an absorption band at 2237 cm^{-1} corresponding to a nitrile function. Its ^{1}H-NMR spectrum displayed a singlet signal at δ = 3.96 ppm corresponding to CH_2 protons, in addition to an aromatic multiplet at δ = 7.04–7.77 ppm, whereas its mass spectrum showed a peak at *m*/*z*: 459 corresponding to its molecular ion.

Pharmacology and toxicology

Anti-estrogenic activity

The anti-estrogenic activity of the newly synthesized compounds as well as letrozole (Femara®), as a reference drug, were evaluated in the Pharmacology and Toxicology Department, Research Units, Hi-Care Pharmaceutical Co., Cairo, Egypt.

The method used is based on the increase of the uterine weight in castrated female rats, induced by repeated administration of estradiol as antagonized by anti-estrogenic compounds. The test compounds (more than 99% purity) in carboxymethylcellulose (CMC) were administered orally by gavage to groups of immature ovarectomized rats. On the 8th day, the animals were sacrificed and the uterine weights were determined.

The mean value of percent reduction in uterine weight was calculated with regard to the control group, which was treated with estradiol alone, and the relative potency to the reference drug was calculated as depicted in Table 1.

Acute toxicity (LD_{50})

The rats were dosed by oral gavage with different doses of an aqueous suspensions of a very fine powder of the tested compounds. Under these conditions, the test compounds were of great safety for the albino rats compared to letrozole as a reference drug, as illustrated in Table 1.

The anti-estrogenic activity of 5-mercaptotriazole **4** was found to be more active and safer than the reference drug letrozole. Addition of a phenyl moiety to N1 of the triazole ring decreases the anti-estrogenic activity and increases the toxicity as shown in compound **6** (Table 1, Figs. 2 and 3). Methyl substitution of the SH group of the latter compound increases the activity by twofold compared to the reference drug and decreases the safety of the resultant molecule as shown in compound **7** (Table 1, Figs. 2 and 3).

Replacement of the mercapto moiety with a hydrazino group showed a slight improvement in the anti-estrogenic activity and decreased the safety to 0.4 of that of letrozole as in case of compound **8** (Table 1, Figs. 2 and 3). Substitution of N1 of the triazole moiety with a polar group such as amino group, as in compound **14**, decreases the activity dramatically and increases the toxicity in comparison with the unsubstituted triazole derivative **4** (Table 1, Figs. 2 and 3).

Replacement of the triazole ring with its bioisostere oxadiazole has no effect for the pharmacological activity

Table 1. The mean anti-estrogenic activity and acute toxicity (LD_{50}) of the synthesized compounds and letrozole.

Compound	% Reduction in uterine weight*	Relative potency to Letrozole	LD_{50} (mg/kg)
Letrozole	21.65	1.00	252.61 ± 0.11
2	43.43	2.01	332.11 ± 0.13
4	36.34	1.67	434.71 ± 0.13
5	16.92	0.78	311.52 ± 0.12
6	19.65	0.90	122.48 ± 0.13
7	43.98	2.03	131.85 ± 0.13
8	23.87	1.10	102.61 ± 0.11
10d	32.43	1.49	122.54 ± 0.16
11	13.98	0.64	232.54 ± 0.15
12	43.54	2.01	431.85 ± 0.13
13	34.75	1.60	323.06 ± 0.11
14	23.76	1.09	121.4 ± 0.011
15	32.45	1.49	873.06 ± 0.19
16	43.46	2.00	234.71 ± 0.13

* Value represents mean of twelve rats.

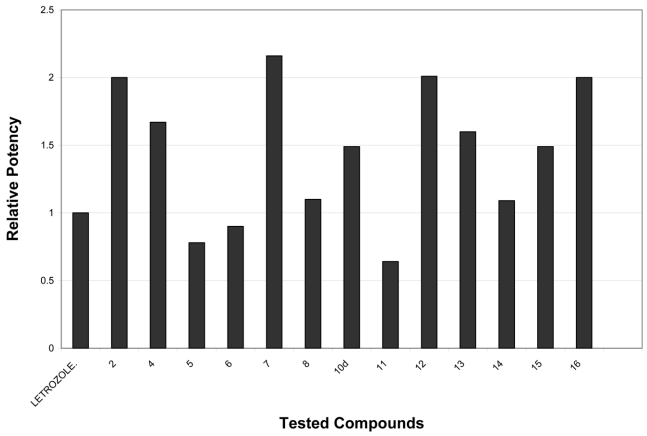


Figure 2. Anti-estrogenic potency of the test compounds relative to letrozole.

Table 2. Growth inhibitory concentration (GI_{50}, μM) of tested compounds on different breast cancer cell lines.

	Cell lines							
	MDA MB-231 ATTC	**MCF-7**	**T-47D**	**NCI ADR-RES**	**HS-578T**	**MDA MB-435**	**MiDA-N**	**BT 549**
2	61.2	85.8	N.T.#	N.T.#	N.T.#	N.T.#	1.34	9.13
4	6.27	I.A.§	6.68	2.48	I.A.	2.64	2.58	I.A.§
5	I.A.§	99.8	45.6	I.A.§	61.8	42.8	34.8	37.7
6	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§
7	I.A.§	68.0	36.3	I.A.§	69.8	41.8	73.8	44.8
8	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§
10a	7.36	4.72	62.5	7.4	6.72	8.62	7.00	4.12
10b	28.0	40.8	22.2	I.A.§	61.8	61.0	56.8	11.8
10c	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§
10d	I.A.§	79.8	26.5	28.0	I.A.§	I.A.§	26.5	11.8
11	5.84	8.53	18.0	99.8	I.A.§	55.8	93.8	I.A.§
12	4.90	I.A.§	63.4	I.A.§	8.04	9.30	2.86	I.A.§
13	38.4	38.0	72.0	99.0	98.0	I.A.§	55.4	26.2
14	I.A.§	23.4	39.9	I.A.§	8.94	2.93	5.00	I.A.§
15	6.21	I.A.§	2.45	2.76	I.A.§	2.75	I.A.§	I.A.§
16	23.8	19.8	54.8	98.0	I.A.§	39.9	23.8	11.8
17a	13.2	19.8	9.08	28.0	I.A.§	48.0	73.0	51.6
17b	5.49	I.A.§	I.A.§	8.4	9.23	1.23	1.19	I.A.§
19a	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§	I.A.§
19b	52.1	41.6	42.6	74.7	8.94	9.23	5.49	I.A.§

§ I.A.: inactive; GI_{50} value >100 μM; # N.T.: not tested.

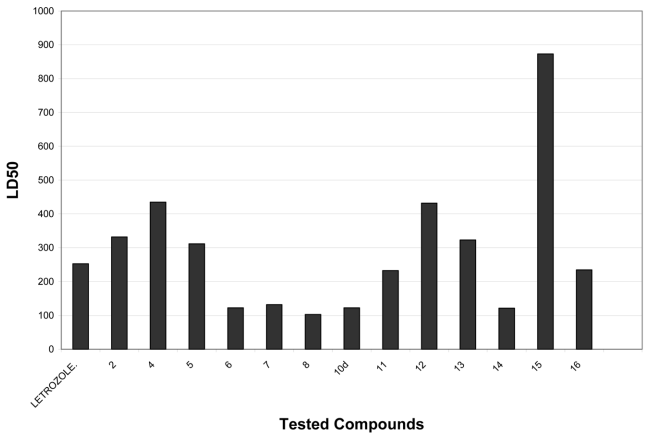


Figure 3. LD_{50} of the tested compounds and letrozole.

and slightly decreases the toxicity of the resultant compound as in the case of compound **13** (Table 1, Figs. 2 and 3). In contrast, the thiadiazole bioisostere **11** showed a lower anti-estrogenic activity than both the reference drug and compound **4** (Table 1, Figs. 2 and 3). Furthermore, the pyrazole derivative **15** was found to be 3.5 times less toxic than letrozole with an anti-estrogenic activity 1.5 times that of the same drug. The pyrazolone derivative **16** was found to be two times more potent than letrozole with almost the same safety range (Table 1, Figs. 2 and 3). Moreover, some of the synthesized intermediates were also investigated for their anti-estrogenic activity. Thus, it was found that both of the acid hydrazide **2** and the potassium salt of **12** are more active than letrozole, while the phenylthiosemicarbazide **5** is less active than the same drug (Table 1, Figs. 2 and 3).

In-vitro disease-oriented primary antitumor screening

All newly synthesized compounds were selected by the National Cancer Institute (NCI), Bethesda, Maryland, USA, for evaluation of their *in-vitro* antitumor activity using 14 breast and ovarian cell lines.

Effect of the newly synthesized compounds on breast cancer

We started the present study with the mercaptotriazole derivative **4** which showed good activity against five breast tumor cell lines, namely MDA/MB-231 ATTC, T-47D, NCI/ADR-RES, MDA/MB-435, and MiDA-N (GI_{50} values between 2.4 and 6.6 μM as depicted in Table 2). Increasing the lipophilic character of such a compound by attaching a phenyl moiety to the triazole ring renders the resultant structure without any cytotoxic properties as in case of compound **6** (Table 2). In contrast, replacement of the phenyl moiety by a more hydrophilic group such as an amino group showed dramatic improvement in the cytotoxic behavior as demonstrated in compound **14** which revealed GI_{50} values against tumor cell lines MDA/MB-

435, MiDA-N, and HS-578T equal to 2.93, 5.00, and 8.94 μM, respectively (Table 2). On the other hand, methylation of the mercapto group of the triazole moiety excerted only a little effect on the cytotoxic properties as shown in compound **7** (Table 2). In addition, replacement of the mercapto group with a hydrazino moiety renders the molecule completely inactive against the tested breast tumor cell lines as in case of compound **8** (Table 2).

In the second line of structural optimization, the triazole ring was replaced with thiadiazole moiety which improved the cytotoxic effect against MDA/MB-231-ATTC and MCF-7 breast tumor cell lines (GI_{50} values are 5.84 and 8.53 ppm, respectively) as shown in compound **11** (Table 2). Whereas, the oxadiazole isostere was found to be less effective as in the case of compound **13** (Table 2). The pyrazole derivative **15** has significant cytotoxic effects against T-47D, NCI/ADR-RES, MDA/MB-435, and MDA/MB-231-ATTC breast cell lines (Table 2). Although the pyrazolone derivative **16** revealed a good anti-estrogenic activity *in vivo*, it has a moderate to weak cytotoxic effect against breast tumor cell lines (Tables 1 and 2).

Since the carboxylic acid hydrazide derivative **2** showed a remarkable cytotoxic effect against two breast tumor cell lines, we decided to modify its structure by attaching different substituted five-membered heterocyclic rings to its N2 position. The first obtained compound is phenylthiazolidene derivative **10a** which showed GI_{50} values against all breast tumor cell lines ranging between 4.1 and 8.6 μM with the exception of T-47D (Table 2). The cytotoxic activity against the latter cell was slightly improved by adding a chlorine atom at the *para*-position of phenyl ring as in compound **10b** (Table 2). In contrast, replacement of the chlorine atom in the latter product with a methyl group renders the resultant compound completely inactive as shown in compound **10c** (Table 2). Furthermore, replacement of the phenyl moiety with a methyl group increased the cytotoxic activity against the BT 549 breast tumor cell line as shown in compound **10d** (Table 2).

In the last line of structural optimization, utilizing both mercapto and amino groups in compound **14** for obtaining the corresponding fused ring systems, **17** and **19** showed high cytotoxic effects. Such modifications afforded a remarkable improvement in the antitumor properties of the resultant molecules. For example, the GI_{50} value against the MiDA-N tumor cell line was reduced from 5.0 to 1.1 μM in case of the triazolothiadiazine derivative **17b** (Table 2). The latter compound showed also significant *in-vitro* cytotoxic activity against MDA/MB-435 (GI_{50} value 1.23 μM; Table 2). In addition, its GI_{50} values were below 10 μM against MDA/MB-231. ATTC, NCI/ADR-RES, and HS-578T cell lines (Table 2). Furthermore, replacement of the thiadiazine **17** with the five-membered thiadiazole ring gave a totally inactive compound as in the case of compound **19a**, in addition to an active compound **19b** against MiDA-N, HS-578T, and MDA/MB-435 tumor cell lines (GI_{50} values between 5.4 and 9.2 μM) as in case of compound **19b** (Table 2).

Table 3. Growth inhibitory concentration (GI_{50}, μM) of the tested compounds on different ovarian cancer cell lines.

	Cell lines					
	IGROVI	**OVCAR-3**	**OVCAR-5**	**OVCAR-8**	**OVCAR-4**	**SK-OV-3**
2	92.8	I.A.§	31.8	71.8	33.8	26.8
4	53.4	I.A.§	3.94	9.94	12.8	56.8
5	91.5	93.8	I.A.§	I.A.§	38.4	36.8
6	I.A.§	63.8	31.8	I.A.§	5.89	I.A.§
7	I.A.§	8.94	9.23	9.23	5.49	I.A.§
8	I.A.§	5.19	I.A.§	7.66	7.66	9.72
10a	7.76	13.3	7.66	9.12	7.26	9.22
10b	80.0	94.5	94.4	I.A.§	32.4	10.8
10c	38.4	7.66	5.12	91.0	60.1	36.4
10d	81.0	91.9	98.0	I.A.§	38.4	36.8
11	91.8	I.A.§	61.8	81.8	73.8	26.8
12	I.A.§	N.T.#	N.T.#	N.T.#	N.T.#	N.T.#
13	0.040	I.A.§	20.3	I.A.§	23.0	I.A.§
14	93.8	I.A.§	61.8	61.8	63.8	36.8
15	95.8	99.8	I.A.§	61.8	18.4	23.8
16	I.A.§	58.9	94.0	I.A.§	94.0	I.A.§
17a	98.0	93.0	I.A.[a]	61.8	63.8	36.8
17b	9.24	I.A.§	7.94	3.23	3.49	I.A.§
19a	94.8	93.8	I.A.§	61.8	7.66	9.72
19b	9.34	I.A.§	8.04	9.33	4.49	I.A.§

§ I.A.: inactive; GI_{50} value >100 μM; # N.T.: not tested.

Beside the designed compounds in the present study, all intermediates were tested for their antitumor activity. Thus, the thiosemicarbazide derivative **5** was found to have moderate to weak activity, while the potassium salt **12** revealed good GI_{50} values between 2.8 and 9.3 μM against MDA/MB-231 ATTC, MiDA-N, HS-578T, and MDA/MB-435 breast tumor cell lines (Table 2).

Effect of the newly synthesized compounds on ovarian cancer

Ovarian OVCAR-5 and eight tumor cell lines were found to be sensitive to triazolethiol derivative **4** (GI_{50} values are 3.94 and 9.94 μM, respectively) (Table 3). The more lipophilic derivative 3-(5-mercapto-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **6** is more active against the OVCAR-4 tumor cell line, while the more hydrophilic derivative 3-(5-mercapto-4-amino-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **14** has moderate, weak, or no activity (Table 3). Methylation of compound **6** afforded a broad-spectrum active compound against all OVCAR types (GI_{50} ranging between 5.4 to 9.2 μM) as shown for compound **7** (Table

3). Replacement of the mercapto group with a hydrazino moiety increases the activity against the SK-OV-3 ovarian tumor cell line (GI_{50} value 9.72 μM) as shown in compound **8** (Table 3).

The oxadiazole derivative **13** revealed cytotoxic activity in the nanomolar range against the IGROVI tumor cell line (GI_{50} value 40 nM; Table 3). In contrast, thiadiazole derivative **11** showed no promising cytotoxic results (Table 3).

Although the carboxylic acid hydrazide derivative **2** showed moderate to weak activity (Table 3), its phenylthiazolidene derivative **10a** has a broad-spectrum activity against all tested ovarian tumor cell lines (GI_{50} values are 10 ± 3 μM; Table 3). In addition, introduction of a methyl group in the *para*-position of the phenyl ring of the latter product improves its antitumor properties against OVCAR-3 and 5 cell lines as shown in compound **10c** (Table 3). Replacement of the methyl group with chlorine atom in the same compound decreases the cytotoxic activity against all ovarian cell lines except SK-OV-3 (Table 3).

The fused triazole derivative **17a** showed insignificant cytotoxic results against the tested ovarian cell lines. Its *para*-methoxy derivative **17b** revealed remarkable antitumor characteristics against OVCAR-8 and 4 with a GI_{50} value around 3.3 μM (Table 3). Finally, both triazolothiadiazoles **19a** and **b** are active against the OVCAR-4 ovarian tumor cell line (GI_{50} values are 7.66 and 4.49 μM, respectively; Table 3).

Conclusion

In this article, different triazole, oxadiazole, thiadiazole, and pyrazole as well as the fused triazole derivatives of 4-cyano-1,5-diphenylpyrazole were synthesized via the intermediates **1**, **2**, **3**, **5**, and **12**. All of the newly designed compounds were tested *in vitro* for there cytotoxic properties against estrogen-dependent tumor cell lines (breast and ovarian tumor types). In addition, the anti-estrogen activity of thirteen selected compounds was determined *in vivo* and their LD_{50} values were determined.

3-(5-Mercapto-1,3,4-oxadiazole-2-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **13** revealed the highest cytotoxic activity in a nanomolar range against the IGROVI ovarian tumor cell line with an anti-estrogen activity 1.6 times that of letrozole and with a greater safety margin. 4-Cyano-1,5-diphenyl-1*H*-pyrazole-[3,4-diphenyl-3*H*-thiazol-(2*E*)-ylidene]-3-carboxylic acid hydrazide **10a** showed significant activity against both breast and ovarian tumor cell lines.

3-(5-(Methylthio)-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **7** showed selective *in-vitro* cytotoxic activity against all OVCAR ovarian tumor cell lines (GI_{50} values below 10 μM) with excellent anti-estrogen properties more than twofold compared to letrozole. Unfortunately, these compounds were found to be more toxic than letrozole.

Experimental

General

All melting points were measured on a Gallenkamp melting point apparatus (Weiss-Gallenkamp, London, UK). The infrared spectra were recorded in potassium bromide disks on a pye Unicam SP 3300 and Shimadzu FT IR 8101 PC infrared spectrophotometers (Pye Unicam Ltd. Cambridge, England and Shimadzu, Tokyo, Japan, respectively). The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer (Varian, Palo Alto, CA, USA). ^{1}H spectra were run at 300 MHz and ^{13}C spectra were run at 75.46 MHz in deuterated chloroform ($CDCl_3$) or dimethyl sulphoxide (DMSO-d_6). Chemical shifts were related to that of the solvent. Mass spectra were recorded on a Shimadzu GCMS-QP 1000 EX mass spectrometer (Shimadzu) at 70 eV. Elemental analyses were carried out at the Micro-analytical Center of Cairo University, Giza, Egypt. Ethyl 4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carboxylate **1** was prepared following the procedures reported in the literature [53].

Chemistry

*4-Cyano-1,5-diphenyl-1H-pyrazole-3-carboxylic acid hydrazide **2***

Hydrazine hydrate (80%, 10 mL) was added to a stirred solution of the 4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carboxylate **1** (5 g, 16.5 mmol) in absolute ethanol (20 mL) and the reaction mixture was stirred for 10 h. The precipitated white solid was collected by filtration, washed with water, and crystallized from diluted ethanol to afford the corresponding acid hydrazide **2** in 78% yield; m. p.: 148–149°C; IR (KBr) ν_{max}/cm^{-1}: 3309, 3160, 3111 (NH, NH_2), 2237 (CN), 1674 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 2.78 (br s, 2H, NH_2 D_2O-exchangeable), 7.21–7.43 (m, 10H, ArH's), 8.50 (br s, 1H, NH, D_2O-exchangeable); MS *m*/*z* (%): 303 [M^+] (100), 180 (11), 141 (10.9), 77 (59.3) Anal. calcd. for $C_{17}H_{13}N_5O$ (303.32): C, 67.31; H, 4.32; N, 23.09. Found: C, 67.35; H, 4.40; N, 23.13.

*1-(4-Cyano-1,5-diphenyl-1H-pyrazole-3-carbonyl)thiosemicarbazide **3***

Method A: To a solution of the pyrazole carboxylic acid hydrazide **2** (6.06 g, 20 mmol) in methanol (50 mL), a solution of potassium thiocyanate (2.91 g, 30 mmol) and hydrochloric acid (3 mL) was added with constant stirring. The mixture was immediately evaporated to dryness under reduced pressure and heated for an additional one hour with another 50 mL of methanol. The resulting solid was treated with water and ethanol, and finally recrystallized from ethanol to afford 1-(4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carbonyl)thiosemicarbazide **3** in 78% yield.

Method B: To a solution of ethyl 4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carboxlate **1** (9.69 g, 30 mmol) and thiosemicarbazide (2.91 g, 30 mmol) in dioxan (30 mL) was added with a few drops of piperidine; the reaction mixture was refluxed for 10 h and then left to cool. The precipitated solid product was filtered off, washed with ethanol, dried, and finally recrystallized from etha-

nol to afford a product in all respects (m. p., mixture m. p., TLC, IR and NMR spectra) identical with that obtained by method A above.

M. p.: 185–186°C; IR (KBr) ν_{max}/cm^{-1}: 3350, 3200 (overlapped NH, NH_2), 2229 (CN), 1670 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 4.34 (br s, 2H, NH_2 D_2O-exchangeable), 7.35–7.47 (m, 10H, ArH's), 7.85, 11.30 (br., s, 2H, 2 NH, D_2O-exchangeable); MS *m/z*: 362 [M^+] (15.6), 272 (100), 244 (10.2), 77 (40.1) Anal. calcd. for $C_{18}H_{14}N_6OS$ (362.41): C, 59.65; H, 3.89; N, 23.19; S, 8.84. Found: C, 59.61; H, 3.87; N, 23.16; S, 8.81.

*3-(5-Mercapto-4H-1,2,4-triazol-3-yl)-1,5-diphenyl-1H-pyrazole-4-carbonitrile **4***

A suspension of the thiosemicarbazide **3** (0.36 g, 1 mmol) in potassium hydroxide solution (10 mL, 7%) was heated under reflux for 3 h. The reaction mixture was allowed to cool and was then adjusted to pH 6 with 10% hydrochloric acid. The formed precipitate was filtered off, washed with water, dried, and finally recrystallized from ethanol to afford the triazolethiol **4** in 68% yield; m. p.: 295–297°C; IR (KBr) ν_{max}/cm^{-1}: 3200 (NH), 2239 (CN); ^{1}H-NMR (DMSO-d_6) δ: 7.21–7.43 (m, 10H, ArH's), 10.5 (s.1H, NH, D_2O-exchangeable), 14.1 (s, 1H, SH); MS *m/z* (%): 344 [M^+] 21.2), 317 (61.2), 245 (10.0), 180 (59.9), 141 (28.1), 77 (100). Anal. calcd. for $C_{18}H_{12}N_6S$ (344.40): C, 62.78; H, 3.51; N, 24.40; S, 9.31. Found: C, 62.83; H, 3.48; N, 24.48; S, 9.33.

*1-(4-Cyano-1,5-diphenyl-1H-pyrazole-3-carbonyl)-4-phenylthiosemicarbazide **5***

An equimolar quantity of the acid hydrazide **2** (6.06 g, 20 mmol) and phenyl isothiocyanate (2.7 g, 20 mmol) in absolute ethanol (40 mL) were refluxed for 3 h and then allowed to cool to room temperature. Fine crystals of the thiosemicarbazide **5** were separated out, filtered off, and recrystallized from ethanol to afford the compound in 85% yield; m. p.: 130°C; IR (KBr) ν_{max}/cm^{-1}: 3310, 3150 (3 NH, overlapped), 2233 (CN), 1667 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 7.03–7.30 (m, 15H, ArH's), 7.93, 8.74, 11.34 (br s, 3H, 3 NH, D_2O-exchangeable); MS *m/z* (%): 439 (57.1), 438 [M^+] (100), 422 (7.3), 180 (10.0), 77 (56.7); Anal. calcd. for $C_{24}H_{18}N_6OS$ (438.51): C, 65.74; H, 4.14; N, 19.16; S, 7.31. Found: C, 65.71; H, 4.13; N, 19.20; S, 7.34.

*3-(5-Mercapto-4-phenyl-4H-1,2,4-triazol-3-yl)-1,5-diphenyl-1H-pyrazole-4-carbonitrile **6***

The thiosemicarbazide **5** (4.38 g, 10 mmol) was refluxed in potassium hydroxide solution (5%, 25 mL) for 3 h. The resulting solution was treated with charcoal, filtered, and cooled. The filtrate was acidified with hydrochloric acid to pH = 5 and the formed solid was filtered off, washed with water, dried, and recrystallized from ethanol to afford the thiazole **6** in 85% yield; m. p.: 250–252°C; IR (KBr) ν_{max}/cm^{-1}: 3265 (NH), 2228 (CN); ^{1}H-NMR (DMSO-d_6) δ: 6.84–7.47 (m, 15H, ArHs), 11.2 (br s, 1H, NH, D_2O-exchangeable), 14.16 (br s, 1H, SH); MS *m/z* (%): 420 [M^+] (47.3), 244 (4.9), 286 (35.8), 127 (3.3), 77 (100). Anal. calcd. for $C_{24}H_{16}N_6S$ (420.50): C, 68.55; H, 3.84; N, 19.98; S, 7.62. Found: C, 68.61; H, 3.88; N, 19.93; S, 7.58.

*3-(5-(Methylthio)-4-phenyl-4H-1,2,4-triazol-3-yl)-1,5-diphenyl-1H-pyrazole-4-carbonitrile **7***

The mercaptotriazol **6** (2.1 g, 5 mmol) was dissolved in an ethanolic solution of sodium ethoxide – prepared from sodium metal (0.11 g, 5 mg/atom) in 20 mL ethanol). Then, methyl iodide (0.28 g, 2 mmol) was added gradually with stirring to the resulting solution. The reaction mixture was refluxed for 2 h, concentrated, cooled, diluted with water, and left to stand overnight. The formed precipitate was filtered off, washed with water, dried, and recrystallized from ethanol to afford compound **7** in 80% yield; m. p.: 236–238°C; IR (KBr) ν_{max}/cm^{-1}: 2225 (CN); ^{1}H-NMR (DMSO-d_6) δ: 3.45 (s, 3H, SCH_3), 7.31–7.65 (m, 15H, ArH's); MS *m/z* (%): 434 [M^+] (16.4), 401 (14.0), 77 (100). Anal. calcd. for $C_{25}H_{18}N_6$ S (434.52): C, 69.10; H, 4.17; N, 19.34; S, 7.37. Found: C, 69.16; H, 4.20; N, 19.30; S, 7.29.

*3-(5-Hydrazino-4-phenyl-4H-1,2,4–triazol-3-yl)-1,5-diphenyl-1H-pyrazole-4-carbonitrile **8***

A mixture of 3-(5-(methylthio)-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **7** (1.3 g, 3 mmol) or 3-(5-mercapto 4-phenyl-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **6** (1.26 g, 3 mmol) and hydrazine hydrate (80%, 5 mL) was refluxed for 5 h. The reaction mixture was evaporated under reduced pressure to remove excess hydrazine hydrate and was allowed to cool. The formed solid product were filtered off, washed with ethanol, and recrystallized from ethanol to give compound **8** in 65% yield; m. p.: 295–297°C; IR (KBr) ν_{max}/cm^{-1}: 3250, 3120, (NH, NH_2), 2225 (CN); ^{1}H-NMR (DMSO-d_6) δ: 2.49 (br s, 2H, NH_2, D_2O-exchangeable), 6.95 (br s, 1H, NH, D_2O-exchangeable), 7.21–7.43 (m, 15H, ArH's); MS *m/z* (%): 418 [M^+] (49.8), 286 (35.8), 77 (100). Anal. calcd. for $C_{24}H_{18}N_8$ (418.46): C, 68.88; H, 4.33; N, 26.78. Found: C, 68.90; H, 4.34; N, 26.75.

*Reaction of 1-(4-cyano-1,5-diphenyl-1H-pyrazole-3-yl)-4-phenylthiosemicarbazide (**5**) with phenacyl bromide derivatives and chloroacetone: General procedure*

To a solution of 1-(4-cyano-1,5-diphenyl-1*H*-pyrazole-3-carbonyl)-4-phenyl thiosemicarbazide **5** (1.31 g, 3 mmol) and the appropriate phenacyl bromide derivatives or chloroacetone (3 mmol) in absolute ethanol (20 mL), a catalytic amount of triethylamine (0.1 mL) was added. Then, the reaction mixture was heated under reflux for 3 h, and was allowed to cool. The precipitated product was filtered off, washed with ethanol, dried, and finally recrystallized from ethanol. The synthesized compounds together with their physical and spectral data are listed below.

*4-Cyano-1,5-diphenyl-1H-pyrazole-[3,4-diphenyl-3H-thiazol-2-ylidene]-3-carboxylic acid hydrazide **10a***

Yield: 68%; m.p.: 155–156°C; IR (KBr) ν_{max}/cm^{-1}: 3128 (NH), 2229 (CN), 1689 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.54 (s, 1H, thiazole-5 C*H*), 721–7.50 (m, 20H, ArH's), 11.01 (br s, 1H, NH, D_2O-exchangeable); MS *m/z* (%): 539 (25.7), 538 [M^+] (56.5), 272 (42.4), 180 (26.2), 135 (25.5), 77 (100). Anal. calcd. for $C_{32}H_{22}N_6OS$ (538.63): C, 71.36; H, 4.11; N, 15.60; S, 5.95. Found: C, 71.40; H, 4.16; N, 15.68; S, 5.89.

*4-Cyano-1,5-diphenyl-1H-pyrazole-[4-(4-chlorophenyl)-3-phenyl-3H-thiazol-2-ylidene]-3-carboxylic acid hydrazid **10b***

Yield: 68%; m. p.: 250–252°C; IR (KBr) ν_{max}/cm^{-1}: 3115 (NH), 2229 (CN), 1688 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.61 (s, 1H, thiazole-5 C*H*), 732–7.63 (m, 19H, ArH's), 10.82 (br s, 1H, NH, D_2O-exchangeable); ^{13}C-NMR (DMSO-d_6) δ: 112.86, 115.64, 120.67, 120.81, 123.21, 125.77, 125.87, 126.12, 126.21, 127.87, 128.57, 129.17, 129.33,

130.32, 130.42, 133.24, 137.26, 138.23, 144.68, 147.25, 149.70, 150.31, 150.46, 153.63, 158.51; MS *m/z* (%): 574 [M$^+$] 18), 572 [M$^+$] (57), 555 (33), 285 (22), 180 (37), 141 (18), 77 (100). Anal. calcd. for $C_{32}H_{21}N_6OSCl$ (573.07): C, 67.07; H, 3.69; N, 14.66; S, 5.59. Found: C, 67.16; H, 3.66; N, 14.70; S, 5.75.

4-Cyano-1,5-diphenyl-1H-pyrazole[4-(4-methoxyphenyl)-3-phenyl-3H-thiazol-2-ylidene]-3-carboxylic acid hydrazide ***10c***

Yield: 65%; m. p.: 206°C; IR (KBr) ν_{max}/cm^{-1}: 3150 (NH), 2229 (CN), 1684 (C=O); ^{1}H-NMR ($CDCl_3$) δ: 3.68 (s, 3H, OCH_3), 6.51 (s, 1H, thiazole-5 C*H*), 7.15-7.37 (m, 19H, ArH's), 7.74 (br s, 1H, NH, D_2O-exchangeable); ^{13}C-NMR (DMSO-d_6) δ: 55.10, 113.85, 120.14, 120.79, 122.99, 125.76, 125.78, 126.00, 126.08, 127.24, 128.50, 129.26, 129.37, 130.19, 130.86, 131.54, 137.68, 137.96, 139.21, 144.18, 150.00, 150.28, 150.40, 151.54, 159.11; MS *m/z* (%): 568 [M$^+$] (28.6), 282 (100), 149 (25.8), 77 (70.7). Anal. calcd. for $C_{33}H_{24}N_6O_2S$ (568.59): C, 69.70; H, 4.25; N, 14.78; S, 5.64. Found: C, 69.65; H, 4.26; N, 14.80; S, 5.58.

4-Cyano-1,5-diphenyl-1H-pyrazole-[4-methyl-3-phenyl-3H-thiazol-2-ylidene)-3-carboxylic acid hydrazide ***10d***

Yield: 68%; m. p.: 265–257°C; IR (KBr) ν_{max}/cm^{-1}: 3120 (NH), 2223 (CN), 1698 (C=O); ^{1}H-NMR ($CDCl_3$) δ: 1.89 (s, 3H, CH_3), 6.02 (s, 1H, thiazole-5 C*H*), 7.11–7.40 (m, 15H, ArH's), 7.87 (br s, 1H, NH, D_2O-exchangeable); MS *m/z* (%): 476 [M$^+$] (34.9), 190 (100), 77 (29.4). Anal. calcd. for $C_{27}H_{20}N_6OS$ (476.55): C, 68.05; H, 4.23; N, 17.63; S, 6.75. Found: C, 67.99; H, 4.25; N, 17.60; S, 6.73.

1,5-Diphenyl-3-[5-(phenylamino)-1,3,4-thiadiazol-2-yl]-1H-pyrazole-4-carbonitrile ***11***

A mixture of the thiosemicarbazide **5** (1.31 g, 0.3 mmol) and concentrated sulphuric acid (10 mL) was stirred for 4 h, then, the reaction mixture was poured into ice-cold water. The formed precipitate was filtered off, washed with water several times, dried, and recrystallized from ethanol. Yield: 70%; m. p.: 185°C; IR (KBr) ν_{max}/cm^{-1}: 3190 (NH), 2229 (CN); ^{1}H-NMR (DMSO-d_6) δ: 7.06–7.50 (m, 15H, ArH's), 7.75 (s, 1H, NH, D_2O exchangeable); MS *m/z* (%): 420 [M$^+$] (43.8), 105 (31.5), 77 (100). Anal. calcd for $C_{24}H_{16}N_6S$ (420.50): C, 68.55; H, 3.84; N, 19.99; S, 7.63. Found: C, 68.53; H, 3.82; N, 19.94; S, 7.65.

Potassium salt of thiosemicarbazide derivative ***12***

To a cold stirred solution of the acid hydrazide **2** (3.03 g, 10 mmol) in absolute ethanol (100 mL) containing potassium hydroxide (0.84 g, 15 mmol), carbon disulphide (1.14 g, 15 mmol) was added gradually. The reaction mixture was stirred at room temperature for 8 h. A yellow precipitate of the corresponding potassium salt **12** was separated. Then, dry ether (100 mL) was added to complete the precipitation of the formed salt which was filtered off and washed with dry ether (100 mL). Yield: 95%; m. p.: >300°C; IR (KBr) ν_{max}/cm^{-1}: 3280, 3150 (2 NH), 2230 (CN) 1675 (CO); ^{1}H-NMR (DMSO-d_6) δ: 7.37–7.49 (m, 10H, ArH's), 7.48 (s, 1H, NH, D_2O exchangeable), 11.19 (s, 1H, NH, D_2O exchangeable). Anal. calcd. for $C_{18}H_{12}KN_5OS_2$ (417.55): C, 51.78; H, 2.90; N, 16.77; S, 15.36. Found: C, 51.85; H, 2.91; N, 16.73; S, 15.39.

3-(5-Mercapto-1,3,4-oxadiazole-2-yl)-1,5-diphenyl-1H-pyrazole-4-carbonitrile ***13***

Method A: A solution of potassium hydroxide (0.84 g, 15 mmol) and the acid hydrazide **2** (3.03 g, 10 mmol) in absolute ethanol (200 mL) were added (1.14 g, 15 mmol). This reaction mixture was refluxed for 5 h, then diluted with water and acidified with HCl. The precipitated solid was filtered off, washed with water, dried, and finally recrystallized with ethanol to afford compound **13** in 89% yield.

Method B: A solution of potassium hydroxide (0.84 g, 15 mmol) and the potassium salt **12** (4.72 g, 10 mmol) in absolute ethanol (200 mL) was refluxed for 4 h, till the evolution of H_2S ceased; then, it was diluted with water and acidified with HCl. The precipitated solid was filtered off, washed with water, dried, and was finally crystallized with ethanol to give compound **13** in 90% yield; m. p.: 137°C; IR (KBr) ν_{max}/cm^{-1}: 2235 (CN). ^{1}H-NMR (DMSO-d_6) δ: 6.48–7.47 (m, 10H, ArH's), 14.0 (s, 1H, SH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6) δ: 115.49, 120.51, 120.54, 120.56, 125.62, 127.85, 128.19, 128.43, 128.75, 128.90, 129.94, 138.40, 143.94, 144.57; MS *m/z* (%): 345 [M$^+$] (40.7), 256 (17.5), 180 (41.2), 77 (100). Anal. calcd for $C_{18}H_{11}N_5OS$ (345.38): C, 62.59; H, 3.21; N, 20.27; S, 9.28. Found: C, 62.51; H, 3.22; N, 20.23; S, 9.23.

3-(5-Mercapto-4-amino-4H-1,2,4-triazol-3-yl)-1,5-diphenyl-1H-pyrazole-4-carbonitrile ***14***

Method A: The potassium salt **12** (4.72 g, 10 mmol) was suspended in 70% hydrazine hydrate (5 mL), then refluxed for 3 h. The formed white solid, which was filtered off, was washed with water, dried, and finally recrystallized with ethanol/DMF to afford compound **14** in 85% yield.

Method B: A solution of the oxadiazole **13** (3.45 g, 10 mmol) in ethanol (20 mL) and 70% hydrazine hydrate (5 mL) was refluxed for 3 h, then allowed to cool, diluted with cold water, and acidified with HCl. The precipitated solid was filtered, washed with water, dried, and recrystallized with ethanol/DMF to give compound **14** in 72% yield; m. p.: 235–236°C; IR (KBr) ν_{max}/cm^{-1}: 3294, 3120 (NH_2), 2585 (SH), 2229 (CN); ^{1}H-NMR (DMSO-d_6) δ: 5.56 (s, 2H, NH_2, D_2O-exchangeable), 7.09–7.47 (m, 10H, ArH's), 14.1 (s, 1H, SH); MS *m/z* (%): 359 [M$^+$] (16.2), 327 (26.3), 269 (25.1), 180 (20.4), 126 (25.7), 90 (65.9), 77 (100). Anal. calcd. for $C_{18}H_{13}N_7S$ (359.41): C, 60.15; H, 3.64; N, 27.28; S, 8.92. Found: C, 60.20; H, 3.58; N, 27.36; S, 8.84.

1,5-Diphenyl-3-(3,5-dimethylpyrazole-1-carbonyl)-1H-pyrazole-4-carbonitrile ***15***

To a mixture of the acid hydrazide **2** (3.03 g, 10 mmol) and acetylacetone (1.0 g, 10 mmol) in ethanol (20 mL), a few drops of piperidine were added. The reaction mixture was refluxed for 6 h and then allowed to cool. The precipitated solid was filtered off, washed with water, dried, and recrystallized from dilute ethanol to afford compound **15** in 67% yield; m. p.: 198°C; IR (KBr) ν_{max}/cm^{-1}: 2234 (CN), 1658 (C=O); ^{1}H-NMR ($CDCl_3$) δ: 1.95 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 5.32 (s, 1H, CH), 7.06–7.60 (m, 10H, ArH's); ^{13}C-NMR (DMSO-d_6) δ: 15.61, 22.98, 112.96, 119.25, 120.77, 125.64, 125.92, 126.11, 128.85, 129.21, 129.28, 130.26, 137.82, 140.21, 140.36, 149.78, 156.93, 158.89; MS *m/z* (%): 367 [M$^+$] (21.4), 338 (31.6), 272 (100), 180 (10.5), 141 (23.9), 95 (22.9), 77 (45.1). Anal. calcd. for $C_{22}H_{17}N_5O$ (367.41): C, 71.92; H, 4.66; N, 19.06. Found: C, 71.98; H, 4.70; N, 19.10.

1,5-Diphenyl-3-(3-methylpyrazol-5-one-1-carbonyl)-pyrazole-1H-4-carbonitrile ***16***

To a mixture of the acid hydrazide **2** (3.03 g, 10 mmol) and ethyl acetoacetate (1.3 g, 10 mmol) in ethanol (20 mL), a few drops of piperidine were added. The reaction mixture was refluxed for 8 h. The precipitated solid was filtered off, washed with water, dried, and recrystallized from ethanol to afford compound **16** in 60% yield; m. p.: 180–181°C; IR (KBr) ν_{max}/cm^{-1}: 2237 (CN), 1665, 1651 (2 C=O); MS *m/z* (%): 369 [M^+] (30.5), 272 (100), 244 (12.1), 77 (23.7). Anal. calcd. for $C_{21}H_{15}N_5O_2$ (369.37): C, 68.28; H, 4.09; N, 18.96. Found: C, 68.35; H, 4.11; N, 18.95.

General procedure for 3-(4-cyano-1,5-diphenyl-1H-pyrazole-3-yl)-6-aryl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine ***17a, b***

To a solution of 3-(5-mercapto- 4-amino-4*H*-1,2,4-triazol-3-yl)-1,5-diphenyl-1*H*-pyrazole-4-carbonitrile **14** (0.72 g, 2 mmol) and the appropriate phenacyl bromide derivatives **9a**, **c** (2 mmol) in absolute ethanol (25 mL), a few drops of triethylamine were added. The reaction mixture was heated under reflux for 5 h, then cooled, adjusted to pH = 8 by the addition of cold saturated solution of sodium acetate, and left to stand overnight. The precipitated product was filtered off, washed with water, dried, and was finally recrystallized from ethanol. The synthesized compounds together with their physical and spectral data are listed below.

3-(4-Cyano-1,5-diphenyl-1H-pyrazole-3-yl)-6-phenyl-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine ***17a***

Yield: 70%; m. p.: 241–242°C; IR (KBr) ν_{max}/cm^{-1}: 2225 (CN); ^{1}H-NMR ($CDCl_3$) δ: 4.43 (s, 2H, CH_2, thiadiazine) 7.02–7.27 (m, 15H, ArH's); ^{13}C-NMR (DMSO-d_6) δ: 40.004 (6CH_2, thiadiazine), 105.138 (5*C*, thiadiazine), 115.661 (CN), 120.04, 121.070, 121.076, 122.38, 124.99, 125.94, 128.11, 129.10, 130.00, 130.135, 130.139, 132.51, 138.09, 138.25, 143.97, 148.40, 155.05 (17 ArC's); MS *m/z* (%): 459 [M^+] (23.6), 370 (10.7), 270 (7.9), 77 (100). Anal. calcd. for $C_{26}H_{17}N_7S$ (459.53): C, 67.95; H, 3.73; N, 21.33; S, 6.97. Found: C, 68.00; H, 3.71; N, 21.34; S, 6.99.

3-(4-Cyano-1,5-diphenyl-1H-pyrazole-3-yl)-6-(4-methoxyphenyl)-7H-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazine ***17b***

Yield: 75%; m. p.: 220°C; IR (KBr) ν_{max}/cm^{-1}: 2230 (CN); ^{1}H-NMR ($CDCl_3$) δ: 3.86 (s, 3H, OCH_3), 4.71 (s, 2H, CH_2, thiadiazine) 7.15–7.37 (m, 14H, ArH's); ^{13}C-NMR (DMSO-d_6) δ: 37.73 (6CH_2 thiadiazine), 55.43 (OCH_3), 106.58 (5*C*, thiadiazine), 113.83 (CN), 120.65, 120.67, 120.68, 125.82, 126.96, 128.09, 128.83, 129.04, 130.15, 130.34, 130.55, 137.57, 138.20, 140.40, 144.14, 150.60, 153.37 (17 ArC's); MS *m/z* (%): 489 [M^+] (30), 370 (26.7), 270 (28.3), 180 (15.9), 135 (73.4), 77 (100). Anal. calcd. for $C_{27}H_{19}N_7SO$ (489.56): C, 66.24; H, 3.91; N, 20.02; S, 6.55. Found: C, 66.25; H, 3.90; N, 19.99; S, 6.60.

*Reaction of 3-(5-(mercapto)-4-amino-4H-1,2,4-triazol-3-yl)-1,5-diphenyl-1H-pyrazole-4-carbonitrile (**14**) with carboxylic acids: General procedure*

A mixture of compound **14** (1.07 g, 3 mmol) and the appropriate carboxylic acid (benzoic or phenylacetic acids; 3 mmol) in phosphorus oxychloride (10 mL) was heated under reflux at 100°C for 2 h. Then, the reaction mixture was cooled, poured gradually with stirring into an ice cold sodium bicarbonate solution. The separated product was filtered off, washed with water, dried, and finally recrystallized from ethanol. The compounds prepared by this method are listed below.

6-Phenyl-3-(4-cyano-1,5-diphenyl-1H-pyrazole-3-yl)-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazole ***19a***

Yield: 84%; m.p.: 235°C; IR (KBr) ν_{max}/cm^{-1}: 2230 (CN); ^{1}H-NMR (DMSO-d_6) δ: 7.11–7.59 (m, 15H, ArH's); MS *m/z* (%): 445 [M^+] (14.1), 372 (74.2), 271 (21.3), 180 (14.1), 77 (100). Anal. calcd. for $C_{25}H_{15}N_7S$ (445.51): C, 67.40; H, 3.39; N, 22.08; S, 7.20. Found: C, 67.42; H, 3.35; N, 22.01; S, 7.19.

6-Benzyl-3-(4-cyano-1,5-diphenyl-1H-pyrazole-3-yl)-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazole ***19b***

Yield: 68%; m.p.: 290°C; IR (KBr) ν_{max}/cm^{-1}: 2237 (CN); ^{1}H-NMR (DMSO-d_6) δ: 3.96 (s, 2H, CH_2), 7.04–7.77 (m, 15H, ArH's); ^{13}C-NMR (DMSO-d_6) δ: 41.33, 112.14 (pyrazole C4), 115.95 (CN), 120.50, 121.01, 124.76, 127.99, 128.00, 128.06, 128.76, 128.90, 129.02, 133.55, 134.07, 134.59, 144.23, 145.20, 149.07, 149.30 (ArC's), 160.66 (thiadiazole-C5); MS *m/z* (%): 459 [M^+] (8.5), 401 (22.4), 271 (32.8), 180 (15.3), 141 (10.7), 77 (100). Anal. calcd. for $C_{26}H_{17}N_7S$ (459.53): C, 67.95; H, 3.73; N, 21.33; S, 6.98. Found: C, 67.90; H, 3.80; N, 21.30; S, 7.00.

Pharmacology

Antagonism of uterus-weight increase due to estrogen treatment (anti-estrogenic activity)

Animals: Immature female Sprague-Dawley rats weighing about 55 g were obtained from Animal House Laboratory, Nile Company, Cairo, Egypt and acclimatized for one week in the animal facility that has a 12 h light/dark cycle with the temperature controlled at 21–23°C. Normal rat chow and water were made available. The animals were housed individually in stainless steel cages in temperature-controlled and humidity-monitored quarters. Test animals were provided with a continuous access to tap water

Procedure: Groups of 12 animals were daily injected subcutaneously for seven days a week with estradiol (0.03–0.05 μg per animal) and various doses (0.0 to 0.06 μg per animal) of the test compounds and letrozole or estradiol alone as reference standard.

The test compound was orally administered in a 0.5% solution of carboxymethylcellulose. On the 8th day, the animals were sacrificed and the uterine weights were determined.

Evaluation: Mean values of each group are calculated and expressed as percent reduction of uterine weight compared to controls treated with estradiol alone.

Evaluation of acute toxicity following a single-dose administration

Animals: Four hundreds adult mice of both sexes weighing 25 ± 3 g were obtained from Animal House Laboratory, Nile Company, Cairo, Egypt and acclimatized for one week in the animal facility that has a 12 h light/dark cycle with the temperature controlled at 21–23°C. Normal rat chow and water were made available.

Procedure: LD_{50} was measured on 30 mice. Animals were fasted for 12 h prior to dosing. Rats were divided into six groups

with five animals in each group. Treated rats were dosed by oral gavage, using a curved, balltipped stainless steel feeding needle, with aqueous suspensions of very fine powder of the test compounds. Animals in each group were given doses of 100, 160, 256, 409, 655, and 1050 mg/kg b.w. After 24 h the results were recorded. The controls received tap water by gavage in the same volume.

Observation: All animals were monitored continuously for 10 h after dosing for signs of toxicity. For the remainder of the 14 days study period, animals were monitored for mortality. At the end of the study, the number of dead animals was expressed in percentage and the LD_{50} value was calculated according to the Weill method (1952; [54]).

Statistical analysis

The data were evaluated for homogeneity of variances and normality by Bartlett's test. Where Bartlett's test indicated homogeneous variances, treated and control groups were compared using a one-way analysis of variance (ANOVA), followed by comparison of the treated groups with the control groups by Dunnett's t-test for multiple comparisons [55], where variances were considered significantly different by Bartlett's test.

In-vitro antitumor screening

Twenty compounds were supplied to the National Cancer Institute, Bethesda, Maryland, USA, for *in-vitro* disease-oriented primary antitumor screening. Fourteen cell lines of breast and ovarian tumor cell lines were utilized. The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96-well microtiter plates in 100 mL at plating densities ranging from 5000 to 40 000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition. Experimental drugs were solubilized in DMSO at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 mg/mL gentamicin. Additional four 10-fold or $^1/_2$log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 mL of these different drug dilutions were added to the appropriate microtiter wells already containing 100 mL of medium, resulting in the required final drug concentrations. Following drug addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 mL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (100 mL) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 mL of 80% TCA (final concentration, 16% TCA). The parameter used here is GI_{50} which is the $\log_{10}$ concentration at which PG is + 50, was calculated for each cell line [56–58].

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