

Synthesis of Novel 1,3,4-Trisubstituted Pyrazole Derivatives and Their Evaluation as Antitumor and Antiangiogenic Agents

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Several 1,3,4-trisubstituted pyrazole derivatives were synthesized and screened for their cytotoxic effect in a primary 3 tumor cell line test at 10^{-4} M drug concentration. Compounds 19 and 20 reduced the growth of one or more of these cell lines to less than 32% and escalated up to evaluation in the full panel of 60 human tumor cell lines at a minimum of 5 concentrations at 10 fold dilutions. Compound *N'*-(1-{1-[4-nitrophenyl]-3-phenyl-1*H*-pyrazol-4-yl}methylene)-2-chlorobenzohydrazide 19 proved to be the most active of these derivatives with full panel median growth inhibition (GI_{50}), total growth concentration (TGI) and median lethal concentration (LC_{50}) mean graph mid-point (MG-MID) of 3.79, 12.5 and 51.5 μ M, respectively. In addition, compounds 19, 39, 40, 41, 43, 45, 47 were tested for their antiangiogenic properties by testing their ability to inhibit human umbilical vein endothelial cells (HUVECs) proliferation, cord formation and migration in response to chemoattractant. 3-Acetyl-2-(1-(4-nitrophenyl)-3-phenylpyrazol-4-yl)-5-(4-pyridyl)-1,3,4-oxadiazoline 39 showed significant antiangiogenic profile at non-cytotoxic doses, with HUVEC proliferation inhibition IC_{50} of 7.60 μ M, chemotaxis IC_{50} of 0.86 μ M and was superior to the reference celecoxib 2 in both tests. Furthermore, in contrary to the references TNP-470 and celecoxib, all the tested compounds interfered with the migratory function of HUVECs in response to vascular endothelium growth factor (VEGF) rather than the endothelial cells proliferation.

Key words 1,3,4-trisubstituted pyrazoles; anticancer; antiangiogenic; endothelial cell

Programmed cell death (apoptosis) proved that rapidly growing tumors are not always tumors exhibiting high levels of cell proliferation but often low levels of cell death as compared to the normal cell population from which these tumor cells issue. Thus, most of the agents used today to combat cancer target tumor cells death rather than their proliferation. However, such agents are mostly highly toxic and tumor cells develop resistance rapidly to them. This limits their clinical use to a relatively low number of administrations per patient. Also, several of these compounds must be combined into a polychemotherapeutic regimen in order to have any effect against cancer.^{1–3)}

A hallmark of malignancy that is as important as cell kinetics, is the development of neovasculatures, through the process known as angiogenesis, to support the growth of a tumor mass and the formation of metastases.⁴⁾ This neovasculature initiation, mediation and propagation requires endothelial cells, the differentiation and functionalities of which are under the control of several soluble factors. Prominent among them is the vascular endothelial growth factor (VEGF), other growth factors and cytokines.^{5,6)}

Antiangiogenic therapy, which targets activated endothelial cells, presents several advantages over therapy directed against tumor cells: it targets genetically stable, normal endothelial cells that should not develop resistance, the targeting of vascular endothelial cells should be useful for many types of cancer. Antiangiogenic therapy targets should be easily accessible by systemic administration. Every 10 to 100 new tumor cells require at least one new endothelial cell (one gram of tumor contains approximately 20 million endothelial cells and 100 million to one billion tumor cells). Therefore, when an angiogenesis inhibitors halts the growth of one endothelial cell, the effect on tumor cells may be amplified.^{7,8)}

By 1992, the first antiangiogenic drug for cancer patients, TNP-470 1, which is a synthetic analogue of the antibiotic fumagillin, entered clinical trials. The first studies were re-

stricted to few kinds of tumors, but the FDA now allows the physician to prescribe TNP-470 in clinical trials for a wide variety of cancer humans.^{7,9)}

Chemotherapeutic drugs such as topoisomerase inhibitors, taxanes, vinca alkaloids, and cisplatin have been reported to have antiangiogenic activity. Some have antiangiogenic effects at very low doses, through a selective interaction with the process of angiogenesis, whereas others show antiangiogenic activity only near fully cytotoxic concentrations. Taxol, one of the most effective and successful antineoplastic drugs introduced in clinical use in recent years, has antiangiogenic activity at subcytotoxic concentrations.^{8,10,11)}

Traditional NSAID's such as aspirin, indomethacin and diclofenac were found to reduce tumor growth, metastasis and angiogenesis, mainly, through the inhibition of prostaglandins (PG) synthesis. Prostaglandins were found to be proangiogenic by themselves and to upregulate the expression of VEGF gene transcription in many cancer types.^{12–14)}

Furthermore, the pyrazole derivative celecoxib 2 and SC-236 3, have been found to exhibit potential antimigratory activity, inhibit neovascularization, decrease VEGF production and increase apoptosis of tumor cells. These actions are mediated through PGE_2 synthesis inhibition.^{12,15)} Celecoxib 2

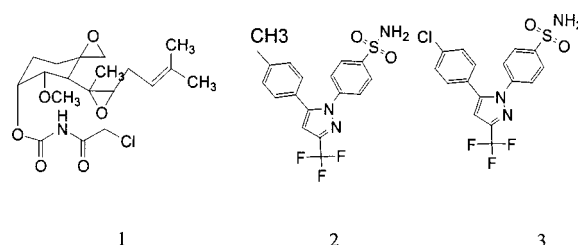


Fig. 1. Chemical Structure TNP-470 (1), Celecoxib (2) and SC-236 (3) as Examples of Pyrazole and Non-pyrazole Containing Antiangiogenic Drugs in Clinical Trials

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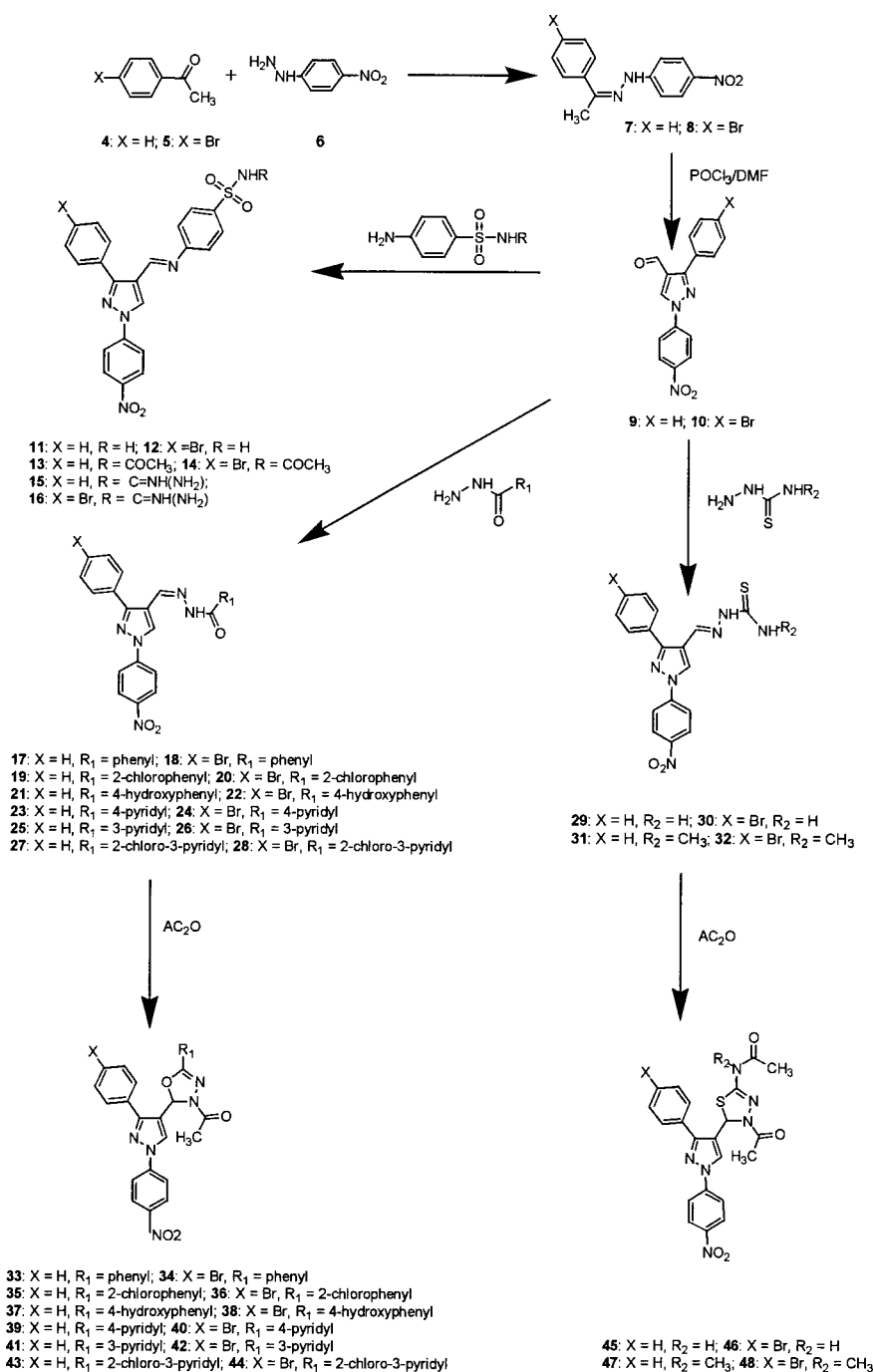


Chart 1

had been approved for the treatment of familial adenomatous polyposis and ongoing clinical trials are currently assessing its potential therapeutic role in both prevention and treatment of a diverse range of human cancers.¹⁶⁾

Encouraged by the above findings and the promising results of celecoxib and SC-236 a number of pyrazole analogues with a 1,3,4-substitution pattern were synthesized and evaluated for their antitumor and antiangiogenic properties.

Results and Discussion

Chemistry The synthesis of the desired 1,3,4-trisubstituted pyrazoles **11**–**48** have been accomplished as described in Chart 1. The key starting materials 1-(4-nitrophenyl)-

3-phenyl-1*H*-pyrazole-4-carbaldehyde¹⁷⁾ **9** and 3-(4-bromophenyl)-1-(4-nitrophenyl)-1*H*-pyrazole-4-carbaldehyde **10**, were prepared from the reaction of 4-nitrophenylhydrazine with acetophenone, 4-bromoacetophenone, followed by Vilsmeier–Haack reaction,^{18,19)} respectively.

Condensation of **9** and **10** with the appropriate sulfonamide, acid hydrazide, *N*⁴-unsubstituted or substituted thiosemicarbazide, gave the corresponding anilide **11**–**16**, hydrazone **17**–**28** and unsubstituted or substituted thiosemicarbazone derivatives, respectively **29**–**32**.

Cyclization of **17**–**28** and **29**–**32** with acetic anhydride yielded the corresponding 1,3,4-oxadiazoline **33**–**44** and 1,3,4-thiadiazoline derivatives **45**–**48**, respectively.

Table 1. Three Cell Panel, Growth Percentages after Incubation with 10^{-4} M Drug Concentration

Compd.	Growth %			Compd.	Growth %		
	MCF7 (breast)	NCI-H460 (lung)	SF268 (CNS)		MCF7 (breast)	NCI-H460 (lung)	SF268 (CNS)
11	104	99	114	28	86	98	113
12	44	86	103	33	103	99	115
13	88	85	108	34	98	99	117
14	68	90	106	35	79	95	104
15	80	92	95	36	98	92	121
16	61	87	67	37	76	96	105
17	106	97	117	38	88	96	108
18	109	98	118	39	107	101	109
19	1	3	12	40	89	100	110
20	20	28	43	41	75	96	108
21	104	100	121	42	80	97	124
22	63	99	112	43	106	99	118
23	104	97	124	44	87	97	116
24	109	99	115	45	106	99	118
25	96	99	104	46	87	99	120
26	98	101	87	47	108	99	120
27	103	100	115	48	84	99	118

Antitumor Activity Thirty-four of the prepared compounds (Table 1) were evaluated for their cytotoxicity in a primary 3-cell line test at 10^{-4} M drug concentration. Cell lines adopted in this prescreen were MCF7 (Breast), NCI-H460 (Lung), and SF-268 (CNS) due to their being good predictors of clinically useful drugs. In this protocol, each cell line is inoculated and preincubated on a microtiter plate. Test agents are then added at a single concentration (10^{-4} M) and the culture incubated for 48 h. Results for each test agent are reported as the percent of growth of the treated cells when compared to the untreated control cells. Compounds which reduce the growth of any one of the cell lines to approximately 32% or less are considered active and passed on for evaluation in the full panel of 60 cell lines over a 5-log dose range.^{20–22)}

It is interesting to point out that compounds **19** and **20** bearing the 2-chlorobenzohydrazide methylene substituent on position 4 of the pyrazole ring were the only active in this test. This emphasizes the role of the chlorine substituent upon activity as shown by the inactivity of the benzohydrazide derivatives **17**, **18**. Further, cyclization of **19** and **20** by acetic anhydride to the corresponding 1,3,4-oxadiazoline derivative **35**, **36** leads to loss of activity in this screen test. The introduction of a bromine to the phenyl on position 3 of the pyrazole ring (**20** vs. **19**) relatively decreased the antitumor activity but still within the acceptable range, justifying the escalation of both compounds up to the next 60 cell lines screening step. For compounds **19** and **20**, the MCF7 (breast) cell line was the most sensitive.

In the second test, both **19** and **20** were evaluated in the NCI *in vitro* disease oriented antitumor screen which determines a test agent's effect on growth against a panel of approximately 60 human tumor cell lines organized into subpanels representing leukemia, melanoma, and cancers of the lung, colon, kidney, ovary, breast, prostate, and central nervous system. The activity of each compound is expressed as the GI_{50} , TGI, and LC_{50} values which represent the molar drug concentrations required to cause half growth inhibition, total growth inhibition or a net 50% loss of initial cells at the end of the incubation period, respectively.^{20–22)}

Both compounds (**19**, **20**) showed appreciable anticancer activity with GI_{50} , TGI, and LC_{50} $<100 \mu\text{M}$ (Tables 2–4). Compound **19** exhibited recognized activity against individual cell lines *e.g.* GI_{50} values of $0.03 \mu\text{M}$ against colon COLO 205, $0.11 \mu\text{M}$ against CNS SNB-19, $0.24 \mu\text{M}$ against ovarian SK-OV-3, $0.026 \mu\text{M}$ against breast T-47D; TGI values of $0.17 \mu\text{M}$ against colon COLO 205, $0.18 \mu\text{M}$ against breast T-47D. The ratio obtained by dividing the compound's full panel MG-MID (μM) by its individual subpanel MG-MID (μM) is considered as an indicator of the compound's selectivity, ratios between 3 and 6 refer to moderate selectivity, while ratios >6 indicate high selectivity towards the corresponding subpanel.^{20–22)}

All the active compounds in this test proved to be non-selective with broad spectrum antitumor activity against the nine tumor subpanels tested with ratios of 0.45–1.46 for GI_{50} , 0.29–1.47 for TGI, and 0.42–1.23 for LC_{50} , respectively (Tables 2–4). Cells of the colon cancer subpanel were the most sensitive ones to compounds **19** and **20** both at the GI_{50} and TGI levels (Tables 2, 3).

Also, we adopted the NCI's computerized, pattern-recognition program COMPARE to analyze the degree of similarity of mean-graph profile produced by compound **19** at the GI_{50} , TGI and LC_{50} levels with that of more than 170 standard compounds to which a putative mechanism of action has been assigned. Compounds high in this ranking may possess a mechanism of action similar to that of the tested compound. Compound **19** showed no good correlation with any of the standard compounds with Pearson Correlation Coefficient <0.6 , indicating a distinct profile from typical chemotherapeutic agents.^{23,24)}

Antiangiogenesis Activity Selective inhibitors of endothelial cells rather than tumor cells are considered as good drug candidates for diseases associated with antiangiogenesis. Crucial endothelial cells activities relevant to angiogenesis, include proliferation, migration, secretion, alignment and formation of capillary like structures. Compounds **19**, **39**, **40**, **41**, **43**, **45** and **47** were selected for the antiangiogenesis testing.

In the human umbilical vein endothelial cell (HUVEC)

Table 2. Median Growth Inhibitory Concentration (GI_{50} , μM) of *in Vitro* Subpanel Tumor Cell Lines

Compd.	Subpanel tumor cell lines ^{a)}									MG-MID ^{b)}
	I	II	III	IV	V	VI	VII	VIII	IX	
19	5.13	8.36	2.60	5.0	8.35	3.10	8.10	5.06	3.75	3.79
20	2.85	6.44	2.80	5.21	5.62	4.68	3.75	5.09	4.48	3.92

a) I, leukemia; II, non-small cell lung cancer; III, colon cancer; IV, CNS cancer; V, melanoma; VI, ovarian cancer; VII, renal cancer; VIII, prostate cancer; IX, breast cancer.
b) GI_{50} (μM) full panel mean-graph mid point.

Table 3. Total Growth Inhibition Concentration (TGI, μM) of *in Vitro* Subpanel Tumor Cell Lines

Compd.	Subpanel tumor cell lines ^{a)}									MG-MID ^{b)}
	I	II	III	IV	V	VI	VII	VIII	IX	
19	25.94	22.13	8.52	18.60	22.58	24.90	20.52	18.40	12.07	12.50
20	11.64	27.16	9.08	17.77	15.18	26.70	13.20	43.50	15.04	12.90

a) For subpanel tumor cell lines, see footnote a) of Table 2. b) TGI (μM) full panel mean-graph mid point.

Table 4. Median Lethal Concentration (LC_{50} , μM) of *in Vitro* Subpanel Tumor Cell Lines

Compd.	Subpanel tumor cell lines ^{a)}									MG-MID ^{b)}
	I	II	III	IV	V	VI	VII	VIII	IX	
19	95.26	60.64	41.70	57.00	51.30	55.89	53.95	61.20	57.38	51.50
20	54.56	68.63	43.39	61.58	41.70	50.28	43.43	— ^{c)}	55.70	42.30

a) For subpanel tumor cell lines, see footnote a) of Table 2. b) LC_{50} (μM) full panel mean-graph mid point. c) Subpanel $LC_{50} > 100 \mu M$.

cells proliferation inhibition test, compounds **19**, **39**, **41** and **43** produced an antiproliferative action superior to celecoxib **2** but less than TNP-470 **1** (Table 5). It is interesting to point out that compounds **39**, **41** and **43** were totally inactive in the preliminary antitumor 3-cell line testing, indicating that they can exert antiangiogenic features at non-cytotoxic concentrations. Structurally, compound **39** with the 4-pyridyl function was nearly 2 times as active as its isostere with the 3-pyridyl one **41**, highlighting the impact of the pyridyl N position upon activity. However, the introduction of a chlorine atom to the carbon between the 3-pyridyl N and the site of attachment to the oxadiazoline rings **43** leads to an improvement of activity. Also, compound **19** which was active in the 60 cell lines antitumor testing was still able to inhibit HUVEC's proliferation at IC_{50} comparable to its full panel GI_{50} (MG-MID).

Also, the selected compounds were tested for their ability to interfere with another endothelial cells function crucial to angiogenesis, the alignment of endothelial cells in a capillary-like structure. Endothelial cells were plated on a 3 dimensional layer of Matrigel where they aligned, forming cords, which were evident a few hours after plating. This test mimics the final events during angiogenesis, when endothelial cells organized in a three-dimensional network of capillaries. Compounds **40** and **41** exhibited cord formation inhibitory properties better than celecoxib but lower than TNP-470. In this test, compound **41** was significantly more potent than its non-brominated analogue **40** (Table 5).

The chemotaxis inhibition test mainly measures the ability to inhibit HUVEC cells migration in response to the specific chemoattractant VEGF. All the tested compounds showed significant activity with IC_{50} 's within the range of 0.86—

Table 5. *In Vitro* Antiangiogenesis Activities of Some Selected Compounds upon HUVEC Cells

Compd.	IC_{50} (μM)		
	Proliferation inhibition	Cord formation inhibition	Chemotaxis
19	8.50	>25	6.40
39	7.60	>25	0.86
40	>5	11.50	4.10
41	12.20	>25	9.20
43	9.60	>25	1.10
45	>12.50	>10	4.10
47	>5	12.30	3.30
Celecoxib	21.00	43.00	>100
TNP-470	0.003	1.00	0.50

9.16 μM , and were superior to the reference celecoxib **2** in this regard. Compound **39** was the most active in this test and its activity was nearly comparable to TNP-470 (Table 5). Again, compound **39** was more active than its isosteric **41**, confirming the impact of the pyridyl N position upon the antiangiogenic activities of this class of compounds. For a second time, the introduction of a chlorine atom to the carbon between the 3-pyridyl N and the site of attachment to the oxadiazoline ring **43** enhanced the antiangiogenic profile.

Most important is the pattern of activity, thus for celecoxib and TNP-470, the antiangiogenic activity was due to its effect on endothelial cells proliferation rather than motility and cord formation. Meanwhile, for compounds **19**, **39**, **40**, **41**, **43**, **45**, **47** the antiangiogenic activity was apparently attributed to cell motility inhibition rather than proliferation, and

that cell motility was inhibited in conditions in which cell proliferation was not affected like low concentration and short exposure time (72 h vs. 5 h). This indicates that the antiangiogenic action of the prepared compounds may be mediated through a novel and different mechanisms of action from that reported to previously known ones.

Conclusion

The reported 1,3,4-trisubstituted pyrazole derivatives represent a novel class of compounds which are non-cytotoxic but of apparent true antiangiogenic profile, mainly through inhibiting the motility of endothelial cells rather than its proliferation. The NCI authority decided to ask the supplier to perform preliminary bioavailability and pharmacokinetic studies to demonstrate that these compounds are orally bioavailable. If these data are favorable, these compounds will be escalated up to *in vivo* antiangiogenesis efficacy studies.

Experimental

Chemistry Melting points were determined on Electrothermal melting point apparatus and are uncorrected. Infrared spectra were recorded on Shimadzu-470 infrared spectrophotometer. ¹H-NMR spectra were obtained on Varian XL-300 MHz and Joel 90 MHz spectrometers, chemical shifts (δ) are given in parts per million (PPM) downfield from TMS as an internal standard. Elemental analyses were performed by the Microanalytical Unit, Faculty of Science, Cairo University; and the Microanalytical Laboratory, Faculty of Science, Ain Shams University; the found values were within $\pm 0.4\%$ of the theoretical ones, unless otherwise indicated. Mass spectra were made on Hewlett Packard GC-MS, model 5890, series II. 1-(4-nitrophenyl)-3-phenyl-1H-pyrazole-4-carbaldehyde **9**¹⁷⁾ and *N*[1-(4-bromophenyl)-ethylidene]-*N'*-(4-nitrophenyl)hydrazine **8**²⁵⁾ were prepared by reported procedures. Arylacid hydrazides were obtained from commercial sources or prepared by reported procedures.^{26–28)} Other chemical and reagents were purchased from commercial sources and used without further purification.

3-(4-Bromophenyl)-1-(4-nitrophenyl)-1H-pyrazole-4-carbaldehyde (10) To an ice cold dimethylformamide (25 g, 30 ml, 0.39 mol) was added dropwise with stirring POCl₃ (10 g, 6 ml, 0.066 mol) over a period of 30 min, stirring was continued for further 60 min, keeping the reaction temperature at 0 °C. Compound **8** (10 g, 0.03 mol) was then added and the reaction mixture was allowed to attain room temperature and stirred for further 4 h. The mixture was refluxed for 2 h, allowed to cool and poured onto ice-H₂O mixture. The obtained mixture was boiled and the precipitate obtained was filtered, dried and crystallized. Yield 70%; mp 228–229 °C; methanol; IR (KBr, cm⁻¹): 1700 (C=O); ¹H-NMR (DMSO-*d*₆): 7.20–8.25 (m, 9H, aromatic+pyrazole C-5 H), 9.20 (s, 1H, CHO); *Anal.* (C₁₆H₁₀BrN₃O₃) C, H and N.

General Procedure for Preparation of Compounds (11–32) A mixture of the appropriate aldehyde (**9**, **10**) (6 mmol) and an equimolar amount of the appropriate sulfonamide (**11–16**), acid hydrazide (**17–28**), thiosemicarbazide or *N*⁴-methylthiosemicarbazide (**29–32**) in ethanol (40 ml) and few drops of acetic acid was refluxed for 4 h, concentrated, cooled and poured onto cold H₂O. The precipitated product was filtered, dried and crystallized

4-({1-[1-(4-Nitrophenyl)-3-phenyl-1H-pyrazol-4-yl]methylene}amino)benzenesulfonamide (**11**): Yield 60%; mp 123–124 °C; DMF-H₂O; IR (KBr, cm⁻¹): 3400, 3350 (NH₂), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 4.00–4.4 (br s, 2H, NH₂), 6.95–8.77 (m, 14H, aromatic+pyrazole C-5 H), 9.40 (s, 1H, CH=N); *Anal.* (C₂₂H₁₇N₅O₄S) C, H and N.

4-({1-[3-(4-Bromophenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-yl]methylene}amino)benzenesulfonamide (**12**): Yield 68%; mp 120–121 °C; DMF-H₂O; IR (KBr, cm⁻¹): 3450, 3360 (NH₂), 1610 (C=N); ¹H-NMR (DMSO-*d*₆): 5.70 (br s, 2H, NH₂), 7.20–8.70 (m, 13H, aromatic+pyrazole C-5 H), 9.35 (s, 1H, CH=N); *Anal.* (C₂₂H₁₆BrN₅O₄S) C, H, N, Br and S.

N-Acetyl-4-({1-(4-nitrophenyl)-3-phenyl-1H-pyrazol-4-ylmethylene}amino)benzenesulfonamide (**13**): Yield 55%; mp 119–120 °C; DMF-H₂O; IR (KBr, cm⁻¹): 3250 (NH), 1700 (C=O), 1610 (C=N); ¹H-NMR (DMSO-*d*₆): 2.10 (s, 3H, CH₃), 6.90–8.35 (m, 14H, aromatic+pyrazole C-5 H), 9.40 (s, 1H, CH=N), 9.70 (br s, 1H, NH); *Anal.* (C₂₄H₁₉N₅O₅S) C, H and N.

N-Acetyl-4-({3-(4-bromophenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-ylmeth-

ylene}amino)benzenesulfonamide (**14**): Yield 46%; mp 243–245 °C; DMF-H₂O; IR (KBr, cm⁻¹): 3200 (NH), 1680 (C=O), 1610 (C=N); ¹H-NMR (DMSO-*d*₆): 2.20 (s, 3H, CH₃), 6.90–8.35 (m, 13H, aromatic+pyrazole C-5 H), 9.40 (s, 1H, CH=N), 9.80 (br s, 1H, NH); *Anal.* (C₂₄H₁₈BrN₅O₅S) C, H, N, Br and S.

N-Amidino-4-({1-(4-nitrophenyl)-3-phenyl-1H-pyrazol-4-ylmethylene}amino)benzenesulfonamide (**15**): Yield 65%; mp 170–171 °C; DMF-H₂O; IR (KBr, cm⁻¹): 3450, 3350 (NH₂), 3250 (NH), 1610 (C=N); ¹H-NMR (DMSO-*d*₆): 6.40–6.75 (cluster, 4H, NH's and NH₂), 6.90–8.35 (m, 14H, aromatic+pyrazole C-5 H), 9.35 (s, 1H, CH=N); *Anal.* (C₂₃H₁₉N₇O₄S) C, H and N.

N-Amidino-4-({3-(4-bromophenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-ylmethylene}amino)benzenesulfonamide (**16**): Yield 59%; mp 288–289 °C; DMF; IR (KBr, cm⁻¹): 3450, 3350 (NH₂), 3200 (NH), 1610 (C=N); ¹H-NMR (DMSO-*d*₆): 6.20–6.80 (cluster, 4H, NH's and NH₂), 7.20–8.40 (m, 13H, aromatic+pyrazole C-5 H), 9.30 (s, 1H, CH=N); *Anal.* (C₂₃H₁₈BrN₇O₄S) C, H, N, Br and S.

N'-(1-{1-[4-Nitrophenyl]-3-phenyl-1H-pyrazol-4-yl}methylene)benzohydrazide (**17**): Yield 55%; mp 290–291 °C; alcohol; IR (KBr, cm⁻¹): 3250 (NH), 1670 (C=O), 1610 (C=N); ¹H-NMR (DMSO-*d*₆): 7.20–8.50 (m, 15H, aromatic+pyrazole C-5 H), 9.29 (s, 1H, CH=N), 11.28 (br s, 1H, NH); *Anal.* (C₂₃H₁₇N₃O₃) C, H and N.

N'-(1-{3-(4-Bromophenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-yl}methylene)benzohydrazide (**18**): Yield 73%; mp 344–346 °C; DMF; IR (KBr, cm⁻¹): 3250 (NH), 1670 (C=O), 1610 (C=N); ¹H-NMR (DMSO-*d*₆): 7.20–8.60 (m, 14H, aromatic+pyrazole C-5 H), 9.40 (s, 1H, CH=N), 11.40 (br s, 1H, NH); *Anal.* (C₂₃H₁₆BrN₃O₃) C, H and N.

N'-(1-{1-[4-Nitrophenyl]-3-phenyl-1H-pyrazol-4-yl}methylene)-2-chlorobenzohydrazide (**19**): Yield 70%; mp 200–201 °C; alcohol; IR (KBr, cm⁻¹): 3200 (NH), 1660 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 7.28–8.60 (m, 14H, aromatic+pyrazole C-5 H), 9.24 (s, 1H, CH=N), 11.80 (br s, 1H, NH); *Anal.* (C₂₃H₁₆ClN₃O₃) C, H and N.

N'-(1-{3-(4-Bromophenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-yl}methylene)-2-chlorobenzohydrazide (**20**): Yield 70%; mp 256–257 °C; alcohol; IR (KBr, cm⁻¹): 3200 (NH), 1660 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 7.28–8.40 (m, 13H, aromatic+pyrazole C-5 H), 9.45 (s, 1H, CH=N), 11.85 (br s, 1H, NH); *Anal.* (C₂₃H₁₅BrClN₃O₃) C, H, N and total halide.

N'-(1-{1-[4-Nitrophenyl]-3-phenyl-1H-pyrazol-4-yl}methylene)-4-hydroxybenzohydrazide (**21**): Yield 65%; mp 267–268 °C; DMF-H₂O; IR (KBr, cm⁻¹): 3500 (OH), 3300 (NH), 1670 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 6.20 (br s, 1H, OH), 7.20–8.55 (m, 14H, aromatic+pyrazole C-5 H), 9.20 (s, 1H, CH=N), 11.20 (br s, 1H, NH); *Anal.* (C₂₃H₁₇N₅O₄) H and N; C: calcd. 64.63, found 63.90.

N'-(1-{3-(4-Bromophenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-yl}methylene)-4-hydroxybenzohydrazide (**22**): Yield 62%; mp 184–186 °C; DMF; IR (KBr, cm⁻¹): 3500 (OH), 3300 (NH), 1660 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 6.40 (br s, 1H, OH), 7.35–8.65 (m, 13H, aromatic+pyrazole C-5 H), 9.17 (s, 1H, CH=N), 11.40 (br s, 1H, NH); *Anal.* (C₂₃H₁₆BrN₅O₄) C, H and N.

N'-(1-[1-(4-Nitrophenyl)-3-phenyl-1H-pyrazol-4-yl]methylene)isonicotinohydrazide (**23**): Yield 75%; mp 269–270 °C; DMF-H₂O; IR (KBr, cm⁻¹): 3250 (NH), 1680 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 7.50–8.85 (m, 14H, aromatic+pyrazole C-5 H), 9.30 (s, 1H, CH=N), 11.30 (br s, 1H, NH); *Anal.* (C₂₂H₁₆N₆O₃) C, H and N.

N'-(1-[3-(4-Bromophenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-yl]methylene)isonicotinohydrazide (**24**): Yield 75%; mp 237–238 °C; DMF; IR (KBr, cm⁻¹): 3250 (NH), 1660 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 7.50–8.85 (m, 13H, aromatic+pyrazole C-5 H), 9.25 (s, 1H, CH=N), 11.20 (br s, 1H, NH); *Anal.* (C₂₂H₁₅BrN₆O₃·H₂O) C, H and N.

N'-(1-[1-(4-Nitrophenyl)-3-phenyl-1H-pyrazol-4-yl]methylene)nicotinohydrazide (**25**): Yield 75%; mp 252–254 °C; alcohol; IR (KBr, cm⁻¹): 3200 (NH), 1690 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 7.36–9.30 (m, 15H, aromatic+pyrazole C-5 H+CH=N), 11.80 (br s, 1H, NH); *Anal.* (C₂₂H₁₆N₆O₃) C, H and N.

N'-(1-[3-(4-Bromophenyl)-1-(4-nitrophenyl)-1H-pyrazol-4-yl]methylene)nicotinohydrazide (**26**): Yield 69%; mp 321–322 °C; DMF; IR (KBr, cm⁻¹): 3200 (NH), 1690 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 7.70–9.30 (m, 14H, aromatic+pyrazole C-5 H+CH=N), 11.80 (br s, 1H, NH); *Anal.* (C₂₂H₁₅BrN₆O₃) C and N; H: calcd. 3.08, found 3.80.

N'-(1-[1-(4-Nitrophenyl)-3-phenyl-1H-pyrazol-4-yl]methylene)-2-chloronicotinohydrazide (**27**): Yield 75%; mp 320–321 °C; DMF-H₂O; IR (KBr, cm⁻¹): 3200 (NH), 1690 (C=O), 1600 (C=N); ¹H-NMR (DMSO-*d*₆): 7.20–8.75 (m, 13H, aromatic+pyrazole C-5 H), 9.15 (s, 1H, CH=N), 12.60 (br s, 1H, NH); *Anal.* (C₂₂H₁₅ClN₆O₃) C, H and N.

N'-{1-[3-(4-bromophenyl)-1-(4-nitrophenyl)-1*H*-pyrazol-4-yl]methyl-ene}-2-chloronicotinohydrazide (**28**): Yield 80%; mp 347—348 °C; DMF; IR (KBr, cm^{-1}): 3300 (NH), 1670 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 7.20—8.80 (m, 12H, aromatic+pyrazole C-5 H), 9.15 (s, 1H, CH=N), 11.80 (br s, 1H, NH); *Anal.* ($\text{C}_{22}\text{H}_{14}\text{BrClN}_6\text{O}_3$) C, H, N and total halide.

1-(4-Nitrophenyl)-3-phenyl-1*H*-pyrazole-4-carbaldehyde Thiosemicarbazone (**29**): Yield 60%; mp 232—233 °C; alcohol; IR (KBr, cm^{-1}): 3450, 3400 (NH₂), 3250 (NH), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 6.90 (s, 1H, pyrazole C-5 H), 7.40—8.70 (m, 11H, aromatic+NH₂), 9.36 (s, 1H, CH=N), 11.40 (br s, 1H, NH); *Anal.* ($\text{C}_{17}\text{H}_{14}\text{N}_6\text{O}_2\text{S}$) C, H and N.

3-(4-Bromophenyl)-1-(4-nitrophenyl)-1*H*-pyrazole-4-carbaldehyde Thiosemicarbazone (**30**): Yield 92%; mp 288—289 °C; DMF; IR (KBr, cm^{-1}): 3500, 3400 (NH₂), 3200 (NH), 1610 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 7.00 (s, 1H, pyrazole C-5 H), 7.40—8.70 (m, 10H, aromatic+NH₂), 9.36 (s, 1H, CH=N), 11.40 (br s, 1H, NH); *Anal.* ($\text{C}_{17}\text{H}_{13}\text{BrN}_6\text{O}_2\text{S}$) C, H, N and Br.

1-(4-Nitrophenyl)-3-phenyl-1*H*-pyrazole-4-carbaldehyde *N*-Methylthiosemicarbazone (**31**): Yield 60%; mp 230—231 °C; alcohol; IR (KBr, cm^{-1}): 3300 (NH), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 3.05 (s, 3H, CH₃), 7.00 (s, 1H, pyrazole C-5 H), 7.35—8.48 (m, 9H, aromatic), 9.20 (s, 1H, CH=N), 11.45 (br s, 1H, NH), 11.70 (br s, 1H, NH); *Anal.* ($\text{C}_{18}\text{H}_{16}\text{N}_6\text{O}_2\text{S}$) C, H and N.

3-(4-Bromophenyl)-1-(4-nitrophenyl)-1*H*-pyrazole-4-carbaldehyde *N*-Methylthiosemicarbazone (**32**): Yield 60%; mp 266—267 °C; alcohol; IR (KBr, cm^{-1}): 3300 (NH), 1590 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 3.03 (s, 3H, CH₃), 7.00 (s, 1H, pyrazole C-5 H), 7.35—8.48 (m, 8H, aromatic), 9.24 (s, 1H, CH=N), 11.20 (br s, 1H, NH), 11.45 (br s, 1H, NH); *Anal.* ($\text{C}_{18}\text{H}_{15}\text{BrN}_6\text{O}_2\text{S}$) H and N, C: calcd. 47.07, found 47.80; EI-MS: m/z =458 (M^+) and 460 ($\text{M}^+ + 2$).

General Procedure for Preparation of Compounds (33—48) A mixture of the appropriate hydrazone (**17—28**), thiosemicarbazone (**29—32**) (3 mmol) and 20 ml of acetic anhydride was refluxed for 4 h, then cooled and added onto crushed ice. The precipitated product was filtered, dried and crystallized.

3-Acetyl-2-(1-(4-nitrophenyl)-3-phenylpyrazol-4-yl)-5-phenyl-1,3,4-oxadiazoline (**33**): Yield 65%; mp 280—281 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1670 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.30 (s, 3H, CH₃), 7.10 (s, 1H, oxadiazoline C-2-H), 7.40—8.30 (m, 15H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{25}\text{H}_{19}\text{N}_5\text{O}_4$) C, H and N.

3-Acetyl-2-(3-(4-bromophenyl)-1-(4-nitrophenyl)pyrazol-4-yl)-5-phenyl-1,3,4-oxadiazoline (**34**): Yield 68%; mp 364—366 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1660 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.26 (s, 3H, CH₃), 7.13 (s, 1H, oxadiazoline C-2-H), 7.59—8.60 (m, 14H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{25}\text{H}_{18}\text{BrN}_5\text{O}_4$) C, H, N and Br.

3-Acetyl-5-(2-chlorophenyl)-2-(1-(4-nitrophenyl)-3-phenylpyrazol-4-yl)-1,3,4-oxadiazoline (**35**): Yield 85%; mp 228—229 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1670 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.26 (s, 3H, CH₃), 7.10 (s, 1H, oxadiazoline C-2-H), 7.27—8.33 (m, 14H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{25}\text{H}_{18}\text{ClN}_5\text{O}_4$) C, H and N.

3-Acetyl-2-(3-(4-bromophenyl)-1-(4-nitrophenyl)pyrazol-4-yl)-5-(2-chlorophenyl)-1,3,4-oxadiazoline (**36**): Yield 85%; mp 216—217 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1690 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.28 (s, 3H, CH₃), 7.10 (s, 1H, oxadiazoline C-2-H), 7.60—8.40 (m, 13H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{25}\text{H}_{17}\text{BrClN}_5\text{O}_4$) C, H, N and total halide.

3-Acetyl-5-(4-hydroxyphenyl)-2-(1-(4-nitrophenyl)-3-phenylpyrazol-4-yl)-1,3,4-oxadiazoline (**37**): Yield 90%; mp 199—200 °C; DMF-H₂O; IR (KBr, cm^{-1}): 3500 (OH), 1670 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.28 (s, 3H, CH₃), 3.9 (br s, 1H, OH), 7.20 (s, 1H, oxadiazoline C-2-H), 7.25—8.35 (m, 14H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{25}\text{H}_{19}\text{N}_5\text{O}_5$) C, H and N.

3-Acetyl-2-(3-(4-bromophenyl)-1-(4-nitrophenyl)pyrazol-4-yl)-5-(4-hydroxyphenyl)-1,3,4-oxadiazoline (**38**): Yield 80%; mp 247—248 °C; DMF-H₂O; IR (KBr, cm^{-1}): 3500 (OH), 1680 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.20 (s, 3H, CH₃), 4.10 (br s, 1H, OH), 7.10 (s, 1H, oxadiazoline C-2-H), 7.60—8.40 (m, 13H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{25}\text{H}_{18}\text{BrN}_5\text{O}_5$) C, H, N and Br.

3-Acetyl-2-(1-(4-nitrophenyl)-3-phenylpyrazol-4-yl)-5-(4-pyridyl)-1,3,4-oxadiazoline (**39**): Yield 60%; mp 180—181 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1700 (C=O), 1620 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.20 (s, 3H, CH₃), 7.15 (s, 1H, oxadiazoline C-2-H), 7.25—8.76 (m, 14 H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{24}\text{H}_{18}\text{N}_6\text{O}_4$) C, H and N.

3-Acetyl-2-(3-(4-bromophenyl)-1-(4-nitrophenyl)pyrazol-4-yl)-5-(4-pyridyl)-1,3,4-oxadiazoline (**40**): Yield 80%; mp 261—262 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1700 (C=O), 1620 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.28 (s,

3H, CH₃), 7.35 (s, 1H, oxadiazoline C-2-H), 7.60—8.75 (m, 13 H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{24}\text{H}_{17}\text{BrN}_6\text{O}_4$) C, H and N.

3-Acetyl-2-(1-(4-nitrophenyl)-3-phenylpyrazol-4-yl)-5-(3-pyridyl)-1,3,4-oxadiazoline (**41**): Yield 65%; mp 246—247 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1660 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.28 (s, 3H, CH₃), 7.25 (s, 1H, oxadiazoline C-2-H), 7.25—8.76 (m, 14 H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{24}\text{H}_{18}\text{N}_6\text{O}_4$) C, H and N.

3-Acetyl-2-(3-(4-bromophenyl)-1-(4-nitrophenyl)pyrazol-4-yl)-5-(3-pyridyl)-1,3,4-oxadiazoline (**42**): Yield 90%; mp 305—307 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1690 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.20 (s, 3H, CH₃), 7.15 (s, 1H, oxadiazoline C-2-H), 7.52—8.80 (m, 13 H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{24}\text{H}_{17}\text{BrN}_6\text{O}_4$) C, H, N and Br.

3-Acetyl-5-(2-chloro(3-pyridyl))-2-(1-(4-nitrophenyl)-3-phenylpyrazol-4-yl)-1,3,4-oxadiazoline (**43**): Yield 85%; mp 201—202 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1700 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.25 (s, 3H, CH₃), 7.10 (s, 1H, oxadiazoline C-2-H), 7.20—8.70 (m, 13 H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{24}\text{H}_{17}\text{ClN}_6\text{O}_4$) C, H and N.

3-Acetyl-2-(3-(4-bromophenyl)-1-(4-nitrophenyl)pyrazol-4-yl)-5-(2-chloro(3-pyridyl))-1,3,4-oxadiazoline (**44**): Yield 85%; mp 341—342 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1670 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.18 (s, 3H, CH₃), 7.10 (s, 1H, oxadiazoline C-2-H), 7.40—8.80 (m, 12 H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{24}\text{H}_{16}\text{BrClN}_6\text{O}_4$) C, H, N and total halide.

N-{4-Acetyl-5-[1-(4-nitrophenyl)-3-phenyl-1*H*-pyrazol-4-yl]-4,5-dihydro-[1,3,4]thiadiazol-2-yl}acetamide (**45**): Yield 95%; mp 252—253 °C; DMF-H₂O; IR (KBr, cm^{-1}): 3200 (NH), 1700 (C=O), 1680 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.02 (s, 3H, NHCOCH₃), 2.63 (s, 3H, COCH₃), 7.06 (s, 1H, thiadiazoline C-2 H), 7.49—8.60 (m, 10H, aromatic+pyrazole C-5 H), 9.57 (br s, 1H, NH); *Anal.* ($\text{C}_{21}\text{H}_{18}\text{N}_6\text{O}_4\text{S}$) C, H and N.

N-{4-Acetyl-5-[3-(4-bromophenyl)-1-(4-nitrophenyl)-1*H*-pyrazol-4-yl]-4,5-dihydro-[1,3,4]thiadiazol-2-yl}acetamide (**46**): Yield 91%; mp 331—332 °C; DMF-H₂O; IR (KBr, cm^{-1}): 3300 (NH), 1700 (C=O), 1680 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.10 (s, 3H, NHCOCH₃), 2.20 (s, 3H, COCH₃), 7.06 (s, 1H, thiadiazoline C-2 H), 7.65—8.41 (m, 9H, aromatic+pyrazole C-5 H), 9.39 (br s, 1H, NH); *Anal.* ($\text{C}_{21}\text{H}_{17}\text{BrN}_6\text{O}_4\text{S}$) C, H and N.

N-{4-Acetyl-5-[1-(4-nitrophenyl)-3-phenyl-1*H*-pyrazol-4-yl]-4,5-dihydro-[1,3,4]thiadiazol-2-yl}-*N*-methylacetamide (**47**): Yield 90%; mp 242—244 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1700 (C=O), 1680 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 1.96 (s, 3H, NHCOCH₃), 2.25 (s, 3H, COCH₃), 3.03 (s, 3H, N-CH₃), 7.02 (s, 1H, thiadiazoline C-2 H), 7.44—8.60 (m, 10H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{22}\text{H}_{20}\text{N}_6\text{O}_4\text{S}$) C, H and N.

N-{4-Acetyl-5-[3-(4-bromophenyl)-1-(4-nitrophenyl)-1*H*-pyrazol-4-yl]-4,5-dihydro-[1,3,4]thiadiazol-2-yl}-*N*-methylacetamide (**48**): Yield 87%; mp 207—208 °C; DMF-H₂O; IR (KBr, cm^{-1}): 1700 (C=O), 1680 (C=O), 1600 (C=N); $^1\text{H-NMR}$ (DMSO- d_6): 2.08 (s, 3H, NHCOCH₃), 2.38 (s, 3H, COCH₃), 3.00 (s, 3H, N-CH₃), 7.10 (s, 1H, thiadiazoline C-2 H), 7.44—8.4060 (m, 9H, aromatic+pyrazole C-5 H); *Anal.* ($\text{C}_{22}\text{H}_{19}\text{BrN}_6\text{O}_4\text{S}$) C, H and N; EI-MS: m/z =542 (M^+) and 544 ($\text{M}^+ + 2$).

In Vitro Cytotoxicity Assay^{20–22} 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 μl at plating densities ranging from 5000 to 40000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5 % CO₂, 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 trichloroacetic acid (TCA), to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in dimethyl sulfoxide 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 $\mu\text{g}/\text{ml}$ 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 μl of these different drug dilutions were added to the appropriate microtiter wells already containing 100 μl 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% CO₂, 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 μl 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 μl) 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 is subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. Dose-response parameters were calculated as described, previously.

Three Cell Lines Prescreen This prescreen is the same as above but would test for the presence of toxicity only at 10^{-4} M drug concentration and could eliminate a large proportion of the inactive agents, and preserve "active" agents for the multi-dose 60 cell line testing. Cell lines adopted in this prescreen were MCF7 Breast, NCI-H460 Lung and SF-268 CNS.

Antiangiogenesis Testings.^{29,30} HUVEC Cells Growth Inhibition Assay HUVEC (1.5×10^3) are plated in a 96-well plate in 100 μ l of EBM-2 (Clonetic #CC3162). After 24 h (day 0), the test compound (100 μ l) is added to each well at 2X the desired concentration (5–7 concentration levels) in EBM-2 medium. On day 0, one plate is stained with 0.5% crystal violet in 20% methanol for 10 min, rinsed with water, and air-dried. The remaining plates are incubated for 72 h at 37 °C. After 72 h, plates are stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The stain is eluted with 1:1 solution of ethanol: 0.1 M sodium citrate (including day 0 plate), and absorbance is measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance is subtracted from the 72 h plates and data is plotted as percentage of control proliferation (vehicle treated cells). IC_{50} (drug concentration causing 50% inhibition) is calculated from the plotted data.

Cord Formation Assay Matrigel (60 μ l of 10 mg/ml; Collaborative Lab #35423) is placed in each well of an ice-cold 96-well plate. The plate is allowed to sit at room temperature for 15 min then incubated at 37 °C for 30 min to permit the matrigel to polymerize. In the mean time, HUVEC are prepared in EGM-2 (Clonetic #CC3162) at a concentration of 2×10^5 cells/ml. The test compound is prepared at 2X the desired concentration (5 concentration levels) in the same medium. Cells (500 μ l) and 2X drug (500 μ l) is mixed and 200 μ l of this suspension are placed in duplicate on the polymerized matrigel. After 24 h incubation, triplicate pictures are taken for each concentration using a Bioquant Image Analysis system. Drug effect (IC_{50}) is assessed compared to untreated controls by measuring the length of cords formed and number of junctions.

Cell Migration Assay Migration is assessed using the 48-well Boyden chamber and 8 μ m pore size collagen-coated (10 μ g/ml rat tail collagen; Collaborative Laboratories) polycarbonate filters (Osmonics, Inc.). The bottom chamber wells receive 27–29 μ l of DMEM medium alone (baseline) or medium containing chemoattractant VEGF 10 ng/ml. The top chambers receive 45 μ l of HUVEC cell suspension (1×10^6 cells/ml) prepared in DMEM+1% BSA with or without test compound. After 5 h incubation at 37 °C, the membrane is rinsed in PBS, fixed and stained in Diff-Quick solutions. The filter is placed on a glass slide with the migrated cells facing down and cells on top are removed using a Kimwipe. The testing is performed in 4–6 replicates and five fields are counted from each well. Negative unstimulated control values are subtracted from stimulated control and drug treated values and data is plotted as mean migrated cell $\pm$ S.D. IC_{50} is calculated from the plotted data.

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