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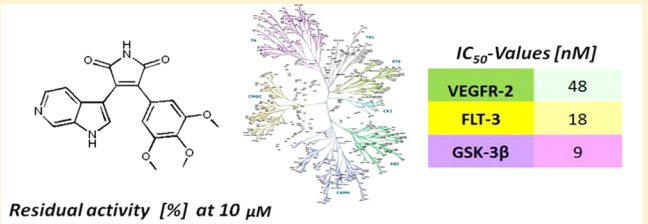
Novel 3-Azaindolyl-4-arylmaleimides Exhibiting Potent Antiangiogenic Efficacy, Protein Kinase Inhibition, and Antiproliferative Activity

Christopher Ganser,[†,∥] Eva Lauermann,[†,∥] Annett Maderer,[‡] Torsten Stauder,[§] Jan-Peter Kramb,[†] Stanislav Plutizki,[†] Thomas Kindler,[§] Markus Moehler,[‡] and Gerd Dannhardt*[,†]

[†]Department of Pharmacy, [‡]Department of Internal Medicine I, and [§]Department of Internal Medicine III, Johannes Gutenberg University of Mainz, Mainz, Germany

ABSTRACT: Tumor growth and metastasis are highly associated with the overexpression of protein kinases (PKs) regulating cell growth, apoptosis resistance, and prolonged cell survival. This study describes novel azaindolyl-maleimides with significant inhibition of PKs, such as VEGFR, FLT-3, and GSK-3β which are related to carcinogenesis. Furthermore, these compounds exhibit high kinase selectivity and potent inhibition of angiogenesis and cell proliferation, offering versatile options in cancer treatment strategies.



Residual activity [%] at 10 μM

Compound	CDK1/ cyclinB	CDK2/ cyclinA	CDK6/ cyclinD3	c-RAF	EGFR	Flt-3	GSK-3β	VEGFR-2
18	80	88	103	117	111	0	2	1
	Met	PDGFR-β	PKC-α	PKC-γ	PKC-ε	PKC-ι	Tie2	
18	83	106	80	83	75	99	116	

INTRODUCTION

Overexpression of protein kinases (PKs), which trigger intracellular signal transduction by phosphorylating tyrosine, threonine, or serine residues in key proteins, is closely related to the process of cancerogenesis. By regulating cell growth, apoptosis resistance, survival, and angiogenesis,[1] PKs such as vascular endothelial growth factor receptor (VEGFR) are of vital relevance in both physiological and pathophysiological angiogenesis. VEGFRs exists in three subtypes VEGFR-1 to VEGFR-3. VEGFR-2 (also known as KDR or FLK-1 in mice) is pivotal for the process of vessel growth. The morphogenetic process of angiogenesis depends on the formation of new blood vessels from endothelial cells of already existing capillary vessels.[2] This physiological process in adolescent organisms is also closely related to a multitude of pathological events in humans including rheumatoid arthritis, psoriasis, ischemia, and malignant diseases.[3] Once tumors have reached a size of about 3 mm^3, their growth is completely dependent on this process.[4] In general, tumors with a high expression of angiogenic factors (e.g., VEGF, basic fibroblast growth factor (bFGF), matrix metalloproteinases, and serine proteases) and low levels of inhibitors of angiogenesis (e.g., thrombospondin and inhibitors of metalloproteinases) have a higher microvessel density and aggressiveness. Also they are locally more advanced and metastasize more frequently than tumors without these angiogenic phenotypes. Because of the limited occurrence of physiological angiogenesis in adults, its inhibition may lead to tumor-specific compound capabilities with a minor toxicity potential.[5] Therefore, angiogenesis inhibitors are considered to be potential therapeutic agents with low toxicity in the treatment of solid tumors such as bronchial carcinoma, mamma carcinoma, prostate carcinoma, glioblastoma, karposi sarcoma, melanoma, and leukemia for which no curative therapies are known so far.

Beside VEGFR-2, more protein kinases such as FMS-like tyrosine kinase 3 (FLT-3)[6] or glycogen synthase kinase 3β (GSK-3β) became attractive drug targets in cancer therapy, e.g., regarding the treatment of acute myeloid leukemia (AML) or colorectal cancer.

Several small molecule inhibitors of protein kinases (imatinib, gefitinib, erlotinib, sorafenib, and sunitinib) showed promising efficacy and acceptable toxicity.[1,7] Despite these first achievements, many tumors are still resistant to therapy and associated with a poor prognosis. Thus, there is an ongoing urgent need for compounds with novel therapeutic profiles amplifying the armamentarium against cancer.

In a previous article[8] we described profile and molecular modeling studies of a highly selective VEGFR-2/3 inhibitor, which illustrate a possible binding mode of indolyl-maleimides into the ATP pocket of VEGFR-2. By implementing electronegative heteroatoms at different positions in the indolyl moiety, lowering the π-electron density, providing a new H-bond acceptor, and increasing $\pi-\pi$-interaction (due to different inhibitor alignment) of the azaindolyl moiety with Phe1045 in the ATP-binding site of VEGFR-2, we aim to enhance the stability of the receptor−inhibitor complex. To get additional SAR data, the aryl moiety of the previously described 7-azaindolyl compound[8] was slightly varied, creating mono- and dimethoxyphenyl derivatives.

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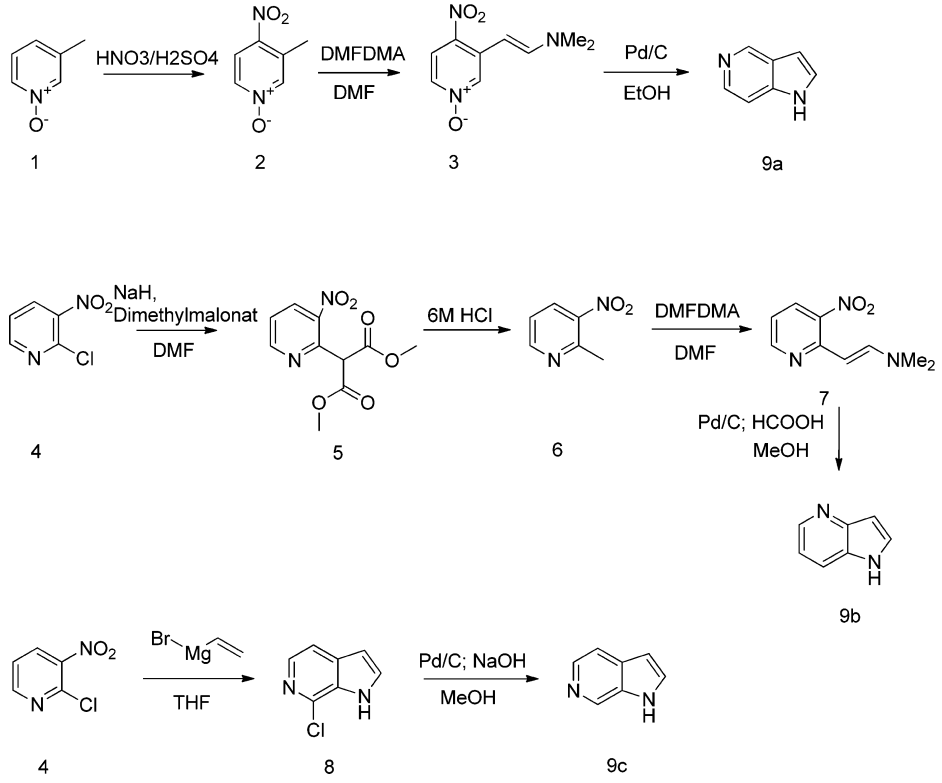
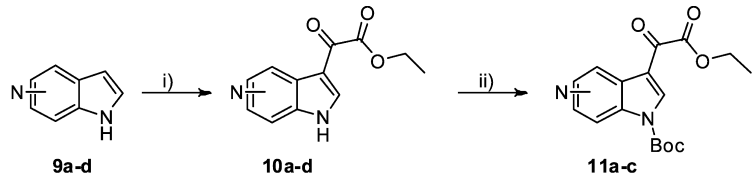
Scheme 1. Synthesis of 4-, 5- and 6-Azaindoles[9−11]



Scheme 2. Acylation and Protection of Azaindole Moieties[a] Using the Methods of Zhang et al.,[9a] Ottoni et al.,[12] and Basel et al.[15]



Entry	Azaindole	Acylation, yield (%)	Protection, yield (%)
1	**9a**	**10a**, 45	**11a**,95
2	**9b**	**10b**, 50	**11b**,86
3	**9c**	**10c**, 42	**11c**, 90
4	**9d***	**10d**, 51	-

[a]Reagents and conditions: (i) (a) azaindole, $AlCl_3$, DCM, 0 °C, 0.5 h; (b) ethyl chlorooxoacetate, rt, 18 h; (c) ice, DCM; (ii) di-*tert*-butyl dicarbonate, DMAP, DCM, rt, 12 h. (∗) **9d**: 7-azaindole (commercially available).

These novel azaindolyl-arylmaleimides show remarkable inhibition of angiogenesis as well as potent inhibitory efficacy against different tyrosine kinases, related to proliferation, survival, cell migration, and angiogenesis in malignant cells. Inhibition of VEGFR-2 and FLT-3 was detected in nanomolar ranges. Further investigations disclosed a likewise inhibitory potency against GSK-3β. In addition, the compounds exhibit potent antiproliferative and proapoptotic properties in different carcinoma cell lines, such as HT-29, Mkn-45, and Molm-14. Because of these results, we focus on selective VEGFR-2, FLT-3, and GSK-3β inhibitors that may be important in the treatment of solid tumors and acute myeloid leukemia (AML), respectively.

RESULTS AND DISCUSSION

Synthesis of 3-(Azaindolyl)-4-arylmaleimides. 4- and 6-Azaindoles (**9b**, **9c**) were obtained by reacting 2-chloro-3-nitropyridine (**4**) with dimethyl malonate, 6 M HCl, *N*,*N*-dimethylformamide dimethyl acetal (DMFDMA), followed by catalytic hydrogenation to yield 4-azaindole (**9b**) and 6-azaindole (**9c**), respectively, using vinylmagnesium bromide and catalytic hydrogenation.[9] In the case of 4-azaindole, procedures by Cash et al.[10] were applied. 5-Azaindole (**9a**) was prepared according to Dormoy et al.[11] by nitration of 3-picoline-1-oxide (**1**) followed by treatment with *N*,*N*-dimethylformamide diethyl acetal (DMFDEA) and reduction with palladium/C, H_2 (Scheme 1). 7-Azaindole (**9d**) is commercially available by Sigma Aldrich. The required

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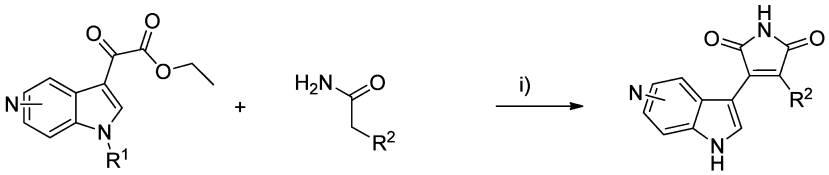
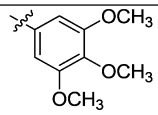
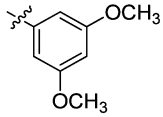
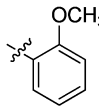
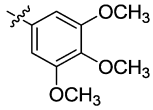
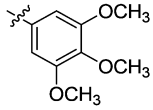
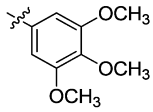
Scheme 3. Synthesis of the Maleimide Moiety[a] According to Faul et al.[13]



10d, 11a-c **12a-c** **13-18**

Entry	Acylated azaindole	R^1	Amide	R^2	Maleimide, yield (%)
1	**10d**	H	**12a**		**13,** 43
2	**10d**	H	**12b**		**14,** 48
3	**10d**	H	**12c**		**15,** 52
4	**11a**	Boc	**12a**		**16,** 36
5	**11b**	Boc	**12a**		**17,** 26
6	**11c**	Boc	**12a**		**18,** 33

[a]Reagents and conditions: (i) (a) potassium *tert*-butoxide (4 equiv), THF, rt, 14 h; (b) NH_4Cl, ethyl acetate.

azaindole glyoxyl esters (**10d**, **11a−c**) proved to be difficult to prepare because of the decreased π-electron density of azaindoles (**9a−d**) in comparison to indole and the therefore minor reactivity toward electrophilic reagents. To acylate at the C-3 position (Scheme 2), we used a modified procedure of Zhang et al.[9a] and Ottoni et al.[12] with excess of aluminum chloride ($AlCl_3$) as Lewis acid in dry dichloromethane (DCM) to increase the electrophilicity. Among the several synthetic methods for preparing maleimides, we modified a method of Faul et al.[13] This procedure results in good yields and appeared to be efficient concerning the tolerance of functional groups. Scheme 3 shows the maleimide formation, carried out in a one-pot reaction with azaindole glyoxyl esters (**10d**, **11a−c**) and arylacetamide derivatives (**12a−c**) in the presence of potassium *tert*-butoxide (^{t}BuOK) and activated molecular sieves in dry THF.[14] Arylacetamides **12a−c** are readily available from the corresponding acetic acids, which are converted to the acid chloride by reacting with phosphorus pentachloride and hydrolyzed with ammonia.

Biological Evaluation. Investigations concerning the inhibition of receptor tyrosine kinases (RTKs) as an innovative strategy in tumor treatment have strongly influenced recent developments of novel drugs in this field.[16]

In previous papers we reported the synthesis and biological evaluation of 3,4-diarylmaleimides as angiogenesis and RTK inhibitors.[14a,8] Motivated by their significant activity, we started to optimize these small molecule compounds with regard to pharmacodynamic and pharmacokinetic demands as drug candidates for the treatment of acute myeloid leukemia and solid tumors. The prepared 3-azaindolyl-4-arylmaleimides (Chart 1) differ from available kinase inhibitors because of their tumor and vascular targeting properties, selective inhibition of relevant kinases (VEGFR-2, FLT-3, GSK-3β), and crucial effects on several cancer cell lines by promoting or inducing apoptosis and/or decreasing cell viability.

In this section we describe the biological evaluation of the synthesized 3-azaindolyl-4-phenylmaleimide derivatives concerning their antiangiogenic activity, their inhibitory efficacy on different protein kinases, and their effect on the viability of vascular endothelial and various cell lines. In addition the combination of maleimide compounds with the topoisomerase I inhibitor irinotecan was investigated.

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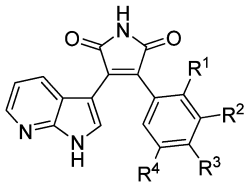
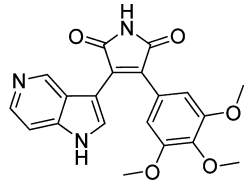
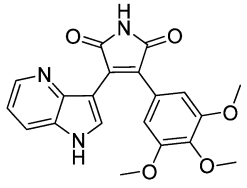
Chart 1. Synthesized 3-azaindolyl-4-arylmaleimide derivatives (13-18) and reference compound 19.[14a]



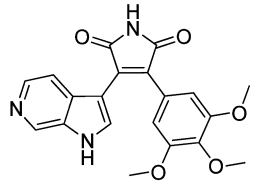
13 R^1 = H, R^2 = R^3 = R^4 = OCH_3
14 R^1 = R^3 = H ,R^2 = R^4 = OCH_3
15 R^1 = OCH_3, R^2 = R^3 = R^4 = H



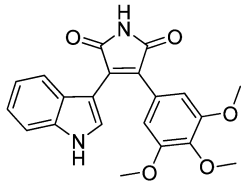
16



17



18



19

The antiangiogenic potency was evaluated using a quantitative in vivo chick embryo assay[17] (Table 1). The azaindolyl compounds at 26 nM inhibit up to 61% the microvessel formation after 24 h of incubation, thus confirming significant antiangiogenic activity.

Table 1. Inhibition of Vessel Growth in the in Vivo Chick Embryo Model by 3-Azaindolyl-4-arylmaleimides (c = 26 nM)

compd	IVG[a] (% inh)
13	61 ± 24
14	25 ± 21
15	30 ± 20
16	40 ± 18
17	10 ± 28
18	32 ± 20
19[b]	82 ± 14

[a]Inhibition of vessel growth versus control in the chick embryo model after incubation for 24 h. Each experiment was performed with 15 samples. For further details see refs 14a and 17. [b]Data from ref 14a.

The compound bearing a 7-azaindole moiety and additional 3,4,5-trimethoxyphenyl substituent **13** is found to be the most potent azaindole (61% inhibition of vessel growth). Related to our previously reported 3-(indol-3-yl)-4-(3,4,5-trimethoxyphenyl)maleimide **19** (82% inhibition of vessel growth),[14a] regioisomere 6- and 5-azaindolyl derivatives as well as monomethoxy- and dimethoxyphenyl derivatives (**18**, **16**, **15**, **14**) revealed moderate inhibition (25−40% inhibition of vessel growth). The 4-azaindole compound **17** showed no significant inhibition in this screening.

To identify the molecular targets, inhibitory effects on VEGFR-2, FLT-3, and GSK-3β were investigated using kinase inhibition assays based on the previous results of Peifer et al.[14a] (Table 2). VEGFRs are key mediators of angiogenesis and play

Table 2. IC_{50} of VEGFR-2, FLT-3, and GSK-3β Inhibition[a]

	IC_{50} (nM)		
compd	VEGFR-2	FLT-3	GSK-3β
13	37 ± 7	132 ± 18	≥10000
16	590 ± 13	≥3000	40 ± 3
17	373 ± 22	≥1500	215 ± 57
18	48 ± 3	18 ± 5	9 ± 1
19	70 ± 2	18 ± 7	71 ± 2

[a]The kinase assays were performed by Millipore using Merck Millipores KinaseProfiler. All compounds have been tested in duplicate.

essential roles by initiating proliferation and migration of endothelial cells, a requirement for tumor growth and metastasis. VEGFR-2 is also strongly involved in the early blood vessel growth in the avian embryo.[18] GSK-3β is known to modulate apoptotic cell signaling,[19] whereas FLT-3 plays a crucial role in cell proliferation and differentiation.[20] The half-maximal inhibitory concentration (IC_{50}) values were determined (Table 2) and discussed in relation to their structural features.

The 6- and 7-azaindole derivatives (**18**, **13**) as well as the indolyl derivative **19** significantly inhibited VEGFR-2 with IC_{50} values in a low nanomolar range. In comparison to **18** and **13**, respectively, IC_{50} values of 4- and 5-azaindolyl derivatives (**17**, **16**) were about 10-fold higher. FLT-3 was most potently inhibited by 6-azaindole derivative **18** (IC_{50}= 18 nm), 7-azaindole derivative **13** (IC_{50}= 132 nm), and the reference compound **19** (indolyl derivative, IC_{50} = 18 nm), whereas **16** and **17** showed only minor inhibition. Compounds **16**, **17**, **18**, and **19** could be identified as potent inhibitors of GSK-3β with IC_{50} values in a low nanomolar range.

The correlation between IC_{50} values of VEGFR-2 inhibition and the antiangiogenic effect in chick embryo assay is

Table 3. Kinase Screening of Selected Compounds 13 and 18[a]

	13	**18**
CDK1/cyclinB	92 ± 1.4	80 ± 0.71
CDK2/cyclinA	94 ± 0.71	88 ± 2.12
CDK6/cyclinD3	99 ± 1.4	103 ± 1.41
c-RAF	111 ± 0.71	117 ± 0.71
EGFR	97 ± 1.4	111 ± 2.12
Flt-3	2 ± 0.00	0 ± 0.00
GSK-3β	50 ± 5.66	2 ± 0.71
VEGFR-2	0 ± 0.00	1 ± 0.00
Met	109 ± 8.49	83 ± 2.83
PDGFR-β	84 ± 1.41	106 ± 8.49
PKC-α	89 ± 0.71	80 ± 3.54
PKC-γ	93 ± 2.1	83 ± 2.12
PKC-ε	77 ± 2.8	75 ± 2.83
PKC-ι	69 ± 0.71	99 ± 2.12
Tie2	77 ± 0.71	116 ± 5.66

[a]Data are presented as residual activity in percent at 10 μM, related to the 100% controls. All compounds have been tested in duplicate.

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Table 4. MTT Assay Using HT-29 Cells, Viability in %[a]

concn (μM)	13	14	16	17	18	19
2.6	64.95 ± 2.16	54.33 ± 4.55	90.76 ± 2.30	114.36 ± 5.2	30.38 ± 1.52	78.50 ± 3.70
7.9	45.55 ± 3.71	49.21 ± 3.18	61.51 ± 2.10	98.31 ± 0.90	25.80 ± 3.52	62.90 ± 1.50
26	38.50 ± 0.99	43.05 ± 1.75	56.55 ± 0.90	92.64 ± 2.80	12.67 ± 2.6	53.90 ± 4.80
52	38.11 ± 5.43	40.50 ± 2.38	47.44 ± 2.10	65.75 ± 1.50	4.22 ± 0.44	42.80 ± 1.60

[a]Samples were incubated for 3 days. Viability of untreated cells is 100%.

Table 5. MTT Assay Using Mkn-45 Cells, Viability in %[a]

concn (μM)	13	14	16	18	19
0.5	113.22 ± 6.16	117.94 ± 4.32	99.07 ± 5.98	99.17 ± 2.22	94.50 ± 0.40
1.0	110.01 ± 4.04	118.13 ± 5.21	100.26 ± 6.39	106.80 ± 5.45	98.60 ± 5.70
5.0	86.84 ± 5.57	60.35 ± 3.97	99.04 ± 5.41	104.94 ± 4.91	93.20 ± 1.00
10.0	58.66 ± 5.21	57.01 ± 0.41	98.61 ± 6.02	107.57 ± 7.78	81.10 ± 6.90
20.0	57.09 ± 4.96	62.12 ± 5.55	89.41 ± 6.78	100.67 ± 5.04	75.90 ± 2.70
50.0	44.68 ± 0.83	59.42 ± 2.91	82.52 ± 1.53	68.58 ± 1.50	65.90 ± 0.90

[a]Samples were incubated for 5 days. Viability of untreated cells is 100%.

remarkable for compounds **13** and **19**. The expected correlation for compound **18** could not be verified, which might be due to its higher inhibitory potency against GSK-3β. **13** (IC_{50}(VEGFR-2) = 37 nM) exhibited 61% inhibition of vessel growth, whereas **18** (IC_{50}(VEGFR-2) = 48 nM) showed only low antiangiogenic effects (32%). In our previous study, we reported an inhibition of 82% for 3-(indol-3-yl)-4-(3,4,5-trimethoxyphenyl)maleimide (**19**) with IC_{50} of 70 nM (VEGFR-2).[14a]

To determine the kinase selectivity profile of two selected compounds, 15 kinases were chosen for screening. The kinase panel consists of members of the CDK family (CDK1/cyclinB, CDK2/cyclinA, CDK6/cyclinD3), based on previous molecular modeling data,[14a] and several isozymes of the PKC family (PKC-α,-γ, -ε, -ι) reported to be affected by staurosporine related diarylmaleimides.[21] Eight kinases bearing cysteine residues in the ATP binding pocket like the addressed FLT-3, GSK-3β, and VEGFR-2 were also included in the panel to verify selectivity (Met, PDGFR, EGFR, c-RAF, Tie2). Residual activity was measured at 10 μM for compounds **13** and **18**, as they both exhibit potent inhibition of VEGFR-2 but differ in inhibiting GSK-3β and angiogenesis assay as mentioned above.

The results of the kinase screening, summarized as residual activity at 10 μM in Table 3, confirmed the selectivity of compound **13** toward FLT-3 and VEGFR-2 and **18** against FLT-3, GSK-3β, and VEGFR-2, respectively. With residual activity between 75% (PKC-ε) and 117% (c-RAF), compound **18** shows no inhibitory efficacy against other tested kinases of the panel. With regard to a weak inhibition of GSK-3β (50% residual activity) maleimide **13** selectively inhibits FLT-3 and VEGFR-2, whereas the selectivity of azaindole **18** comprises FLT-3, GSK-3β, and VEGFR-2.

Because of these results, the antiproliferative activity of new compounds was evaluated by using a MTT assay in various cell lines. The viability was determined 3 days (HT-29, Molm-14) and 5 days (Mkn-45) after incubation with compounds. As outlined in Table 4, the viability of human colon adenocarcinoma cells (HT-29) decreases dose-dependently for compounds **18** and **19** while applying concentrations in the low micromolar range. Whereas **18** offers potent activity, the previously reported compound **19** shows minor efficacy against HT-29 cells. For maleimide **14** no dose-dependency could be observed in the MTT assay using HT-29 cells, indicating possible cytotoxic effects.

Table 6. MTT Assay Using Molm-14 Cells, Viability in %[a]

concn (μM)	13	18
0.5	65.89 ± 6.34	73.37 ± 3.41
1.0	38.88 ± 3.38	54.20 ± 4.53
10.0	4.41 ± 0.29	9.35 ± 0.57
20.0	3.56 ± 0.20	5.39 ± 0.75
50.0	1.77 ± 0.10	2.40 ± 0.24

[a]Samples were incubated for 3 days. Viability of untreated cells is 100%.

The human gastric carcinoma cell line Mkn-45 (Table 5) offers marginal activity in the MTT assay and no dose-dependency for all tested compounds. Viability was measured 5 days after treatment of compounds, as no effects could be seen after 3 days. The herein presented data suggest a generally higher response of synthesized maleimides against HT-29 cells than Mkn-45 cells.

Taking the kinase inhibition and selectivity profile of the compounds into account (see Tables 2 and 3) we subsequently measured potential inhibitory effects of compounds **13** and **18** using the leukemic cell line Molm-14, which carries an activating FLT3-ITD (internal tandem duplication) mutation.[22] Both inhibitors were capable of remarkably reducing cell proliferation at low micromolar concentration (Table 6).

In summary, the highest activity for compounds **13** and **18** was found in Molm-14 cells followed by the decrease of viability using HT-29 cells.

To determine proapoptotic effects, we investigated human umbilical vein endothelial cells (HUVECs) as well as gastric cancer cells Mkn-45 with Nicoletti FACS analysis. As illustrated in Table 7, a substantial, dose-dependent proapoptotic activity in HUVECs was observed, indicated by a significant increase of the sub-G1 cell fraction. Among the tested maleimides this effect was most pronounced for indolyl derivative **19** (87.7% sub-G1 fraction, 2.6 μM) and 6-azaindolyl derivative **18** (66.3%, sub-G1 fraction, 2.6 μM), whereas 7-azaindole (**13**) and 5-azaindole (**16**) derivatives showed only moderate induction of apoptosis. For compound **19** these results are in agreement with the IC_{50} values for the VEGFR-2 inhibition (Table 2, **19**, 70 nM) and the antiangiogenic effect (chick

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Table 7. Induction of Apoptosis in HUVEC[a]

	sub-G1 fraction (%)				
concn (μM)	**13**	**16**	**17**	**18**	**19**
0.79	26.7 ± 1.9	31.3 ± 1.7	14.2 ± 2.4	48.0 ± 2.3	25.7 ± 0.9
2.6	49.2 ± 2.2	46.3 ± 3.2	16.3 ± 1.3	66.3 ± 1.0	87.7 ± 2.3

[a]Samples were incubated for 4 days. Sub-G1 fraction of untreated cells: 15.4 ± 1.5%.

Table 8. Induction of Apoptosis in Mkn-45 Cells[a]

	sub-G1 fraction (%)				
concn (μM)	**13**	**14**	**16**	**18**	**19**
1.0	9.3 ± 0.8	12.7 ± 0.9	8.8 ± 2.4	12.8 ± 0.3	14.2 ± 2.4
5.0	16.3 ± 0.6	24.8 ± 0.6	15.7 ± 3.6	15.8 ± 2.5	20.3 ± 0.7
10.0	37.8 ± 2.4	31.8 ± 3.2	21.5 ± 1.2	16.5 ± 0.6	29.8 ± 3.5
15.0	50.7 ± 1.6	45.8 ± 5.6	25.7 ± 3.3	22.9 ± 4.1	45.9 ± 0.9
20.0	65.9 ± 2.7	55.7 ± 4.2	31.6 ± 2.1	28.3 ± 2.7	55.3 ± 2.6

[a]Samples were incubated for 7 days. Sub-G1 fraction of untreated cells: 10.9% ± 3.2%.

Table 9. Enhancement of Apoptosis in HT-29 Human Colon Adenocarcinoma Cells by Combining Compounds and Irinotecan[a]

	sub-G1 fraction (%)			
concn	**13**	**17**	**18**	**19**
7.9 μM test compound	2.32 ± 1.16	4.22 ± 0.74	79.9 ± 4.13	4.7 ± 0.5
7.9 μM test compound plus 1.18 μM irinotecan	22.36 ± 2.80	25.71 ± 1.97	75.69 ± 1.77	53.1 ± 3.3

[a]Samples were incubated for 7 days. Induction of apoptosis at 1.18 μM irinotecan single application: 18.3 ± 1.1%. Induction of apoptosis by drug-free control: 3.4 ± 0.9%.

embryo assay, Table 1, 82% IVG). The expected correlation for compounds **13** and **18** could not be verified, which might be due to its high inhibitory potency against FLT-3 and GSK-3β. Hence, various mechanisms may be responsible for the induction of apoptosis.

Table 8 indicates a significant dose-dependent induction of apoptosis in Mkn-45 cells for azaindoles **13**, **14**, and the previously reported indolyl derivative **19**. In comparison to VEGF dependent HUVECs, a lower increase of sub-G1 fraction was observed for all tested compounds in the gastric cell line Mkn-45, e.g., **13** (2.6 μM, 49.2% sub-G1 fraction in HUVEC versus 5.0 μM, 16.3% sub-G1 fraction in Mkn-45). The findings of the antiproliferative and proapoptotic assays using different cell lines support the hypothesis that the potent effects of maleimides are mediated through different mechanisms of action.

We further investigated the efficacy of the synthesized maleimides and the topoisomerase I inhibitor irinotecan alone and in combination to induce apoptosis using human HT-29 cells. As illustrated in Table 9, indolyl derivative **19** demonstrated a synergistic effect on induction of apoptotic cell death upon combination with irinotecan. Contrary to the 4-azaindole derivative (**17**) and 7-azaindole (**13**), both of which slightly increase the potency of irinotecan (1.18 μM, 18.3 ± 1%), the 6-azaindole (**18**) did not increase the efficacy of irinotecan.

These results demonstrate a possible benefit of combining synthesized maleimides and topoisomerase I inhibitors for cancer treatment.

CONCLUSION

This study evaluated the antiangiogenic and kinase inhibition efficacy as well as the kinase selectivity profile of 3-azaindolyl-4-arylmaleimides in an in vivo chick embryo assay and kinase assays. The 7-azaindolyl derivate **13** demonstrated a strong inhibition of vessel growth. **18** (6-azaindole) and **13** (7-azaindole) are highly potent inhibitors of VEGFR-2 kinase with IC_{50} in the low nanomolar range, similar to the previously reported indolyl derivative **19**. Furthermore maleimide **18** was identified as potent GSK-3β inhibitor. Kinase selectivity of compounds **13** and **18** was confirmed representatively by screening 15 chosen kinases of different classes.

The 6-azaindole **18** is highly effective in leukemic cells and endothelial cells, as well as against HT-29 cells in monotherapy. Regarding proapoptotic efficacy in combination with topoisomerase I inhibitor irinotecan, no synergistic increase was detected because of its high efficacy at 7.9 μM in monotherapy. 7-Azaindole **13** shows minor potency against endothelial cells in comparison to the above-mentioned 6-azaindole **18**. In addition, they offer no dose dependency in cell viability assays using colon and gastric tumor cells lines. The indolyl derivative **19** in combination with irinotecan possesses a synergistic effect on apoptosis induction using human endothelial cells.

5-Azaindole derivative **16** and 4-azaindole derivate **17** indicate moderate to weak activity in kinase and cellular assays.

On the basis of the high potency of the presented maleimides both in vitro and in vivo, prospective studies are on the way to improve the pharmacological profile leading to novel drug candidates.

EXPERIMENTAL SECTION

Chick Embryo Assay as in Vivo Angiogenesis Model. The assay was performed according to Peifer et al.[17] Test compounds were dissolved in DMSO and used as 26 mM stock solution. For the preparation of agarose pellets, the stock solution was mixed with 2% agarose solution at 80 °C and 10 μL pellets were produced (final concentration, 26 nM). Fertilized chicken eggs (white leghorn) were

incubated at 37.5 °C with 62% relative humidity in an automatically turning egg incubator (Ehret). After 70 h of incubation time, 8 mL of albumin was removed and a window was opened in the shell to uncover the developing germination layer. The window was sealed with transparent tape. The eggs were returned to the incubator and stored at 90% relative humidity without turning. After 75 h of incubation the angiogenic experiment was initiated (start time, 0 h) and 15 agarose pellets were placed on the area vasculosa preparations. After 24 h, images of the control and sample-treated areas were taken and digitized via Leica QWin software. The angiogenic response was measured using the automatic image analysis system programmed in a routine. The digitized images were analyzed for total, major (capillaries with a diameter of more than 50 μm), and minor blood vessel areas using the customized image analysis software Leica QWin (Leica Microsystems, Bensheim, Germany). Calculated data were sent automatically via dynamic data exchange to an Excel table and processed statistically. An antiangiogenic response due to agent-treated samples was considered positive if the mean of minor blood vessel area showed >20% inhibition compared to control samples. Differences between treated and control samples were evaluated using Student's *t* test.

MTT Assay. The test compounds were determined for their antiproliferative activity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann et al.[23] Cells in the exponential growth phase were transferred to 96-well flat-bottom plates. Then 10 000 viable cells resuspended in 200 μL of RPMI medium were plated into each well and incubated overnight. Cells were then exposed to various concentrations of test compounds (100 μL/well) for 3 or 5 days at 37 °C with 5% CO_2. Subsequently, 10 μL/well MTT stock solution (5 mg/mL; Biomol, Germany) was added and the cells were incubated at 37 °C with 5% CO_2 for 4 h. An amount of 100 μL solubilization solution (10% SDS in 0.01 M HCl) was added, and the cells were incubated at 37 °C with 5% CO_2 overnight. Plates were read on an ELISA reader ELX 800 (Bio-Tek KC4 software) at 562 nm absorbance. Each experiment was done in triplicate.

Nicoletti Cell Cycle Analysis. HUVEC or cancer cells (HT-29) were transferred to 12-well flat-bottomed plates. Then 1.5×10^5 viable cells contained in 1 mL of cell suspension were plated into each well, incubated overnight before exposure to various concentrations of drugs. Subsequently, cells were incubated in 1 mL of medium containing various concentrations of test compounds at 37 °C with 5% CO_2. After incubation the cells were washed with PBS, trypsinized, pelleted, mixed with PI buffer (containing 0.1% sodium citrate, 0.1% Triton X-100, 50 mg/mL propidium iodide (PI)), and incubated for 1 h at 4 °C. Cell cycle sub-G1 fraction analysis was performed as described previously (Nicoletti et al.)[24] using a flow cytometer (BD FACS Calibur, BD Biosciences, Heidelberg, Germany). Each experiment was done in triplicate. The same analysis was performed with HUVEC cells. Then 1.5×10^4 cells were plated into each well and treated with test compounds for 7 days.

GSK-3β, VEGFR-2, FLT-3 Kinase Assays and Kinase Screening. IC_{50} values and residual activity determination were carried out at Millipore UK Ltd. (Gemini Crescent, Dundee Technology Park, Dundee DD2 1SW, U.K. (IC_{50} Profiler)).[25] Details of each assay protocol can be found on Millipore's Web site at www.millipore.com/drugdiscovery/dd3/assayprotocols. Other kinase assay data are taken from Peifer et al.[8]

Cell Lines. The human colon cancer cell line HT-29 was obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). The cells were routinely cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany) and supplemented with 10% FCS (Cölbe, Germany), 100 units/mL penicillin, and 100 μg/mL streptomycin (1%, Cambrex, Germany).

Human gastric cancer cell line Mkn-45 was obtained from the DSMZ and from the European Collection of Cell Cultures (ECACC, Salisbury, U.K.) and cultured in RPMI 1640 medium supplemented with 20% FCS and 1% penicillin/streptomycin.

The human leukemia cell line Molm-14 was kindly provided by Scott Armstrong (Boston Children's Hospital, U.S.) and cultured in RPMI 1640 supplemented with 10% FCS and L-glutamine. All cells were maintained at 37 °C in an incubator under an atmosphere containing 5% CO_2. Each experiment was performed with exponentially growing cells.

Chemistry. Infrared spectra were recorded on a Thermo Nicolet Avatar 330 FT-IR spectrometer. ^1H (300 MHz, digital resolution 0.3768 Hz) and ^{13}C (75 MHz, digital resolution 1.1299 Hz) NMR spectra were recorded on a Bruker AC 300. The data are reported as follows: chemical shift in ppm from Me_4Si as external standard, multiplicity, and coupling constant (Hz). EI mass spectra were recorded on a Varian MAT 44S (80 eV) and FD mass spectra on a Finnigan MAT 7 (5 kV). For clarity only the highest measured signal is given for FD mass spectra. Elemental analyses were performed on a Haereus CHN rapid analyzer, Carlo Erba Strumentazione 1106. Combustion analysis results agreed with the calculated data within ±0.4% unless otherwise stated. Melting points/decomposition temperatures were determined on a Büchi apparatus according to Dr. Tottoli and are uncorrected. Where appropriate, column chromatography was performed for crude precursors with Merck silica gel 60 (0.063−0.200 mm). Column chromatography for test compounds was performed using a MPLC system B-680 (Büchi) with Merck silica gel (0.015−0.040 mm). The progress of the reactions was monitored by thin layer chromatography (TLC) performed with Merck silica gel 60 F-254 plates. Where necessary, reactions were carried out in a nitrogen atmosphere using 4 Å molecular sieves. All reagents and solvents were obtained from commercial sources and used as received. On the basis of NMR and combustion analysis data, all final compounds reported are ≥95% pure.

General Procedure A for the C-3 Acylation of Azaindoles (Modified from Zhang et al.).[9a] Azaindole (1 equiv) was added to a mixture of aluminum chloride (5 equiv) in 100 mL of dry dichloromethane at 0 °C under argon atmosphere. After 30 min at 0 °C, the mixture was warmed to room temperature and ethyl chlorooxoacetate (5 equiv) was added dropwise. The reaction mixture was stirred vigorously overnight, and then carefully ice was added. After addition of dichloromethane the organic layer was separated, washed carefully with cold $NaHCO_3$ solution, dried over $MgSO_4$, filtered, and concentrated.

General Procedure B for Protection of Azaindoles (Modified from Basel et al.).[15] Di-*tert*-butyl dicarbonate (1.5 equiv) and DMAP were added to a stirred suspension of acylated azaindole (1 equiv). After being stirred for 12 h, the mixture was concentrated in vacuo and purified by column chromatography (petroleum ether/ethyl acetate, 1/1).

General Procedure C for the Preparation of Phenylacetamide Derivates (12a−c). To the phenylacetic acid derivative (1 equiv) was added phosphorus pentachloride (5 equiv), and the mixture was stirred for 2 h in dichloromethane at room temperature. In a second step ammonia (25%) was added via septum and the mixture was stirred for another 2 h, then given into ice and extracted with dichloromethane (4 times). The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. The crude product was recrystallized from ethanol.

General Procedure D for the Preparation of 3-Azaindolyl-4-arylmaleimides (Modified from Peifer et al.).[14a] Potassium *tert*-butoxide (4 equiv) in dry THF was added via septum to a stirred solution of phenylacetamide derivative and acylated azaindole derivative in dry THF, containing 2−3 g of molecular sieves (4 Å). After the mixture was stirred overnight, the reaction was quenched with saturated NH_4Cl-solution. The residue was filtered, extracted with ethyl acetate, and the combined organic layers were dried over $MgSO_4$, concentrated, and purified by column chromatography (ethyl acetate/ethanol, 9/1).

3-Methyl-4-nitropyridine-l-oxide (2) (Modified from Dormoy et al.).[11] To 3-picoline-1-oxide **1** (10 g, 91.6 mmol) was slowly added a solution of HNO_3 (27.5 mL) and H_2SO_4 (35 mL) via syringe at 25 °C. The mixture was heated to 100−105 °C for 3 h, cooled to room temperature, followed by slow addition of ice and a pH adjustment to 2−3 with sodium carbonate. The mixture was filtered and washed with chloroform. The organic layers were dried and concentrated in vacuo

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to give a crude orange solid which was washed with acetone to give the desired compound as yellow crystals (48.2 mmol, 52%). For further data see ref 11.

3-Dimethylaminovinylene-4-nitropyridine-l-oxide (3) (Modified from Dormoy et al.).[11] A solution of 3-methyl-4-nitropyridine-l-oxide **2** (2.64 g, 17.1 mmol) and DMFDMA (3.82 g, 26 mmol) in DMF was heated to 100 °C for 1.5 h. The mixture was concentrated in vacuo, and the crude residue was washed with methanol. The title compound was obtained as dark purple crystals (16.76 mmol, 98%). For further data see ref 11.

Dimethyl 2-(3-Nitropyridin-2-yl)malonate (5) (Modified from Cash et al.).[10] 2-Chloro-3-nitropyridine **4** (2 g, 12.5 mmol) was added to a stirred suspension of sodium hydride (0.5 g, 12.5 mmol, mixture of 60% NaH in mineral oil) in 20 mL of dry DMF under nitrogen. Dimethyl malonate (1.43 mL, 1.65 g, 12.5 mmol) was added cautiously dropwise. After the mixture was stirred for 5 h at room temperature, the solution was diluted with water. After addition of diethyl ether, the mixture was washed four times with brine to remove DMF. The organic phase was dried over Na_2SO_4, filtered, concentrated, and purified by column chromatography (petroleum ether/ethyl acetate, 2/1). The title compound was obtained as a pale brown oil (5.5 mmol, 44%). For further data see ref 26.

2-Methyl-3-nitropyridine (6) (Modified from Cash et al.).[10] Dimethyl 2-(3-nitropyridin-2-yl)malonate **5** (1.4 g, 5.5 mmol) was dissolved in 70 mL of 6 M HCl and refluxed for 8 h. After neutralization with saturated Na_2CO_3 solution, the aqueous portion was extracted three times with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, concentrated, and purified by bulb tube distillation (0.35 mbar, 70−80 °C). The title compound was obtained as a pale yellow oil (5.1 mmol, 92%). For further data see ref 26.

***N*,*N*-Dimethyl-2-(3-nitropyridin-2-yl)ethenamine (7) (According to Cash et al.).**[10] 2-Methyl-3-nitropyridine **6** (0.7 g, 5.1 mmol) was dissolved in 15 mL of dry DMF and stirred under nitrogen. *N*,*N*-Dimethylformamide dimethyl acetal (1.35 mL, 1.22 g, 10.2 mmol) was added dropwise. The mixture was heated to 90 °C for 4 h. After approximately 15 min a deep reddish color appeared. After evaporation of the solvent, the title compound was obtained as a red oil (0.8 mmol, 73%) which can be used without further purification. For further data see ref 26.

7-Chloro-6-azaindole (8) (According to Zhang et al.).[27] 2-Chloro-3-nitropyridine **4** (5.0 g, 31 mmol) was dissolved in 200 mL of dry THF under nitrogen atmosphere and cooled to −78 °C. After dropwise addition of 100 mL of 1 M vinylmagnesium bromide solution the mixture was stirred for 8 h, quenched with 20% NH_4Cl solution, extracted with ethyl acetate, dried over Na_2SO_4, and concentrated in vacuo. Crude product was purified by column chromatography (isopropyl ether/ethyl acetate, 1/1) to obtain a brown solid (10.23 mmol, 33%). Mp 188−190 °C. IR ν [cm^{-1}] = 3077 ν(NH); 1618; 1548; 1483. For further data see ref 27.

5-Azaindole (9a) (Modified from Dormoy et al.).[11] To a solution of 3-dimethylaminovinylene-4-nitropyridine-l-oxide **3** (1 g, 4.78 mmol) in dry ethanol was added 10% Pd/charcoal (0.93 g, 26 mmol) under argon atmosphere. Then the mixture was put in a miniautoclave under H_2 pressure (2−3 bar) and stirred for 8 h at 65 °C and overnight at room temperature. The slurry was filtered, concentrated, and further purified by column chromatography (ethanol) to obtain yellow crystals (3.56 mmol, 74.5%). Mp 112 °C. IR ν [cm^{-1}] = 3657 ν(NH); 2978; 2901; 1618; 1462. For further data see ref 11.

4-Azaindole (9b) (According to Cash et al.).[10] An amount of 0.2 g of 10% Pd/charcoal was flashed with nitrogen before 10 mL of a mixture of 8.8% formic acid in methanol was added cautiously. **7** was also dissolved in 10 mL of a mixture of 8.8% formic acid in methanol before it was added to the reaction mixture. The mixture was stirred for 4 h until the red color completely disappeared. The Pd catalyst was removed by filtration through Celite, and the filtrate was concentrated. The title compound crystallized overnight (1.8 mmol, 71%). For further data see ref 28.

6-Azaindole (9c) (Modified from Mahadevan et al.).[9b] An amount of 0.1 g of Pd/charcoal (10% Pd) was placed in a miniautoclave with 20 mL of alkaline ethanol. A solution of 7-chloro-6-azaindole **8** (0.5 g, 3.3 mmol) in 80 mL of alkaline ethanol was added. The miniautoclave was put under H_2 pressure (1 bar), and the mixture was stirred for about 8 h at room temperature. The slurry was filtered through Celite, washed with alkaline ethanol, and concentrated in vacuo. The crude residue was absorbed with 40 mL of water, extracted three times with dichloromethane, dried over Na_2SO_4, and concentrated in vacuo. Purification was achieved by recrystallization (cyclohexane/dichloromethane, 1/1) to obtain light brown crystals (2.74 mmol, 83%). Mp 131−132 °C. IR ν [cm^{-1}] = 3060 ν(aromat CH); 1622; 1495; 1458. FD-MS: m/z (rel intens) = 118.2 (74.69%; $M^{+\bullet}$). For further data see ref 9b.

Ethyl 2-(5-Azaindol-3-yl)-2-oxoacetate (10a). General procedure A was followed, using 5-azaindole **9a** (0.5 g, 4.23 mmol) to obtain the title compound as a pale yellow powder (1.9 mmol, 45%). For further data see ref 14b.

Ethyl 2-(4-Azaindol-3-yl)-2-oxoacetate (10b). General procedure A was followed, using 4-azaindole **9b** (0.38 g, 4.60 mmol) to obtain a yellow oily residue which solidified to yellow crystals. After a washing with cold petroleum ether the title compound was obtained (2.30 mmol, 50%) as a light yellow powder. For further data see ref 28.

Ethyl 2-(6-Azaindol-3-yl)-2-oxoacetate (10c). General procedure A was followed, using 6-azaindole **9c** (0.25 g, 2.12 mmol) to obtain a yellow oily residue which solidified to a greenish-yellow powder. After a washing with cold diethyl ether the title compound was obtained (0.89 mmol, 42%) as light yellow powder. For further data see ref 14b.

Ethyl 2-(7-Azaindol-3-yl)-2-oxoacetate (10d). General procedure A was followed, using 7-azaindole **9d** (1.20 g, 10.16 mmol) to obtain a yellow oily residue which solidified to a greenish-yellow powder. After a washing with cold diethyl ether the title compound was obtained (5.13 mmol, 50.5%) as a light yellow powder. For further data see ref 14b.

Ethyl 2-[1-(*tert*-Butoxycarbonyl)-5-azaindol-3-yl]-2-oxoacetate (11a). General procedure B was followed, using **10a** (0.3 g, 1.4 mmol) to yield the title compound as colorless crystals (1.33 mmol, 95%). For further data see ref 14b.

Ethyl 2-[1-(*tert*-Butoxycarbonyl)-4-azaindol-3-yl]-2-oxoacetate (11b). General procedure B was followed, using **10b** (0.48 g, 2.20 mmol) to obtain light yellow crystals (1.9 mmol, 86%). For further data see ref 28.

Ethyl 2-[1-(*tert*-Butoxycarbonyl)-6-azaindol-3-yl]-2-oxoacetate (11c). General procedure B was followed, using **10c** (0.55 g, 2.5 mmol) to yield the title compound as colorless crystals (2.25 mmol, 90%). Mp 152 °C. For further data see ref 14b.

3-(7-Azaindol-3-yl)-4-(3,4,5-trimethoxyphenyl)maleimide (13). General procedure D was followed using **10d** (1.03 g, 4.70 mmol) and 3,4,5-trimethoxyphenylacetamide **12a** (0.67 g, 2.98 mmol). Purification was achieved by column chromatography (petroleum ether/ethyl acetate/isopropanol, 7/2/1) to yield the title compound (1.26 mmol, 42.6%) as yellow crystals. Anal. ($C_{20}H_{17}N_3O_5 \cdot H_2O$), calcd C 61.85, H 4.67, N 10.82; found C 61.76, H 4.29, N 11.12. For further data see ref 14b.

3-(7-Azaindol-3-yl)-4-(3,5-dimethoxyphenyl)maleimide (14). General procedure D was followed using **10d** (1.5 g, 6.84 mmol) and **12b** (1.54 g, 6.84 mmol) to yield the title compound as yellow crystals (3.28 mmol, 48%). Mp >270 °C. ^{1}H NMR (300 MHz, DMSO) δ [ppm] = 12.35 (s, 1H), 11.13 (s, 1H), 8.18 (s, 1H), 8.05 (s, 1H), 6.84 (s, 1H), 6.75 (s, 1H), 6.53 (s, 3H), 3.53 (s, 6H). Anal. ($C_{19}H_{15}N_3O_4 \cdot 0.5H_2O$), calcd C 63.68, H 4.50, N 11.73; found C 63.94, H 4.16, N 11.86.

3-(7-Azaindol-3-yl)-4-(2-Methoxyphenyl)maleimide (15). General procedure D was followed using **10d** (0.5 g, 2.3 mmol) and **12c** (0.51 g, 2.5 mmol). Purification with chromatography on silica gel (petroleum ether/ethyl acetate, 1/4) gave the title compound as a yellow solid (1.19 mmol, 52%). Mp >280 °C. ^{1}H NMR (DMSO-d_6), 300 MHz, δ [ppm] = 3.36 (s, 3 H); 6.64−6.80 (m, 2 H); 6.96−7.08 (m, 2 H); 7.27 (d, J = 8.82 Hz, 1 H); 7.38−7.48 (m, 1 H); 8.01 (s, 1

H); 8.15 (d, J = 2.62 Hz, 2 H); 11.07 (s, 1 H); 12.39 (s, 1 H). IR $\tilde{\nu}$ [cm^{-1}] = 3154 ν(NH); 1744 ν(CO); 1691 ν(CO). FD-MS (m/z) = 319.4 ($M^{+\bullet+1}$). Anal. ($C_{18}H_{13}N_3O_3 \cdot 0.5H_2O$), calcd C 66.76, H 4.20, N 12.98; found C 66.99, H 4.06, N 12.86

3-(5-Azaindol-3-yl)-4-(3,4,5-trimethoxyphenyl)maleimide (16). General procedure D was followed using **12a** (0.32 g, 1.4 mmol) and **11a** (0.3 g, 1.4 mmol) to obtain the title compound as yellow crystals (0.5 mmol, 36%). IR $\tilde{\nu}$ [cm^{-1}] = 3281 ν(NH); 3060; 2993; 1765, 1698. Anal. ($C_{20}H_{17}N_3O_5 \cdot H_2O$), calcd C 60.45, H 4.82, N 10.57; found C 60.23, H 4.45, N 10.38. For further data see ref 14b.

3-(4-Azaindol-3-yl)-4-(3,4,5-trimethoxyphenyl)maleimide (17). General procedure D was followed, using **11b** (0.54 g, 1.7 mmol) and **12a** (0.38 g, 1.7 mmol) to obtain yellow crystals (0.45 mmol, 26%). Anal. ($C_{20}H_{17}N_3O_5 \cdot H_2O$), calcd C 60.45, H 4.82, N 10.57; found C 60.12, H 4.53, N 10.42. For further data see ref 28.

3-(6-Azaindol-3-yl)-4-(3,4,5-trimethoxyphenyl)maleimide (18). General procedure D was followed using **11c** (0.54 g, 1.7 mmol) and **12a** (0.38 g, 1.7 mmol). Purification was achieved by column chromatography (petroleum ether/ethyl acetate/isopropanol, 5/2/3). The obtained oil was recrystallized from chloroform, forming yellow crystals (0.5 mmol, 33%). Anal. ($C_{20}H_{17}N_3O_5 \cdot H_2O$), calcd C 60.45, H 4.82, N 10.57; found C 60.13, H 4.56, N 10.24. For further data see ref 14b.

AUTHOR INFORMATION

Corresponding Author

*Phone: +49-6131-39-25726. Fax: +49-6131-3330477. E-mail: dannhardt@uni-mainz.de.

Author Contributions

[‖]Authors C.G. and E.L. contributed equally.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

bFGF, basic fibroplast growth factor; DMFDEA, *N*,*N*-dimethylformamide diethyl acetal; DMFDMA, *N*,*N*-dimethylformamide dimethyl acetal; FLK, fetal liver kinase; FLT-3, FMS-like tyrosine kinase 3; GSK, glycogen synthase kinase; HT-29, human colon adenocarcinome grade II cell line; HUVEC, human umbilical vein endothel cell; ITD, internal tandem duplication; KDR, kinase insert domain receptor; Mkn-45, human gastric cancer cell line; Molm-14, human leukemia cell line; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaH, sodium hydride; RTK, receptor tyrosine kinase; ^{t}BuOK, potassium *tert*-butoxide

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