Donated Chemical Probe

ROCK1/2 Inhibitor Probe BAY-549

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Presenters:
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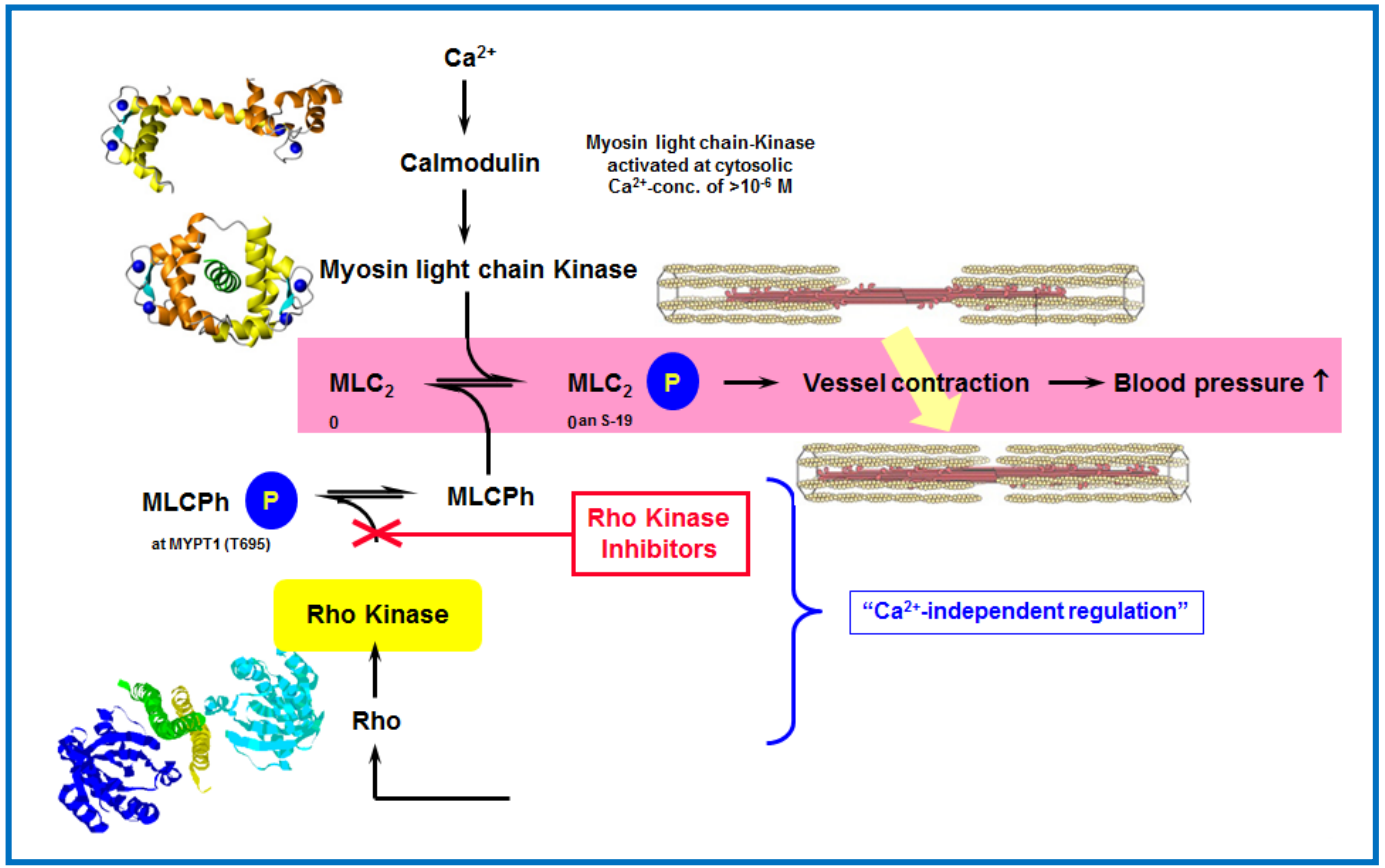
Raimund Kast

ROCK1/2 Inh Probe BAY-549

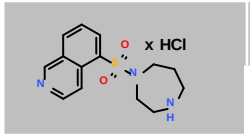
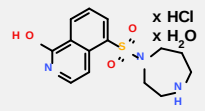
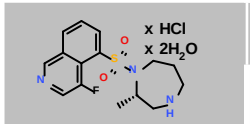
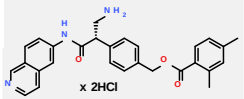
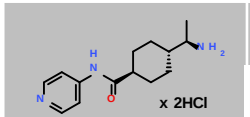
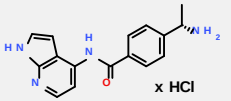
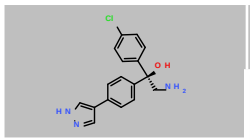
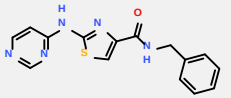
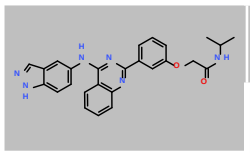
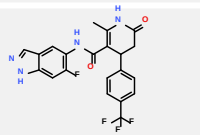
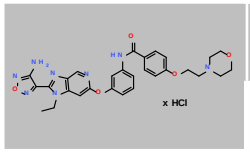
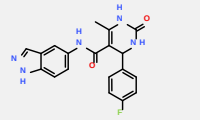
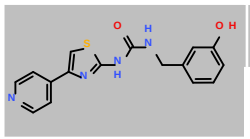
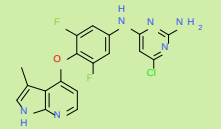
Scientific rationale

- The Rho-associated coiled coil containing protein kinase (ROCK) is a ~160 kDa serine/threonine kinase from the AGC kinase family.
- ROCK is an effector of the small GTP-binding protein RhoA, and is implicated in a multitude of fundamental cellular processes including smooth muscle contraction, cell growth and migration, endothelial barrier maintenance, and apoptosis.
- ROCK mediates the phosphorylation of the regulatory myosin-binding subunit of myosin light chain phosphatase (MLCPh). This inhibits the phosphatase activity causing an increase in the level of phosphorylated MLC and the contractile tone of the vascular smooth muscle apparatus independently of any change in intracellular Ca^{2+} concentration, a phenomenon known as “calcium sensitization”.
- ROCK inhibitors counterbalance this process leading to net vasodilation.



ROCK1/2 Inh Probe BAY-549

Comparison with commercially available compounds[1)]

	Name	ROCK	ROCK1	ROCK2	others	
	Fasudil			330 nM (Ki)	PKA 1.6 µM, PKG 1.6 µM, PKC 3.3 µM	
	Hydroxyfasudil		730 nM	720 nM	PKA 37 µM	
	Ripasudil (K-115)		51 nM	19 nM		
	Netarsudil (AR-13324)	2 nM				
	Y-27632 x 2HCl		140 nM	300 nM		
	Y-39983 x HCl	3.6 nM				
	AT13148		6 nM	4 nM	p70S6K 8 nM, Akt1 38 nM, PKA 3 nM	
	Thiazovivin	ca. 500 nM				
	KD025 (SLx-2119(			60 nM		
	GSK429286A		14 nM	63 nM		
	GSK269962A HCl		1.6 nM	4 nM	MSK1 49 nM, RSK1 132 nM	
	GSK180736A		100 nM		GRK2 770 nM	
	RKI-1447		14.5 nM	6.2 nM		
	BAY-549		**0.6 nM[2)]**	**1.1 nM[2)]**		

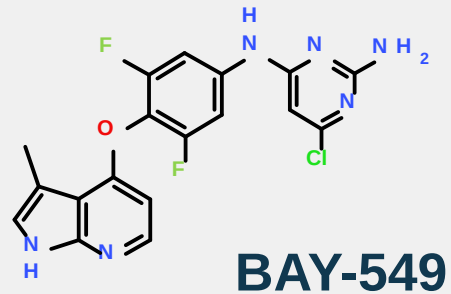
1) https://www.selleckchem.com/ROCK.html
2) BAYER data

BAY-549:

- highly potent
- in-depth characterized
- novel scaffold
- good selectivity
- inactive structural analog

ROCK1/2 Inh Probe BAY-549

Technical *in vitro* profile



BAY-549

POTENCY (IC_{50} [nM])	
Biochem. ROCK-1 (h) IC_{50} [nM]	0.6
Biochem. ROCK-2 (h) IC_{50} [nM]	1.1
Biochem. ROCK-2 (m) IC_{50} [nM]	2.4
Biochem. ROCK-2 (r) IC_{50} [nM]	0.8
Arteria Saphena rabbit IC_{50} [nM]	65

Properties & Physchem	
logD @ pH 7.5	2.3
BEI / LLE_d (calc, ROCK-2 (h))	25 / 8.6
Sw @ pH 6.5 [mg/L]	0.5
MW / MW corr / TPSA [g*mol^{-1} / Å^2]	403 / 359 / 102
Stability (r /h plasma, 4h) [%]	nd

Solubility	mg/L
pH 6.5	0.5
pH 2	1.8
pH 4	0.8
pH 8	0.3
pH 10	0.2
PEG400	>30
PEG400/H_2O 80/20	120
Solutol/EtOH /H_2O 40/10/50	240
0.1 M HCl	247
Acetone	6331

in vitro DMPK Properties						
Caco2 Permeability	P_{app}(A-B) [nm/s]		P_{app}(B-A) [nm/s]		efflux ratio	
	59		23		0.4	
metabolic stability			CL [L/h/kg]		F_{max} [%]	
	liver mics (r)		2.3		44	
	rat hepatocytes		2.0		53	
	human hepatocytes		0.82		38	
CYP inhibition IC_{50} [µM]	1A2	2C8	2C9	2D6	3A4	3A4 preinc.
	>50	nd	6.0	>50	41	6.6
PXR	nd					

Selectivity	
In-house kinase panel	High selectivity see next slides
Upstate @ 10 µM (kinase panel)	High selectivity see next slides

SAFETY	
Cytotox (HuH-7 cells) [µM]	1.9
hERG IC_{50} [µM]	>30

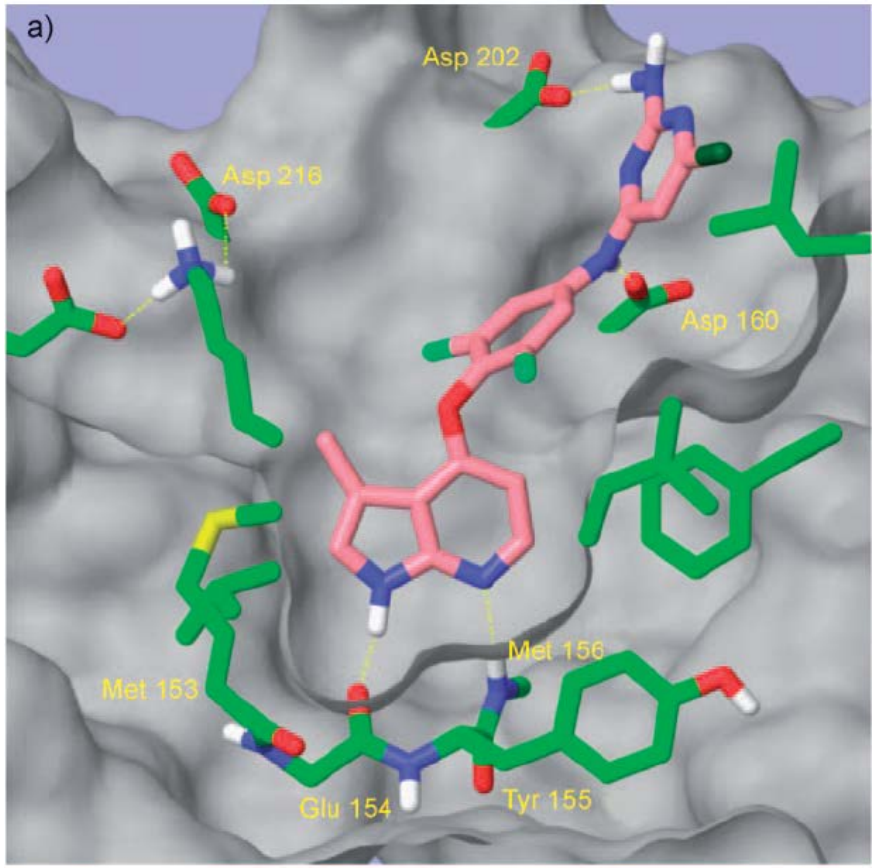
- BAY-549 has high *in vitro* potency and selectivity.
- BAY-594 has low solubility and intermediate permeability with no efflux in Caco2 assay.

ROCK1/2 Inh Probe BAY-549

Docking study with BAY-549

Molecular modeling images of BAY-549 docked and minimized into ROCK-1 (2esm.pdb, subunit A, Asp216 side chain minimized):
The inhibitors is shown in pink, while the protein surface is given in grey with key amino acids labeled and highlighted in green. Heteroatoms and polar hydrogens are color coded (N, blue; O, red; S, yellow; F, light green; Cl, dark green; polar H, white) and hydrogen bonds are shown as yellow dotted lines.

from: *J. Med. Chem.* **2008**, *3*, 1893 – 1904.
DOI: 10.1002/cmdc.200800211



ROCK1/2 Inh Probe BAY-549

Selectivity profile in more detail – kinase panel

Kinase Profiling at Upstate
114 Kinases (10 µM)

23 Different kinases	IC_{50} : 1-10 µM
TrkA	IC_{50} : 252 nM
Flt3	IC_{50} : 303 nM
PKA	IC_{50} : 734 nM
ROCK-2 (rat);	IC_{50} : <1 nM
ROCK-2 (human);	IC_{50} : 1 nM

Inhouse Kinase Data:
111 Kinases (10 µM)

Mps1	IC_{50} : 0.54 µM
AKT1	IC_{50} : 0.82 µM
FLT4	IC_{50} : 1.04 µM
NUAK1 (10 µM ATP)	IC_{50} : 1.66 µM
TrkA	IC_{50} : 3.84 µM
KDR	IC_{50} : 3.89 µM
FMS	IC_{50} : 4.21 µM
PDGFRβ	IC_{50} : 4.90 µM

Bub1, CDK2, CDK9, cKit, EGFR, FGFR1, FGFR3, FGFR4, GSK3β, IKKβ, IRAK4, MKNK1, NEK2, SRPK1, TAO2, Tie2: IC_{50} > 20 µM

As usual, full profile will be delivered after probe acceptance

ROCK1/2 Inh Probe BAY-549

Selectivity profile in more detail - safety pharmacology *in vitro*

Off-Target	Species	Inhibition in % @ 10 µmol	IC50 [µmol/l]	Ki [µmol/l]
Adenosine A1	Human	55		
Adenosine A1	Human	57		
Dopamine D1	Human	62		
Dopamine D1	Human	61		
Opiate kappa (OP2, KOP)	Human	69		
Opiate kappa (OP2, KOP)	Human	65		
Opiate mu (OP3, MOP)	Human	51		
Transporter, Dopamine (DAT)	Human		32	0.25
Transporter, Dopamine (DAT)	Human		34	0.0027
Transporter, Dopamine (DAT)	Human		39	0.31
Transporter, Dopamine (DAT)	Human	98		
Transporter, Norepinephrine (NET)	Human	55		
Transporter, Norepinephrine (NET)	Human	56		
Calcium Channel L-Type, Dihydropyridine	Rat		599	0.039
Calcium Channel L-Type, Dihydropyridine	Rat		690	4.4
Calcium Channel L-Type, Dihydropyridine	Rat		703	4.5
Calcium Channel L-Type, Dihydropyridine	Rat	72		
Sodium Channel, Site 2	Rat		656	6.0
Sodium Channel, Site 2	Rat		664	6.1
Sodium Channel, Site 2	Rat		704	6.4
Sodium Channel, Site 2	Rat	70		

Biochemical Screen at Panlabs

63 Radioligand binding assays (10 µM)

Values of inhibition >70% were followed up with determination of IC_{50}

Proliferation Assays at Panlabs

24 tumour cell lines (see at the end of the document)

Growth inhibition; IC_{50} : >1 µM

No significant off target effects identified

Safety pharmacology details

In vivo tests at Pharmacology Discovery Servies (Eurofins)

Off-Target	Species	Test concentration [µmol/l]	Inhibition in % @ 10 µmol	IC50 [µmol/l]	Ki [µmol/l]
Body Temperature - Agonist - Body temperature - 1 Hour	Mouse		98		
Body Temperature - Agonist - Body temperature - 2 Hours	Mouse		99		
Body Temperature - Agonist - Body temperature - 30 Minutes	Mouse		100		
Cholesterol, Normal Serum (Total, HDL, TG) - Agonist - Cholesterol of serum	Mouse		85		
Cholesterol, Normal Serum (Total, HDL, Triglyceride) - Agonist - HDL of serum	Mouse		76		
Cholesterol, Normal Serum (Total, HDL, Triglyceride) - Agonist - Triglyceride (TG) of serum	Mouse		91		
Cholesterol, Normal Serum (Total, HDL, Triglyceride) - Agonist - Triglyceride (TG) of serum	Mouse		92		
Depression, Behavior	Mouse		100		
Gastrointestinal Motility - Agonist - G.I Motility Decrease	Mouse		79		
Gastrointestinal Motility - Agonist - G.I Motility increase	Mouse		79		
Glucose, Serum, Fasted - Glucose	Mouse		139		
Hepatotoxicity, SGPT PharmaProfile - Agonist - S-Glutamic pyruvic transaminase	Mouse		95		
Cardiovascular, Postural Hypotension,Tilt Response - Agonist - Heart Rate - 1 Hour	Rat		94		
Cardiovascular, Postural Hypotension,Tilt Response - Agonist - Tilt Blood Pressure	Rat		89		
Gastric Acidity, Basal - increase - Gastric acidity measurement	Rat		102		

100% = normal

ROCK1/2 Inh Probe BAY-549

In-vivo pharmacokinetics and histopathological findings

		Mouse	Rat	Dog
dose *iv*	[mg/kg]	0.5^{bolus}	0.5^{inf}	0.05^{inf}
CL_{plasma}	[L/(h•kg)]	1.9	1.2	0.23
CL_{blood}	[L/(h•kg)]	3.2	1.7	0.41
V_{ss}	[L/kg]	2.2	1.6	0.83
$t_{1/2}$	[h]	1.5	1.2	2.5
dose *po*	[mg/kg]	nd	0.91	0.10
$AUC_{norm,\ po}$	[kg•h/L]	nd	0.39	3.2
$C_{max,norm}$	[kg/L]	nd	0.089	0.43
BA	[%]	nd	48	73

- The compound is well suited to perform *in-vivo* studies with oral application.

Pharmacokinetics

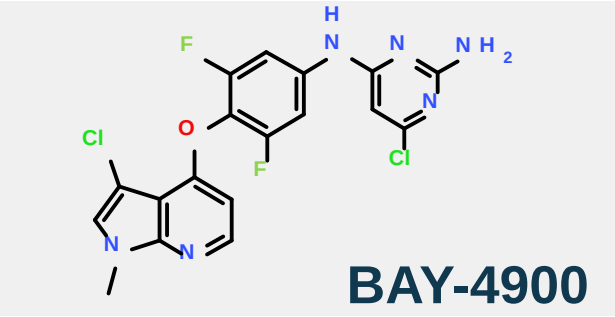
- BAY-549 is a low to medium clearance drug in conscious female NMRI mice, male Wistar rats and female beagle dogs. The **clearance** decreased with increasing size of the animal species. Mice had the highest volume of distribution of 2.2 L/kg. However, its half-life in dogs was longer than in rats and mice.
- The **oral bioavailability** amounted approximately to 50% in male Wistar rats and 75% in female beagle dogs. In dogs, the half-life was significantly longer after oral administration compared to that after i.v. administration indicating a slower absorption by the oral route.

Histopathological findings in rats (SHR), 10 mg/kg, gavage, 2 weeks, n = 5

- Heart: Focal myocardial degeneration (grade 1 or 2)
- Liver: Centrilobular hypertrophy
- Kidneys: Hyperplasia of cortical tubules, basophilic tubules
- Mesenteric vessels: minimal proliferation of vasa vasorum. In SHR controls no such findings were found. However, the number and severity score is comparable to the Wistar controls of this study. An inflammatory reaction was absent.
- Mesenteric lymph node: Erythrophagocytosis/blood resorption.
- Nephrotoxicity similar to that observed in Wistar rats, borderline vascular lesions of equivocal toxicological relevance

ROCK1/2 Inh Probe BAY-549

In vitro profile of Negative Control BAY-4900



POTENCY (IC_{50} [nM])	
Biochem. ROCK-1 (h) IC_{50} [nM]	
Biochem. ROCK-2 (h) IC_{50} [nM]	17.700
Biochem. ROCK-2 (m) IC_{50} [nM]	
Biochem. ROCK-2 (r) IC_{50} [nM]	
Arteria Saphena rabbit IC_{50} [nM]	

Properties & Physchem	
LogD @ pH 7.5	3.0
BEI / LLE	
Sw @ pH 6.5 [mg/L]	0.57
MW / MW corr / TPSA [g*mol^{-1} / Å^2]	437 / 377 / 91

in vitro DMPK Properties						
Caco2 Permeability	P_{app}(A-B) [nm/s]		P_{app}(B-A) [nm/s]		efflux ratio	
	76		41		0.54	
metabolic stability			CL [L/h/kg]		F_{max} [%]	
	liver mics (m / r / d / h)					
	rat hepatocytes					
	human hepatocytes					
CYP inhibition IC_{50} [µM]	1A2	2C8	2C9	2D6	3A4	3A4 preinc.
PXR						

Selectivity	
In-house kinase panel	ongoing
Eurofins @ 1 µM (kinase panel)	

SAFETY	
Cytotox	
hERG IC_{50} [µM]	ongoing

- BAY-4900 has high structural similarity to BAY-4900 and similar Caco2 permeability
- BAY-4900 does not inhibit ROCK

ROCK1/2 Inh Probe BAY-549

Summary / Conclusion

Probe criteria	
Inhibitor/agonist potency: goal is < 50 nM (IC_{50}, Kd)	**Surpasses criteria**; high potency in biochemical ROCK1 assay with $IC_{50} < 1$ nM
Selectivity within target family: goal is > 30-fold	**Surpasses criteria**; selectivity >250 fold vs all other kinases (Upstate panel , Trk-A $IC_{50} = 252$ nM)
Selectivity outside target family: describe the off-targets (which may include both binding and functional data)	**Surpasses criteria**; LeadProfilingScreen Dopamine transporter $IC_{50} = 0.4$ µM
On target cell activity for cell-based targets: goal is < 1 µM IC_{50}/EC_{50}	**Surpasses criteria**; mechanistic tissue assay (arteria saphena rabbit), $IC_{50} = 65$ nM;
On target cell activity for secreted targets: appropriate alternative such as mouse model or other mechanistic biological assay, e.g., explant culture	**Surpasses criteria**; suitable pharmacokinetic profile for *in vivo* studies; *in vivo* efficacy in experimental animal models (rat, dog)
Neg ctrl: *in vitro* potency – > 100 times less; Cell activity – >100 times less potent than the probe	**Surpasses criteria**;

We ask for acceptance of ROCK inhibitor BAY-549 as chemical probe, accompanied by BAY-4900 as negative control

ROCK1/2 Inh Probe BAY-549

Project Team / Acknowledgement

Chemistry
Marcus Bauser
Samir Bennabi
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Jaques Dumas
Jens Ergüden
Achim Feurer
Santiago Figueroa-Pérez
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Joachim Hütter

Physiology
Heimo Ehmke (Univ. Hamburg)

Toxicology
Volker Geiß

Safety Pharmacology
Michael Hoffmann

Pharmaceutical Technology
Alfons Grunenberg
Susanne Zuleger

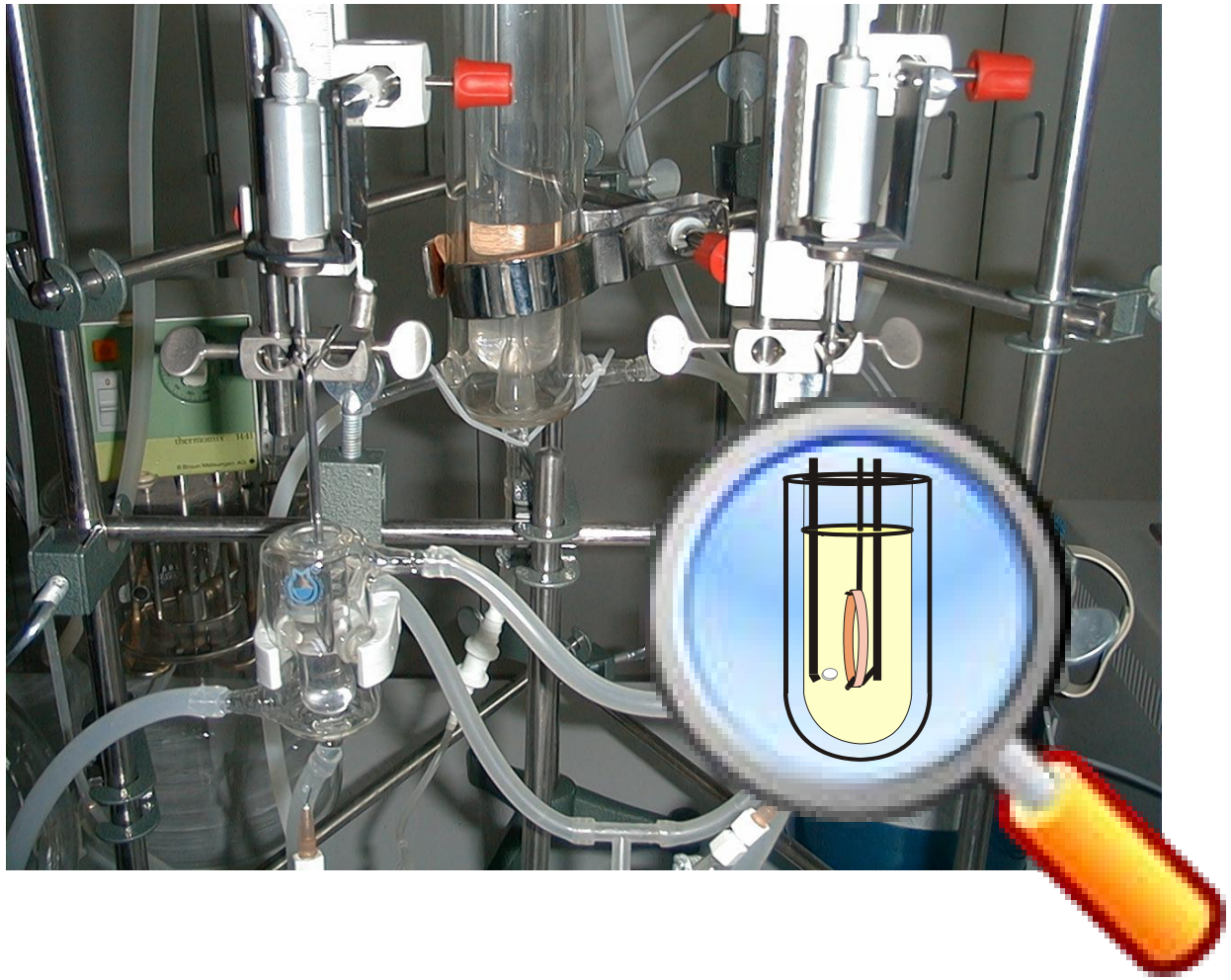
Patents
Gabriele Handke-Ergüden
Alan Graff

PD-CV
Sabine Gelfert-Peukert

Thank You

ROCK1/2 Inh Probe BAY-549

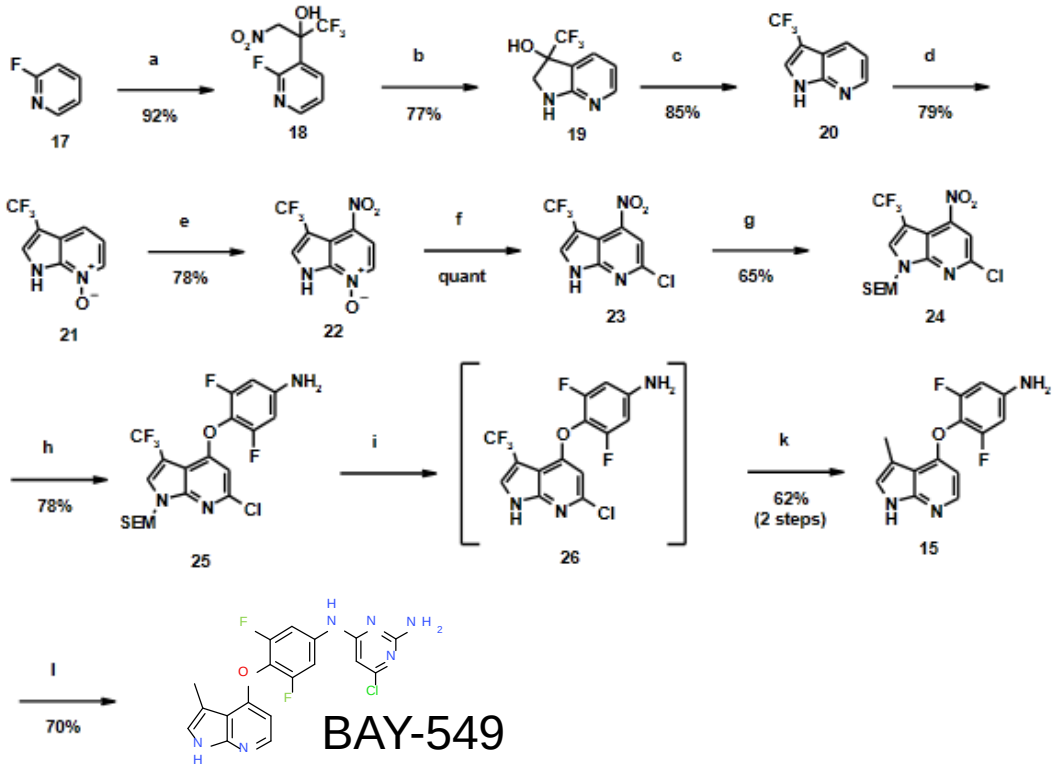
Assay description



Effects of vasoactive compounds on contractions of isolated rabbit saphenous aterery rings induced by phenylephrine: Rabbit saphenous artery rings were connected to isometric force transducers and placed in carbogen-gassed Krebs-Henseleit solution at 37 °C. Contractions in response to phenylephrine treatment are carried out several times. Test compound-dependent changes in subsequent contractions are evaluated as percent of previous control. The IC_{50}-value determines the concentration of test compound needed to cause 50% inhibition of the control contraction.

ROCK1/2 Inh Probe BAY-549

Chemical Synthesis of BAY-549



(a) LDA, THF, -75 °C; CF_3CO_2Et, -78 °C f rt; CH_3NO_2, rt, 92%. (b) H_2 (1 atm), PtO_2, EtOH, rt; filtration, reflux, 77%. (c) $SOCl_2$, Pyr, CH_2Cl_2, rt, 85%. (d) *m*-CPBA, EtOAc, 0 °C, 79%. (e) HNO_3, TFA, 70 °C, 78%. (f) Cl_3CCOCl, HMDS, THF, 0 °C; quant. (g) SEMCl, NaH, DMF, rt, 65%. (h) K_2CO_3, DMSO, 120 °C, 78%. (i) TFA, CH_2Cl_2, rt. (k) $LiAlH_4$, THF, reflux, 62%. (l) 4,6-Dichloropyrimidine-2-amine, water, aq HCl, reflux, 70%.

<u>Numbering of compounds from</u>
H. Schirok, H. Paulsen, W. Kroh, G. Chen, P. Gao, *Org. Proc. Res. Devel.* **2010**, *14*, 168-173.

<u>Starting Materials</u>
2-Fluoropyridine CAS-RN 372-48-5
Ethyl trifluoroacetate CAS-RN 383-63-1
Nitromethane CAS-RN 75-52-5
4-Amino-2,6-difluorophenol CAS-RN 126068-97-7
4,6-Dichloropyrimidine-2-amine CAS-RN 156-83-2

BAY-549 was synthesized in a linear sequence of 11 steps;

ROCK1/2 Inh Probe BAY-549

Chemical Synthesis of BAY-549

Step 1

1,1,1-Trifluoro-2-(2-fluoropyridin-3-yl)-3-nitropropan-2-ol (18). To a solution of freshly prepared LDA (1.48 mol) in THF (3.2 L) at −75 °C was added 2-fluoropyridine (120 g, 1.24 mol), and the mixture was stirred for 4 h at this temperature. To the resulting suspension, ethyl trifluoroacetate (246 g, 1.73 mol) was added, during which the internal temperature should not rise above −45 °C. The reaction was warmed to rt. Nitromethane (134 mL, 2.47 mol) was added, and the reaction was stirred overnight. The solution was poured into HCl (aq 2 N, 17 L), and the mixture was extracted with EtOAc (2 × 8 L). (*Remark:* The retro-nitro-aldol reaction takes place under basic conditions. Therefore, the reaction mixture must be poured into an acidic medium.) The combined organic layers were washed with brine (5 L), dried (Na_2SO_4), and the solvent was evaporated. The crystalline residue was triturated with PE, and the product was collected by suction filtration to yield 290 g (92%) of the title compound. (*Remark:* The DTA analysis of **18** revealed a strongly exothermic decomposition beginning at 140 °C with 2300 kJ/kg.) ^{1}H NMR (300 MHz, DMSO-d_6): δ = 5.10−5.16 (m, 1H), 5.68 (d, J = 13.2 Hz, 1H), 7.25 (ddd, J = 7.7, 4.8, 2.3 Hz, 1H), 8.27 (ddd, J = 10.0, 7.7, 1.9 Hz, 1H), 8.33−8.38 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 73.8 (dq, $^2J_{C,F}$ = 30.0 Hz, $^3J_{C,F}$ = 6.9 Hz), 77.1 (d, $^4J_{C,F}$ = 8.3 Hz), 116.7 (d, $^2J_{C,F}$ = 27.8 Hz), 122.6 (d, $^4J_{C,F}$ = 4.2 Hz), 123.8 (q, $^1J_{C,F}$ = 286 Hz), 141.6 (d, $^3J_{C,F}$ = 3.2 Hz), 148.9 (d, $^3J_{C,F}$ = 15.7 Hz), 159.0 (d, $^1J_{C,F}$ = 236 Hz). HRMS calcd for $C_8H_6F_4N_2O_3$: 254.0315; found: 254.0321.

Step 2

3-(Trifluoromethyl)-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-3-ol (19). Compound **18** (100 g, 393 mmol) was dissolved in EtOH (1.5 L) and stirred under H_2 (1 atm) with PtO_2 (2.23 g, 7.87 mmol) as catalyst. After the consumption of the theoretical amount of H_2, the solution was filtered, and the filtrate was refluxed overnight. Subsequently, the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (1 L) and washed with aq sat. $NaHCO_3$ solution (0.8 L). The aqueous phase was extracted with EtOAc (0.5 L), and the organic layer was dried (Na_2SO_4). The solvent was removed under reduced pressure, and the oily residue was triturated with CH_2Cl_2 (0.3 L). The crystalline product was collected by suction filtration and washed with CH_2Cl_2 (150 mL) to yield 57.2 g (71%) of the title compound. An additional 4.5 g (6%) was obtained after chromatographic purification on silica gel (1.0 kg, eluent: CH_2Cl_2/MeOH, 30:1 to 10:1) of the mother liquor. (*Remark:* Fluoride is liberated during the reaction and etches the glassware.) ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.45 (d, J = 11.7 Hz, 1H), 3.71 (d, J = 11.7 Hz, 1H), 6.59 (dd, J = 7.3, 5.1 Hz, 1H), 6.84 (s, 1H), 6.97 (s, 1H), 7.53 (d, J = 7.3 Hz, 1H), 7.97 (dd, J = 5.1, 1.2 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 52.2, 77.7 (q, $^2J_{C,F}$ = 29.9 Hz), 112.4, 117.7, 125.3 (q, $^1J_{C,F}$ = 284 Hz), 132.9, 149.9, 163.4. HRMS calcd for $C_8H_7F_3N_2O$: 204.0510; found: 204.0515.

Step 3

3-(Trifluoromethyl)-1*H*-pyrrolo[2,3-*b*]pyridine (20). Compound **19** (211 g, 1.03 mol) was dissolved in CH_2Cl_2 (3.2 L). Pyridine (164 g, 2.07 mmol) and thionyl chloride (246 g, 2.07 mmol) were added, and the reaction was stirred for 2 h. Then ice was added, and the reaction was neutralized to pH 5.7 with aq NaOH solution. The solution was extracted with CH_2Cl_2 (2 × 1.5 L), and the combined organic layers were washed with water (1.5 L) and dried (Na_2SO_4). The solvent was removed in vacuo to yield tan crystals. The crude product was triturated with PE (600 mL) for 15 min, and the crystals were collected by suction filtration to yield 164 g (85%) of the title compound. (*Remark:* The DTA analysis of **20** showed a strongly exothermic decomposition beginning at >160 °C with ~850 kJ/kg.) ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.26 (dd, J = 7.9, 4.7 Hz, 1H), 8.05 (d, J = 7.9 Hz, 1H), 8.16 (s, 1H), 8.39 (dd, J = 4.7, 1.3 Hz, 1H), 12.51 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 102.8 (q, $^2J_{C,F}$ = 36.8 Hz), 115.3, 117.2, 124.2 (q, $^1J_{C,F}$ = 266 Hz), 126.9, 127.2 (q, $^3J_{C,F}$ = 5.0 Hz), 144.5, 148.0. HRMS calcd for $C_8H_5F_3N_2$: 186.0405; found: 186.0407.

Step 4

3-(Trifluoromethyl)-1*H*-pyrrolo[2,3-*b*]pyridine 7-Oxide (21). A solution of *m*-chloroperbenzoic acid (335 g, 1.45 mol) in EtOAc (3 L) was dried (Na_2SO_4) and cooled to 0 °C. Compound **20** (180 g, 969 mmol) was added in portions. The mixture was stirred for 1 h during which time white crystals precipitated. They were collected by suction filtration and washed with EtOAc (600 mL) to yield 155 g (79%) of the desired *N*-oxide. (*Remark:* The DTA analysis revealed a weakly exothermic reaction of the mixture.) ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 7.25 (dd, J = 8.0, 6.2 Hz, 1H), 7.67 (d, J = 8.0 Hz, 1H), 8.16 (s, 1H), 8.31 (d, J = 6.2 Hz, 1H), 13.40 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 105.4 (q, $^1J_{C,F}$ = 37.4 Hz), 117.5 118.5, 119.5 (q, $^3J_{C,F}$ = 2.2 Hz), 123.5 (q, $^1J_{C,F}$ = 266 Hz), 127.5 (q, $^1J_{C,F}$ = 5.0 Hz), 132.7, 138.5. HRMS calcd for $C_8H_5F_3N_2O$: 202.0354; found: 202.0348.

Numbering of compounds and procedures from

H. Schirok, H. Paulsen, W. Kroh, G. Chen, P. Gao, *Org. Proc. Res. Devel.* **2010**, *14*, 168-173.

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Step 5

4-Nitro-3-(trifluoromethyl)-1*H*-pyrrolo[2,3-*b*]pyridine 7-Oxide (22). A solution of compound **21** (162 g, 801 mmol) in trifluoroacetic acid (1.9 L) was heated to 70 °C. HNO_3 (65%, 111 mL, 1.60 mol) was added within 10 min. (*Remark:* The reaction was slightly exothermic, and the heating bath was removed for 30 min upon HNO_3 addition during which time the internal temperature was 65−75 °C.) The reaction was heated to 70 °C for 2 h. Then it was poured into an ice/water mixture (5.4 L). The precipitate was collected by suction filtration and washed with water (1.8 L). The product was dried in vacuum to yield 156 g (78%) of the title compound. (*Remark:* The DTA analysis of the nitration mixture resulted in a maximal tolerable internal temperature of 30 °C and a recommended maximal reaction volume of 2 L. The DTA analysis of **22** showed a strongly exothermic decomposition beginning at >105 °C with >2900 kJ/kg. However, the compound was not sensitive to impact or friction.) ^{1}H NMR (DMSO-d_6, 500 MHz): δ = 8.09 (d, J = 6.9 Hz, 1H), 8.46 (s, 1H), 8.49 (d, J = 6.9 Hz, 1H), 14.2 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 105.5 (q, $^2J_{C,F}$ = 37.9 Hz), 110.7, 115.4, 122.6 (q, $^1J_{C,F}$ = 266 Hz), 132.4, 132.7 (q, $^3J_{C,F}$ = 6.5 Hz), 137.0, 141.3. HRMS calcd for $C_8H_4F_3N_3O_3$: 247.0205; found: 247.0209.

Step 6

6-Chloro-4-nitro-3-(trifluoromethyl)-1*H*-pyrrolo[2,3-*b*]pyridine (23). Compound **22** (152 g, 615 mmol) was dissolved in THF (2.6 L). Hexamethyldisilazane (130 mL, 615 mmol) was added, and the mixture was cooled to 0 °C. An orange precipitate was formed. Trichloroacetyl chloride (279 g, 1.54 mol) was added dropwise during which the precipitate dissolved, and the color changed to yellow. The mixture was subsequently warmed to rt. The mixture was stirred for 2 h and then poured into water (13 L) and extracted with EtOAc (2 × 5.3 L). The combined organic layers were washed with brine (2.6 L) and dried (Na_2SO_4). The solvent was evaporated, and the residue was triturated with PE. The product was collected by suction filtration to give the desired compound in quantitative yield (200 g). ^{1}H NMR (DMSO-d_6, 500 MHz): δ = 8.08 (s, 1H), 8.63 (s, 1H), 13.62 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 102.5 (q, $^2J_{C,F}$ = 38.2 Hz), 105.2 (q, $^3J_{C,F}$ = 1.8 Hz), 111.6, 129.9 (q, $^1J_{C,F}$ = 266 Hz), 133.6 (q, $^3J_{C,F}$ = 5.7 Hz), 144.3, 148.5, 149.9. HRMS calcd for $C_8H_3ClF_3N_3O_2$: 264.9866; found: 264.9872.

Step 7

6-Chloro-4-nitro-3-(trifluoromethyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-pyrrolo[2,3-*b*]pyridine (24). To compound**23** (204 g, 634 mmol) and [2-(chloromethoxy)ethyl](trimethyl)silane (116 g, 697 mmol) in DMF (2.5 L) was added NaH (60% suspension in mineral oil, 25.4 g, 634 mmol) in portions, and the mixture was stirred at rt for 45 min. The mixture was poured into brine and extracted with EtOAc (2 × 4 L). The combined organic layers were washed with brine (2 L), dried (Na_2SO_4), and evaporated. The crude product was purified by column chromatography on silica gel (6.0 kg, eluent: PE/EtOAc, 95:5) to yield 162 g (65%) of the title compound. ^{1}H NMR (DMSO-d_6, 300 MHz): δ = −0.10 (s, 9H), 0.84 (dd, J = 8.1, 8.0 Hz, 2H), 3.58 (dd, J = 8.1, 8.0 Hz 2H), 5.70 (s, 2H), 8.16 (s, 1H), 8.83 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = −1.51, 17.0, 66.4, 73.9, 102.7 (q, $^2J_{C,F}$ = 38.6 Hz), 105.8 (q, $^3J_{C,F}$ = 1.9 Hz), 112.8, 122.7 (q, $^1J_{C,F}$ = 266 Hz), 136.1 (q, $^3J_{C,F}$ = 6.0 Hz), 145.1, 148.8, 148.9. HRMS calcd for $C_{14}H_{17}ClF_3N_3O_3Si$ + [H^+]: 396.0753; found: 396.0753.

Numbering of compounds and procedures from

H. Schirok, H. Paulsen, W. Kroh, G. Chen, P. Gao, *Org. Proc. Res. Devel.* **2010**, *14*, 168-173.

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Step 8

4-[(6-Chloro-3-(trifluoromethyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-pyrrolo[2,3-*b*]-pyridin-4-yl)oxy]-3,5-difluoroaniline (25). Compound **24** (71.6 g, 181 mmol) was dissolved in DMSO (0.7 L) under argon. K_2CO_3 (75.0 g, 543 mmol) and 4-amino-2,6-difluorophenol (39.4 g, 271 mmol) were added, and the mixture was heated to 120 °C for 3 h. The mixture was poured into water (3.5 L) and extracted with EtOAc (2 × 1.5 L). The combined organic layers were washed with brine (1 L) and dried (Na_2SO_4), and the solvent was evaporated. The crude product was purified by column chromatography on silica gel (4.0 kg, eluent: PE/EtOAc, 4:1) to yield 72.5 g (96% pure, 78% yield) of the desired product. ^{1}H NMR (DMSO-d_6, 500 MHz): $\delta = -0.09$ (s, 9H), 0.84 (dd, $J = 8.1$, 8.0 Hz, 2H), 3.58 (dd, $J = 8.1$, 8.0 Hz, 2H), 5.62 (s, 2H), 5.90 (s, 2H), 6.40 (d, $J = 11.0$ Hz, 2H), 6.53 (s, 1H), 8.39 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): $\delta = -1.50$, 17.0, 66.1, 73.4, 96.9 (dd, $^2J_{C,F} = 23.1$ Hz, $^3J_{C,F} = 4.0$ Hz), 101.3, 102.9 (q, $^2J_{C,F} = 38.8$ Hz), 104.7 (q, $^3J_{C,F} = 1.6$ Hz), 117.0 (t, $^2J_{C,F} = 16.4$ Hz), 123.1 (q, $^1J_{C,F} = 266$ Hz), 130.2 (q, $^3J_{C,F} = 5.6$ Hz), 146.7, 148.3, 148.7 (t, $^3J_{C,F} = 13.4$ Hz), 155.2 (dd, $^1J_{C,F} = 244$ Hz, $^3J_{C,F} = 6.9$ Hz), 159.2. HRMS calcd for $C_{20}H_{21}ClF_5N_3O_2Si$ + [H$^+$]: 494.1085; found: 494.1086.

Step 9 and 10

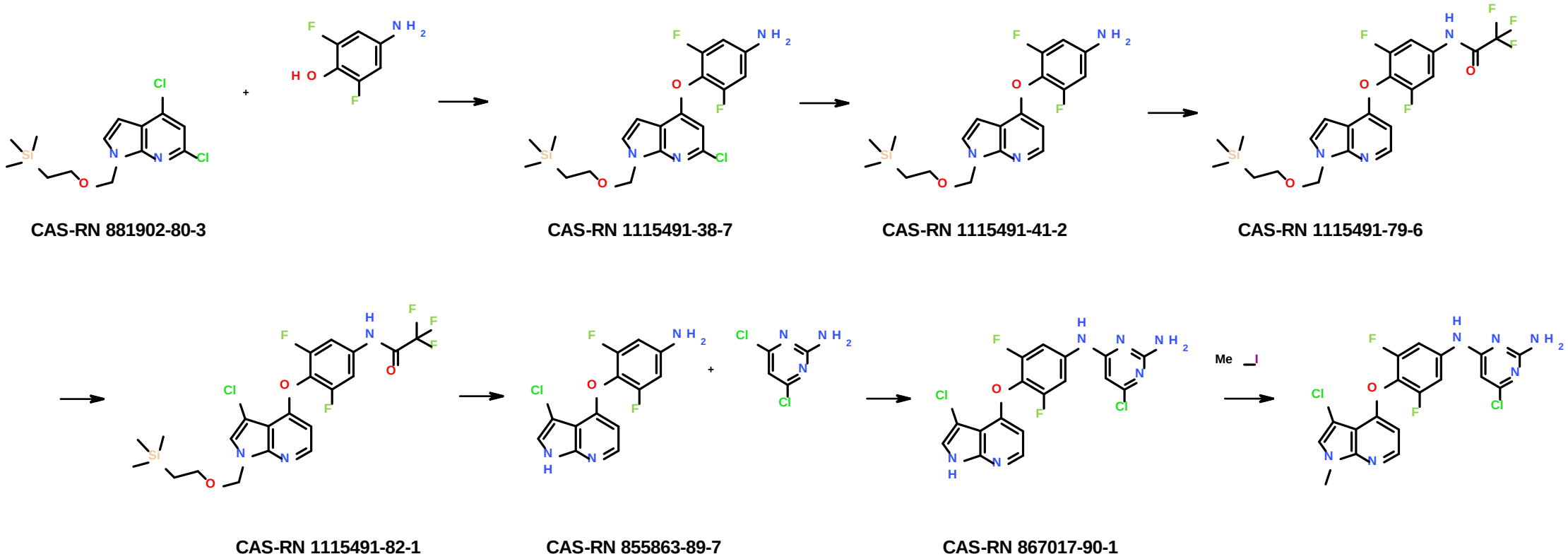
3,5-Difluoro-4-[(3-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)oxy]aniline (15). Compound **25** (20.0 g, 40.5 mmol) was dissolved in CH_2Cl_2 (200 mL) and trifluoroacetic acid (200 mL) was added. The mixture was stirred at rt for 1.5 h and then concentrated in vacuo. The residue was diluted with EtOAc (400 mL), and the solution was washed with brine (300 mL), dried (Na_2SO_4), and the solvent was evaporated. To the residue was added toluene (100 mL), and the mixture was concentrated again. This procedure was repeated three times to give the SEM-deprotected compound. The crude product (14.7 g, 40.4 mmol) was dissolved in THF (200 mL) under nitrogen and slowly treated with $LiAlH_4$ (2.4 M in THF, 170 mL, 408 mmol). The reaction was heated to reflux for 10 h. Then a second portion of $LiAlH_4$ (2.4 M in THF, 170 mL, 408 mmol) was added, and the mixture was heated to reflux for additional 14 h. Excess of $LiAlH_4$ was then hydrolyzed by the addition of aq 10% NaOH solution (100 mL). The solid was removed by filtration, and the filtrate was concentrated in vacuo to leave an aqueous solution which was extracted with EtOAc (2 × 200 mL). The combined organic layers were dried (Na_2SO_4), the solvent was evaporated, and the residue was purified by column chromatography on silica gel (500 g, eluent: ethyl acetate/petroleum ether, 1:2) to give the title compound (7.00 g, 62% over two steps). (*Remark:* Fluoride is liberated during the reaction and etches the glassware.) ^{1}H NMR (500 MHz, DMSO-d_6): $\delta = 2.42$ (s, 3H), 5.77 (s, 2H), 6.17 (d, $J = 5.4$ Hz, 1H), 6.40 (d, $J = 10.7$ Hz, 2H), 7.13 (s, 1H), 7.98 (d, $J = 5.4$ Hz, 1H), 11.36 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): $\delta = 12.2$, 97.1 (dd, $^2J_{C,F} = 19.3$ Hz, $^4J_{C,F} = 4.6$ Hz), 98.5, 108.0, 109.2, 118.2 (t, $^2J_{C,F} = 16.4$ Hz), 122.2, 144.4, 148.0 (t, $^3J_{C,F} = 13.2$ Hz), 151.1, 155.9 (dd, $^1J_{C,F} = 243$ Hz, $^3J_{C,F} = 7.3$ Hz), 159.3. HRMS calcd for $C_{14}H_{11}F_2N_3O$ + [H$^+$]: 276.0943; found: 276.0948.

Step 11

6-Chloro-N^4-{3,5-difluoro-4-[(3-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)oxy]phenyl}pyrimidin-2,4-diamine (16). Compound **15** (6.00 g, 21.8 mmol) and 4,6-dichloropyrimidine-2-amine (3.93 g, 24.0 mmol) were suspended in water (80 mL). HCl (4 N aq, 11 mL) was added, and the mixture was heated to reflux for 20 h. Subsequently, the mixture was basified with conc. aq NaOH solution. Some dmf was added, and the aqueous phase was extracted with EtOAc. The organic layer was washed with water and dried (Na_2SO_4), and the solvent was evaporated. The crude product was triturated with a small volume of ice-cold methanol. The precipitate was collected by suction filtration and washed with CH_2Cl_2 to yield 4.50 g (51%) of the title compound. The mother liquor was concentrated and purified by column chromatography on silica gel (200 g, eluent: CH_2Cl_2/MeOH, 100:4 with increasing proportion of MeOH) to yield further 1.70 g (19%) of the title compound. ^{1}H NMR (400 MHz, DMSO-d_6): $\delta = 2.44$ (s, 3H), 6.04 (s, 1H), 6.21 (d, $J = 5.4$ Hz, 1H), 6.99 (br s, 2H), 7.16 (s, 1H), 7.74 (d, $J = 10.6$ Hz, 2H), 7.99 (d, $J = 5.4$ Hz, 1H), 9.77 (s, 1H), 11.43 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): $\delta = 12.0$, 94.3, 98.3, 103.1 (dd, $^2J_{C,F} = 21.3$ Hz, $^4J_{C,F} = 4.8$ Hz), 107.7, 108.9, 122.3, 123.0 (t, $^2J_{C,F} = 16.1$ Hz), 138.6 (t, $^3J_{C,F} = 13.0$ Hz), 144.1, 151.1, 154.9 (dd, $^1J_{C,F} = 245$ Hz, $^3J_{C,F} = 6.8$ Hz), 158.3, 158.4, 161.3, 162.6. HRMS calcd for $C_{18}H_{13}ClF_2N_6O$ + [H$^+$]: 403.0881; found: 403.0865.

ROCK1/2 Inh Probe BAY-549

Chemical Synthesis of Negative Control BAY-4900



CAS-RN 881902-80-3 **CAS-RN 1115491-38-7** **CAS-RN 1115491-41-2** **CAS-RN 1115491-79-6**

CAS-RN 1115491-82-1 **CAS-RN 855863-89-7** **CAS-RN 867017-90-1**

BAY-4900 was synthesized in a linear sequence of 8 steps starting from CAS-RN 881902-80-3

ROCK1/2 Inh Probe BAY-549

Selectivity profile in more detail

Kinase Profiling at Upstate

114 Kinases (10 µM)

23 Different kinases	IC_{50} : 1-10 µM
TrkA	IC_{50} : 252 nM
Flt3	IC_{50} : 303 nM
PKA	IC_{50} : 734 nM
ROCK-2 (rat);	IC_{50} : <1 nM
ROCK-2 (human);	IC_{50} : 1 nM

Inhouse Kinase Data:

Mps1	IC_{50} : 0.54 µM
AKT1	IC_{50} : 0.82 µM
FLT4	IC_{50} : 1.04 µM
NUAK1 (10 µM ATP)	IC_{50} : 1.66 µM
TrkA	IC_{50} : 3.84 µM
KDR	IC_{50} : 3.89 µM
FMS	IC_{50} : 4.21 µM
PDGFRβ	IC_{50} : 4.90 µM

Bub1, CDK2, CDK9, cKit, EGFR, FGFR1, FGFR3, FGFR4, GSK3β, IKKβ, IRAK4, MKNK1, NEK2, SRPK1, TAO2, Tie2: IC_{50} > 20 µM

	BR 4463 @ 10 µM
Abl(h)	86
Abl(m)	67
Abl(T315I)(h)	72
ALK(h)	22
AMPK(r)	92
Arg(h)	63
Arg(m)	64
Aurora-A(h)	23
Axl(h)	15
Blk(m)	84
Bmx(h)	82
BTK(h)	99
CaMKII(r)	93
CaMKIV(h)	108
CDK1/cyclinB(h)	122
CDK2/cyclinA(h)	80
CDK2/cyclinE(h)	60
CDK3/cyclinE(h)	67
CDK5/p35(h)	93
CDK6/cyclinD3(h)	91
CDK7/cyclinH/MAT1(h)	43
CHK1(h)	91
CHK2(h)	36
CK1δ(h)	35
CK1(y)	56
CK2(h)	94
c-RAF(h)	24
CSK(h)	121
cSRC(h)	104
EGFR(h)	115
EphA2(h)	96
EphB2(h)	112
EphB4(h)	109
ErbB4(h)	95
Fes(h)	72
FGFR3(h)	88
Fgr(h)	73
Flt3(h)	2
Fms(h)	36
Fyn(h)	86
GSK3α(h)	87
GSK3β(h)	101
IGF-1R(h)	59
IKKα(h)	141
IKKβ(h)	128
IR(h)	41
JNK1α1(h)	78
JNK2α2(h)	84
JNK3(h)	92
Lck(h)	50
Lyn(h)	96
Lyn(m)	69

MAPK1(h)	99
MAPK2(h)	83
MAPK2(m)	79
MAPKAP-K2(h)	106
MEK1(h)	91
Met(h)	18
MKK4(m)	121
MKK6(h)	94
MKK7β(h)	80
MSK1(h)	14
MST2(h)	41
NEK2(h)	81
p70S6K(h)	16
PAK2(h)	98
PAR-1Bα(h)	67
PDGFRα(h)	97
PDGFRβ(h)	128
PDK1(h)	72
Pim-1(h)	80
PKA(b)	6
PKA(h)	5
PKBα(h)	16
PKBβ(h)	15
PKBγ(h)	13
PKCα(h)	94
PKCβII(h)	92
PKCβII(h)	91
PKCγ(h)	67
PKCδ(h)	84
PKCε(h)	72
PKCη(h)	76
PKCι(h)	82
PKCμ(h)	40
PKCθ(h)	58
PKCζ(h)	107
PKD2(h)	72
Plk3(h)	103
PRAK(h)	64
PRK2(h)	30
Ret(h)	48
ROCK-II(h)	0
ROCK-II(r)	-2
Ros(h)	90
Rse(h)	85
Rsk1(h)	28
Rsk1(r)	21
Rsk2(h)	39
Rsk3(h)	52
SAPK2a(h)	79
SAPK2b(h)	99
SAPK3(h)	96
SAPK4(h)	84
SGK(h)	24
Syk(h)	80
Tie2(h)	74
TrkA(h)	1
TrkB(h)	16
Yes(h)	77
ZAP-70(h)	108

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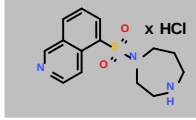
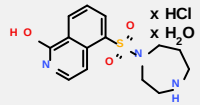
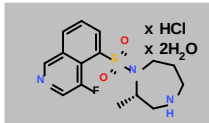
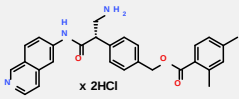
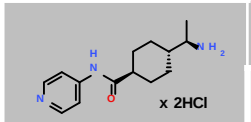
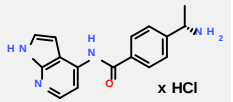
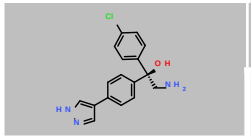
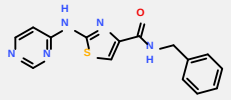
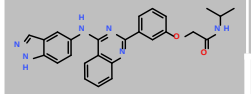
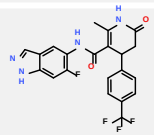
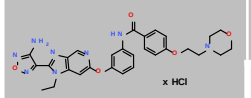
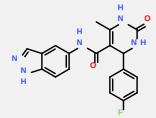
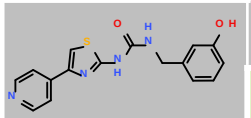
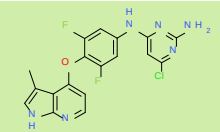
Comparison with commercially available compounds[1)]

	Name	ROCK	ROCK1	ROCK2	others	Sol. Water	
	Fasudil			330 nM (Ki)	PKA 1.6 µM, PKG 1.6 µM, PKC 3.3 µM	66 mg/mL	
	Hydroxyfasudil		730 nM	720 nM	PKA 37 µM	60 mg/mL	
	Ripasudil (K-115)		51 nM	19 nM		79 mg/mL	
	Netarsudil (AR-13324)	2 nM				54 mg/mL	
	Y-27632 x 2HCl		140 nM	300 nM		64 mg/mL	
	Y-39983 x HCl	3.6 nM				70 mg/mL	
	AT13148		6 nM	4 nM	p70S6K 8 nM, Akt1 38 nM, PKA 3 nM	insoluble	
	Thiazovivin	ca. 500 nM				insoluble	
	KD025 (SLx-2119(			60 nM		insoluble	
	GSK429286A		14 nM	63 nM		insoluble	
	GSK269962A HCl		1.6 nM	4 nM	MSK1 49 nM, RSK1 132 nM	insoluble	
	GSK180736A		100 nM		GRK2 770 nM	insoluble	
	RKI-1447		14.5 nM	6.2 nM		insoluble	
	BAY-549		**0.6 nM[2)]**	**1.1 nM[2)]**			

1) https://www.selleckchem.com/ROCK.html
2) BAYER data

BAY-549:
highly potent and in-depth characterized dual ROCK1/2 inhibitor with novel scaffold, good selectivity and an inactive structural analog

PHARMACOLOGY DATA REPORT

Bayer HealthCare AG

MDSPS PT#: 1044288 and 1044289
WO#: 1013906

ALT CODE 1:

ALT CODE 2:

ALT CODE 3:

SAMPLE(S): BAY-549 = BR-4474

M.W.: Assumed as 300

STRUCTURE:

MDS Pharma Services
Discovery

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E. METHODS

2. Determination of IC_{50}, TGI and LC_{50}

The measured results was calculated by the following formula:

PG (%)=100 × (Mean F_{test} - Mean F_{time0})/(Mean F_{ctrl} - Mean F_{time0})

If (Mean F_{test} - Mean F_{time0}) < 0, then

PG (%)= 100 × (Mean F_{test} - Mean F_{time0})/(Mean F_{time0} - Mean F_{blank})

Where:

PG: percent growth

Mean F_{time0} = The average of 2 measured fluorescent intensities of reduced alamarBlue at the time just before exposure of cells to the test substance.

Mean F_{test} = The average of 2 measured fluorescent intensities of alamarBlue after 72-hour exposure of cells to the test substance.

Mean F_{ctrl} = The average of 2 measured fluorescent intensities of alamarBlue after 72-hour incubation without the test substance.

Mean F_{blank} = The average of 2 measured fluorescent intensities of alamarBlue in medium without cells after 72-hour incubation.

A decrease of 50% or more (≥50%) in fluorescent intensity relative to vehicle-treated control indicated significant cell growth inhibition, cytostatic or cytotoxic activity, and a semi-quantitative IC_{50}, TGI and LC_{50} were then determined by nonlinear regression using GraphPad Prism (GraphPad Software, USA).

IC_{50} (50% Inhibition Concentration): Test compound concentration where the increase from $time_0$ in the number or mass of treated cells was only 50% as much as the corresponding increase in the vehicle-control at the end of experiment.

TGI (Total Growth Inhibition): Test compound concentration where the number or mass of treated cells at the end of experiment was equal to that at $time_0$.

LC_{50} (50% Lethal Concentration): Test compound concentration where the number or mass of treated cells at the end of experiment was half that at $time_0$.

F. TABLES OF RESULTS

Table 1-2

Effect of Test Substance on the Growth of 24 Tumor Cell Lines

Treatment	Assay Name	Percent Growth (Mean ± SEM, n = 2)							
		Blank	$Time_0$	Vehicle	Concentration (µM)				
					100	10	1	0.1	0.01
PT# 1044289	370000, Breast, MCF-7	-100	0	100	6±4	57±2	87±1	89±9	92±3
(BR-4474)	370100, Breast, T-47D	-100	0	100	-45±2	-2±4	86±1	97±8	106±6
=BAY-549	370200, Colon, DLD-1	-100	0	100	5±1	24±1	66±4	94±2	101±0
	370300, Colon, HT-29	-100	0	100	-38±9	10±4	75±1	95±1	100±8
	370400, Kidney, A-498	-100	0	100	-1±2	26±2	98±5	93±5	99±2
	370500, Kidney, ACHN	-100	0	100	-25±4	27±0	60±10	106±2	103±1
	370600, Leukemia, HL-60	-100	0	100	35±2	53±6	89±5	88±4	98±4
	370700, Leukemia, K562	-100	0	100	32±6	42±3	69±4	92±5	95±7
	370800, Liver, HC-4	-100	0	100	-56±8	12±8	59±5	94±1	102±5
	370850, Liver, Hep 3B	-100	0	100	-4±5	11±0	51±6	82±3	90±8
	370900, Liver, HepG2	-100	0	100	-3±6	18±4	62±2	95±6	101±4
	371000, Lung, A549	-100	0	100	-15±3	7±2	42±2	97±4	103±4
	371050, Lung, NCI-H460	-100	0	100	-45±12	2±1	50±2	82±3	98±1
	371100, Lung, PC-6	-100	0	100	-65±6	32±7	70±9	84±5	105±9
	371200, Lymphoma, H33HJ- JA1	-100	0	100	80±7	98±1	97±6	103±5	96±8
	371300, Lymphoma, U937	-100	0	100	17±5	56±7	104±2	103±2	101±1
	371350, Melanoma B16-F0	-100	0	100	-46±5	53±9	106±1	94±4	98±5
	371400, Melanoma, SK-MEL-5	-100	0	100	-32±1	65±6	98±3	89±8	106±3
	371500, Neuroepithelioma, SK-N-MC	-100	0	100	-72±8	38±3	98±5	103±4	103±6
	371700, Pancreas, MIA PaCa-2	-100	0	100	-23±5	45±0	88±3	98±1	95±3
	371800, Pancreas, PANC-1	-100	0	100	-31±9	55±1	87±2	86±3	98±8
	371900, Prostate, PC-3	-100	0	100	29±3	48±1	58±3	102±5	97±2
	372000, Skin, A431	-100	0	100	-36±5	35±3	77±3	103±6	92±9
	372100, Stomach, KATO III	-100	0	100	19±7	27±7	66±8	99±11	98±7

A decrease of 50% or more (≥50%) in fluorescent intensity relative to vehicle-treated control indicates significant growth inhibition, cytostatic or cytotoxic activity.

F. TABLES OF RESULTS

Table 1-3

Effect of Test Substance on the Growth of 24 Tumor Cell Lines

Treatment	Assay Name	Percent Growth (Mean ± SEM, n = 2)							
		Blank	$Time_0$	Vehicle	Concentration (μM)				
					10	1	0.1	0.01	0.001
Mitomycin	370000, Breast, MCF-7	-100	0	100	-67±9	11±2	31±4	81±6	98±3
	370100, Breast, T-47D	-100	0	100	-75±0	5±5	37±8	107±4	97±5
	370200, Colon, DLD-1	-100	0	100	-9±9	34±0	53±4	100±1	100±5
	370300, Colon, HT-29	-100	0	100	-44±9	25±7	67±7	107±4	104±3
	370400, Kidney, A-498	-100	0	100	-93±2	-23±5	45±1	75±4	95±2
	370500, Kidney, ACHN	-100	0	100	-86±2	-33±7	30±1	89±2	93±2
	370600, Leukemia, HL-60	-100	0	100	-92±9	-17±11	31±2	81±4	93±4
	370700, Leukemia, K562	-100	0	100	-42±4	10±1	67±5	80±8	97±8
	370800, Liver, HC-4	-100	0	100	-72±0	-5±8	14±2	85±9	100±9
	370850, Liver, Hep 3B	-100	0	100	-68±0	8±2	22±7	97±10	96±9
	370900, Liver, HepG2	-100	0	100	-99±2	-19±0	34±2	91±1	99±6
	371000, Lung, A549	-100	0	100	-92±2	-15±3	22±2	76±2	99±1
	371050, Lung, NCI-H460	-100	0	100	-100±0	-28±6	8±2	79±4	104±3
	371100, Lung, PC-6	-100	0	100	-73±3	9±5	58±6	98±5	94±10
	371200, Lymphoma, H33HJ- JA1	-100	0	100	-90±6	-58±0	38±3	53±0	77±2
	371300, Lymphoma, U937	-100	0	100	-84±8	-16±4	19±1	88±5	96±2
	371350, Melanoma B16-F0	-100	0	100	-90±7	-29±9	25±8	68±8	89±9
	371400, Melanoma, SK-MEL-5	-100	0	100	-91±1	-18±2	54±5	76±8	93±10
	371500, Neuroepithelioma, SK- N- MC	-100	0	100	-91±5	-63±9	12±1	91±1	101±2
	371700, Pancreas, MIA PaCa-2	-100	0	100	-96±5	12±1	40±7	94±5	97±2
	371800, Pancreas, PANC-1	-100	0	100	-61±2	-1±1	19±1	91±7	91±3
	371900, Prostate, PC-3	-100	0	100	-47±3	31±4	60±4	93±6	102±2
	372000, Skin, A431	-100	0	100	-95±2	-14±9	29±1	83±9	97±7
	372100, Stomach, KATO III	-100	0	100	-59±6	-4±9	53±2	85±5	101±2

A decrease of 50% or more (≥50%) in fluorescent intensity relative to vehicle-treated control indicates significant growth inhibition, cytostatic or cytotoxic activity.

F. TABLES OF RESULTS

Table 2-2

The Summary of IC_{50}, TGI and LC_{50} Values

Treatment	Asssay #	Assay Name	[a]IC_{50}	[b]TGI	[c]LC_{50}
PT# 1044289	370000	Tumor, Breast, MCF-7	11 μM	>100 μM	>100 μM
(BR-4474)	370100	Tumor, Breast, T-47D	2.9 μM	15 μM	75 μM
	370200	Tumor, Colon, DLD-1	2.4 μM	>100 μM	>100 μM
BAY-549	370300	Tumor, Colon, HT-29	2.9 μM	19 μM	>100 μM
	370400	Tumor, Kidney, A-498	7.8 μM	70 μM	>100 μM
	370500	Tumor, Kidney, ACHN	3.6 μM	32 μM	>100 μM
	370600	Tumor, Leukemia, HL-60	21 μM	>100 μM	>100 μM
	370700	Tumor, Leukemia, K562	8.3 μM	>100 μM	>100 μM
	370800	Tumor, Liver, HC-4	2.1 μM	13 μM	82 μM
	370850	Tumor, Liver, Hep 3B	1.3 μM	45 μM	>100 μM
	370900	Tumor, Liver, HepG2	3.1 μM	54 μM	>100 μM
	371000	Tumor, Lung, A549	1.5 μM	25 μM	>100 μM
	371050	Tumor, Lung, NCI-H460	1.1 μM	11 μM	>100 μM
	371100	Tumor, Lung, PC-6	4.2 μM	18 μM	76 μM
	371200	Tumor, Lymphoma, H33HJ-JAI	>100 μM	>100 μM	>100 μM
	371300	Tumor, Lymphoma, U937	14 μM	>100 μM	>100 μM
	371350	Tumor, Melanoma, B16-F0	12 μM	36 μM	>100 μM
	371400	Tumor, Melanoma, SK-MEL-5	16 μM	50 μM	>100 μM
	371500	Tumor, Neuroepithelioma, SK-N-MC	7.9 μM	20 μM	52 μM
	371700	Tumor, Pancreas, MIA PaCa-2	8.9 μM	48 μM	>100 μM
	371800	Tumor, Pancreas, PANC-1	11 μM	45 μM	>100 μM
	371900	Tumor, Prostate, PC-3	9.4 μM	>100 μM	>100 μM
	372000	Tumor, Skin, A431	5.5 μM	31 μM	>100 μM
	372100	Tumor, Stomach, KATO III	3.2 μM	>100 μM	>100 μM

[a]IC_{50} (50% Inhibition Concentration): Test compound concentration where the increase from $time_0$ in the number or mass of treated cells was only 50% as much as the corresponding increase in the vehicle-control at the end of experiment.

[b]TGI (Total Growth Inhibition): Test compound concentration where the number or mass of treated cells at the end of experiment was equal to that at $time_0$.

[c]LC_{50} (50% Lethal Concentration): Test compound concentration where the number or mass of treated cells at the end of experiment was half that at $time_0$.

A semi-quantitative determination of IC_{50}, TGI and LC_{50} was carried out by nonlinear regression analysis using GraphPad Prism (GraphPad Software, USA).

F. TABLES OF RESULTS

Table 2-3

The Summary of IC_{50}, TGI and LC_{50} Values

Treatment	Assay #	Assay Name	[a]IC_{50}	[b]TGI	[c]LC_{50}
Mitomycin	370000	Tumor, Breast, MCF-7	0.081 μM	0.76 μM	7.1 μM
	370100	Tumor, Breast, T-47D	0.13 μM	0.76 μM	4.4 μM
	370200	Tumor, Colon, DLD-1	0.36 μM	5.6 μM	>10 μM
	370300	Tumor, Colon, HT-29	0.38 μM	2.2 μM	>10 μM
	370400	Tumor, Kidney, A-498	0.079 μM	0.40 μM	2.0 μM
	370500	Tumor, Kidney, ACHN	0.060 μM	0.31 μM	1.6 μM
	370600	Tumor, Leukemia, HL-60	0.061 μM	0.36 μM	2.2 μM
	370700	Tumor, Leukemia, K562	0.22 μM	1.7 μM	>10 μM
	370800	Tumor, Liver, HC-4	0.048 μM	0.43 μM	3.8 μM
	370850	Tumor, Liver, Hep 3B	0.082 μM	0.69 μM	5.8 μM
	370900	Tumor, Liver, HepG2	0.082 μM	0.38 μM	1.8 μM
	371000	Tumor, Lung, A549	0.047 μM	0.32 μM	2.1 μM
	371050	Tumor, Lung, NCI-H460	0.036 μM	0.20 μM	1.2 μM
	371100	Tumor, Lung, PC-6	0.21 μM	1.0 μM	4.9 μM
	371200	Tumor, Lymphoma, H33HJ-JAI	0.027 μM	0.17 μM	1.1 μM
	371300	Tumor, Lymphoma, U937	0.052 μM	0.34 μM	2.3 μM
	371350	Tumor, Melanoma, B16-F0	0.035 μM	0.25 μM	1.8 μM
	371400	Tumor, Melanoma, SK-MEL-5	0.10 μM	0.50 μM	2.3 μM
	371500	Tumor, Neuroepithelioma, SK-N-MC	0.042 μM	0.15 μM	0.54 μM
	371700	Tumor, Pancreas, MIA PaCa-2	0.15 μM	0.72 μM	3.5 μM
	371800	Tumor, Pancreas, PANC-1	0.056 μM	0.59 μM	6.1 μM
	371900	Tumor, Prostate, PC-3	0.32 μM	2.1 μM	>10 μM
	372000	Tumor, Skin, A431	0.064 μM	0.37 μM	2.1 μM
	372100	Tumor, Stomach, KATO III	0.12 μM	0.87 μM	6.1 μM

[a]IC_{50} (50% Inhibition Concentration): Test compound concentration where the increase from $time_0$ in the number or mass of treated cells was only 50% as much as the corresponding increase in the vehicle-control at the end of experiment.

[b]TGI (Total Growth Inhibition): Test compound concentration where the number or mass of treated cells at the end of experiment was equal to that at $time_0$.

[c]LC_{50} (50% Lethal Concentration): Test compound concentration where the number or mass of treated cells at the end of experiment was half that at $time_0$.

A semi-quantitative determination of IC_{50}, TGI and LC_{50} was carried out by nonlinear regression analysis using GraphPad Prism (GraphPad Software, USA).

H. REFERENCES

1. Ahmed, S. A., Gogal Jr., R. M. and Walsh, J. E. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: An alternative to [^{3}H]-thymidine incorporation assay. Journal of Immunological Methods 170: 211-224, 1994.

2. Boyd, M. R., Status of the NCI preclinical antitumor drug discovery screen. (Published by J. B. Lippincott Company, Philadelphia, PA 19105, USA) Principles & Practices of Oncology Updates 3 # 10: 1-12, 1989.

3. Boyd, M. R. et al. Data display and analysis strategies for the NCI disease-oriented *in vitro* antitummor drug screen. In: Cytotoxic anti-cancer drugs: models and concepts for drug discovery and development. Boston: Kluwer Academic Page: 11-34, 1992.