Supporting Information

SAR156497 an exquisitely selective inhibitor of Aurora kinases

Jean-Christophe Carry,[¶] François Clerc,[¶] Hervé Minoux,[§] Laurent Schio,[¶] Jacques Mauger,[#] Anil Nair,[#] Eric Parmantier,[¶] Ronan Le Moigne,^{¶,¶} Cécile Delorme,[¶] Jean-Paul Nicolas,[¶] Alain Krick,[†] Pierre-Yves Abécassis,[†] Véronique Crocq-Stuerga,[⊥] Stéphanie Pouzieux,[§] Laure Delarbre,[§] Sébastien Maignan,[§] Thomas Bertrand,[§] Kirsten Bjergarde,[#] Nina Ma,[#] Sylvette Lachaud,[¶] Houlfia Guizani,[¶] Rémi Lebel,[¶] Gilles Doerflinger,[¶] Sylvie Monget,[¶] Sébastien Perron,[¶] Francis Gasse,^{¶×} Odile Angouillant-Boniface,[‡] Bruno Filoche-Rommé,[¶] Michel Murer,^{‡×} Sylvie Gontier,^{‡×} Céline Prévost,[‡] Marie-Line Monteiro[‡] and Cécile Combeau[¶]*

[¶]Oncology Drug Discovery, [§] Structure Design Informatics, [†] Disposition Safety Animal Research, [⊥] Chemical Development, [‡] Analytical Sciences, Sanofi, 13 quai Jules Guesde, 94403 Vitry-sur-Seine, France. [#] Combinatorial Techno Center, Sanofi, 2090 East Innovation Park Dr., Oro Valley, AZ 85755-1965, USA

Supporting Information Content

- S-1 Selectivity data
- S-2 Cellular evaluation of cpd. **47** (SAR156497)
- S-3 Overall profile of cpd. **47** (SAR156497)
- S-4 Chemistry
- S-5 *In vitro* assays
- S-6 *In vivo* assays

- S-7 Crystallographic studies

S-1 Selectivity Data

Selectivity Data of Compound 16.

Table 1: Compound 16 selectivity data

	IC50 (nM)		IC50 (nM)
Cell Cycle Kinases		Ser/Thr Kinases	
Aurora A	3	CDK5/p25	>10000
AuroraB/Incenp	4	GSK3b	>10000
AuroraC/Incenp	4	ALK4	>10000
CDK1/B1	>10000	PKA	>10000
CDK2/E	>10000	PKCb	>10000
CDK2/A2	>10000	PKCd	>10000
CDK4/D1	>10000	PKG1a	>10000
CDK7/H	>10000	ASK1	>10000
CDK9/T1	>10000	CAMK2	>10000
PLK1	>10000	CHK1	>10000
CDC7/Dbf4	>10000	CK1d	>10000
Tyrosine Kinases		CK1e	>10000
EGFR	>10000	CK2	>10000
FGFR1	>10000	c-RAF	>10000
FAK	>10000	DAPK2	>10000
PYK2	>10000	IRAK1	>10000
BTK	>10000	MAPK1	>10000
IGF1R	>10000	MEK1	>10000
IRK	>10000	MEKK1	>10000
ITK	>10000	MLK1	>10000
VEGFR2	>10000	MNK2	>10000
PDGFRb	>10000	PI3Kb	>10000
SYK	>10000	AKT	>10000
TIE2	>10000	bARK1	>10000
AXL	>10000	JNK3	>10000
c-MET	>10000	PAK3	>10000
CSK	>10000	PDK4	>10000
DYRK2	>10000	RHODK	>10000
EPHB2	>10000	ROCK2	>10000
FES	>10000	S6K1	>10000
FLT1	>10000	SGK	>10000
JAK3	>10000	SLK1	>10000
ABL	>10000	WNK3	>10000
SRC	>10000	p38	>10000

Selectivity Data of Compound 47 (SAR156497).

Table 2: Compound 47 (SAR156497) selectivity data

	IC50 (nM)		IC50 (nM)
Cell Cycle Kinases		Ser/Thr Kinases	
Aurora A	0.6	CDK5/p25	>10000
AuroraB/Incenp	1	GSK3b	>10000
AuroraC/Incenp	3	ALK4	>10000
CDK1/B1	>10000	PKA	>10000
CDK2/E	>10000	PKCa	>10000
CDK4/D1	>10000	PKCbI	>10000
CDK7/H	>10000	PKCbII	>10000
CDK9/T1	>10000	PKCd	>10000
PLK1	>10000	PKCg	>10000
CDC7/Dbf4	>10000	PKCz	>10000
Tyrosine Kinases		PKG1b	>10000
EGFR	>10000	ASK1	>10000
FGFR1	>10000	CAMK2d	>10000
FGFR4	>10000	CHK1	>10000
PYK2	>10000	CK1d	>10000
BTk	>10000	CK1e	>10000
IGF1R	>10000	CK2	>10000
IRK	>10000	c-RAF	>10000
VEGFR2	>10000	DAPK2	>10000
PDGFRa	>10000	IRAK1	>10000
PDGFRb	>10000	IRAK4	>10000
SYK	>10000	MAPK1	>10000
TIE2	>10000	MEK1	>10000
AXL	>10000	MEKK1	>10000
c-MET	>10000	MLK1	>10000
CSK	>10000	MNK2	>10000
DYRK2	>10000	PI3Ka	>10000
EPHB2	>10000	AKT1	>10000
FES	>10000	bARK1	>10000
FLT2	>10000	PAK1	>10000
FLT3	>10000	PAK2	>10000
JAK1	>10000	PDK4	>10000
JAK2	>10000	ROCK2	>10000
JAK3	>10000	S6K1	>10000
ABL	>10000	SGK	>10000
SRC	>10000	WNK3	>10000
TrkB	>10000	p38	>10000
AMPK(r)	>10000	mTOR	>10000
cKit	>10000	SIK	>10000
		ERK1	>10000

S-2 Cellular evaluation of compound 47 (SAR156497)

Figure 2: Cellular evaluation of compound 47. **A)** Tumor cell line proliferation inhibition by cpd. 47 IC₅₀ vs. solid tumor and haematological cell lines. **B)** DNA content of HCT116 cells treated for 48 h with different concentrations of compound 47. **C)** Inhibition of Aurora A and Aurora B biomarker phosphorylation (p-Aurora A and p-histone H3, respectively) in HCT116 cells treated for 4 h with cpd 47.

S-3 Overall profile of cpd. 47

Table 3: Properties of cpd. 47

Biochemistry		
Aurora A : IC ₅₀ = 0.6 nM	LE = 0.361	kCal/Mol
Aurora B/Incenp : IC ₅₀ = 1 nM	LE = 0.353	kCal/Mol
Aurora C/Incenp : IC ₅₀ = 3 nM	LE = 0.334	kCal/Mol
Selective vs. 70 kinases (see table 2)		
No effect on tubulin polymerization at 25 µM		
PDE3 inhibition IC ₅₀ = 4 µM		
Cell biology		
Cpd. 47 inhibits tumor cell line proliferation with IC ₅₀ = 5-500 nM, induces polyploidy at 30 nM in HCT116 cells, inhibits Aurora A autophosphorylation with IC ₅₀ = 80 nM and histone H3 phosphorylation by Aurora B with IC ₅₀ = 80 nM too (see figure 2, section S2).		
SAR156497 has no effect on quiescent cells (peripheral blood mononuclear cells) viability.		
Physicochemistry		
pKa: experimental: 3.4 and 10.5, calculated (ADCv11): 4.92, 5.17 and 10.45		
log P: experimental: 2.42, calculate (ACDv11): 5.1 +/- 1.08		
log D (pH = 7.4): 2.36 (evaluated by HPLC)		
Rule of five compliant		
Solubility (sulfate salt): 2 mg/l in PBS (pH = 7.4), >2g/l in 0.1 N HCl (pH = 1.2), 0.68 g/l in 0.6% methylcellulose, 0.5% Tween 80 (pH = 2.6)		
ADMET		
Microsome metabolism: 53% in 20 min. in mice microsomes, 32% in 20 min. in human microsomes.		
Moderate clearance by human hepatocytes (Cl = 0.130 l.h ⁻¹ per 10 ⁶ cells)		

CYP 3A4 inhibition: recombinant human CYP 3A4: $k_{\text{inact}} = 0.076 \text{ min}^{-1}$, $K_i = 0.99 \text{ }\mu\text{M}$;

human Liver-derived CYP 3A4: $k_{\text{inact}} = 0.028 \text{ min}^{-1}$, $K_i = 2.47 \text{ }\mu\text{M}$.

High Caco2 absorption: $P_{\text{tot}} = 60 \cdot 10^{-7} \text{ cm/sec}$

hERG $\text{IC}_{50} = 3.7 \text{ }\mu\text{M}$ at 37°C . No major haemodynamic or electrophysiological effect in anaesthetized dogs at 10 mg/kg dosage by iv route

Ames negative, Micronucleus negative.

Pharmacokinetics

PK in female Balb/C mice after single 10 mg/kg iv administration $\text{AUC} (0\text{-}\infty) = 158000$

ng.h/mL , $T_{1/2} = 2.4 \text{ h}$ (moderate); $\text{Cl}_t = 0.063 \text{ L/h/kg}$ (low); $V_{\text{dss}} = 0.17 \text{ L/kg}$ (low). Single

10 mg/kg p.o. administration: $\text{AUC} = 101000 \text{ ng.h/mL}$; $T_{1/2} = 2.3 \text{ h}$ (moderate); $F = 64\%$

S-4 Chemistry

Experimental Methods.

The nomenclature of the compounds was generated with the ACDLABS software, Version 11.01. All solvents and reagents were obtained from commercial sources and were used without further purification. Thin layer chromatography was carried out on Merck silica gel 60 F254 glass plates. Flash chromatography was performed using column filled with $15\text{-}40 \text{ }\mu\text{m}$ Merck silica gel or pre-packed Merck silica gel cartridges ($15\text{-}40 \text{ }\mu\text{m}$). The microwave oven used was a Biotage, InitiatorTM 2.0, 400 W max, 2450 MHz instrument. The ^1H NMR spectra were performed on Bruker DPX-300, Bruker Avance DRX-400 or Bruker Avance DPX-500 spectrometers respectively operating at 300 , 400 and 500 MHz , with the chemical shifts (δ in ppm) in dimethylsulphoxide- d_6 ($\text{DMSO-}d_6$) referenced at 2.5 ppm at a temperature of 303 K and coupling constants (J) given in Hertz. The optical rotations were recorded on a Perkin-Elmer 341 polarimeter.

The mass spectra (MS) were obtained by methods A-E:

Analytical LC/MS method A:

Analysis was conducted using Shimadzu LC-10AD HPLC pumps; a Gilson 215 well plate autosampler; a Shimadzu SPD-10A UV detector; the mass spectrometer was a PE Sciex API 100LC instrument model on a YMC basic S5 column eluted with a gradient of acetonitrile containing 0.1% TFA in water at a flow rate of 0.1mL/min, gradient details are given for each example. Compounds eluting off the column were detected by electrospray ionisation mass spectrometry (EIMS).

Analytical LC/MS method B:

Analysis was conducted on a Waters model ZQ mass spectrometer working in positive and negative ion electrospray mode (mass range = 100-1200 amu) fitted on an Agilent HP1100 HPLC instrument. Separation was performed on a Waters Xbridge C18 column (3x50 mm, 2.5 µm particle diameter) maintained at a temperature of 60°C and eluted by a gradient of acetonitrile in water containing 0.1% (v/v) formic acid at a flow rate of 1.1 mL/min. The gradient had the following shape: 5 to 100% acetonitrile in 5 min, plateau 100% acetonitrile for 0.5 min, then back to 5% acetonitrile in 1 min. Total run time was 7 min. In addition to mass spectrometry, UV diode array detection was performed at 210 to 400 nm wavelengths and evaporative light scattering was carried out using a Sedere Sedex 85 instrument.

Analytical LC/MS method C:

Analysis was conducted on a Waters model ZQ mass spectrometer working in positive and negative ion electrospray mode (mass range = 100-1200 amu) fitted on a Waters Acquity UPLC Instrument. Separation was performed on a Waters UPLC BEH C18 column (2.1x50 mm, 1.7 µm particle diameter) maintained at a temperature of 55°C and eluted by a gradient of acetonitrile in water containing 0.1% (v/v) formic acid at a flow rate of 1.2 mL/min. The gradient had the following shape: 5 to 100% acetonitrile in 3 min, then back to 5% acetonitrile in 1 min. Total run time was 4.5 min. In addition to mass spectrometry, UV diode array detection was performed from 210 to 400 nm wavelengths.

Analytical LC/MS method D:

Analysis was conducted on an Acquity BEH C18 UPLC column, 1.7 µm 2.1x50 mm (Waters)

thermostated at 70°C and eluted at a flow rate of 1 mL/min with a gradient from acetonitrile containing 0.1% formic acid (solvent B) into water containing 0.1% formic acid (solvent A); elution program: isocratic stage at 5% of solvent B for 0.15 min, gradient from 5% to 100% of solvent B in 3.15 min then return to the initial conditions over 0.1 min. The products were detected by an Acquity PDA diode-array UV/vis detector (Waters, wavelength range scanned: 192-400 nm), a Sedex 85 light scattering detector (Sedere, nebulizing gas: nitrogen, nebulizing temperature: 32°C, nebulizing pressure 3.8 bar) and an Acquity SQD mass spectrometer (Waters, operating in positive and negative mode, mass range scanned: 80 to 800 amu).

Analytical LC/MS method E:

Analysis was conducted on a Waters UPLC-SQD instrument in positive and/or negative electrospray ionization mode (ES+/-), under the following liquid chromatography conditions: column: ACQUITY BEH C18 1.7 μ m, 2.1x50 mm; T_{column} : 50°C; flow rate: 1 mL/min; solvents: A: H₂O (0.1% formic acid); B: CH₃CN (0.1% formic acid); gradient (2 min): 5% to 50% B in 0.8 min; 1.2 min: 100% B; 1.85 min 100% B; 1.95 min 5% B.

Analytical LC/MS method F:

Analysis was conducted on a Waters WATERS QUATTRO PREMIER instrument in positive and/or negative electrospray ionization mode (ES+/-), under the following liquid chromatography conditions: column: ACQUITY MSS T3 – 1.8 μ m – 2.1 x 50 mm; T_{column} : 55°C; flow rate: 0.9 mL/min; solvents: A: H₂O (0.1% formic acid); B: CH₃CN (0.1% formic acid); gradient (3.7 min): 5% to 100% B in 3 min; back to 5% B for in 0.1 min.; maintain 5% B for 0.6 min.

Preparative LC/MS method A:

Preparative LC/MS separations were carried out on Waters HPLC instruments: 515 HPLC Pump; 2525 Binary Gradient Module; 2487 DAD (Dual Absorbance Detector); 2767 Sample Manager connected with a Micromass mass spectrometer. Products were separated on a YMC Combi Prep Pro C18 column eluted with a gradient acetonitrile containing 0.1% TFA in water containing 0.1% TFA at a

flow rate of 32 mL/min. For each separation gradient programming was adapted on the basis of an analytical LC/MS chromatogram of the sample.

Preparative LC/MS method B:

Preparative LC/MS separations were carried out on a Waters FractionLynx system composed of a Waters model 600 gradient pump, a Waters model 515 regeneration pump, a Waters Reagent Manager make-up pump, a Waters model 2700 autoinjector, two Rheodyne model LabPro switches, a Waters model 996 photodiode array detector, a Waters model ZMD mass spectrometer and a Gilson model 204 fraction collector. The instrument was controlled by a Waters FractionLynx software. At the output of the separating column the flow was split to the 1/1000 ratio using a LC Packing AccuRate splitter; 1/1000 of the flow was mixed with methanol (0.5 mL/min flow rate) and sent to the detectors, this flow was split again: $\frac{3}{4}$ of the flow was sent to the photodiode array detector and $\frac{1}{4}$ to the mass spectrometer; the rest of the output of the column (999/1000) was sent to the fraction collector where the flow was normally directed to waste unless the expected mass signal was detected by the FractionLynx software. The FractionLynx software was supplied with molecular formulas of expected compounds and triggered the collection of compounds when mass signal corresponding to $[M+H]^+$ and $[M+Na]^+$ were detected. In certain cases (depending on analytical LC/MS results, when $[M+2H]^{++}$ was detected as an intense ion) the FractionLynx software was additionally supplied with calculated half molecular weight (MW/2), in these conditions collection was also triggered when mass signal corresponding to $[M+2H]^{++}$ and $[M+Na+H]^{++}$ were detected. Compounds were collected in tarred glass tubes. After collection, solvent was evaporated in a Jouan model RC 10.10 centrifuge evaporator and the weight of compound was determined by weighing of the tubes after solvent evaporation. Column and gradient details are given for each example.

Purities of final compounds were measured by HPLC and/or LC/MS using UV detection at 220 nm and were $\geq 95.0\%$.

Abbreviations used

DIEA, diisopropylethylamine.

Compounds Synthesis and Characterization

General synthesis of 4-Aryl-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one derivatives

To a mixture of 1 mmol of N-Boc-3,5-diketopiperidine¹ and 1 mmol of 3-aminopyrazole in 5 mL of ethanol was added 1 mmol of benzaldehyde derivative. The mixture was heated at reflux temperature for 2 h and cooled to rt. The precipitate was collected by filtration and washed with ethanol to give the desired product.

The isolated compound was dissolved in 5 mL of DCM and treated with 5 mL of TFA for 1 hr at rt. After evaporation of the solvent the crude product was directly purified via preparative reverse phase HPLC. The desired compound was isolated after lyophilisation of the fractions.

General synthesis of 4-[3-(Phenoxy)-phenyl]-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one derivatives

To a mixture of 0.5 mmol of N-Boc-3,5-diketopiperidine¹ and 0.5 mmol of 3-aminopyrazole in 2.5 mL of ethanol was added 0.5 mmol of 3-(phenoxy)-benzaldehyde derivative (0.5 mmol). The mixture was heated at reflux temperature for 1/2 h and cooled to rt. The solution was concentrated under vacuum. The resulting oily residue was dissolved in 2.5 mL of DCM and treated with 2.5 mL of TFA at rt for 1 h. After evaporation of the solvent, the crude product was directly purified using preparative reversed phase HPLC. The desired compound was isolated after lyophilisation of the fractions.

The following examples were prepared using the above procedures (Table 3):

Table 4: Synthesis of 4-[3-(Phenoxy)-phenyl]-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one derivatives 1-7.

Cpd.	Compound name	MW of expected compound	EIMS ([M+H] ⁺)	LC/MS RT (min) ^a
1	4-(2-Fluoro-phenyl)-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one; compound with trifluoro-acetic acid	284.11	285.32	2.67 ^b
2	4-[3-(3,5-Dichloro-phenoxy)-phenyl]-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one; compound with trifluoro-acetic acid	427.07	428.32	3.29
3	4-(3-Phenoxy-phenyl)-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one; compound with trifluoro-acetic acid	358.14	359.43	2.79
4	4-[3-(4-Chloro-phenoxy)-phenyl]-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one; compound with trifluoro-acetic acid	392.85	393.17	3.91 ^b
5	4-[3-(4-Methoxy-phenoxy)-phenyl]-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one; compound with trifluoro-acetic acid	388.15	389.45	2.79
6	4-[3-(3,4-Dichloro-phenoxy)-phenyl]-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one; compound with trifluoro-acetic acid	427.07	428.32	3.28
7	4-[5-(1H-Benzimidazol-2-ylsulfanyl)-furan-2-yl]-2,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]-1,7-naphtyridin-5-one; compound with trifluoro-acetic acid	404.11	405.48	1.85

^aAnalytical LC/MS conditions: method A gradient 2 to 80 % acetonitrile in 7 min. ^bAnalytical LC/MS conditions: method A gradient 5 to 85 % acetonitrile in 7 min.

Scheme 1: Synthesis of cpds bearing a 5-(1H-benzimidazol-2-ylsulfanyl)-furan-2-yl side chain.

^aReagents and conditions: (a) EtOH or 1-butanol, reflux, 5-87%; (b) TFA, CH₂Cl₂ or 4N HCl, dioxane.

4-[5-(1H-Benzimidazol-2-ylsulfanyl)-furan-2-yl]-1,4,6,7,8,9-hexahydro-pyrazolo[3,4-b]quinolin-5-one (8)

To a mixture of 225 mg of 1,3-cyclohexanedione (2 mmol) and 183 mg of 3-aminopyrazole (2.2 mmol) in ethanol was added 490 mg of 5-(1H-benzimidazol-2-yl-sulfanyl)-furan-2-carbaldehyde (2 mmol). The mixture was heated at reflux temperature for 1/2 h and cooled to rt. The precipitate was collected by filtration and washed with ethanol to give 460 mg (58%) of **8** as a pale yellow solid.

Analytical LC/MS (method A): (2-85% ACN/H₂O gradient over 7 min) ^tR = 3.13 min EIMS [M+H]⁺: m/z 404. ¹H NMR (300MHz, DMSO-*d*₆): 1.96 (m, 2H); 2.26 (m, 2H); 2.54 (m, 2H); 4.11 (s, 1H); 5.22 (s, 1H); 5.97 (s, 1H); 6.85 (s, 1H); 7.16 (m, 2H); 7.37 (m, 1H); 7.46 (s, 1H); 7.55 (m, 1H); 10.0 (s, 1H); 12.2 (s, 1H); 12.47 (s, 1H).

The following examples were prepared using the above procedure:

9-[5-(1H-Benzimidazol-2-ylsulfanyl)-2-furyl]-1,4,5,6,7,9-hexahydropyrazolo[4,3-b]quinolin-8-one (9)

Yield: 80 mg (11%). Analytical LC/MS (method B): ^tR = 2.28 min; [M+H]⁺: m/z 404.0. ¹H NMR (300 MHz, DMSO-*d*₆): 1.79 to 1.92 (m, 2H); 2.19 to 2.28 (m, 2H); 2.48 to 2.57 (partially masked m, 2H); 5.30 (broad s, 1H); 6.02 (d, *J* = 3.0 Hz, 1H); 6.83 (d, *J* = 3.0 Hz, 1H); 7.11 to 7.19 (m, 2H); 7.26 (broad s, 1H); 7.42 to 7.50 (m, 2H); 9.45 (broad s, 1H); 12.10 to 12.7 (broad m, 2H).

Ethyl 9-[5-(1H-benzimidazol-2-ylsulfanyl)-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrazolo[4,3-b]quinoline-3-carboxylate (10)

Yield: 55 mg (7%). Analytical LC/MS (method B): t_R = 2.79 min; $[M+H]^+$: m/z 476.0. 1H NMR (300 MHz, DMSO- d_6): 1.30 (t, J = 7.1 Hz, 3H); 1.75 to 1.95 (m, 2H); 2.21 to 2.29 (m, 2H); 2.48 to 2.63 (partially masked m, 1H); 2.72 to 2.84 (m, 1H); 4.32 (q, J = 7.1 Hz, 2 H); 5.32 (s, 1H); 6.11 (d, J = 3.0 Hz, 1H); 6.89 (d, J = 3.0 Hz, 1H); 7.13 to 7.21 (m, 2H); 7.43 to 7.51 (m, 2H); 8.85 (s, 1H).

9-[5-(1H-Benzimidazol-2-ylsulfanyl)-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrazolo[4,3-b]quinoline-3-carboxamide (11)

Yield: 110 mg (21%). Analytical LC/MS (method B): t_R = 2.72 min; $[M+H]^+$: m/z 447; $[M-H]^-$: m/z 445. 1H NMR (400 MHz, DMSO- d_6): 1.79 to 1.92 (m, 2H); 2.21 to 2.28 (m, 2H); 2.48 to 2.61 (partially masked m, 1H); 2.75 to 2.88 (m, 1H); 5.43 (s, 1H); 6.11 (d, J = 3.0 Hz, 1H); 6.90 (d, J = 3.0 Hz, 1H); 7.11 to 7.19 (m, 2H); 7.26 (broad s, 1H); 7.40 (d, J = 8.0 Hz, 1H); 7.49 (broad s, 1H); 7.52 (d, J = 8.0 Hz, 1H); 8.86 (s, 1H); 12.36 (broad s, 1H); 13.20 (s, 1H).

2-Amino-5-[5-(1H-benzimidazol-2-ylsulfanyl)-2-furyl]-4-hydroxy-7,8,9,10-tetrahydro-5H-pyrimido[4,5-b]quinolin-6-one (12)

Yield: 410 mg (57%). Analytical LC/MS (method B): t_R = 2.48 min; $[M+H]^+$: m/z 447; $[M-H]^-$: m/z 445. 1H NMR (400 MHz, DMSO- d_6): 1.72 to 1.91 (m, 2H); 2.18 to 2.27 (m, 2H); 2.45 to 2.54 (partially masked m, 2H); 5.01 (s, 1H); 6.03 (d, J = 3.0 Hz, 1H); 6.42 (broad s, 2H); 6.81 (d, J = 3.0 Hz, 1H); 7.15 (m, 2H); 7.49 (m, 2H); 9.45 (s, 1H); 10.50 (broad s, 1H); 12.10 (broad s, 1H).

Methyl 9-[5-(1H-benzimidazol-2-ylsulfanyl)-2-furyl]-8-oxo-5,6,7,9-tetrahydro-4H-thieno[3,2-b]quinoline-3-carboxylate (13)

Yield: 87 mg (14%). Analytical LC/MS (method B): t_R = 4.20 min; $[M+H]^+$: m/z 478; $[M-H]^-$: m/z 476. 1H NMR (400 MHz, DMSO- d_6): 1.85 to 1.95 (m, 2H); 2.15 to 2.21 (m, 2H); 2.51 to 2.64 (m, 1H); 2.78 to 2.83 (m, 1H); 3.82 (s, 3H); 5.41 (s, 1H); 6.11 (d, J = 3.0 Hz, 1H); 6.87 (d, J = 3.0 Hz, 1H); 7.12 to 7.19 (m, 2H); 7.45 (broad m, 2H); 8.19 (s, 1H); 8.68 (s, 1H); 12.44 (broad s, 1H).

(+)-9-[5-(1H-Benzimidazol-2-ylsulfanyl)-2-furyl]-2,4,5,6,7,9-hexahydropyrrolo[3,4-b]quinolin-8-one (14)

Yield: 12 mg. Analytical LC/MS (method B): $t_R = 2.71$ min; $[M+H]^+$: m/z 403; $[M-H]^-$: m/z 401. 1H NMR (400 MHz, DMSO- d_6): 1.85 to 1.93 (m, 2H); 2.18 to 2.26 (m, 2H); 2.45 to 2.56 (partially masked m, 1H); 2.65 to 2.74 (m, 1H); 5.12 (s, 1H); 5.89 (d, $J = 3.0$ Hz, 1H); 6.27 (t, $J = 2.0$ Hz, 1H); 6.50 (t, $J = 2.0$ Hz, 1H); 6.80 (d, $J = 3.0$ Hz, 1H); 7.13 to 7.23 (m, 2H); 7.43 to 7.53 (broad m, 2H); 9.38 (s, 1H); 10.40 (broad s, 1H); 13.50 (broad m, 1H).

9-[5-(1H-Benzimidazol-2-ylsulfanyl)-furan-2-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (15)

A 2 L conical flask was charged, under magnetic stirring, with 50 g (0.26 mol) of 3-amino-2-ethoxycarbonylpyrrole hydrochloride and 0.204 L (0.41 mol) of 2N sodium hydroxide solution. The mixture was stirred for 15 min at rt, and then extracted with 3x0.3 L of DCM. The organic phases were combined, dried over $MgSO_4$ and concentrated to dryness under reduced pressure. The residue was triturated with *n*-pentane, filtered and dried under reduced pressure to a constant weight, to give 36.4 g of crude 3-amino-2-ethoxycarbonylpyrrole as a brown solid. MS EI: m/z 154: $[M]^+$; m/z 126: $[M]^+ - C_2H_5$; m/z 108: $[M]^+ - OC_2H_5$ (base peak); m/z 80:108 - CO.

3-Amino-2-ethoxycarbonyl-pyrrole (31.5 g, 204 mmol), 5-(1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde (50 g, 204 mmol) and 1,3-cyclohexanedione (23 g, 204 mmol) were combined in 2.5 L of 1-butanol and the reaction mixture was heated at reflux temperature for 3 h and then concentrated under reduced pressure. The residue was resuspended in 1 L of ethanol and heated at reflux temperature for 2 h and let to cool to rt. The insoluble material was collected by filtration, washed with ethanol (0.4 L), diisopropyl ether (0.4 L), pentane (0.4 L) and dried under vacuum. The residue was resuspended in 2 L of acetonitrile, heated at reflux temperature for 2 h and let to cool to rt. The insoluble material was collected by filtration, washed with acetonitrile (0.6 L), diisopropyl ether (0.6 L), pentane (0.6 L) and dried under vacuum to yield 41.5 g (43%) of **15** as a light gray powder. Analytical LC/MS (method B):

$t_R = 3.32$ min; $[M+H]^+$: m/z 475.06. 1H NMR (400 MHz, DMSO- d_6): 1.28 (t, $J = 7.0$ Hz, 3H); 1.88 (m, 2H); 2.25 (m, 2H); 2.58 (m, 1H); 2.79 (m, 1H); 4.26 (q, $J = 7.0$ Hz, 2H); 5.15 (s, 1H); 5.97 (d, $J = 3.5$ Hz, 1H); 6.79 (d, $J = 3.5$ Hz, 1H); 6.82 (d, $J = 3.5$ Hz, 1H); 7.12-7.17 (m, 2H); 7.47 (broad m, 2H); 8.40 (s, 1H); 11.4 (broad s, 1H); 12.4 (broad m, 1H).

The following examples were prepared using the above procedure:

(+) Methyl-9-[5-(1H-benzimidazol-2-ylsulfanyl)-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b]quinoline-3-carboxylate (18)

Yield: 12 mg. Analytical LC/MS (method B): $t_R = 1.3$ min; $[M+H]^+$: m/z 461; $[M-H]^-$: m/z 459. 1H NMR (400 MHz, DMSO- d_6): 1.82 to 1.92 (m, 2H); 2.21 to 2.28 (m, 2H); 2.45 to 2.62 (partially masked m, 1H); 2.75 to 2.84 (m, 1H); 3.76 (s, 3H); 5.14 (s, 1H); 5.97 (d, $J = 3.0$ Hz, 1H); 6.81 (d, $J = 3.0$ Hz, 2H); 7.10 to 7.20 (broad m, 2H); 7.37 to 7.58 (broad m, 2H); 8.49 (s, 1H); 11.46 (d, $J = 3.0$ Hz, 1H); 12.43 (broad m, 1H).

(+)-9-[5-(1H-Benzimidazol-2-ylsulfanyl)-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b]quinoline-3-carboxylic acid (19)

Yield: 17 mg. Analytical LC/MS (method B): $t_R = 2.86$ min; $[M+H]^+$: m/z 447. 1H NMR (400 MHz, DMSO- d_6): 1.82 to 1.92 (m, 2H); 2.20 to 2.28 (m, 2H); 2.45 to 2.60 (partially masked m, 1H); 2.67 to 2.78 (m, 1H); 5.12 (s, 1H); 5.92 (d, $J = 3.0$ Hz, 1H); 6.53 (broad s, 1H); 6.80 (d, $J = 3.0$ Hz, 1H); 7.10 to 7.20 (broad m, 2H); 7.35 to 7.58 (broad m, 2H); 8.48 (broad m, 1H); 10.83 (broad m, 1H); 12.35 (broad m, 2H).

9-[5-(1H-Benzimidazol-2-ylsulfanyl)-furan-2-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]-1,7-naphthyridine-3-carboxylic acid ethyl ester; trifluoroacetate (20)

To a mixture of 42 mg (0.2 mmol) of N-Boc-3,5-diketopiperidine¹ and 38 mg (0.2 mmol) of 3-amino-2-ethoxycarbonylpyrrole hydrochloride in 2.5 mL of ethanol was added 51 mg (0.2 mmol) of 5-(1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde and 33 μ L of DIEA (0.2 mmol). The mixture was heated at reflux temperature for 1/2 h and cooled to rt. The solution was concentrated and the residue

was purified by silica gel chromatography. The enantiomers of the Boc-protected intermediate were separated on a Welk 01 SS column eluted with 2-propanol/THF (98:2, v/v) and the dextrorotatory enantiomer (i.e. $[\alpha]_D^{20} +198.6 \pm 2.7$ (c 0.4, MeOH) was collected and concentrated under vacuum. The resulting oily residue was dissolved in 2.5 mL of 4M HCl in dioxane and reacted for 4 h to yield 8.5 mg (7%) of desired product **20**. Analytical LC/MS (method F): $t_R = 0.97$ min; $[M+H]^+$: m/z 476. 1H NMR (400 MHz, DMSO- d_6): 1.29 (t, $J = 7.1$ Hz, 3H); 3.70 to 3.86 (m, 2H); 4.22 (broad m, 1H); 4.28 (q, $J = 7.1$ Hz, 2H); 4.45 (d, $J = 16.1$ Hz, 1H); 5.21 (s, 1H); 6.34 (broad m, 1H); 6.87 (d, $J = 3.0$ Hz, 1H); 6.90 (broad d, $J = 3.0$ Hz, 1H); 7.21 (m, 2H); 7.51 (m, 2H); 9.37 (s, 1H); 9.88 (broad m, 1H); 10.12 (broad m, 1H); 11.63 (d, $J = 3.0$ Hz, 1H).

General synthesis of 5-(1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde derivatives (B)

Scheme 2: Synthesis of aldehydes (B).

^aReagents and conditions: (a) $SnCl_2/NaBH_4$, AcOEt/ tBuOH, 60°C; (b) CS_2 , DMF, 25°C; (c) Di-2-pyridylthionocarbonate, THF, 25°C; (d) 1,1'-thiocarbonyldiimidazole, THF, 25°C; (e) NaH, THF.

5-(5-fluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde

2-Mercapto-5-fluoro-benzimidazole:

Di-2-pyridylthionocarbonate (3.68 g, 15.9 mmol) was added by portions to a solution of 4-fluoro-*ortho*-phenylenediamine (2 g, 15.9 mmol) in 20 mL of tetrahydrofuran and the mixture was stirred at rt for 16 h. The reaction mixture was then concentrated under reduced pressure and the residue was

dissolved in 200 mL of EtOAc and washed with water (2x60 mL). The organic phase was then dried over MgSO₄, filtered and concentrated. The residue was triturated in diisopropylether and pentane and dried under vacuum to yield 2.24 g (84%) of 2-mercapto-5-fluoro-benzimidazole as a brown powder. Analytical LC/MS (method B): ¹R = 2.32 min; [M+H]⁺: m/z 168.97.

5-(5-Fluoro-1H-benzoimidazol-2-ylsulfanyl)-furan-2-carbaldehyde:

A solution of 2-mercapto-5-fluoro-benzimidazole (2.24 g, 13.3 mmol) in THF (10 mL) was added dropwise to a mixture of sodium hydride (60% dispersion in mineral oil, 0.852 g, 21.3 mmol) and THF (5 mL). The mixture was stirred at rt for 2 h. A solution of 5-nitro-2-furaldehyde (1.88 g, 13.3 mmol) in THF (15 mL) was then added dropwise over 15 min and the mixture was stirred for 2 h at rt. The reaction mixture was then concentrated under reduced pressure and the residue was dissolved in 200 mL of EtOAc and washed with water (2x 60 mL). The organic phase was dried over MgSO₄ and concentrated under reduced pressure. The residue was purified on a silica gel column (40 g) eluted with DCM to yield 700 mg (20%) of 5-(5-fluoro-1H-benzoimidazol-2-ylsulfanyl)-furan-2-carbaldehyde as an orange powder. Analytical LC/MS (method B): ¹R = 3.08 min; [M+H]⁺: m/z 263.05.

5-(4,6-difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde

2-Mercapto-4,6-difluorobenzimidazole:

A mixture of 1,2-diamino-3,5-difluorobenzene (1 g, 6.94 mmol) and 1,1'-thiocarbonyldiimidazole (2.05 g, 11.52 mmol) in 10 mL of THF was stirred at rt for 16 h. The reaction mixture was then concentrated under reduced pressure and the residue was dissolved in 100 mL of EtOAc and washed with water (2x30 mL). The organic phase was dried over MgSO₄, filtered and concentrated under reduced pressure to yield 763 mg (61%) of 2-mercapto-4,6-difluorobenzimidazole. Analytical LC/MS (method B): ¹R = 2.49 min; [M+H]⁺: m/z 186.95.

5-(4,6-Difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde:

2-mercapto-4,6-difluorobenzimidazole (16 g, 86 mmol) in THF (80 ml) was added dropwise to a mixture of sodium hydride (60% dispersion in mineral oil, 5.5 g, 86 mmol) and THF (20mL) at 0 °C.

The reaction mixture was stirred at rt for 3 h. 5-Nitro-2-furaldehyde (12.1 g, 86 mmol) in 50 mL of THF was then added dropwise over 15 min and the mixture was stirred for 16 h at rt. Water (10 mL) was then added and the reaction mixture was stirred for 30 min and concentrated under reduced pressure. The residue was dissolved in a minimal volume of EtOAc and the solution was filtered on a plug (50 g) of silica gel. The silica gel plug was washed with EtOAc (1L) and the filtrate was concentrated under reduced pressure. The residue was purified on a silica gel column (300g) eluted successively with cyclohexane/EtOAc (9/1 v/v) and cyclohexane/EtOAc (7/3 v/v). The fractions containing the expected product were concentrated under reduced pressure to yield 6.7 g (28%) of 5-(4,6-difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde as an orange powder. Analytical LC/MS (method B): $t_R = 3.34$ min; $[M+H]^+$: m/z 281.0.

5-(6,7-Difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde

6,7-Difluoro-1H-benzimidazole-2-thiol:

To a solution of 1,2-diamino-3,4-difluoro-benzene (996 mg, 6.9 mmol) in 20 mL of ethanol in a 25 mL microwave tube, were added 2.0 mL (33.3 mmol) of carbon disulfide. The tube was capped and the reaction mixture was heated under microwaves at 120 °C twice for 20 min, upon which 1.0 mL (16.6 mmol) of carbon disulfide was added and heating under microwaves was pursued for 20 min. Carbon disulfide 2.0 mL (33.3 mmol) was added and heating was pursued for 30 min under microwaves at 150 °C. The reaction mixture was then cooled to rt and concentrated to dryness under reduced pressure. The residue was dissolved into EtOAc and concentrated to dryness under reduced pressure. The residue was triturated into diisopropylether, filtered and washed once with diisopropylether. The solid was dried under reduced pressure to yield 0.35 g (27%) of 6,7-difluoro-1H-benzimidazole-2-thiol as a mauve solid. Analytical LC/MS (method B): $[M+H]^+$: m/z 187; $[M-H]^-$: 185.

5-(6,7-Difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde:

To 6,7-Difluoro-1H-benzimidazole-2-thiol (0.34 g, 1.81 mmol) suspended into 20 mL of THF under argon, was added 130 mg (2.71 mmol) of sodium hydride. The reaction mixture was stirred at rt until

gas evolution had ceased. 5-Nitro-2-furaldehyde (0.25 g, 1.81 mmol) was then added and the reaction mixture was stirred at rt for 3 h, upon which it was poured on ice and extracted twice with EtOAc. The organic extracts were combined, washed with brine, dried on magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on a prepacked 70 g 15-40 μ m silica gel cartridge (eluting solvent: DCM/THF from 100/0 to 80/20 v/v; rate : 40 mL/min). The fractions containing the desired product were combined and concentrated to dryness under reduced pressure. The residue was repurified by chromatography on a prepacked 30 g 15-40 μ m silica gel cartridge (eluting solvent: DCM/methanol from 100/0 to 95/5 v/v; rate : 20 mL/min). The fractions containing the desired product were combined and concentrated to dryness under reduced pressure to yield 0.28 g (55%) of 5-(6,7-difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde as a pale yellow meringue. Analytical LC/MS (method B): $[M+H]^+$: m/z 281.

9-[5-(5-Fluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (21)

A mixture of 3-amino-2-ethoxycarbonyl-pyrrole (0.235 g, 1.52 mmol), 5-(5-fluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-carbaldehyde (0.40 g, 1.52 mmol) and 1,3-cyclohexanedione (0.171 g, 1.52 mmol) in 5 mL of 1-butanol was heated at reflux temperature for 2 h. The reaction mixture was then concentrated under reduced pressure. The residue was resuspended in 5 mL of acetonitrile and the mixture was heated at reflux temperature for 30 min. The mixture was allowed to cool to rt and the insoluble material was collected by filtration, washed with diisopropylether (20 mL), pentane (20 mL) and dried under vacuum to yield 377 mg (50%) of **21** as a white powder. Analytical LC/MS (method B): $[M-H]^-$: m/z 491; $[M+H]^+$: m/z 493. ^1H NMR (400MHz, DMSO- d_6): 1.28 (t, $J = 7.0$ Hz, 3H); 1.89 (m, 2H); 2.26 (m, 2H); 2.58 (m, 1H); 2.80 (m, 1H); 4.25 (q, $J = 7.0$ Hz, 2H); 5.14 (s, 1H); 5.96 (d, $J = 3.5$ Hz, 1H); 6.79 (d, $J = 3.5$ Hz, 1H); 6.82 (d, $J = 3.5$ Hz, 1H); 7.00 (dt, $J = 2.5, 9.0$ Hz, 1H); 7.27 (broad d, $J = 9.5$ Hz, 1H); 7.45 (broad m, 1H); 8.42 (s, 1H); 11.4 (broad s, 1H); 12.5 (broad m, 1H).

The following example was prepared using the above procedure:

Ethyl 9-[5-[(6-fluoro-1H-benzimidazol-2-yl)sulfanyl]-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b]quinoline-3-carboxylate (22)

Yield: 300 mg (73%). Analytical LC/MS (method B): $t_R = 3.75$ min; $[M+H]^+$: m/z 493; $[M-H]^-$: m/z 491. 1H NMR (300 MHz, DMSO- d_6): 1.28 (t, $J = 7.1$ Hz, 3H); 1.80 to 1.92 (m, 2H); 2.21 to 2.28 (m, 2H); 2.51 to 2.62 (m, 1H); 2.72 to 2.82 (m, 1H); 4.26 (q, $J = 7.1$ Hz, 2H); 5.16 (s, 1H); 5.98 (d, $J = 3.0$ Hz, 1H); 6.77 (d, $J = 3.0$ Hz, 1H); 6.83 (d, $J = 3.0$ Hz, 1H); 6.95 (broad dd, $J = 8.3, 10.8$ Hz, 1H); 7.13 (dt, $J = 4.9, 8.3$ Hz, 1H); 7.28 (broad d, $J = 8.3$ Hz, 1H); 8.40 (s, 1H); 11.38 (d, $J = 3.0$ Hz, 1H); 12.80 (broad m, 1H).

9-[5-(4,6-Difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (23)

Yield: 3.8 g (36%) as a white powder. Analytical LC/MS (method B): $[M-H]^-$: m/z 509; $[M+H]^+$: m/z 511. 1H NMR (400 MHz, DMSO- d_6): 1.28 (t, $J = 7.0$ Hz, 3H); 1.89 (m, 2H); 2.24 (m, 2H); 2.57 (m, 2H); 2.79 (m, 1H); 4.25 (q, $J = 7.0$ Hz, 2H); 5.14 (s, 1H); 5.98 (d, $J = 3.5$ Hz, 1H); 6.78 (d, $J = 3.5$ Hz, 1H); 6.84 (d, $J = 3.5$ Hz, 1H); 7.02 (broad m, 1H); 7.13 (broad m, 1H); 8.45 (s, 1H); 11.4 (broad s, 1H); 13.0 (broad m, 1H).

9-[5-(5,6-Difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (24)

Yield: 189 mg (40%) as an off-white crystalline powder. mp: 160-170°C. Analytical LC/MS (method B): $t_R = 3.56$ min; $[M+H]^+$: m/z 511 (base peak); $[M-H]^-$: m/z 509 (base peak). 1H NMR (300 MHz, DMSO- d_6): 1.28 (t, $J = 7.1$ Hz, 3H); 1.82 to 1.95 (m, 2H); 2.20 to 2.30 (m, 2H); 2.52 to 2.64 (m, 1H); 2.72 to 2.85 (m, 1H); 4.26 (q, $J = 7.1$ Hz, 2H); 5.14 (s, 1H); 5.96 (d, $J = 3.0$ Hz, 1H); 6.79 (d, $J = 3.0$ Hz, 1H); 6.82 (d, $J = 3.0$ Hz, 1H); 7.53 (m, 2H); 8.41 (s, 1H); 11.41 (d, $J = 3.0$ Hz, 1H); 12.70 (broad m, 1H).

9-[5-(6,7-Difluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (25)

Yield: 127 mg (26%) as a light-yellow crystalline powder. mp: 144-148°C. Analytical LC/MS (method B): t_R = 3.89 min; $[M+H]^+$: m/z 511. 1H NMR (500 MHz, $CDCl_3$): 1.40 (broad t, J = 6.9 Hz, 3H); 2.01 to 2.13 (m, 2H); 2.41 to 2.57 (m, 2H); 2.60 to 2.70 (m, 2H); 4.38 (broad q, J = 6.9 Hz, 2H); 5.36 (s, 1H); 6.00 (d, J = 3.1 Hz, 1H); 6.70 (d, J = 3.1 Hz, 1H); 6.74 (broad d, J = 3.1 Hz, 1H); 7.00 (ddd, J = 6.7, 8.6, 10.1 Hz, 1H); 7.21 (ddd, J = 1.5, 3.7, 8.6 Hz, 1H); 7.61 (broad m, 1H); 8.59 (broad m, 2H); 10.84 (broad s, 1H).

8-Oxo-9-[5-(4,5,6-trifluoro-1H-benzimidazol-2-ylsulfanyl)-furan-2-yl]-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (26)

Yield: 144 mg (22%) as a yellow crystalline powder. mp: 186°C. Analytical LC/MS (method B): t_R = 3.95 min; $[M+H]^+$: m/z 529; $[M-H]^-$: m/z 527. 1H NMR (400 MHz, $DMSO-d_6$): 1.28 (t, J = 7.1 Hz, 3H); 1.83 to 1.94 (m, 2H); 2.20 to 2.29 (m, 2H); 2.52 to 2.63 (m, 1H); 2.75 to 2.83 (m, 1H); 4.25 (q, J = 7.1 Hz, 2H); 5.14 (s, 1H); 5.98 (d, J = 3.0 Hz, 1H); 6.75 (d, J = 3.0 Hz, 1H); 6.83 (d, J = 3.0 Hz, 1H); 7.35 (broad m, 1H); 8.40 (s, 1H); 11.37 (broad m, 1H); 13.10 (broad m, 1H).

Ethyl 9-[5-[(7-chloro-1H-benzimidazol-2-yl)sulfanyl]-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b]quinoline-3-carboxylate (27)

Yield: 620 mg (61%). Analytical LC/MS (method B): t_R = 3.76 min; $[M+H]^+$: m/z 509; $[M-H]^-$: m/z 507. 1H NMR (400 MHz, $DMSO-d_6$): 1.28 (t, J = 7.1 Hz, 3H); 1.80 to 1.93 (m, 2H); 2.21 to 2.28 (m, 2H); 2.51 to 2.62 (m, 1H); 2.73 to 2.82 (m, 1H); 4.26 (q, J = 7.1 Hz, 2H); 5.16 (s, 1H); 5.99 (broad s, 1H); 6.78 (d, J = 3.0 Hz, 1H); 6.86 (broad s, 1H); 7.17 (t, J = 7.8 Hz, 1H); 7.22 (d, J = 7.8 Hz, 1H); 7.40 (broad d, J = 7.8 Hz, 1H); 8.40 (s, 1H); 11.38 (broad s, 1H); 12.70 (broad s, 1H).

9-[5-(5-Chloro-1H-benzimidazol-2-ylsulfanyl)-furan-2-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (28)

The product was purified using preparative LC/MS method B on an Xbridge C18 column (Waters, 30*100 mm) eluted with a 0 to 50% gradient of acetonitrile in aqueous 10 mM ammonium formate pH=9.0 at a 30 mL/min. flow rate in 12 min. The fractions containing the expected product were

concentrated under reduced pressure. The residue was recrystallized in 2 mL of acetonitrile, collected by filtration, washed with diisopropyl ether (2 x 2 mL) and dried under vacuum to yield 115 mg (12%) of **28** as a white powder. Analytical LC/MS (method B): $[M+H]^+$: m/z 509 (1 Cl present); $[M-H]^-$: m/z 507 (1 Cl present). 1H NMR (400 MHz, DMSO- d_6): 1.28 (t, $J = 7.0$ Hz, 3H); 1.89 (m, 2H); 2.25 (m, 2H); 2.58 (m, 1H); 2.79 (m, 1H); 4.26 (q, $J = 7.0$ Hz, 2H); 5.15 (s, 1H); 5.98 (d, $J = 3.5$ Hz, 1H); 6.79 (s, 1H); 6.83 (d, $J = 3.5$ Hz, 1H); 7.16 (dd, $J = 2.5, 8.5$ Hz, 1H); 7.46 (d, $J = 8.5$ Hz, 1H); 7.52 (broad s, 1H); 8.43 (s, 1H); 11.0 (broad m, 1H); 11.4 (broad s, 1H).

9-[5-(3H-Imidazo[4,5-b]pyridin-2-yl)sulfanyl]-furan-2-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (29)

Yield: 37 mg (17%) as a yellow powder. mp: 190-202°C. Analytical LC/MS (method B): $t_R = 2.82$ min; $[M+H]^+$: m/z 476 (base peak); $[M-H]^-$: m/z 474 (base peak). 1H NMR (400 MHz, DMSO- d_6): 1.28 (t, $J = 7.1$ Hz, 3H); 1.84 to 1.95 (m, 2H); 2.19 to 2.31 (m, 2H); 2.52 to 2.62 (m, 1H); 2.79 (td, $J = 4.6, 17.1$ Hz, 1H); 4.26 (q, $J = 7.1$ Hz, 2H); 5.15 (s, 1H); 5.99 (d, $J = 3.0$ Hz, 1H); 6.79 (d, $J = 3.0$ Hz, 1H); 6.85 (d, $J = 3.0$ Hz, 1H); 7.18 (dd, $J = 4.7, 8.1$ Hz, 1H); 7.87 (broad d, $J = 8.1$ Hz, 1H); 8.26 (broad d, $J = 4.7$ Hz, 1H); 8.40 (s, 1H); 11.39 (d, $J = 3.0$ Hz, 1H); 13.04 (broad m, 1).

Ethyl 9-[5-[(4-hydroxy-1H-benzimidazol-2-yl)sulfanyl]-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b]quinoline-3-carboxylate (30)

Yield: 164 mg (55%). Analytical LC/MS (method B): $t_R = 3.27$ min; $[M+H]^+$: m/z 491; $[M-H]^-$: m/z 489. 1H NMR (400 MHz, DMSO- d_6): 1.28 (t, $J = 7.1$ Hz, 3H); 1.82 to 1.92 (m, 2H); 2.22 to 2.28 (m, 2H); 2.48 to 2.63 (partially masked m, 1H); 2.75 to 2.82 (m, 1H); 4.25 (q, $J = 7.1$ Hz, 2H); 5.12 (s, 1H); 5.93 (d, $J = 3.0$ Hz, 1H); 6.50 (broad m, 1H); 6.78 (m, 2H); 6.82 to 6.97 (broad m, 2H); 8.39 (broad s, 1H); 11.37 (broad m, 1H); 12.31 (broad m, 1H).

Ethyl 9-[5-[(4-amino-1H-benzimidazol-2-yl)sulfanyl]-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b]quinoline-3-carboxylate (31)

Yield: 289 mg (35%). Analytical LC/MS (method B): $t_R = 1.23$ min; $[M+H]^+$: m/z 490; $[M-H]^-$: m/z

488. ¹H NMR (400 MHz, DMSO-*d*₆): 1.28 (t, *J* = 7.1 Hz, 3H); 1.80 to 1.92 (m, 2H); 2.20 to 2.29 (m, 2H); 2.53 to 2.62 (m, 1H); 2.73 to 2.83 (m, 1H); 4.25 (q, *J* = 7.1 Hz, 2H); 5.13 (s, 1H); 5.95 (d, *J* = 3.0 Hz, 1H); 6.42 (broad m, 1H); 6.72 (broad m, 1H); 6.79 (d, *J* = 3.0 Hz, 2H); 6.91 (t, *J* = 7.8 Hz, 1H); 8.42 (s, 1H); 11.38 (d, *J* = 3.0 Hz, 1H); 12.26 (very broad m, 1H).

Ethyl 9-[5-(1H-benzimidazol-2-ylsulfanyl)-2-furyl]-6-ethyl-8-oxo-4,5,7,9-tetrahydro-2H-pyrrolo[3,4-b][1,7]naphthyridine-3-carboxylate (32)

Yield: 230 mg (27%). Analytical LC/MS (method B): ^tR = 2.57 min; [M+H]⁺: m/z 504; [M-H]⁻: m/z 502. ¹H NMR (400 MHz, DMSO-*d*₆): 1.01 (t, *J* = 7.1 Hz, 3H); 1.28 (t, *J* = 7.1 Hz, 3H); 2.43 to 2.52 (partially masked m, 2H); 2.89 (dd, *J* = 1.5, 15.7 Hz, 1H); 3.15 (d, *J* = 15.7 Hz, 1H); 3.28 (d, *J* = 16.1 Hz, 1H); 3.85 (d, *J* = 16.1 Hz, 1H); 4.26 (q, *J* = 7.1 Hz, 2H); 5.15 (s, 1H); 5.98 (d, *J* = 3.0 Hz, 1H); 6.80 (d, *J* = 3.0 Hz, 1H); 6.84 (d, *J* = 3.0 Hz, 1H); 7.12 to 7.17 (m, 2H); 7.47 (broad m, 2H); 8.76 (s, 1H); 11.47 (d, *J* = 3.0 Hz, 1H); 12.43 (broad s, 1H).

Ethyl (9S)-6-(2-aminoacetyl)-9-[5-(1H-benzimidazol-2-ylsulfanyl)-2-furyl]-8-oxo-4,5,7,9-tetrahydro-2H-pyrrolo[3,4-b][1,7]naphthyridine-3-carboxylate hydrochloride (33)

Yield: 370 mg (87%). Analytical LC/MS (method B): ^tR = 0.50 min; [M+H]⁺: m/z 533; [M-H]⁻: m/z 531. ¹H NMR (400 MHz, DMSO-*d*₆): 1:1 mixture of conformers 1.29 (2 t, *J* = 7.1 Hz, 3H); 3.57 (s, 1H); 3.2 to 4.50 (m, 6H); 4.91 (d, *J* = 17.6 Hz, 0.5H); 5.20 to 5.28 (m, 1.5H); 6.03 (2 d, *J* = 3.0 Hz, 1H); 6.85 (2 d, *J* = 3.0 Hz, 1H); 6.90 (d, *J* = 3.0 Hz, 1H); 7.22 (m, 2H); 7.52 (m, 2H); 8.12 to 8.24 (broad m, 3H); 9.12 (s, 0.5H); 9.26 (s, 0.5H); 11.57 (d, *J* = 3.0 Hz, 1H).

Ethyl (9S)-6-(2-aminoacetyl)-9-[5-[(4-fluoro-1H-benzimidazol-2-yl)sulfanyl]-2-furyl]-8-oxo-4,5,7,9-tetrahydro-2H-pyrrolo[3,4-b][1,7]naphthyridine-3-carboxylate hydrochloride (34)

Yield: 300 mg (72%). Analytical LC/MS (method B): ^tR = 2.76 min; [M+H]⁺: m/z 551; [M-H]⁻: m/z 549. ¹H NMR (400 MHz, DMSO-*d*₆): 1:1 mixture of conformers 1.29 (2 t, *J* = 7.1 Hz, 3H); 3.57 (s, 3H); 3.80 to 4.50 (m, 6H); 4.93 (d, *J* = 17.6 Hz, 0.5H); 5.21 (s, 0.5H), 5.22 (s, 0.5H); 5.25 (d, *J* = 17.6 Hz, 0.5H); 6.02 (2 d, *J* = 3.0 Hz, 1H); 6.82 (2 d, *J* = 3.0 Hz, 1H); 6.89 (d, *J* = 3.0 Hz, 1H); 6.97 (dd, *J* = 8.3,

10.8 Hz, 1H); 7.16 (dt, $J = 4.9, 8.3$ Hz, 1H); 7.29 (d, $J = 8.3$ Hz, 1H); 8.15 to 8.29 (broad m, 3H); 9.14 (s, 0.5H); 9.26 (s, 0.5H); 11.56 (d, $J = 3.0$ Hz, 1H).

Ethyl 9-[5-[(7-chloro-1H-benzimidazol-2-yl)sulfanyl]-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b][1,7]naphthyridine-3-carboxylate (35)

Yield: 350 mg (70%). Analytical LC/MS (method B): $t_R = 1.17$ min; $[M+H]^+$: m/z 510; $[M-H]^-$: m/z 508. 1H NMR (400 MHz, DMSO- d_6): 1.28 (t, $J = 7.1$ Hz, 3H); 3.17 (d, $J = 17.2$ Hz, 1H); 3.22 (d, $J = 17.2$ Hz, 1H); 3.61 (d, $J = 17.2$ Hz, 1H); 3.90 (d, $J = 17.2$ Hz, 1H); 4.26 (q, $J = 7.1$ Hz, 2H); 5.15 (s, 1H); 6.12 (d, $J = 3.0$ Hz, 1H); 6.81 (d, $J = 3.0$ Hz, 1H); 6.87 (d, $J = 3.0$ Hz, 1H); 7.16 (t, $J = 8.3$ Hz, 1H); 7.23 (broad d, $J = 8.3$ Hz, 1H); 7.42 (broad d, $J = 8.3$ Hz, 1H); 8.67 (s, 1H); 11.43 (d, $J = 3.0$ Hz, 1H).

Ethyl 9-[5-[(4-fluoro-1H-benzimidazol-2-yl)sulfanyl]-2-furyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b][1,7]naphthyridine-3-carboxylate (36)

Yield: 163 mg (50%). Analytical LC/MS (method B): $t_R = 2.61$ min; $[M+H]^+$: m/z 494. 1H NMR (400 MHz, DMSO- d_6): 1.28 (t, $J = 7.1$ Hz, 3H); 2.99 (broad m, 1H); 3.11 to 3.27 (m, 2H); 3.60 (d, $J = 17.3$ Hz, 1H); 3.89 (d, $J = 17.3$ Hz, 1H); 4.25 (q, $J = 7.1$ Hz, 2H); 5.15 (s, 1H); 6.11 (d, $J = 3.0$ Hz, 1H); 6.79 (d, $J = 3.0$ Hz, 1H); 6.85 (d, $J = 3.0$ Hz, 1H); 6.95 (broad m, 1H); 7.12 (broad m, 1H); 7.27 (broad d, $J = 8.3$ Hz, 1H); 8.66 (s, 1H); 11.41 (d, $J = 3.0$ Hz, 1H); 12.82 (broad m, 1H).

9-[5-(1H-Benzimidazol-2-ylsulfanyl)-furan-2-yl]-6,6-dimethyl-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (37)

Yield: 330 mg (47 %). Analytical LC/MS (method B): $t_R = 3.61$ min; $[M+H]^+$: m/z 503.3.

O6-*tert*-butyl O3-ethyl 9-[5-(1H-benzimidazol-2-ylsulfanyl)-2-furyl]-8-oxo-4,5,7,9-tetrahydro-2H-pyrrolo[3,4-b][1,7]naphthyridine-3,6-dicarboxylate (38)

Yield: 6.9 g (N/A). Analytical LC/MS (method B): $t_R = 3.79$ min; $[M+H]^+$: m/z 576. 1H NMR (400 MHz, DMSO- d_6): 1:1 mixture of conformers 1.28 (t, $J = 7.1$ Hz, 3H); 1.41 (broad s, 9H); 3.76 (broad m, 1H); 4.11 (d, $J = 17.6$ Hz, 1H); 4.18 (broad m, 1H); 4.27 (q, $J = 7.1$ Hz, 2H); 4.96 (d, $J = 17.6$ Hz, 1H);

5.20 (s, 1H); 5.92 (d, $J = 3.0$ Hz, 1H); 6.81 (d, $J = 3.0$ Hz, 1H); 6.83 (d, $J = 3.0$ Hz, 1H); 7.14 (broad m, 2H); 7.35 to 7.58 (broad m, 2H); 9.13 to 9.28 (broad m, 1H); 11.52 (broad s, 1H); 12.44 (broad s, 1H).

Ethyl 9-[5-(1H-benzimidazol-2-ylsulfanyl)-2-furyl]-6-benzyl-8-oxo-4,5,7,9-tetrahydro-2H-pyrrolo[3,4-b][1,7]naphthyridine-3-carboxylate (39)

Yield: 220 mg (24%). Analytical LC/MS (method B): $t_R = 3.07$ min; $[M+H]^+$: m/z 566. 1H NMR (400 MHz, DMSO- d_6): 1.26 (t, $J = 7.1$ Hz, 3H); 2.97 (d, $J = 15.7$ Hz, 1H); 3.13 (d, $J = 15.7$ Hz, 1H); 3.36 (d, $J = 16.2$ Hz, 1H); 3.61 (d, $J = 13.2$ Hz, 1H); 3.68 (d, $J = 13.2$ Hz, 1H); 3.81 (d, $J = 16.2$ Hz, 1H); 4.24 (q, $J = 7.1$ Hz, 2H); 5.16 (s, 1H); 6.01 (d, $J = 3.0$ Hz, 1H); 6.80 (d, $J = 3.0$ Hz, 1H); 6.85 (d, $J = 3.0$ Hz, 1H); 7.14 (m, 2H); 7.25 to 7.39 (m, 5H); 7.46 (broad m, 2H); 8.85 (s, 1H); 11.45 (d, $J = 3.0$ Hz, 1H); 12.44 (broad m, 1H).

Ethyl 9-[5-(1H-benzimidazol-2-ylsulfanyl)-2-thienyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-b]quinoline-3-carboxylate (40)

Yield: 250 mg (25%). Analytical LC/MS (method B): $t_R = 3.36$ min; $[M+H]^+$: m/z 491. 1H NMR (400 MHz, DMSO- d_6): 1.28 (t, $J = 7.1$ Hz, 3H); 1.80 to 1.94 (m, 2H); 2.22 to 2.29 (m, 2H); 2.52 to 2.62 (m, 1H); 2.80 (td, $J = 4.6, 17.1$ Hz, 1H); 4.26 (q, $J = 7.1$ Hz, 2H); 5.37 (s, 1H); 6.75 (d, $J = 3.4$ Hz, 1H); 6.88 (d, $J = 3.0$ Hz, 1H); 7.12 (m, 2H); 7.21 (d, $J = 3.4$ Hz, 1H); 7.44 (broad m, 2H); 8.48 (s, 1H); 11.44 (d, $J = 3.0$ Hz, 1H); 12.49 (broad m, 1H).

9-[2-(1H-Benzimidazol-2-ylsulfanyl)-thiazol-5-yl]-8-oxo-4,5,6,7,8,9-hexahydro-2H-pyrrolo[3,4-b]quinoline-3-carboxylic acid ethyl ester (41)

Yield: 313 mg (32%) as a yellow powder. mp: 194°C. Analytical LC/MS (method B): $[M+H]^+$: m/z 492 (base peak); $[M-H]^-$: m/z 490 (base peak). 1H NMR (400 MHz, DMSO- d_6): 1.29 (t, $J = 7.1$ Hz, 3H); 1.71 to 1.92 (m, 2H); 2.19 to 2.27 (m, 2H); 2.48 to 2.60 (m, 1H); 2.77 (td, $J = 4.6, 17.1$ Hz, 1H); 4.26 (q, $J = 7.1$ Hz, 2H); 5.37 (s, 1H); 6.86 (d, $J = 3.0$ Hz, 1H); 7.22 (m, 2H); 7.33 (s, 1H); 7.55 (broad m, 2H); 8.56 (s, 1H); 11.52 (d, $J = 3.0$ Hz, 1H); 13.08 (broad m, 1H).

Ethyl 9-[4-(1H-benzimidazol-2-ylsulfanyl)-2-thienyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-

b]quinoline-3-carboxylate (42)

Yield: 220 mg (65%). Analytical LC/MS (method B): $t_R = 3.23$ min; $[M+H]^+$: m/z 491. 1H NMR (400 MHz, DMSO- d_6): 1.29 (t, $J = 7.1$ Hz, 3H); 1.76 to 1.95 (m, 2H); 2.21 to 2.29 (m, 2H); 2.47 to 2.62 (m, 1H); 2.75 to 2.84 (m, 1H); 4.27 (q, $J = 7.1$ Hz, 2H); 5.36 (s, 1H); 6.80 (broad, 1H); 6.86 (d, $J = 3.0$ Hz, 1H); 7.16 (m, 2H); 7.45 (broad m, 2H); 7.47 (d, $J = 1.5$ Hz, 1H); 8.49 (s, 1H); 11.44 (d, $J = 3.0$ Hz, 1H); 12.54 (broad s, 1H).

Ethyl 9-[3-(1H-benzimidazol-2-ylsulfanyl)phenyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-**b]quinoline-3-carboxylate (43)**

Yield: 144 mg (19%). 1H NMR (400 MHz, DMSO- d_6): 1.29 (t, $J = 7.1$ Hz, 3H); 1.60 to 1.71 (m, 1H); 1.76 to 1.86 (m, 1H); 2.04 to 2.21 (m, 2H); 2.48 to 2.63 (partially masked m, 2H); 4.26 (q, $J = 7.1$ Hz, 2H); 5.03 (s, 1H); 6.68 (d, $J = 3.0$ Hz, 1H); 7.12 to 7.24 (m, 6H); 7.50 (broad s, 2H); 8.22 (s, 1H); 11.29 (d, $J = 3.0$ Hz, 1H); 12.77 (broad s, 1H).

Ethyl 9-[6-(1H-benzimidazol-2-ylsulfanyl)-2-pyridyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-**b]quinoline-3-carboxylate (44)**

Yield: 35 mg (5%). Analytical LC/MS (method B): $t_R = 3.25$ min; $[M+H]^+$: m/z 486.

Ethyl 9-[2-(1H-benzimidazol-2-ylsulfanyl)-4-pyridyl]-8-oxo-2,4,5,6,7,9-hexahydropyrrolo[3,4-**b]quinoline-3-carboxylate (45)**

Yield: 85 mg (15%). Analytical LC/MS (method B): $t_R = 2.87$ min; $[M+H]^+$: m/z 486. 1H NMR (400 MHz, DMSO- d_6): 1.29 (t, $J = 7.1$ Hz, 3H); 1.45 to 1.57 (m, 1H); 1.68 to 1.78 (m, 1H); 1.94 to 2.03 (m, 1H); 2.07 to 2.18 (m, 1H); 2.36 to 2.53 (partially masked m, 2H); 4.26 (q, $J = 7.1$ Hz, 2H); 4.97 (s, 1H); 6.74 (d, $J = 3.0$ Hz, 1H); 6.86 (broad s, 1H); 7.14 (dd, $J = 1.5, 5.4$ Hz, 1H); 7.26 (m, 2H); 7.59 (broad m, 2H); 8.24 (s, 1H); 8.25 (d, $J = 5.4$ Hz, 1H); 11.38 (d, $J = 3.0$ Hz, 1H); 13.07 (broad s, 1H).

S-5 In Vitro Assays

In Vitro Aurora Enzyme Assay. The inhibitory effect of compounds with respect to the Aurora A, B

and C kinases was determined with a radioactivity scintillation assay. The kinase activity of Aurora was measured by the phosphorylation of NuMA-histidine substrate in the presence of radiolabelled ATP ($[^{33}\text{P}]$ ATP) using 96-well nickel chelate Flash plates. The amount of ^{33}P incorporated to the substrate NuMA is proportional to the Aurora activity. Protein production was performed in house:

- Aurora A: the full length recombinant protein including an N-terminal poly-histidine tag was expressed in *E. coli* and purified to 82%.
- Aurora B : the full length protein (poly-His tagged in N-terminal) was co-expressed in SF9 cells with the C3 fragment of INCENP protein fused to GST protein. The complex was purified using the N-terminal poly-histidine tail to 50% homogeneity.
- Aurora C: the A25-S309 Aurora C (poly-His tagged in N-terminal with a tobacco etch virus protease cleavage sequence) was co-expressed in SF9 cells with the C3 fragment of INCENP protein fused to GST protein in a similar manner to Aurora B INCENP.
- NuMA, (a nuclear protein which binds to the mitotic apparatus): fragment of 424 amino acids (position 1687-2101) was expressed in *E. coli* (tagged on the N-terminal end with a poly-histidine tail for use as a the substrate for Aurora enzymes).

The Flash plates used were nickel-chelate 96 well plates (Perkin Elmer, model SMP107). The products to be evaluated were incubated in a 100 μL reaction volume per well in the presence of 10 nM of Aurora A, or Aurora B, or 20 nM of Aurora C, 500nM of NuMA substrate in the following buffer: 50 mM tris-HCl pH 7.5, 50 mM NaCl, 5 mM MgCl_2 , (Aurora B) or 10 mM MgCl_2 (Aurora A, Aurora C), and 1mM of DTT at 37°C. 80 μL of enzyme/substrate incubation buffer was distributed in each well, followed by 10 μL of solution of compound to be measured with various concentrations. The reaction was started by adding 1 μM of ATP (final concentration) containing 0.2 μCi of $[\gamma^{33}\text{P}]$ ATP (10 μL) in the case of Aurora A / B and 0.3 μM of ATP (final concentration) containing 1 μCi of $[\gamma^{33}\text{P}]$ ATP (150 μL) in the case of Aurora C. After 30 min of incubation the reaction was stopped by removal of the reaction mixture and each well was washed twice with 300 μL of buffer Tris/HCl. The radioactivity was

measured in each well using a Packard Top count scintillation counter instrument. The enzymatic activity was expressed as counts per minute obtained in 30 min after subtraction of the background noise (reaction medium without enzyme). The measurement was expressed as percentage of inhibition of Aurora activity versus the control. To generate IC₅₀ values, compounds were tested at different concentrations and the percentages of inhibition were plotted as function of compound concentration, IC₅₀s were calculated using the Xlfit 4 curve fitting software.

***In Vitro* Kinase Selectivity Assays.** The selectivity profiles of cpds. **16** and **47** were evaluated in a panel of biochemical kinase assays at Sanofi, Upstate and CEREP/Millipore and are summarized in Section S-1.

***In Vitro* PDE3 Enzyme Assay.** The effect of the cpds on the activity of the enzyme PDE3 was evaluated by the company CEREP (Le bois l'Evêque, 86600 Celle l'Evescault, France; <http://www.cerep.fr>) in accordance with its standard protocol:² the enzyme PDE3A in recombinant form was expressed in Sf9 cells, the substrate was cAMP and the residual cAMP was measured by HTRF. The reference inhibitor in the test was milrinone, the IC₅₀ of which was 270 nM. The residual activity percentages were related to the control without inhibitor. The results were expressed either as the concentration which induced inhibition by 50% (IC₅₀) or as a percentage inhibition measured at a set concentration of the compound.

Cell Proliferation Assays (solid tumor cell lines). Cells were grown in the presence of the test cpd. for 96 h, with ¹⁴C-thymidine added during the last 24 h. The cell proliferation was estimated by the amount of ¹⁴C-thymidine incorporated in the cells. The test cpd. was dissolved to form a stock solution at 10 mM in DMSO, and the latter was used to produce a range of serial dilutions, generally from 10 000 µM to 0.3 µM, these serial dilutions being themselves diluted 1/50 in the cell culture medium (20x solution) which was later used for 1/20 dilution in the cell culture plates. The final concentrations of the test compound were generally between 10 000 and 0.3 nM.

The cells were seeded in 96-well Cytostar plates in 180 µL of culture medium. The plates were then

placed in an incubator at 37°C, 5% CO₂ for 4 h. The test products were then added in a volume of 10 µL per well, starting from a 20x solution. This solution contained 2% of DMSO in the culture medium. The final concentration of DMSO was therefore 0.1%. The plates were then placed in an incubator at 37°C/5% CO₂ for 72 h. Then, 10 µL per well of ¹⁴C-thymidine at 10 µCi/mL in the culture medium were added. The plates were then placed in an incubator at 37°C, 5% CO₂ for 24 h. The incorporation of ¹⁴C thymidine was finally measured on a Micro-Beta radioactivity counter (Perkin-Elmer) after this 24 h, "pulse" period. The total time of treatment of the cells with the test product was 96 h.

The percentage inhibitions IC₅₀ were calculated using the following formula:

$$I\% \text{ Inhibition} = 100 * (1 - (\frac{X - Blank}{CC - Blank}))$$

X = Measurement for the sample

CC = Cell Control

Blank = Measurement in the wells without cells

IC₅₀ were calculated using the XLfit software (IDBS, UK) with the aid of formula 205, with the parameter D (Hill number) locked to a value of 1.

Viability assay (hematological tumor cell lines, PBMC).

Viability of hematological cell lines was estimated using a MTS assay.

Viability of peripheral blood mononuclear cells (PBMC) was evaluated using a Fluorescein diacetate (FDA) / Propidium iodide (PI) assay. Briefly, PBMC (Biowhyttaker) were seeded in 96-well plates (TPP) at 0.1 10⁶ cells per well in 190 µL of RPMI medium (Invitrogen) supplemented with 10% heat inactivated FBS (Invitrogen) and 2 mM L-Glutamine (Invitrogen). Serial dilutions of the cpds. ranging from 10µM to 0.3 nM were added and cells were incubated for 96 h in the presence of cpds., at 37°C under 5% CO₂. Cells were then treated with 0.25µg/mL of FDA (Sigma) and 10 µg/mL of PI (Sigma) for 5 to 10 min at rt and then analyzed on a FC500 cytometer (Beckman Coulter). IC₅₀s were calculated using XLfit (IDBS). FDA diffuses into cells and is hydrolyzed to fluorescein in live cells whereas PI is incorporated in DNA of cells with impaired membrane permeability (dead cells).

Evaluation of DNA content in cultured cells (polyploidy assay). Cells were seeded in 6-well plates at 0.2×10^6 cells per well in 2 mL of DMEM medium supplemented with 10% heat-inactivated FBS, 1% glutamine. 24 h later, cpds. were added. Cells were incubated for 48 h in the presence of cpds., at 37°C under 5% CO₂. Cells were harvested by trypsinization, fixed and permeabilized in 100% cold ethanol for 30 min at 4°C. Cells were then washed in PBS and resuspended in PBS containing 10 µg/mL propidium iodide and 0.1 mg/mL boiled RNase. Cells were then filtered and analyzed on a FACSCalibur (Becton Dickinson) for the estimation of polyploidy.

Monitoring of Aurora A and B biomarkers in cultured cells. Human colon adenocarcinoma HCT116 were treated with serial dilutions of cpd. 47 for 4 h and the extent of phosphorylation of Aurora A (Aurora A biomarker) and histone H3 (Aurora B biomarker) was evaluated in a Icyte high content cell profiling assay.

***In Vitro* Metabolism Assay Using Human and Murine Liver Microsomal Preparations.**

The cpds. (5 µM) were incubated at physiological temperature with human and murine microsomal liver fractions (1 mg/mL of proteins), diluted in a phosphate buffer in the presence of bovine serum albumin (1 mg/mL BSA), and 1 mM NADPH. To terminate incubation, 4 volumes of acetonitrile containing corticosterone as internal standard (IS) were added. The samples were centrifuged and the supernatants were analyzed by LC/MS-MS. The LC/MS-MS analysis was performed on a QTRAP API4000 mass spectrometer (Sciex) equipped with a 1100 series chromatography system (Agilent) and a Pal CTC automatic injector. The data were acquired and analyzed using Analyst 1.4.1 software. The samples were separated on a 3 µm C18 Polaris column, eluted at a flow rate of 0.7 mL/min with a gradient from acetonitrile (solvent B) into water containing 0.1% formic acid (solvent A). The elution programme was composed of the gradient from 20 to 90% of solvent B in 2 min, an isocratic stage at 90% of solvent B, for 0.9 min, and a return to the initial conditions in 0.1 min. The areas of the chromatographic peaks for the compound and for the internal standard were integrated using the Analyst-Classic algorithm. The metabolic stability of cpds. was estimated by comparing the integration

ratios (ion currents of the compounds/ion current IS) measured after 0 min (t_0) and 20 min (t_{20}) of incubation. The metabolic stability was then expressed as a percentage disappearance in accordance with the following formula:

$$\text{Metabolism\%} = (\text{ratio of peaks at } t_0 - \text{ratio of peaks at } t_{20}) / \text{ratio of peaks at } t_0$$

Cytochrome P-450 (CYP) 3A4 inhibition.

Recombinant CYP3A4 inhibition screening assay.

The inhibition assay was conducted with recombinant CYP3A4 co-expressed with human P450 reductase and human b5 reductase from BD Gentest at 3 pmoles of P450/ well. The substrate was 7-benzyloxyquinoline (7BQ) used at 10 μM ($\sim K_m$). IC_{50} values were determined using 10 inhibitor concentrations from 0.2 nM to 40 μM . 0.1 mg/mL protein was added in the incubation mixture. Final concentration of DMSO was 0.4%. The production of 7-Hydroxyquinoline was measured by fluorescence. Incubation at 37°C was started by the addition of 1 mM NADPH. Measurements were taken every 30 seconds for 15 min by reading the fluorescence at an excitation wavelength of 410 nm and an emission wavelength of 538 nm using a Labsystem™ Fluoroskan Ascent Fluorimeter. Itraconazole and Mifepristone (RU486) were used as references for reversible and irreversible CYP3A inhibition respectively. IC_{50} values for inhibition of CYP activity were determined from the initial velocity of the kinetic curve. This value reflects the affinity of the inhibitor toward the enzyme.

Time course analysis of inhibition was also examined in order to assess whether the tested cpds. could exhibit a reversible or irreversible interaction with the CYP enzyme. Cpds. showing a slope lower than – 3%/min were defined as irreversible inhibitors. For compound with IC_{50} higher than 40 μM , no slopes could be determined.

Characterization of CYP3A4 inhibition by cpd. 47 (SAR156497).

Microsomes containing CYP enzyme cDNA (Supersomes™) were purchased from BD Biosciences and stored at approximately -80°C. Human liver microsomes (HLM) were obtained from BD Gentest. Each batch of microsomes was characterized for protein and cytochrome P-450 concentrations. Studies

were performed with a pool of 50 different human donors.

Reaction mixtures were prepared in 0.1 M phosphate buffer pH 7.4, with SupersomesTM containing 100 pmol/mL of cDNA expressed CYP3A4 or with 1 mg protein/mL of HLM, with 1 mM NADPH and generating system containing 3.3mM G-6-P and 1U/mL G-6-P dehydrogenase and with SAR156497 at 0.78, 1.56, 3.13, 6.25, 12.5 or 25 μ M. The reaction mixtures were pre-incubated at 37°C for 5 different time periods at 0, 5, 10, 15 and 30 min. 10 μ L of each reaction mixture, resulting in a 20-fold dilution, were transferred into a second set of reaction mixtures, containing Midazolam in excess at saturating concentration of CYP3A4 enzyme activity defined at 70 μ M (10-fold higher than the apparent K_m concentration) and containing 1 mM NADPH in 0.1 M phosphate buffer pH 7.4 with 0.1 % BSA final concentration. The final volume of the second set of reaction mixture was 200 μ L and Midazolam 1'-hydroxylase activity was carried out at 37°C and was stopped after 7 min by addition of 2 volumes of ice-cold acetonitrile containing 1 μ M corticosterone, as internal standard. Acetonitrile extracts were analyzed by LC/MS/MS (LC instrument Agilent1100, column Interchim C₁₈ HDO 250x2.1 mm, flow rate: 0.3 mL/min, gradient 30 to 95% acetonitrile in aqueous 52 mM formic acid 10 mM ammonium acetate in 2.4 min total analysis time 6 min, MS/MS instrument ABSciex API4000 qTrap). If time-dependent inhibition of the CYP3A4 activity was observed, then the kinetic constants of the irreversible inactivation, k_{inact} and K_i , were determined. Positive control was performed in the same run with Mifepristone at 10 μ M (4-5). Negative control was top SAR156497 concentration without NADPH cofactor. In same conditions, Mifepristone as positive comparator was evaluated with an adapted range of concentrations (0.313, 0.625, 1.25, 2.5, 5, 10 and 20 μ M). All experiments were performed in duplicate.

S-6 *In Vivo* Assays

Pharmacokinetic (PK) study in non-tumor bearing mice.

Female balb-C mice (25-35 g, 3 animals per group, Charles River, France) were treated with cpd. 47

(SAR156497) at a 10 mg/kg dose either by iv or po routes. For iv route, **47** was formulated at a 2 mg/mL concentration in 4% glycofurol, 2% PS-80 and 5% glucose and injected in the tail vein. For po route **47** was formulated at a 1 mg/mL concentration in 2% glycofurol, 1% PS-80 and 5% glucose and administered by gavage. Blood was sampled in orbitary plexus, heparinized on ice and centrifugated. The plasma samples were stored at -20°C before analysis. For analysis, plasma samples were treated with acetonitrile to precipitate plasma proteins, centrifuged for 10 min at 4000 RPM, the supernatant fractions were diluted with 1 volume of 22 mM formic acid and **47** was quantified by LC/MS/MS (LC instrument Perkin-Elmer 200 Series, column AIT C₁₈ ACE 5µm 50x2.1 mm, flow rate: 0.3 mL/min, gradient 30 to 95% acetonitrile in aqueous 26 mM formic acid in 0.5 min total analysis time 4 min, MS/MS instrument ABSciex API4000 qTrap). The pharmacokinetic parameters were calculated from the arithmetic mean of plasma concentration using the program WinNonLin 5.2, non-compartment model 200/201.

Pharmacokinetic/pharmacodynamic (PK/PD) and efficacy study in HCT116 xenograft bearing mice.

Human colon HCT116 adenocarcinoma were implanted in the flank of female SCID mice (4-5 weeks, ~20 g) and grown up to a size of ~200 mm³. ALZET[®] minipumps were then implanted to continuously infuse cpd. **47** or vehicle alone at a flow rate of 8 µL/h and at a dose of 25 mg.kg⁻¹.day⁻¹ for 24 and 48 h and at a dose of 50 mg.kg⁻¹.day⁻¹ for 24 h. For the 48 h infusion, the pumps were changed after the first 24 h in less than 5 min. Cpd. **47** sulfate salt was formulated at a concentration of 2.5 mg/mL (for the 25 mg.kg⁻¹.day⁻¹ dose) or 5 mg/mL (for the 50 mg.kg⁻¹.day⁻¹ dose) in 2.5% ethanol, 7.5% PS80, 5% glucose, 0.15% ascorbic acid, pH = 3. For the 50 mg.kg⁻¹.day⁻¹ dose, cpd. **47** plasma concentration was assayed during the time course of treatment. Blood was collected at t = 1, 4, 8, 24, 25, 28, 48 h on isoflurane anesthetized mice by retro orbital puncture in heparinized microtubes. After immediate centrifugation (3 min, 7600g) at 4°C, the plasma was collected and immediately frozen and stored at -20°C until analysis. After addition of 100 µL acetonitrile containing an internal standard to precipitate

proteins to 10 μ L of plasma, the sample was centrifuged for 10 min at 4000 RPM; 80 μ L of supernatant was diluted with 80 μ L of 26 mM formic acid and 50 μ L of acetonitrile / water (50/50 v/v) and cpd. **47** was quantified by LC-MS/MS (LC instrument Perkin-Elmer 200 Series, column AIT C₁₈ ACE 5 μ m 50x2.1 mm, flow rate: 0.3 mL/min, gradient 30 to 95% acetonitrile in aqueous 26 mM formic acid in 0.5 min total analysis time 4 min, MS/MS instrument ABSciex API4000 qTrap). At indicated time points some animals were sacrificed, their tumors were excised and phospho-histone H3 positive cells were quantified by immunohistochemistry on tumor slices. The volumes of tumors were measured by caliper at indicated time points. The primary efficacy end points was tumor volume changes from baseline summarized by the ratio of medians between treated and control groups ($\Delta T/\Delta C$) expressed in percentage. Interpretation of the $\Delta T/\Delta C$ expressed in percentage was based on the following criteria: >40%: inactive; $\leq$ 40% that corresponds to an obvious tumor growth delay: active; <10% very active that corresponds to a tumor stasis; <0%: highly active that corresponds to a tumor regression.

S-7 Crystallographic studies

Human AuroraA (125-399)-His was expressed and purified from *E. Coli* BL21 cells via Ni-affinity and gel filtration chromatography and its sequence verified by mass spectrometry. For compd **1**, native crystals were obtained from 28% PEG 400, 200 mM NaAcetate, 5mM DTT, 100 mM Tris pH 8, then soaked in 2 mM compd. For compd. **16**, native crystals were obtained from 21% PEG 3350, 50 mM Tris pH 8, then soaked in 2 mM compd. For compd **47**, cocrystals were obtained from 4% PEG 4000, 50 mM Hepes pH 7. Crystals were cryoprotected and flash-frozen in liquid nitrogen prior to data collection. Diffraction data were collected at the European Synchrotron Radiation Factory and were processed with Mosflm³ and SCALA.⁴ The structures were solved by molecular replacement using Amore, MOLREP⁴ and Phaser for compounds **1**, **16** and **47**, respectively. The structure was refined using CNX for compd **1** and BUSTER/TNT⁵ for compds **16** and **47**. Model building was performed in COOT.⁶

Table 5: X-ray data collection statistics and PDB codes for the structures of Aurora A bound to compds 1, 16 and 47, respectively

	Compound 1	Compound 16	Compound 47
Data Collection			
Space group	P2 ₁	P2 ₁	P3 ₁ 21
Unit cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	48.17-45.56-59.41	48.35-45.91-59.59	90.45-90.45-206.88
α , β , γ (°)	90.00-98.38-90.00	90.00-97.32-90.00	90.00-90.00-120.00
Resolution (Å)	39.8-2.0(2.11-2.00)	59.0 -1.9(2.01-1.90)	78.3-3.2(3.37-3.20)
Total reflections	42,626	71,772	95,464
Unique reflections	16,871	20,475	16,476
Completeness (%)	96.8(97.5)	99.3(97.3)	97.9 (99.3)
R _{sym} (%)	11.6(23.4)	6.0(14.0)	7.5 (41.1)
$\langle I/\sigma(I) \rangle$	8.5(3.2)	20.8(6.3)	19.9 (3.1)
Refinement			
Reflections used	16,868	20,466	16,434
R / R _{free} (%)	20.5 / 23.5	20.1 / 23.4	21.5 / 26.2
Average B-values (Å ²)	28	31	94
Rms deviations			
Bond lengths (Å)	0.06	0.01	0.01
Bond angles (°)	1.23	1.36	1.05
PDB ID	4UZH	4UYN	4UZD

Data in parentheses correspond to the highest resolution shells. To calculate R_{free}, 5-10% of the reflections were excluded from the refinement. R_{sym} is defined as $R_{sym} = \sum_{hkl} \sum_i |I_i(hkl)| - \langle I(hkl) \rangle / \sum_{hkl} \sum_i I_i(hkl)$.

Table 6: X-ray structures of Aurora A bound to compds 1 in orange (A), 16 in orange (B) and 47 in green (C). Helix C is colored in red.

Table 7: Zoom on the binding site of X-ray structures of Aurora A bound to compds 1 in orange (A), 16 in orange (B) and 47 in green (C). Helix C is colored in red. Hydrogen-bonds are depicted as yellow dashed lines. A) Pyrazole moiety of compd 1 makes 2 hydrogen bonds with the hinge region of Aurora A, with residues Glu211 and Ala213. A third water –mediated hydrogen bond is observed between ring C and the carbonyl moiety of Ala213. The fluorophenyl group is oriented toward residue Leu210. B) Compd 16 makes two hydrogen bonds with the hinge region of Aurora A, the first one between the pyrrole moiety and Glu211 and the second one between the ester

moiety and Ala213. Two additional hydrogen bonds are observed with Lys162, one from the cyclohexenone moiety and a second one from the imidazole moiety. Finally a fifth hydrogen bond is observed between the imidazole moiety and the side chain of Gln185 on helix C. C) The pattern of interactions observed between compd 47 and Aurora A is similar to what is observed for compd 16 in (B).

References for Supporting Information.

- (1) Knudsen, S. M.; Pettersson, I.; Lau, J.; Behrens, C.; Petersen, A. K.; Lau, J.; Garibay, P. W. Condensed thiophene derivatives and their use as cyclic GLP-1 agonists. PCT Int. Appl. WO2006003096, **2006**.
- (2) Bender, A.T.; Beavo, J.A. Cyclic Nucleotide Phosphodiesterases: Molecular Regulation to Clinical Use. *Pharmacol. Rev.* **2006**, 58, 488-520.
- (3) Leslie, A. G. W.; Powell, H. R. Processing diffraction data with mosflm. In *Evolving Methods for Macromolecular Crystallography*; Read, R. J., Sussman, J. L., Eds.; Springer Netherlands, 2007; pp 41-51.
- (4) Collaborative Computational Project, Number 4. The CCP4 Suite: Programs for Protein Crystallography *Acta Cryst.* **1994**, D50, 760-763.
- (5) Bricogne, G. *Ab Initio* Macromolecular Phasing: Blueprint for an Expert System Based on Structure Factor Statistics with Built-in Stereochemistry. *Methods Enzymol.* **1997**, 277, 14-18.
- (6) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and development of *Coot*. *Acta Cryst.* **2010**, D66, 486-501.