Supplementary Material

Discovery of a potent and isoform selective targeted covalent inhibitor of the lipid kinase PI3Kα

Mariana Nacht, Lixin Qiao, Michael P. Sheets, Thia St. Martin, Matthew Labenski, Hormoz Mazdiyasni, Russell Karp, Zhendong Zhu, Prasoon Chaturvedi, Deepa Bhavsar, Deqiang Niu, William Westlin, Russell C. Petter, Aravind Prasad Medikonda and Juswinder Singh*

Celgene Avilomics Research, 45 Wiggins Ave, Bedford MA 01730

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Experimental Section

Protein production for crystallography

Suitable constructs for human PI3Kα expression had been previously established by Proteros (Martinsried, Germany). Expression of human PI3Kα was performed according to previously established protocols. A purification protocol was established and homogeneous protein was produced in preparative amounts. The protein was purified through a process comprising affinity and gel filtration chromatography steps. This procedure yielded homogeneous protein with a purity greater than 95% as judged from coomassie stained SDS-PAGE.

Crystallization

The purified protein was used in crystallization trials employing both a standard screen with approximately 1200 different conditions, as well as crystallization conditions identified using literature data. Conditions initially obtained have been optimized using standard strategies, systematically varying parameters critically influencing crystallization, such as temperature, protein concentration, drop ratio, and others. These conditions were also refined by systematically varying pH or precipitant concentrations.

Data collection and processing

A cryo-protocol was established using Proteros' standard protocols. Crystals have been flash-frozen and measured at a temperature of 100 K. The x-ray diffraction data have been collected from complex crystals of human PI3Kα with the ligand **3** at the SWISS LIGHT SOURCE (SLS, Villigen, Switzerland) using cryogenic conditions. The crystals belong to space group P 21 21 21. Data were processed using the programs XDS and SCALA.

Structure modeling and refinement

The phase information necessary to determine and analyze the structure was obtained by molecular replacement. A previously solved structure of human PI3Kα was used as a search model. Subsequent model building and refinement were performed according to standard protocols with the software packages CCP4 and COOT. For the calculation of the free R-factor, a measure to cross-validate the correctness of the final model, about 2.6 % of measured reflections were excluded from the refinement procedure (see **Table S2** in Supporting Information). TLS refinement (using REFMAC5, CCP4) has been carried out, which resulted in lower R-factors and higher quality of the electron density map. The ligand parameterization and generation of the corresponding library files were carried out with CORINA. The

water model was built with the "Find waters"-algorithm of COOT by putting water molecules in peaks of the Fo-Fc map contoured at 3.0 σ followed by refinement with REFMAC5 and checking all waters with the validation tool of COOT. The criteria for the list of suspicious waters were: B-factor greater 80 Å2, 2Fo-Fc map less than 1.2 σ, distance to closest contact less than 2.3 Å or more than 3.5 Å. The suspicious water molecules and those in the active site (distance to inhibitor less than 10 Å) were checked manually. The occupancy of side chains, which were in negative peaks in the Fo-Fc map (contoured at -3.0 σ), were set to zero and subsequently to 0.5 if a positive peak occurred after the next refinement cycle. The Ramachandran Plot of the final model shows 90.2 % of all residues in the most favoured region, 9.8 % in the additionally allowed region, and 0.0 % in the generously allowed region. No residues are found in the disallowed region (see **Table S2** in Supporting Information). Statistics of the final structure and the refinement process are listed in **Table S2** in Supporting Information.

Figure S1. 2D chemical structure of **8**

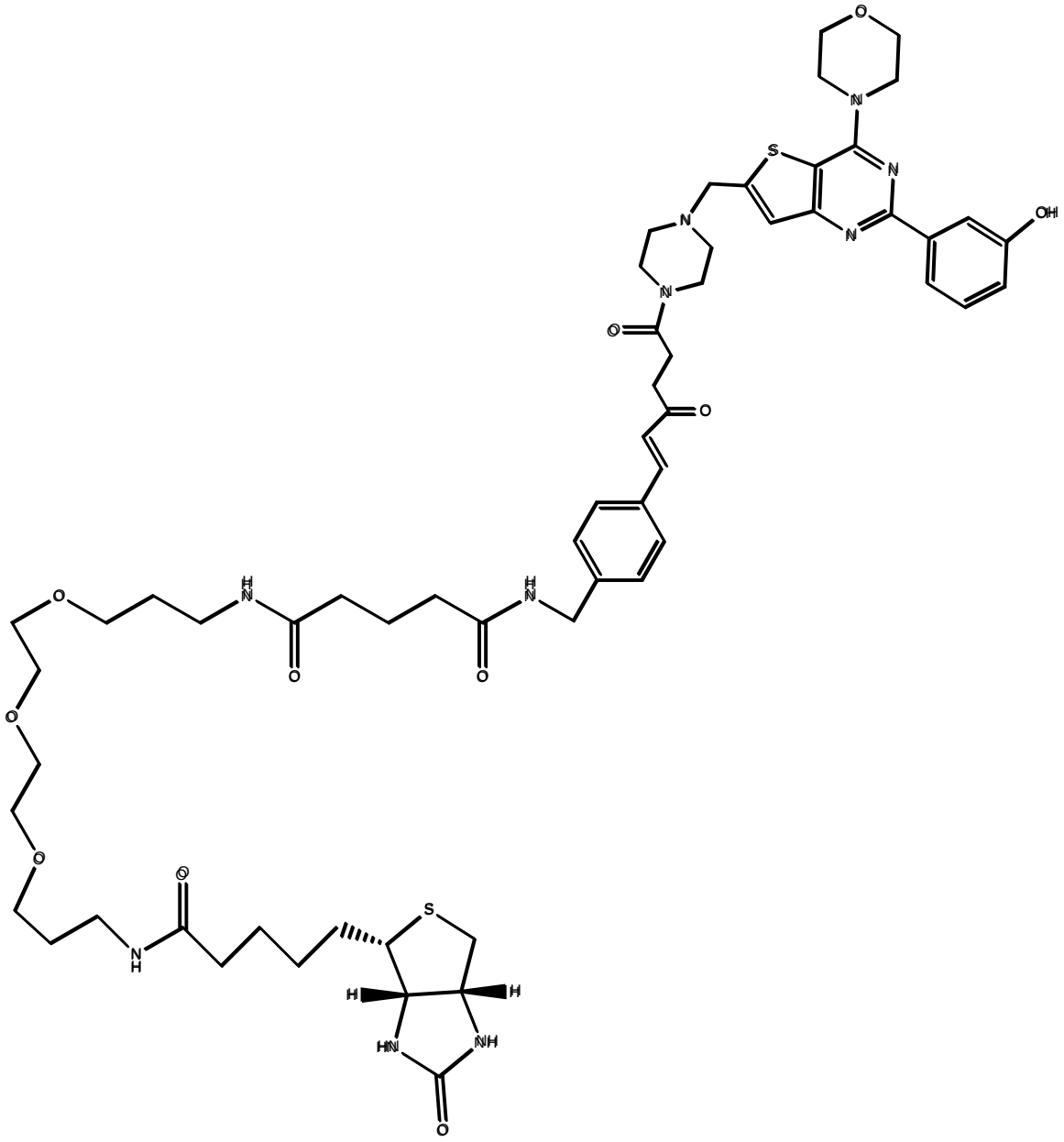
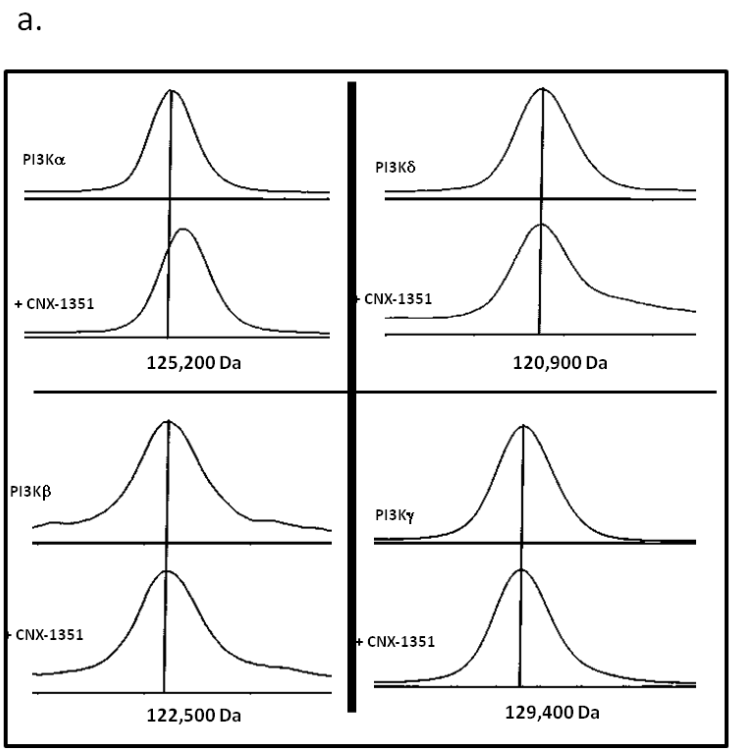
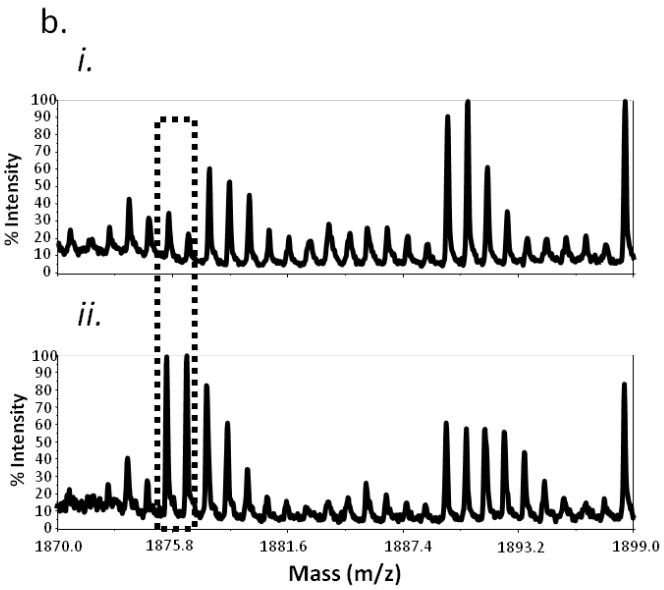


Figure S2. **3** (CNX-1351) bonds specifically with PI3Kα at C862

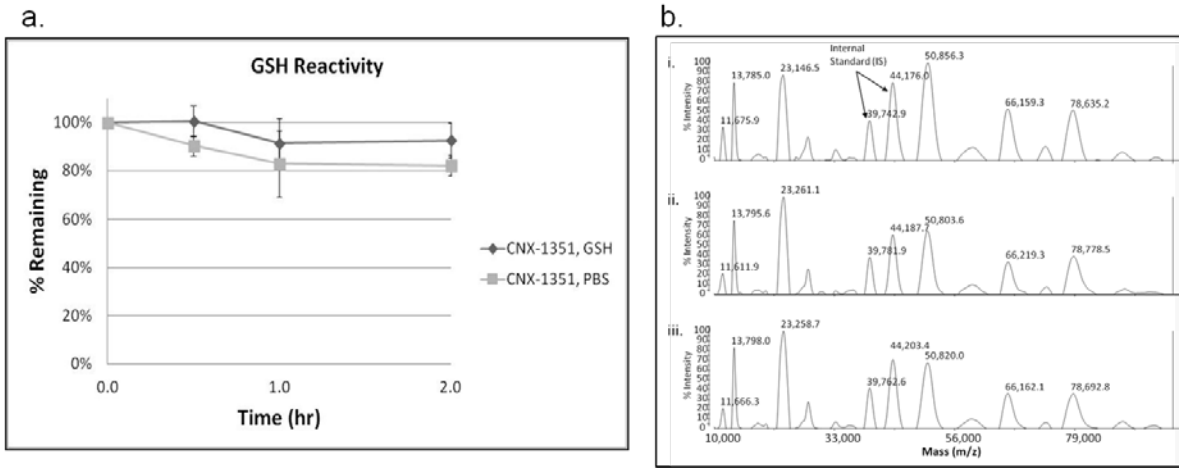


A. Covalent PI3K Inhibitor, CNX-1351, is Selective for p110α. Mass spectrometry confirmed that CNX-1351 bonds only to PI3Kα. The mass shift seen with PI3Kα is consistent with the mass of CNX-1351.



B Tryptic Digest to Confirm that CNX-1351 Selectively Bonds Cys862. PI3Kα was incubated alone *(i)* or with *(ii)* CNX-1351 for 1 hr (10:1 CNX-1351:protein). Sample was then run on 4-12% BT gel. Band of interest was excised and subjected to an in-gel trypsin digest. Analysis was performed on MALDI TOF/TOF 4800. The targeted Cys862 in peptide NSHTIMQIQCK was clearly modified (boxed in *ii*).

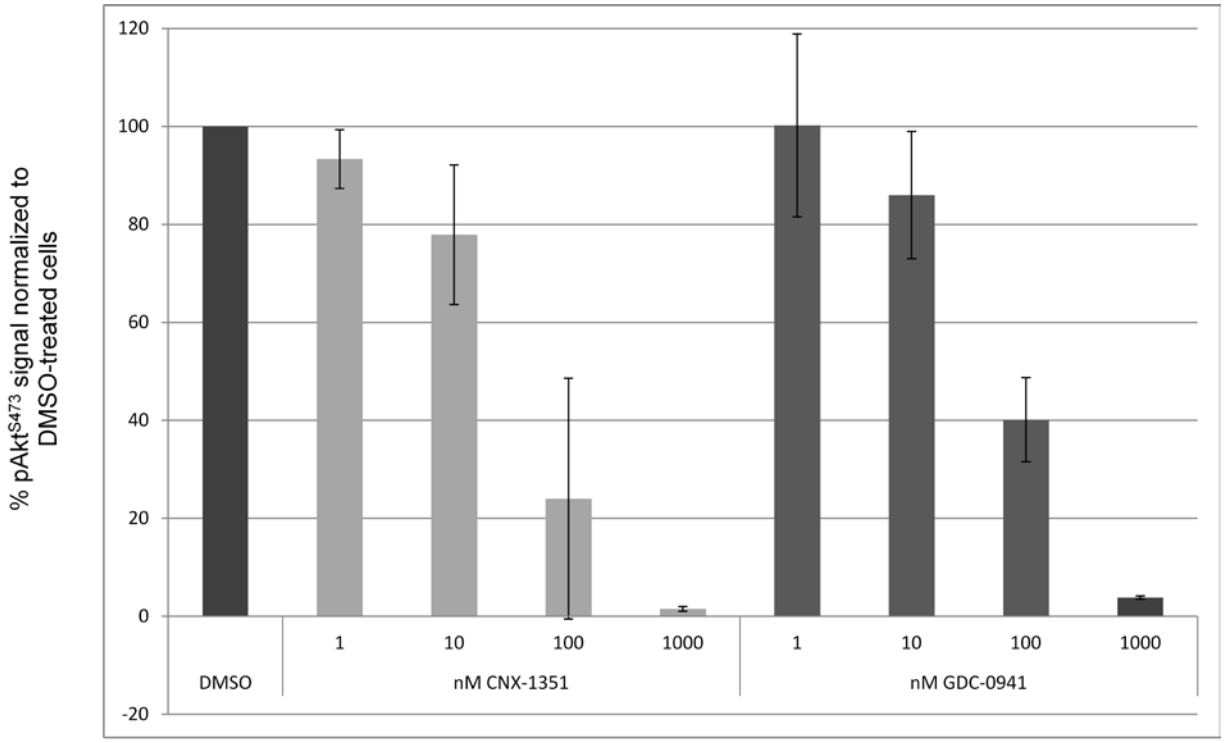
Figure S3. Reactivity of **3** with glutathione and plasma proteins



a. CNX-1351 has no appreciable reactivity towards GSH. CNX-1351 was reacted with or without 500-fold molar excess of GSH for up to 2 hours at 37°C in PBS. Samples were analyzed by triple quadrupole mass spectrometry, and values represent the average of 3 replicates.

b. CNX-1351 does not bond to proteins in human plasma. CNX-1351 (1mM) was incubated in 10 mg of albumin-depleted human plasma for 1 or 3 hours at 37°C. Panel (i) shows the untreated albumin-depleted human plasma; (ii) and (iii) show the albumin-depleted human plasma after a 1 or 3 hour incubation with CNX-1351, respectively.

Figure S4. **3** inhibits P-Akt ^{Ser473} in SKOV3 with an EC_{50}<100nM



CNX-1351 Inhibits PI3K Signaling in SKOV3 cells. SKOV3 were incubated with the covalent PI3Kα-selective inhibitor, CNX-1351, or the non-covalent pan-PI3K inhibitor, GDC-0941, (1-1000 nM) for 1hr. Cell lysates were immunoblotted for P-Akt^{Ser473}. Data shown for 3 experiments.

Figure S5. a. Mass spectrometry was used to confirm that the covalent probe, **8**, bonds only to recombinant PI3Kα, but not to the other PI3K isoforms. The mass shift detected with PI3Kα protein is consistent with the molecular weight of **8**. b. SKOV3 cell lysate was incubated for one hour with the biotinylated covalent probe, **8**. The protein lysate was then incubated with PI3K isoform specific antibodies (α or β) to immunoprecipitate the specific isoform bound or not bound to the covalent probe. The immunoprecipitated proteins were run on an SDS polyacrylamide gel and immoblotted using antibodies to detect PI3Kα, PI3Kβ or biotin. The co-localization (merge) of the streptavidin signal with the PI3Kα signal confirms that **8** bond to PI3Kα but not PI3Kβ.

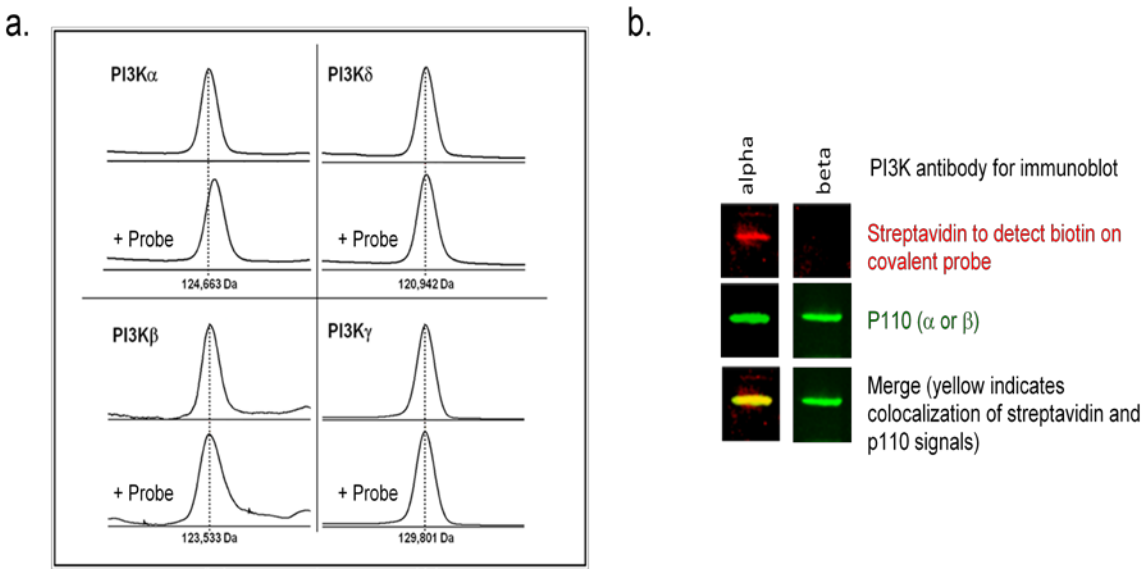


Table S1. Biochemical Selectivity of **3** against a panel of kinases

Kinase	Average % Inhibition At 1μM CNX-1351
ABL1	2.1
AKT1	0.7
AKT2	7.5
Aurora A	25.1
BLK	-2.1
BMX/ETK	24.0
BTK	31.0
c-Kit	28.1
c-Kit (D816V)	18.5
c-MET	-12.4
c-Src	7.6
CAMK2b	9.6
CDK1/cyclin B	1.3
CDK2/cyclin A	3.9
CHK2	3.6
CK2a2	5.8
DAPK1	-16.8
DYRK2	9.1
EGFR	-16.6
EGFR (L858R, T790M)	13.8
EPHA3	11.4
ERBB4/HER4	12.5
ERK1	-7.2
FAK/PTK2	-0.5
FGFR3	-1.1
FLT1/VEGFR1	7.3
FLT3	35.5
FMS	-5.1
GSK3b	-1.1
IKKa/CHUK	-3.5
IRAK4	10.4
ITK	-1.2

Kinase	Average % Inhibition At 1μM CNX-1351
JAK2	-3.6
JAK3	1.2
JNK1	2.3
KDR/VEGFR2	-6.6
LCK	-0.8
LYN	19.6
MINK/MINK1	8.7
MST2/STK3	5.8
NEK2	4.1
PAK2	-2.2
PDGFRa	-3.5
PIM1	-5.9
PKA	8.1
PKCa	3.3
PLK1	3.0
RET	2.3
ROCK1	8.7
RON/MST1R	13.8
SYK	-7.0
TAK1	3.2
TAOK2/TAO1	-1.3
TEC	7.4
TIE2/TEK	-5.6
TRKA	8.2
TXK	7.8
WNK2	0.6
YES/YES1	-3.4
ZAP70	31.3

Table S2. Bioanalytical method for the pharmacokinetic study of **3**

Method Type:	LC-MS/MS
Matrix:	Mouse EDTA plasma
Internal Standard:	Carbutamide
HPLC System:	Pump: Shimadzu LC-20AD Autosampler: CTC Autosampler (Leap Technologies; Carrboro, NC) System Controller: Shimadzu CBM-20A
Mass Spectrometer: **Conditions:**	API 4000 Q Trap LC-MS system (Applied Biosystems; Carlsbad, CA). Collision Gas - Medium Curtain Gas - 40 Temperature - 600^{0}C Gas I - 60 Gas II - 40 Ion voltage - 5500
LC-MS/MS Conditions:	**Column:** Luna C-8 (30x 2 mm; Phenomenex; Torrance, CA) **Mobile Phase:** A. Water with 0.1 % formic acid B. Acetonitrile with 0.1% formic acid Flow Rate: 0.7 mL/min **Gradient Program (Linear):** Time (min): 0 2 3 3.1 5 %B: 5 95 95 5 5 Stop Injection Volume: 10 µl MS Mode: Positive ion electrospray Scan Type: Multiple Reaction Monitoring MS/MS Ion Pair: CNX-1351: 574.2 → 350.1 Carbutamide: 272.2 → 74.0
Sample Preparation:	The analytes were extracted from mouse EDTA plasma by acetonitrile protein precipitation.
Assay Range:	The assay range was 1 – 5000 ng/mL
LLOQ and ULOQ	2 ng/ml and 5000 ng/ml
Regression,Weighting:	Quadratic. 1/x

Table S3. Pharmacokinetic study of **3**

Dose (mg/kg)	Route	C_{max} (ng/mL)	T_{max} (hr)	$t_{1/2}$ (hr)	AUC_{last} (hr*ng/mL)
10	IP	3292	0.083	2.4	1791

Table S4.Data collection and refinement statistics (molecular replacement)

*Highest-resolution shell is shown in parentheses

	CNX-1351
Data collection	
Space group	P 21 21 21
Cell dimensions	
a, *b*, *c* (Å)	59.34 135.03 142.92
α, β, γ (°)	90.00 90.00 90.00
Resolution (Å)	2.85 (3.04 - 2.85) *
R_{sym} or R_{merge}	3.9 (43.1)
$I / \sigma I$ or **Mn(I)/sd**	21.9(3.1)
Completeness (%)	99.8 (99.4)
Redundancy	5.9 (5.7)
Refinement	
Resolution (Å)	98.15-2.85
No. reflections	27540
R_{work} / R_{free}	24.1 / 28.5
No. atoms	
Protein	7238
Ligand/ion	41 / 0
Water	9
B-factors	
Protein	94.5
Ligand/ion	107.7
Water	67.3
R.m.s. deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.16

1 Crystal used
[Ramachandran Plot (calculated with PROCHECK):
Most favoured regions: 90.2
Additional allowed regions: 9.8
Generously allowed regions: 0.0
Disallowed regions: 0.0
REMARK 200 SYNCHROTRON (Y/N) : Y
REMARK 200 RADIATION SOURCE : SLS
REMARK 200 BEAMLINE : PXI/X06SA
REMARK 200 MONOCHROMATIC OR LAUE (M/L) : M
REMARK 200 WAVELENGTH OR RANGE (A) : 1.00002
REMARK 200 TEMPERATURE (KELVIN) : 100

Table S5.Completeness of the x-ray model of PI3Kα and **3**

Amino acid residues defined by electron density

A. 107-1046

Except for amino acid residues $

234-244, 310-324, 348-351, 409-418, 940-948, 966-970

Except for side chains#

- 107-112, 115, 132, 174, 176-177, 179, 181, 184, 186-187, 190-191, 194, 201-202, 204, 206, 210-211, 220, 223,226-230, 232-233, 245-247, 249, 264, 281, 290, 296, 306, 309, 325, 327, 329, 331, 334, 337, 339, 353, 365, 367,374-375, 377, 379-380, 382, 391-392, 404, 422, 437, 440, 450, 453, 455, 459, 474, 476, 481, 485, 494, 497, 500, 502, 519-526, 528-529, 532, 540, 542-543, 545-546, 561, 564, 573, 588, 594, 621, 627, 648, 655, 678, 697, 700, 723-725, 729, 733, 765, 774, 784, 788, 795, 809, 818, 822, 826-827, 852, 860, 863, 867, 871-873, 875-877, 879, 881-884, 886, 888-889, 894, 896, 905, 913, 915, 918, 924, 939, 949, 951, 955-956, 958-959, 961-962, 964, 973, 975-976, 978-979, 981-982, 985-986, 989, 992, 999, 1012, 1014-1015, 1022-1024, 1026, 1030, 1032, 1034, 1036-1037, 1040-1041

$Residues not included in the model

Occupancy set to 0.00 in the model