Abstract # 757

Identification and Structure-guided Optimisation of Novel Inhibitors of Checkpoint Kinase 1 (Chk1) through Combined Biochemical and Crystallographic Screening

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Abstract

The purpose of this study was to identify novel small molecule inhibitors of Chk1. Chk1 is a serine/threonine kinase that is phosphorylated and activated in response to DNA damage, initiating a cascade culminating in cell cycle arrest in the S and G2/M phases. Inhibition of Chk1 has been shown to abrogate cell cycle arrest leading to enhanced tumor cell death following DNA damage by a range of chemotherapeutics. Cells lacking intact G1 checkpoints are particularly dependent on S and G2/M checkpoints and are therefore expected to be more sensitive to chemotherapeutic treatment in the presence of a Chk1 inhibitor, whereas normal cells with functional G1 checkpoints would be predicted to undergo less cell death.
We used a fragment-based approach and ligand template screening to identify multiple novel low potency compounds suitable for optimisation into potent and selective Chk1 inhibitors. In this approach, fragments and lead-like small molecules were screened in a high-concentration biochemical assay, and compounds demonstrating a certain level of inhibition were soaked into crystals of Chk1 and the co-complex structures solved by x-ray crystallography. Validated hits having a known binding mode were optimised in a highly-focused structure-guided approach using iterative cycles of design, synthesis, assay and crystallography into potent inhibitors of Chk1. Using this technique we have been able to generate compounds with sub-micromolar mechanism-based cellular activity, excellent selectivity against Chk2 and CDK1 and good pharmacokinetic properties.
Thus multiple series of low-potency Chk1 inhibitors have been discovered and optimised into potent and selective compounds using a structure-based approach.

Methods

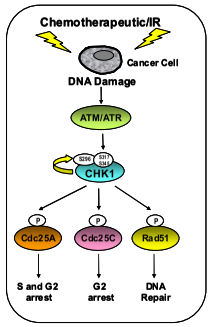
Measurement of Inhibition of CHK1 Kinase Function
The in vitro activities of CHK1 kinase inhibitors were determined by measuring the phosphorylation of a CDC25C peptide using DELFIA technology (PerkinElmer). Briefly, 1nM CHK1, 1 µM Biotin-KKKVSRSGLYRSPSMPENLNRPR, 30 µM ATP, 2mM MgCl2, 40mM Tris-HCl, pH 7.5, 40mM NaCl, 1mM DTT, 0.1% Tween-20 and compound in 2.5% DMSO were incubated for 30 mins at room temperature then stopped and transferred to a neutravidin-coated plate. Detection was performed using anti-phospho CDC25C (Cell Signalling Technology) and europium-labelled anti-rabbit IgG followed by addition of enhancement solution (PerkinElmer) and a time-resolved fluorescence measurement at 615 nm.

Cytotoxicity Assay
HT29 colon carcinoma cells (ATCC, Rockville, MD, USA) were plated at 1.6×10^3 per well in 96-well plates and treated with compound 36 hours later. After 96 hours, proliferation was determined by fixing the cells in 10% TCA, staining with Sulforhodamine B (SRB, 0.4% in 1% acetic acid, Sigma, UK) resolubilising SRB in 10 mM Tris base then measuring the OD at 490 nm. Results were expressed relative to untreated controls and the concentration of compound required to inhibit growth by 50% (SRB IC50) was calculated.

Mitosis Inhibition Assay (MIA)
Checkpoint abrogation by CHK1 kinase function inhibitors in combination with genotoxic agents was assessed using a europium based ELISA assay designed to quantify the number of cells trapped in mitosis after treatment with a genotoxic agent (to induce G2 arrest) followed by a test compound in combination with nocodazole to abrogate this arrest. HT29 colon carcinoma cells (ATCC, Rockville, MD, USA) were plated at 104 cells per well in 96-well plates and allowed to attach for 36 hours. Cells then then incubated with 50 µM etoposide for 1 hour and after medium replacement 100 ng/mL nocodazole and test compound added for 21 hours. Cells were then fixed in 4% formaldehyde followed by 100% methanol and immunodetection of MPM-2 performed using anti-MPM-2 (Upstate) and europium labelled anti-mouse (PerkinElmer) followed by treatment with enhancement solution (PerkinElmer) and
Europium emission measurement on a Wallac, Victor2 counter. Appropriate controls were included and results were expressed as the concentration of test compound required to allow 50% of cells to enter mitosis (MIA IC50).

Checkpoint kinase-1 (CHK1) Liganded Structure Method
The catalytic domain of Chk1 with a hexahistidine tag was expressed for 72 hours at 27°C in Sf9 insect cells using baculovirus. Protein was purified by NINTA affinity (Qiagen) followed by size exclusion chromatography (Superdex 75 16/10, GE Healthcare). Purified protein was concentrated to 8 mg/ml and crystallised by 1µl protein + 1µl well solution hanging drop vapour diffusion in 24-well plates using a 0.5 ml reservoir solution of DL-Malic acid/PEG 3350. Protein crystals were soaked in reservoir1 mM ligand for 3 hours at 20°C, flash frozen in liquid nitrogen then x-ray data collected at 100K on a Rigaku 944 CCD mounted on a 007HF generator with an X-2000 cryosystem. Crystals belonged to the space group P21 and routinely diffracted from 2.5 to 1.8 Å. Data were processed and scaled using MOSFLM/SCALA with rigid body refinement performed using MOLREP (CCP4).

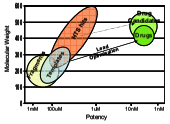
DNA Damage Response Pathway



Template Library Selection

- Templates are low-molecular weight compounds
- Ideal start points for Lead Optimisation
- Selected computationally by Template Descriptors, kinase pharmacophore model & volume constraint
- Templates are screened biochemically and hits are validated by X-ray crystallography
- 361 Templates were screened at 250uM using AlphaScreen™ format

Min	Property	Max
8	# Atoms	24
-2	AlogP	4
1	H-Bonding	6
1	Rings	3
0	Rot Bonds	6



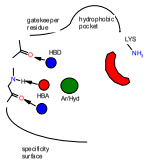
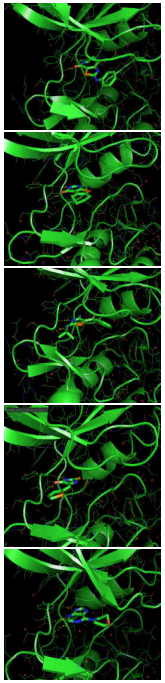


Figure 1: Template descriptors, Mol. Wt. vs potency range and generic kinase pharmacophore model used for Template selection

Template Screen Results

- 20 Templates inhibited Chk1 > 25% at 250uM
- 20 Templates assessed by x-ray crystallography
- 13 Template / Protein complexes solved

Example Results



SAR-000610
48% inhibition @ 250uM

SAR-004467
IC_{50} = 46uM

SAR-007236
45% inhibition @ 250uM

SAR-011076
49% inhibition @ 250uM

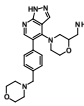
SAR-011797
IC_{50} = 49uM

Evaluation of SAR-011797

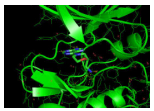
SAR-011797
IC_{50} = 49uM

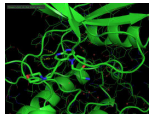
Compound 1
IC_{50} = 7uM

Compound 2
IC_{50} = 0.89uM



Compound 3
IC_{50} = 0.42uM





Optimisation of SAR-011797 series

Structure-guided Lead Optimisation

Compound 4 – an example from the optimised lead series:

• Chk-1 potency:	IC_{50} = 30nM (DELFIA in-house)
• G2/M Abrogation:	IC_{50} = 120nM (MIA in-house)
• Chemistry:	MW = 383, ClogP = 3.8, TPSA = 85Å^2
• Selectivity:	Chk-2 IC_{50} = 84,000 nM (DELFIA in-house)
	CDK1 IC_{50} = >100,000 nM (DELFIA in-house)
	IC_{50} > 10,000nM @ 44/49 kinases tested (Reaction Biology Corp)
• *in vivo* PK (mouse)	Well tolerated at 50 mg/kg ip (MTD not reached)
	Well distributed to tissue and tumor (e.g. spleen:plasma = 20:1) with sustained levels
• hERG inhibition:	59% at 10uM (DMSO = 9% - Millipore)
• X-ray structure:	1.8Å (in-house)

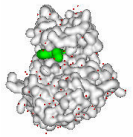


Figure 2: Compound 4 bound to active Chk1 kinase domain

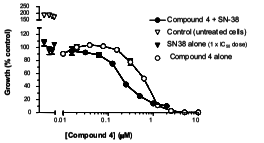


Figure 3: Potentiation of SN-38 cytotoxicity
Compound 4 Cytotoxicity IC_{50} = 0.6uM
SN-38 Cytotoxicity IC_{50} = 11.6nM
Combined cytotoxicity IC_{50} = 0.16uM
Potentiation = 3.8-fold

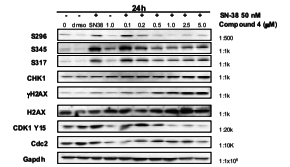


Figure 4: Effect on SN-38 induced DNA damage in HT-29 cells
Chk1 pS296 signal diminished
Robust γH2AX signal at higher concentrations
A decrease in the CDK1 pY15 signal is observed

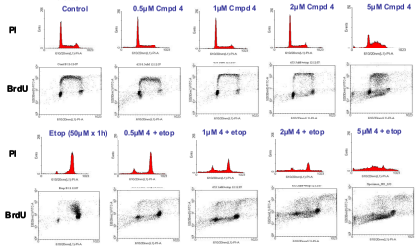


Figure 5: Effect on etoposide induced G2/M Arrest (21h)
Compound 4 abolished etoposide induced G2/M arrest at 2-5uM

Summary

- Template screening identified 13 hits validated by crystallography
- Lead series progressed by iterative synthesis/crystallography
- Lead series example:
  - Potent and selective
  - Good PK properties
  - Biomarker modulation
  - G2 checkpoint abrogation
  - Potentiation of SN-38-induced cytotoxicity