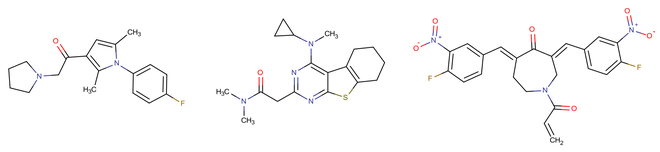
Evaluation and Characterization of Small Molecule Inhibitors of Deubiquitinating Enzyme USP14 as Potential Anti-Cancer Agents

Stina Lundgren, Ewa Odrzywol, Kerstin Böhm, Eldar Abdurakhmanov, Klara Acs, Mark Albertella, Oscar Belda, Erwin Brenndörfer, Dean Derbyshire, Ian Henderson, Daniel Jönsson, Sofia Karlström, Helen Kylefjord, Kevin Parkes, Ralf Paul, Sofia Unnerståle, Hongtao Zhao, Fredrik Öberg

Medivir AB, Huddinge, Sweden

BACKGROUND

- Ubiquitin-specific protease 14 (USP14) is one of three proteasome-associated deubiquitinating enzymes, responsible for the removal and reutilization of ubiquitin (Ub) molecules as well as regulating proteasome activity.
- USP14 binds reversibly with the proteasome and this stimulates USP14 catalytic activity. However, pools of free USP14 and proteasomes exists.
- Structure of the USP14 catalytic domain resembles that of other members of the ubiquitin specific protease family. The catalytic triad is in a productive conformation, even in absence of substrate, although two surface loops are re-ordered upon ubiquitin-aldehyde binding.[1]
- USP14 has been reported to regulate multiple cellular processes, not only by controlling the stability of proteins but also by modulating signal transduction pathways *via* nondegradative mechanism. One such example would be the described USP14 positive regulation of Wnt pathway by modulating ubiquitination (K-63 ubiquitin chains) of Dishevelled.[2]
- USP14 has been associated with tumorigenesis and its aberrant expression was reported in a variety of cancers, including colorectal and liver cancer[3], lung cancer[4], multiple myeloma[5] and epithelial ovarian cancer.[6]
- There are three different previously published USP14 inhibitors. IU1 was identified in a HTS by the research group of Daniel Finley.[7] The IU1 series was out-licensed and further developed by Proteostatis Therapeutics.[8] The tricyclic USP14 inhibitor IU2 was also developed by Proteostatis Therapeutics (IU2).[9]
- The research group of Stig Linder published a series of compounds reported to be dual UCHL5 and USP14 inhibitors.[5] In collaboration with Vivolux this series of compounds was further developed resulting in VLX-1570 proceeding into clinical Phase I/II for myeloma.[10]



IU1 IU2 VLX-1570

- As part of Medivir DUB drug discovery efforts, we have characterized a set of published and in-house developed small molecule USP14 inhibitors. We have evaluated their cytotoxic/cytostatic potential in cancer cell line models and effect on the Wnt signalling pathway.

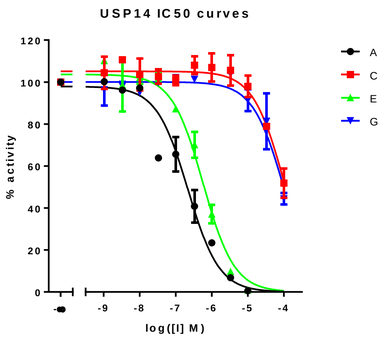
COMPOUND CHARACTERIZATION

Biochemical, physiochemical and DMPK assays

Compound	IU1	IU2	VLX-1570	A	B	C	D	E	F	G
Series				1	1	1	1	2	2	2
USP14 IC_{50} (µM)	**38**	**0.99**	**>100**	**0.50**	**0.54**	**96**	**93**	**0.50**	**0.68**	**66**
MW	300	345	469	357	358	343	353	399	385	304
Log D	1.5	>4	2.5	2.2	2.8*	3.3*	4.4*	2.7	1.5*	2.1
Kinetic Solubility (µM)	>100	85	6	98	>100	86	19	98	90	97
CACO-2 Papp (cm/s*10^-6)	15	20	nd#	12	9.2	11	10	32	5.9	15
HLM CLint (µL/min*mg)	9	250	>300	23	<6	19	>300	>300	52	52

Low recovery, *ADMET predictor 8.1 Simulations Plus

- We have characterized the published USP14 inhibitors in biochemical, physicochemical and DMPK assays.
- In addition, we have developed and profiled a number of potential USP14 inhibitors from two structurally distintict series; series 1 and 2 (Compound structures not disclosed).
- Both Series 1 and 2 that have sub-µM USP14 potencies and are good starting points for further optimization.



Selectivity

Enzyme Activity IC_{50} (µM)

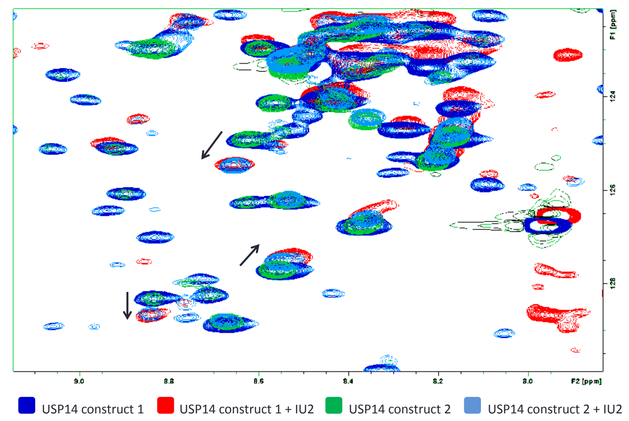
Compound	IU1	IU2	VLX-1570	A	E
USP1/UAF1	31	>100	16	>100	>100
USP5#	nd	>100	>10	2.6	>100
USP7	37	>100	10	>100	>100
USP28	>100	>100	>10	>100	96
USP35	nd	>100	>10	19	>100
USP47	>100	>100	6.6	>100	>100

#Ubiquitine @Bmax

- Although claimed to be selective against USP14,[7] the IU1 compound was active on USP1 and USP7.
- Compound A also inhibited USP5 and USP35, while IU2 and E showed good selectivity profile for USP14.

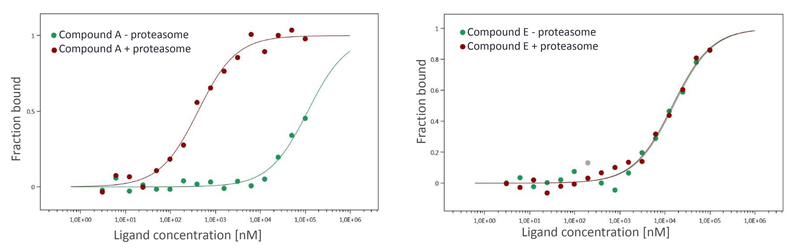
BINDING VALIDATION

Nuclear Magnetic Resonance (NMR)



- $^{1}H^{15}N$ TROSY spectra of two distinct USP14 catalytic domain constructs were recorded.
- Chemical shift perturbation upon adding the presumed allosteric inhibitor IU2 confirms binding.

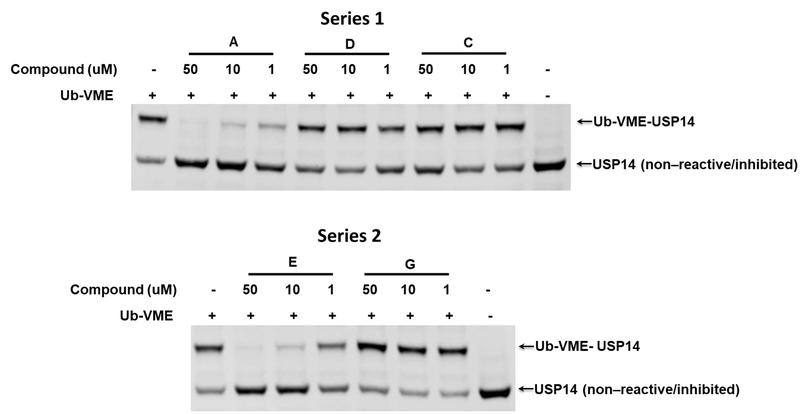
MicroScale Thermophoresis (MST)



Compound	K_D (µM) - proteasome	K_D (µM) + proteasome
A	110 ± 60	0.4 ± 0.1
E	15.6 ± 2.7	14.7 ± 2.2

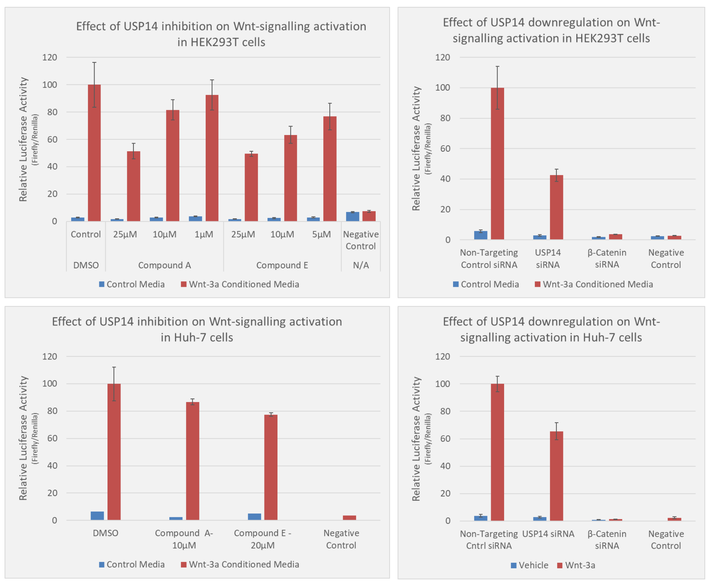
- MST confirmed binding of compounds to FL USP14.
- In contrast to compound E (Series 2), affinity of compound A (Series 1) increased in the presence of proteasome. This could indicate different binding sites of the two series.

PROFILING TARGET OCCUPANCY IN CELLS



- Concentration dependent, low µM (Series 1) to sub–µM (Series 2) target engagement was observed.
- Data are in agreement with the biochemical compound activity profiling.

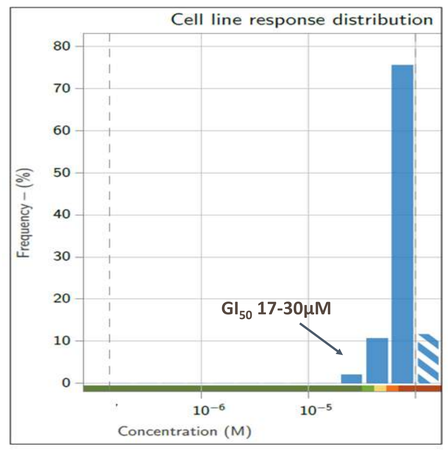
EFFECT OF USP14 INHIBITION ON WNT-SIGNALING



- Downregulation of USP14 protein levels or inhibition of its catalytic activity had moderate effect on Wnt-3a induced transcriptional activity of β-Catenin as measured by Luciferase Reporter Assay.

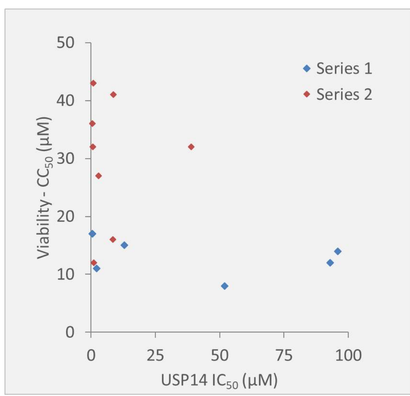
EFFECT ON USP14 INHIBITION ON CELL VIABILITY

Profiling compound activity in a panel of cancer cell lines



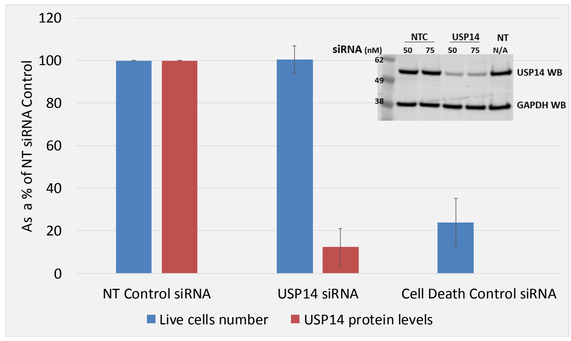
- Representative, USP14 selective compound, decreased cell viability in a subset of cell lines.
- Narrow range of GI_{50} values was observed.

Compound *in vitro* activity vs effect on cell viability



- Poor correlation of the compounds *in vitro* activity with their effect on cell proliferation in MV4-11 cells was found.

Effect of USP14 protein downregulation on cell viability



- siRNA-mediated USP14 protein knockdown in selected sensitive cell line (MV4-11) did not affected cell viability.

SUMMARY

- We have developed sub-µM USP14 inhibitors with acceptable DMPK properties. Series 2 showed good selectivity over other USPs tested.
- Biophysical methods confirmed binding of both series of compounds to FL USP14.
- Compounds showed in-cell target engagement.
- Moderate modulation of Wnt-signalling by USP14 inhibition was observed, indicating its non-essential role in this pathway.
- The cytotoxic potential of the tested USP14 compounds is limited. However, taking into account USP14 involvement in multiple cellular phenomena, other anti-tumour effects by USP14 inhibitors could be explored further.
- The described selective, cell-permeable inhibitors provide the opportunity for further development and can be used to test other therapeutic hypotheses based on USP14 inhibition.

METHODS

- Ub-VME-proteasome activated USP14 (produced in-house) was assayed using ubiquitin rhodamine 110 (Life Sensors) as substrate. USP7 (produced in-house) and USP47 (Life Sensors) were assayed using DiUb48-1 FRET substrate (Life Sensors). USP1/UAF1 from Boston Biochem was assayed using DiUb48-4 FRET substrate (Life Sensors).
- USP5, USP28 and USP35 enzyme assays were performed by Ubiquigent (Ubiquigent DUB*profiler*™).
- For target engagement assay, HCT116 cells were treated with compounds for 1 h, before lysing and labelling with HA-Ub-VME active probe.
- Luciferase Reporter assay was performed using TCF/LEF assay kit (Qiagen) and Dual-Glo Luciferase System (Promega).

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