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

Stina Lundgren, Ewa Odrzywoł, Kerstin Böhm, Eldar Abdurahmanov, Klara Acu, Mark Albertella, Oscar Belda, Erwin Brenndörfer, Dean Derbyshire, Ian Henderson, Daniel Jönsson, Sofia Karlström, Helen Kylejord, Kevin Parkes, Ralf Paul, Sofia Unnerstål, 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 proteasome exist.
- 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.
- USP14 has been reported to regulate multiple cellular processes, not only by controlling the stability of proteins but also by modulating signaling transduction pathways via non-degradative mechanism. One such example would be the described USP14 positive regulation of Wnt pathway by modulating ubiquitination (K6-Ub ubiquitin chains) of Dishevelled.
- USP14 has been associated with tumorigenesis and its aberrant expression was reported in a variety of cancers, including colorectal and liver cancer, lung cancer, multiple myeloma and epithelial ovarian cancer.
- There are three different previously published USP14 inhibitors. IU1 was identified in a HTS by the research group of Daniel Finley. The IU1 series was out-licensed and further developed by Protextic Therapeutics. The tricyclic USP14 inhibitor IU2 was also developed by Protextic Therapeutics (IU2).
- The research group of Stig Linder published a series of compounds reported to be dual UCHL3 and USP14 inhibitors. In collaboration with Vivitox this series of compounds was further developed resulting in VX-1570 proceeding into clinical Phase II for myeloma.

**COMPOUND CHARACTERIZATION**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IU1</th>
<th>IU2</th>
<th>VX-1570</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Series</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**BINDING VALIDATION**

Nuclear Magnetic Resonance (NMR)

- NTROSY spectra of two distinct USP14 catalytic domain constructs were recorded.
- Chemical shift perturbation upon adding the presumed allosteric inhibitor IU2 confirms binding.

**PROFILING TARGET OCCUPANCY IN CELLS**

- 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**

- 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.

**EFECT 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.

**SUMMARY**

- We have developed sub-mM USP14 inhibitors with acceptable DMPK properties. Several showed 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 cytosolic 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-VWE-protease substrate USP14 (produced in-house) was assayed using ubiquitin-His28 (Silenus) as substrate. USP14 (produced in-house) and USP7 (Life Sciences) were assayed using DiUb-4-FRET substrate (Life Sciences). USP14/USP7 from Boston Biochem was assayed using DiUb-4-FRET substrate (Life Sciences).
- USP14 inhibition was performed by Ubiquitase (DiOligo DiUbprotease).
- For target engagement, NCT1/16 cells were treated with compounds for 2 h, before lysis and labeling with HA-Ub-VWE active probe.
- Luciferase Reporter assay was performed using TGF/US assay kit (Diagen) and Dual-Glo luciferase system (Promega).

**REFERENCES**

1. Hu et al., EMBO (2000) 24, 3747-3756
5. Tien et al., BioMed (2016) 1, 76-116
8. WO2012075723
9. WO2010133127, WO2010133269, WO2011312693