Mapping and Linking Druggable Sites on Rad6 Using Proteomimetic Fragments and AI-assisted Design

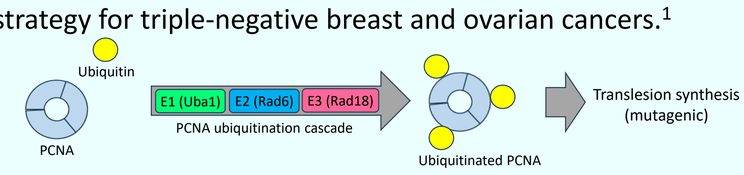
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Identifying druggable sites and modulating interactions on protein surfaces that lack well-defined cavities remain challenging for traditional drug-like small molecules. Our target, Rad6, containes a shallow, solvent-exposed catalytic region and broad protein–protein interaction (PPI) surfaces. These features make the development of Rad6 inhibitors difficult.

We present a **modular proteomimetic strategy** to disrupt Rad6-mediated PPIs and covalently engage the active-site Cys88. After screening for fragments capable of binding Rad6, we linked proteomimetic building blocks and warheads using dynamic covalent library (DCL) experiments and an AI-assisted linker optimization strategy.

Rad6 plays an essential role in the PCNA ubiquitination cascade and the DNA damage tolerance pathway translesion synthesis (TLS). TLS is error-prone, and promotes tumor cell survival and chemoresistance. Rad6 inhibition is a promising therapeutic strategy for triple-negative breast and ovarian cancers.[1]

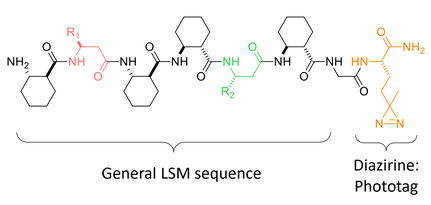


Mapping druggable sites – identifying potential building blocks

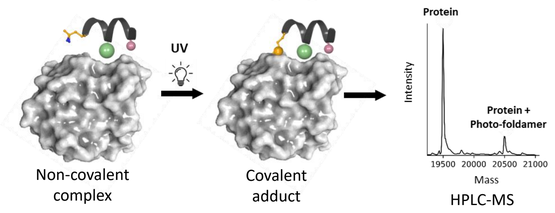
Druggable PPI sites on Rad6: Screen proteomimetic foldamer fragment library

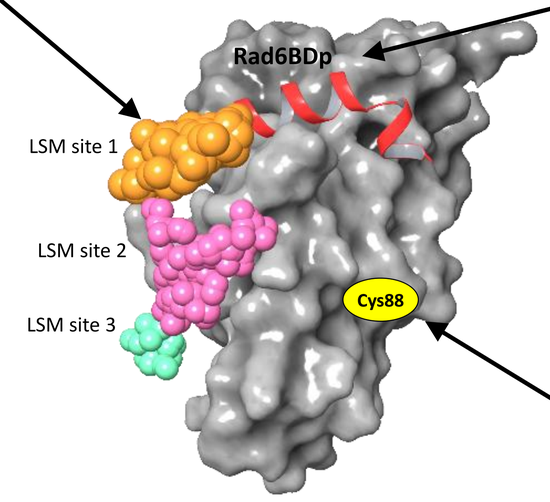
We screened a 100-membered local surface mimetic (LSM) foldamer library[2,3] using a photoscreening assay. Best Rad6-binders contained positively charged side chains, e.g. **RW**, **RF**, **RR**, **RL**, **WR**, **QR** (LSM names correspond to R_1 and R_2 side chains).

LSM foldamer library



Screening against Rad6





Helical peptide segment from the native interacting partner

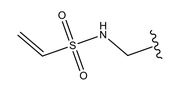
Mimicking a native binding partner is a straightforward strategy in peptidomimetics design. The 21mer helical peptide segment of the Rad6-binding domain of the Rad18 protein (**Rad6BDp)** binds to Rad6 relatively weakly (K_d~ 60 µM).

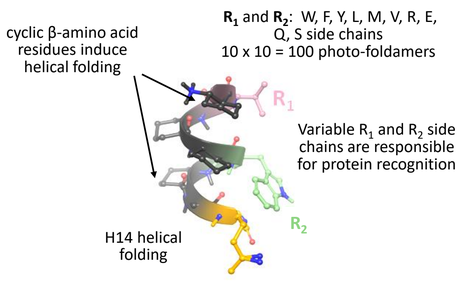
Rad6BDp KYRKKHKSEFQLLVDQARKGY

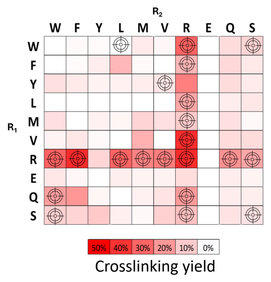
We exploited **Rad6BDp** as a building block of larger Rad6-binding constructs.

Covalent targeting of Cys88 with warheads

Rad6 accepts ubiquitin at its active-site Cys88. Targeting Cys88 with covalent inhibitors (warheads) can therefore inactivate Rad6. After screening a library of cysteine-selective covalent inhibitors by HPLC-MS, we selected vinylsulfonamide (VSA) for incorporating into Rad6 ligands.

VSA 





Localization of LSM hits

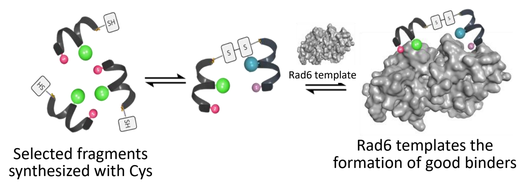
Three main LSM sites were determined (spheres represent the center of mass of the docked foldamer structures).

- **Proteomics**: covalent Rad6-LSM adducts were digested by trypsin/chymotrypsin and analysed by HPLC-MS
- **NMR**: ^{15}N HSQC NMR titrations
- **Docking** with the ab initio mode of the HADDOCK program.[4,5]

Connecting Rad6-binding peptide fragments and warheads

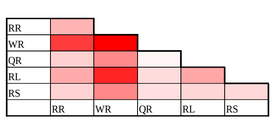
Linking nearby sites – templated DCL experiments

LSM hits (**RR, WR, QR, RS, RL**) and **Rad6BDp** bind to adjacent and partially overlapping sites on Rad6. To obtain higher-affinity compounds by linking two LSM hits or an LSM and **Rad6BDp,** and to identify the best combination of building blocks, we used protein-templated dynamic covalent library (DCL) experiments that enable selective enrichment of fragment combinations with enhanced target binding.

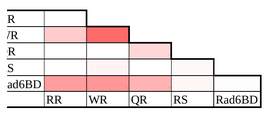


60 µM for each fragment in the DCL
60 µM Rad6 template
20 mM borate buffer (pH 8.2), 50 mM NaCl,
glutathione redox buffer: 500 µM GSH, 125 µM GSSG
incubation for 3 days at 37 °C
HPLC-MS analysis

Linking two LSM hits



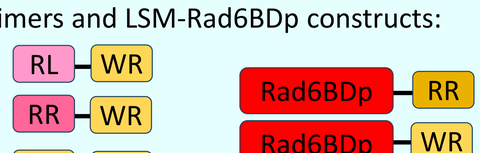
Linking LSMs with **Rad6BDp**



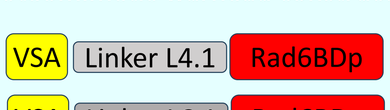


$$\text{Amplification factor (AF)} = \frac{\text{AUC (Rad6-templated)}}{\text{AUC (control without Rad6)}}$$

LSM dimers and LSM-Rad6BDp constructs:

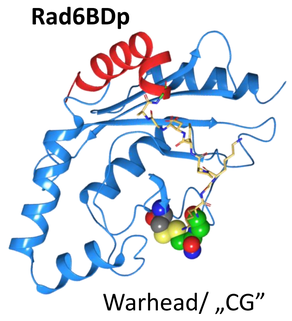


Proteomimetic warhead constructs:



Linking distant sites – linker optimization by AI tools

Rad6BDp

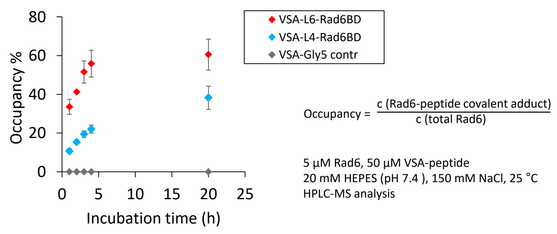


The linker between the Rad6BDp and the warhead moiety was designed using recently developed AI tools:

1. The backbone structure of the linker was generated using the RFDiffusion code.[6]
2. The sequence and 3D structure of the side chains were designed using the full-atom MPNN.[7]
3. The structures were then refined and ranked using Rosetta tools from Baker's lab.[8]

Selected linker sequences:
L4.1 sequence: PYTKAGDGQ
L6.1 sequence: KGKPIDGA

While the VSA-Gly_5 control did not label Rad6, VSA-linker-Rad6BDp constructs were effective



Summary and outlook

The identified LSM hits, acting as proteomimetic fragments, recognize PPI sites on Rad6. However, they do not fully cover the Rad6 surface interacting with Rad6BDp and did not engage the catalytic site around Cys88. Therefore, we used not only LSM hits, but also the native binding partner Rad6BDp and the cysteine-selective warhead VSA as building blocks to engineer a range of Rad6-binding constructs. Compound characterization is ongoing, but the initial results indicate that combining distinct chemical modalities is a worthwhile modular strategy for inhibitor development.

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