Novel Small Molecule Antagonists of XIAP, cIAP1/2 Generated by Fragment-Based Drug Discovery

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INTRODUCTION

- The inhibitor of apoptosis (IAP) proteins are important regulators of cancer cell survival, which makes them attractive targets for cancer therapy.
- IAP proteins are characterized by one to three baculovirus IAP repeat (BIR) domains, and most of them also possess a carboxyterminal ubiquitin ligase RING domain. BIRs are small (~70 aa) Zn-coordinated domains, which are necessary for the antiapoptotic activity of most IAPs. The majority of BIR domains present a surface groove with affinity toward N-terminal epitopes of defined sequence. A variety of proteins use their N-terminal region to interact with BIR domain grooves. Some of these protein-protein interactions contribute to oncogenesis and resistance to therapy.
- X-Chromosome-linked IAP (XIAP) has antiapoptotic activity as a result of its potent inhibition of caspases 3, 7 and 9 via its BIR domains.
- Cellular IAP proteins, cIAP1 and cIAP2, are also able to interact with tumor necrosis factor receptor-associated factor 2 (TRAF2). This unique property among IAP proteins enables recruitment of cIAP1 and cIAP2 to TNFR-signaling complexes where they regulate the activation of caspase-8.
- Small molecule BIR antagonists that mimic the N-terminal sequence of SMAC (an endogenous inhibitor of the IAPs) have the ability to sensitise and/or promote apoptosis in cancer cells and inhibit tumor growth in vivo. Binding of IAP antagonists to the BIR domains of cIAP1/2 and XIAP leads to the release of caspases from XIAP inhibition and also to the induction of c-IAP autoubiquitination activity and rapid proteasomal degradation of the c-IAP proteins. Besides neutralizing these antiapoptotic proteins, the IAP antagonists activate canonical and non-canonical NF-κB pathways and induce cell death that is dependent on TNF signaling.
- Fragment-based drug discovery is a rapidly growing alternative to high throughput screening in which very small molecules are screened by specialised techniques such as NMR and X-ray crystallography. Only relatively small libraries of fragments are required and observed fragment hits often possess high potency when normalised to their size (high ligand efficiency).
- Here we describe an application of Astex Pyramid™ fragment screening to BIR domains, which led to the identification of novel, non-peptidic and non-alanine lead series.

ASTEX PYRAMID™ FRAGMENT SCREENING PLATFORM FOR INHIBITOR OF APOTOSIS PROTEIN FAMILY

X-Ray Crystallography and NMR Spectroscopy

- Different constructs of the BIR domains have been investigated in order to obtain suitable crystal systems, which are amenable for high throughput crystallography and fragment screening. Four IAP crystal systems have been identified and used to acquire structural information on a range of bound peptides, tool compounds and fragments.
- 1D and 2D NMR spectroscopy has been used to investigate the BIR3 domain of XIAP. The up-field region of ^{1}H protein spectra presents chemical shifts, which are very sensitive to ligand binding in the BIR3 groove. Those signals have been used to detect binding events with Kds up to 30 mM.

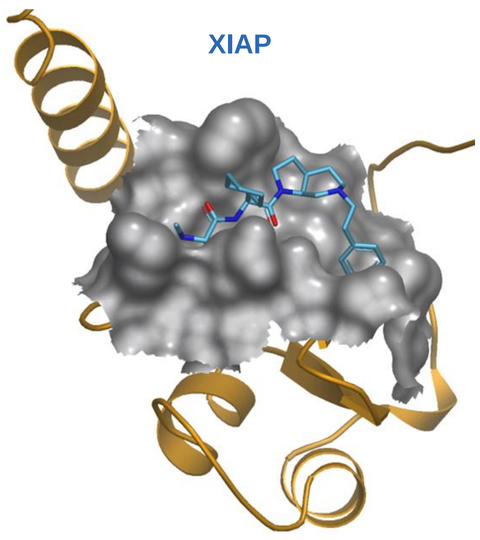


Figure 1. X-ray crystal structure of Novartis lead molecule (LBW242) with XIAP-BIR3 domain.

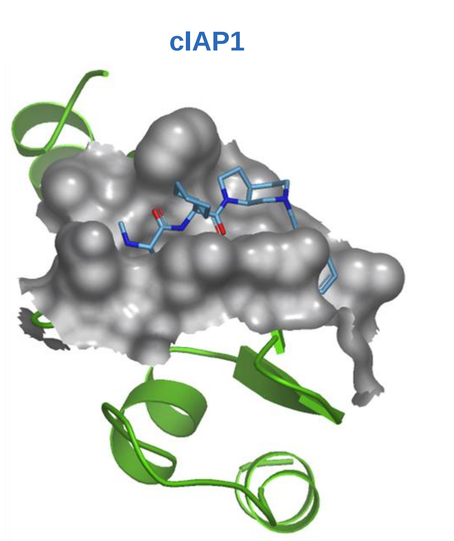


Figure 2. X-ray crystal structure of Novartis lead molecule (LBW242) with cIAP1-BIR3 domain.

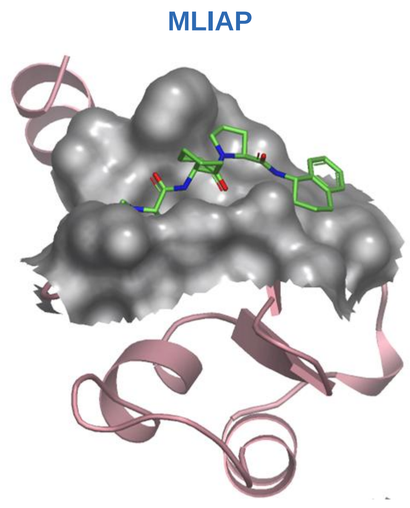


Figure 3. X-ray crystal structure of Abbott lead molecule with MLIAP-BIR domain.

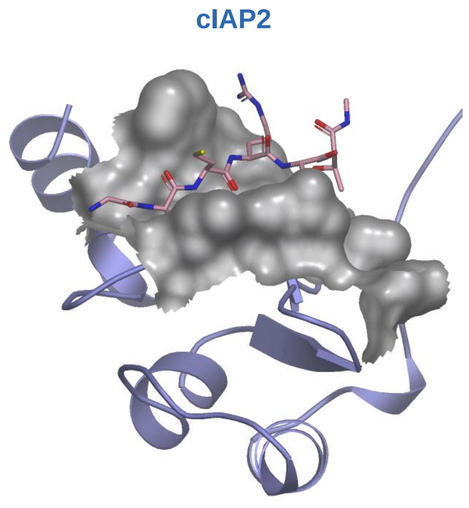


Figure 4. X-ray crystal structure of a peptide with cIAP2-BIR3 domain.

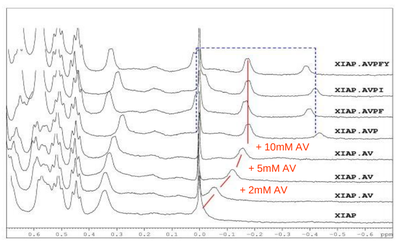


Figure 5. Changes in chemical shift in the up-field region of 1H NMR spectra due to peptide binding.

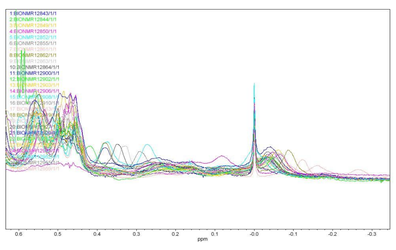


Figure 6. Changes in chemical shift in the up-field region of 1H NMR spectra due to fragment binding.

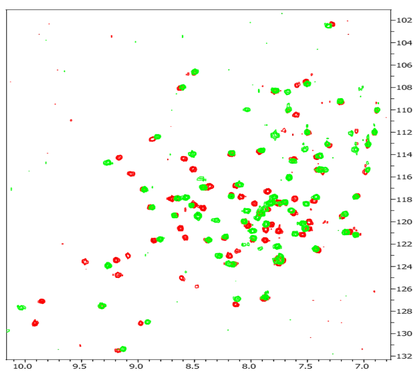


Figure 7. Overlay of two ^{15}N HSQC spectra of XIAP BIR3 domain with (red) and without (green) a ligand. 2D NMR was used to obtain structural information on ligand binding.

Pyramid™ Fragment Screening

- The Astex fragment library and a targeted set were screened against XIAP-BIR3 domain via X-ray crystallography and 1D-NMR. The binding mode of the hits was investigated using X-ray crystallography and 2D-NMR. Isothermal calorimetry (ITC) and bioassay were used to estimate the binding affinity.

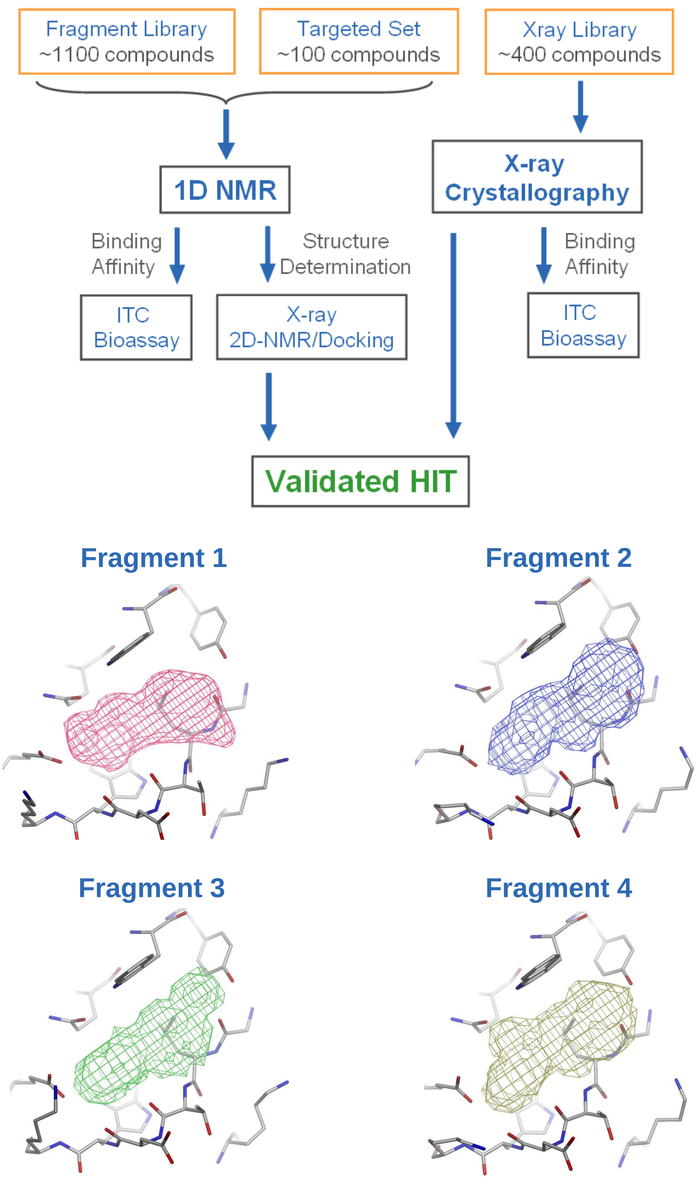
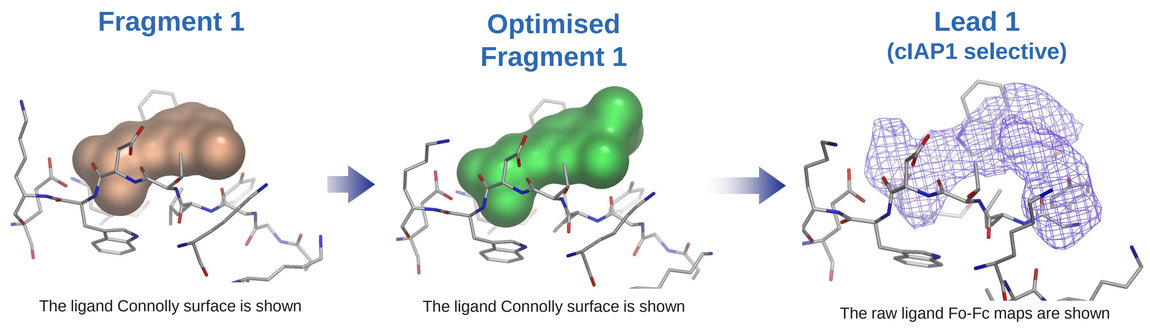


Figure 8. Crystal structures of four different fragment hits interacting with the SMAC binding groove in XIAP-BIR3 domain. The raw ligand Fo-Fc maps are shown.

FRAGMENTS TO LEADS

Optimisation of Fragment Hits into Lead Molecules

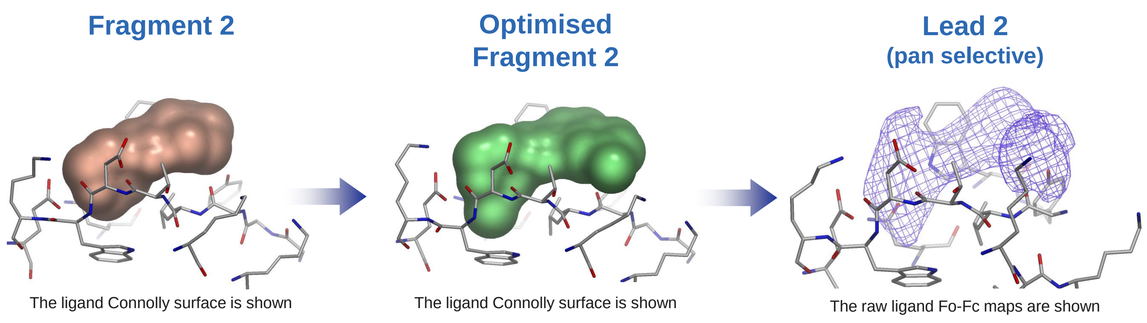
- Structure based drug design has been used to develop fragment hits into lead molecules.
- **Fragment 1** has an intrinsic selectivity towards the BIR3 domain of cIAP1. Its optimisation generated **Lead 1**, which is a selective cIAP1-BIR3 antagonist.



The ligand Connolly surface is shown — cIAP1-BIR3 8 μM, XIAP-BIR3 140 μM

The ligand Connolly surface is shown — cIAP1-BIR3 0.9 μM, XIAP-BIR3 52 μM

The raw ligand Fo-Fc maps are shown — cIAP1-BIR3 0.01 μM, XIAP-BIR3 26 μM



The ligand Connolly surface is shown — cIAP1-BIR3 16 % at 5 mM, XIAP-BIR3 17 % at 5 mM

The ligand Connolly surface is shown — cIAP1-BIR3 29 μM, XIAP-BIR3 13 μM

The raw ligand Fo-Fc maps are shown — cIAP1-BIR3 0.06 μM, XIAP-BIR3 0.09 μM

- **Fragment 2** binds very weakly to both cIAP1-BIR3 and XIAP-BIR3. However, its X-ray crystal structure suggested areas of improvement, which allowed us to improve the potency. Further optimisation yielded **Lead 2**, which is a equipotent cIAP1-BIR3 and XIAP-BIR3 antagonist.

Cell based data

- Compounds were tested in 72h proliferation assays using two sensitive human breast cancer cell lines, EVSA-T and MDA-MB-231 and in the insensitive human colon cancer cell line, HCT116, to control for off-target cytotoxicity.
- Both **Lead 1** and **Lead 2** led to significant induction of apoptosis with EC_{50} values of 250 nM and 300 nM on the more sensitive EVSA-T cell line. The Novartis lead, LBW242, was included as a control.

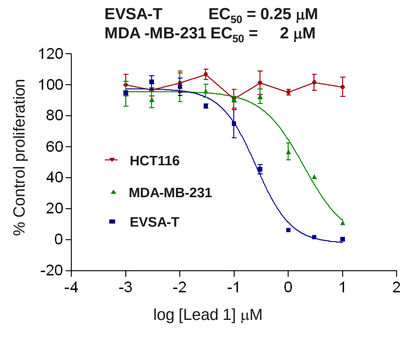


Figure 9. Lead 1 tested in 72h proliferation assays.

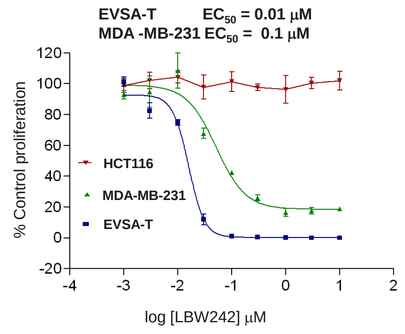


Figure 10. LBW242 tested in 72h proliferation assays.

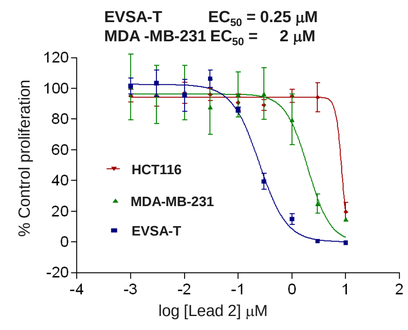


Figure 11. Lead 2 tested in 72h proliferation assays.

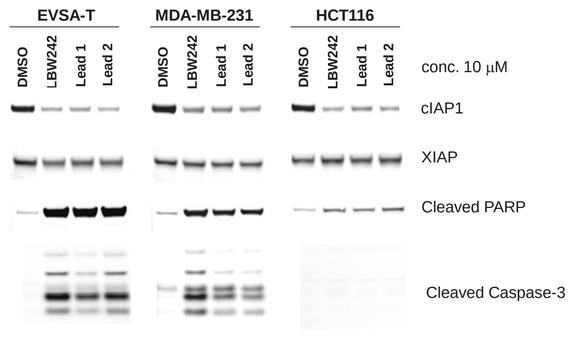


Figure 12. Western blot analysis of levels of IAPs, PARP and cleaved Caspase-3. **Lead** 1 and **Lead 2** were compared to **LBW242**.

- To confirm the mechanism of action of our compounds, the three cell lines were tested by Western blot analysis of levels of cIAP1 and XIAP and induction of apoptosis markers (cleaved PARP and cleaved caspase-3) after 8 h treatment with 10 μM compound.
- All compounds led to a drop in levels of cIAP1 (but not XIAP as expected) in all three cell lines.
- Apoptosis markers were induced in both the sensitive cell lines (EVSA-T and DA-MB-231) but not in the insensitive cell line (HCT116).

CONCLUSIONS

- We have developed a platform to perform fragment screening and structure based drug design on four different members of the IAP protein family.
- A Pyramid™ screening cascade based on high throughput X-ray crystallography and NMR has allowed us to identify fragment hits against the BIR3 domain of XIAP.
- Careful structure-based optimisation based on the initial Fragment 1 and very weak Fragment 2 delivered two lead series with distinct selectivity profiles.
- Both lead series show anti proliferative activity and biomarker response in two cancer cell lines (EVSA-T and MDA-MB231).
- Lead optimisation on both series is ongoing.

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