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Characterisation of Novel, Small Molecule Antagonists of XIAP, cIAP1 and cIAP2 Generated By Fragment Based Drug Discovery (FBDD)

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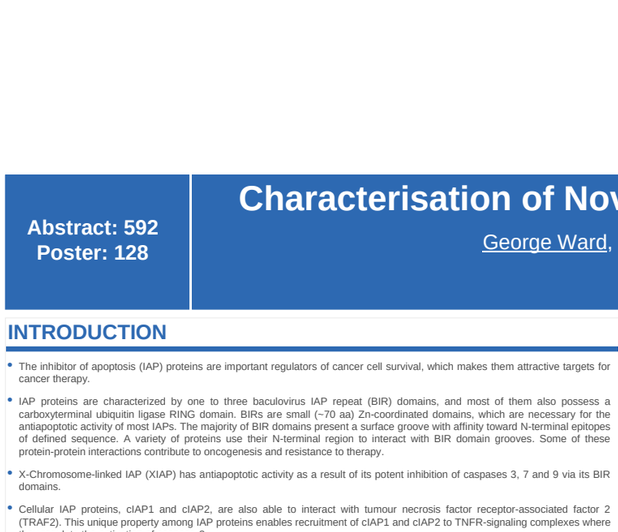
INTRODUCTION

- 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 tumour 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 tumour 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- B pathways and induce cell death that is dependent on TNF-a 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).
- Novel non-peptidic and non-alanine lead series were identified from application of Astex Pyramid™ fragment screening to BIR domains.
- Here we describe the characterisation of these novel IAP antagonists in cellular assays.

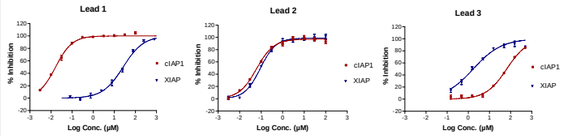
Figure 1. Optimisation of Fragment Hits into Lead Molecules

- 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.



- **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 cIAP-BIR3 and XIAP-BIR3 antagonist and **Lead 3**, which is a XIAP-BIR3 selective antagonist.
- Binding potencies were measured in a fluorescence polarisation (FP) binding assay.

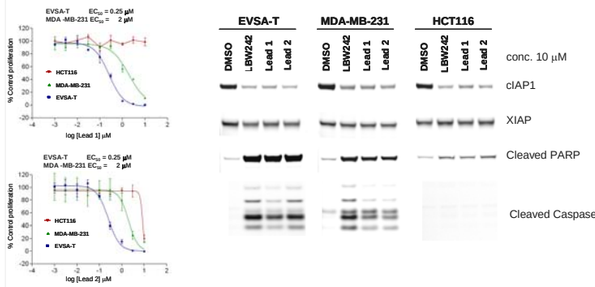
Figure 2. Fluorescence Polarisation (FP) Binding Assay Data



- Fluorescence polarisation binding assays were performed with AbuRPFK(5&6FAM)-amide (Peptide Protein Research) as the tracer for both cIAP1 and XIAP.
- Binding was assessed with purified recombinant BIR3 domains from cIAP1 and XIAP.
- **Lead 1** is > 2000 fold selective for cIAP1 over XIAP.
- **Lead 2** is similar in its binding of cIAP1 and XIAP.
- **Lead 3** is 38 fold selective for XIAP over cIAP1, but was inactive on cells due its high IC_{50} against cIAP1 (60 µM).

Figure 3. Cellular Activity

- 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 both with an EC_{50} values of 250 nM on the more sensitive EVSA-T cell line. The Novartis lead, LBW242, was included as a control.



- 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).1111

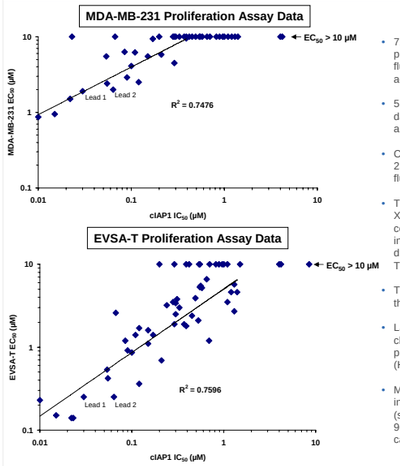
Table 1. Lead Compound Summary

- Comparison of *in vitro* binding assay data and cell proliferation assay data:

	Fluorescence Polarisation Assay IC_{50} (µM)			Cell Proliferation Assay EC_{50} (µM)		
	cIAP1	cIAP2	XIAP	EVSA-T	MDA-MB-231	HCT116
Lead 1	**0.010**	**0.32**	**26**	**0.25**	**1.9**	**>10**
Lead 2	**0.054**	**ND**	**0.085**	**0.25**	**2.0**	**8.5**

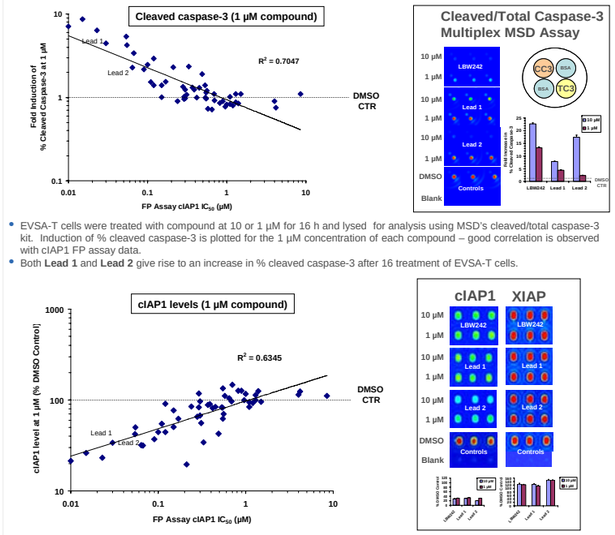
ND = not determined

Figure 4. Anti-Proliferative Profile of Compounds



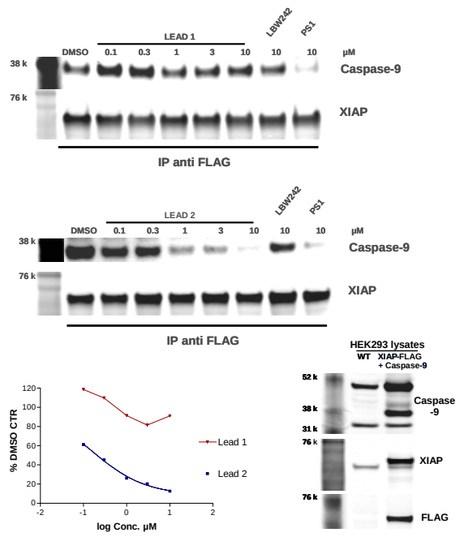
- 72 h proliferation assays were run as a primary cellular screen of compounds with fluorescence polarisation binding assay activity.
- 57 compounds have been tested on cells to date from multiple series (including non-alanine series compounds).
- Cell killing in the sensitive cell lines (MDA-MB-231 and EVSA-T) correlates with cIAP1 fluorescence polarisation assay data.
- There is no correlation with cell killing and XIAP fluorescence polarisation assay data confirming published data that *in vitro* induction of apoptosis in a sensitive cell line is driven by cIAP1/2 inhibition and induction of TNF-α.
- The EVSA-T cell line is more sensitive than the MDA-MB-231 cell line.
- Lack of non-specific off-target cytotoxicity was checked by running the compounds in proliferation assays with an insensitive cell line (HCT116).
- Mechanism of action of cytotoxicity via IAP inhibition was verified by biomarker modulation (shown in Figure 5 below) using parallel MSD 96-well plate assays for induction of cleaved caspase-3 and reduction in cIAP1 levels.

Figure 5. Biomarker Modulation



- EVSA-T cells were treated with compound at 10 or 1 µM for 16 h and lysed for analysis using MSD's cleaved/total caspase-3 kit. Induction of % cleaved caspase-3 is plotted for the 1 µM concentration of each compound – good correlation is observed with cIAP1 FP assay data.
- Both **Lead 1** and **Lead 2** give rise to an increase in % cleaved caspase-3 after 16 treatment of EVSA-T cells.
- EVSA-T cells were treated with compound at 10 or 1 µM for 2 h and lysed for analysis using an in house MSD assay for cIAP1 and XIAP levels. There was correlation in the reduction in levels of cIAP1 observed and cIAP1 FP assay data. XIAP levels were not significantly altered at 10 or 1 µM.
- Both **Lead 1** and **Lead 2** give rise to a drop in cIAP1 (but not XIAP) levels after 2 h treatment of EVSA-T cells.

Figure 6. Immunoprecipitation (IP) Assay for XIAP Inhibition



- A stable HEK293 cell line transfected with both full length XIAP (FLAG-tagged) and caspase-9 cDNA clones (Origene) was generated – overexpression of XIAP and caspase-9 was confirmed by comparison of lysates with those from the parental cell line.
- IP assays were set up with each compound added for 2 h at 37 °C before lysing the cells and setting up the IP anti-FLAG resin (Sigma) for 2 h at RT. The resin was spun down and washed 3 times with 1XTBS before boiling in SDS-PAGE sample buffer and setting up Western blots.
- The cIAP1 selective compound, **Lead 1**, is poor at inhibiting the interaction between XIAP and caspase-9 in transfected HEK293 cells (similar to LBW242 which is a weak XIAP inhibitor).
- The pan selective inhibitor, **Lead 2**, inhibits the interaction between XIAP and caspase-9 in transfected HEK293 cells (similar to PS1 which is a good XIAP inhibitor).
- Work is under way to look at the interaction between XIAP BIR3 domain and caspase-9, and also the interaction between SMAC and full length XIAP or XIAP BIR3, with a view to adapting one of these assays to the MSD analyser.

CONCLUSIONS

- We have developed a platform to perform fragment screening and structure based drug design on 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 compound series with distinct selectivity for cIAP1 or XIAP or both cIAP1 and XIAP.
- Lead series compounds show anti proliferative activity and biomarker response in two cancer cell lines (EVSA-T and MDA-MB231). This correlates closely with cIAP1 inhibition as expected.
- An immunoprecipitation assay has been developed to monitor XIAP inhibition in cells.
- Lead optimisation is ongoing.

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