Development and preclinical assessment of a first-in-class small molecule inhibitor of the major cell death regulator protein FLIP

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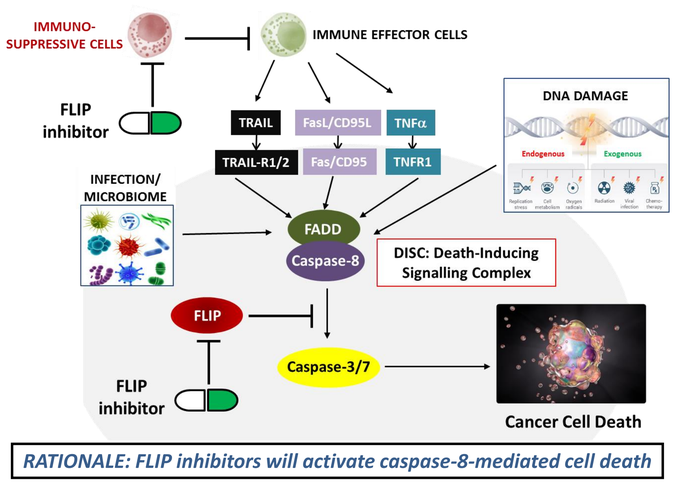
Introduction: FLIP, the DISC and Drug Resistance



RATIONALE: FLIP inhibitors will activate caspase-8-mediated cell death

Figure 1. ***Figure 1. Schematic overview of programme.*** The complex formed following ligation of the Fas and TRAIL-R1/R2 (DR4/DR5) death receptors by their ligands (FasL/CD95 and TRAIL) is called the death-inducing signalling complex (DISC), which consists of the receptors, the adaptor molecule FADD, procaspase-8 and its regulator FLIP[1]. Similar complexes are formed downstream of TNFR1 and in response to infection and DNA damage. Interaction with death receptors exposes the FADD death effector domain (DED), which recruits procaspase-8 by interacting with its tandem DEDs[2]. Procaspase-8 homo-dimerization results in conformational changes in its catalytic domains that lead to its activation and initiation of apoptosis[3].

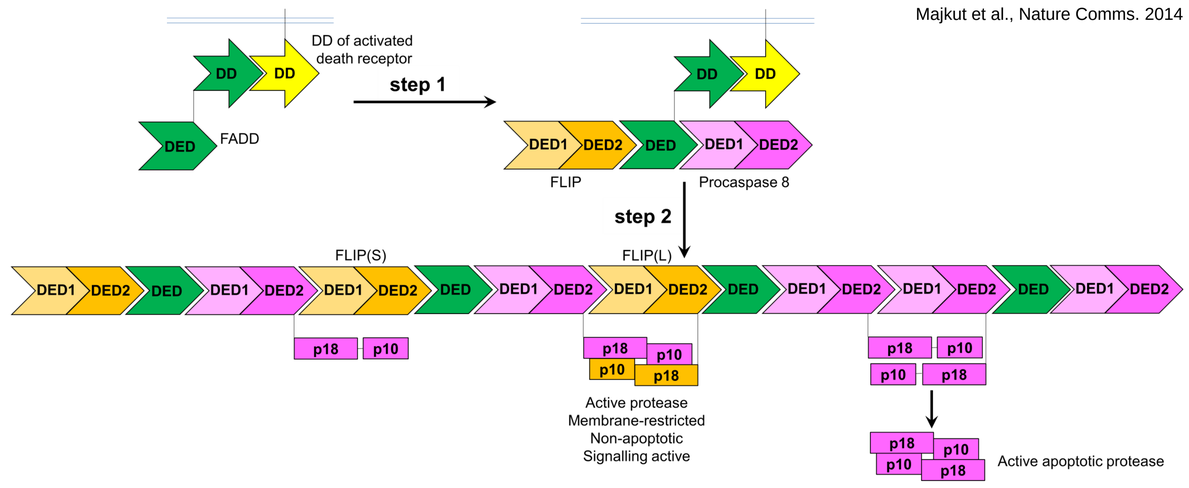
FLIP can also bind at the DISC and regulate procaspase-8 processing: its short splice form FLIP(S) blocks procaspase-8 processing and activation; however the long spice form FLIP(L) can activate or inhibit procaspase-8 processing depending on its expression levels[4]. FLIP is frequently over-expressed in NSCLC, colorectal, prostate and other cancers and correlates with adverse disease outcome[1].

FLIP overexpression promotes drug resistance, and RNAi-mediated FLIP down-regulation leads to:

- The induction of caspase-8-dependent apoptosis in FLIP "addicted" cancer models[5-7]
- Enhanced TRAIL- and IAP-antagonist-induced apoptosis[8-9]
- Enhanced chemotherapy-induced apoptosis[5,6]

FLIP has also been demonstrated to play a critical role in maintaining the viability of immunosuppresive, tumor-promoting immune cells, *e.g.* Tregs and MDSCs.

Model of DISC Assembly



Majkut et al., Nature Comms. 2014

Figure 3. Model of DISC assembly.
1. Upon death receptor engagement, FADD is recruited to the intracellular part of the death receptor through homotypic death domain (DD) interactions, making its DED available for interaction with other DED proteins;
2. FLIP preferentially binds to the α1/α4 surface of FADD's DED, whereas procaspase-8 binds to FADD's α2/α5 surface (***step 1***);
3. Individual co-localized FLIP-FADD-procaspase-8 intermediates interact to form higher order structures via interactions between the α1/α4 surface of FLIP and the α2/α5 surface of procaspase-8 (***step 2***);
4. FLIP(S)/caspase-8 heterodimers are membrane-restricted and cannot activate apoptosis signalling; however, FLIP(L)/caspase-8 heterodimers are catalytically active and can cleave local substrates such as RIPK1;
5. At higher DISC stimulation, the more lowly expressed FLIP becomes depleted, and the more highly expressed procaspase-8 is recruited to the α1/α4 face of FADD as well as the α2/α5 surface;
6. Under these conditions, interactions between co-localized procaspase-8-FADD-procaspase-8 intermediate trimers results in procaspase-8 dimerization bringing the catalytic domains together, resulting in processing and full activation of the enzyme and initiation of the apoptotic process.

Differential affinity of FLIP and procaspase-8 for FADD

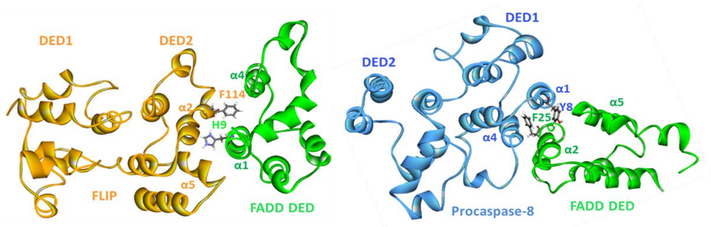
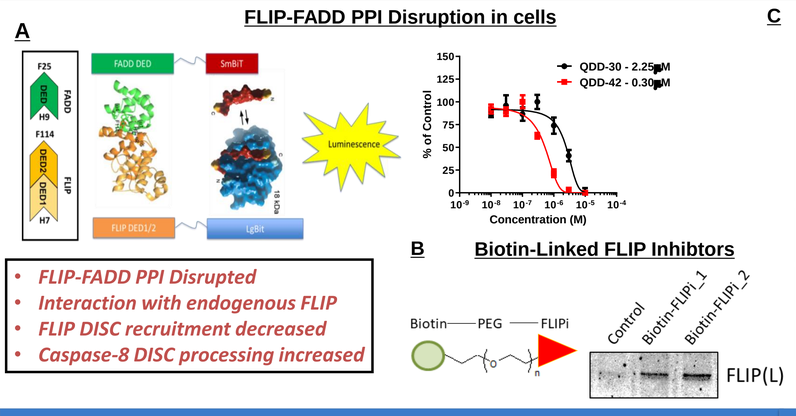


Figure 2. Models of the inter-molecular interactions between FADD/FLIP and FADD/procaspase 8. FLIP preferentially binds to the DED of FADD via its DED2, whereas procaspase-8 preferentially binds to FADD via its DED1. The main residues which are important for the interactions are highlighted: **FLIP** uses its **F114** residue on the α2 helix to bind into a groove between α1/α4 helices in the DED of FADD with a reciprocal interaction from **FADD H9** into the α2/α5 hydrophobic patch of FLIP; **procaspase-8** uses the **Y8** residue on its α1 helix to bind into the hydrophobic patch between the α2/α5 helices in FADD, with a reciprocal interaction from **FADD F25** into the α1/α4 groove in procaspase-8.

Confirmation of On-target Activity



- *FLIP-FADD PPI Disrupted*
- *Interaction with endogenous FLIP*
- *FLIP DISC recruitment decreased*
- *Caspase-8 DISC processing increased*

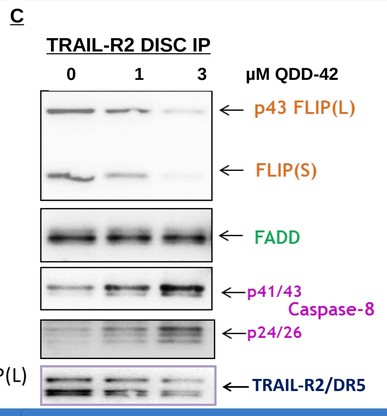
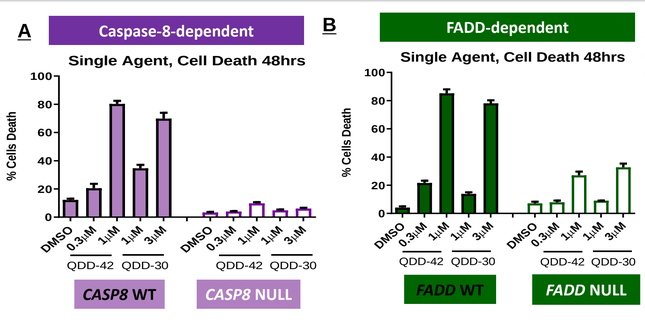


Figure 4. Confirmation of On-target activity.
(A) FLIP/FADD protein-protein interaction (PPI) assay demonstrating a dose-dependent disruption of the protein-protein interaction by FLIP inhibitors using the NanoBiT™ split luciferase assay system (Promega), where smBiT and LgBiT of the nanoluciferase enzyme are fused to DED proteins and expressed in U20S cells. Upon PPI, the two parts of the enzyme come together resulting in reconstitution of the nanoluciferase enzyme and the generation of a luminescent signal which can be inhibited in a dose-dependent manner by FLIP inhibitors.
(B) Biotin-linked FLIP inhibitors interact with endogenous FLIP.
(C) FLIP inhibitors block FLIP's recruitment to the TRAIL-R2 DISC. A549 cells were treated for 1h with indicated concentrations of QDD-42 and formation of the DISC was stimulated for 30 minutes using an anti-TRAIL-R2 agonistic antibody. Proteins were assessed by Western blot.

Confirmation of On-target Mechanism-of-Action



Activation of apoptosis is caspase-8 and FADD-dependent

Figure 5. Impact of FLIP inhibitors on Caspase-8 and FADD CRISPR KO models.
(A) A549 parental and CRISPR *CASP8* KO cells were treated with QDD-42 or QDD-30. Cell death was assessed 48h post-treatment by PI/Annexin V staining using high content microscopy.
(B) A549 parental and CRISPR *FADD* KO cells were treated with QDD-42 or QDD-30. Cell death was assessed 48h post-treatment by PI/Annexin V staining using high content microscopy.

Lead Compound Profiles

Parameter	QDD-30	QDD-42	QDD-62
FLIP-FADD protein-protein interaction : 50% Inhibition (μM)	2.0	0.3	0.2
Caspase 3 activation in A549 *KRAS* MT NSCLC: 50% increase in activation (6h + TRAIL) (μM)	2.7	0.4	0.1
Cell viability A549 *KRAS* MT NSCLC: 96h + TRAIL, IC_{50} (mM)	0.5	0.1	0.02
Kinetic solubility (μM)	3250	1000	TBD
MWt/log$D_{7.4}$	<330/1.3	<410/1.0	<400/2.4 (calc)
Caco-2 A:B/(efflux ratio) x10^{-6}cm^{-1}	8.5 (1.7)	2.5 (9.5)	0.9 (4.1)
Hep CL_{int} (m/r/h) (mL/min/10^6 cells)	77/55/39	15/9/10	>70/-/>70
CYP Inh @10 mM (5-isoforms)	<55% (all)	86% (2C19)	in progress
PPB% m/h (%); WBB m/h	73/83; 77/75	81/79; 99/72	in progress
Cl (mL/min/kg) m/r/d	50/25/12	in progress	in progress
t1/2 m/r/d	<1/3.9/7.9 (*p.o.*)	>5h (m, *s.c.*), High [QDD-42] lung,	in progress
F (%) m/r/d	18/7/74	ND	in progress
hERG IC_{50} (μM)	31	>33	in progress

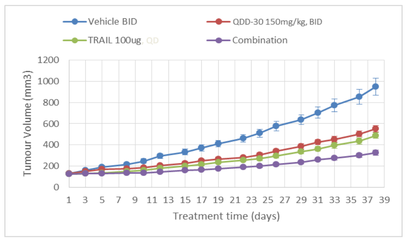
Table 1. Profiles of Lead compounds

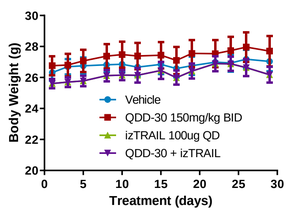
Lead compounds have drug-like properties

In Vivo efficacy and Tolerability

***KRAS* mutant NSCLC – high clinical unmet need**

IZ-TRAIL mimics human immune effector cells



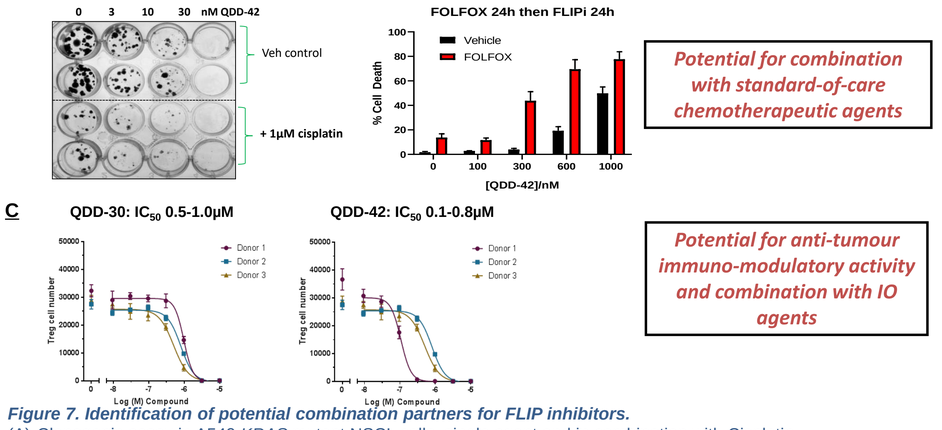


T/C FLIPi alone ~0.5
T/C FLIPi + IZ-TRAIL ~ 0.15

Well-tolerated (no weight loss)

Figure 6. QDD-30 retards the growth of A549 NSCLC xenografts in Balb/c nude mice as a single agent and this is further potentiated in combination with IZ-TRAIL. Balb/c nude mice were inoculated with $2x10^6$ A549 cells in each flank. After 8 days, mice were treated PO with 150 mg/kg FLIPi BID or vehicle. IZ-TRAIL was administered by daily i.p. injections of 100μg. Treatment was administered daily, and tumor volume recorded as indicated. IZ-TRAIL was purified as previously described[10].

Clinical Positioning

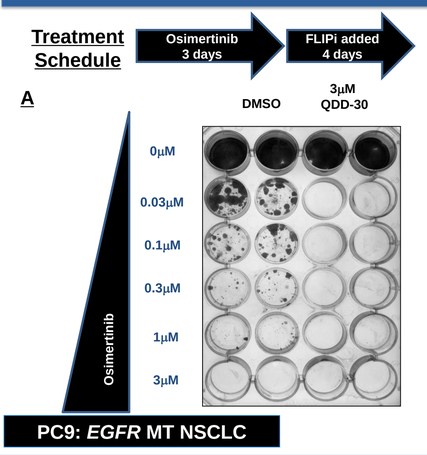


Potential for combination with standard-of-care chemotherapeutic agents

Potential for anti-tumour immuno-modulatory activity and combination with IO agents

Figure 7. Identification of potential combination partners for FLIP inhibitors.
(A) Clonogenic assay in A549 *KRAS* mutant NSCL cells, single agent and in combination with Cisplatin.
(B) Annexin V/PI Cell Death assay (high content microscopy), single agent and combination with FOLFOX (5μM 5-FU and 1μOxaliplatin).
(C) Anti-proliferative activity of single agent FLIPi in T_{reg} cells (Horizon Discovery).

Combination with EGFRi in *EGFR* mutant NSCLC



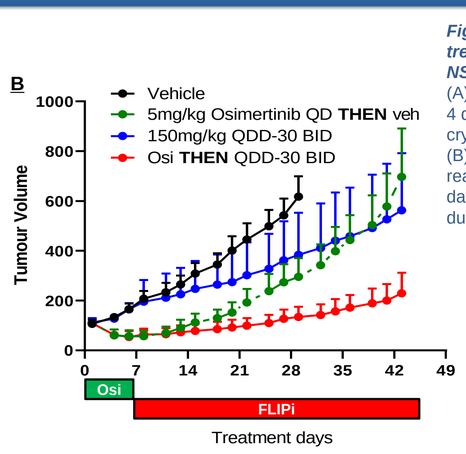
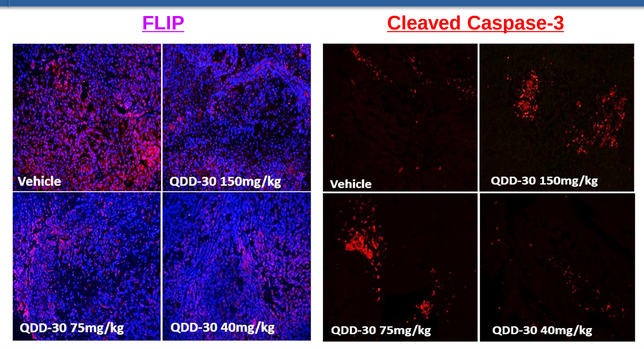


Figure 8. QDD-30 ablates colony formation of cells surviving Osimertinib treatment in vitro and suppresses in vivo tumor regrowth in PC9 EGFR mutant NSCLC cells.
(A) PC9 cells were treated with Osimertinib for 3 days followed by FLIPi for an additional 4 days. Colonies were left to form and were fixed with methanol and stained with 0.5% crystal violet solution.
(B) Balb/c nude mice were inoculated with $1x10^6$ PC9 cells. When tumor volume reached ~$100mm^3$, mice were treated PO with Osimertinib (5 mg/kg) or vehicle for 7 days. At day 8, 150 mg/kg FLIPi po BID or vehicle was administered daily for the duration of the study.

A FLIP inhibitor ablates colony formation of cells surviving Osimertinib treatment

B FLIP inhibitor ablates colony formation of cells surviving Osimertinib treatment

PD Readouts



QDD-30 decreases FLIP expression and increases Caspase-3 cleavage in A549 xenografts

Figure 9. QDD-30 downregulates FLIP protein expression and increases Cleaved Caspase-3 in tumor tissue (day 21). Formalin-fixed paraffin-embedded (FFPE) tumor tissue sections were stained for FLIP (Abcam ab8421) and Cleaved Caspase-3 (Abcam ab13847) by standard IHC protocols.

Summary

- **FLIP is a frequently overexpressed, key regulator of cell death and drug resistance in many cancers.**
- **A medicinal chemistry programme has identified first-in-class drug-like compounds capable of:**
  - **Binding to FLIP with low and sub-micromolar activity AND disrupting FLIP's recruitment to the DISC in cancer cells**
  - **Inducing apoptosis as a single agent AND promoting TRAIL- and TNF- induced apoptosis**
  - **Retarding growth of NSCLC xenografts as a single agent and in combination with a multivalent TRAIL agonist**
  - **Enhancing standard-of-care chemotherapy in *KRAS* mutant CRC and NSCLC**
  - **Demonstrating anti-proliferative activity against key immunosuppressive T_{reg} cells**
  - **Ablating colony formation and suppressing tumour regrowth following Osimertinib treatment in *EGFR* mutant NSCLC**

References

1. Safa, A. R. & Pollok, K. E. Targeting the Anti-Apoptotic Protein c-FLIP for Cancer Therapy. *Cancers* **3**, 1639-1671, (2011).
2. Scott, F. L. *et al.* The Fas-FADD death domain complex structure unravels signalling by receptor clustering. *Nature* **457**, 1019-1022, (2009).
3. Lavrik, I. *et al.* The active caspase-8 heterotetramer is formed at the CD95 DISC. *Cell death and differentiation* **10**, 144-145, (2003).
4. Majkut, J. *et al.* Differential affinity of FLIP and procaspase 8 for FADD's DED binding surfaces regulates DISC assembly. *Nature communications* **5**, 3350, (2014).
5. Longley, D. B. *et al.* c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. *Oncogene* **25**, 838-848, (2006).
6. Wilson, T. R. *et al.* Procaspase 8 overexpression in non-small-cell lung cancer promotes apoptosis induced by FLIP silencing. *Cell death and differentiation* **16**, 1352-1361, (2009).
7. Wilson, C. *et al.* IL-8 signaling attenuates TRAIL- and chemotherapy-induced apoptosis through transcriptional regulation of c-FLIP. *Mol. Canc. Therap.* **7**, 2649-2661, (2008).
8. Wilson, T. R. *et al.* c-FLIP: a key regulator of colorectal cancer cell death. *Cancer research* **67**, 5754-5762, (2007).
9. Crawford, N. *et al.* SAHA overcomes FLIP-mediated inhibition of SMAC mimetic-induced apoptosis in mesothelioma. *Cell death & disease* **4**, e733, (2013).
10. Ganten, T.M. *et al.* Preclinical differentiation between apparently safe and potentially hepatoxic applications of TRAIL. Clin. Cancer Res. **12**, 2640-6, (2006).

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