Induction of immunogenic cell death and enhancement of dendritic cell function: Development of an *in vitro*, *ex vivo* ICD platform for the identification of novel ICD inducers

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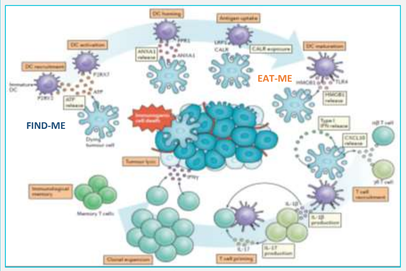
1 CONTEXT & OBJECTIVES

Immunogenic cell death and Nanocyclix®



Galluzzi *et al*, Nat Rev Immunol (2017) 17-97

DAMPs	Localization and mode-of-emission	Referent cell death pathway	Receptors
ATP	Actively or passively released	ICD, apoptosis or secondary necrosis and necrosis	P2Y2 and P2X1
Heat shock proteins (HSPs)	Surface exposure, active secretion or passive release		CD91, TLR2, TLR4, SREC-1 and FEEL-1
High mobility group box 1 (HMGB1)	Mostly passively released; sometimes actively released	ICD, secondary necrosis, necrosis	TLR2, TLR4, RAGE and TIM3
Calreticulin (CRT)	Mostly surface exposed; sometimes passively released	ICD	CD91

Garg *et al*, Front Immunol (2015) 6-588

DAMPs (ATP, CRT, HSPs and HMGB1) released during immunogenic cell death (ICD) recruit and activate immune cells (DC, monocytes, T cells) to recognize tumor (neo)-antigens.

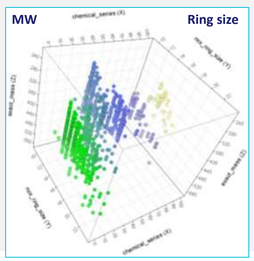
Some single-agent ICD inducers in cancer:

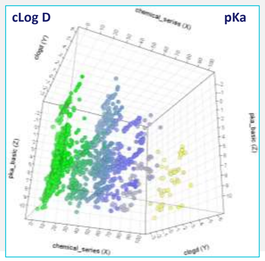
ICD inducers	Associated ICD-relevant DAMPs	
	DAMP	Stage of cell death
Anthracyclines (mitoxantrone, doxorubicin, etc.)	Surface CRT Surface HSP70 Secreted ATP Released HMGB1	Pre-apoptotic Mid-apoptotic Early/mid apoptotic Post-apoptotic
Bortezomib	Surface HSP90 Surface CRT Surface HSP70	Early/mid apoptotic Early/mid apoptotic Early/mid apoptotic
Cyclophosphamide	Surface CRT Released HMGB1	Early/mid apoptotic Post-apoptotic

Garg *et al*, Front Immunol (2015) 6-588

Several ICD inducers were tested in DAMP-associated assays following which mitoxantrone and doxorubicin were chosen as positive controls.

Nanocyclix® is a chemistry technology based on the macrocyclization of small molecule hinge binders of kinase ATP active site. This leads to low MW kinase inhibitors with a unique binding mode and mode of action. The shape complementarity between the inhibitor and the active site of the kinase is believed to result in high potency and selectivity.





A Lead-like set of 2318 compounds was selected to screen for novel ICD inducers.

2 RESULTS

In vitro detection of ICD inducers

ICD, a non-conventional type of apoptosis is associated with the activation of an adaptive immune response against dead cell-associated antigens. Anthracyclines exert immunostimulatory effects that rely on ICD. It is desirable to explore if other molecules can increase cancer cell immunogenicity and be attractive candidates for (combination) immunotherapy. Based on this knowledge, we developed a high throughput *in vitro* screening platform enabling the identification of compounds that induce ATP secretion, CRT exposure and HMGB1 release. We first tested this platform on our Lead-like set, unveiling several Nanocyclix® molecules that render cell death immunogenic.

SCREENING STRATEGY for IDENTIFICATION of HITS – *in vitro*

Step 1: Identify lowest toxic dose (384-well plate)
- Cell lines : U-2 OS, MDA-MB-231 and MDA-MB-436 (human), Hepa 1-6, CT26 and Pan02 (mouse)
- 24-72h incubation followed by assessment of cell viability (CellTiter Glo) using EnVision plate reader

144 hits

Step 2: Identify compounds that result in secreted ATP at non-toxic dose (96-well plate)
- Cell lines : U-2 OS, MDA-MB-231 and MDA-MB-436 (human), Hepa 1-6, CT26 and Pan02 (mouse)
- 5 doses : highest concentration chosen from Step 1
- 24-72h incubation followed by evaluation of cell viability and secreted ATP (Enliten)

24 hits

		U-2 OS	MDA-MB-231	Hepa 1-6
Compound	Conc	Secreted ATP (72h)		
DMSO 0.2%	0.20%	100%	100%	100%
ODS336 (µM)	0.625	230%	109%	122%
	1.250	460%	218%	294%
	2.500	392%	271%	392%
	5.000	318%	271%	369%
	10.000	767%	246%	394%

		CT26	Pan02 (24h)	MDA-MB-436
Conc		Secreted ATP (48h)		
0.20%		100%	100%	100%
0.500		188%	169%	147%
1.000		448%	160%	192%
2.500		289%	172%	175%
5.000		246%	167%	209%
10.000		284%	205%	155%

ODS336 identified as a hit

Color code:
Activity without toxicity
Toxicity

ODS336 treatment results in an increase in secreted ATP at non-toxic concentration.

Step 3: Identify ICD inducers (96-well plate)
- Cell lines : U-2 OS, MDA-MB-231 and MDA-MB-436 (human), Hepa 1-6, CT26 and Pan02 (mouse)
- 5-8 doses : highest concentration chosen from Step 2
- 24-72h incubation followed by assessment of:
  - cell viability, secreted ATP, HMGB1 release (ELISA) and surface CRT (IF / FACS)

➢ **HMGB1 release: ELISA**

		MDA-MB-231	Hepa 1-6
Compound	Conc	HMGB1 (48h)	
DMSO 0.2%	0.20%	100%	100%
ODS336 (µM)	0.625	163%	178%
	1.250	230%	313%
	2.500	248%	671%
	5.000	151%	428%
	10.000	397%	146%

	CT26	Pan02 (24h)	MDA-MB-436
Conc	HMGB1 (48h)		
0.20%	100%	100%	100%
0.500	211%	95%	295%
1.000	609%	153%	501%
2.500	580%	114%	613%
5.000	246%	187%	306%
10.000	690%	95%	199%

U-2 OS cells:
- **At non-toxic doses, MTX and Dox (positive controls) treatment did not result in an increase in HMGB1 release.**
- **High concentrations of ODS336 leads to HMGB1 release.**

ODS336 treatment results in HMGB1 release in several cell lines at non-toxic concentration.

➢ **Surface calreticulin detection: IF / FACS (CT26)**

		U-2 OS	MDA-MB -231	Hepa 1-6
Compound	Conc	Calreticulin (72h)		
DMSO	0.20%	100%	100%	100%
ODS336 (µM)	0.625	125%	113%	96%
	1.250	291%	137%	126%
	2.500	453%	200%	237%
	5.000	409%	317%	365%
	10.000	293%	191%	319%

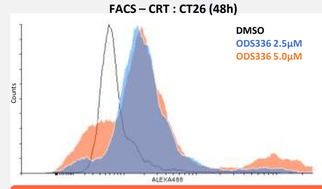
	CT26	Pan02 (24h)	MDA-MB-436
Conc	Calreticulin (48h)		
0.20%	100%	100%	100%
0.500	N/A	104%	134%
1.000	166%	152%	202%
2.500	269%	205%	304%
5.000	275%	209%	165%
10.000	N/A	142%	94%

	Cut-off
Cell viability	>70%
Secreted ATP	>150%
Released HMGB1	>150%
Surface CRT	>150%

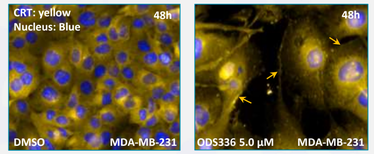
3 RESULTS

➢ **Surface calreticulin detection: IF / FACS (CT26)**

FACS – CRT : CT26 (48h)



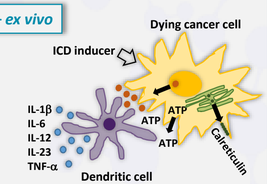
IF image capture and analysis: Operetta High-Content Analysis System (PerkinElmer)



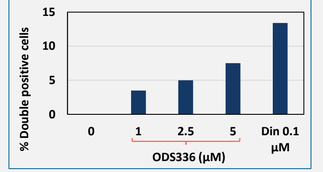
ODS336 treatment results in an increase in surface calreticulin at non-toxic concentration.

CONFIRMATION of HITS (Dendritic cell function) – *ex vivo*

As dendritic cells (DCs) play a key role in the recognition of DAMPs associated with ICD and the subsequent uptake and presentation of tumor antigens, we examined the phagocytosis of ICD inducer-treated tumor cells by DCs.

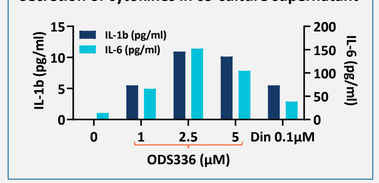


CT26 cell phagocytosis by mDCs



Din = Dinaciclib (Control)
Hossain *et al*, JCI (2018) 128 (2)-644

Secretion of cytokines in co-culture supernatant



ODS336 treated CT26 cells enhance DC function.

- Secretion of IL-1β from DCs in response to purinergic receptor agonists (ATP) and TLR4 ligands (HMGB1) plays an important role in antitumor T cell priming.
- In addition, pro-inflammatory cytokine IL-6 that promotes T cell differentiation and NK cell activation was detected.

4 CONCLUSION

- **Here, we describe a general strategy for the identification of ICD inducers within large chemical libraries.**
- **We have validated the capability of our ICD screening platform by identifying ODS336, a compound that elicits an *in vitro* ICD response – secreted ATP, HMGB1 release and surface CRT.**
- **An *ex-vivo* co-culture assay demonstrated enhanced DC function suggesting that ICD activates both innate and adaptive arms of the immune system.**

Cancer immunotherapy: ICD process elicits enhanced adjuvanticity and antigenicity from dying cancer cells and consequently promotes the development of clinically desired antitumor immunity.

Next step (on-going): Cancer cell- DC-T cell co-culture to demonstrate tumor specific T cell activation.