Development of a high throughput *in vitro* screening platform to identify novel inducers of immunological cell death

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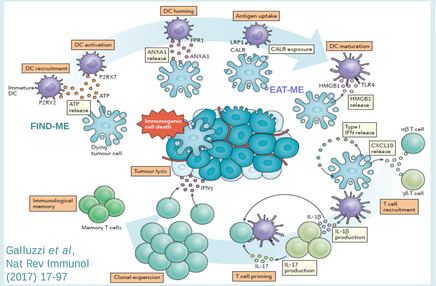
INTRODUCTION

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/secondary necrosis and necrosis	P2Y2 and P2X1
Calreticulin (CRT)	Mostly surface exposed; sometimes passively released	ICD	CD91
Heat shock proteins (HSPs)	Surface exposure, active secretion or passive release	ICD, apoptosis/secondary necrosis, necrosis	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

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.

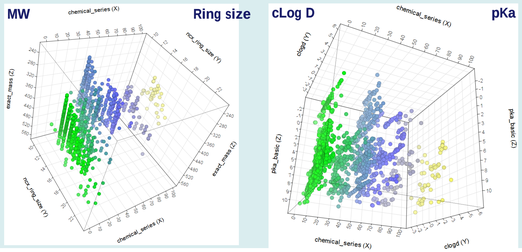
Some single-agent ICD inducers in cancer:

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 compound library: Nanocyclix® is a proprietary medicinal chemistry technology based on the macrocyclization of small Lead-like molecules. 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.

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 to render cell death immunogenic.

SCREENING STRATEGY for IDENTIFICATION of HITS

Step 1: Identify lowest toxic dose

- 3 cell lines : U-2 OS (human), MDA-MB-231 (human) and Hepa 1-6 (mouse)
- 5 doses : 10, 5, 2.5, 1.25, 0.61 µM
- 72h incubation followed by assessment of cell viability (CellTiter Glo) using EnVision plate reader
- Assay format: 384-well plate

➢ Cut-off: >75% viability

144 hits

Step 2: Identify compounds that result in secreted ATP at non-toxic dose

- 3 cell lines : U-2 OS (human), MDA-MB-231 (human) and Hepa 1-6 (mouse)
- 5 doses : highest concentration chosen from Step 1
- 72h incubation followed by evaluation of cell viability (CellTiter Glo) and secreted ATP (Enliten)
- Assay format: 96-well plate

➢ Cut-off: >2x secreted ATP with >75% viability

24 hits

	U-2 OS	
Cpd (µM)	Viability	Secreted ATP
DMSO 0.2%	100%	100%
MTX 0.2	92%	801%
MTX 0.3	81%	1214%
Dox 0.2	79%	627%
Dox 0.25	85%	847%

	MDA-MB-231	
Cpd (µM)	Viability	Secreted ATP
DMSO 0.2%	100%	100%
MTX 0.1	90%	629%
MTX 0.25	90%	441%
Dox 0.1	99%	289%
Dox 0.25	96%	429%

	Hepa 1-6	
Cpd (µM)	Viability	Secreted ATP
DMSO 0.2%	100%	100%
MTX 0.25	91%	312%
MTX 0.5	105%	445%
Dox 0.25	91%	240%
Dox 0.5	98%	487%

Compound	U-2 OS		MDA-MB-231		Hepa 1-6	
	Conc	Secreted ATP	Conc	Secreted ATP	Conc	Secreted ATP
DMSO 0.2%	0.2%	**100%**	0.2%	**100%**	0.2%	**100%**
ODS142 (µM)	0.050	**116%**	0.001	**50%**	0.010	**78%**
	0.100	**266%**	0.0025	**130%**	0.050	**201%**
	0.250	**313%**	0.005	**536%**	0.100	**392%**
	0.500	**523%**	0.0075	**560%**	0.500	**394%**
	0.750	**647%**	0.010	**636%**	0.750	**477%**
	1.000	**841%**	0.100	**1070%**	1.000	**438%**
	5.000	**1094%**	1.000	**835%**	5.000	**370%**
	10.000	**901%**	10.000	**238%**	10.000	**369%**

ODS142 identified as a hit

Color code:
Activity without toxicity
Toxicity

ODS142 treatment results in an increase in secreted ATP at non-toxic concentration.

Step 3: Identify ICD inducers

- 3 cell lines : U-2 OS (human), MDA-MB-231 (human) and Hepa 1-6 (mouse)
- 5 doses : highest concentration chosen from Step 2
- 72h incubation followed by assessment of cell viability (CellTiter Glo), secreted ATP (Enliten), HMGB1 release (ELISA - 48h), surface CRT (IF)
- Assay format: 96-well plate

In vitro detection of ICD inducers

➢ **HMGB1 release: ELISA** (IBL international)

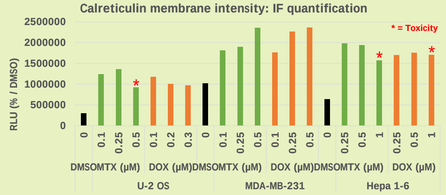
	MDA-MB-231		Hepa 1-6	
Cpd (µM)	Viability	HMGB1	Viability	HMGB1
DMSO 0.2%	100%	100%	100%	100%
MTX 0.25	102%	240%	90%	171%
MTX 0.5	87%	274%	84%	240%
MTX 1	79%	327%	75%	331%
Dox 0.5	102%	228%	83%	193%
Dox 1	87%	273%	68%	309%
Dox 5	60%	600%	14%	630%

Cpd	MDA-MB-231		Hepa 1-6	
	Conc	HMGB1	Conc	HMGB1
DMSO	0.2%	**100%**	0.2%	**100%**
ODS142 (µM)	0.001	**97%**	0.010	**98%**
	0.0025	**140%**	0.050	**113%**
	0.005	**155%**	0.100	**122%**
	0.0075	**184%**	0.500	**157%**
	0.010	**186%**	0.750	**171%**
	0.100	**276%**	1.000	**182%**
	1.000	**324%**	5.000	**269%**
	10.000	**682%**	10.000	**286%**

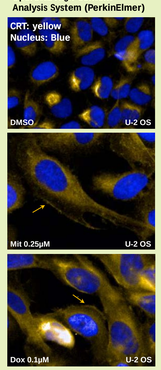
U-2 OS cells:
- At non-toxic doses, MTX and Dox treatment did not result in an increase in HMGB1 release.
- High concentrations of ODS142 lead to HMGB1 release.

ODS142 treatment results in HMGB1 release in 3 cell lines at non-toxic concentration.

➢ **Surface calreticulin detection: IF** (ThermoFisher antibody)



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



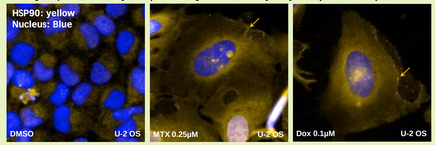
Cpd	U-2 OS		MDA-MB-231		Hepa 1-6	
	Conc	Surface CRT	Conc	Surface CRT	Conc	Surface CRT
DMSO	0.2%	**100%**	0.2%	**100%**	0.2%	**100%**
ODS142 (µM)	0.050	**123%**	0.001	**113%**	0.010	**98%**
	0.100	**127%**	0.0025	**163%**	0.050	**120%**
	0.250	**261%**	0.005	**246%**	0.100	**126%**
	0.500	**247%**	0.0075	**269%**	0.500	**262%**
	0.750	**258%**	0.010	**260%**	0.750	**268%**
	1.000	**269%**	0.100	**323%**	1.000	**280%**
	5.000	**285%**	1.000	**233%**	5.000	**241%**
	10.000	**339%**	10.000	**256%**	10.000	**208%**

	Cut-off
Viability	>75%
Secreted ATP	>150%
Released HMGB1	>150%
Surface CRT	>150%
Surface HSP90	>150%

ODS142 treatment results in an increase in surface CRT at non-toxic concentration.

➢ **Surface HSP90: IF** (abcam antibody)

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



U-2 OS		MDA-MB-231		Hepa 1-6	
Cpd (µM)	HSP90	Cpd (µM)	HSP90	Cpd (µM)	HSP90
DMSO 0.2%	**100%**	DMSO 0.2%	**100%**	DMSO 0.2%	**100%**
MTX 0.1	**397%**	MTX 0.25	**240%**	MTX 0.25	**329%**
MTX 0.25	**425%**	MTX 0.5	**230%**	MTX 0.5	**343%**
Dox 0.1	**429%**	Dox 0.25	**250%**	Dox 0.25	**224%**
Dox 0.2	**441%**	Dox 0.5	**251%**	Dox 0.5	**311%**

Surface HSP90 is detectable after MTX and Dox treatment and can be used as an ICD read-out.

Conclusions

- 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 ODS142, a compound that elicits an ICD response – secreted ATP, HMGB1 release and surface CRT.

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