

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

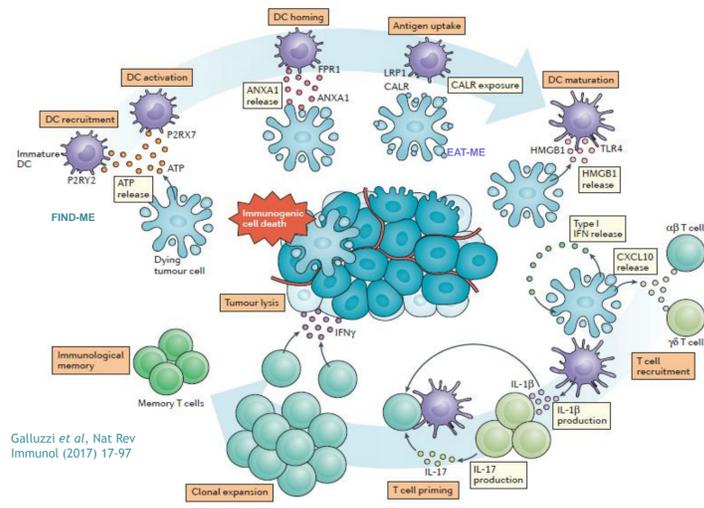


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Immunogenic cell death and Nanocyclix



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.

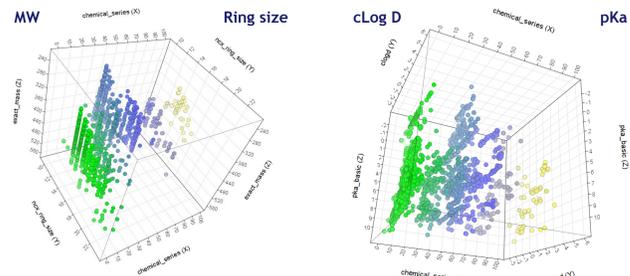
Some single-agent ICD inducers in cancer:

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

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.

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



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

In vitro detection of ICD inducers - Strategy

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			MDA-MB-231			Hepa 1-6		
Cpd (µM)	Viability	Secreted ATP	Cpd (µM)	Viability	Secreted ATP	Cpd (µM)	Viability	Secreted ATP
DMSO 0.2%	100%	100%	DMSO 0.2%	100%	100%	DMSO 0.2%	100%	100%
MTX 0.2	92%	801%	MTX 0.1	90%	629%	MTX 0.25	91%	312%
MTX 0.3	81%	1214%	MTX 0.25	90%	441%	MTX 0.5	105%	445%
Dox 0.2	79%	627%	Dox 0.1	99%	289%	Dox 0.25	91%	240%
Dox 0.25	85%	847%	Dox 0.25	96%	429%	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%

Color code: Activity without toxicity (green), Toxicity (red)

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 - Results

HMGB1 release: ELISA (IBL international)

Cpd (µM)	MDA-MB-231		Hepa 1-6	
	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%

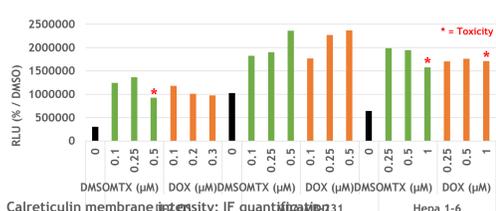
U-2 OS cells:

- 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.

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

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

Surface calreticulin detection: IF (ThermoFisher antibody)



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%

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

Surface HSP90: IF (abcam antibody)

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%

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



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.