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

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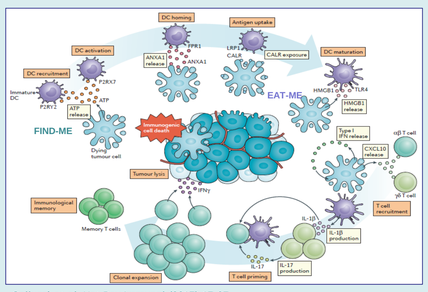
## Immunogenic cell death and Nanocyclix

### INTRODUCTION




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.

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

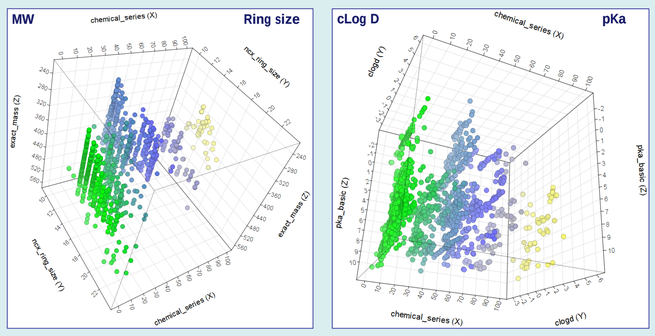
Some single-agent ICD inducers in cancer:

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

## *In vitro* detection of ICD inducers

### RESULTS

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

**ODS142**

**identified as a hit**

| | U-2 OS | | MDA-MB-231 | | Hepa 1-6 | |
|---|---|---|---|---|---|---|
| Compound | 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**
**Toxicity**

| | U-2 OS | | Hepa 1-6 | |
|---|---|---|---|---|
| Cpd (µM) | Viability | Secreted ATP | Viability | Secreted ATP |
| DMSO 0.2% | 100% | 100% | 100% | 100% |
| MTX 0.2 | 92% | **801%** | | |
| MTX 0.3 | 81% | **1214%** | | |
| MTX 0.25 | | | 91% | **312%** |
| MTX 0.5 | | | 105% | **445%** |
| Dox 0.2 | 79% | **627%** | | |
| Dox 0.25 | 85% | **847%** | 91% | **240%** |
| Dox 0.5 | | | 98% | **487%** |

| | 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%** |

**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

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

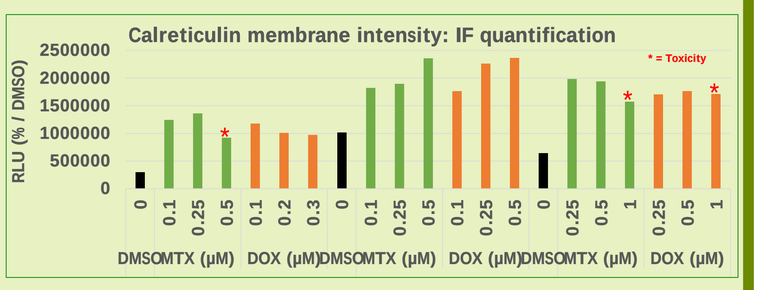
| | MDA-MB-231 | | Hepa 1-6 | |
|---|---|---|---|---|
| Cpd (µM) | Viability | HMGB1 | Viability | HMGB1 |
| DMSO 0.2% | 100% | 100% | 100% | 100% |
| MTX 0.25 | 102% | **240%** | 90% | **171%** |
| MTX 0.5 | 87% | **274%** | 84% | **240%** |
| MTX 1 | 79% | **327%** | 75% | **331%** |
| Dox 0.5 | 102%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

| | MDA-MB-231 | | Hepa 1-6 | |
|---|---|---|---|---|
| Cpd | 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.**

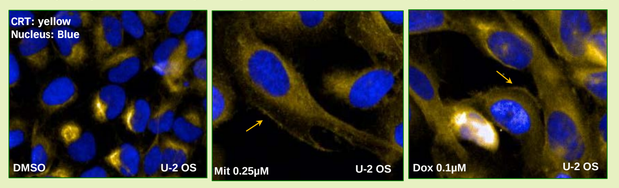
**ODS142 treatment results in HMGB1 release in 3 cell lines at non-toxic concentration.**

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




| | U-2 OS | | MDA-MB-231 | | Hepa 1-6 | |
|---|---|---|---|---|---|---|
| Cpd | 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%** |

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



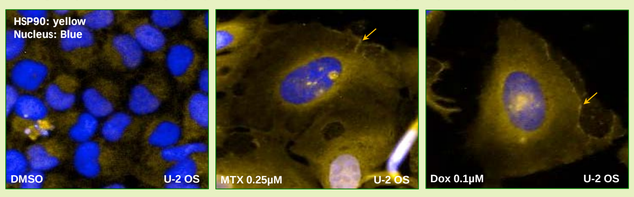

| | Cut-off |
|---|---|
| Viability | >75% |
| Secreted ATP | >150% |
| Released HMGB1 | >150% |
| Surface CRT | >150% |
| Surface HSP90 | >150% |

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

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

| 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.