Identification of small-molecule inhibitors of ricin using a cell-based high-throughput screen

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Wadsworth Center, Albany NY
Discovery of ricin inhibitors

Target = Ricin (and Shiga toxins)

Discovery method = Cell-based high-throughput screening
Ricin

A toxic protein found in the seeds of the castor bean plant (*Ricinus communis*)

Castor bean plant

Castor beans
Ricin is very toxic

- LD$_{50}$ is ~1 µg/kg body weight for mice, rats and dogs.
- Ricin can be weaponized for biochemical warfare purposes.
- Ricin is a select agent.
Surface representation of ricin and Shiga toxin

RTA and StxA – catalytic subunits
RTB and StxB – receptor binding subunits

PDB:2AAI
PDB:1DM0
Cytotoxicity of ricin (and Shiga toxin)

- Receptor binding
- Endocytosis
- Trafficking
  - RTA N-glycosidase activity
  - Ribotoxic stress response
  - Apoptosis and inflammation
Ricin is an RNA $N$-glycosidase

28S rRNA

Protein synthesis inhibition
Limitations of current inhibitors of ricin

1. Pteroic acid

\[ \text{IC}_{50} \sim 600 \, \mu \text{M} \]

- Pteroic acid is cytotoxic and insoluble in aqueous media.

2. Transition state mimics

- Inhibit RTA only at low pH (~ 4.0)

\[ \text{IC}_{50} \sim 1 \, \mu \text{M} \]

Currently there is no effective inhibitor of ricin
To identify **effective small molecule inhibitors** of ricin using high throughput screening (HTS)

**GOAL**

Cell-based HTS assay

Virtual screening

**Confirmatory**

Cell-based assay

**In vitro** translation assay

Analogues and SAR

Animal studies
Chemical library screening

• **NSRB** – Screening facility is located in Harvard Medical School

• **Chemical libraries**: ~300,000 pure compounds and natural product extracts

**HTS EQUIPMENT**

- Liquid handler
- Pin transfer robot
- Plate reader
Ricin cytotoxicity assay

Vero cell

+ ricin

ricin inhibitors

protein synthesis inhibition

apoptosis

dead cell

low ATP levels

high ATP levels

cell proliferation

No ricin addition

Luciferin + ATP + O₂ $\xrightarrow{\text{luciferase } \text{Mg}^{2+}}$ Oxyluciferin + AMP + PPi + CO₂ + light
Summary of HTS and follow-up studies

1° Screened 20025 compounds
   ~ 1500 primary screen hits

2° Cherry picked 600 compounds

3° 64 hit compounds

6 potential inhibitors
### New inhibitors of ricin

<table>
<thead>
<tr>
<th>No.</th>
<th>2D structure</th>
<th>EC$_{50}$ (μM)</th>
<th>2D structure</th>
<th>EC$_{50}$ (μM)</th>
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<td><img src="image" alt="Structure 10" /></td>
<td>&gt;100</td>
<td><img src="image" alt="Structure 72" /></td>
<td>31</td>
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</table>
Ricin inhibition by compound PW66

EC$_{50}$ = 8 µM

PW66
Potential target sites of ricin inhibitors

- Receptor binding: ×
- Endocytosis: ×
- RTA N-glycosidase activity: √
- Ribotoxic stress response: √
- Apoptosis and inflammation: √
3 compounds interfered with the enzymatic activity of RTA

RTA inhibition by compound PW5

\[ IC_{50} = 30 \mu M \]
Compound 5 docks at the active site of RTA
<table>
<thead>
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<th>2D structure</th>
<th>RTA IC(_{50}) (μM)</th>
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<td>10</td>
<td><img src="image3.png" alt="Structure 10" /></td>
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3 compounds inhibited ricin cytotoxicity by interfering with stress signaling

- Receptor binding
- Endocytosis
- Trafficking
- RTA N-glycosidase activity
- Ribotoxic stress response
- Apoptosis and inflammation
MAPK signaling

- MAPK pathways - p38, JNK, and ERK1/2

**Cellular stress**
- ricin
- other kinases

**Cytokines, EGF**
- ASK1, MLK3, TAK1

**MAPKKK**
- MLK7
**MAPKK**
- MKK4/MKK7

MAPK
- JNK
- p38

Transcription factors
- (c-jun, ATF-2)
PW66 and PW72 reduced levels of ricin-induced TNF-α secretion by J774 cells
Activation of p38 MAPK by ricin

A

Kinase assay

[p38 MAPK (phospho)]

ATF-2~P

CellSignaling Technology®

B

<table>
<thead>
<tr>
<th>Cpd</th>
<th>DMSO</th>
<th>DMSO</th>
<th>PW72</th>
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C

<table>
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<tr>
<th>PW66 (µM)</th>
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<th>-</th>
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<th>20</th>
<th>10</th>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tbody>
</table>

ATF-2~P
Proposed target for PW66

Ricin

MLK7 (ZAK)

p-MKK3/MKK6 (active)

PW66 ?

p-p38 MAPK (active)

Apoptosis

PW66

Proposed target for PW66
Ricin mediated apoptosis

- Ricin
- TNF-α
- TNF-R1
- Calcium↑
- mitochondria
- Apaf1
- Caspase-9
- TRADD
- FADD
- Caspase-8
- Caspase-3
- Apoptosis
Compounds PW69 and PW72 interfered with Caspases 3/7 activities.

- Vero cells
- Test compound: Ricin
- Add caspase 3/7 substrate
- Measure luminescence

<table>
<thead>
<tr>
<th>ricin</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
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</table>
Proposed target(s) for PW69 and PW72

Ricin → TNF-α → TNF-R1

Calcium ↑ → mitochondria

? → Apaf1 → Caspase-9

PW69, PW72 ? → Caspase-9, Caspase-8, Caspase-3 → Apoptosis

TNF-R1 → TRADD → FADD

Apoptosis

Calcium ↑

PW69

PW72
Potential target sites of ricin inhibitors

- Receptor binding: ×
- Endocytosis: ×
- Trafficking: ×
- RTA N-glycosidase activity: ✓
- Ribotoxic stress response: ✓
- Apoptosis and inflammation: ✓

Potential inhibitors:
- PW2
- PW5
- PW10
- PW66
- PW69
- PW72
Conclusions

• Developed a robust cell based HTS assay for identifying inhibitors of ricin.

• Identified 6 compounds with significant anti-ricin activity and low to moderate cytotoxicity.

• Identified 3 compounds that inhibit RTA and StxA1. One compound showed \( \sim 10\times \) higher activity than pteroic acid.

• Identified 3 compounds that interfered with ricin induced stress signaling.
Future directions

• Determine mode of ricin inhibition by the inhibitors.
  - X-ray crystallography
  - Solution NMR
  - Cell-based and biochemical kinase assays

• Test analogues of the compounds to establish structure activity relationship (SAR).

• Initiate *in vivo* studies using established animal models of ricin intoxication.
## Acknowledgements

<table>
<thead>
<tr>
<th>Location</th>
<th>Institutions</th>
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</thead>
<tbody>
<tr>
<td><strong>Wadsworth Center (NY)</strong></td>
<td>University of Texas (Austin)</td>
</tr>
<tr>
<td>Dr. Nicholas Mantis</td>
<td>Dr. Jon Robertus</td>
</tr>
<tr>
<td>Dr. Sarita Alahwat</td>
<td></td>
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<td>Mantis lab</td>
<td></td>
</tr>
<tr>
<td><strong>NSRB (Harvard Medical School)</strong></td>
<td>NIH</td>
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<tr>
<td>Dr. Su Chiang</td>
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<tr>
<td>Dr. Ren Tao</td>
<td>Postdoctoral Chapter of Northeast Branch of ASM</td>
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<tr>
<td>Ruchir Shah</td>
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<td>Andrew Daab</td>
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</table>
Is activation of ATF-2 inhibited by PW66 or PW72?

ATF-2 + ATP $\xrightarrow{\text{P38~P MAPK}}$ ATF-2$\sim$P + ADP

<table>
<thead>
<tr>
<th>Cpd (µM)</th>
<th>DMSO</th>
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$\xrightarrow{\text{PW66, PW72 or SB203580}}$
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## SUMMARY OF PHASE I STUDIES

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<th>Cherry picks(^b)</th>
<th>2º hits</th>
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<td><strong>252</strong></td>
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\(^a\) Number of compounds that gave ≥ 30% cell protection in HTS assay.
\(^b\) Compounds from the primary screen were retested to confirm their anti-ricin activity.
\(^c\) Individual stock well contained undefined number of compounds here simply referred as “compound”.
<table>
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<tr>
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</table>

| Total compounds | 118697 | 403 | 356 | 44 |

<sup>a</sup> Number of compounds that gave ≥ 50% cell protection in HTS assay.

<sup>b</sup> Compounds from the primary screen were retested to confirm their anti-ricin activity.

<sup>c</sup> Compounds whose anti-ricin activity was confirmed in the secondary screen.
- R 180 donates a proton to adenine (substrate) and then through resonance the C1’-N9 glycosidic bond is cleaved leading to formation of an oxacarbenium ion.

- Hydroxonium ion generated by E177 abstraction of H⁺ from H₂O stabilizes the oxacarbenium ion.

Robertus and Monzingo, 2004
Ricin inhibitory activity of PW5 (▲), and cytotoxicity (△)

A

Cell viability, %

PW5 (μM)

△ ricin + PW5

----- ricin

B

Cell viability, %

PW5 (μM)

△ PW5
Transition state mimics
RNA N-glycosidase activity

28S rRNA
Primary screen

Screened 81328 stock wells in duplicate from 17 chemical libraries

Selection criteria for hit compounds

• $Z' \geq 0.5$ ($Z'$ measures robustness of an HTS assay)

• $Z$-score $> 2.0$

• $\geq 30\%$ ricin inhibition

Hits were classified into three groups

1. Strong ($\geq 80\%$ inhibitory activity, 25 compounds)

2. Medium ($\geq 50\% < 80\%$, 61 compounds)

3. Weak ($\geq 30\% < 50\%$, 214 compounds)
Summary of Phase II primary screen

• Screened ~118700 compounds

97 (0.082%) compounds showed strong (≥ 80%) anti-ricin activity

307 (0.259%) compounds showed moderate (≥ 50% < 80%) anti-ricin activity

• Selected 356 hit compounds for secondary screening

• Compounds were retested at ~ 1, 5 and 15 µM
Ricin inhibitory activity of compound PW69

- **▲** ricin + PW69
- **Δ** ricin only

**PW69**

- **△** ricin + PW69
- **Δ** ricin only
Ricin inhibitory activity of compound PW72

\begin{align*}
\text{Cell viability, } \% \\
\text{PW 72 (μM)}
\end{align*}

\begin{align*}
\text{Log}_{10} [\text{ricin}] (\text{ng/ml})
\end{align*}

- \text{△ ricin + PW72}
- \text{--- ricin only}
- \text{△ ricin only}
Cellular stress signaling

Acute injury, Oxidant Stress and Hydrogen Peroxide

p-MKK3/MKK6 (active)

PW66 ?

p-p38 MAPK (active)

PW72 ?

p-HSP27

PW69 and 72 ?

IκB

NFκB

TNF gene transcription

IL-1β

Apoptosis

TNF
<table>
<thead>
<tr>
<th>No.</th>
<th>2D structure</th>
<th>EC$_{50}$ (μM)</th>
<th>2D structure</th>
<th>EC$_{50}$ (μM)</th>
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## Inhibitors of ricin

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<th>EC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
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<tbody>
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</tr>
<tr>
<td>72</td>
<td><img src="image3" alt="2D structure" /></td>
<td>31</td>
</tr>
</tbody>
</table>
No compound inhibited ricin binding to cell surface receptor

Vero or THP-1 cell

+ Test compound

Ricin-FITC

---

Analyse with flow cytometry or microscopy

---

Ricin-FITC bound

Ricin-FITC unbound
Overview of HTS and follow-up studies

Primary screen
- test chemical libraries

Secondary screen
- retest hit compounds to confirm activity
- counterscreen to eliminate false positives

Tertiary studies
- dose response studies
- *in vitro* translation assay
Ischemia/reperfusion, Hypoxia, Reactive Oxygen Species

Calcium overload  TNFα or other death stimuli

mitochondria  Death receptors

Apafl  Caspase-9  Caspase-8

Caspase-3

Apoptosis
Ricin induces release of TNF-α by macrophages

1. Ricin
2. MAPKKK → MLK7 (ZAK)
3. MAPKK → p-MKK3/MKK6 (active)
4. MAPK → p-p38 MAPK (active)
5. NFκB
6. TNF gene transcription
7. TNF
8. Apoptosis
Signaling pathway of TNF-R1
Ricin inhibitory activity of PW2 (▲), and cytotoxicity (△)

▲ ricin + PW2
----- ricin
EC_{50} = 23 \mu M

△ PW2

PW2
3 compounds inhibited the enzymatic activity of ricin

In vitro protein translation assays

Vero cells

ricin or ricin and test compound

radiolabeled cystine

Incorporation of radiolabeled amino acids to nascent polypeptides

luc mRNA 5' → Protein synthesis

Luciferase + substrate

Light

PW2

PW5

PW10
Induction of apoptosis by TNF

- TNF-α
- TNFR1
- TRADD
- FADD
- Calcium ↑
- mitochondria
- Apaf1
- Caspase-9
- Caspase-8
- Caspase-3
- Apoptosis

Ricin
Inhibition of p38 MAPK activation by PW66 in J774 cells

ATF-2 + ATP $\xrightarrow{\text{P38}^{\sim}P \text{ MAPK}}$ ATF-2$^{\sim}P$ + ADP

<table>
<thead>
<tr>
<th>Cpd</th>
<th>DMSO</th>
<th>DMSO</th>
<th>PW72</th>
<th>PW69</th>
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<tbody>
<tr>
<td>Ricin</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

PW66 (µM)  40  20  10  5  
Ricin  +  +  +  +  +  +  

$\Rightarrow$ ATF-2$^{\sim}P$
To identify **effective small molecule inhibitors** of ricin toxin using high throughput screening (HTS)

**GOAL**

Cell-based HTS assay ← Virtual screening

\[ A \]

\[ B \]

\[ In vitro \text{ translation assay} \]

\[ A \]

Kinetic studies ← Development

\[ A \]

Animal studies → Cell based and *in vitro* translation assays

Animal studies
Cell-based high-throughput screening

Chemical libraries

~ 300,000 pure compounds and natural product extracts

• **Known bioactives** e.g., brefeldin A, adenosine, zidovudine, etc

• **Commercially available compounds**

  Vendors: ChemDiv, Enamine, Peakdale, Chembridge, Maybridge
Surface representation of ricin

A subunit (RTA) – catalytic subunit

B subunit (RTB) – receptor binding subunit

PDB: 2AAI
Ricin cellular binding, trafficking and ribosome inactivation

Ricin exposure

- ingestion
- injection
- inhalation

Ricin exposure

Cell membrane

Early endosome

Golgi apparatus

Endoplasmic reticulum

Nucleus

Cytosol

Lysosome

Late endosome

Receptor

Ribosome

mRNA
Ricin is an RNA N-glycosidase

N-glycosidic bond cleaved by ricin or Shiga toxin

28S rRNA

Adenosyl group

Adenine

Protein synthesis inhibition