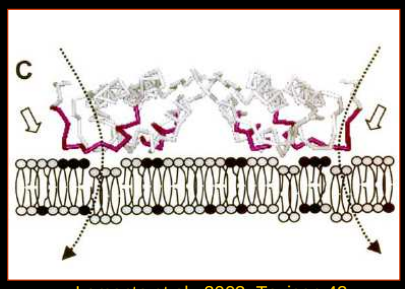
Institut
"Jožef Stefan"
Ljubljana, Slovenija

Enzymatic Activity Restored in Ammodytin L, an Inactive Phospholipase A_2 Homologue, Increases its Toxic and Membrane Damaging Activities

Toni Petan
Jozef Stefan Institute
Slovenia

Ammodytin L (AtnL), a Ser-49 group IIA $sPLA_2$ homologue

- Ser-49/Lys-49 homologues
  - Inability to bind Ca^{2+}
  - Myonecrosis *in vivo*
  - Ca^{2+}-independent membrane damage *in vitro*
- Protein-membrane interactions on the sarcolemma



Lomonte et al., 2003, Toxicon 42.

Ammodytin L (AtnL) is a myotoxic structural homologue of group IIA sPLA2s from snake venom and is one of the two known Ser-49 sPLA2 isoforms.

Similarly to the growing group of Lys-49 sPLA2 snake venom myotoxins, AtnL has been predicted to be enzymatically inactive due to substitution of the conserved Asp-49 and the consequential inability to coordinate the essential Ca2+-ion.

Despite having little or no enzymatic activity, these sPLA2 homologues are very active in induction of myonecrosis *in vivo* and show a potent Ca2+-independent membrane damaging activity *in vitro.*

Current evidence indicates that the myotoxic effects of Lys-49 and Ser-49 sPLA2 homologues are a consequence of this catalytically-independent mechanism involving protein-membrane interactions on the plasma membrane of target muscle cells.

S49/K49 $sPLA_2$ (in)activity controversy

- "$sPLA_2$s with no or very low catalytic activity" – (still) a controversial issue?
- First recombinant BthTX I proved inactivity of K49 $sPLA_2$s (Ward et al., (2002) *Biochem. J.* 362)
- K49D mutant of BthTX I remained inactive

  => Lys-49 is not the only residue responsible for inactivity

The question whether Lys-49 and Ser-49 sPLA2s have retained some low levels of catalytic activity has been a subject of considerable controversy over the last two decades.

Recently, in a study with the first recombinant Lys-49 sPLA2, bothropstoxin I, a strong argument was made in favor of the proposed lack of catalytic activity of Lys-49 sPLA2s.

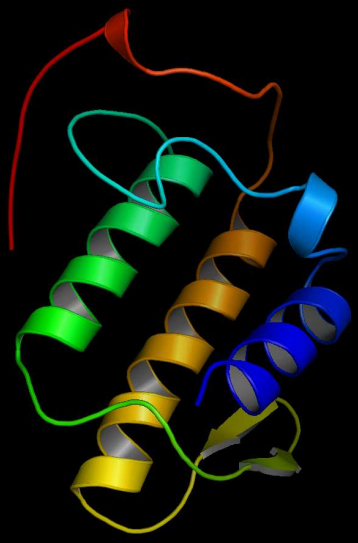
However, the preparation of the K49D mutant of bothropstoxin I, where the completion of the catalytic motif did not result in restoration of enzymatic activity, indicated that the residue at position 49 is not the only one responsible for their inactivity.

(Nevertheless, a number of structural studies have indicated that the substrate binding site has retained the ability to bind fatty acids and other long chain ligands in their "active site".

Moreover, a direct structural connection between ligand binding in the phospholipase "active site" and the C-terminal "myotoxic site" of Lys-49 sPLA2s has been proposed recently (Ambrosio *et al.,* JBC, 2005). However, a convincing evidence of phospholipid binding in the active site has not been provided yet.)

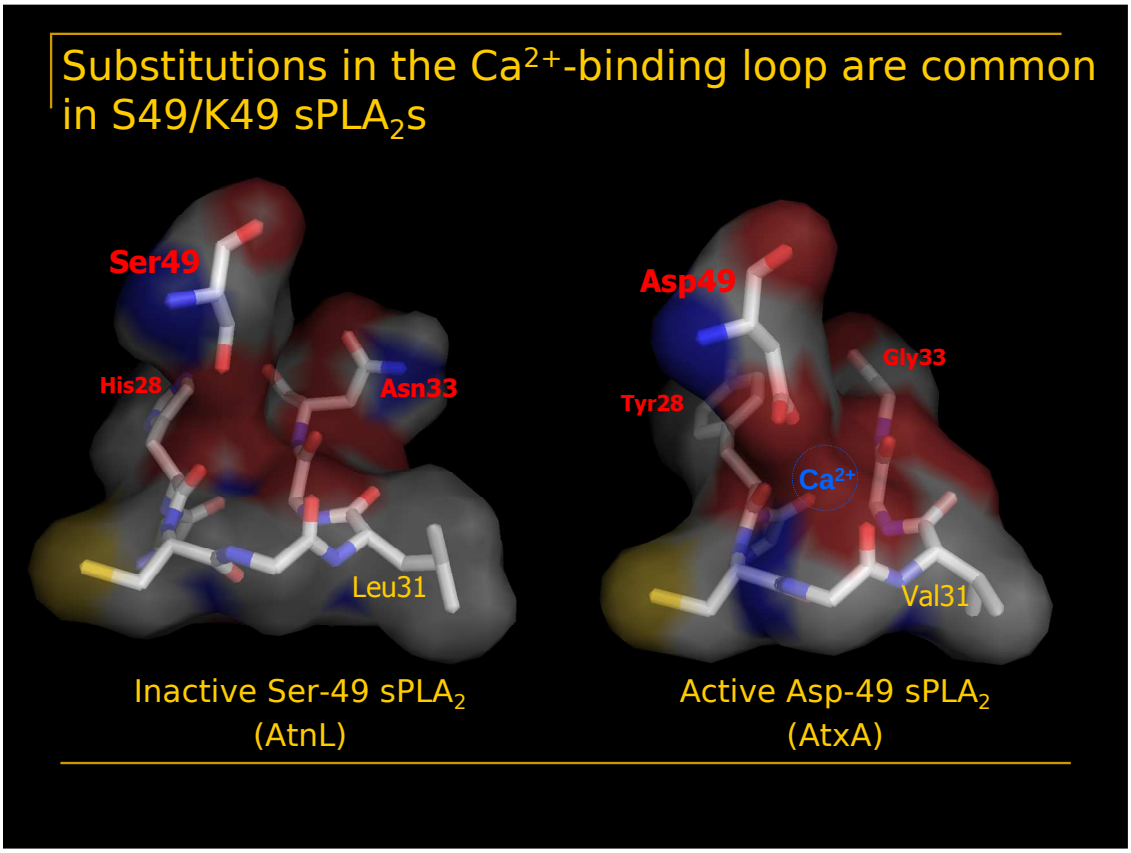
Our aim

- Confirm the inactivity of AtnL
- Restore a working catalytic machinery in AtnL
- Determine its impact on the toxic and membrane damaging activities



Ammodytin L (AtnL)

The main goal of our study was to restore the catalytic machinery of Ammodytin L and to determine the impact of enzymatic activity on its toxic and membrane damaging properties.



Besides the substitution of Asp-49, in virtually all Lys-49 and Ser-49 sPLA2 homologues we find substitutions also in the calcium binding loop, especially at positions 28, 32 and 33, in comparison to active sPLA2s.

Therefore, besides Ser49, we decided to substitute three residues in the calcium binding loop of AtnL (His28, Leu31 and Asn33) with those present in AtxA, a highly specific neurotoxin and an efficient sPLA2 enzyme, which shares a 74% amino acid identity with AtnL.

Mutagenesis of AtnL

AtnL: ^{28}**H..L.N**^{33}**...S**^{49}

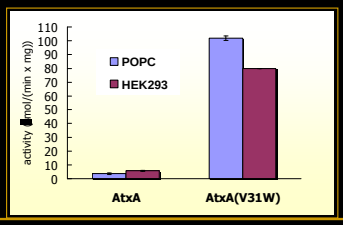
AtxA: ^{28}**Y..V.G**^{33}**...D**^{49} (74% identity to AtnL)

Two mutants of AtnL:

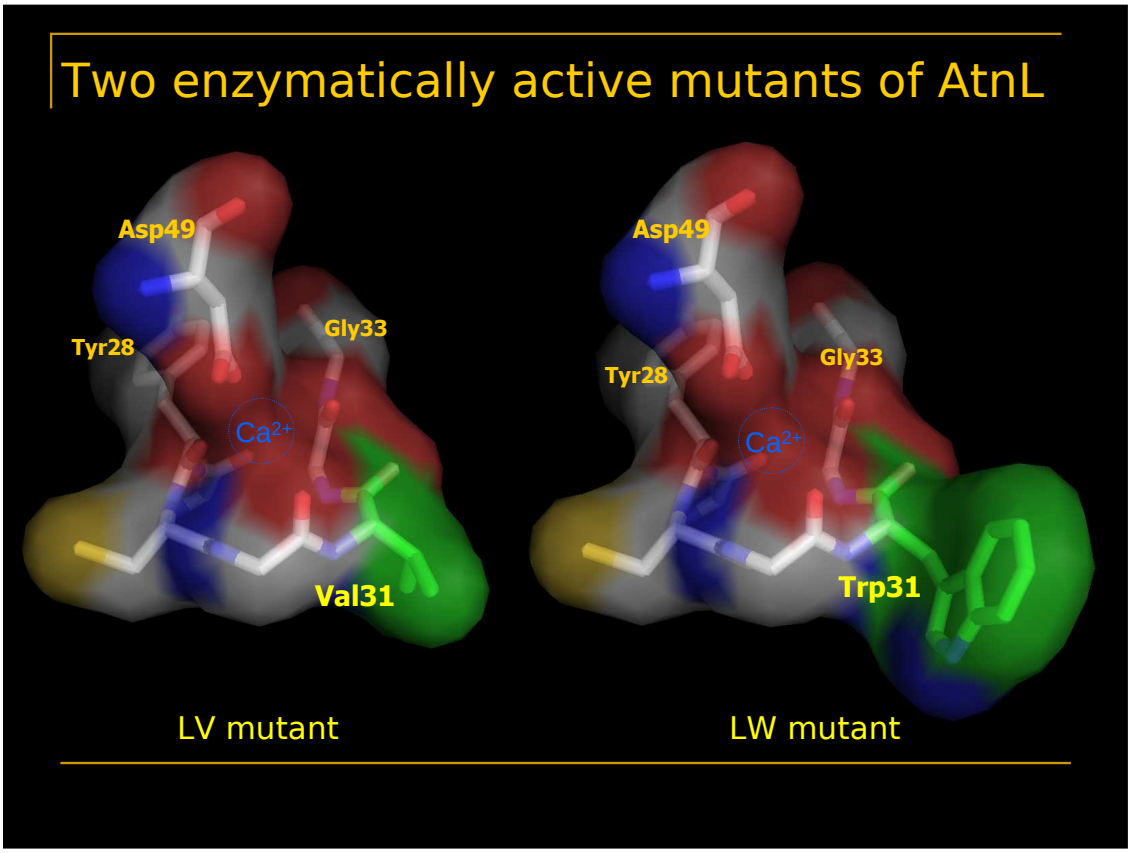
AtnL(YVGD): ^{28}**Y..V.G**^{33}**...D**^{49} (in short LV)

AtnL(YWGD): ^{28}**Y..W.G**^{33}**...D**^{49} (in short LW)

Precautionary step:
Introduction of Trp-31 in the LW mutant to increase interfacial binding affinity



Petan et al., (2005) *Biochemistry*, 44.

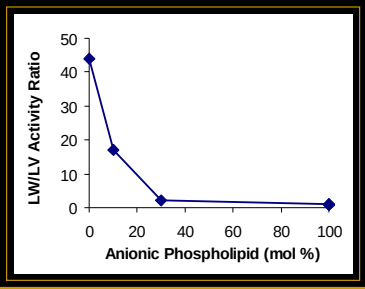


In this way we prepared the first enzymatically active mutants of AtnL, which differed only in the presence or absence of Trp-31. This is the first example of a restoration of catalytic activity in a K49/S49 sPLA2 homologue.

Trp-31 enhances the activity of LW on PC-rich vesicles

specific enzymatic activity, v_0 (μmol min^{-1} mg^{-1})

	POPC	10% PS/PC	30% PS/PC	POPS	POPG
AtnL	0	0	0	0	0
LV	~0.005	0.5	43	90	225
LW	0.22	8.3	96	90	184



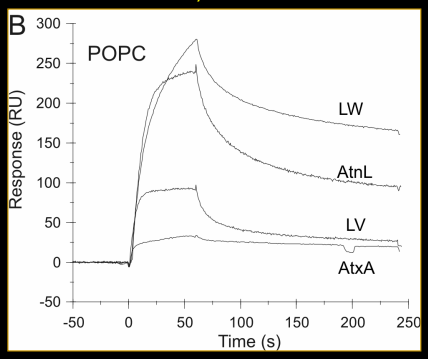
While rec. AtnL did not show any activity on phospholipid vesicles, both enzymatically active mutants of AtnL were especially effective in hydrolyzing phospholipid vesicles containing high amounts of anionic phospholipid.

On the other hand, the hydrolytic potency of LV and LW differed considerably on zwitterionic POPC vesicles, the Trp mutant being about 44-fold more effective than the Val mutant, and the difference between their activities decreased with increasing anionic content.

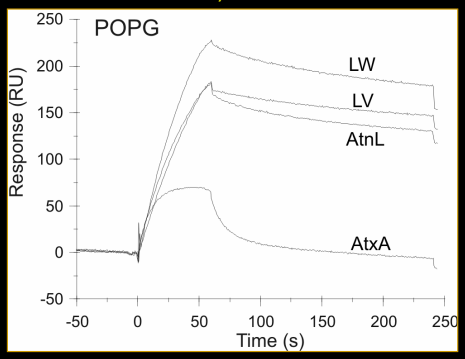
Their similar activities on anionic vesicles indicate that their catalytic mechanism is equally effective and that the difference in activity on PC-rich vesicles is a consequence of differential binding of the two mutants, due to the presence or absence of Trp-31.

LW binds very well to PC vesicles

100 nM toxins, POPC vesicles



50 nM toxins, POPG vesicles

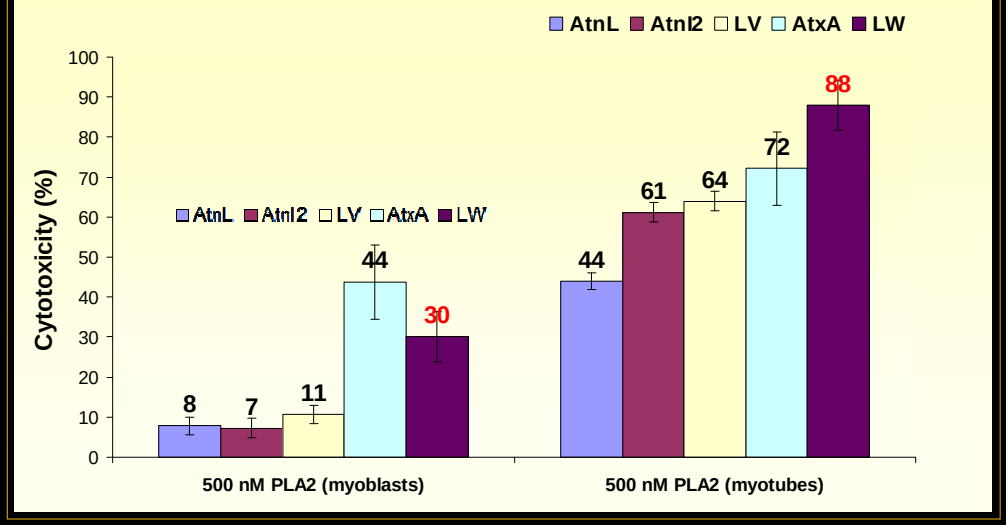


100 nM extruded LUVs deposited on a Pioneer L1 chip
Running buffer: HBSS w/o Ca^{2+}, Mg^{2+}

Indeed, the qualitative analysis of interfacial binding to phospholipid vesicles performed by SPR, confirmed that the LW mutant has a higher apparent affinity for binding to POPC vesicles in comparison to the LV mutant, but that both mutants bind with similar affinity to anionic POPG vesicles.

In both cases the highly basic AtnL and its mutants were able to bind very well to both anionic and zwitterionic membranes, their apparent binding affinities being much higher than that of AtxA.

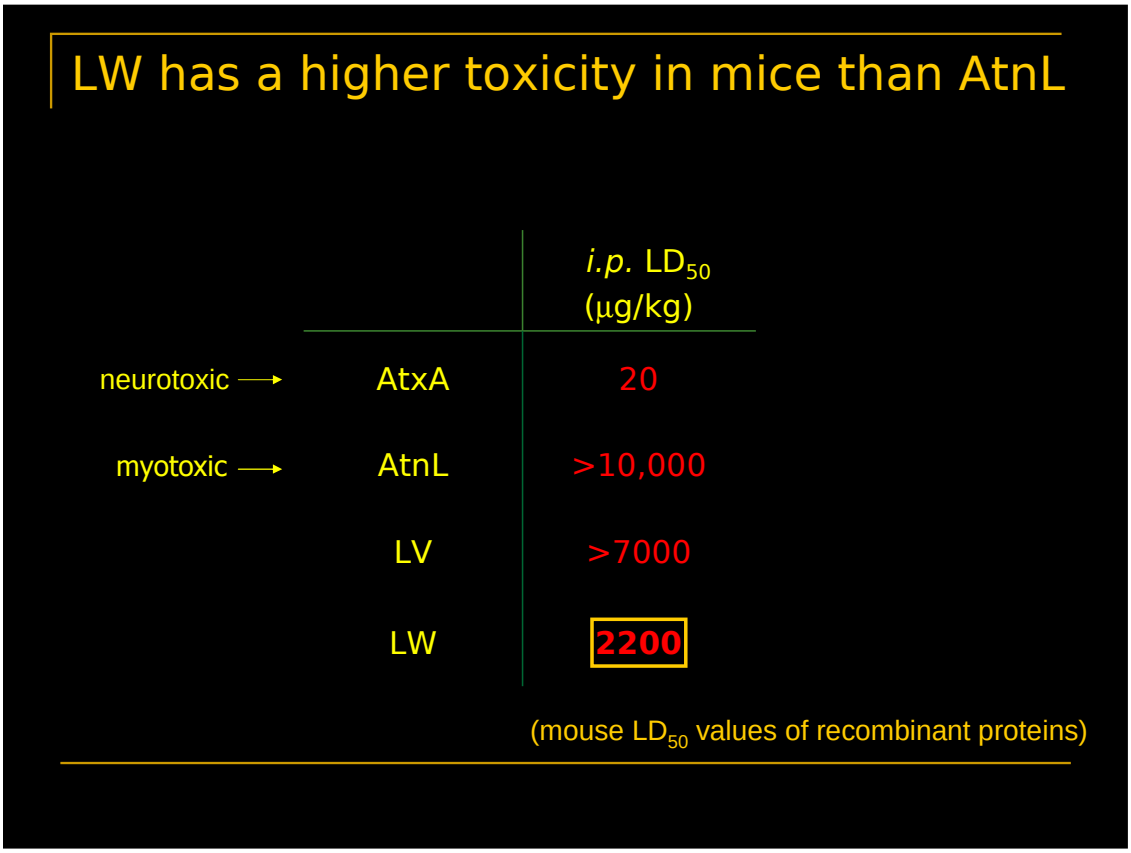
LW displays high cytotoxicity towards C2C12 myoblasts and myotubes



Next we looked at the cytotoxic potential of Atxs and Atns on the basis of LDH release from the cytosol of damaged C2C12 myoblasts or myotubes.

While AtnL and LV had a very weak potency in damaging undifferentiated C2C12 myoblasts, the enzymatically active LW mutant displayed a significantly higher cytotoxicity on C2C12 myoblasts, similar to that of AtxA. (indicating a major impact of enzymatic activity)

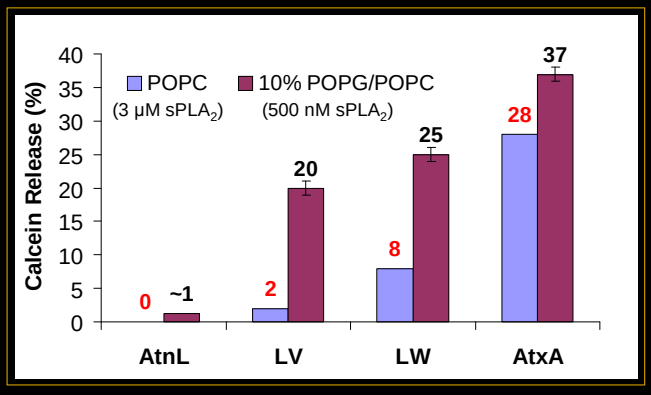
Differentiated C2C12 cells were much more susceptible to the actions of both Atxs and Atns, but the LW mutant was the most cytotoxic of all, displaying even higher cytotoxicity than AtxA, (and the lack of correlation with enzymatic activity was evident.)



Interestingly, by enabling the enzymatic activity of AtnL and adding a Trp at position 31, we obviously produced a molecule with increased toxicity.

The Trp mutant displayed at least 5-fold higher toxicity than AtnL itself.

LV and LW cause calcein release in a Ca^{2+}-dependent manner



- Good correlation with enzymatic activity
- No release in the absence of calcium for any of the toxins
  => crucial role of enzymatic activity

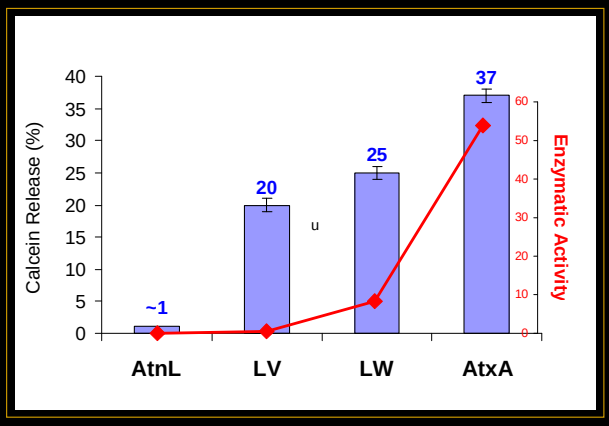
To determine the membrane damaging potency of Atxs and Atns we used a classic method based on release of calcein entrapped in phospholipid vesicles.

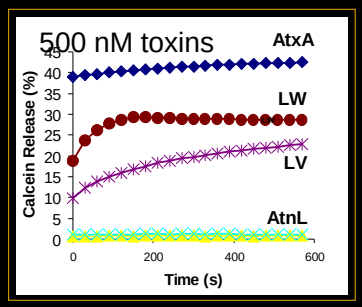
AtnL was not able to induce calcein release from POPC or 10% POPG/POPC LUVs, while LW and LV induced some calcein release already from pure POPC vesicles, but they displayed a significant release when tested on 10% POPG/POPC vesicles.

Additionally, there was a good correlation with enzymatic activities of the toxins on vesicles of equal composition and calcein leakage was not detected for any of the enzymes in the absence of calcium, indicating that enzymatic activity is responsible for the calcein release effects observed.

(Clearly, apart from AtnL, the calcein releasing potency of the enzymatically active sPLA2s increased significantly in the presence of anionic phospholipids in vesicles, which is in accordance with their higher interfacial binding affinity and consequently enzymatic activity on vesicles containing anionic phospholipids.)

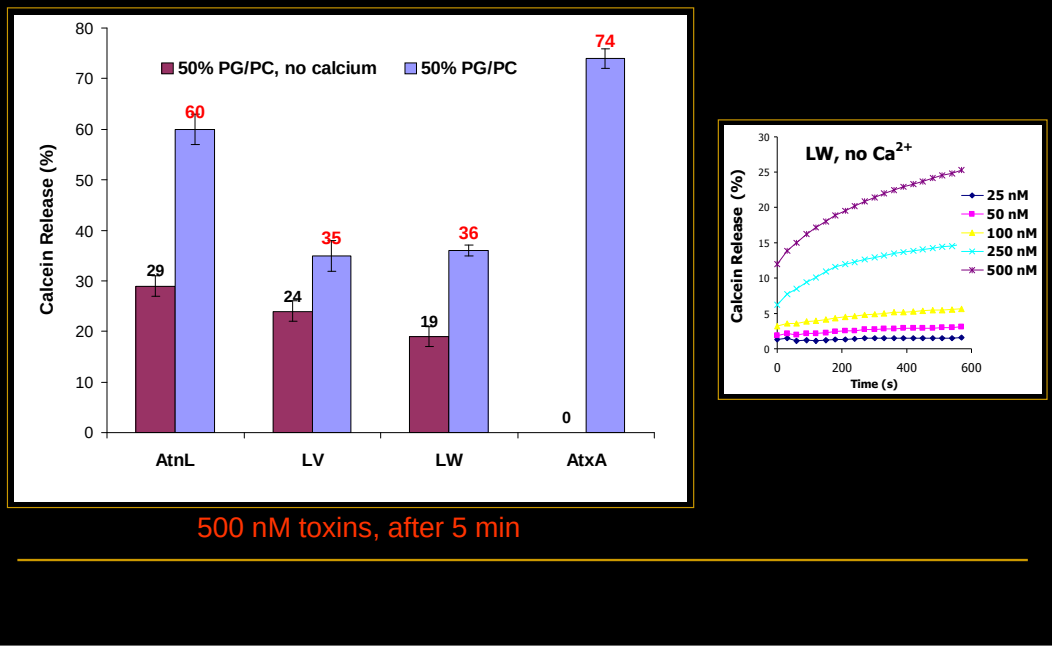
Calcein release by LW and LV from 10% PG/PC vesicles detected only in the presence of Ca^{2+}





Calcein leakage could not be detected in the absence of calcium for any of the toxins!

LW and LV cause calcein release from 50% PG/PC vesicles in the presence or absence of calcium

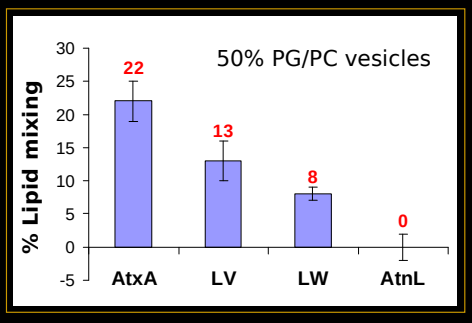


500 nM toxins, after 5 min

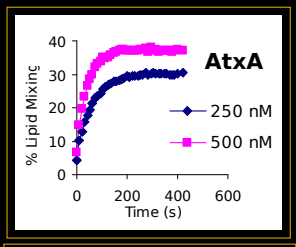
The incorporation of 50% anionic POPG phospholipids into charge-neutral POPC vesicles enabled AtnL as well as its mutants, LV and LW, to release calcein both in the presence and absence of calcium. *This indicates that a threshold concentration of anionic POPG higher than 10% in the zwitterionic environment of POPC phospholipids is necessary for initiation of the Ca-independent mechanism of membrane damage by AtnL, as well as its mutants.*

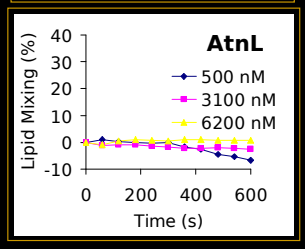
On the other hand, with AtxA calcein release could not be detected in the absence of calcium, suggesting that its membrane damaging potency is dependent on enzymatic activity.

$sPLA_2$-induced lipid mixing is dependent on enzymatic activity



NBD/Rh FRET lipid mixing assay with 500 nM toxin, 50% POPG/POPC vesicles, HBSS



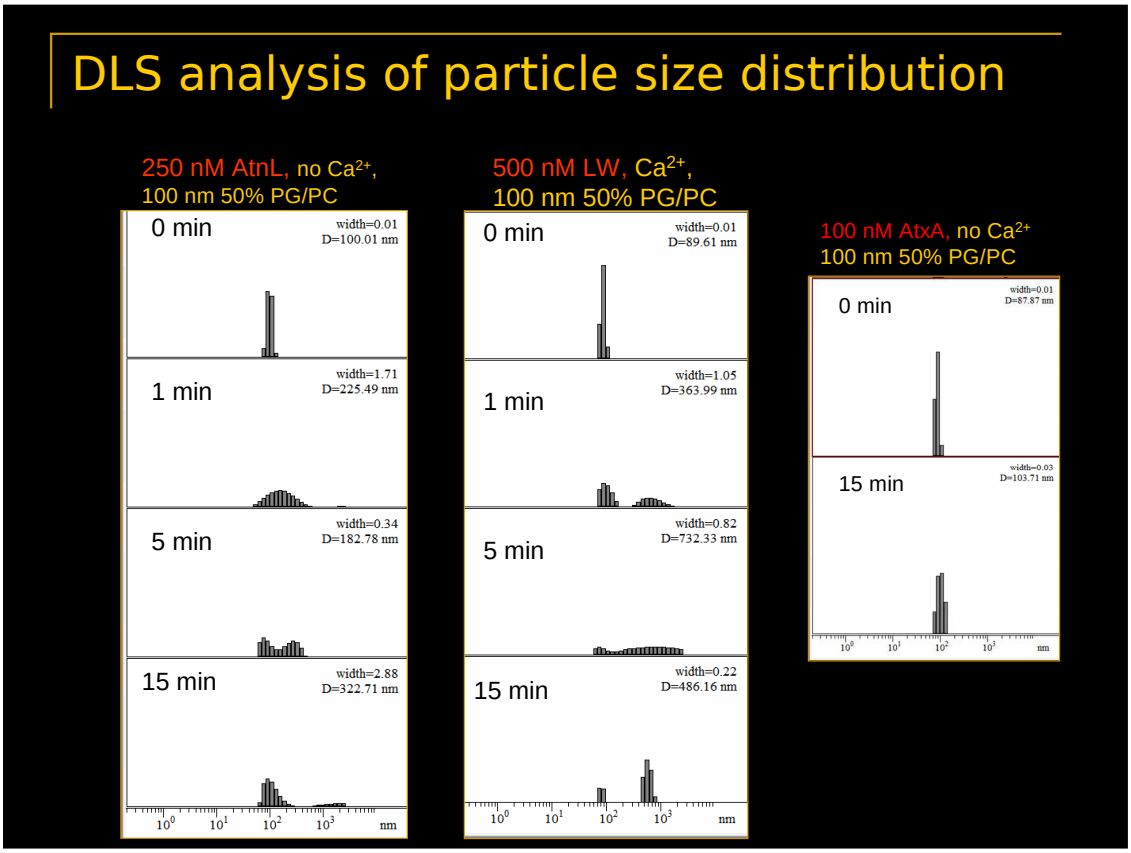


All $sPLA_2$s induced lipid mixing only in the presence of Ca^{2+}!

The fusogenic activity of Atxs and Atns was estimated by their ability to induce lipid mixing between two populations of vesicles.

We readily detected lipid mixing with all of the enzymes tested only in the presence of calcium, but the inactive AtnL failed to induce any lipid mixing up to 6.2 μM regardless of the absence or presence of up to 5 mM calcium (or 1 mM EGTA or 0.1 mg/ml BSA).

This strongly suggests that the fusogenic activity of these sPLA2 toxins is strictly dependent on their enzymatic activity.



By performing a dynamic light scattering analysis of the effects of Atxs and Atns on vesicles we confirmed that the actions of AtnL does not include vesicle fusion regardless of the presence or absence of calcium, while the effects of LW in the presence of calcium were similar to that of AtxA, resulting in the appearance of particles displaying a diameter around 600 nm (400-800), which have most probably resulted from vesicle fusion.

(AtxA did not cause any notable changes in vesicle size in the absence of calcium.)

Conclusions

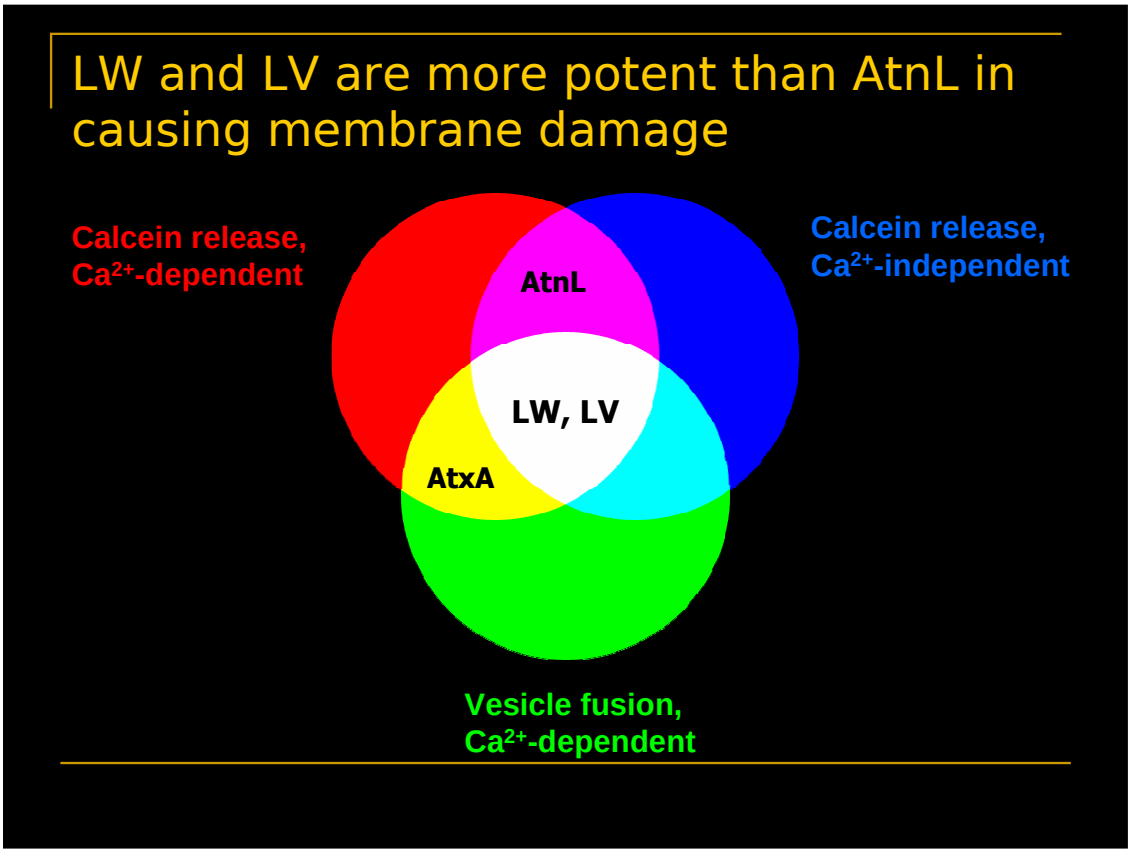
- S49 $sPLA_2$ homologues are enzymatically inactive
- Besides S49/K49, calcium binding loop residues are crucial for inactivity
- the substrate binding and catalytic networks of S49/K49 $sPLA_2$ homologues are well conserved

The lack of enzymatic activity in the first recombinant Ser-49 sPLA2, AtnL, proves the hypothesis that Ser-49 sPLA2 homologues are incapable of phospholipid hydrolysis.

Apart from the absence of Asp-49, several substitutions in the calcium binding loop, which are frequent and unique in the Lys-49/Ser-49 sPLA2s, are responsible for the lack of enzymatic activity of these sPLA2 homologues.

Our results further indicate that in AtnL, apart from the residues that take part in calcium binding, the rest of the substrate binding and catalytic network has been well conserved, since relatively small changes in the molecule were enough to restore its long lost enzymatic activity.

(This provides strong evidence that accommodation of a substrate molecule in the “active site” of Ser-49/Lys-49 sPLA2 homologues is indeed possible, supporting the proposed connection between ligand binding and their mechanism of membrane damage.)



In general, the ability of LW and LV to cause membrane damage was higher than AtnL, since:

> *they have the ability to use both the Ca2+-dependent and Ca2+-independent mechanisms – the one similar to AtxA, dependent on enzymatic activity and involving fusion and leakage, and the other, similar to AtnL, where contents leakage is caused by disruption of vesicle integrity that does not include membrane fusion.*

Conclusions

- LW and LV require a lower threshold of anionic lipid for membrane damage
- higher enzymatic activity of LW on PC-rich membranes,

=> All these factors might have enabled LW, rather than LV, to express the highest cytotoxicity for C2C12 cells as well as much higher toxicity *in vivo* in comparison with wild-type AtnL.

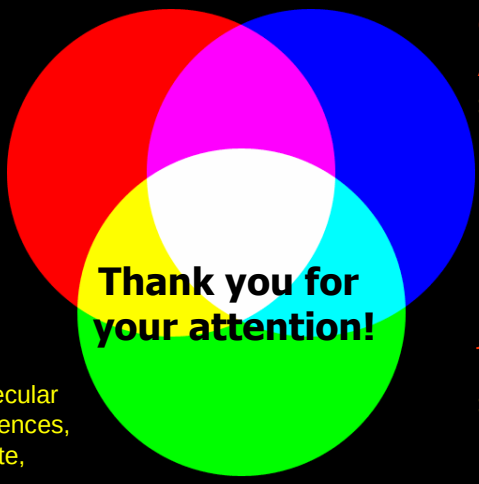
Additionally, the mutants had the ability to release calcein at lower concentrations of anionic lipid, while AtnL requires a critical, higher concentration to effectively permeabilise the membrane.

=> These factors, along with its higher enzymatic activity on PC-rich membranes, might have enabled LW, rather than LV, to express the highest cytotoxicity for C2C12 myotubes as well as much higher toxicity *in vivo* in comparison with other Atns.

Zala Jenko
Petra Prijatelj
Jernej Šribar
Lidija Kovačič
Uroš Logonder
Jože Pungerčar
Igor Križaj

Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana

Institut "Jožef Stefan" Ljubljana, Slovenija



Gregor Anderluh
Andrej Bavdek
SPR Center,
Biotechical Faculty,
Ljubljana

Michael H. Gelb
Farideh Ghomashchi
Jim Bollinger
University of Washington, Seattle, USA

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SLOVENIAN BIOCHEMICAL SOCIETY