Cyclic peptides as Connexin43 and Pannexin1 inhibitors for the treatment of inflammatory disorders

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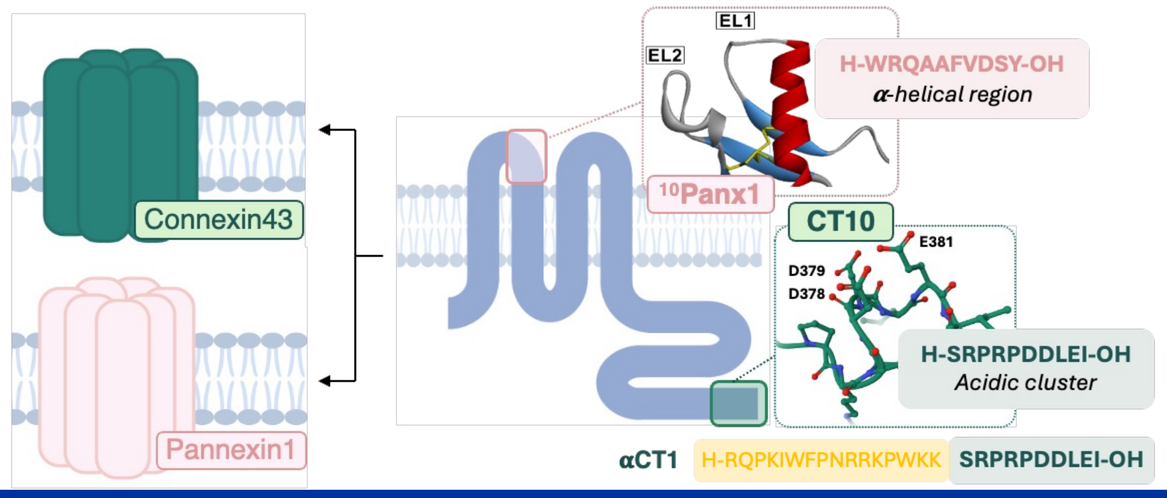
Debora Iaculli,[1] Arthur Lamouroux,[1] Malaury Tournier,[2] Jade Montgomery,[2] Anne Caufriez,[1,3] Mathieu Vinken,[3] Filippo Molica,[2] Brenda R. Kwak,[2] Steven Ballet[1]

[1] Research Group of Organic Chemistry, Vrije Universiteit Brussel, Brussels, Belgium; [2] Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland; [3] Department of in Vitro Toxicology and Dermato-Cosmetology, Vrije Universiteit Brussel, Brussels, Belgium.

Debora.iaculli@vub.be

CONNEXIN43 AND PANNEXIN1

Connexins (Cxs) and Pannexins (Panxs) are ubiquitous channel-forming membrane proteins which play an important role in a variety of both physiological and pathological processes. In the cardiac system, Connexin43 (**Cx43**) hemichannels (HCs) and Pannexin1 (**Panx1**) channels have shown to be critical mediators of inflammation in the context of **cardiovascular diseases**, including **ischemia-reperfusion (I/R) injury** and **atherosclerosis**. Selective modulation of Cx43 HCs and Panx1 channels represents then a potential therapeutic approach.[1]



CT10 AND 10Panx1

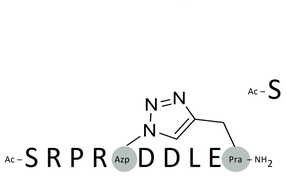
Peptide inhibitors mimicking a segment of the sequences of the proteins, in particular **CT10** and **αCT1** (including a cell-penetrating peptide (CPP)) for Cx43 and **10Panx1** for Panx1, have shown to be promising **therapeutic agents** both by *in vitro* and *in vivo* experiments.[2] However, their rapid proteolytic cleavage and low bioavailability make them poor systemically-applied drug candidates.[3] To overcome these limitations and fine-tune the properties of the **10Panx1** and **CT10** peptides, a series of chemical strategies, including **cyclization** and the introduction of **lipidic motifs**, were investigated.[4,5]

DESIGN AND SYNTHESIS

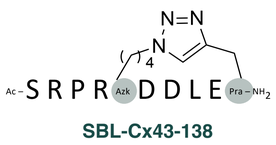
CT10 — H-SRPRPDDLEI-OH

- **Alanine scan** did not pinpoint any residues as essential for biological activity
- Cyclic peptide **SBL-Cx43-125** designed based on the NMR solution structure of the *C*-terminal domain[6]

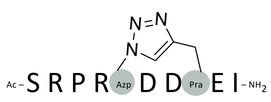
Variation of linker position and flexibility allowed for generation of a set of cyclic peptide inhibitors



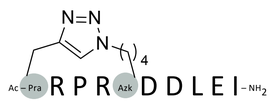
SBL-Cx43-125



SBL-Cx43-138

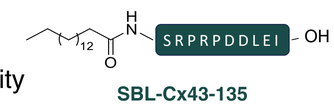


SBL-Cx43-139



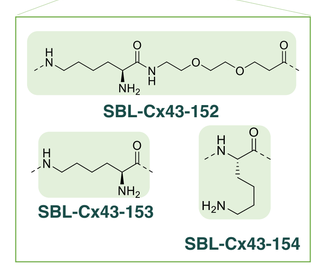
SBL-Cx43-140

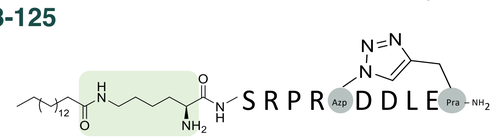
- The use of **lipidic motifs** to improve cell permeability was investigated
- **SBL-Cx43-135** shows reduced water solubility



SBL-Cx43-135

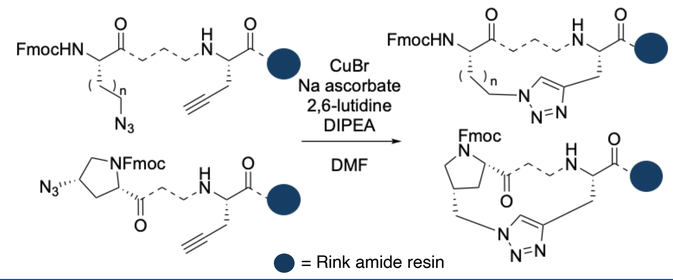
- Different polar linkers were introduced to **improve solubility**
- The best linker was used to design **SBL-Cx43-157**, a **water-soluble**, lipidated cyclic peptide based on **SBL-Cx43-125**





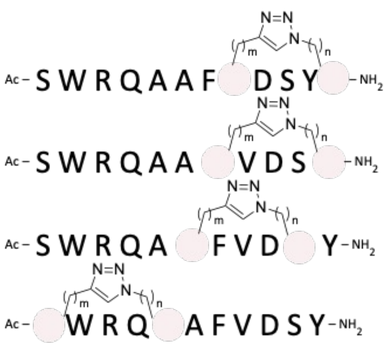
SBL-Cx43-157

CYCLIZATION STRATEGY



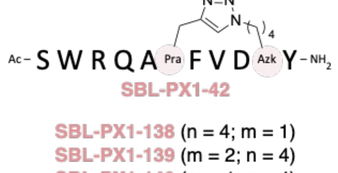
10Panx1 — H-WRQAAFVDSY-OH

- **Cyclization scan** at (*i*,*i*+4) distance to stabilize helical conformation



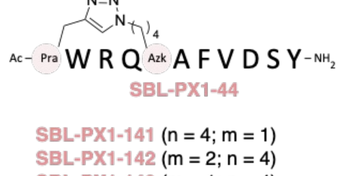
SBL-PX1-38 (m = 1; n = 4)
SBL-PX1-39 (m = 1; n = 3)
SBL-PX1-40 (m = 1; n = 4)
SBL-PX1-41 (m = 1; n = 3)
SBL-PX1-42 (m = 1; n = 4)
SBL-PX1-43 (m = 1; n = 3)
SBL-PX1-44 (m = 1; n = 4)
SBL-PX1-45 (m = 1; n = 3)

- Lead peptides **SBL-PX1-42** and **SBL-PX1-44**
- Variation of linker orientation, stereochemistry and length



SBL-PX1-42

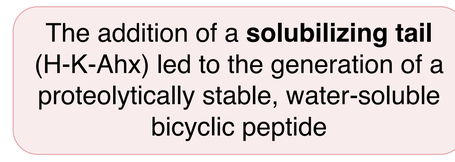
SBL-PX1-138 (n = 4; m = 1)
SBL-PX1-139 (n = 2; n = 4)
SBL-PX1-140 (m = 1; n = 4)



SBL-PX1-44

SBL-PX1-141 (n = 4; m = 1)
SBL-PX1-142 (m = 2; n = 4)
SBL-PX1-143 (m = 1; n = 4)

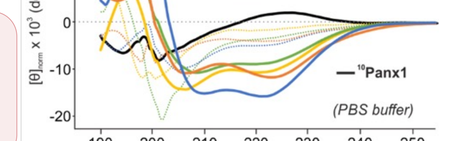
- The structures of lead peptides **SBL-PX1-42** and **SBL-PX1-44** were combined into a "**double stapled**" structure



SBL-PX1-206

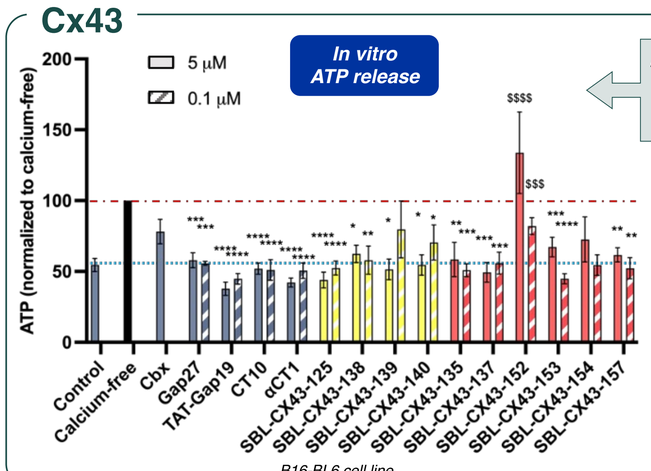
The addition of a **solubilizing tail** (H-K-Ahx) led to the generation of a proteolytically stable, water-soluble bicyclic peptide

The **α**-helical secondary structure is stabilized



BIOLOGICAL ACTIVITY

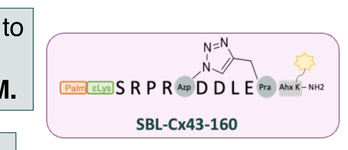
Cx43

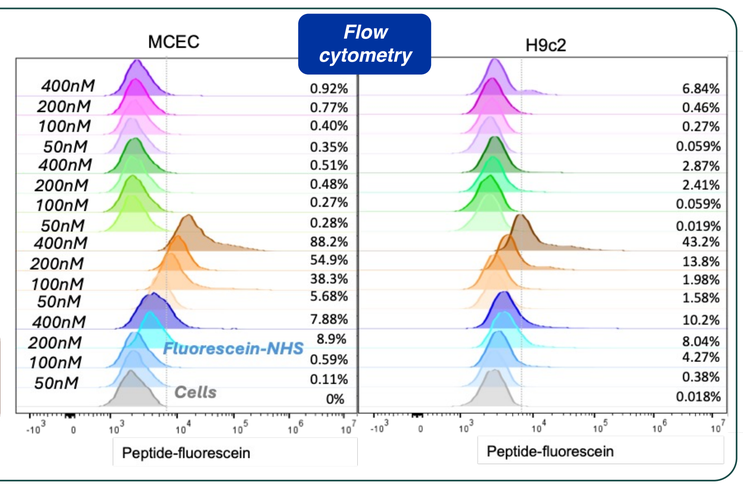


At **5 μM**, all the cyclic peptides have an inhibitory activiity comparable to that of **αCT1** without a CPP.
SBL-Cx43-125 retains its activity at a concentration as low as **100 nM.**

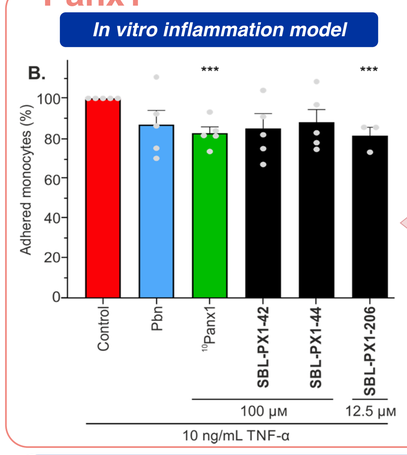
The **length** of the linker affects activity of the lipidated peptides **SBL-Cx43-157** shows a higher inhibitory capacity compared to the peptide based on **CT10 (SBL-Cx43-135).**

The cyclic, lipidated peptide was linked to a **cardiac endothelium-targeting peptide**[7] and binding was tested by flow cytometry together with two control peptides in different cardiac cell lines. **SBL-Cx43-158** binds preferentially to a mouse cardiac endothelial cell line (MCEC).





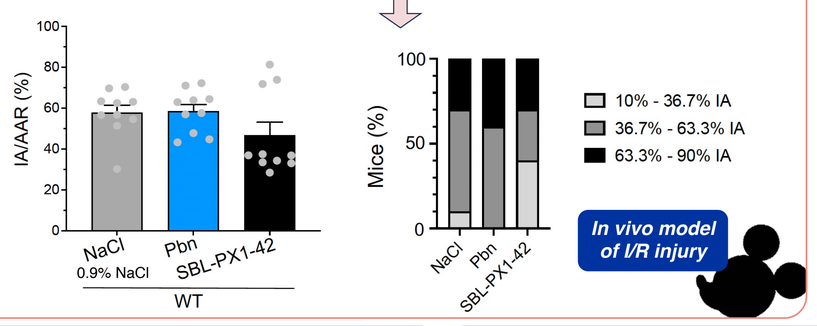
Panx1



SBL-PX1-42 and **SBL-PX1-44** reduce the adhesion of THP-1 monocytes to a TNF-α-activated endothelial monolayer by about 20% at 100 μM, similarly to **10Panx1**. The same effect was reached by the water-soluble "double stapled" **SBL-PX1-206** at **12.5 μM**.

SBL-PX1-42 was tested in an *in vivo* mouse model of I/R injury. 30 min ischemia followed by 24h reperfusion was induced in WT and Panx1-/- mice by ligation of the left anterior descending coronary artery. 90μM **SBL-PX1-42** was injected intravenously at 5 min before reperfusion.

SBL-PX1-42 decreased the infarct size by 40% in 7 out of 10 WT mice. No reduction in infarct size was observed in Panx1-/- mice.



PLASMA STABILITY

CT10	t½ = 75.86 ± 4.01 min

The half-life of **SBL-Cx43-125** largely **exceeds 24h**

10Panx1	t½ = 2.27 ± 0.11 min
SBL-PX1-42	t½ = 66.13 ± 0.52 min
SBL-PX1-44	t½ = 62.42 ± 2.51 min

For **SBL-PX1-206**, after **24h**, **20%** of the intact peptide is still intact in the blood plasma. The only metabolite is the peptide without the solubilizing tail. The stability of the bicyclic structure **exceeds 24h**.

CONCLUSIONS

- **SBL-Cx43-125** is a potent and proteolytically stable inhibitor of Cx43 HC activity *in vitro*, showing an activity comparable to that of **αCT1** at submicromolar concentrations **without requiring a CPP.**
- The designed **solubilizing linkers** allowed to obtain **water-soluble lipidated peptides** of increased lipophilicity, which might be favourable for *in vivo* bioavailability.
- The presence of an endothelial cell-targeting motif in **SBL-Cx43-158** increased the binding of this peptide to MCECs compared to the cardiomyocyte-like cell line H9c2.
- Cyclic peptides **SBL-PX1-42** and **SBL-PX1-44** **reduce monocyte adhesion to the endothelium** (the first step in the inflammatory cascade) by 20%, similarly as 10Panx1, with a 30-fold **longer half-life**.
- The combination of the staples in the peptides led to **SBL-PX1-206**. This bicyclic peptide reaches a similar anti-inflammatory activity *in vitro* at **low concentration (12.5 μM).**
- Preliminary *in vivo* results on **SBL-PX1-42** show its ability to **decrease infarct size by 40%** in 7 out of 10 WT mice in an acute model of **myocardial I/R injury.**

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