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Development of an *in vitro* assay for protein-phosphatase 1

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Background:

A newly identified element of the β-adrenergic signaling pathway is the specific protein phosphatase-1 inhibitor-1 (I-1). I-1 acts as a conditional amplifier of β-adrenergic signaling downstream of PKA by inhibiting type-1 phosphatases only in its PKA-phosphorylated form. I-1 is like β1-adrenoceptors downregulated in failing hearts and presumably contributes to protect against excessive catecholamine levels in heart failure. Disruption of the I-1 gene results in protection from catecholamine induced lethal arrhythmias and hypertrophy (El-Armouche et al. Cardiovasc Res 2008). Therefore, we postulated that a therapeutic window could be downstream in the β-adrenergic signaling cascade, in which interventions could extend extracellular (classical beta-blocker) with "intracellular beta-blockade". In this project, we aimed to develop a reliable *in vitro* assay and have screened diverse libraries of chemical compounds that may have inhibitory effects on I-1 activity.

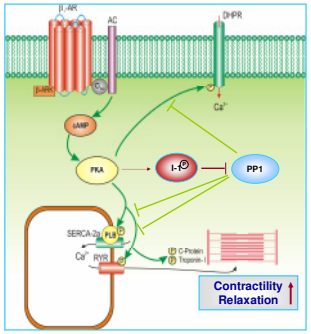

Figure 1: Scheme of inhibitor-1 (I-1) as an amplifier of β-adrenergic signaling.

Methods & Results:

Fluorescence assay

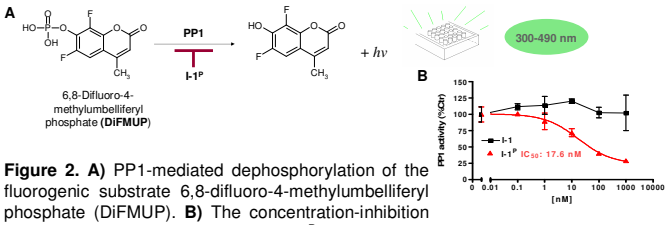

Figure 2. A) PP1-mediated dephosphorylation of the fluorogenic substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP). **B)** The concentration-inhibition curve of I-1 (non-active) vs. I-1^P (active) on recombinant PP1.

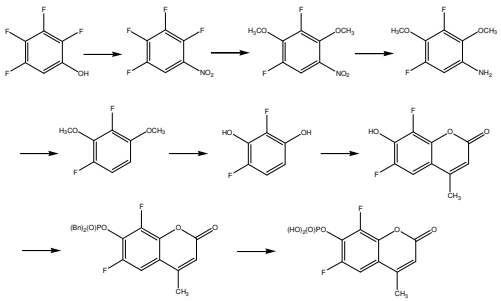

Figure 3. Synthesis procedure of DiFMUP, applicable in g quantities

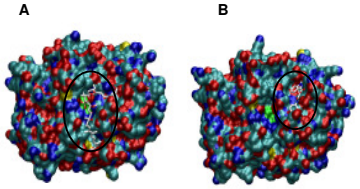

Figure 4. Bioinformatic evaluation of libraries of chemical structures for single molecules that most likely bind to PP1c. **A)** Virtual screening for compounds, which may interact with the active site of PP1, using calyculin-A as reference structure (circle). **B)** The localization (circle) of each compound in the acidic groove and evaluation of potential compounds that bind to PP1.

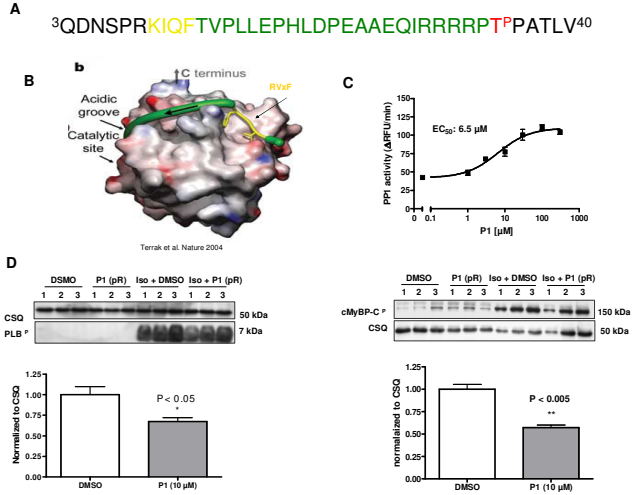

Figure 5. The synthetic peptide of I-1. **A)** The N-terminal sequence of I-1 around the KIQF binding motif (yellow) and inhibitory P-Thr 35 (red). **B)** The crystal structure of PP1. I-1 may follow the C-terminal path from RVxF motif (yellow) through acidic groove to position the inhibitory P-Thr 35. **C)** The effects of various concentrations of the peptide containing KIQF motif (P1) in presence of I-1^P (50 nM). **D)** Western blot analysis representative of the effect of P1-poly arginines on phosphorylation level of PLB, & cMyBP-C, in neonatal rat cardiomyocytes in the presence of 10 nM isoprenalin.

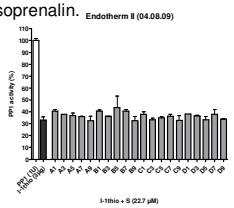

Figure 6. Screening of a diversified library of 300 compounds, selected according to their ADMET profile in accordance with "Lipinski-rules of 5" have not shown a significant effect

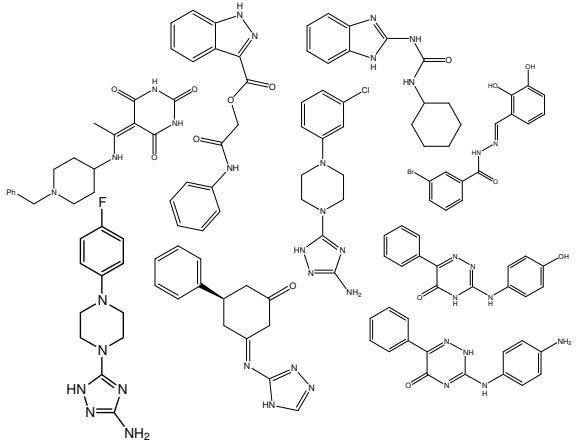

Figure 7. Synthesized compounds which resulted from bioinformatics studies

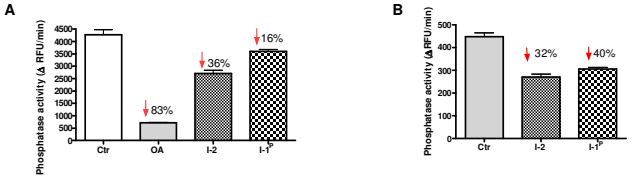

Figure 8. Determination of endogenous PP1 activity in mouse heart homogenate using phosphatase inhibitors. **A)** The total phosphatase activities were measured after preincubation of heart homogenates with okadaic acid (OA, 10 nM) in a concentration specific for PP2A (+ PP4) or with the PP1 specific inhibitors I-2 (1 µM) and I-1^P (1 µM). **B)** The inhibitory effects of I-2 and I-1^P on heart homogenate after preincubation with OA (10 nM).

Conclusion:

- We have developed an appropriate fluorescence protein phosphatase-inhibition assay, and screened various compound libraries, in turn generated by their chemical diversity (including a promising ADMET profile), and by bioinformatics studies.
- The synthetic peptide of I-1 containing the PP1c binding motif prevented I-1^P inhibitory effect on PP1 activity with an EC_{50} value of 6.5 µM. Hence, the peptide attenuated the phosphorylation levels of PLB and cMyBP-C in the presence of 10 nM isoprenalin in neonatal rat cardiomyocytes.
- None of the libraries consisting of non-peptide small molecules, neither those selected by their diversity nor those obtained according bioinformatics studies have shown a significant effect.
- The fluorescence assay, using okadaic acid in a PP2A –specific concentration and specific inhibitors I-2 & I-1^P enabled us to quantify PP1 activity in the tissue/cell extracts.

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