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Identification and *in vitro* characterisation of a novel inhibitor of vascular calcification

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1 Introduction

Loss of mineral homeostasis may lead to **vascular calcification (VC)**, which is highly prevalent in chronic kidney disease and associated with **major adverse cardiovascular events**[1]. Presently, there are no pharmacological therapies approved for the **prevention or treatment** of VC[2]. Circulating **calciprotein particles** (CPPs) are recognised as a marker for mineral stress[3]. Therefore, we aimed at:

1. Synthesizing and screening a series of inositol phosphates as novel inhibitors of VC.
2. Investigating hit molecules for markers of *in vitro* efficacy and metabolic stability.

2 Materials and Methods

Multi-step syntheses Starting from protected *myo*-inositol species, PEGylation reaction followed by deprotection and phosphorylation/sulfation of the free hydroxyls were employed to afford a library of novel inositol derivatives.

Screening assay A serum calcification propensity assay was adapted for compound screening needs in a 96-well plate set-up[4]. In brief, human serum was spiked with 10 mM Ca^{2+} and 6 mM PO_4^{3-} to induce CPP formation. Increasing concentrations of compounds were added, and the time required for primary CPPs to develop into larger, crystalline secondary CPPs, denoted T_{50} (min), was detected by time-resolved changes of light scattering at 37°C.

***In vitro* characterization** Compounds were incubated with 3-phytase at pH 7.4 and 37 °C. PO_4^{3-} hydrolysis was monitored via formation of a phosphomolybdate malachite green complex.

Human vascular smooth muscle cells (VSCMs) were incubated with either 50 µg/mL CPPs (referred to Ca^{2+} content)[5] or 2.7 mM Ca^{2+} and 2.5 mM PO_4^{3-} supplemented medium (CaP), with and without increasing concentrations of INS or IP6. Calcification was assessed qualitatively by Alizarin Red S (ARS) staining and quantitatively by the *o*-cresolphthalein method and normalized to total protein. VSMC viability was assessed by the MTS assay.

Statistics Level of significance was calculated by ordinary one-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3 IP4-di-PEG2 (INS-3001) efficiently prevents serum calcification propensity

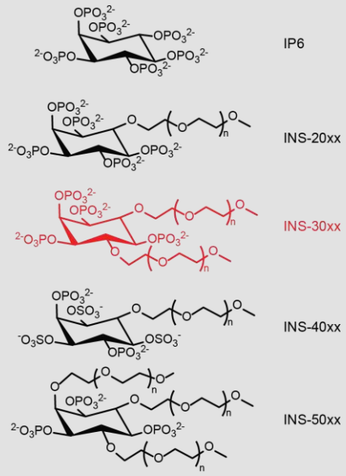

Fig. 1. INS compound series legend: xx=01: n=1; xx=31: n=6 and xx=41: n=10.

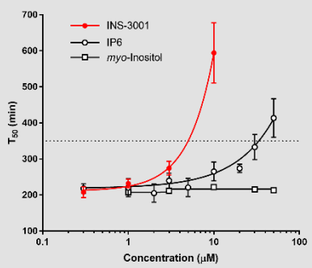

Fig. 2. The compounds' activity was defined as the concentration necessary to delay T_{50} to 350 min (dotted line). *Myo*-inositol (no phosphate, no PEG) did not delay serum calcification. IP6 demonstrated modest activity. High efficacy was achieved by fine-tuning degree of phosphorylation and PEGylation. IP4-di-PEG2 (INS-3001) was superior to all investigated molecules in delaying serum calcification propensity.

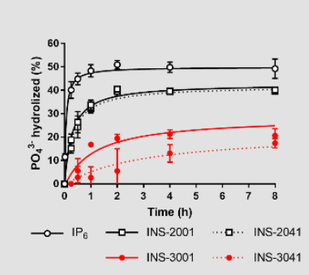

Fig. 3. Phytase degradation of compounds. The protective effect increased with number, but not with length of functionalized PEG.

Table 1. INS compound ranking in the calcification propensity screening assay.

Compound	Concentration (mg/L) at T_{50} = 350 min mean ± s.d. (n)
INS-2001	7.1 ± 2.4 (3)
INS-2031	7.6 ± 1.0 (3)
INS-2041	6.8 ± 0.9 (3)
INS-2042	6.6 ± 0.9 (5)
INS-3001	4.3 ± 0.7 (6)
INS-3031	6.0 ± 0.6 (3)
INS-3041	6.3 ± 0.6 (3)
INS-4001	9.8 ± 1.6 (4)
INS-4031	40.0 ± 4.8 (3)
INS-4041	22.9 ± 2.3 (5)
INS-5001	5.4 ± 0.7 (3)
INS-5031	14.3 ± 1.0 (3)
IP6	30.9 ± 8.7 (26)

4 INS-3001 rescues VSMCs from CPP or CaP-induced calcification

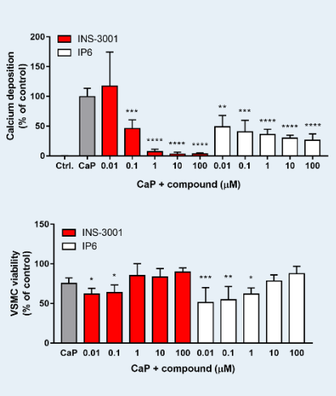

Fig. 4. CaP medium induced VSMC calcification. INS-3001 significantly and dose dependently prevented calcification thereof. Data are expressed as mean ± s.d. from n = 3 independent experiments.

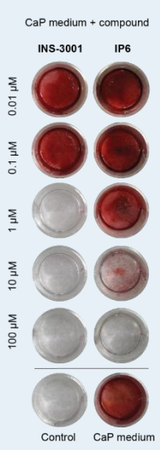

Fig. 5. ARS staining of cells in a 24-well plate

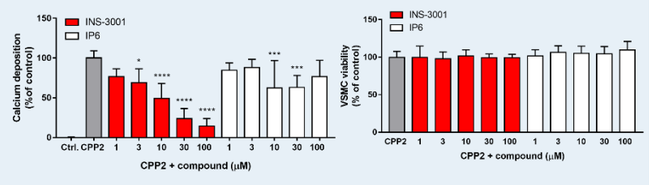

Fig. 6. CPP2 (50 µg/mL) induced VSMC calcification. INS-3001 significantly and dose dependently prevented calcification of cell monolayers. Data are expressed as mean ± s.d. from n = 3 independent experiments.

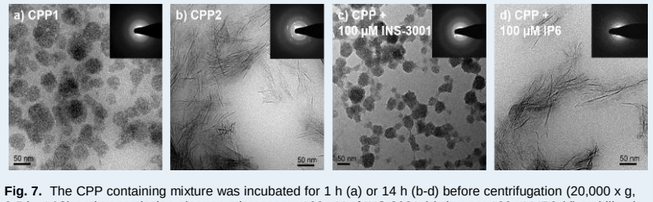

Fig. 7. The CPP containing mixture was incubated for 1 h (a) or 14 h (b-d) before centrifugation (20,000 x g, 2.5 h, 4 °C) and transmission electron microscopy. 100 µM of INS-3001 (c), but not 100 µM IP6 (d) stabilized CPPs in the primary, amorphous state. Crystalline material was evident from selected area diffraction analysis only in samples CPP2 (b) and CPP + 100 µM IP6 (d).

5 Conclusion

The herein presented data provides support for the further development and potential clinical utility of **INS-3001** as an **inhibitor of VC**.

- Compound screening revealed IP4-di-PEG2 (INS-3001) to be almost 10-fold more potent than IP6 in delaying human serum calcification propensity, with **activity in the low µM range**.
- **VSMC calcification** was largely **abolished** by 1 µM INS-3001 in the CaP setup (Fig. 4.), and 30 µM INS-3001 in the CPP setup (Fig. 6.), respectively.
- TEM analysis demonstrated that INS-3001 potently **stabilizes CPP** in their small, amorphous and potentially less pathogenic form.

6 Outlook

Study the effect of INS-3001 on expression of osteoblast markers in calcifying VSMCs.

Further investigate the molecule's mechanism of action.

References: [1]Vervloet & Cozzolino, Kidney Int., 2017; [2]Schantl et al., Adv. Therap., 2018; [3]Kuro-O, Nat. Rev. Nephrol., 2013; [4]Cai et al., Calcif Tissue Int, 2017; [5]Pasch et al., J. Am. Soc. Nephrol., 2012;
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