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A phytic acid analog INS-3001 prevents ectopic calcification in an *Abcc6*^{-/-} mouse model of pseudoxanthoma elasticum

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Abstract

Pseudoxanthoma elasticum (PXE), a prototype of heritable ectopic calcification disorders, affects the skin, eyes, and the cardiovascular system due to inactivating mutations in the *ABCC6* gene. There is no effective treatment for the systemic manifestations of PXE. In this study, the efficacy of INS-3001, an analog of phytic acid, was tested for inhibition of ectopic calcification in an *Abcc6*^{-/-} mouse model of PXE. In prevention study, *Abcc6*^{-/-} mice, at 6 weeks of age, the time of onset of ectopic calcification, were treated with INS-3001 with 0.16, 0.8, 4, 20 or 100 mg/kg/day administered by subcutaneous implantation of osmotic pumps, as well as 4 mg/kg/day by subcutaneous injection thrice weekly or 14, 4, and 0.8 mg/kg/day once weekly subcutaneous injection. Mice were necropsied at 12 weeks of age. Histologic examination and quantitative calcium assay revealed that mice receiving 6 weeks of continuous INS-3001 administration via osmotic pumps showed dose-dependent inhibition of muzzle skin calcification with complete response at 4 mg/kg/day and a minimum effective dose at 0.8 mg/kg/day. INS-3001 plasma concentrations were dose-dependent and largely consistent during treatment for each dose. Thrice weekly and once weekly subcutaneous injections of INS-3001 also prevented calcification. In established disease study, 12-week-old *Abcc6*^{-/-} mice with extensive calcification were continuously administered INS-3001 at 4 mg/kg/day for a follow-up of 12 weeks. INS-3001 treatment was found to stabilize existing calcification that had developed at start of treatment. These results suggest that INS-3001 may provide a promising preventive treatment strategy for PXE, a currently intractable ectopic calcification disorder.

Keywords

animal model; ectopic calcification; phytic acid; INS-3001; pseudoxanthoma elasticum

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AUTHORS' CONTRIBUTIONS

IJ and DL carried out experiments. MI, JU, and QL designed the research study. IJ and QL analyzed the data. QL wrote the paper. All authors reviewed the manuscript and approved the version to be published.

CONFLICT OF INTEREST

MI is an inventor of patents licensed to Inositec Inc. and a shareholder thereof. INS-3001 is in development by Inositec Inc. The other authors have declared no conflicting interests.

1. BACKGROUND

Pseudoxanthoma elasticum (PXE) is a heritable multisystem ectopic calcification disorder which manifests with profound deposition of calcium phosphate complexes in the extracellular matrix of connective tissues in several organs. The disease presents with late-onset, progressive development of clinical manifestations in the skin, eyes, and cardiovascular system.¹⁻³ Mineral deposition in the skin is primarily of aesthetic concern, but may portend development of serious eye and cardiovascular problems, causes of significant morbidity and occasional mortality. The classic forms of PXE are caused by loss-of-function mutations in the *ABCC6* gene encoding a putative efflux transporter protein ABCC6.⁴⁻⁶ *ABCC6* mutations can also cause generalized arterial calcification of infancy (GACI) type 2^{7, 8}, a severe early-onset vascular calcification disorder that affects the fetus *in utero* or in infancy. While the substrate of ABCC6 is unknown, recent studies demonstrated that insufficient plasma levels of inorganic pyrophosphate, a potent anti-calcification factor, underlies the pathophysiology in PXE.^{1, 9, 10} Despite significant progress in understanding the disease pathogenesis, there are no effective treatments for the systematic manifestations of PXE.

Phytic acid (*myo*-inositol hexakisphosphate; IP6) is the phosphate ester of inositol, a naturally occurring substance found in nuts and seeds, known as a protective agent against pathological calcification.¹¹⁻¹⁴ To overcome the poor pharmacokinetics of IP6¹⁵, a series of inositol phosphate analogs were synthesized from *myo*-inositol, through controlled derivatization with oligo (ethylene glycol) chains, sulfate groups and/or phosphate groups, and subsequently screened for their ability to inhibit calcification. INS-3001 was found to be superior to all investigated compounds in delaying human serum calcification propensity, almost 10-fold more potently than IP6.¹⁶ *In vitro* assays also demonstrated that INS-3001 rescued human vascular smooth muscles cells from calciprotein and calcium phosphate-induced calcification.¹⁶ INS-3001 displayed an almost 10-fold longer plasmatic half-life compared to IP6 after intravenous bolus injection in rats (78 ± 32 min vs. 8 ± 2 min, respectively). INS-3001 inhibited vitamin D3-induced and adenine diet-induced cardiovascular calcification in Sprague Dawley rats with favorable pharmacokinetic and safety profiles.¹⁶ Although inhibition of induced vascular calcification *in vitro* and *in vivo* was demonstrated for INS-3001, its efficacy on spontaneous tissue calcification has yet to be demonstrated.

1.1. Question addressed

In this study, we investigated the inhibitory effects of INS-3001 on spontaneous connective tissue calcification in an *Abcc6*^{-/-} mouse model of PXE.

2. EXPERIMENTAL DESIGN

Abcc6 knock-out mice (*Abcc6*^{-/-}) on C57BL/6J background were generated as described previously.¹⁷ C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME) were used as wild-type controls (WT). In prevention study, 6-week-old *Abcc6*^{-/-} mice were administered with INS-3001 by periodic subcutaneous injections or continuous administration via

subcutaneous ALZET osmotic pump model 2006 (DURECT Corp, Cupertino, CA) for duration of 6 weeks (Table 1). In established disease study, 12-week-old *Abcc6*^{-/-} mice were administered INS-3001 at 4 mg/kg/day via osmotic pumps for 12 weeks (Table 1). All mice were maintained on a standard diet and sacrificed at 12 weeks of age, 6 weeks after initiation of treatment (prevention study), or at 24 weeks of age, 12 weeks after initiation of treatment (established disease study). All protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Muzzle skin biopsies (left side) from euthanized mice were collected and fixed in 10% phosphate-buffered formalin and embedded in paraffin. Paraffin sections (6 μ m) were stained with Hematoxylin and Eosin or von Kossa using standard procedures. To quantify the mineral deposition, muzzle skin biopsies (right side) were harvested and decalcified with 1.0 M HCl for 48 hours at room temperature. Solubilized calcium was then determined by a colorimetric assay.^{18, 19} The values were normalized to tissue weight. Whole blood was collected by retro-orbital bleeding or cardiac puncture. For subcutaneously injected mice, whole blood was collected 30 minutes after injection of INS-3001. Whole blood was collected at 2 days, 3 weeks, and 6 weeks after treatment initiation in mice receiving INS-3001. The concentrations of INS-3001 in heparin plasma were analyzed on an ultra-fast liquid chromatography, as described previously.¹⁶ The data were analyzed using Kruskal-Wallis nonparametric test. Statistical significance was reached with $P < 0.05$.

3. RESULTS

Prevention study: Continuous delivery of INS-3001 in young *Abcc6*^{-/-} mice

The *Abcc6*^{-/-} mice were administered INS-3001 by subcutaneous implantation of osmotic pumps at doses of 0.16, 0.8, 4, 20, and 100 mg/kg/day. The untreated wild-type and *Abcc6*^{-/-} mice served as negative and positive controls of ectopic calcification. Treatment was initiated at 6 weeks of age, a time point at the earliest stages of ectopic calcification in the *Abcc6*^{-/-} mice.¹⁷ The extent of ectopic calcification after 6 weeks of treatment was analyzed by two independent assays measuring calcification in the dermal sheath of vibrissae in muzzle skin, an early and reliable biomarker in this mouse model of PXE.¹⁷ One piece of muzzle skin was processed for semi-quantitative histological evaluation. A calcium-specific stain, von Kossa staining, revealed robust calcification in the dermal sheath of vibrissae in the untreated *Abcc6*^{-/-} mice at 12 weeks of age. The *Abcc6*^{-/-} mice receiving INS-3001 at concentrations at and above 4 mg/kg/day showed little, if any, calcification in the muzzle skin (Fig. 1A). There is no dose correlation with INS-3001 at the range of 4–100 mg/kg/day, as the lowest dose of 4 mg/kg/day completely prevented ectopic calcification to the same extent as the highest dose of 100 mg/kg/day (almost 100% inhibition). Significantly reduced calcification was noted at 0.8 mg/kg/day; by contrast, 0.16 mg/kg/day showed no effects on prevention of muzzle skin calcification (Fig. 1A). The therapeutic effects of INS-3001 were substantiated by the calcium content in the other muzzle skin biopsy measured using a quantitative chemical assay (Fig. 2A). The results showed that the levels of calcium in the INS-3001 treated *Abcc6*^{-/-} mice at 4 mg/kg/day and above were similar to those of WT mice devoid of ectopic calcification (Fig. 2A). The partial inhibition of ectopic calcification at 0.8 mg/kg/day was corroborated by calcium

assay corresponding to 59% inhibition (Fig. 2A), thus 0.8 mg/kg/day was identified as the minimum active dose.

INS-3001 was undetectable in plasma of untreated *Abcc6*^{-/-} mice. Administration of INS-3001 at doses from 0.16 to 100 mg/kg/day resulted in dose-dependent plasma concentrations (Fig. 2B). In addition, the levels of INS-3001 remained largely constant at 2 days, 3 weeks, and 6 weeks after implantation of the osmotic pump (Fig. S1), attributed by the nature of the consistent and continuous release of INS-3001 via osmotic pump.

Prevention study: Intermittent subcutaneous injections of INS-3001 in young *Abcc6*^{-/-} mice

INS-3001, when delivered continuously by osmotic pump at 4 mg/kg/day, completely prevented ectopic calcification in the muzzle skin of *Abcc6*^{-/-} mice. We next examined whether periodic, bolus subcutaneous injections of INS-3001 at the same dose of 4 mg/kg/day, would have similar therapeutic benefits. Six-week-old *Abcc6*^{-/-} mice were administered INS-3001 by subcutaneous injections three times per week corresponding to 4 mg/kg/day. These mice showed significantly reduced calcification in the muzzle skin 6 weeks after start of treatment but residual calcification was still evident (Fig. 1A), corresponding to 63% inhibition (Fig. 2A). Thus, subcutaneous injection of INS-3001 is effective, albeit less than with continuous delivery at the same dose of 4 mg/kg/day on inhibition of ectopic calcification.

Three additional dose levels of INS-3001 at weekly subcutaneous injections for a duration of 6 weeks were tested to evaluate the inhibitory effects of INS-3001 (Fig. 1A). The efficacy of weekly injection at 14 mg/kg/day was similar to 4 mg/kg/day three injections per week, suggesting that transient increase of plasma levels of INS-3001 might be beneficial. The *Abcc6*^{-/-} mice receiving INS-3001 injection at weekly frequency of 4 mg/kg/day showed significantly reduced calcification, similar to the effects with the same dose but at frequency of three injections per week. Weekly injection of INS-3001, at dose as low as 0.8 mg/kg/day, also significantly reduced ectopic calcification, suggesting that INS-3001 is a potent inhibitor of ectopic calcification with pharmacological activity that is maintained over days. The semi-quantitative histologic evaluations of INS-3001 treatment were consistent with the calcium contents in the muzzle skin measured by a chemical assay (Fig. 2A).

The T_{\max} of INS-3001 in plasma after subcutaneous administration in Sprague Dawley rats was reported to be about 30 minutes after injection¹⁶, we therefore measured INS-3001 concentrations in plasma 30 minutes after injection in the *Abcc6*^{-/-} mice. INS-3001 levels in plasma 30 minutes after injection were much higher than that in the pump administered groups, as expected for a bolus administration (Fig. 2B). INS-3001-mediated prevention of ectopic calcification was more effective with continuous delivery reaching steady-state plasma concentrations, whereas bolus injections resulted in transient increase of INS-3001 concentrations but was still significantly potent when administered periodically.

Established disease study: Continuous delivery of INS-3001 in old *Abcc6*^{-/-} mice

In established disease study, *Abcc6*^{-/-} mice were aged to 12 weeks, the time at which ectopic calcification was extensive in the muzzle skin containing vibrissae (Fig. 1B).

INS-3001 was administered at 4 mg/kg/day by osmotic pump, a dose and delivery route that resulted in a complete response in the young mice (Fig. 1A). The degree of ectopic calcification was evaluated 12 weeks later. Histopathology and calcium assay of the muzzle skin in the INS-3001 treated mice demonstrated calcification similar to 12-week-old *Abcc6*^{-/-} control mice (Fig. 1, 2A). These results demonstrated that INS-3001, for a total of 12 weeks of follow-up, stabilized existing calcification and stopped the further growth of calcification.

4. CONCLUSIONS

Our results demonstrated that INS-3001 treatment prevented muzzle skin calcification in the *Abcc6*^{-/-} mice with concurrent increases in its plasma levels, suggesting that pharmacologic therapy with INS-3001 might provide a promising treatment strategy for spontaneous calcification in PXE and GACI type 2, diseases that currently lack preventive or therapeutic options. The mechanism of action of INS-3001 as a new calcification inhibitor are not through chelation of calcium but rather coating of early nidus of calcification to hinder apposition of further mineralization and deposition of hydroxyapatite crystals.¹⁶ This mode of action is similar to that of the natural compound IP6, which was recently reported to stop the progression, albeit without regression, of cardiovascular calcifications in patients on hemodialysis following a 1-year treatment.²⁰ INS-3001 arrested further deposition of mineral at the point when the treatment was initiated, suggesting that if INS-3001 is to be used to treat patients with PXE, it should be started as soon as the diagnosis is made. In contrast to chaperone-based allele-specific therapies^{21, 22}, INS-3001 might have the potential to treat PXE regardless of the types of mutations in *ABCC6*. Compared to pyrophosphate and bisphosphonates, INS-3001 has high subcutaneous bioavailability, good local tolerability, high potency and no anti-osteoclastic activity.^{16, 23-27} However, further work is necessary to confirm the compound's clinical potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

| | |
|------------|--|
| PXE | pseudoxanthoma elasticum |
| IP6 | phytic acid, myo-inositol hexakisphosphate |

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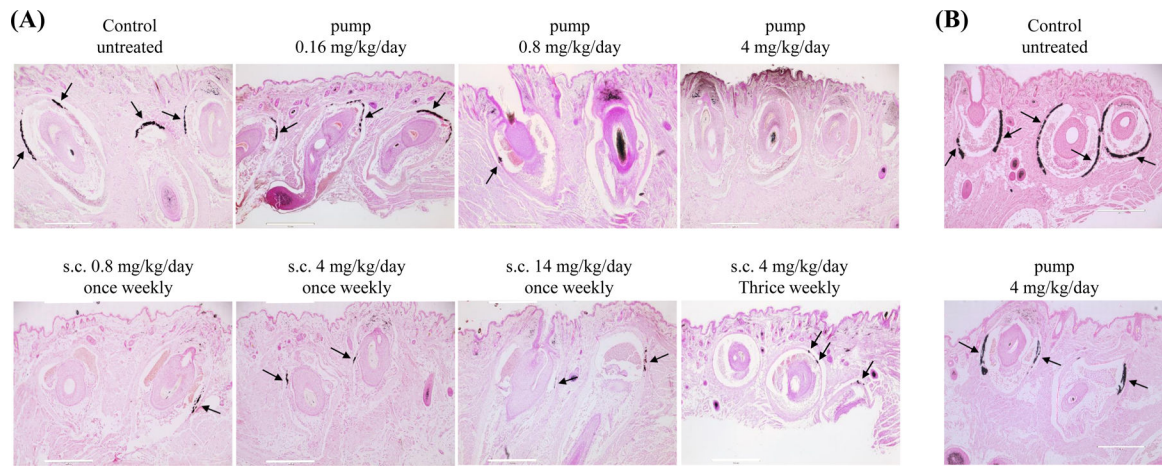


Figure 1. Histopathologic examination of vibrissae calcification in the muzzle skin of *Abcc6*^{-/-} mice in different groups.

(A), Prevention study. All mice were analyzed at 12 weeks of age. von Kossa stains revealed robust calcification in the dermal sheath of vibrissae in untreated *Abcc6*^{-/-} mice. The *Abcc6*^{-/-} mice receiving INS-3001 via osmotic pumps at 4 mg/kg/day had very little, if any, calcification in the vibrissae. The *Abcc6*^{-/-} mice receiving INS-3001 via osmotic pumps at 0.16 mg/kg/day had no effects on calcification in the vibrissae. Osmotic pump delivery of INS-3001 at 0.8 mg/kg/day had evident but significantly reduced calcification. Subcutaneous injections of INS-3001 at different doses and injection frequencies all resulted in decreased calcification. (B), Established disease study. All mice were analyzed at 24 weeks of age. von Kossa stains revealed extensive vibrissae calcification in untreated *Abcc6*^{-/-} mice and INS-3001 treatment prevented further calcification which was stabilized at the time of treatment. Arrows indicate ectopic calcification. Scale bar, 0.4 mm.

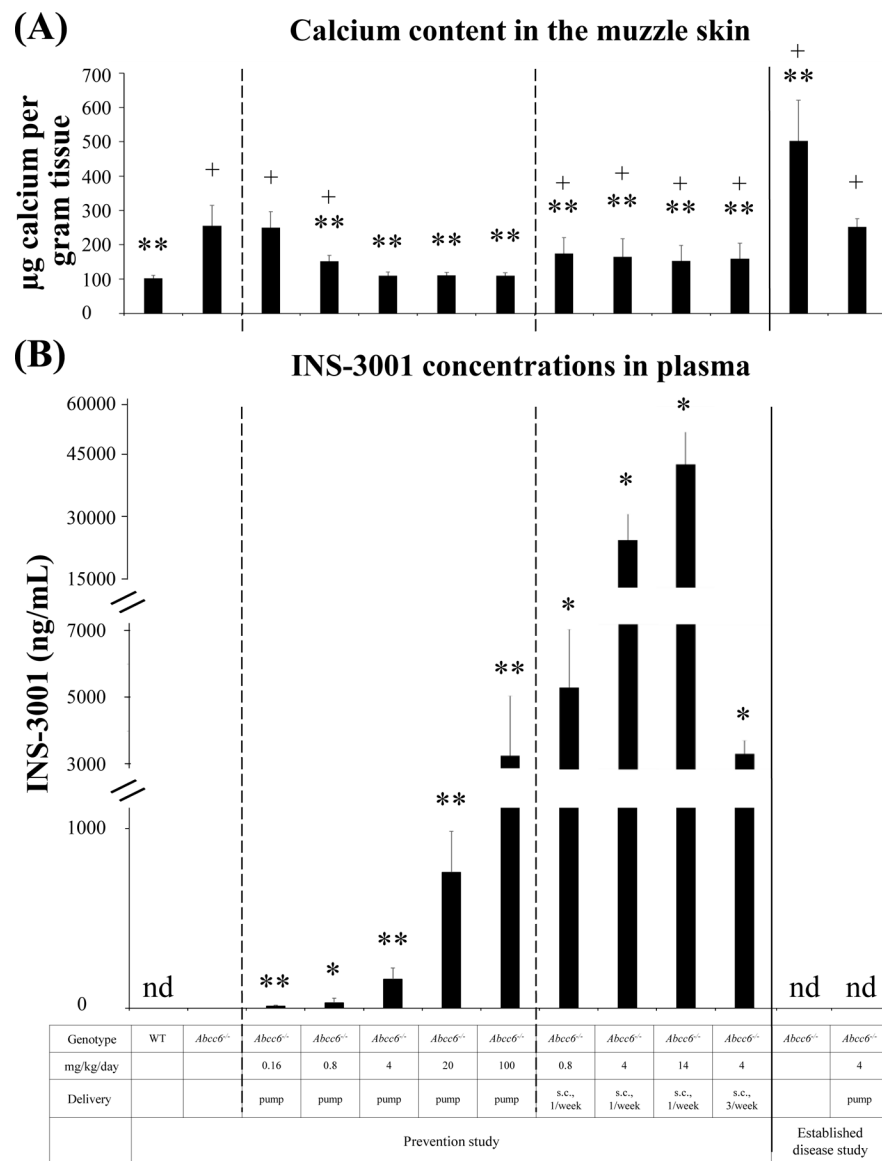


Figure 2. Chemical assay of calcium in muzzle skin biopsies and plasma concentrations of INS-3001 in mice receiving INS-3001 administration. (A), The calcium content in the muzzle skin containing vibrissae. 6–12 mice per group. (B), Plasma concentrations of INS-3001. 5 mice per group. Blood was collected either prior to sacrifice for mice implanted with osmotic pumps or 30 minutes after a subcutaneous injection. Overall, the plasma concentrations scaled relatively well with the dose administered despite variabilities among different groups. The variability may be due to the fact that a single sampling time point was chosen around the anticipated T_{max} of 30 minutes, which leads to greater variability than if a complete pharmacokinetic profile had been obtained. * $P < 0.05$, ** $P < 0.01$, compared to untreated 12-week-old *Abcc6*^{-/-} mice; + $P < 0.01$, compared to untreated WT mice. Data were presented as mean ± SD. nd, not determined.

Table 1.

Experimental groups of mice by genotype and treatment regimen

| Mice | Treatment | Delivery route | Dose (mg/kg/day) [†] | Dose (mg/kg/per injection) [†] | Frequency | No. of mice (F+M) [‡] |
|----------------------------------|-----------|----------------|-------------------------------|---|---------------|--------------------------------|
| <u>Prevention study</u> | | | | | | |
| <i>Abcc6</i> ^{+/+} | – | – | – | – | – | 6+6 |
| <i>Abcc6</i> ^{-/-} | – | – | – | – | – | 5+6 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | Osmotic pump | 0.16 | – | Continuous | 5+5 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | Osmotic pump | 0.8 | – | Continuous | 4+6 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | Osmotic pump | 4 | – | Continuous | 3+3 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | Osmotic pump | 20 | – | Continuous | 5+4 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | Osmotic pump | 100 | – | Continuous | 3+4 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | s.c. injection | 4 | 9 | Thrice weekly | 4+4 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | s.c. injection | 14 | 100 | Once weekly | 5+5 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | s.c. injection | 4 | 28 | Once weekly | 5+5 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | s.c. injection | 0.8 | 5.6 | Once weekly | 5+5 |
| <u>Established disease study</u> | | | | | | |
| <i>Abcc6</i> ^{-/-} | – | – | – | – | – | 5+4 |
| <i>Abcc6</i> ^{-/-} | INS-3001 | Osmotic pump | 4 | – | Continuous | 0+6 |

[†]INS-3001 was administered in sodium salt but the doses refer to the acid form of the compound.

[‡]F, female; M, male; s.c., subcutaneous.