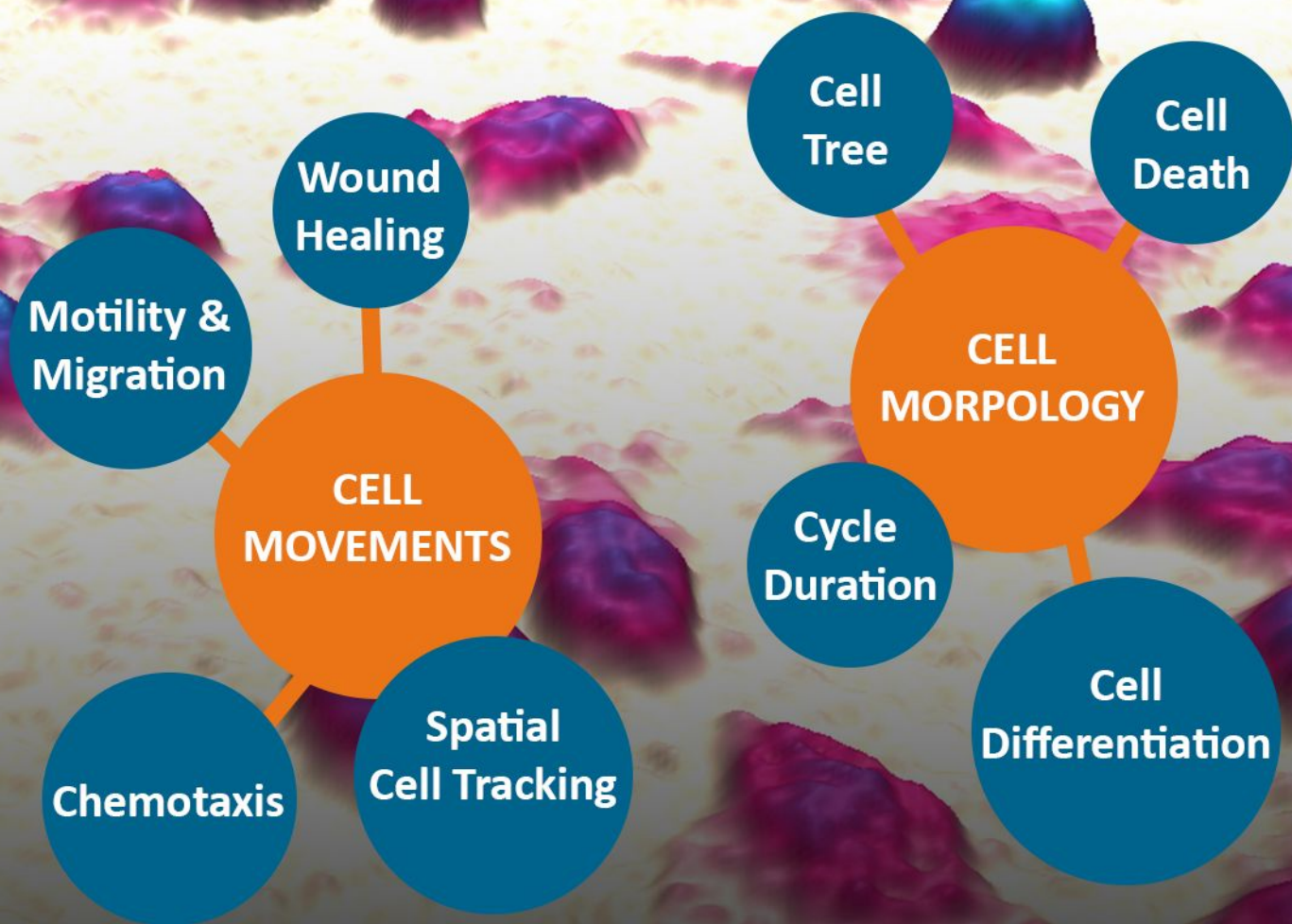


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2-Oxothiazolidine-4-carboxylic acid inhibits vascular calcification via induction of glutathione synthesis

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Abstract

Arterial medial calcification (AMC), the deposition of hydroxyapatite in the medial layer of the arteries, is a known risk factor for cardiovascular events. Oxidative stress is a known inducer of AMC and endogenous antioxidants, such as glutathione (GSH), may prevent calcification. GSH synthesis, however, can be limited by cysteine levels. Therefore, we assessed the effects of the cysteine prodrug 2-oxothiazolidine-4-carboxylic acid (OTC), on vascular smooth muscle cell (VSMC) calcification to ascertain its therapeutic potential. Human aortic VSMCs were cultured in basal or mineralising medium (1 mM calcium chloride/sodium phosphate) and treated with OTC (1–5 mM) for 7 days. Cell-based assays and western blot analysis were performed to assess cell differentiation and function. OTC inhibited calcification $\leq 90\%$, which was associated with increased ectonucleotide pyrophosphatase/phosphodiesterase activity, and reduced apoptosis. In calcifying cells, OTC downregulated protein expression of osteoblast markers (Runt-related transcription factor 2 and osteopontin), while maintaining expression of VSMC markers (smooth muscle protein 22 α and α -smooth muscle actin). GSH levels were significantly reduced by 90% in VSMCs cultured in calcifying conditions, which was associated with declines in expression of gamma-glutamylcysteine synthetase and GSH synthetase. Treatment of calcifying cells with OTC blocked the reduction in expression of both enzymes and prevented the decline in GSH. This study shows OTC to be a potent and effective inhibitor of in vitro VSMC calcification. It appears to maintain GSH synthesis which may, in turn, prevent apoptosis and VSMCs gaining osteoblast-like characteristics. These findings may be of clinical relevance and raise the possibility that treatment with OTC could benefit patients susceptible to AMC.

KEYWORDS

arterial medial calcification, glutathione, reactive oxygen species, vascular smooth muscle cells, 2-oxothiazolidine-4-carboxylic acid

Abbreviations: AMC, arterial medial calcification; CKD, chronic kidney disease; GCL, gamma-glutamylcysteine synthetase; GCLC, glutamate-cysteine ligase catalytic subunit; GSH, glutathione; GSS, glutathione synthetase; LDH, lactate dehydrogenase; NPP, ectonucleotide pyrophosphatase/phosphodiesterase; OTC, 2-oxothiazolidine-4-carboxylic acid; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TNAP, tissue nonspecific alkaline phosphatase; VC, vascular calcification; VSMC, vascular smooth muscle cells.

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1 | INTRODUCTION

Vascular calcification (VC) is a common complication of chronic kidney disease (CKD), diabetes, and atherosclerosis. It is the pathological deposition of the calcium phosphate mineral, hydroxyapatite, in the intimal and/or medial layers of the arterial wall (Durham, Speer, Scatena, Giachelli, & Shanahan, 2018). Arterial medial calcification (AMC), defined as calcification within the tunica media of blood vessels, is associated with arterial stiffness, reduced blood flow, heart failure, and mortality (Vervloet & Cozzolino, 2017). In CKD, coronary artery calcification is six times more prevalent than in the general population (Kramer, Toto, Peshock, Cooper, & Victor, 2005; Roy, Cespedes, Li, Choi, & Budoff, 2011), and is known to contribute to nearly 50% of deaths in patients with end-stage renal disease due to cardiovascular complications (Chen et al., 2017; Parikh et al., 2006). Despite these known adverse effects of AMC, there are currently no specific treatments to prevent or retard calcium deposition. Given that the prevalence of diseases associated with AMC are on the rise (Heidenreich et al., 2011; Hoerger et al., 2015; Rowley, Bezold, Arikian, Byrne, & Krohe, 2017), novel therapies to manage this condition are acutely required.

Currently, the mechanisms leading to AMC are insufficiently understood. However, the traditional concept of passive calcification resulting from elevated phosphate and calcium levels is now known to be false (Mizobuchi, Towler, & Slatopolsky, 2009). Instead, AMC is considered an active cell-mediated process, sharing some outward similarities with bone mineralisation, driven by vascular smooth muscle cells (VSMCs). Mechanisms considered to play a role in VC include loss of calcification inhibitors (e.g., pyrophosphate [PPi], ectonucleotide pyrophosphatase/phosphodiesterase (NPP), fetuin A, and matrix gla protein), gain of calcification inducers (e.g., tissue nonspecific alkaline phosphatase [TNAP]), and apoptosis (Mizobuchi et al., 2009; Patel, Bourne, Davies et al., 2019; Patel, Bourne, Millan et al., 2019). In addition, when maintained in a calcifying environment (elevated calcium and/or phosphate), VSMCs undergoes phenotypic transdifferentiation to gain limited osteoblast-like characteristics (Patel, Bourne, Davies et al., 2019).

Oxidative stress, the imbalance between production and accumulation of reactive oxygen species (ROS), is a known mediator of AMC (Mody, Parhami, Sarafian, & Demer, 2001; Sudo, Sato, Azechi, & Wachi, 2015; Zhao et al., 2011). ROS, such as hydrogen peroxide, have been shown to promote VSMC calcification *in vitro* via VSMC transdifferentiation to an osteoblast-like cell (Byon et al., 2008; Mody et al., 2001). In addition, oxidative stress has been implicated in the induction of VSMC apoptosis (Cui, Li, Chang, Cong, & Hao, 2017), which could play a role in AMC pathogenesis, as mentioned above. Antioxidants, which inhibit ROS and oxidative stress, can protect against VSMC calcification. Plant polyphenols, such as curcumin and silybin, known to have antioxidant properties, have been shown to reverse VSMC calcification *in vitro* by reducing ROS levels and VSMC transdifferentiation (Roman-Garcia, Barrio-Vazquez, Fernandez-Martin, Ruiz-Torres, & Cannata-Andia, 2011). The synthetic vitamin E analogue, Trolox, has also been found to reverse VSMC phenotypic

changes and inhibit calcification *in vitro* as well as *in vivo* using a uremic calcification model (Mody et al., 2001; S. Yamada et al., 2012).

In addition to exogenous antioxidants, cells have evolved mechanisms to counteract localised increases in oxidative stress. One such example is glutathione (GSH), which acts as a reducing agent or binds to electrophiles to neutralise ROS (Lushchak, 2012; Siauquinaite, Foulkes, Calabrò, & Vallone, 2019). Intracellular levels of GSH range from 0.5 to 10 mM and it is the most abundant low-molecular-mass thiol in cells (Lushchak, 2012). Though synthesised in nearly every cell, GSH is prominently produced in the liver. GSH synthesis from glutamate, cysteine, and glycine requires two enzyme reactions, the first (rate-limiting) gamma-glutamylcysteine synthetase (GCL) and, subsequently, glutathione synthetase (GSS). In addition to GCL, another rate-limiting factor for GSH synthesis is cysteine availability (Lu, 2009; Wu, Fang, Yang, Lupton, & Turner, 2004). Prodrugs of cysteine, such as 2-oxothiazolidine-4-carboxylic acid (OTC) and *N*-acetylcysteine have been shown to increase cellular cysteine and GSH levels (Gould & Pazdro, 2019). OTC is converted to cysteine via intracellular 5-oxoprolinase and has been shown to be more effective than *N*-acetylcysteine in replenishing GSH levels (Williamson, Boettcher, & Meister, 1982). In clinical trials, cysteine levels are elevated in HIV patients given OTC at doses up to 100 mg/kg *iv* (Kalayjian et al., 1994). In patients with CKD on peritoneal dialysis, OTC (500 mg orally, every 8 hr for 14 days), increased circulating GSH levels (Moberly et al., 1998). Furthermore, OTC reversed dialysate-induced acceleration of cellular senescence and inflammation in peritoneal mesothelial cells (Sosinska-Zawierucha, Mackowiak, Begier-Krasinska, Hoppe, & Breborowicz, 2018). OTC (63 mg/kg *iv*, every 8 hr for 10 days) also mediated GSH repletion in patients with acute respiratory distress syndrome and led to reductions in acute lung injury (Bernard et al., 1997).

Given CKD and diabetes have been associated with elevated oxidative stress (Dounousi et al., 2006; Seghrouchni et al., 2002), ROS may play a pivotal role in causing AMC in these patients. Therefore, reductions in oxidative stress via intracellular antioxidants, such as GSH, may serve to inhibit or slow calcification progression. Here, we examine the effects of the cysteine prodrug OTC on VSMC calcification and elucidate the potential effects of GSH in AMC.

2 | MATERIALS AND METHODS

2.1 | Reagents

Tissue culture reagents were purchased from Life Technologies (Paisley, UK). Unless otherwise stated, chemicals and reagents were purchased from Sigma-Aldrich (Poole, UK).

2.2 | VSMC calcification assay

Human aortic VSMCs (Lonza, Slough, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine,

supplemented with 10% foetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete mixture abbreviated to DMEM). Passages 5–8 cells were utilised and seeded into 24-well plates at a density of 5×10^4 cells/well. VSMCs were cultured in either control medium (DMEM) or calcifying medium (DMEM + 1 mM calcium chloride and sodium phosphate), for up to 7 days at 37°C in 5% CO₂, with half medium changes taking place every 3–4 days. Cells were treated with 1.5 mM 2-oxo-thiazolidine-4-carboxylate (Alfa Aesar, Heysham, UK), or 1–20 µM l-buthionine-sulfoximine throughout. Stock solutions of 2-oxo-thiazolidine-4-carboxylate and l-buthionine-sulfoximine were prepared in water.

2.3 | Determination of VSMC calcification

To quantify calcification, calcifying VSMCs cultured for 7 days were washed twice with phosphate-buffered saline (PBS), then incubated with 0.6 M hydrochloric acid at room temperature for 24 hr to solubilise deposited calcium. Calcium content was measured colorimetrically by stable interaction with o-cresolphthalein using the Calcium Colorimetric Assay Kit (Sigma-Aldrich), as per manufacturer's instructions. Calcium content was corrected for total protein concentration using the Bradford assay.

For visualisation of calcium deposition, monolayers were fixed with 2.5% glutaraldehyde, and stained for calcium with alizarin red S (1% solution wt/vol in water) for 5 min and rinsed with 50% ethanol to remove the excess stain. In addition to alizarin red, which measures general calcium deposition, the OsteoImage (Lonza, Slough, UK) Mineralisation Assay Kit was also utilised. This assay utilises a fluorescent stain that binds to hydroxyapatite, the calcium-phosphate salt of bone mineral. Assays were performed as per manufacturer's instructions. Briefly, fixed cells were initially washed in wash buffer and incubated with staining reagent for 30 min at room temperature in the dark. Cells were subsequently incubated with wash buffer for 5 min and rinsed; this was repeated three times. After the final wash, wash buffer was added to keep monolayers wet for microscopy. Hydroxyapatite deposition was visualised using an EVOS FL Imaging System (Life Technologies), with a GFP light cube (Ex: 470/22 nm, Em: 510/42 nm).

2.4 | Determination of total TNAP and NPP activity

TNAP enzyme activity was assessed in VSMCs cultured for 7 days, utilising a Colorimetric Assay Kit (Anaspec, Fremont), as previously described (Orriss, Key et al., 2012). Briefly, monolayers were washed in PBS and collected in water using a scraper. Samples were centrifuged at 500g at 4°C, and TNAP activity of the supernatant measured using a Clariostar microplate reader (Aylesbury, UK) set at 405 nm. TNAP activity was normalised to cell protein using the Bradford assay.

Total cellular NPP activity was assessed as previously described (Patel, Bourne, Davies et al., 2019). In brief, VSMCs cultured for

7 days were lysed in a buffer containing 1% Triton X-100 in 0.2 M Tris base with 1.6 mM MgCl₂, pH 8.1. Following centrifugation at 500g at 4°C, NPP activity of collected supernatants was measured on a Clariostar microplate reader set at 405 nm using 5 mM p-nitrophenyl-thymidine 5'-monophosphate as a substrate. The total protein in cell lysates was determined using the Bradford assay.

2.5 | Cell number and viability assay

Cell number and viability were determined at Day 7 of culture using the CytoTox 96® Colorimetric Cytotoxicity Assay (Promega, Southampton, UK), as described previously (Patel, Bourne, Davies et al., 2019). Briefly, cell supernatants were collected to determine medium lactate dehydrogenase (LDH) levels (cell viability); to establish total cellular LDH levels, cells were lysed with 1% Triton X-100 in water for 1 hr. Cell supernatant and lysates were measured as per manufacturer's instructions using a Clariostar microplate reader set at 490 nm. Cell lysate absorbance values were compared to a standard curve of known cell density, allowing quantification of cell number. Cell viability was calculated by expressing medium LDH as a percentage of the total cellular LDH cell.

2.6 | Quantification of apoptosis by flow cytometry

VSMC were cultured in the noncalcifying or calcifying medium for 7 days. Apoptosis was assessed via flow cytometry using an annexin V antibody conjugated to fluorescein (Life Technologies), as per manufacturer's instructions. Briefly, cells were detached using trypsin-ethylenediaminetetraacetic acid (EDTA; 0.05%) and the resultant pellet washed in ice-cold PBS. This suspension was centrifuged and resuspended in 1X annexin-binding buffer (Life Technologies). A sample of this suspension was incubated with the annexin V antibody for 15 min and analysed using a Guava easyCyte 8HT Flow Cytometer (Merck-Millipore, Watford, UK). Data were processed to calculate percentage apoptosis using Flowing Software (version 2.5.1; Turku University, Finland).

2.7 | Western blot analysis

Protein was extracted from VSMCs cultured in control and calcification medium at Days 4 and 7 of culture. Cell monolayers were lysed in ice-cold radioimmunoprecipitation lysis buffer (50 mM tris hydrochloride, pH 7.4, 150 mM sodium chloride, 5 mM EDTA, and 0.1% sodium dodecyl sulfate [SDS]), with protease inhibitor cocktail (Sigma-Aldrich). Cell homogenates were sonicated for 5 min and centrifuged at 10,000 rpm at 4°C, before protein concentration determination using the Bradford assay. Loading samples were prepared using Bolt lithium dodecyl sulfate sample buffer and Bolt sample reducing agent (Life Technologies), as per manufacturer's instructions. Samples were denatured at 70°C for 10 min.

Protein samples were loaded into Bolt Bis-Tris Plus Gels (8%) (Life Technologies), and run alongside a protein ladder (Precision Plus Protein Kaleidoscope Standard; Bio-Rad, Watford, UK). Gel electrophoresis was run at 160 V for 40 min in Bolt MES SDS running buffer (Life Technologies). Protein samples were subsequently transferred onto a nitrocellulose membrane using the iBlot 2 Transfer System (Life Technologies). Membranes were then blocked with 5% nonfat milk for 1 hr, and, subsequently, incubated with Runx-related transcription factor 2 (Runx2), α -smooth muscle actin (ACTA2; Cell Signaling Technology, London, UK), smooth muscle protein 22 α (SM22 α), glutamate-cysteine ligase (glutamate-cysteine ligase catalytic subunit [GCLC]), GSS (Abcam, Cambridge, UK), or osteopontin (OPN; Bio-Rad, Kidlington, UK) antibodies overnight at 4°C. After washing, blots were incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, London, UK/Bio-Rad, Kidlington), for 1 hr at room temperature. A β -actin-HRP conjugate antibody (Abcam) was used as a loading control, with blots incubated for 1 hr at room temperature. A peroxidase detection system (Radiance ECL; Azure Biosystems, Dublin) and myECL Imager (Thermo Fisher Scientific, Hemel Hempstead, UK) were used for the visualisation of immunoreactivity.

2.8 | Measurement of total GSH

Total GSH was assessed in VSMCs cultured in control or calcification medium for 7 days, using a colorimetric assay based on a method by Rahman, Kode, and Biswas (2006). After the removal of culture media, monolayers were washed with PBS and subsequently collected in PBS using a cell scraper. Samples were centrifuged at 1,000g at 4°C for 5 min, after which the pellet was washed in fresh PBS and resuspended. The pellet was resuspended in ice-cold lysis buffer (0.1% Triton-X and 0.6% sulfosalicylic acid in 0.1 M potassium phosphate buffer with 5 mM EDTA, pH 7.5), and homogenised with a Teflon pestle. Lysed cells were subsequently sonicated for 2–3 min

with vortexing every 30 s. Following centrifugation at 3,000g at 4°C for 5 min, total GSH of collected supernatants was measured following addition of 5,5-dithiobis(2-nitrobenzoic acid), GSH reductase, and NADPH using a Clariostar microplate reader set at 412 nm. Absorbance values were compared to a standard curve of known GSH concentration and corrected for total protein concentration using the Bradford assay.

2.9 | Statistical analysis

Data were analysed using GraphPad Prism 7 software (San Diego, CA). Statistical comparisons were made using a *T* test or a one-way analysis of variance with a post hoc Bonferroni correction for multiple comparisons. Results are expressed as mean \pm standard error of the mean for six technical replicates. Results are representative of experiments performed at least three times.

3 | RESULTS

3.1 | OTC inhibits VSMC calcification

VSMCs cultured with calcifying medium (1 mM Ca/P) for 7 days, and stained with alizarin red, showed abundant regions of calcification (Figure 1a). To ascertain whether this deposition was similar in composition to in vivo calcification, calcifying cells were stained with the hydroxyapatite specific fluorescence stain OsteoImageTM. Fluorescence staining (Figure 1e) confirmed calcification of VSMCs as seen with alizarin red.

The addition of OTC to calcifying VSMCs led to a strong inhibition of calcification at concentrations of ≥ 2.5 mM (Figures 1b–d and 1f–h). Quantification of calcium deposition showed up to 90% inhibition in OTC-treated cells, compared to untreated cells (Figure 2).

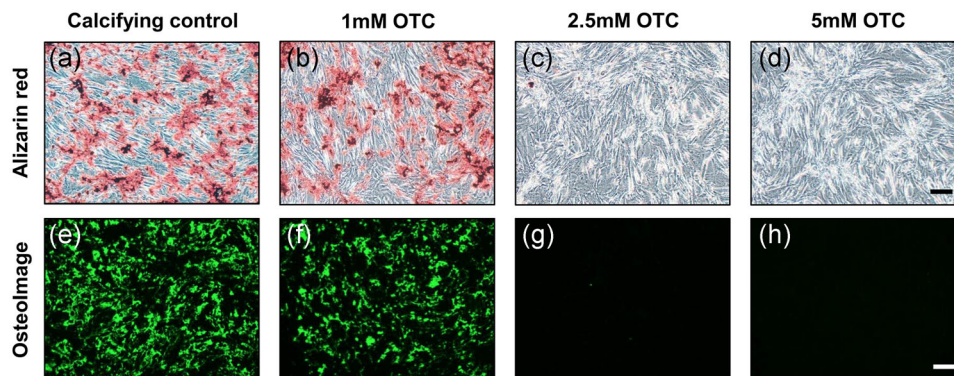


FIGURE 1 OTC inhibits VSMC calcification. The pictures show phase contrast (alizarin red stained) and fluorescent (OsteoImageTM) images of VSMCs after 7 days in culture. Abundant regions of calcification are present in calcium/phosphate treated cells (a), the deposition was confirmed to be hydroxyapatite utilising the specific fluorescent dye (e). Treatment with OTC inhibited calcification with increasing dose (b–d, f–h). Scale bars: Phase contrast and fluorescent microscopy images = 250 μ m. OTC, 2-Oxothiazolidine-4-carboxylic acid

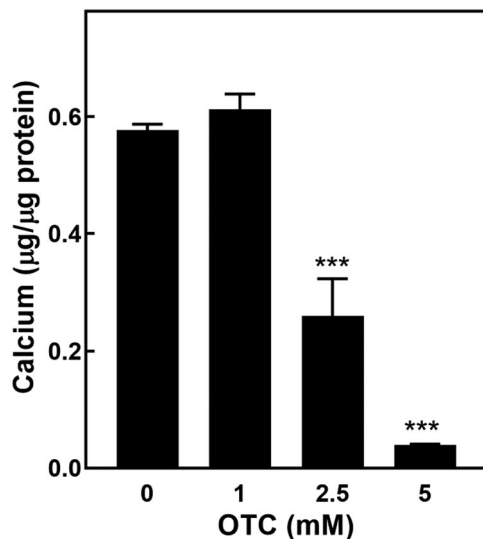


FIGURE 2 Inhibition of VSMC calcification by OTC. Calcium deposition was inhibited concentration dependently by OTC in calcifying VSMCs treated with the drug for 7 days. Values are mean \pm standard error of the mean ($n = 6$ replicate wells), *** $p < .001$. OTC, 2-oxothiazolidine-4-carboxylic acid

3.2 | OTC treatment increased total NPP activity while reducing TNAP activity

PPI, the potent inhibitor of calcification, acts via the prevention of hydroxyapatite formation and crystal growth. The inhibitor is formed by NPP, which hydrolyses nucleotide triphosphates to generate PPI (Orriss, Arnett, & Russell, 2016; Villa-Bellosta & Egidio, 2015). PPI can be degraded to phosphate by TNAP leading to the induction of mineralisation, in addition to the removal of the inhibitor (Villa-Bellosta & Egidio, 2015). To establish whether OTC

affects NPP and/or TNAP activity, calcifying VSMCs were treated with OTC for 7 days and enzyme activity assessed. OTC concentration-dependently enhanced the activity of NPP but decreased that on TNAP by about threefold when compared to respective controls (Figure 3).

3.3 | OTC inhibits apoptosis and enhances cell viability in calcifying VSMCs

OTC concentration-dependently increased cell number by up to 40%, compared to untreated calcifying cells (Figure 4a); this was associated with a rise in cell viability (measured as the percentage of dead cells). OTC treatment reduced the percentage of dead cells by up to 85%, compared to untreated calcifying cells (Figure 4b). To ascertain whether the effects of OTC on cell number and viability were mediated via inhibition of apoptosis, a known factor in the induction of AMC (Proudfoot, 2019), the early apoptosis marker annexin V was assessed utilising flow cytometry. Apoptosis was significantly increased by 75% in calcifying VSMCs when compared to noncalcifying cells. Treatment with OTC (2.5 mM) decreased apoptosis in calcifying cells, reducing this back to control levels (Figure 4b).

3.4 | OTC reverses acquisition of osteoblast-like characteristics in calcifying VSMCs

Previous studies have shown that calcification is associated with VSMCs gaining limited osteoblast-like characteristics (e.g., Runx2 and OPN expression), and reduced expression of VSMC markers (SM22 α and ACTA2) (Patel, Bourne, Davies et al., 2019; Steitz et al., 2001). At Days 4 and 7 of culture, expression of Runx2 and OPN were

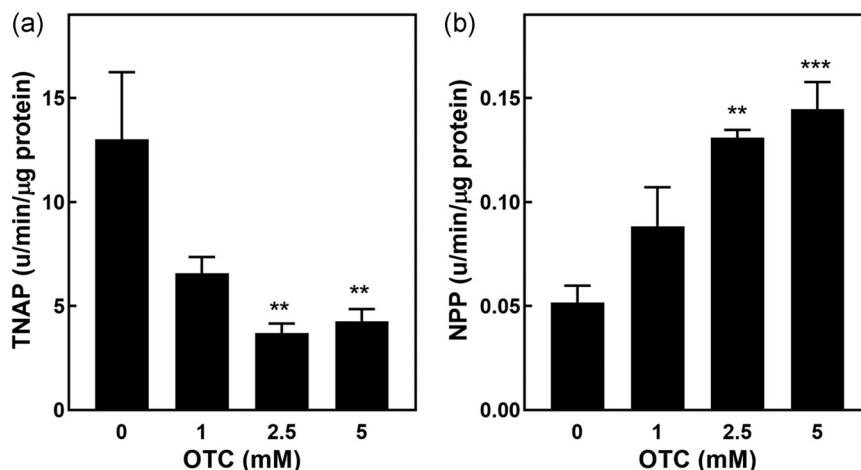


FIGURE 3 Reduced TNAP and increased NPP activity with OTC treatment. Calcifying VSMCs treated with OTC for 7 days exhibited a threefold reduction in TNAP activity (a) and a threefold increase in NPP activity (b) compared to untreated cells. Values are mean \pm standard error of the mean ($n = 6$ replicate wells), *** $p < .001$, ** $p < .01$. NPP, ectonucleotide pyrophosphatase/phosphodiesterase; OTC, 2-oxothiazolidine-4-carboxylic acid; TNAP, tissue nonspecific alkaline phosphatase; VSMC, vascular smooth muscle cell

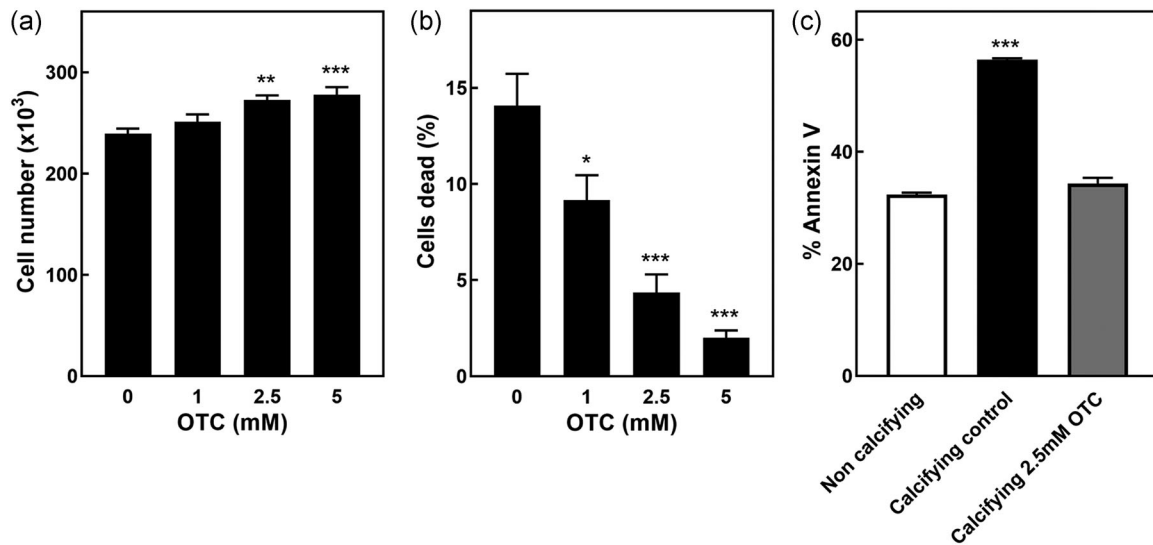


FIGURE 4 OTC increases VSMC number and viability while reducing apoptosis. OTC concentration-dependently increased VSMC number by up to 40% compared to untreated cells (a). Treatment with OTC was associated with an increase in VSMC viability; the percentage of dead cells was up to 85% lower in treated cells compared to untreated (b). The culture of VSMCs in calcifying conditions was associated with a 75% increase in apoptosis when compared to noncalcifying cells. Treatment with OTC (2.5 mM) led to a reduction in apoptosis to levels seen in control noncalcifying cells (c). Values are mean \pm standard error of the mean ($n = 6$ replicate wells), * $p < .05$, ** $p < .01$, *** $p < .001$. OTC, 2-oxothiazolidine-4-carboxylic acid; VSMC, vascular smooth muscle cell

upregulated, while SM22 α and ACTA2 were downregulated in calcifying VSMCs when compared to noncalcifying cells. The addition of OTC (2.5 mM) to calcifying cells reversed these effect, particularly at Day 7, leading to a similar expression of both osteoblast and VSMC markers to those seen in noncalcifying cells (Figure 5).

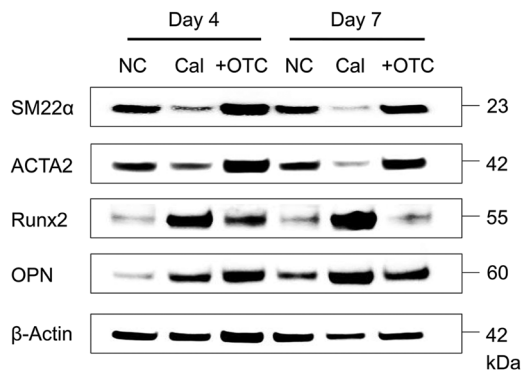


FIGURE 5 OTC reverses the acquisition of osteoblast-like phenotype in calcifying VSMCs. At Days 4 and 7 of culture, expression of VSMC markers (SM22 α and ACTA2) were downregulated in calcifying VSMCs (Cal) compared to noncalcifying controls (NC). In contrast, osteoblast markers (Runx2 and OPN) were upregulated. Treatment of calcifying VSMCs with OTC (2.5 mM; +OTC) reversed these changes. Blots shown are representative of experiments performed three times with different protein sets. ACTA2, α -smooth muscle actin; OPN, osteopontin; OTC, 2-oxothiazolidine-4-carboxylic acid; Runx2, Runt-related transcription factor 2; SM22 α , smooth muscle protein 22 α

3.5 | Diminished GSH levels promote calcification

To ascertain whether calcification and OTC treatment are associated with changes in GSH levels, intracellular GSH was assessed in VSMCs cultured for 7 days. GSH levels were reduced by 90% in calcifying cells compared to noncalcifying VSMCs. Treatment of calcifying cells with OTC (2.5 mM) rescued GSH levels to those seen in controls (Figure 6a).

Given the reduction in GSH levels in calcifying VSMC, the effects of diminished GSH on calcium deposition were assessed. Calcifying VSMCs were treated with the GSH depletion agent l-buthionine-sulfoximine, an inhibitor of GCL (Bailey, 1998). l-buthionine-sulfoximine ($\geq 10 \mu\text{M}$) increased calcification by up to 50% compared to untreated cells. Addition of OTC (2.5 mM) to these cultures abolished increases in calcification due to l-buthionine-sulfoximine (Figure 6b). From this, we sought to ascertain whether OTC regulates the expression of both GSH synthesis enzymes, namely GCL and GSS. At Day 7 of culture, expression of GCL (measured via GCLC) and GSS were downregulated in calcifying VSMCs when compared to noncalcifying cells. The addition of OTC (2.5 mM) reversed the inhibition in protein expression and restored levels similar to those seen in noncalcifying cells (Figure 6c).

4 | DISCUSSION

AMC, common in patients with CKD, diabetes, and atherosclerosis, has been linked to elevated oxidative stress (Mody et al., 2001; Sudo et al., 2015; Zhao et al., 2011). Antioxidants, which inhibit ROS and

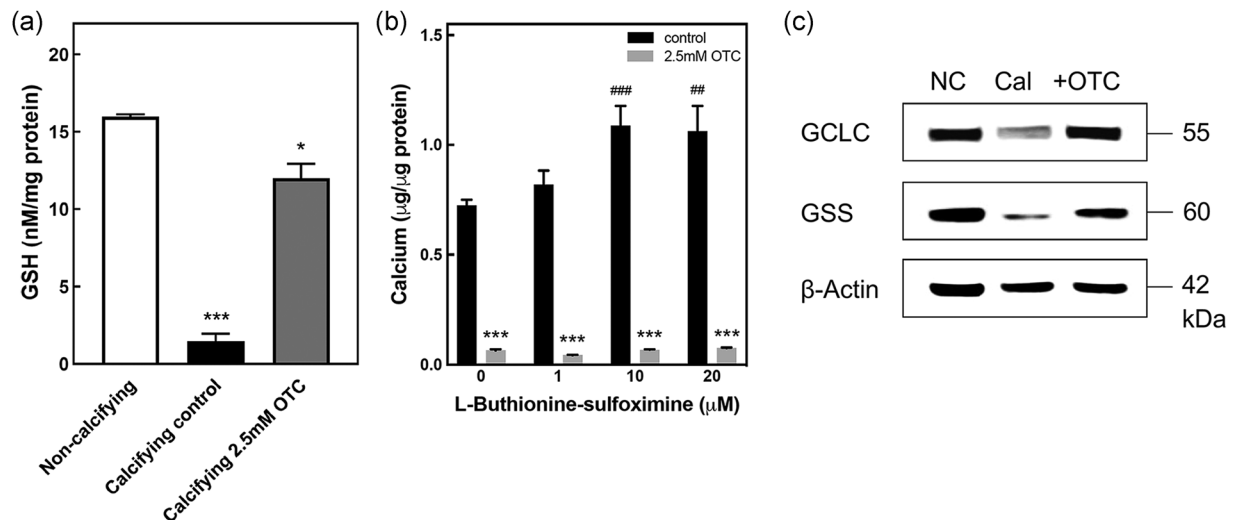


FIGURE 6 Diminished GSH levels promote calcification in VSMC. GSH was reduced by 90% in calcifying VSMCs compared to noncalcifying cells; OTC (2.5 mM) reversed this decline (a). L-buthionine-sulfoximine, a specific inhibitor of GCL, increased calcification by up to 50% compared to untreated cells. OTC (2.5 mM) inhibited this increase in calcification (b). Expression of protein for glutamate-cysteine ligase catalytic subunit (GCLC) and for GSS were downregulated in calcifying VSMC (Cal) compared to noncalcifying controls (NC). Treatment of calcifying VSMCs with OTC (2.5 mM; +OTC) reversed this inhibition (c). Values are mean \pm standard error of the mean ($n = 6$ replicate wells), *** $p < .001$, ** $p < .01$, * $p < .05$ versus control; ### $p < .001$, ## $p < .01$ versus untreated. Blots shown are representative of experiments performed three times with different protein sets. GCL, gamma-glutamylcysteine synthetase; GSH, glutathione; GSS, glutathione synthetase; OTC, 2-oxothiazolidine-4-carboxylic acid; VSMC, vascular smooth muscle cell

reduce oxidative stress, have been shown to inhibit calcification. Given the lack of treatment options currently available for VC, boosting intracellular antioxidants, such as GSH, may provide a novel treatment option. We have demonstrated, for the first time, that the cysteine prodrug OTC strongly inhibits VSMC calcification in vitro. This inhibitory effect was associated with increased total NPP activity as well as prevention of apoptosis and VSMC gaining osteoblast-like characteristics. These effects were subsequently found to be associated with preserving intracellular GSH levels by maintaining GSH synthesis enzyme expression, in addition to the ability of OTC to boost cysteine levels and thus GSH formation.

Initial staining of VSMC cultures with alizarin red highlighted significant calcium deposition in calcifying VSMCs. Alizarin red is, however, a nonspecific calcium stain, which together with excessive phosphate levels used in some VSMC methods, can lead to calcium/phosphate precipitation and overestimation of quantified calcification (Orriss, Taylor, & Arnett, 2012). To ascertain whether our calcifying VSMCs form hydroxyapatite, the calcium phosphate mineral found in bone, monolayers were stained with the hydroxyapatite specific OsteoImage fluorescent dye. The fluorescent dye exhibited a similar pattern of staining to that with alizarin red, confirming that our model is appropriate for investigating in vitro calcification. Utilising this model, OTC (≥ 2.5 mM) was found to strongly inhibit calcification by up to 90%, suggesting it to be a potent inhibitor of in vitro VSMC calcification.

Given the inhibitory effect of OTC on calcification and that OTC contains a carboxylic acid moiety, OTC may simply elicit its inhibitory effect by either inhibition of Ca/P precipitation or via a reduction in

extracellular pH. However, M. Yamada et al. (2013) showed *N*-acetylcysteine (similar to OTC in structure), promoted in vitro bone formation in osteoblasts, and accelerated bone repair in vivo. Given calcium phosphate mineral in bone and calcifying VSMCs is the same (hydroxyapatite), it would be unlikely that OTC affects precipitation. pH has been shown to be an important regulator of bone formation and small changes in pH (from 7.4 to 6.9) can inhibit physiological bone mineralisation (Arnett, 2010; Brandao-Burch, Utting, Orriss, & Arnett, 2005). Given that *N*-acetylcysteine can promote bone formation and itself contains a carboxylic acid moiety, pH is therefore unlikely to play a role. To validate this, calcifying VSMCs were treated with a stock solution with a similar pH to that of OTC but this did not induce any calcification of VSMCs even after 7 days of culture (data not shown).

A key regulator of mineralisation is the ratio of phosphate and PPI within the local environment (Fleisch & Bisaz, 1962). As mentioned above, NPP hydrolyses nucleotide triphosphates to generate PPI, while TNAP degrades PPI to phosphate (Orriss et al., 2016; Villa-Bellosta & Egado, 2015). Here, TNAP activity was reduced whereas NPP activity increased by about threefold in OTC-treated cells when compared to untreated controls. Given the similar changes in TNAP and NPP activity, it could be argued that OTC may not cause any overall change in pyrophosphate levels. However, we previously showed that TNAP activity in calcifying VSMCs was 100-fold lower than in bone-forming osteoblasts (Patel, Bourne, Davies et al., 2019). Thus, it is unlikely that TNAP activity plays a significant role in mediating VSMC calcification in vitro but the increased NPP activity induced by OTC is likely to lead to a larger pool of pyrophosphate and subsequent inhibition of mineralisation.

Cell death via increased VSMC apoptosis has been found to promote both intimal and medial calcification. Apoptosis is believed to induce the release of apoptotic bodies, which in addition to containing high calcium levels, act as nucleation sites for calcification (Proudfoot et al., 2000; Reynolds et al., 2004). Initial assessment of cell number and viability showed OTC treatment increased cell number by up to 40% and viability by 85% when compared to untreated cells. From this, it is reasonable to suggest that the inhibitory effects of OTC on calcification may be via the prevention of cell death. To validate this, we subsequently assessed apoptosis utilising measurement of annexin V, an early marker of apoptosis, via flow cytometry. Consistent with previous findings (Patel, Bourne, Davies et al., 2019), calcifying VSMCs exhibited a 75% increase in annexin V positive cells which was inhibited by pretreated with OTC (2.5 mM). Taken together, this suggests that OTC may act to inhibit calcification via a reduction in apoptosis.

As discussed above, VSMC calcification has been associated with VSMCs gaining osteoblast-like characteristics; this transdifferentiation is associated with downregulation of contractile smooth muscle cell markers (SM22 α and ACTA2) and upregulation of osteogenic markers (Runx2 and OPN) (Mizobuchi et al., 2009; Steitz et al., 2001). Consistent with these previous findings, expression of proteins for Runx2 and OPN were upregulated, while SM22 α and ACTA2 were downregulated in calcifying VSMC when compared to noncalcifying cells cultured for 4 and 7 days. The addition of OTC (2.5 mM) rescued these cells and reversed transdifferentiation to an osteoblast-like cell. Thus, OTC appears to prevent VSMC phenotypic changes to an osteoblast-like cell and this is in addition to its effects on pyrophosphate homeostasis and VSMC apoptosis.

Given the beneficial effects of OTC on inhibiting calcification, cell death, and VSMC transdifferentiation, we assessed potential mediators of this effect. As discussed above, OTC is a prodrug of cysteine, which is the rate-limiting amino acid for GSH production. GSH within cells can exist in either the reduced (GSH) or the oxidised form glutathione disulfide (GSSG). The ratio of GSH/GSSG is 50:1 and is affected by increased oxidative stress which leads to increased GSSG formation (Owen & Butterfield, 2010). Calcifying VSMCs exhibited a significant reduction (90%) in GSH levels and treatment with OTC was found to rescue GSH levels similar to that in noncalcifying cells. It is therefore likely that a calcifying environment, containing high levels of ROS, shifts GSH to the oxidised form to aid in neutralising oxidative stress and OTC may reverse this by increasing production of GSH. To ascertain whether reduced GSH is linked to increased calcification, we employed the commonly used GSH depletion agent, l-buthionine-sulfoximine, which specifically inhibits GCL and shown to reduce GSH levels at $\sim 20 \mu\text{M}$ (Bailey, 1998; Bohl et al., 2012; Kachadourian, Leitner, & Day, 2007). In calcifying VSMCs, l-buthionine-sulfoximine increased calcium deposition by up to 50%, suggesting that reductions in GSH induce calcification. The addition of OTC effectively blocked the inhibition and prevented increased calcification. Taken together, these findings suggest that reductions in GSH levels induce calcification and that treatment of VSMCs with OTC rescues GSH formation, which may subsequently lead to potential reductions in oxidative stress and thus calcification.

The mechanisms leading to reduced GSH in calcifying VSMCs are not known. As mentioned above, oxidative stress is a known inducer of VC and it could be that reduced GSH may be due to increased conversion to GSSG in an attempt to counteract elevated ROS. However, studies have linked higher oxidative stress to increased GSH via upregulation of GCL, as well as GCL activity (Rahman et al., 1966; Ravuri, Svineng, & Huseby, 2013). In contrast, clinical studies have highlighted ageing and certain diseases associated with lower GSH levels and reduced synthesis. In diabetic individuals, insulin deficiency has been shown to downregulate GCL expression and reduce GSH levels (Lu, 2009). In addition, elevated glucose has been linked to reduced messenger RNA expression of GCL in mouse endothelial cells (Urata et al., 1996). In patients with CKD, GSH levels and GCL activity were found to be significantly reduced in uremic patients and those on dialysis; however, the factors behind this effect could not be established (Alhamedani, 2005). We, therefore, wanted to assess if GSH synthesis enzyme expression was affected in VSMC calcification. Western blot analysis was performed to determine changes in expression of GCL and GSS. At Day 7 of culture, expression of both enzymes was downregulated in calcifying VSMC compared to noncalcifying cells. and this could explain the diminished GSH levels observed. From these findings, we subsequently assessed the effects of OTC on GCLC and GSS and found that OTC significantly induced the expression of both enzymes when compared to untreated calcifying cells. In the case of GCLC, protein expression showed a recovery to a level similar to untreated VSMCs. Thus, OTC may have a dual effect on GSH, acting via its well-established role as a cysteine prodrug and via enhancing GCLC and GSS protein expression. More importantly, OTC may inhibit calcification of VSMCs through maintaining GSH levels in cells.

In summary, we have shown for the first time that OTC can inhibit VSMC calcification via increased NPP activity and pyrophosphate homeostasis, reduced apoptosis and VSMC transdifferentiation. In addition, OTC was also able to rescue GSH levels which were diminished in calcifying VSMCs due to the inhibition in expression of its synthesis enzymes. These enzymes were induced by OTC. Taken together, our findings indicate that OTC could be a novel inhibitor of AMC and could provide potential clinical solutions for patients susceptible to calcification. For this to be the case, the concentrations of OTC used in our studies should reflect those achievable in vivo. On the basis of previous OTC dosage regimes from clinical trials that were not associated with adverse effects, single intravenous doses led to levels of OTC of between 6 and 9 mM (Bernard et al., 1997; Kalayjian et al., 1994). These concentrations are well above the 1–5 mM used in our experiments and gives confidence that OTC may be effective in inhibiting calcification in vivo and may do so at lower doses than have been utilised clinically.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Study conception and design: J. J. P., I. R. O., and A. R. B. Acquisition of data: J. J. P. Data analysis and interpretation: J. J. P., L. E. B., S. T., K. F., D. A. G., I. R. O., and A. R. B. Manuscript preparation and revision: J. J. P., K. F., D. A. G., I. R. O., and A. R. B.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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