RESEARCH ARTICLE

The Sirt1 Activators SRT2183 and SRT3025 Inhibit RANKL-Induced Osteoclastogenesis in Bone Marrow-Derived Macrophages and Down-Regulate Sirt3 in Sirt1 Null Cells

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Abstract

Increased osteoclast-mediated bone resorption is characteristic of osteoporosis, malignant bone disease and inflammatory arthritis. Targeted deletion of Sirtuin1 (Sirt1), a key player in aging and metabolism, in osteoclasts results in increased osteoclast-mediated bone resorption in vivo, making it a potential novel therapeutic target to block bone resorption. Sirt1 activating compounds (STACs) were generated and were investigated in animal disease models and in humans however their mechanism of action was a source of controversy. We studied the effect of SRT2183 and SRT3025 on osteoclastogenesis in bone-marrow derived macrophages (BMMs) in vitro, and discovered that these STACs inhibit RANKL-induced osteoclast differentiation, fusion and resorptive capacity without affecting osteoclast survival. SRT2183 and SRT3025 activated AMPK, increased Sirt1 expression and decreased RelA/p65 lysine310 acetylation, critical for NF-κB activation, and an established Sirt1 target. However, inhibition of osteoclastogenesis by these STACs was also observed in BMMs derived from sirt1 knock out (sirt1−/−) mice lacking the Sirt1 protein, in which neither AMPK nor RelA/p65 lysine 310 acetylation was affected, confirming that these effects require Sirt1, but suggesting that Sirt1 is not essential for inhibition of osteoclastogenesis by these STACs under these conditions. In sirt1 null osteoclasts treated with SRT2183 or SRT3025 Sirt3 was found to be down-regulated. Our findings suggest that SRT2183 and SRT3025 activate Sirt1 and inhibit RANKL-induced osteoclastogenesis in vitro however under conditions of Sirt1 deficiency can affect Sirt3. As aging is associated with reduced Sirt1 level and activity, the influence of STACs on Sirt3 needs to be investigated in vivo in animal and human disease models of aging and osteoporosis.
Introduction

Increased bone resorption by osteoclasts is characteristic of osteoporosis, inflammatory arthritis, hyperparathyroidism, malignant bone disease and other metabolic bone diseases. Currently available therapies to suppress osteoclast-mediated bone resorption include the bisphosphonates which induce osteoclast apoptosis and may result in suppression of bone formation and anti receptor activator of nuclear factor-κB ligand (RANKL)-antibody. These therapeutic agents are precluded from long term use due to side effects. Sirtuin 1 (Sirt1), a nicotinamide adenine dinucleotide (NAD⁺)-dependent lysine deacetylase, a key player in aging, inflammation and metabolism [1] regulates bone mass, and its targeted deficiency in osteoclasts results in increased bone resorption [2–6]. Enhancing Sirt1 activity is a plausible novel approach to inhibit bone resorption while concurrently ameliorating other age-related pathologies.

Resveratrol, the first Sirt1 activator to be studied, inhibits osteoclast generation and function [7], but this effect may be mediated via its cellular targets beyond Sirt1 such as estrogen receptor alpha, a key regulator of osteoclast generation [8] and influenced by resveratrol [9]. Synthetic Sirtuin 1 activating compounds (STACs), structurally different than resveratrol with a higher potency and bioavailability were generated, however their mechanism of action was a source of ongoing debate [10–13]. The controversy seemed to have been resolved by a study showing an allosteric activation of Sirt1 by STACs requiring hydrophobic motifs in the substrates and glutamic acid at position 230 of the Sirt1 N-terminal domain [14]. Different STACs were extensively tested in a wide spectrum of disease models in animals and over the past few years in humans in patients with type 2 diabetes mellitus and inflammatory conditions [15–17].

Osteoclast-mediated bone resorption is a high energy demanding process [18] and sensors of cellular energy are likely to play a role in it. In this study we investigated the effects of second and third generations STACs [19] on osteoclast generation and function in vitro, and discovered that SRT2183 and SRT3025 inhibit RANKL-induced osteoclastogenesis in bone marrow-derived macrophages (BMMs) by activating AMPK and deacetylating RelA/p65 lysine 310, critical for activation of the NF-κB signaling pathway. However, inhibition of osteoclastogenesis was also observed in SRT2183 and SRT3025-treated bone marrow macrophages derived from sirt1 knock-out mice in which neither AMPK nor RelA/p65 lysine 310 acetylation was affected but Sirt3 was down-regulated. Our findings suggest that these STACs inhibit osteoclastogenesis and can down-regulate Sirt3 under conditions of Sirt1 deficiency.

Methods

Animals

8-week-old female 129/Sv mice were used for this study. Inbred 129/Sv Sirt1+/Δ mice [20] were a generous gift (see Acknowledgments), and were used for generating Sirt1 Δ/Δ (Sirt1−/−) mice and the littermates of the parental WT strain. Genotyping of mice was performed at 4 weeks of age using ear genomic DNA as templates. All mice were maintained under specific pathogen-free conditions. Mice were housed in a constant temperature room with a 12-hour dark/12-hour light circle and were allowed free access to standard chow and water. Mice were sacrificed by isoflurane inhalation (Minrad INC, USA). All experiments were performed with the approval of the Animal Study Committee of the Hebrew University-Hadassah Medical School (MD-12-13154-3).

In vitro assays of osteoclast differentiation

Bone marrow-derived macrophages (BMMs) from femurs and tibias were collected, plated, and non-adherent cells were re-plated 24-hrs later in a 96-well plate at a concentration of
20,000 cells/well unless otherwise specified. The cells were cultured for 3 days in 5% CMG14–12 culture supernatant as a source of macrophage-colony stimulating factor (M-CSF) [21] in minimum essential medium α (α–MEM) containing 15% FBS. The plated cells were then induced to differentiation with 10% M-CSF and 10 ng/ml RANKL (PeproTech, Rocky Hill, New Jersey) for 4 days with a medium change every 3 days. Cells were TRAP-stained using a commercial kit (Sigma-Aldrich product 387-A, St. Louis, MO). Four non-overlapping images representing 80% of the area of each well were photographed with the Nikon DS Fi1 camera attached to Nikon Eclipse 80i microscope. Osteoclasts, defined as TRAP-positive multi-nucleated (≥3 nuclei) cells, were manually counted.

Compounds
SRT2183 (Fig 1A) and SRT3025, kindly provided by Sirtris-GSK (see Acknowledgments), were dissolved in DMSO and were co-administrated with RANKL, unless otherwise specified. The compounds or the vehicle (0.01% DMSO) were added upon each medium exchange. All experiments were conducted with SRT2183 and some key experiments were repeated with SRT3025. Initial dose-response experiments with 0.5, 1, 2 μM SRT2183 and 1, 2, 5 μM SRT3025 were conducted based on the manufacturer recommendation, and TRAP staining suggested that the 2μM and 5μM concentrations are toxic for SRT2183 and SRT3025, respectively. All experiments were therefore conducted with 1μM SRT2183 and 2μM SRT3025. For the time course studies SRT2183, SRT3025 or a vehicle was added in the proliferation phase (co-administrated with M-CSF on day of plating and removed 3 days post plating), differentiation phase (co-administrated with RANKL on day 4 and removed on day 7), maturation phase only (day 7 post plating for 24 hrs) or differentiation and maturation phases (co-administrated with RANKL on day 4).

Pit formation assay
BMMs were harvested, plated and 24-hrs later non-adherent cells were re-plated on Osteo Assay Plate with an inorganic crystalline calcium phosphate coating (Corning, NY-cat no CI-3988) [22] at a density of 20,000 cells/well in α–MEM/15%FBS/5% M-CSF. On day 4 cells were induced to differentiation with 10% M-CSF/20ng/ml RANKL in the presence of SRT2183, SRT3025 or a vehicle for 7 days. Higher doses of RANKL compared to the differentiation assays were used for this experiment as cells were maintained for a longer period of time in culture. On day 11 adherent osteoclasts were removed using sodium hypochlorite solution (Sigma-Aldrich, St. Louis, MO) and the resorption area was determined by a Nikon eclipse 80i microscope coupled color camera Nikon DS (Digital sight)-Fi1 and IMAGE-PRO EXPRESS 4.0 software (Media Cybernetics, Silver Spring, MD). Resorption area was quantified by MATLAB Image Processing Toolbox (MATLAB R2013a) and is presented as percentage of well area.

Gene expression analyses
RNA was extracted from osteoclasts using peqGOLD TriFast (PeqLab, Erlangen, Germany) at the indicated time points, reverse transcribed into cDNA and analyzed with SYBR Green-based quantitative Real-Time PCR in triplicates. mRNA expression level was normalized to Gapdh, β-actin or RNA polymerase II Polr2a. Gapdh is commonly used as a reference gene in osteoclast studies and indeed was stable in our experiments. To confirm that it does not affect the results when analyzing genes involved in energy metabolism we also used the mentioned above genes.
Fig 1. SRT2183 inhibits RANKL-induced osteoclastogenesis in bone marrow-derived macrophages (BMMs). (A) Chemical structure of SRT2183. (B-C) TRAP staining of SRT2183 or vehicle-treated BMMs induced to osteoclastogenesis 4 days post RANKL stimulation (B). Total number of osteoclasts (C). (D) Enzyme-sulphation of vehicle and SRT2183 treated BMMs. (E) M-CSF and RANKL treatment of BMCs leading to the formation of osteoclasts. (F) Caspase activity and cell viability in Absorbance at 405 nm. (G) Caspase3 activity and cell viability comparison between vehicle and SRT2183 treated BMMs. (H) Caspase3 activity and cell viability comparison between vehicle and SRT2183 treated BMMs.
Antibodies to the following proteins were used for western blotting: NFATc1 (sc-7294, Santa Cruz), DC-STAMP (MABF39, Millipore), Sirt1 (07–131, Millipore), Sirt3 (#5490, Cell signaling), acetylated Sod2 (acetyl-superoxide dismutase K68, ab137037, Abcam), Sod2 (ab16956, Abcam), phosphorylated AMPKα (AMP-activated protein kinase, #2535, Cell Signaling), AMPKα (#2793, Cell Signaling), phosphorylated ACC (Acetyl CoA Carboxylase, #3661, Cell Signaling), ACC (#3662, Cell Signaling), acetylated p65 (Acetyl-NF-κB p65 (Lys310) #3045, Cell signaling), p65 (#3987, Cell signaling), IκBα (#4814, Cell Signaling), HSP90 (610419, BD Transduction laboratories) and GAPDH (ab8245, Abcam, UK) were used as reference proteins.

Proliferation assay
BMMs were harvested, plated and 24-hrs later non-adherent cells were re-plated at a density of 20,000 cells/well in α–MEM/15%FBS/5% M-CSF. For determining cell proliferation, cells were treated with SRT2183 or a vehicle for 72 hours post plating and BrdU reagent (Abcam, UK) was added 48hrs post SRT2183 or vehicle administration according to the manufacturer’s instructions to determine cell proliferation 3 days post plating.

Cell viability assay
BMMs were harvested, plated and 24-hrs later non-adherent cells were re-plated at a density of 20,000 cells/well in α–MEM/15%FBS/5% M-CSF. For determining cell viability during the proliferation phase, cells were treated with SRT2183 or a vehicle for 72 hours post plating and cell-Titer-Blue reagent (Promega, Madison, Wis) was added according to the manufacturer’s instructions to determine cell survival on day 4. For determining cell viability during the differentiation and maturation phases, cells were induced to differentiation on day 4 with 10% M-CSF/10ng/ml RANKL in the presence of SRT2183 or a vehicle, and cellTiter-Blue reagent was added to determine cell survival on day 8 post plating.

Apoptosis assay by Caspase 3 activity
BMMs were harvested, plated and 24-hrs later non-adherent cells were re-plated at a density of 20,000 cells/well in α–MEM/15%FBS/5% M-CSF. For apoptosis determination during the proliferation phase, cells were treated with SRT2183 or a vehicle for 72 hours and Caspase 3 activity within the cells was assessed by using the Apo-ONE Homogeneous Caspase 3/7 Assay Kit (Promega) on day 4.

For apoptosis determination during the differentiation and maturation phases, cells were induced to differentiation on day 4 with 10% M-CSF/10ng/ml RANKL in the presence of SRT2183 or a vehicle. Caspase 3 activity within the cells was assessed with the same kit on day 8. Experiments were carried out 3 times in parallel with cell viability assays.
Statistical analysis

Results are presented as Mean ± SEM. Data was analyzed by 2-way ANOVA, one or two sample Student’s t-test as appropriate to compare treated versus untreated cells using graphPad Prism version 6 (software San Diego CA). Each experiment was repeated at least 3 times. P-values less than 0.05 were considered significant.

Results

SRT2183 inhibits RANKL-induced osteoclast generation and resorptive capacity in bone marrow macrophages

The generation of multi-nucleated osteoclasts as determined by TRAP staining was significantly hampered when BMMs were induced to osteoclastogenesis in the presence of SRT2183 in a dose-dependent manner (Fig 1B). Total osteoclast number was not affected by SRT2183 administration, but the generation of large osteoclasts with a high nuclei number was markedly decreased (Fig 1C). Importantly, SRT2183 dramatically inhibited osteoclast resorptive capacity as indicated by a marked reduction in the area eroded by treated cells (Fig 1D).

Time course experiments revealed that SRT2183 treatment at the proliferation phase did not affect osteoclast generation, however osteoclast differentiation and maturation were markedly reduced when SRT2183 was administered at the differentiation stages (Fig 1E a-d). The proliferation of osteoclast precursors was not altered by SRT2183 treatment (Fig 1F). To understand if SRT2183 affects cell survival, viability and apoptosis studies were conducted at the proliferation and differentiation phases. The administration of SRT2183 at the proliferation and differentiation phases did not decrease cell viability or increased apoptosis (Fig 1G and 1H). Accordingly, total protein was unchanged in SRT2183 versus vehicle-treated cells (S1 Fig). These results suggest that SRT2183 inhibits osteoclast differentiation and function but not precursors’ proliferation or cell survival. Consistently, nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), a master transcription factor in osteoclast differentiation [23] as well as its downstream target, dendritic cell-specific transmembrane protein (DC-STAMP), necessary for osteoclast multi-nucleation [24] were reduced in SRT2183-treated cells (Fig 2A and 2B). Similarly, mRNA expression of osteoclast markers and specifically key osteoclast fusion-related genes, Tm7sf4 (encoding for DC-STAMP) and the gene encoding for osteoclast stimulatory trans-membrane protein (OC-STAMP) were significantly decreased in SRT2183-treated osteoclasts (Fig 2C).

SRT2183 activates AMPK in osteoclasts

To gain insight into the mechanism by which SRT2183 inhibits RANKL-induced osteoclast differentiation and function major signaling pathways downstream of RANK were screened. Early phosphorylation of: c-jun N-terminal kinase (JNK), mitogen-activated protein kinase 14 (p38), ERK and p65 were not affected by SRT2183 administration (S2 Fig).

Previous work has shown that Sirt1 is closely coupled to AMP-activated protein kinase (AMPK) activity in a mutually enforcing mechanism [25]. Moreover, AMPK regulates osteoclast differentiation and function, and AMPKα1 deficiency in mice causes enhanced osteoclast differentiation and fusion [26]. We therefore investigated AMPK activation and discovered increased phosphorylation of AMPKα and its target acetyl CoA carboxylase (ACC) in SRT2183-treated cells, indicating AMPK stimulation (Fig 3A and 3B). Of note, increased Sirt1 level in SRT2183-treated cells was also observed (Fig 3C), and can result from AMPK activation, as AMPK was shown to positively regulate Sirt1 level [27].
SRT2183 regulates factors of the NF-κB signaling pathway in osteoclasts

Both Sirt1 and AMPK were shown to inhibit NF-κB signaling, a key pathway in RANKL-induced osteoclastogenesis [28]. Sirt1 represses NF-κB transcriptional activity by deacetylating RelA/p65 at lysine 310, critical for NF-κB activation [29]. AMPK was shown to inactivate the NF-κB pathway by activating Sirt1 and deacetylating RelA/p65 at lysine 310.

Fig 2. SRT2183 inhibits RANKL-induced NFATc1 activation in bone marrow-derived macrophages (BMMs). (A) The effect of SRT2183 on NFATc1 protein level. Western blot analysis of NFATc1 and HSP90 in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. NFATc1- nuclear factor of activated T-cell cytoplasmic 1. (B) The effect of SRT2183 on DC-STAMP protein level. Western blot analysis of DC-STAMP and GAPDH in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. DC-STAMP- dendritic cell-specific transmembrane protein. (C) The effect of SRT2183 on mRNA expression of osteoclast markers and fusion-related genes. SRT2183 or vehicle were co-administrated with RANKL. Gene expression analysis by quantitative Real-Time PCR 4 days post RANKL stimulation is shown. Results are relative to GAPDH. Data are Mean ± SEM (n = 3 independent experiments), analyzed by one-sample Student’s t-test, *P<0.05; **P<0.01; ***P<0.001 compared to vehicle-treated BMMs.

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Fig 3. SRT2183 activates AMPK and deacetylates RelA/p65 K310 in RANKL-induced BMMs. (A) The effect of SRT2183 on AMPKα phosphorylation (Thr172). Western blot analysis of pAMPKα and AMPKα in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. p- phosphorylated; AMPKα- AMP-activated protein kinase alpha. (B) The effect of SRT2183 on ACC phosphorylation. Western blot analysis of pACC and ACC in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. p- phosphorylated; ACC- Acetyl CoA Carboxylase. (C) The effect of SRT2183 on Sirt1 protein level in RANKL-stimulated osteoclasts. Western blot analysis of Sirt1 and HSP90 in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. (D) The effect of SRT2183 on IκBα protein level. Western blot analysis of IκBα and GAPDH in SRT2183- and vehicle-treated BMMs 24 hours post RANKL stimulation. (E) The effect of SRT2183 on p65 acetylation (Lys310). Western blot analysis of p65K310 ac and p65 in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. Data are Mean ± SEM (n = 3 independent experiments), analyzed by one-sample Student’s t-test; *P<0.05 versus vehicle-treated BMMs.

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NF-κB pathway via inhibition of IκB kinase and IκBα degradation. IκBα is an inhibitory subunit complexed to NF-κB/Rel proteins in the cytoplasm, preventing the release and movement of NF-κB into the nucleus [30]. Along these lines, decreased IκBα was reported in osteoclasts derived from AMPKα1/- mice [26]. Indeed, IκBα was markedly increased and RelA/p65 K310 acetylation was significantly decreased in SRT2183-treated osteoclasts (Fig 3D and 3E). These results suggest that SRT2183 activates Sirt1 and AMPK in bone marrow-derived osteoclasts leading to inhibition of RANKL-induced NF-κB activation and NFATc1 expression.

SRT2183 inhibits osteoclastogenesis in sirt1−/−-derived bone marrow macrophages

To understand the role of Sirt1, the influence of SRT2183 on osteoclastogenesis was evaluated in bone marrow cells derived from sirt1−/− mice. Sirt1Δ/Δ (Sirt1−/−) mice lacking Sirt1 protein were generated from inbred 129/Sv Sirt1+/Δ mice, whereas their littermates WT served as the controls. These KO mice are lacking sirt1 exons 5–7 resulting in no sirt1 protein production (Fig 4A) and have a birth rate lower than 3% [20]. Strikingly, SRT2183 abolished the generation of large multi-nucleated osteoclasts and their resorptive capacity in sirt1−/− BMMs similar to the effect observed in WT-derived osteoclasts, indicating that Sirt1 is not essential for inhibition of osteoclast generation and function under these conditions (Fig 4B and 4C). As expected, RelA/p65 K310 acetylation was not changed in SRT2183-treated sirt1−/− osteoclasts (Fig 4D), as this is a direct Sirt1 target. Furthermore, AMPKα phosphorylation was not affected by SRT2183 administration in sirt1−/−-derived osteoclasts (Fig 4E), suggesting that Sirt1 is upstream of and is required for AMPK activation by SRT2183 under these conditions. Consistently IκBα level was unchanged (Fig 4F).

SRT2183 down-regulates Sirt3 in sirt1−/−-derived bone marrow macrophages

To better understand the mechanism by which osteoclastogenesis is inhibited in SRT2183-treated sirt1−/− osteoclasts, we first asked if Sirtuins 2–7 protein level is changed in sirt1−/−-derived bone marrow macrophages or osteoclasts. No change was detected (S3 Fig). Next, we asked if Sirt2-7 protein level is modulated by SRT2183 administration in WT or sirt1 KO cells. Strikingly, while no change was observed in treated WT osteoclasts (S4 Fig), Sirt3 protein but not mRNA expression was significantly reduced in sirt1−/−-treated osteoclasts (Fig 4G and 4H). Moreover, decreased Sirt3 activity was detected in SRT2183-treated sirt1 null cells as indicated by increased acetylation of its target manganese superoxide dismutase (MnSOD, Sod2) [31] (Fig 4I). Finally, we asked if inhibition of osteoclastogenesis occurs also with a more advanced STAC, such as SRT3025, a third generation STAC [19]. Similar effects of SRT3025 on inhibition of osteoclastogenesis were observed in both WT (S5 Fig) and in sirt1 null cells (S6 Fig). Consistently, Sirt3 was reduced in sirt1 null cells treated with SRT3025. Thus, both SRT2183 and SRT3025 inhibited RANKL-induced osteoclastogenesis independently of Sirt1 and down-regulated Sirt3 in sirt1 null cells.

Discussion

This study demonstrates that Sirt1 activating compounds (STACs), SRT2183 and SRT3025, inhibit RANKL-induced osteoclast differentiation, multi-nucleation and resorptive capacity in bone marrow derived-macrophages in vitro without hampering cell survival. SRT2183 and SRT3025 inhibited RANKL-induced osteoclast differentiation by promoting deacetylation of RelA/p65 at lysine 310, a well recognized direct Sirt1 target [29] critical for NF-κB activation
[32], and by activating AMPK. RelA/p65 was previously shown to promote osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway, leading to enhanced OC differentiation [33]. Moreover, mice lacking RelA/p65 in the hematopoietic compartment have a deficient osteoclastogenic response to RANKL [33]. RelA/p65 contains seven lysine acetylation sites of which lysine 310 acetylation is required for NF-κB transcription activation and was shown to be deacetylated by Sirt1 [34]. In agreement with our results, targeted sirt1 deletion in

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**Fig 4. SRT2183 inhibits RANKL-induced osteoclastogenesis and pit formation in sirt1−/− BMMs.** (A) Sirt1 expression in WT- and in Sirt1−/−-derived osteoclasts. PCR amplification of exons 1–9 of the sirt1 gene (left panel) and Western blot analysis with Sirt1 antibody (right panel) demonstrates complete loss of Sirt1 protein in osteoclasts obtained from Sirt1−/−/− (Sirt1−/−) mice. (B) The effect of SRT2183 on osteoclast differentiation in Sirt1−/−-derived BMMs. BMMs were induced to osteoclastogenesis with RANKL in the presence or absence of SRT2183. TRAP staining performed 4 days post induction. (C) The effect of SRT2183 on pit formation in Sirt1−/−-derived BMMs stimulated with RANKL. An eroded area (left panel) and pit formation assay (right) are shown. (D) The effect of SRT2183 on p65 acetylation (Lys310). Western blot analysis of p65K310 ac and p65 in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. (E) The effect of SRT2183 on AMPKα phosphorylation (Thr172). Western blot analysis of pAMPKα and AMPKα in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. (F) The effect of SRT2183 on IkBα protein level. Western blot analysis of IkBα and GAPDH in SRT2183- and vehicle-treated BMMs 24 hours post RANKL stimulation. (G-H) The effect of SRT2183 on Sirt3 protein (G) and gene expression (H). Western blot analysis of Sirt3 and GAPDH in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation (G). Gene expression analysis by quantitative Real-Time PCR 4 days post RANKL stimulation is shown. Results are relative to Polr2a (H). (I) The effect of SRT2183 on superoxide dismutase 2 (Sod2) Lys68 acetylation. Western blot analysis of acetylated (ac) Sod2K68 and Sod2 in SRT2183- and vehicle-treated osteoclasts 4 days post RANKL stimulation. Data are Mean ± SEM (n = 3 independent experiments), analyzed by paired Student's t-test paired (C) or one-sample Student's t-test (H-I); ***P<0.001, ****P<0.0001, versus vehicle-treated BMMs. Magnification X40; scale bar 1mm.

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osteoclasts leads to increased osteoclastogenesis and bone resorption accompanied by elevated osteoclast RelA/p65 lysine 310 acetylation [6]. Consistently, deacetylation of RelA/p65 K310 by STACs was previously described in U20S or HEK293 cells [35]. SRT2183 and SRT3025 activated AMPK via Sirt1 stimulation, as indicated by lack of effect on AMPK in sirt1 null cells. An intimate interplay between Sirt1 and AMPK was previously shown in a number of cell types. Sirt1 deacetylases and activates serine-threonine liver kinase 1 (LKB1), the primary AMPK kinase and activator [36]. On the other hand, AMPK increases Sirt1 expression and function via increasing its co-factor NAD⁺ [27,37]. AMPK inhibits osteoclastogenesis by inhibiting the NF-κB pathway in part by preventing the degradation of IκBα a repressor of NF-κB which holds it quiescent in the cytoplasm [38]. The physiologic significance of this effect is illustrated by the phenotype in IκBα haplo-insufficient mice which display increased osteoclastogenesis [6].

Suppression of osteoclastogenesis by SRT2183 and SRT3025 occurred also in BMMs derived from sirt1−/− mice. These findings are in disagreement with some previously published studies which reported no effect of STACs in sirt1 deficient cells [14,39]. However, other previously published studies reported Sirt1-independent effects of STACs [10,40]. The discrepancy may be explained at least in part by the fact that in those studies claiming no off target effects only sirt1 exons 4–5, encoding for the enzyme catalytic domain, were deleted, allowing for STACs binding to inactive existing Sirt1 protein and precluding their binding to other targets, whereas in our study cells with complete deletion of sirt1 were investigated. It is also plausible that sirt1 specificity of these compounds is cell-dependent and osteoclasts were never studied before.

We discovered Sirt3 to be a target of SRT2183 and SRT3025 in sirt1 null cells resulting in its down-regulation, thus these STACs had an inhibitory action rather than being activators under these conditions. Supporting our findings, SRT1720 was previously shown to inhibit mouse and human Sirt3 by partially occupying the acetyl-lysine binding site, thus competing with the peptide substrate [41,42]. Furthermore, resveratrol, the first described Sirt1 activator, was also shown to inhibit Sirt3 [43]. The mechanism by which reduced Sirt3 in sirt1−/− osteoclasts leads to inhibition of osteoclastogenesis is not completely understood, however an increase in the acetylated inactive form of MnSod2 was found in SRT2183− treated sirt1 null cells. The role of Sod2 in osteoclasts is unknown. Sod2 was identified as a susceptibility gene for osteoporosis in humans. SNPs in the Sod2 gene were to be translated into changes in mRNA transcription and protein expression, and Sod2 protein expression is inversely associated with BMD in the Chinese population, suggesting that low Sod2 may be bone protective [44,45].

In summary, this study demonstrates that the STACs SRT2183 and SRT3025 inhibit osteoclast generation and function in vitro. These compounds did not cause osteoclast apoptosis and therefore are unlikely to impair the coupling between osteoclasts and osteoblasts. Whether these STACs or other STACs inhibit osteoclast-mediated bone resorption or influence other Sirtuins in vivo remains to be investigated in disease models of osteoporosis, aging and impaired metabolism as these conditions are associated with reduced Sirt1 level and function [46,47]. Importantly, our findings may have implications beyond osteoclast biology as they shed novel light on the STACs mechanism of action.

Supporting Information

S1 Fig. The effect of SRT2183 administration on protein content in RANKL-induced BMMs. SRT2183 or vehicle were co-administrated with RANKL. Protein content was determined 4 days post RANKL stimulation. Data are Mean ± SEM (n = 3 independent
experiments).

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S2 Fig. SRT2183 does not influence early MAP Kinase and NFκB phosphorylation. Western blot analysis of phosphorylated and total JNK, Erk1/2, p38 and p65 in SRT2183- and vehicle-treated BMMs 0, 10, 20, 30, 60 minutes post RANKL administration. p- phosphorylated; JNK/ Jun-amino-terminal kinase (mitogen-activated protein kinase 8/ mitogen-activated protein kinase 9); Erk1/2, extracellular signal regulated kinase 1/ extracellular signal regulated kinase 2 (mitogen-activated protein kinase 3/ mitogen-activated protein kinase 1); p38 (mitogen-activated protein kinase 14); p65 (RelA, v-rel reticuloendotheliosis viral oncogene homolog A).

Data are Mean ± SEM (n = 3 independent experiments).

(TIF)

S3 Fig. Sirtuin 2–7 protein expression in RANKL-induced WT and Sirt1-/BMMs. Western blot analysis of Sirt2-7 and GAPDH in vehicle-treated BMMs obtained from WT and sirt1 knockout (Sirt1-) mice 4 days post RANKL stimulation. Data are Mean ± SEM (n = 3 mice of each genotype).

(TIF)

S4 Fig. SRT2183 does not influence Sirt2, 4–7 protein level in WT and in Sirt1-/derived osteoclasts. (A-B) The effect of SRT2183 on protein levels in (A) WT and (B) Sirt1-/derived osteoclasts. Western blot analysis of Sirt2-7, GAPDH and HSP90 in SRT2183- or vehicle-treated osteoclasts 4 days post RANKL stimulation. Data are Mean ± SEM (n = 3 mice of each genotype).

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S5 Fig. SRT3025 inhibits RANKL-induced osteoclastogenesis in bone marrow-derived macrophages (BMMs). (A) The effects of SRT3025 on osteoclast differentiation. BMMs were inducted to osteoclastogenesis with RANKL in the presence or absence of SRT3025. TRAP staining was performed 4 days post RANKL stimulation. (B) The effect of SRT3025 on pit formation by RANKL-induced osteoclasts. BMMs were inducted to osteoclastogenesis in the presence or absence of SRT3025. A pit formation assay (left panel) and eroded area (right) are shown. (C-D) The effect of SRT3025 on NFATc1 (C) and DC-STAMP (D) expression. Western blot analysis of NFATc1 and GAPDH in SRT3025 or vehicle-treated osteoclasts 4 days post RANKL stimulation. (E) The effect of SRT3025 on expression of osteoclast markers and fusion-related genes. Gene expression analysis by quantitative RT-PCR 4 days post induction to osteoclastogenesis is shown. Results are relative to GAPDH. (F) The effect of SRT3025 on AMPK phosphorylation. Western blot analysis of pAMPKα and AMPKα in SRT3025 or vehicle-treated osteoclasts 4 days post RANKL stimulation. (G) The effect of SRT3025 on ACC phosphorylation. Western blot analysis of pACC and ACC in SRT3025 or vehicle-treated osteoclasts 4 days post RANKL stimulation. (H) The effect of SRT3025 on Sirt1 expression in RANKL-stimulated osteoclasts. Western blot analysis of Sirt1 and HSP90 in SRT3025 or vehicle-treated osteoclasts 4 days post RANKL stimulation. (I) The effect of SRT3025 on IκBα expression. Western blot analysis of pIκBα and IκBα in SRT3025 or vehicle-treated BMMs 24 hours post RANKL stimulation. (J) The effect of SRT3025 on p65 acetylation (Lys310). Western blot analysis of p65K310 ac and p65 in SRT3025 or vehicle-treated osteoclasts 4 days post RANKL stimulation. Data are Mean ±SEM (n = 3), analyzed by paired sample Student’s t-test (B) or one-sample Student’s t-test (C-J) *P<0.05, **P<0.01, ***P<0.001 compared to vehicle-treated BMMs. Magnification X40; Scale bar 1mm.

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S6 Fig. SRT3025 inhibits RANKL-induced osteoclastogenesis in Sirt1−/− bone marrow-derived macrophages (BMMs). (A) The effect of SRT3025 on osteoclast differentiation. Sirt1−/−-derived BMMs were induced to osteoclastogenesis with RANKL in the presence or absence of SRT3025. TRAP staining was performed 4 days post RANK stimulation. (B) The effect of SRT3025 on pit formation by RANKL-induced BMMs. Sirt1−/−-derived BMMs were induced to osteoclastogenesis in the presence or absence of SRT3025. A pit formation assay (left panel) and eroded area (right) are shown. (C) The effect of SRT3025 on Sirt3 protein level. Western blot analysis of Sirt3 and HSP90 in SRT3025- and vehicle-treated osteoclasts 4 days post RANKL stimulation. Data are Mean ±SEM (n = 3 independent experiments), analyzed by paired Student’s t-test (B) and one-sample Student’s t-test (C–E); *P<0.01; **P<0.001 compared to vehicle-treated BMMs. Magnification X40; Scale bar 1mm.

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S1 Text. Supplementary Methods.

(DOC)

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Author Contributions

Conceived and designed the experiments: IG RDP. Performed the experiments: IG ECK GH GBS MEH. Analyzed the data: IG HA GH BF RDP. Contributed reagents/materials/analysis tools: IG HA BF. Wrote the paper: IG RDP.

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