Suppressive effects of N-bisphosphonate in osteoblastic cells mitigated by non-N-bisphosphonate but not by sodium-dependent phosphate cotransporter inhibitor

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SHORT TITLE: Mitigation of N-BP effects on osteoblasts by non-N-BP

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ABSTRACT

There are two types of bisphosphonates (BPs), nitrogen-containing (N-BPs) and those free from nitrogen (non-N-BPs). Although N-BPs show greater inhibition of bone resorption than non-N-BPs, their effects are likely accompanied with inflammation, which non-N-BPs mitigate. We examined the competitive effects of zoledronate (ZOL), an N-BP, and etidronate (ETI), a non-N-BP, in osteoblasts. ZOL, but not ETI, markedly reduced alkaline phosphatase activity and cell viability in osteoblastic MC3T3-E1 and Saos2 cells, while that inhibition was relieved by simultaneous administration of ETI, possibly because of competition with ZOL for cellular uptake. However, phosphonoformate, an inhibitor of the phosphonate transporters SLC20A and SLC34A, did not mitigate the reducing effects of ZOL, suggesting that those transporters are not involved in BP uptake in osteoblastic cells. Additionally, ZOL reduced fibroblastic NIH3T3 and C3H10T1/2 cell viability, which was relieved by administration of both ETI and phosphonoformate. Transporter gene expression levels were significantly lower in osteoblasts as compared to fibroblasts, which may account for the distinct effects of phosphonoformate with different cell types. Together, our results suggest existence of a common uptake route of N-BPs and non-N-BPs into osteoblastic cells that is unrelated to the SLC20A and SLC34A families.

Significance of the study

N-BP ZOL was shown to suppress differentiation and viability of osteoblasts. ZOL-induced cell viability suppression was also observed in fibroblasts, which was markedly relieved by addition of the non-N-BP ETI. Additionally, mitigation of the effects of ZOL was achieved with phosphonoformate, a sodium-phosphate cotransporter inhibitor, in fibroblastic cells but not osteoblasts. Expression levels of SLC20A and SLC34A family genes were significantly lower in osteoblasts as compared to fibroblasts. These observations suggest that incorporation of N-BPs and non-N-BPs in osteoblasts is mediated via common transporters that appear to be distinct from SLC20A and 34A, which operate in fibroblasts.

KEYWORDS

bisphosphonate, cell differentiation, cell viability, etidronate, fibroblast, osteoblast, SLC transporter family, zoledronate

1. INTRODUCTION

Bisphosphonates (BPs) are chemical analogs of inorganic pyrophosphate that carry a P-C-P instead of a P-O-P bond, making them resistant to biological degradation.[1] BPs inhibit bone destruction and lead to increased bone mineral density by suppression of bone resorption and turnover.[2] Accordingly, these reagents are clinically used as anti-bone resorption agents for treating osteoporosis, multiple myeloma, and malignant hypercalcemia.[3]

At the cellular level, the main target of BPs is osteoclasts, bone-resorbing cells,[4-6] by inhibition of farnesyl pyrophosphate synthase, which is essential for cholesterol biosynthesis in those cells.[7] However, since cholesterol biosynthesis commonly occurs in eukaryotic cells, the effect of BPs may not be limited to osteoclasts. In fact, BP oral administration sometimes causes inflammation in digestive mucosa[8] as well as osteonecrosis of the jaw, known as bisphosphonate-associated osteonecrosis of the jaw (BRONJ).[9] Moreover, osteoblasts have also been reported to be affected by BPs, though currently available experimental data remain controversial.[10, 11] This ambiguity seems to be due to the fact that the effects of BP are reversed at different concentrations.[11]

BPs are classified into two types, depending on the existence of nitrogen,[12] of which nitrogen-containing BPs (N-BPs) remarkably induced increased bone mass and occasionally accompany inflammation, while non-nitrogen-containing BPs (non-N-BPs) have a weak bone mass generation potential coupled with a low inflammatory effect. Recently, Endo and colleagues reported that non-N-BPs have an effect to inhibit intracellular transport of N-BPs in mouse soft tissues. They found that injection of etidronate (ETI) or clodronate, representative non-N-BPs, together with zoledronate (ZOL), an N-BP, into mouse ear skin significantly reduced the inflammatory response caused by ZOL alone.[1, 13] Furthermore, mitigation of the effects of ZOL was also achieved by an inhibitor of the sodium-dependent phosphate transporter family,

thus they proposed that ETI competes with ZOL for uptake via phosphate transporters, resulting in decreased intracellular concentration of ZOL.[14, 15]

The sodium-dependent phosphate transporter group, coded by *SLC20A* and *SLC34A*, is involved in active transport of phosphate into cells, with BP incorporation also considered to occur via its members.[14] The SLC34A family, composed of the SLC34A1, SLC34A2, and SLC34A3 isoforms, plays predominant roles in phosphate homeostasis. Among those, SLC34A1 and SLC34A3 are mainly expressed in renal proximal tubules, while SLC34A2 is expressed in intestine, liver, salivary gland, and lung tissues. All 3 isoforms exhibit a high affinity for inorganic phosphate (K_m < 100 μM) and are specifically inhibited by phosphonoformate.[14, 16] SLC20A contains 2 members, SLC20A1 and SLC20A2, which show broad tissue distribution and exhibit lower responsiveness to phosphonoformate as compared to members of the SLC34 family.[17-19] To the best of our knowledge, the transport systems used by BPs in osteoblastic cells have yet to be well elucidated.

In the present study, we examined the competitive effects of N-BP and non-N-BP members on cell differentiation and viability of osteoblastic cell lines. Furthermore, the involvement of phosphate transporter-family members SLC20A and SLC34A in BP transport into these cells was examined, with the findings compared with those of fibroblastic cell lines.

2. MATERIALS AND METHODS

2.1 Reagents

ZOL, ETI, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium phosphonoformate hexahydrate came from Alfa Aesar (Heysham, Lancashire, UK).

2.2 Cell culture

MC3T3-E1 cells, derived from mouse calvaria, Saos2 cells, from a human osteogenic sarcoma specimen, NIH3T3-3 mouse embryonic fibroblast-like cells, and C3H10T1/2 cells, from mouse fibroblasts, were obtained from RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained under an enviroment of 5% CO_2 at 37°C in minimum essential medium α modification (Sigma Chemical Co.) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin (Life Technology Inc., Grand Island, NY), and 80 µg/ml L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Corp., Osaka, Japan), as previously described.[20] Culture medium was exchanged every 2 or 3 days until the cells reached confluence.

2.3 Determination of alkaline phosphatase (ALP) activity

ALP activity was determined according to a protocol previously reported.[20] Briefly, cells were plated at a density of 1.0 x 10^4 per cm^2 in 24-well plates. After reaching confluence, reagents were added to the medium, then at various time points the cultures were lysed in 80 µl of solubilization buffer (10 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100) and subjected to centrifugation at 12,000 x *g* for 20 minutes. Supernatant aliquots (4 µl) were then incubated in 50 mM of carbonate buffer (pH 10) (total, 150 µl) containing 4 mM *p*-nitrophenyl phosphate and 5 mM $MgCl_2$ at room temperature. After 6 minutes, the reaction was stopped by addition of 2 M

NaOH (50 µl), then absorbance at 405 nm was determined using a microplate reader. We defined 1 unit as activity that produced 1 µmol of *p*-nitrophenol after 1 hour.

2.4 ALP staining

After cells were washed with phosphate buffer, ALP staining reagent (1 mg naphthol AS-MX phosphate, 50 µl dimethylformamide and 6 mg fast blue BB salt in 10 ml of 0.1 M Tris-HCl, pH 8.5) was added. After 30-min incubation at room temperature, cells were washed with distilled water.

2.5 MTT assay

Cell viability was determined using an MTT assay method previously reported.[21] Cells were plated at a density of 1.0 x 10^4 per cm^2 in 96-well plates. After reaching confluence, ZOL and ETI were added to the culture medium, then medium was removed at various time points and replaced with 50 µl of a solution containing 12.5 µg MTT, then further incubation was performed at 37°C. After 3 hours, the solution was removed and then insoluble formazan crystals were dissolved in 100 µl DMSO at room temperature for 5 minutes. Colorimetric changes were measured using a microplate reader at a wavelength of 570 nm (Model 550, Bio-Rad, Hercules, CA).

2.6 Quantitative RT-PCR

Total RNA was isolated using Isogen reagent (Nippon Gene Co., Tokyo, Japan). cDNA was prepared from 1 µg of RNA with reverse transcriptase (TAKARA BIO Inc., Otsu, Japan) in a

reaction mixture (20 μl) containing 1.25 mM dNTPs and 0.3 μM oligo-dT (Life Technology Inc.), as previously described.[20] Mixtures containing 5 ng of cDNA and 500 nM of primers as well as THUNDERBIRD SYBR qPCR MIX (TOYOBO, Osaka, Japan) were subjected to reactions using a LightCycler 480 real-time PCR system (Roche, Basel, Switzerland). mRNA levels were quantified using a $\Delta\Delta C_t$ method, with the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA utilized as the standard. The expression level of sodium phosphate cotransporter as compared to that of GAPDH genes in MC3T3-E1 cells was expressed as 1. The primers used have been previously reported.[14, 22]

2.6 Statistical analysis

All values are presented as the mean ± SD. Differences between groups were assessed using Student's *t*-test.

3 RESULTS

3.1 ALP activity in osteoblastic cells suppressed by ZOL and effect relieved by ETI

Initially, we examined the effects of BPs on cell differentiation of osteoblastic cell lines; MC3T3-E1 cells derived from mouse calvaria and Saos2 cells, from a human osteosarcoma (Figure 1). After reaching confluence, cells were cultured with or without ZOL as the representative N-BP and ETI as the non-N-BP, then ALP activity was determined as a marker of osteoblast differentiation. ZOL at 25 and 50 μM significantly reduced that activity to 26% and 21%, respectively, as compared to without its addition, indicating an effect to suppress

osteoblastic cell differentiation, though that effect did not occur in a clear dose-dependent manner. Interestingly, the reduction was markedly recovered up to 87% by simultaneous administration of 50 μM ETI, whereas ETI administered alone had scant effects to the change of ALP activity. Similarly, ZOL inhibited ALP activity and simultaneous addition of ETI relieved that inhibition in Saos2 cells.

This effect of ETI to abrogate ZOL-induced suppression of cell differentiation was further examined by reagent administration at various time points (Figure 2). Following a 6-day culture of confluent Saos2 cells, 10 μM of ZOL added on Day 1 and Day 3 resulted in a significant decrease in ALP activity. In accordance with the results shown in Figure 1, no such remarkable reduction was seen with simultaneous addition of ETI on Day 1 and Day 3, with ALP activity restored to 71% with 50 μM and 90% with 100 μM of ETI (Figure 2). On the other hand, when ETI was added separately from ZOL on Days 2 and 4, its restoration effect of ETI was less notable (50% at 50 μM, 71% at 100 μM) as compared to when the N-BP and non-N-BP were simultaneously administered. Thus, coexistence of ETI is apparently effective for mitigation of the ZOL-inducing suppressive effects towards osteoblasts. These results suggest that ETI competitively inhibits uptake of ZOL into cells, resulting in relief of its effects on differentiation of osteoblasts.

3.2 Effect of phosphonoformate on suppression of ALP activity by ZOL

It was previously reported that phosphonoformate, an inhibitor of the sodium phosphate cotransporter family members SLC20A and SLC34A, alleviated inflammation induced by ZOL in mouse ear skin tissues.[14] Hence, we examined whether SLC family transporters are also involved in transport of ZOL and ETI into osteoblastic cells using the same inhibitor.

Phosphonoformate alone had no effect on ALP activity in MC3T3-E1 (data not shown) or Saos2 cells (Figure 3). When the phosphonoformate was administered simultaneously with ZOL, the suppressed ALP activity was not restored in MC3T3-E1 cells (Figure 3). In addition, simultaneous administration of phosphonoformate at a minimum concentration of 0.5 mM with ZOL significantly reduced ALP activity further in Saos2 cells, though the reason for this inhibitory phenomenon was ambiguous. Nevertheless, these results strongly suggested that incorporation of ZOL and ETI in osteoblastic cells is not mediated by SLC family transporters.

Expression modulation of ALP was also examined by activity staining on Saos2 cells (Figure 4). Ten-μM ZOL reduced the number of ALP positive cells, and this reduction was canceled by 100-μM ETI, but not by PFA.

3.3 Effects of ZOL on cell viability of osteoblasts and fibroblasts, and examination of competitive effects of ETI and phosphonoformate on ZOL

We speculated that the uptake route of BPs may vary among cell types. To examine this possibility, the response of confluent cells other than osteoblasts, i.e., mouse fibroblastic NIH3T3 and C3H10T1/2 cells, towards the examined BPs was investigated. First, we examined the effects of ETI and ZOL on cell viability of osteoblasts and fibroblasts, since ALP activity is not available for evaluation of the effects of BPs effect in fibroblasts. Following a 6-day culture with 10 μM of ZOL, the number of MC3T3-E1 cells was significantly declined to 50% of that of the control, indicating that ZOL had a suppressive effect on osteoblast viability by inhibition of cell differentiation (Figure 5). Similar to our finding with ALP activity, the suppression caused by ZOL was recovered to 81% and 94% by administration of 50 and 100 μM ETI, respectively. With Saos2 cells, the number was decreased to 50% of the control with 10 μM ZOL and then

recovered up to 70% with simultaneous addition of ETI. On the other hand, ETI alone did not have an effect on cell number. Furthermore, in accordance with our examination of ALP activity, phosphonoformate did not relieve the inhibitory effects of ZOL on cell viability in either MC3T3-E1 or Saos2 cells (Figure 5).

ZOL also markedly decreased the numbers of NIH3T3 and C3H10T1/2 cells as compared to the control cultures at the same concentration (Figure 6). The number of NIH3T3 cells was decreased to 44% and of C3H10T1/2 cells to 54%. As with MC3T3-E1 and Saos2 cells, this suppression by ZOL was relieved by coadministration of ETI to the NIH3T3 cells, with recovery up to at least 80%. Similarly, the number of C3H10T1/2 cells was recovered by up to 80% with simultaneous addition of ETI. Taken together, we concluded that the reducing effects of N-BP on cell viability of both osteoblastic and fibroblastic cells were obviously mitigated by the coexistence of non-N-BP.

Next, we examined whether phosphonoformate cancels the suppressive effects of ZOL towards fibroblasts. Notably, in contrast to osteoblasts, suppression of ZOL in NIH3T3 cells was markedly recovered by up to 70% with simultaneous administration of phosphonoformate (Figure 6), while mitigation of that effect of ZOL by phosphonoformate was also observed in C3H10T1/2 cells. Taken together, these results strongly suggested that transport of ZOL and ETI into mouse fibroblasts is mediated by SLC family transporters, and inhibited by phosphonoformate. Also, the BP transport system in osteoblastic cells seems to be distinct from this transporter family. Thus, to examine this issue in more detail, expression levels of SLC family transporter genes in osteoblastic and fibroblastic cells were compared using quantitative RT-PCR.

3.4 Expressions of sodium phosphate cotransporter family members in osteoblastic and fibroblastic cells

Both the SLC20A and SLC34A families are involved in active transport of phosphate into cells, and BPs seem to be incorporated into soft tissue cells by these transporters.[14] Two members of the SLC20A family, *SLC20A1* and *SLC20A2*, and 3 of SLC34A, *SLC34A1*, *SLC34A2*, and *SLC34A3*, are known to be expressed in mouse cells.[17, 18] We determined the expression levels of these SLC genes in osteoblastic MC3T3-E1 and fibroblastic NIH3T3 and C3H10T1/2 cells using quantitative RT-PCR. As shown in Figure 7, expression levels of *SLC20A1* and *SLC20A2* in MC3T3-E1 cells were reduced to 1/5 to 1/9 as compared to those in NIH3T3 fibroblastic cells. In addition, mRNA levels of *SLC34A1*, *SLC34A2,* and *SLC34A3* were substantially reduced to 60 to 140 times lower in osteoblastic MC3T3-E1 as compared to C3H10T1/2 cells. These findings showed that the expression levels of SLC transporter family genes were substantially low in osteoblasts and indicate that BPs are possibly transported via a transporter other than an SLC family member in those cells, though that entity was not revealed in our examinations and should be addressed in a future study.

4 DISCUSSION

BPs exhibit high affinity for bone minerals and induce apoptosis of osteoclasts, thus are widely used as anti-bone resorption agents. N-BPs have greater effects to inhibit bone resorption as compared to non-N-BPs, though the molecular mechanisms for those different effects have not been fully elucidated. Presumably because of their potent effects, adverse conditions such as inflammation and BRONJ frequently arise in patients who receive N-BP therapy.

Since N-BPs inhibit farnesyl pyrophosphate synthase in the essential metabolic mevalonate pathway, they apparently have effects on cells other than osteoclasts and possibly produce adverse effects. In accordance with a previous report,[23] the present findings revealed that high concentrations (10 - 20 μM) of ZOL inhibited the activity of ALP, a marker of osteoblast differentiation, and reduced cell viability of both osteoblasts and fibroblasts in a confluent state. Thus, we speculated that inhibition of the mevalonate pathway by a higher concentration of an N-BP results in cell function decrement or defects. In addition, it was noteworthy that relatively higher concentrations (20 - 100 μM) of ETI alone did not induce such inhibitory effects. These distinct differences between ZOL and ETI may be due to their potency to inhibit responsible enzymes. Alternatively, transport of ZOL into cells may be faster than that of ETI, thus quickly reaching an intracellular concentration adequate to force inhibition.

The present results also demonstrated that ZOL-induced inhibition was markedly relieved by simultaneous treatment with ETI in both osteoblasts and fibroblasts. Should the inhibitory effects of ZOL be dependent on its intracellular concentration, ETI seems to compete for incorporation into cells.[13, 14] Our analysis further revealed that mitigation of the effects of ZOL on fibroblastic NIH3T3 and C3H10T1/2 cells was achieved by use of phosphonoformate, an SLC inhibitor, while that inhibitor had no effect on osteoblasts. The present finding with fibroblasts was in accordance with a previous report that noted that the transporter inhibitor displaced ETI and mitigated inflammation caused by ZOL in soft tissues of mice.[14] Therefore, transportation of BPs seems to be differently mediated by osteoblasts and fibroblasts. In the latter, the phosphate transporter family members SLC20A and SLC34A, which are inhibited by phosphonoformate, may be involved in BP incorporation, though that is not the case with osteoblastic cells.

Should ZOL be incorporated into cells via SLC20A and SLC34A family members, then phosphonoformate would likely reduce that incorporation, diminishing the effects of ZOL taken up by osteoblasts. However, no blocking by phosphonoformate was observed in the present

study, which suggests that ZOL is incorporated into MC3T3-E1 cells via transporters other than SLC20A and SLC34A. Coincidently, expression levels of the SLC20A and SLC34A family members were significantly low in those cells, *i.e.*, phosphonoformate-sensitive transporter expression was subtle. This substantially low expression of those transporters implicates nearly no effect of phosphonoformate on suppression of cell differentiation and viability caused by ZOL in osteoblastic cells. Since osteoblasts are involved in mineralization, phosphate may be more potently incorporated into those as compared with other types of cells. Hence, it seems reasonable to speculate that yet unknown dedicated transporters mediate the incorporation of phosphate as well as BPs in osteoblasts.

5 CONCLUSION

Our results showed that the N-bisphosphonate zoledronate suppresses differentiation and cell viabilty of osteoblastic cells. Those inhibitory effects were markedly relieved by co-existence of the non-N-bisphosphonate etidronate, suggesting competition between zoledronate and etidronate for incorporation into cells via common phosphate transporters. Since the effects of zoledronate were not relieved by phosphonoformate, incorporation of bisphosphonates seems to be mediated by yet unknown transporters distinct from SLC20A and 34A family members in mouse osteoblasts.

CONFLICT OF INTEREST

None of the authors have a conflict of interest to declare.

CONSENT FOR PUBLICATION

All of the authors have read the final version of the manuscript, agreed with its contents, and have provided consent for publication.

AVAILABILITY OF DATA AND MATERIALS

All relevant raw data are freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

AUTHORS' CONTRIBUTIONS

Tomomi T. Baba designed the experiments, conducted data collection, and prepared the manuscript.

Toshihiro Miyazaki conducted real-time PCR analyses.

Yuko Ohara-Nemoto gave technical support and conceptual advice, and assisted with preparing the manuscript.

Takayuki K. Nemoto gave technical support and conceptual advice, and assisted with preparing the manuscript.

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FIGURE LEGENDS

FIGURE 1 Effects of BPs on differentiation of osteoblastic cells. ALP activities of MC3T3-E1 (A, B) and Saos2 (C, D) cells were determined following 9 days of culture with ZOL, ETI, or a combination of those. *p < 0.01, as compared to control; #p < 0.01, for comparisons between indicated groups

FIGURE 2 Mitigation of differentiation-suppression effect of ZOL by ETI. ALP activities of Saos2 cells were determined following 6 days of culture under various conditions, as indicated below the panel. Medium with or without ZOL and ETI was changed daily. *p < 0.01, as compared to control; #p < 0.01, for comparisons between indicated groups

FIGURE 3 Effect of phosphonoformate on reduction of differentiation of osteoblastic cells induced by ZOL. ALP activities modulated by ZOL alone or in combination with phosphonoformate (PFA) in MC3T3-E1 (A) and Saos2 (B) cells were determined after 10-day cultures. *p < 0.01, as compared to control; #p < 0.01, for comparisons between indicated groups

FIGURE 4 Representative images of ALP activity staining of Saos2 cells. Saos2 was cultured for 6 days in the absence (A) and presence of (B) 10 μM zoledronate, (C) 10 μM zoledronate with 100 μM etidronate, or (D) 10 μM zoledronate with 20 μM PFA. Bar: 500 μm.

FIGURE 5 Effects of BPs and phosphonoformate on cell viability of osteoblastic cells. Cell viability of MC3T3-E1 (A, B) and Saos2 (C, D) cells was determined using MTT assay findings obtained after 6 days of culture with or without ZOL, ETI, and phosphonoformate (PFA). *p < 0.01, as compared to control; #p < 0.01, for comparisons between indicated groups

FIGURE 6 Effects of BPs on cell viability of fibroblastic cells. Cell viability of NIH3T3 (A) and C3H10T1/2 (B) cells was determined using MTT assay findings obtained after 7 days of culture with or without ZOL, ETI, and phosphonoformate (PFA). *$p < 0.01$, as compared to control; $^{\#}p < 0.01$, for comparisons between indicated groups

FIGURE 7 Expression level of sodium phosphate cotransporter mRNA in osteoblastic and fibroblastic cells. Expression levels of *SLC34A1*, *SLC34A2*, *SLC34A3*, *SLC20A1*, and *SLC20A2* mRNAs were determined using quantitative RT-PCR, with expression of the *GAPDH* gene used as the internal control. The level of expression of the sodium phosphate cotransporter as compared to GAPDH in MC3T3-E1 cells was expressed as 1

FIGURE 1

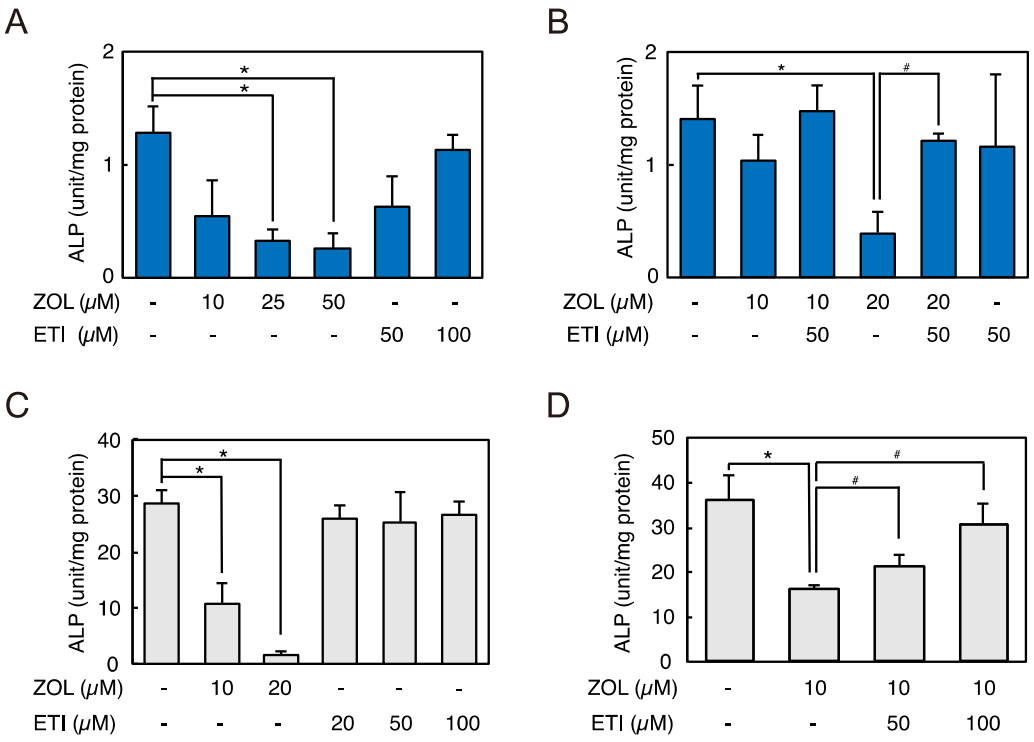
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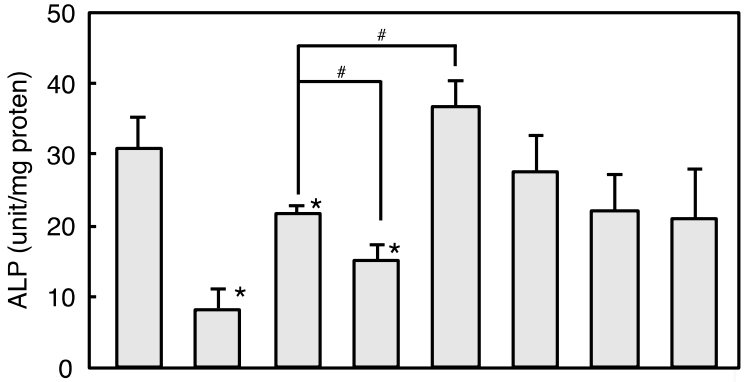
FIGURE 2



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	ETI (μM)	-	-	50	-	50	100	-	100
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	ETI (μM)	-	-	-	50	-	-	100	-
3	ZOL (μM)	-	10	10	10	50	10	10	-
	ETI (μM)	-	-	50	-	-	100	-	100
4	ZOL (μM)	-	-	-	-	-	-	-	-
	ETI (μM)	-	-	-	50	-	-	100	-

FIGURE 3

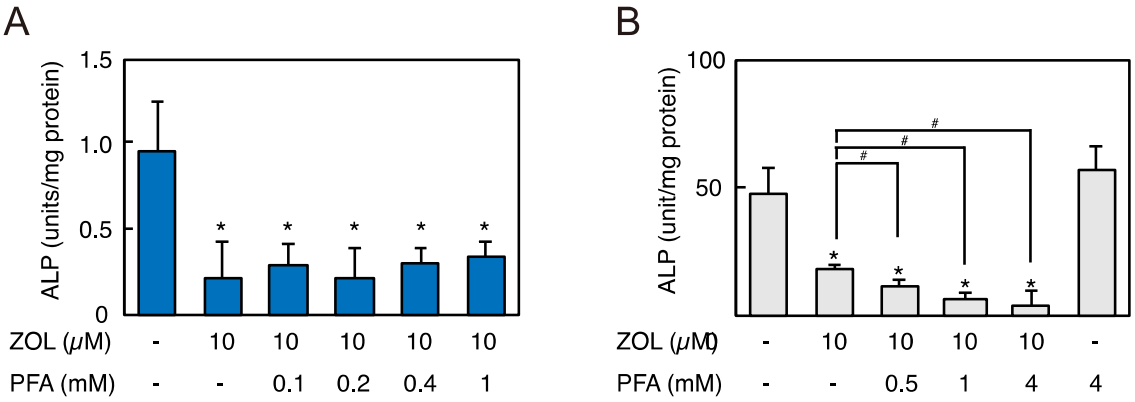

A
ALP (units/mg protein)
1.5
1.0
0.5
0
ZOL (μM) - 10 10 10 10 10
PFA (mM) - - 0.1 0.2 0.4 1
B
ALP (unit/mg protein)
100
50
0
ZOL (μM) - 10 10 10 10 -
PFA (mM) - - 0.5 1 4 4

FIGURE 4

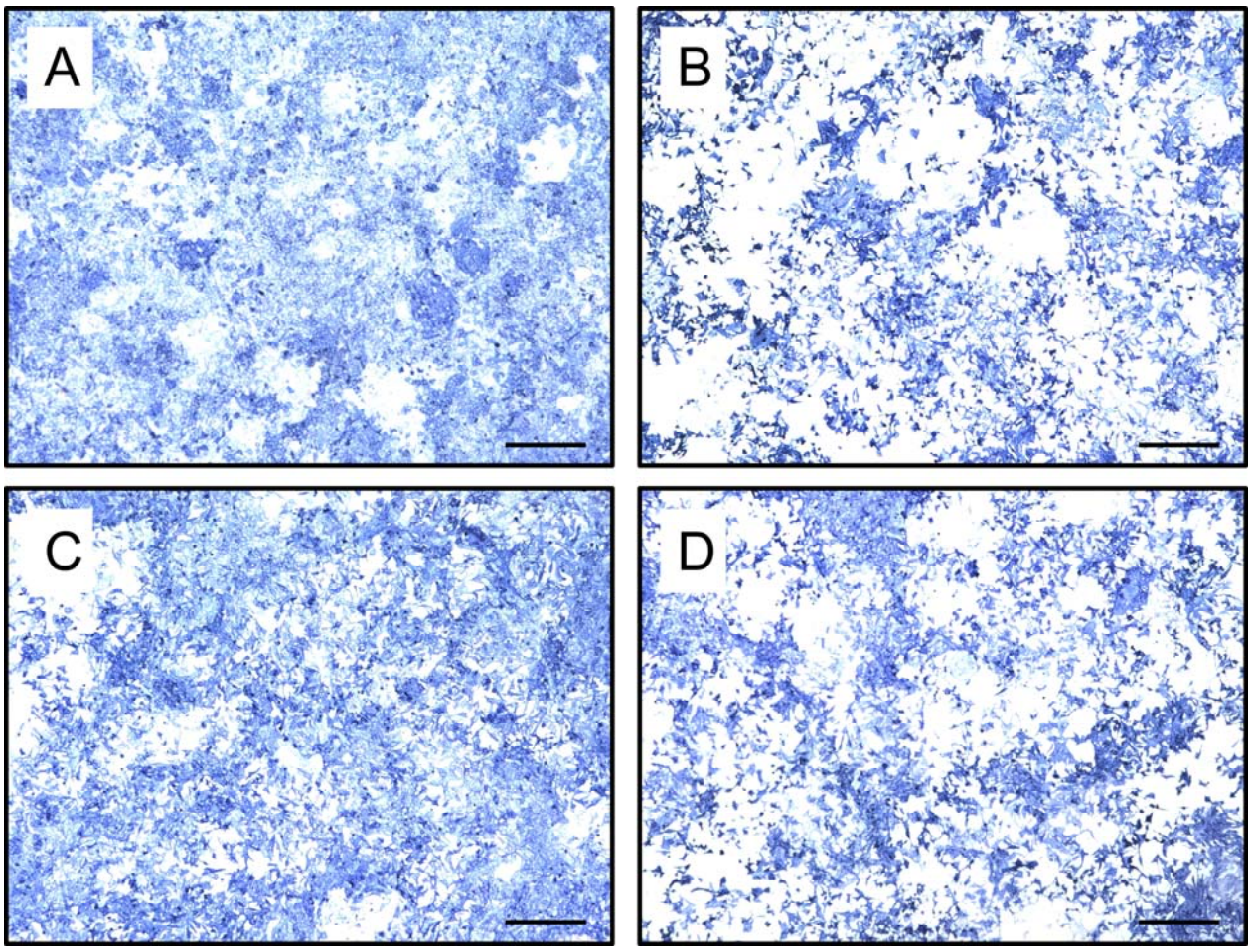


FIGURE 5

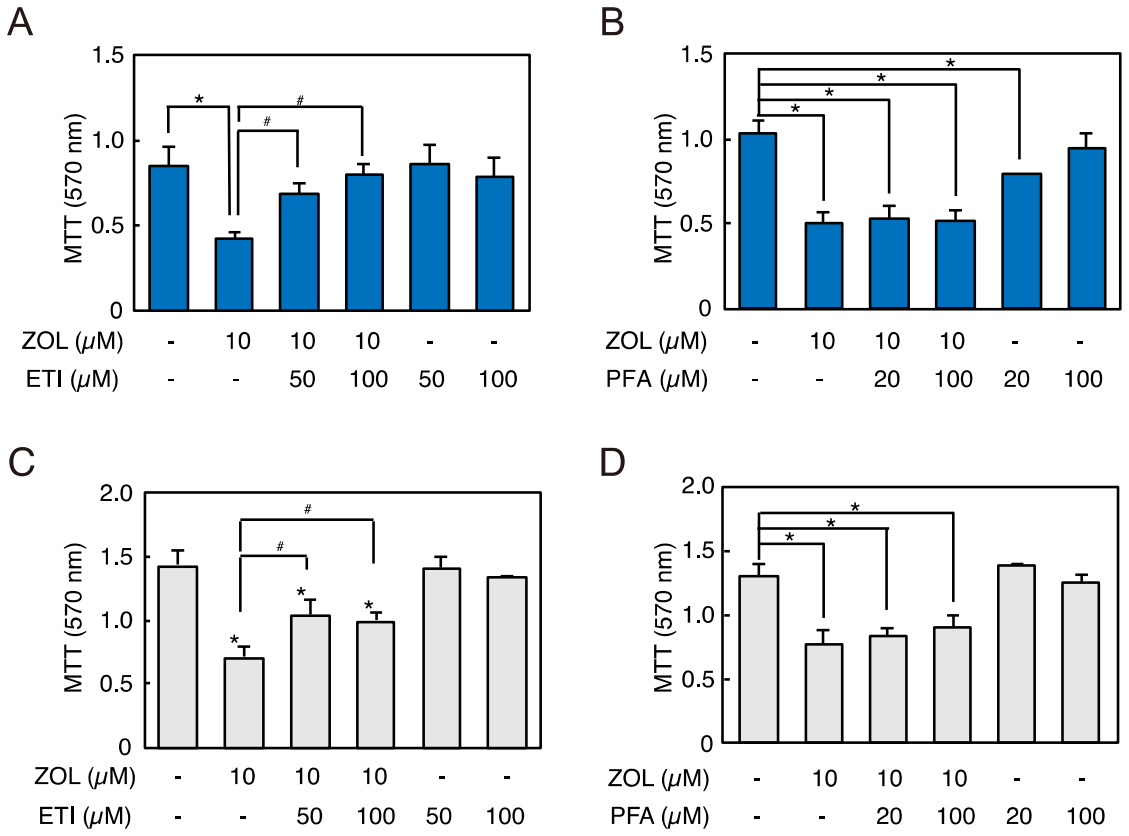


FIGURE 6

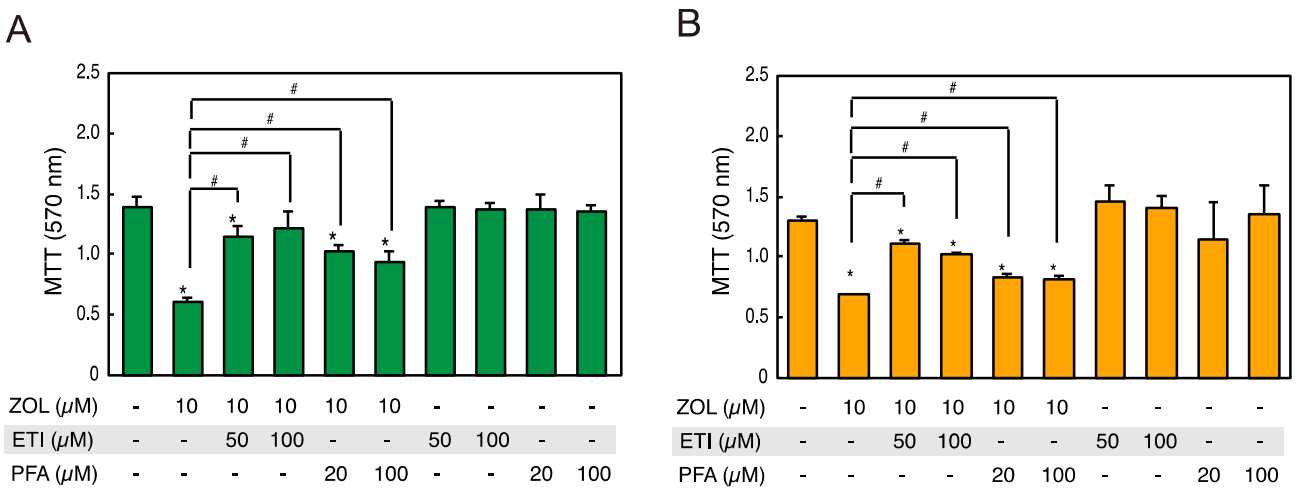
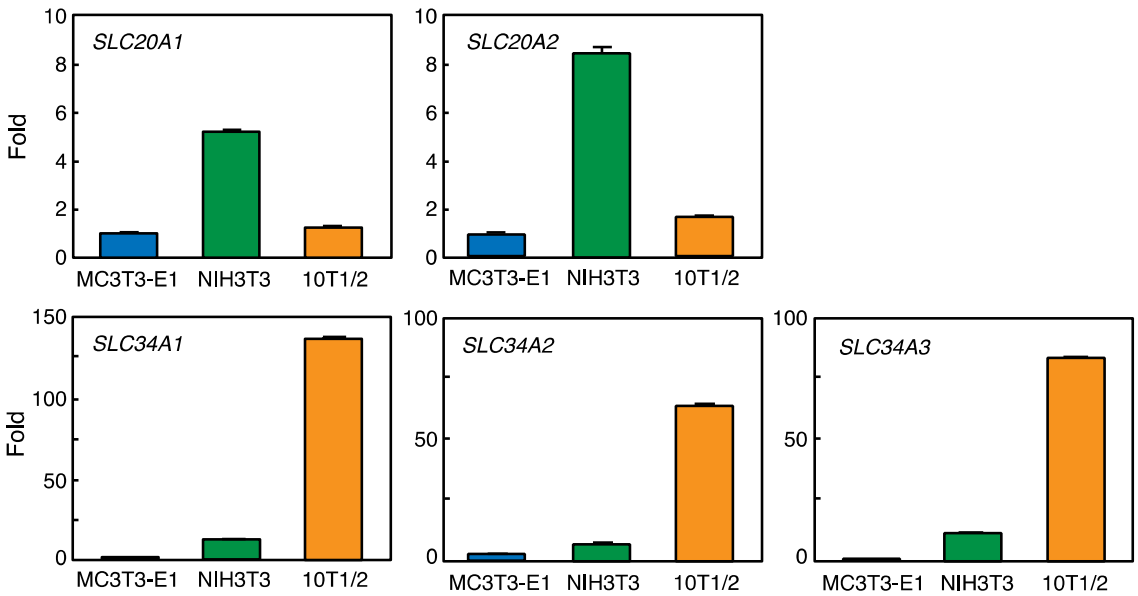

A
B
MTT (570 nm)
2.5
2.0
1.5
1.0
0.5
0
ZOL (μM) - 10 10 10 10 10 - - - -
ETI (μM) - - 50 100 - - 50 100 - -
PFA (μM) - - - - 20 100 - - 20 100

FIGURE 7


SLC20A1
SLC20A2
SLC34A1
SLC34A2
SLC34A3
Fold
MC3T3-E1
NIH3T3
10T1/2