Extensive protein expression changes induced by pamidronate in RAW 264.7 cells as determined by IP-HPLC

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Background. Bisphosphonate therapy has become a popular treatment for osteoporosis, Paget’s disease, multiple myeloma, osteogenesis imperfecta, myocardial infarction, and cancer despite its serious side effects. Bisphosphonate-induced molecular signaling changes in cells are still not clearly elucidated. Methods. As bisphosphonates are primarily engulfed by macrophages, we treated RAW 264.7 cells (a murine macrophage cell line) with pamidronate and investigated global protein expressional changes in cells by immunoprecipitation high performance liquid chromatography (IP-HPLC) using 218 antisera. Results. Pamidronate upregulated proliferation-activating proteins associated with p53/Rb/E2F and Wnt/β-catenin pathways, but downregulated the downstream of RAS signaling, pAKT1/2/3, ERK-1, and p-ERK-1, and subsequently suppressed cMyc/MAX/MAD network. However, in situ proliferation index of pamidronate-treated RAW264.7 cells was slightly increased by 3.2% versus non-treated controls. Pamidronate-treated cells showed increase in the expressions of histone- and DNA methylation-related proteins but decrease of protein translation-related proteins. NFkB signaling was also suppressed as indicated by the down-regulations of p38 and p-p38 and the up-regulation of mTOR, while the protein expressions related to cellular protection, HSP-70, NRF2, JNK-1, and LC3 were upregulated. Consequently, pamidronate downregulated the protein expressions related to immediate inflammation, cellular differentiation, survival, angiogenesis, and osteoclastogenesis, but upregulated PARP-1 and FAS-mediated apoptosis proteins. These observations suggest pamidronate affects global protein expressions in RAW 264.7 cells by stimulating cellular proliferation, protection, and apoptosis but suppressing immediate inflammation, differentiation, osteoclastogenesis, and angiogenesis. Accordingly, pamidronate appears to affect macrophages in several ways eliciting not only its therapeutic effects but also atypical epigenetic modification, protein translation, RAS and NFkB signalings. Therefore, our observations suggest pamidronate-induced protein expressions are dynamic, and the
affected proteins should be monitored by IP-HPLC to achieve the therapeutic goals during treatment.
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Short title: Pamidronate-induced protein expressions

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Abstract

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Bisphosphonate therapy has become a popular treatment for osteoporosis, Paget’s disease, multiple myeloma, osteogenesis imperfecta, myocardial infarction, and cancer despite its serious side effects. Bisphosphonate-induced molecular signaling changes in cells are still not clearly elucidated.

Methods.

As bisphosphonates are primarily engulfed by macrophages, we treated RAW 264.7 cells (a murine macrophage cell line) with pamidronate and investigated global protein expressional changes in cells by immunoprecipitation high performance liquid chromatography (IP-HPLC) using 218 antisera.

Results.

Pamidronate upregulated proliferation-activating proteins associated with p53/Rb/E2F and Wnt/β-catenin pathways, but downregulated the downstream of RAS signaling, pAKT1/2/3, ERK-1, and p-ERK-1, and subsequently suppressed cMyc/MAX/MAD network. However, in situ proliferation index of pamidronate-treated RAW264.7 cells was slightly increased by 3.2% versus non-treated controls. Pamidronate-treated cells showed increase in the expressions of histone- and DNA methylation-related proteins but decrease of protein translation-related proteins. NFkB signaling was also suppressed as indicated by the down-regulations of p38 and p-p38 and the up-regulation of mTOR, while the protein expressions related to cellular protection, HSP-70, NRF2, JNK-1, and LC3 were upregulated. Consequently, pamidronate downregulated the protein expressions related to immediate inflammation, cellular
differentiation, survival, angiogenesis, and osteoclastogenesis, but upregulated PARP-1 and FAS-mediated apoptosis proteins. These observations suggest pamidronate affects global protein expressions in RAW 264.7 cells by stimulating cellular proliferation, protection, and apoptosis but suppressing immediate inflammation, differentiation, osteoclastogenesis, and angiogenesis. Accordingly, pamidronate appears to affect macrophages in several ways eliciting not only its therapeutic effects but also atypical epigenetic modification, protein translation, RAS and NFkB signalings. Therefore, our observations suggest pamidronate-induced protein expressions are dynamic, and the affected proteins should be monitored by IP-HPLC to achieve the therapeutic goals during treatment.

Introduction

Bone undergoes constant remodeling maintained by a balance between osteoblasts and osteoclasts. Bisphosphonates inhibit the digestion of bone by causing osteoclasts to undergo apoptosis (Ito et al. 2001) and impair osteoclasts’ ability to form a ruffled border (Sato et al. 1991), to adhere to the bone surface, and to synthesize protons necessary for bone resorption. Furthermore, bisphosphonates suppress osteoclast activity by decreasing osteoclast progenitor development and recruitment (Cecchini et al. 1987; Endo et al. 1993). These diphosphate analogs inhibit intermediate enzymes of mevalonate pathway and are used to treat osteoporosis and Paget’s disease (historically osteitis deformans) (Abelson 2008). In osteoporosis and Paget’s disease, IV zoledronic acid is the first-choice treatment for Paget disease because of its efficacy.
and ease of administration (Wat 2014). The choice of zoledronic acid as the initial agent for most patients with active Paget disease is consistent with both the 2014 clinical practice guidelines of the Endocrine Society and the 2019 Paget’s Association guidelines (Singer et al. 2014).

Bisphosphonates bind calcium and are readily deposited in bone. They also change bone ultrastructures, e.g., they obliterate Haversian canals and deposit irregular and thick reversal lines (Acevedo et al. 2015; Carmagnola et al. 2013; Kim et al. 2017c; Lee 2013). The common side effects of bisphosphonates include bone pain, low blood calcium levels, nausea, and dizziness. In addition, bisphosphonate-related osteonecrosis of the jaw (BRONJ) may develop in patients who have used bisphosphonates long term (Marx et al. 2005; Ruggiero et al. 2004). Total 37 BRONJ cases out of 1014 patients using bisphosphonates for osteoporosis treatment showed 62.6% were associated with intravenous and 37.4% with oral application (Hansen et al. 2012). The incidence of BRONJ is known to be low among patients treated with oral bisphosphonates (Sarasquete et al. 2009). The estimated prevalence of oral BRONJ was 0.05-0.07%. And the average oral bisphosphonate treatment duration was 43.1 months (range, 5-120 months) (Hong et al. 2010). Among the 320 osteoporotic patients who underwent tooth extraction, 11 developed BRONJ, reflecting an incidence rate of 3.44%. And the incidence of BRONJ increased with age, was greater in the mandible than the maxilla, and was associated with a duration of administration of more than 3 years (Jeong et al. 2017; Marx et al. 2005; Ruggiero et al. 2004). The pathophysiology of BRONJ is currently unclear. BRONJ has been attributed to infection (Chirappapha et al. 2017; Choi et al. 2017; Park et al. 2009), bisphosphonate-related osteonecrosis.
(Guimaraes et al. 2013), quantitative reduction of the vascularization (Kun-Darbois et al. 2018), local immune dysfunction (Hoefert et al. 2016b), genetic predisposition like polymorphisms on CYP2C8 gene (Sarasquete et al. 2009), etc.

In addition, to the anti-osteoporotic effect of bisphosphonates, adjunctive bisphosphonate therapy appears to be effective at managing periodontitis (Akram et al. 2017), fibrous dysplasia (Majoer et al. 2017), and Gorham-Stout disease (Hammer et al. 2005; Kim et al. 2015). Therefore, it is believed bisphosphonates may have several systemic effects such as anti-inflammatory, anti-proliferative, and anti-angiogenesis effects (Kamel et al. 2012; Ohlrich et al. 2016; Ribatti et al. 2008). However, the biological effects of bisphosphonates in different cells have not been clearly elucidated at the molecular level.

Pamidronate (pamidronate disodium or pamidronate disodium pentahydrate) is a nitrogen-containing bisphosphonate and used to prevent bone loss due to steroid use like glucocorticotoid-induced low bone mineral density in children (Jayasena et al. 2015) or to inhibit calcium release from bone by impairing osteoclast-mediated bone resorption (Miyazaki et al. 2011), pamidronate is frequently used to treat high calcium levels (Polyzos et al. 2011). In addition, it has also been used as an experimental treatment for osteogenesis imperfecta and been studied for the treatment of complex regional pain syndrome (Chevreau et al. 2017).

Immunoprecipitation high-performance liquid chromatography (IP-HPLC) had been used previously by several authors to detect organic compounds including peptides quantitatively, but the technique used was complicated and of limited applicability (Clarke et al. 1998; Luo et al. 2013). Recently, a new IP-HPLC protocol was developed
to determine protein expression levels in different biological fluids, such as blood serum, urine, saliva (Kim & Lee 2015), inflammatory exudates (Kim et al. 2017a; b; 2018), and different protein extracts from cells (Kim et al. 2019; Yoon et al. 2018b), liver (Yoon et al. 2018a), and cancer tissues (Kim et al. 2017d). The IP-HPLC is comparable to enzyme-linked immunosorbent assay (ELISA). The former uses protein A/G agarose beads in buffer solution and UV spectroscopy to determine protein concentrations, whereas the latter uses fluorescence-conjugated antibodies fixed in plastic wells and fluoroscopy. Furthermore, multiple trials have shown that IP-HPLC can be used to rapidly determine multiple protein levels accurately (<±5% standard deviation) and reproducibly. In the previous study (Yoon et al. 2018b), 64 proteins were assessed by IP-HPLC 4-8 times repeatedly and their results showed low error range <±5% standard deviation (shown in the raw data sheets of supplementary dataset 5).

When pamidronate is injected into blood vessels, it immediately chelates Ca$^{++}$ (Ebetino et al. 2011; Fernandez et al. 2002) and is bound to serum albumin (90% of tiludronate) (Sansom et al. 1995), and subsequently recognized by macrophages, which suggests its various pharmacologic effects may be associated with the cellular functions of pamidronate-laden macrophages. Therefore, the present in vitro study was undertaken to investigate the effects of pamidronate on protein expressions in RAW 264.7 macrophages by IP-HPLC.

Materials & Methods

RAW264.7 cell culture with pamidronate treatment
RAW 264.7 cells, an immortalized murine macrophage cell line (ATCC, USA), were cultured as previously described (Yoon et al. 2018b). About 70% confluent RAW 264.7 cells grown on Petri dish surfaces were treated with 6.5 μM disodium pamidronate (similar to the therapeutic dose, 1.5 mg/kg) (Sigma, USA) for 12, 24, or 48 h; control cells were treated with 1 mL of normal saline. Cultured cells were harvested with protein lysis buffer (PRO-PREP™, iNtRON Biotechnology INC, Korea) and immediately preserved at -70°C until required.

**In situ proliferation index of RAW 264.7 cells after 24 h of pamidronate treatment**

RAW 264.7 cell proliferations were directly observed on plastic surfaces of Petri dishes after treatment with pamidronate at 6.5 μM for 12, 24, or 48 h, and compared with non-treated controls. When cells formed clusters containing 20-30 cells after 24 h of pamidronate treatment, ten representative histological images (taken at areas photographed before pamidronate treatment) were obtained using an inverted microscope (DP-73, Olympus Co., Japan). Cell numbers were obtained using the iSolution Lite program (IMT i-Solution Inc., Canada), proliferation indices were calculated by dividing increases in cell numbers after 24 h and 48 h of culture by initial cell numbers and compared between pamidronate treatment groups and non-treated controls.

**Immunoprecipitation high-performance liquid chromatography (IP-HPLC)**

Protein extracts (about 100μg) were subjected to immunoprecipitation using a protein A/G agarose column (Amicogen, Korea). Protein A/G agarose columns were...
separately pre-incubated with 1 μg of 218 different antisera (147 monoclonal antibodies and 71 polyclonal antibodies (36 antibodies were purified with affinity columns) specific to target amino acid motifs (product companies were listed in Table 1); for proliferation-related proteins (n=11), cMyc/MAX/MAD signaling proteins (n=3(1)), p53/Rb/E2F signaling proteins (n=4(2)), Wnt/β-catenin signaling proteins (n=6), epigenetic modification-related proteins (n=7), protein translation-related proteins (n=5), growth factor-related proteins (n=18), RAS signaling proteins (n=22), NFκB signaling proteins (n=12(6)), up-regulated inflammatory proteins (n=17), down-regulated inflammatory proteins (n=27(1)), p53-mediated apoptosis-related proteins (n=15(2)), FAS-mediated apoptosis-related proteins (n=5(3)), cell survival-related proteins (n=5(11)), protection-related proteins (n=12(13)), differentiation-related proteins (n=11(11)), oncogenesis-related proteins (n=10(10)), angiogenesis-related proteins (n=14(9)), osteogenesis-related proteins (n=11(4)), and control housekeeping proteins (n=3) (numbers in parenthesis indicate number of overlapping antibodies, Table 1).

Briefly, each protein sample (about 100μg) was mixed with 5 mL of binding buffer (150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA, 0.2mM sodium vanadate, 0.2mM PMSF, 0.5% NP-40, and mixture of protein inhibitors (Sigma, USA)) and incubated with protein A/G agarose beads (200 μL, Amicogen, Korea) bound with objective antibody on a rotating stirrer for 1 hour at 4°C. After washing beads with PBS (phosphate buffered saline solution), target proteins were eluted using 150μL of IgG elution buffer (Pierce, USA). Immunoprecipitated proteins were analyzed using a HPLC unit (1100 series, Agilent, USA) equipped with a reverse phase column and a micro-analytical detector system (SG Highteco, Korea), using 0.15M NaCl/20% acetonitrile
solution at 0.4 mL/min for 30 min, and proteins were detected using a UV spectrometer at 280 nm. Control and experimental samples were run sequentially to allow comparisons. For IP-HPLC, whole protein peak areas (mAU*s) were mathematically calculated with analytical algorithm (see Supplementary data 1) by subtracting negative control antibody peak areas, and protein expression levels (mAU) were compared and normalized using the square roots of protein peak areas. Analyses were repeated two to six times to achieve mean standard deviations of ≤ ±5% (RAW data, Supplementary data 2). Objective protein expression level (%) between experiment and control groups were calculated and results were analyzed using the chi-squared test program (Kim et al. 2019; Yoon et al. 2018a; b).

The housekeeping proteins β-actin, α-tubulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also used as internal controls. Expressional changes of housekeeping proteins were adjusted to < ±5% using a proportional basal line algorithm. Protein expressional changes of ≤ ±5%, ±5-10%, ±10-20%, and ≥±20% change were defined as minimal, slight, meaningful, or marked, respectively.

When the IP-HPLC results were compared with the western blot data of cytoplasmic housekeeping protein (β-actin), the former exhibiting minute error ranges less than ±5% and could be analyzed statistically, while the latter showed a large error range of more than 20%, and thus it was almost impossible to analyze them statistically (see Supplementary data 3). Therefore, the present study utilized IP-HPLC to statically analyze global protein expression changes in pamidronate-treated RAW 264.7 cells rather than Western blot method (Seo et al. 2019).
Statistical analysis

Proportional data (%) of experimental and control groups were plotted, and analyses were repeated two to six times until standard deviations were $\leq \pm 5\%$. Results were analyzed by measuring standard error ($s = \pm \frac{\sigma}{\sqrt{n}}$). The expressions of control housekeeping proteins, that is, $\beta$-actin, $\alpha$-tubulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) non-responsive ($\leq 5\%$) to 12, 24, or 48 h of pamidronate treatment.

Results

In situ proliferation index of RAW 264.7 cells after 24 h of pamidronate treatment

Both 6.5 $\mu$M pamidronate-treated RAW 264.7 cells and non-treated controls proliferated on Petri dishes and formed large cell clusters after 24 h of culture (Figs. 1 A-F). The in situ proliferation index of pamidronate-treated RAW 264.7 cells was 73.1 $\pm$ 2.32 % at 24 h, 74.7 $\pm$ 2.8 % at 48 h, and that of non-treated RAW 264.7 cells was 69.9 $\pm$ 2.46 % by the in situ proliferation assay (Fig. 1 G). These results indicate pamidronate slightly elevated mitosis of RAW 264.7 cells, murine macrophages, by 3.2% in 24 h and 4.8% in 48 h of culture.

Effects of pamidronate on the expressions of proliferation-related proteins in RAW 264.7 cells

RAW 264.7 cells treated with 6.5 $\mu$M pamidronate for 12, 24, or 48 h exhibited increases in levels of proliferation-activating proteins, Ki-67 (by 12.6%), proliferation cell
nuclear antigen (PCNA, 4%), cyclin dependent kinase 4 (CDK4, 10.1%), mitosis phase
promoting factor (MPF) recognized by a mitosis-specific monoclonal antibody (MPM-2, 10.7%), polo-like kinase 4 (PLK4, 7.3%), cyclin D2 (4.9%), and lamin A/C (8.7%) and
also increases in proliferation-inhibiting proteins, p14 (21.1%), p15/16 (14%), p27 (18.7%) levels versus non-treated controls. These expressional changes of proliferation-activating proteins became noticeable after 24 and 48 h of pamidronate treatment but remained at < ±15%, but the proliferative activity of RAW 264.7 cells was limited by the increase of protein expressions of proliferation-inhibiting proteins (Figs. 2 A and B).
These results suggest pamidronate might have a mild proliferative effect on RAW 264.7 cells.

**Effects of pamidronate on the expressions of cMyc/MAX/MAD network proteins in RAW 264.7 cells**

The expressions of cMyc and MAX decreased by 12.6% and 7.9%, respectively, after 12 h of pamidronate treatment and consistently decreased by 7.4% and 1.8%, respectively, at 48 h versus non-treated controls, whereas MAD-1 expression decreased by a maximum of 15.7% after 12 h of treatment and slightly increased by 1.7% at 48 h. On the other hand, p27 expression increased by 18.7% after 48 h of treatment (Figs. 2 C and D). These results indicate pamidronate suppressed cMyc/MAX/MAD network expressions and resulted low level of Myc-Max heterodimers which are strongly binding to E-box (CACGTG). These expressional changes of cMyc/MAX/MAD network proteins may negatively contribute to the proliferative effect of pamidronate on RAW 264.7 cells.
Effects of pamidronate on the expressions of p53/Rb/E2F signaling proteins in RAW 264.7 cells

Pamidronate increased the expression of p53 in RAW 264.7 cells by 14.5% at 12 h but its increase was diminished by 8.7% at 48 h versus non-treated controls, and decreased the expression of negative regulator of p53, MDM2, by 4.3% at 12 h. Rb-1 expression was also slightly increased by 7.9%, 7.3%, 15.8% at 12, 24, and 48 h, respectively. Notably, the expression of CDK4, activator of Rb-1 was increased by 16.6% at 12 h, although p21, CDK inhibitor was also increased by 11% at 12 h concurrent with the elevation of p53 expression. Resultantly, the expression of the objective transcription factor, E2F-1, increased by 12.8% at 24 h and by 9.1% at 48 h (Figs. 2 E and F). This up-regulation of p53/Rb/E2F signaling by pamidronate may indicate the increase in the level of Rb-1 phosphorylation and positively affect RAW 264.7 cell proliferation.

Effects of pamidronate on the expressions of Wnt/β-catenin signaling proteins in RAW 264.7 cells

The expressions of Wnt1, β-catenin, and adenomatous polyposis coli (APC) in RAW 264.7 cells were increased by 25.2%, 12.9%, and 8.7%, respectively, by pamidronate at 24 h versus non-treated controls, while the expression of E-cadherin was reduced by 13.8% coincident with slight increase of snail expression by 2.2% at 48 h. Resultantly, the expression of the objective transcription factor T-cell factor 1 (TCF-1) was increased by 9.3% at 12 h and by 13.3% at 48 h (Figs. 2 G and H). These findings regarding the
up-regulation of Wnt/β-catenin signaling and downregulation of E-cadherin by pamidronate may have significantly increased RAW 264.7 proliferation.

**Effects of pamidronate on the expressions of epigenetic modification-related proteins in RAW 264.7 cells**

Histone H1 expression increased in pamidronate treated cells to 131.3% at 24 h and to 122.3% at 48 h versus non-treated controls. Regarding histone modification, the expression of lysine-specific demethylase 4D (KDM4D) was 5% lower at 24 h, but that of histone deacetylase 10 (HDAC10) showed little change. With respect to DNA modification, DNA (cytosine-5)-methyltransferase 1 (DNMT1) expression was 10.4% higher at 48 h and those of DNA methyltransferase 1-associated protein 1 (DMAP1) and methyl-CpG binding domain 4 (MBD4) were 18.2% and 15.9% higher at 24 h, respectively, and were maintained at 8.6% and 21% higher at 48 h (Figs. 3 A and B). These results suggest pamidronate increased histone and DNA methylation and subsequently hindered DNA transcription in RAW 264.7 cells, and that this epigenetic effect of pamidronate might be related to the down-regulation of various proteins.

**Effects of pamidronate on the expressions of translation-related proteins in RAW 264.7 cells**

RAW 264.7 cells treated with pamidronate showed gradual reductions in protein translation-related protein levels versus non-treated controls. Although deoxyhypusine hydroxylase (DOHH) expression slightly increased by 17% and 5.4% after 24 and 48 h of treatment, respectively, deoxyhypusine synthase (DHS) expression was consistently
reduced by 18.8% and 16.8%, respectively, at these times. The protein expressions of
objective factors of protein translation, that is, eukaryotic translation initiation factor 5A-1
(eIF5A-1) and eIF5A-2, were also reduced by 2.9% and 3.2% at 48 h, respectively,
while that of eukaryotic translation initiation factor 2-α kinase 3 (eIF2AK3; an inactivator
of eIF2) was increased by 6.8% at 24 h (Figs. 3 C and D). We considered that the
pamidronate-induced reductions in the expressions of translation-related proteins might
cause global inactivation of cellular signaling. However, changes in the levels of these
protein levels which are normally abundant in cells tended to remain at < ±15% after 48
h of pamidronate treatment.

**Effects of pamidronate on the expressions of growth factor-related proteins in**
**RAW 264.7 cells**

RAW 264.7 cells treated with pamidronate for 48 h showed increases in the
expressions of growth hormone (by GH, 13.5%), growth hormone-releasing hormone
(GHRH, 6.6%), platelet-derived growth factor-A (PDGF-A, 13.2%), insulin-like growth
factor-1 (IGF-1, 12.8%), IGF-2 receptor (IGFIIR, 22.5%), epidermal growth factor
receptor (ErbB-1, HER1, 19.2%), HER2 (receptor tyrosine-protein kinase ErbB-2, 13%),
transforming growth factor-β1 (TGF-β1, 16.4%), TGF-β2 (27.7%), TGF-β3
(20.7%), SMAD4 (18.4%), fibroblast growth factor-7 (FGF-7 known as a keratinocyte
growth factor, 20.7%), and estrogen receptor β (ERβ, 14%) over 48 h versus non-
treated controls whereas the expressions of FGF-1, FGF-2, and CTGF decreased by
14%, 13.9%, and 9.6%, respectively. The expressions of other growth factor-related
proteins, including those of hepatocyte growth factor α (HGFα) and Met, changed
minimally (by ±5%) like the expressions of housekeeping proteins (Figs. 3 E and F).

These results indicate pamidronate influenced the expressions of many growth factors necessary for the growth and differentiation of RAW 264.7 cells, that is, it increases the expressions of GH, GHRH, PDGF-A, IGF-1, IGFIIR, HER1, HER2, TGF-β1, TGF-β2, TGF-β3, SMAD4, FGF-7, and ERβ, while reduces the expressions of extracellular matrix maturation, that is, FGF-1, FGF-2, and CTGF.

Effects of pamidronate on the expressions of RAS signaling proteins in RAW 264.7 cells

Although many RAS upstream signaling proteins were upregulated by pamidronate, RAS downstream effector proteins were significantly downregulated. The increase in the expressions of KRAS (by 16.8%), NRAS (7.7%), HRAS (12.6%), phosphatidylinositol 3-kinase (PI3K, 12.3%), Jun N-terminal protein kinase-1 (JNK-1, 12.4%), mammalian target of rapamycin (mTOR, 14.2%), phosphatase and tensin homolog (PTEN, 11.2%), RAF-B (serine/threonine-protein kinase B-Raf, 18%), Rab 1 (GTPase, 10.5%), neurofibromin 1 (NF-1, 16%), protein kinase C (PKC, 10%), p-PKC (14%), son of sevenless homolog 1 (SOS-1, 22.7%), SOS-2 (14.1%), and signal transducer and activator of transcription-3 (STAT3, 7.2%) were found over 48 h of treatment versus non-treated controls, while RAS downstream expressions of pAKT1/2/3, 5' AMP-activated protein kinase (AMPK), extracellular signal–regulated kinase 1 (ERK-1), and p-ERK-1 were decreased by 8.8%, 2.9%, 7.9%, and 8%, respectively. And the expressions of A-kinase anchoring protein (AKAP) and caveolin-1 were also reduced by 16.6% and 15.4%, respectively (Figs. 3 G and H). These results
indicate pamidronate significantly reduced the expressions of the downstream effector proteins, ERK-1 and p-ERK-1, albeit many upstream proteins (KRAS, NRAS, HRAS, PI3K, JNK-1, mTOR, PTEN, RAF-B, Rab 1, NF-1, PKC, p-PKC, SOS-1, SOS-2, and STAT3, and thus, suggest RAS signaling (a major signal for cellular growth) was gradually attenuated in RAW 264.7 macrophages.

**Effects of pamidronate on the expressions of NFkB signaling proteins in RAW 264.7 cells**

Pamidronate had different effects on the expressions of NFkB signaling proteins in RAW 264.7 cells. The expressions of NFkB upstream signaling proteins were increased by pamidronate, that is; nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB, by 11%), PTEN (11.2%), mTOR (14.2%), peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α, 10.4%), and nuclear factor (erythroid-derived 2)-like 2 (NRF2, 12.1%), while the expressions of NFkB downstream effector proteins, pAKT1/2/3, growth arrest and DNA damage 45 (GADD45), GADD153, p38, p-p38, steroid receptor coactivator-1 (SRC1), and multi-drug resistance (MDR) were reduced by 8.8%, 13.4%, 17.3%, 10.2%, 10.2%, 15.5%, and 19.1%, respectively. The expression of ikappaB kinase (IKK) expressions was decreased by 15.5% after 48 h of treatment, and those of tumor necrosis factor α (TNFα) and activating transcription factor 6 (ATF6) only decreased by < 5%. These results indicate the expressions of many proteins that enhance NFkB signaling tended to be downregulated by treatment with pamidronate for 48 h, that is, pAKT1/2/3, GADD45, GADD153, p38, p-p38, SRC1, MDR, and more, proteins suppressing NFkB signaling tended to be upregulated by
pamidronate, that is, PTEN, mTOR, PGC-1α, and NRF2 (Figs. 4 A and B). These results indicate pamidronate effectively suppressed NFκB signaling in RAW 264.7 cells.

**Effects of pamidronate on the expressions of upregulated inflammatory proteins in RAW 264.7 cells**

The proteins upregulated by pamidronate were; CD3 (a T cell co-receptor constituting T cell receptor (TCR) complex, by 21.6%), CD4 (a co-receptor of the T cell receptor (TCR), 12%), neural cell adhesion molecule (NCAM, CD56, detecting natural killer cells, gamma delta (γδ) T cells, activated CD8+ T cells, and dendritic cells, 32.8%), CD80 found on the surface of dendritic cells, B cells, monocytes and antigen-presenting cells (16.9%), programmed cell death protein 1 (Pdcd-1/1, CD279, 18.9%), IL-8 (a chemoattractant cytokine, 18.3%), IL-12 (a T cell-stimulating factor, 11.4%), MMP-3 stromelysin-1 involved in wound repair, progression of atherosclerosis, and tumor initiation (17.7%), MMP-9 (a regulating factor for neutrophil migration, angiogenesis, and wound repair, 13.3%), MMP-12 (a macrophage metalloelastase contributing to elastin degradation, 10.4%), cathepsin C (a lysosomal exo-cysteine protease degrading various extracellular matrix components, 21.6%), C-X-C chemokine receptor type 4 (CXCR4, 17%), monocyte chemotactic protein-1 (MCP-1, an eotaxin, 33.5%), cyclooxygenase 2 (COX2, an important mediator of inflammation, 13.8%), versican (a large extracellular matrix proteoglycan that is involved with tissue homeostasis and inflammation, 26.5%), and kininogen (a constituent of the blood coagulation system as well as the kinin-kallikrein system, 13%) (Figs. 4 C and D).
These results indicate pamidronate stimulated cell-mediated immunity and chronic inflammation by upregulation of CD3, CD4, NCAM, CD80, Pdcd-1/1, IL-8, IL-12, MMP-3, MMP-9, MMP-12, cathepsin C, CXCR4, MCP-1, COX2, versican, and kininogen in RAW 264.7 cells.

Effects of pamidronate on the expressions of downregulated inflammatory proteins in RAW 264.7 cells

Proteins downregulated were tumor necrosis factor α (TNFα, by 11.6%), IL-1α (a “dual-function cytokine”, which means it plays a role in the nucleus by affecting transcription, apart from its extracellular receptor-mediated effects as a classical cytokine, 24.1%), IL-6 (an important mediator of fever and of the acute phase response, 8.6%), IL-10 (an anti-inflammatory cytokine, 15.2%), IL-28 which play a role in the adaptive immune response (6%), B-lymphocyte antigen CD20 (6%), CD28 which is necessary for T cell activation and survival (6.8%), PECAM-1 (CD31, a role for leukocyte transmigration, angiogenesis, and integrin activation, 11.3%), CD34 (a transmembrane phosphoglycoprotein protein which is expressed in early hematopoietic and vascular-associated tissues, 25.4%), CD40 (a costimulatory protein found on antigen presenting cells, 8.5%), intercellular adhesion molecule 1 (ICAM-1, CD54, 19.8%), CD68 (a marker for the various cells of the macrophage lineage, 21.6%), CD99 (MIC2, a heavily O-glycosylated transmembrane protein which is expressed in all leukocytes, 7.6%), vascular cell adhesion molecule-1 (VCAM, CD106, a role in leukocyte-endothelial cell signal transduction, 12.7%), cathepsin G (an important role in eliminating intracellular pathogens and breaking down tissues at inflammatory sites,
7.8%), cathepsin K (a lysosomal cysteine protease involved in bone remodeling and resorption, 27.9%), COX1 (prostaglandin G/H synthase 1 involved in cell signaling and maintaining tissue homeostasis, 15%), lysozyme (17.1%), macrophage colony-stimulating factor (M-CSF, 16.1%), MMP-1 (an interstitial collagenase, 23.3%), MMP-2 (a role for lymphangiogenesis, 22%), MMP-10 (stromelysin-2, 14.1%), leukotriene A4 hydrolase (LTA4H, 4.9%), cathelicidin antimicrobial peptides LL-37 (an antimicrobial peptide, 23.6%), α1-antitrypsin (a protease inhibitor, 22.1%), β-defensin 1 (a microbicidal and cytotoxic peptide, 7.4%), β-defensin 2 (a microbicidal and cytotoxic peptide, 4.8%), and β-defensin 3 (a microbicidal and cytotoxic peptide, 7.6%) over 48 h of pamidronate treatment (Figs. 4 E and F).

These results indicate pamidronate inhibited innate immunity, immediate inflammatory reaction, and wound repair processes by downregulation of TNFα, IL-1α, IL-6, IL-10, IL-28, CD20, CD28, PECAM-1, CD34, CD40, CD68, CD99, VCAM, cathepsin G, cathepsin K, COX1, lysozyme, M-CSF, MMP-1, MMP-2, MMP-10, LTA4H, LL-37, α1-antitrypsin, β-defensin 1, β-defensin 2, and β-defensin 3 in RAW 264.7 cells.

**Effects of pamidronate on the expressions of p53-mediated apoptosis-related proteins in RAW 264.7 cells**

Pamidronate affected the expressions of p53-mediated apoptosis-related proteins, particularly p53 protein, which was increased by 14.5% after treatment for 24 h, while the expression of E3 ubiquitin-protein ligase MDM2 was decreased by 4.3% at 12 h versus non-treated controls. After treatment for 48 h, the expressions of pro-apoptotic proteins, Bcl-2-associated death promoter (BAD), Bcl-2 homologous antagonist/killer
(BAK), pro-apoptotic member of the Bcl-2 protein family NOXA, apoptosis regulator BAX, and apoptosis inducing factor (AIF) were decreased by 12.4%, 12.2%, 26.6%, 23.5%, and 16%, respectively, but the expressions of p53 upregulated modulator of apoptosis (PUMA) and apoptotic protease activating factor 1 (APAF-1) were increased by 12.4% and 5.4%. The expressions of apoptosis executor proteins, caspase 9, c-caspase 9, caspase 3, c-caspase 3, and poly [ADP-ribose] polymerase 1 (PARP-1) increased by 28%, 20.9%, 27.5%, 14.6%, and 26.5% at 48 h, whereas that of cleaved PARP-1 (c-PARP-1) was reduced by 18.2% at 24 h. On the other hand, the expression of the anti-apoptosis protein, BCL2 gradually decreased by 12.9% at 48 h (Figs. 5 A and B). These results indicate pamidronate induced PARP-1/caspase 9/caspase 3-mediated apoptosis independently of p53/BAX and AIF signalings and in RAW 264.7 cells, which suggests pamidronate might induce PARP-1-mediated non-apoptotic cell death.

**Effects of pamidronate on the expressions of FAS-mediated apoptosis-related proteins in RAW 264.7 cells**

RAW 264.7 cells treated with pamidronate showed increases in the expressions of FAS-mediated apoptosis-related proteins as compared with non-treated controls. After treatment with pamidronate for 48 h, the expressions of death receptors on cell surfaces, that is, of FAS, FAS ligand (FASL), and FAS-associated protein with death domain (FADD), were increased by 4.6%, 15.3%, and 24.4%, respectively, and those of caspase 8, caspase 3, and c-caspase 3 were also increased by 30.8%, 27.5%, and 14.6%, respectively. On the other hand, the expressions of FLICE-like inhibitory protein (FLIP) and BH3 interacting-domain death agonist (BID) were minimally changed (<
These findings indicate pamidronate might induce apoptosis via caspase 8 and 3 through FASL/FAS/FADD signaling in RAW 264.7 cells.

**Effects of pamidronate on the expressions of cell survival-related proteins in RAW 264.7 cells**

RAW 264.7 cells treated with pamidronate showed variable changes in the expressions of cell survival-related proteins as compared with non-treated controls. The expressions of PTEN, telomerase reverse transcriptase (TERT), NRF2, PGC-1α, PKC, p-PKC, and focal adhesion kinase (FAK) were increased by 11.2%, 8.6%, 12.1%, 10.4%, 10%, 14%, and 13.7%, respectively, after 48 h of pamidronate treatment, while those of pAKT1/2/3, survivin, BCL2, p38, p-p38, and SP-1 were reduced by 9.1%, 10.9%, 12.9%, 10.2%, 10.2%, and 9.6%, respectively. On the other hand, the expressions of SP-3, AMPK, and ATF6 hardly changed (< ±5%) (Figs. 5 E and F).

These results suggested cell survival was enhanced by the up-regulations of NRF2/PGC-1α and PKC/FAK signaling, which are features of mitochondrial biogenesis and the signal transduction cascade, respectively, but reduced by the down-regulations of AKT/survivin/BCL2 and p38/SP-1 signalings, which are features of cell exposure to stressors, such as oxidative damage. These results suggest that pamidronate increases energy metabolism and signal transduction in RAW 264.7 cells, but that the abilities of their cells to overcome different cytological stressors is relatively poor.

**Effects of pamidronate on the expressions of cell protection-related proteins in RAW 264.7 cells**
The expressions of cell protection-related proteins in RAW 264.7 cells were increased by pamidronate; heat shock protein-70 (HSP-70) by 21.7% at 12 h, 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase β-2 (PLC-β2) by 30.6% at 48 h, PI3K by 12.3% at 48 h, master regulator of mitochondrial biogenesis PGC-1α by 10.4% at 12 h, mitogen-activated protein kinase JNK-1 by 12.4% at 48 h, PKC by 10% at 48 h, p-PKC by 14% at 12 h, focal adhesion kinase (FAK) by 13.7% at 24 h, mucin 1 by 3.3% at 48 h, and mucin 4 by 10.8% at 24 h versus non-treated controls, whereas the expressions of HSP-27, HSP-90, and 5’AMP-activated protein kinase (AMPK) were decreased by pamidronate; by 3.6% at 48 h, by 12.7% at 12 h, and by 2.9% at 12 h, respectively.

The expressions of anti-oxidative proteins in RAW 264.7 cells were increased by pamidronate; detoxifying enzyme glutathione S-transferase ω 1 (GSTO1) by 9.9% at 24 h, autophagy substrate LC3 by 30.9% at 12 h, NRF2 by 12.1% at 24 h, while the expressions of Cu-Zn superoxide dismutase-1 (SOD-1), nitric oxide synthase 1 (NOS1), heme oxygenase 1 (HO-1), and endoplasmic reticulum (ER) stress-regulated transmembrane transcription factor ATF6 were decreased by pamidronate; by 13% at 24 h, by 19.2% at 48 h, by 5.5% at 48 h, and by 5.5% at 48 h, respectively. And sodium-dependent vitamin C transporter 2 (SVCT2) and cross-linking enzyme transglutaminase 2 (TGase-2) were decreased by 11.1% and 14.6% at 12 h, respectively, but gradually increased by 8.1% and 17.6% at 48 h, respectively (Figs. 6 A and B).

Although RAW 264.7 cells treated with pamidronate appeared to be silent, as they exhibited reduced NFκB signaling and had low levels of antioxidant-related proteins, SOD-1, NOS1, and HO-1 in their cytoplasms, they had higher levels of the cell
protection-related proteins, HSP-70, PLC-β2, PI3K, PGC-1α, JNK-1, PKC, p-PKC, FAK, and mucin 1 and 4 than non-treated controls. These observations suggest the expressions of cellular protection-related proteins, such as those involved in detoxification and autophagy, are upregulated by pamidronate in RAW 264.7 cells despite reduced RAS and NFkB signalings.

Effects of pamidronate on the expressions of differentiation-related proteins in RAW 264.7 cells

RAW 264.7 cells treated with pamidronate for 48 h showed increases in the expressions of the differentiation-related proteins p63 (25.4%), vimentin (39.8%), transglutaminase 2 (TGase-2, 17.6%), PLC-β2 (30.6%), PI3K (12.3%), PKC (10%), p-PKC (14%), FAK (20.7%), integrin α5 (21%), neural cell adhesion molecule (NCAM, CD56, 32.9%), cysteine rich protein-1 (CyRP-1, 19.6%), AP-1 complex subunit mu-1 (AP1M1, 12.8%), transcription factor SP-3 (5.3%), sonic hedgehog (SHH, 22%), and S-100 (27.1%) as compared with non-treated controls, but reductions in the expressions of the differentiation-related proteins, caveolin-1 (15.4%), α-actin (10.8%), intercellular adhesion molecule 1 (ICAM-1, CD54, 19.8%), homing cell adhesion molecule (HCAM, CD44, 27.5%), platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31, 11.3%), receptor for sonic hedgehog PTCH-1 (12.9%), transcription factor SP-1 (9.6%), and cystatin A (28.9%) (Figs. 6 C and D).

The proteins essential for the differentiation, migration, adhesion, and endocytosis of RAW 264.7 cells, that is, p63, vimentin, TGase-2, PLC-β2, PI3K, PKC, p-PKC, FAK, integrin α5, NCAM (CD56), CyRP-1, SP-3, SHH, and S-100 were upregulated by
treatment with pamidronate for 48 h, whereas some proteins required for further
differentiation into active macrophages or dendritic cells, that is, caveolin-1, α-actin,
ICAM-1 (CD54), HCAM (CD44), PECAM-1 (CD31), PTCH-1, SP-1, and cystatin A, were
downregulated. These observations suggest pamidronate-treated RAW 264.7 cells
maintain major signal transduction organelles for cellular proliferation and protection but
are defective in terms of advanced cytological differentiation due to reductions in the
expressions of caveolin-1, α-actin, PTCH-1, and SP-1.

Effects of pamidronate on the expressions of oncogenic proteins in RAW 264.7
cells
RAW 264.7 cells treated with pamidronate showed increases in the expressions of
the oncogenic proteins, carcinoembryonic antigen (CEA, 21.2%), conserved regulatory
molecule 14-3-3 (31.4%), deleted in malignant brain tumors 1 protein (DMBT1, 22.8%),
telomerase reverse transcriptase (TERT, 8.6%), transmembrane subunit containing
three EGF-like domains mucin 4 (10.8%), and serine protease inhibitor maspin (22.7%)
as compared with non-treated controls, and also increases in the expressions of the
tumor suppressor proteins, phosphatase and tensin homolog (PTEN, 11.2%), mTOR
(14.2%), GTPase-activating protein neurofibromin 1 (NF-1, 16%), breast cancer type 1
susceptibility protein (BRCA 1, 16%), BRCA 2 (6.3%), and methyl-CpG binding protein-
4 (MBD4, 21%). Whereas the expressions of strong oncogenic proteins, BCL2, SP-1,
proto-oncogene serine/threonine-protein kinase PIM-1, and Yes-associated protein
(YAP) were reduced by 12.9%, 9.6%, 17.4%, and 34.3%, respectively, after treatment
for 48 h. In addition, the expression of tumor suppressor protein ATM was also
diminished by 5.2% after treatment for 12 h (Figs. 6 E and F).

Concomitant increases in the expressions of oncogenic proteins and tumor
suppressor proteins indicate RAW 264.7 cells were stimulated by pamidronate and
reacted by initiating oncogenic signaling for cellular proliferation, survival, and
apoptosis.

**Effects of pamidronate on the expressions of angiogenesis-related proteins in**
**RAW 264.7 cells**

RAW 264.7 cells treated with pamidronate showed rapid reductions in the
expressions of angiogenesis-related proteins, as follows, HIF-1α (12%), angiogenin
(13.2%), vascular endothelial growth factor A (VEGF-A, 14.7%), VEGFR2 (12.5%), p-
VEGFR2 (22.1%), von Willebrand factor (vWF, 16%), capillary morphogenesis protein 2
(CMG2, 18.5%), COX1 (11.6%), FGF-1 (14%), FGF-2 (13.9%), MMP-2 (22%), MMP-10
(14.1%), plasminogen activator inhibitor-1 (PAI-1, 12.4%), PECAM-1 (CD31, 11.3%),
and vascular cell adhesion molecule-1 (VCAM-1, CD106, 12.7%) after treatment with
pamidronate for 48 h versus non-treated controls. The expressions of endothelin 1 (21-
amino acid vasoconstricting peptide, ET-1) and PDGF-A were increased by 18.6% and
13.2%, respectively, whereas the expressions of VEGF-C, lymphatic vessel endothelial
hyaluronan receptor 1 (LYVE-1), Fms-related tyrosine kinase 4 (FLT-4), and
plasminogen barely changed (< ±5%) (Figs. 7 A and B).

Among the angiogenesis-related proteins, the expressions of the blood vessel-
forming proteins, angiogenin, VEGF-A, VEGFR2, vWF, and CMG2 were markedly
reduced by pamidronate, while those of the lymphatic vessel-forming proteins, VEGF-C and LYVE-1 tended to increase slightly (< 5%). Pamidronate also reduced the expressions of the extracellular matrix proteins, FGF-1, FGF-2, MMP-2, and MMP-10, which are required for de novo angiogenesis and wound healing. These results suggest pamidronate significantly suppresses the expressions of angiogenesis-related proteins in RAW 264.7 cells, and that it might be able to potently inhibit blood vessel formation in vivo.

**Effects of pamidronate on the expressions of osteogenesis-related proteins in RAW 264.7 cells**

Treatment with pamidronate for 48 h decreased the expressions of the osteogenesis-related proteins; osteoprotegerin (OPG, 30.7%), osterix (4.5%), mammalian Runt-related transcription factor 2 (RUNX2, 23.8%), osteocalcin (16.2%), and connective tissue growth factor (CTGF, 9.6%) and those of the osteoclastogenesis-related proteins; receptor activator of nuclear factor kappa-B ligand (RANKL, 31.6%), cathepsin K (27.9%), and HSP-90 (12.7%) versus non-treated controls. On the other hand, the expressions of osteopontin and TGF-β1 were increased by pamidronate by 19.4% and 16.4% and the expressions of bone morphogenetic protein-2 (BMP-2, 8.3%), BMP-3 which negatively regulates bone density (16.8%), BMP-4 (6.8%), osteonectin (5.7%), and alkaline phosphatase (ALP, 5.3%), tended to be increased (Figs. 7 C and D).

The expressions of the major osteoblast differentiation proteins; OPG, osteocalcin, and RUNX2, and of the osteoclast differentiation proteins; RANKL, HSP-90, and
cathepsin K, were markedly reduced by 48 h of pamidronate treatment, whereas the expressions of the bone matrix proteins, osteopontin, BMP-2, BMP-4, osteonectin, and ALP tended to increase. In particular, the expressions of BMP-3 (an antagonist to other BMP’s in the differentiation of osteogenic progenitors) and TGF-β1 (an inhibitor of osteoclast activity) were markedly increased by pamidronate treatment. These results suggest pamidronate-treated RAW 264.7 cells are hardly differentiated into osteoclasts and give sparse influence on adjacent osteoblastic cells by expression of bone matrix proteins.

Global protein expressions in pamidronate-induced RAW 264.7 cells

Global protein expression changes of representative proteins (n=73) from above 19 different protein signaling pathways are illustrated as a star plot in Fig. 8. Although pamidronate is low molecular weight entity, it was found to widely affect the expressions of proteins in different signaling pathways in RAW 264.7 cells. In particular, pamidronate inactivated epigenetic modification and protein translation and subsequently down-regulated the expressions of some proteins required for the proliferation, differentiation, protection, and survival of RAW 264.7 cells.

The increases observed in the expressions of proliferation-related proteins were presumably related to the up-regulations of p53/Rb/E2F and Wnt/β-catenin signaling by pamidronate albeit suppression of cMyc/MAX/MAD network signaling. The suppression of RAS signaling induced by pAKT1/2/3, ERK-1, and p-ERK-1 down-regulations was followed by cMyc/MAX/MAD network down-regulation and by a subsequent inhibition in RAW 264.7 cell proliferation. Furthermore, the marked suppression of NFκB signaling
appeared to be associated with elevation of PARP-1- and FAS-mediated apoptosis and reduction of cellular differentiation, survival, immediate inflammatory reaction, and wound repair.

Overall changes in protein expressions induced by pamidronate affected the differentiation of RAW 264.7 cells and resulted in the productions of immature and/or inactive macrophages expressing lower levels of M2 macrophage differentiation proteins (wound healing proteins, TNFα, IL-1α, IL-6, IL-10, PECAM-1, CD99, VCAM, cathepsin G, cathepsin K, COX1, lysozyme, M-CSF, MMP-1, MMP-2, MMP-10, LL-37, α1-antitrypsin, β-defensin 1, β-defensin 2, and β-defensin 3), angiogenesis-related proteins (HIF-1α, angiogenin, VEGF-A, VEGFR2, p-VEGFR2, vWF, CMG2, COX1, FGF-1, FGF-2, MMP-2, MMP-10, PAI-1, PECAM-1, and VCAM-1), and osteoclast/osteoblast differentiation proteins (OPG, osterix, RUNX2, osteocalcin, and CTGF) and those of the osteoclastogenesis-related proteins (RANKL, cathepsin K, and HSP-90) than non-treated controls. Thus, pamidronate-treated RAW 264.7 cells simultaneously exhibited anti-inflammatory, anti-angiogenesis, and bone resorption inhibitive effects. However, the essential protein expression changes for cell proliferation, RAS signaling, and NFκB signaling rarely exceeded ±20%, which suggests pamidronate-treated cells exhibit relatively benign nature and be under homeostatic control.

Highly up- and down-regulated proteins by pamidronate in RAW 264.7 cells

In dot graphs plotted with highly up- and down-regulated proteins (> ± 10%, n=155, Fig. 9), pamidronate-treated RAW 264.7 cells showed reactive upregulation (10-30%) of
some proteins for cellular proliferation (CDK4, E2F-1, and TCF-1), protection (HSP-70, LC3, PLC-β2, and p-PKC), differentiation (vimentin, NCAM, p63, S-100, and SHH), RAS signaling proteins (KRAS, HRAS, SOS1, SOS2, RAF-B, JNK-1, and Rab), NFkB signaling proteins (NFkB, mTOR, NRF2, PGC-1α, and PTEN), and oncogenic proteins (DMBT1, 14-3-3, and CEA) versus non-treated controls, and were tended to be proliferative but their cellular activities became abortive by the downregulation (10-20%) of some essential proteins (p14, p15/16, cMyc, MAX, MAD-1, E-cadherin, FGF-1, FGF-2, CTGF, AKAP, caveolin-1, MDR, IKK, GADD45, GADD153, SRC1, and p-p38) and by increases in the expression of histone and DNA methylation-related proteins (histone H1, MBD4, and DMAP1) and by decreases in the expressions of protein translation-related proteins (eIF2AK3 and DHS) (Fig. 9 A and C).

On the other hand, pamidronate-treated RAW 264.7 cells appeared to be degenerated by marked downregulation (10-30%) of M2 macrophage differentiation-related inflammatory proteins (M-CSF, lysozyme, α1-antitrypsin, CD34, cathepsin K, and MMP-2) and survival-related proteins (BCL2, survivin, SP-1, and p-p38) and by marked upregulation (10-40%) of apoptosis-related proteins (caspase 9, c-caspase 9, caspase 3, c-caspase 3, PARP-1, p53, and PUMA) versus non-treated controls.

Subsequently, the major protein expressions for angiogenesis (VEGF-A, p-VEGFR2, angiogenin, HIF-1α, VCAM-1, FGF-1, FGF-2, PECAM-1, MMP-2, and MMP-10) and osteoclastogenesis (OPG, RANKL, cathepsin K, RUNX2, osteocalcin, and HSP-90) were dramatically suppressed (10-40%) by pamidronate (Fig. 9 A-C).

Discussion
Pamidronate is a nitrogen-containing, synthetic bisphosphonate, and its phosphate groups are believed to interfere with phosphorylation processes or interact with proteins in cells (Chen et al. 2012; Nishida et al. 2003; Stefanucci et al. 2015). Pamidronate is not sequestered as a waste material but relatively well adapted in cells, and thus, it is presumed pamidronate is maintained as a metabolite and influences not only the intracellular mevalonate pathway and protein isoprenylation but also signaling molecules and genetic materials (Henneman et al. 2011; Iguchi et al. 2010; Kaiser et al. 2013; Tatsuda et al. 2010). It has been shown pamidronate has considerable impact on cells such as macrophages, osteoclasts, and endothelial cells, and that its long-time usage is associated with the risk of BRONJ (Hoefert et al. 2015; Sharma et al. 2016; Zhang et al. 2013). In the present study, we assessed the effects of a therapeutic dose of pamidronate on the expressions of proteins in RAW 264.7 cells by IP-HPLC. As RAW 264.7 cells are derived from murine macrophages, and their immunological roles to dialysed coffee extract were assessed by IP-HPLC (Yoon et al. 2018b), and this study also explored RAW 264.7 cells for their macrophage roles to pamidronate.

Pamidronate-induced proliferation of RAW 264.7 cells was examined by counting cell numbers directly on Petri dishes, and protein expressional changes were determined by IP-HPLC. The in situ proliferation index of pamidronate-treated RAW 264.7 cells over 24 h was 73.1 ± 2.32 %, whereas that of non-treated cells was 69.9 ± 2.46 %, thus the pamidronate-induced increase was 3.2%. Furthermore, this increase in in situ proliferation index matched the pamidronate-induced increases in the expressions of different proliferation-related proteins as determined by IP-HPLC. These
data suggest pamidronate can slightly activate mitosis of murine macrophages, RAW 264.7 cells.

When we explored cellular mechanism responsible for altering protein expressions in RAW 264.7 cells, we noticed that the epigenetic environment was generally inactivated by pamidronate due to the up-regulations of DMNT1, MBD4, and DMAP1 and the down-regulation of KDM3D, which would tend to increase histone and DNA methylation levels. Protein translation was also inactivated by a marked reduction in DHS expression and an increase in eIF2AK3 (an inactivator of eIF2) expression versus non-treated controls. We suggest the concurrent inactivations of epigenetic modification and protein translation by pamidronate may have reduced global RAW 264.7 cell activity.

Pamidronate-treated RAW 264.7 cells showed a marked reduction in cMyc/MAX/MAD network signaling during culture, and this was followed by the up-regulation of p27 (a negative regulator of G1 progression) by 16.7% at 48 h. Whereas p53/Rb/E2F signaling was enhanced by the up-regulations of p53, Rb-1, and CDK4 resulted in an increase in the expression of the objective transcription factor, E2F-1. Also, Wnt/β-catenin signaling was also enhanced by the up-regulations of Wnt-1, β-catenin, and snail, which led to the up-regulation of the objective transcription factor, TCF-1. As a result, the expressions of the proliferation-activating proteins Ki-67, PCNA, MPM2, CDK4, cyclin D2, and lamin A/C, were increased by pamidronate, and concurrently the expressions of the proliferation-inhibiting proteins p14, p15/16, p21, and p27 were compensatory increased during 48 h of pamidronate treatment. These results indicate pamidronate-treated RAW 264.7 cells were partly activated and
proliferative due to increased p53/Rb/E2F and Wnt/β-catenin signaling despite a marked reduction in cMyc/MAX/MAD network signaling.

Pamidronate-treated RAW 264.7 cells showed increases in the expressions of some growth factors and associated proteins, such as IGF-1, IGFIIR, GH, HER1, HER2, TGF-β1, -β2, -β3, SMAD4, and ERβ, and subsequently, the expressions of upstream RAS signaling proteins including KRAS, HRAS, SOS-1, SOS-2, PI3K, JNK-1, and RAF-B were increased. However, downstreams of AKT and ERK signaling were reduced by the down-regulations of pAKT1/2/3, ERK-1, and p-ERK-1 and by the up-regulations of PTEN and mTOR. Consequently, RAS signaling was attenuated by the down-regulations of pAKT1/2/3, ERK-1, and p-ERK-1 in pamidronate-treated RAW 264.7 cells. Therefore, it appeared the pamidronate-induced negative regulation of RAS signaling might significantly reduce the expression of cMyc/MAX/MAD-1 network proteins.

Although the expressions of NFkB, NRF2, PGC-1α, PTEN, and mTOR tended to increase (< 10%) after pamidronate treatment, the expressions of p38, p-p38, GADD45, GADD153, ATF6, MDR, and SRC-1 were reduced after 48 h of treatment. In addition, the expressions of the reactive oxygen and nitrogen species-related proteins SOD-1, NOS1, and HO-1 were consistently reduced by pamidronate. These observations indicate NFkB signaling was reduced due to pamidronate-induced suppression of the downstream effector protein p38 (p-p38) in RAW 264.7 cells, and that treated cells were less reactive to oxidative or endoplasmic reticulum stress than non-treated controls.

Although pamidronate suppressed RAS and NFkB signalings simultaneously, RAW 264.7 cells expressed higher levels of the protection-related proteins HSP-70, JNK-1,
PLC-β2, LC3, and FAK, the cell survival-related proteins TERT, NRF2, PGC-1α, p-PKC, and FAK, and the oncogenesis-related proteins CEA, 14-3-3, and DMBT1 than non-treated controls. In particular, increases in the expressions of HSP-70 (protects against thermal and oxidative stress), JNK-1 (a mitogen-activated protein kinase responsible to different stress stimuli), LC3 (autophagosome biogenesis protein), NRF2 (transcription factor for many antioxidant genes), 14-3-3 (a regulator of diverse signaling proteins), DMBT1 (a glycoprotein containing multiple cysteine-rich domains that interact with tumor cells), and TERT (an RNA-dependent polymerase that lengthens telomeres in DNA strands) indicated pamidronate stressed RAW 264.7 cells and stimulated them to respond by expressing protection- and oncogenesis-related proteins.

Macrophages constitute a component of the front line of host defense and mediate innate immune responses by triggering; the productions of cytokines, chemokines, and cytotoxic molecules, the mobilizations of cells such as neutrophils and other leukocytes, the phagocytosis of pathogens and their delivery to lysosomes for degradation, and the induction of autophagy (Zhang et al. 2016). Many authors have reported macrophage functions are reduced after pamidronate treatment in vitro and in vivo (Escudero & Mandalunis 2012; Hoefert et al. 2015; Hoefert et al. 2016a; Mian et al. 1994). In the present study, although the general cytodifferentiation proteins, p63, vimentin, PLC-β2, PI3K, PKC, FAK, integrin α5, SHH, and S-100 were upregulated by pamidronate, the M2 macrophage differentiation-related proteins, TNFα, lysozyme, cathepsin G, cathepsin K, M-CSF, ICAM-1, and α1-antitrypsin were consistently downregulated, which suggested pamidronate prevented the differentiation of RAW 264.7 cells into
active M2 macrophages, and resulted retarded wound healing after pamidronate
treatment in vivo (Ariza Jimenez et al. 2018; Chen et al. 2018).

Pamidronate-treated RAW 264.7 cells also showed increases in the expressions of
the apoptosis executor proteins, caspase 8, caspase 3, and c-caspase 3, which are
activated by the FAS-mediated apoptosis signaling cascade, and that the expressions
of caspase 9 and c-caspase 9 were also increased by p53 upregulated modulator of
apoptosis (PUMA) and APAF-1 even though the expressions of the upstream p53-
mediated apoptosis signaling proteins, BAD, BAK, BAX, NOXA, and BCL2 were
suppressed. In addition, the expression of PARP-1 was increased by pamidronate
whereas the expression of cleaved PARP-1 (c-PARP-1) was decreased. These results
suggest pamidronate-treated RAW 264.7 cells underwent FAS/caspase 3/PARP-1-
mediated apoptosis, that is, parthanatos, due to the accumulation of polymeric
adenosine diphosphate ribose (poly (ADP-ribose) or PAR) caused by severe DNA
damage. Actually, pamidronate-treated RAW 264.7 cells were continuously proliferative
as evidenced by the up-regulations of p53/Rb/E2F and Wnt/β-catenin signaling, though
they only showed a slight increase in cell numbers after 24 h of pamidronate treatment
versus non-treated controls, which suggests some cells unable to differentiate into
mature macrophages may have succumbed to FAS-mediated or PARP-1-associated
apoptosis.

Pamidronate reduced the expressions of the osteoclastogenesis-related proteins,
RANKL and cathepsin K in RAW 264.7 cells, indicating it inhibited osteoclast
differentiation, which is in-line with the reported disappearance of osteoclasts in
bisphosphonate-treated animals (Kameka et al. 2014; Kawata et al. 2004; Mayahara &
Sasaki 2003) and has implications regarding the effects of pamidronate effects on osteolytic diseases such as including osteoporosis, fibrous dysplasia, Paget’s disease, and Gorham’s disease (Hammer et al. 2005; Kravets 2018; Saraff et al. 2018), etc. Pamidronate also downregulated the osteoblast differentiation proteins OPG, RUNX2, osterix, and osteocalcin but slightly induced the expressions of bone matrix proteins such as osteopontin, BMP-2, BMP-4, osteonectin, and ALP together with BMP-3 which negatively regulates bone density. These findings may be relevant to the osteoinductive effects of low-dose bisphosphonate reported in chronic periodontitis and after dental implantation (Alqhtani et al. 2017; Ata-Ali et al. 2016; Bhavsar et al. 2016; Khojasteh et al. 2019). However, pamidronate-treated RAW 264.7 cells may negatively regulate cytodifferentiation to osteoblasts in vivo and their abnormal bone production can contribute to the disruption of Haversian system canaliculi, which leads osteocyte death and increases the risk of osteonecrotic infections like BRONJ (Acevedo et al. 2015; Favia et al. 2009; Park et al. 2009).

Interestingly, pamidronate altered expressions of inflammatory proteins in RAW 264.7 cells both positively and negatively. The expressions of inflammatory proteins that participate in immediate inflammatory reaction, e.g., TNFα, IL-1, lysozyme, CD68, LL-37, and β-defensin-1, -2, -3, were markedly reduced, whereas those that participate in delayed inflammatory reaction, e.g., CD3, CD80, Pdcd-1/1, IL-12, and MCP-1, were elevated. The inhibition of immediate inflammatory reaction results the failure of innate immunity, and is relevant to severe necrotic infection of BRONJ involved with reduction of granulation tissue (Burr & Allen 2009; Carmagnola et al. 2013; Marx & Tursun 2012; Ziebart et al. 2011). Actually, pamidronate markedly suppressed the expressions of the
angiogenesis-related proteins, HIF-1α, VEGF-A, VERFR2, p-VEGFR2, vWF, CMG2, FGF-1, FGF-2, MMP-2, MMP-10, COX-1, PAI-1, VCAM-1, and PECAM-1 in RAW 264.7 cells versus non-treated controls but had relatively little effect on the expressions of the lymphatic vessel-related proteins, VEGF-C, LYVE-1, and FLT-4. These observations suggest that pamidronate-treated RAW 264.7 cells do not participate in immediate inflammatory reactions and vascular capillary production, but that they still provide some support for lymphatic drainage.

Pamidronate was found to widely affect the expressions of proteins in different signaling pathways in RAW 264.7 cells. Its global protein expression changes were illustrated in Fig. 8, exhibiting dynamic impacts on epigenetic modification, protein translation, RAS signaling, NFkB signaling, cellular proliferation, protection, differentiation, survival, apoptosis, inflammation, angiogenesis, and osteoclastogenesis. Highly up- and down-regulated proteins for each cellular functions were summarized in Fig. 9. Pamidronate induced marked over- and under-expression of some elective proteins more than 20% compared to non-treated controls, which may play pathogenetic roles (biomarkers) for cellular differentiation, inflammation, apoptosis, angiogenesis, and osteoclastogenesis in RAW 254.7 cells.

Conclusions

Summarizing, pamidronate was found to alter the expressions of many important proteins in RAW 264.7 cells. It upregulated proliferation-related proteins associated with p53/Rb/E2F and Wnt/β-catenin signaling and inactivated epigenetic modification and protein translation. In addition, RAS (cellular growth) and NFkB (cellular stress)
Signalings were markedly affected by pamidronate. Pamidronate-treated cells showed that upstream of RAS signaling was stimulated by up-regulation of some growth factors, while downstream of RAS signaling was attenuated by down-regulation of ERK-1 and p-ERK-1, resulted in reduction of cMyc/MAX/MAD network expression. They also showed suppression of NFkB signaling by downregulating p38 and p-p38 and upregulating mTOR. Consequently, pamidronate affects global protein expression in RAW 264.7 cells by downregulating the expressions of immediate inflammation, cellular differentiation, survival, angiogenesis, and osteoclastogenesis-related proteins, but by upregulating PARP-1- and FAS-mediated apoptosis, protection, and proliferation-related proteins. These findings suggest pamidronate has potent anti-inflammatory, anti-angiogenesis, and anti-osteoporotic effects together with cellular stresses dysregulating RAS signaling, NFkB signaling, apoptosis, and proliferation. The present study explored the global expressions of representative essential proteins (n=218) in pamidronate-treated RAW 264.7 cells, but some affected proteins were so dynamic and variable that they should be continuously monitored by IP-HPLC, if pamidronate treatment will be prolonged. Finally, we suggest further molecular biologic studies be undertaken on interactions between pamidronate and target proteins.

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References
Abelson, A., 2008. A review of Paget’s disease of bone with a focus on the efficacy and safety of zoledronic acid 5 mg. *Curr Med Res Opin, 24*(3):695-705

Acevedo, C., Bale, H., Gludovatz, B., Wat, A., Tang, S.Y., Wang, M., Busse, B., Zimmermann, E.A., Schaible, E., Allen, M.R., Burr, D.B., and Ritchie, R.O., 2015. Alendronate treatment alters bone tissues at multiple structural levels in healthy canine cortical bone. *Bone, 81*(3):352-363

Akram, Z., Abduljabbar, T., Kellesarian, S.V., Abu Hassan, M.I., Javed, F., and Vohra, F., 2017. Efficacy of bisphosphonate as an adjunct to nonsurgical periodontal therapy in the management of periodontal disease: a systematic review. *Br J Clin Pharmacol, 83*(3):444-454

Alqhtani, N.R., Logan, N.J., Meghji, S., Leeson, R., and Brett, P.M., 2017. Low dose effect of bisphosphonates on hMSCs osteogenic response to titanium surface in vitro. *Bone reports, 6*(6):64-69

Ariza Jimenez, A.B., Nunez Cuadros, E., Galindo Zavala, R., Nunez Caro, L., Diaz-Cordobes Rego, G., and Urda Cardona, A., 2018. Recurrent multifocal osteomyelitis in children: Experience in a tertiary care center. *Reumatologia clinica, 14*(6):334-338

Ata-Ali, J., Ata-Ali, F., Penarrocha-Oltra, D., and Galindo-Moreno, P., 2016. What is the impact of bisphosphonate therapy upon dental implant survival? A systematic review and meta-analysis. *Clinical oral implants research, 27*(2):e38-46

Bhavsar, N.V., Trivedi, S.R., Dulani, K., Brahmbhatt, N., Shah, S., and Chaudhri, D., 2016. Clinical and radiographic evaluation of effect of risedronate 5 mg as an adjunct to treatment of chronic periodontitis in postmenopausal women (12-month study). *Osteoporos Int, 27*(8):2611-2619

Burr, D.B., and Allen, M.R., 2009. Mandibular necrosis in beagle dogs treated with bisphosphonates. *Orthodontics & craniofacial research, 12*(3):221-228

Carmagnola, D., Canciani, E., Sozzi, D., Biglioli, F., Moneghini, L., and Dellavia, C., 2013. Histological findings on jaw osteonecrosis associated with bisphosphonates (BONJ) or with radiotherapy (ORN) in humans. *Acta odontologica Scandinavica, 71*(6):1410-1417
Cecchini, M.G., Felix, R., Fleisch, H., and Cooper, P.H., 1987. Effect of bisphosphonates on proliferation and viability of mouse bone marrow-derived macrophages. *J Bone Miner Res, 2(2):135-142*

Chen, C., Xia, M., Wu, L., Zhou, C., and Wang, F., 2012. Modeling the interaction of seven bisphosphonates with the hydroxyapatite(100) face. *Journal of molecular modeling, 18(9):4007-4012*

Chen, Z., Cheng, L., and Feng, G., 2018. Bone inflammation and chronic recurrent multifocal osteomyelitis. *Eur Rev Med Pharmacol Sci, 22(5):1380-1386*

Chevreau, M., Romand, X., Gaudin, P., Juvin, R., and Baillet, A., 2017. Bisphosphonates for treatment of Complex Regional Pain Syndrome type 1: A systematic literature review and meta-analysis of randomized controlled trials versus placebo. *Joint Bone Spine, 84(4):393-399*

Chirappapha, P., Kitudomrat, S., Thongjood, T., and Aroonroch, R., 2017. Bisphosphonate-related osteonecrosis of jaws in advanced stage breast cancer was detected from bone scan: a case report. *Gland surgery, 6(1):93-100*

Choi, W.S., Lee, J.I., Yoon, H.J., Min, C.K., and Lee, S.H., 2017. Medication-related osteonecrosis of the jaw: a preliminary retrospective study of 130 patients with multiple myeloma. *Maxillofacial plastic and reconstructive surgery, 39(1):1-7*

Clarke, N.J., Tomlinson, A.J., Ohyagi, Y., Younkin, S., and Naylor, S., 1998. Detection and quantitation of cellularly derived amyloid beta peptides by immunoprecipitation-HPLC-MS. *FEBS letters, 430(3):419-423*

Ebetino, F.H., Hogan, A.M., Sun, S., Tsoumpra, M.K., Duan, X., Triffitt, J.T., Kwaasi, A.A., Dunford, J.E., Barnett, B.L., Oppermann, U., Lundy, M.W., Boyde, A., Kasmiehov, B.A., McKenna, C.E., and Russell, R.G., 2011. The relationship between the chemistry and biological activity of the bisphosphonates. *Bone, 49(1):20-33*

Endo, Y., Nakamura, M., Kikuchi, T., Shinoda, H., Takeda, Y., Nitta, Y., and Kumagai, K., 1993. Aminoalkylbisphosphonates, potent inhibitors of bone resorption, induce a prolonged stimulation of
histamine synthesis and increase macrophages, granulocytes, and osteoclasts in vivo. *Calcif Tissue Int*, 52(3):248-254

Escudero, N.D., and Mandalunis, P.M., 2012. Influence of bisphosphonate treatment on medullary macrophages and osteoclasts: an experimental study. *Bone marrow research*, 2012(1):526236-526243

Favia, G., Pilolli, G.P., and Maiorano, E., 2009. Histologic and histomorphometric features of bisphosphonate-related osteonecrosis of the jaws: an analysis of 31 cases with confocal laser scanning microscopy. *Bone*, 45(3):406-413

Fernandez, D., Vega, D., and Goeta, A., 2002. The calcium-binding properties of pamidronate, a bone-resorption inhibitor. *Acta crystallographica Section C, Crystal structure communications*, 58(Pt 10):m494-497

Guimaraes, E.P., Pedreira, F.R., Jham, B.C., de Carli, M.L., Pereira, A.A., and Hanemann, J.A., 2013. Clinical management of suppurative osteomyelitis, bisphosphonate-related osteonecrosis, and osteoradionecrosis: report of three cases and review of the literature. *Case reports in dentistry*, 2013(402096)

Hammer, F., Kenn, W., Wesselmann, U., Hofbauer, L.C., Delling, G., Allolio, B., and Arlt, W., 2005. Gorham-Stout disease--stabilization during bisphosphonate treatment. *J Bone Miner Res*, 20(2):350-353

Hansen, P.J., Knitschke, M., Draenert, F.G., Irle, S., and Neff, A., 2012. Incidence of bisphosphonate-related osteonecrosis of the jaws (BRONJ) in patients taking bisphosphonates for osteoporosis treatment-a grossly underestimated risk? *Clin Oral Investig*,

Henneman, L., van Cruchten, A.G., Kulik, W., and Waterham, H.R., 2011. Inhibition of the isoprenoid biosynthesis pathway; detection of intermediates by UPLC-MS/MS. *Biochimica et biophysica acta*, 1811(4):227-233

Hoefert, S., Hoefert, C.S., Albert, M., Munz, A., Grimm, M., Northoff, H., Reinert, S., and Alexander, D., 2015. Zoledronate but not denosumab suppresses macrophagic differentiation of THP-1 cells. An
aetiologic model of bisphosphonate-related osteonecrosis of the jaw (BRONJ). Clin Oral Investig, 19(6):1307-1318

Hoefert, S., Sade Hoefert, C., Munz, A., Northoff, H., Yuan, A., Reichenmiller, K., Reinert, S., and Grimm, M., 2016a. Altered macrophagic THP-1 cell phagocytosis and migration in bisphosphonate-related osteonecrosis of the jaw (BRONJ). Clin Oral Investig, 20(5):1043-1054

Hoefert, S., Sade Hoefert, C., Munz, A., Schmitz, I., Grimm, M., Yuan, A., Northoff, H., Reinert, S., and Alexander, D., 2016b. Effect of bisphosphonates on macrophagic THP-1 cell survival in bisphosphonate-related osteonecrosis of the jaw (BRONJ). Oral surgery, oral medicine, oral pathology and oral radiology, 121(3):222-232

Hong, J.W., Nam, W., Cha, I.H., Chung, S.W., Choi, H.S., Kim, K.M., Kim, K.J., Rhee, Y., and Lim, S.K., 2010. Oral bisphosphonate-related osteonecrosis of the jaw: the first report in Asia. Osteoporos Int, 21(5):847-853

Iguchi, K., Tatsuda, Y., Usui, S., and Hirano, K., 2010. Pamidronate inhibits antiapoptotic bcl-2 expression through inhibition of the mevalonate pathway in prostate cancer PC-3 cells. Eur J Pharmacol, 641(1):35-40

Ito, M., Amizuka, N., Nakajima, T., and Ozawa, H., 2001. Bisphosphonate acts on osteoclasts independent of ruffled borders in osteosclerotic (oc/oc) mice. Bone, 28(6):609-616

Jayasena, A., Atapattu, N., and Lekamwasam, S., 2015. Treatment of glucocorticoid-induced low bone mineral density in children: a systematic review. International journal of rheumatic diseases, 18(3):287-293

Jeong, H.G., Hwang, J.J., Lee, J.H., Kim, Y.H., Na, J.Y., and Han, S.S., 2017. Risk factors of osteonecrosis of the jaw after tooth extraction in osteoporotic patients on oral bisphosphonates. Imaging science in dentistry, 47(1):45-50

Kaiser, T., Teufel, I., Geiger, K., Vater, Y., Aicher, W.K., Klein, G., and Fehm, T., 2013. Bisphosphonates modulate vital functions of human osteoblasts and affect their interactions with breast cancer cells.
Breast cancer research and treatment, 140(1):35-48

Kameka, A.M., Haddadi, S., Jamaldeen, F.J., Moinul, P., He, X.T., Nawazdeen, F.H., Bonfield, S., Sharif, S., van Rooijen, N., and Abdul-Careem, M.F., 2014. Clodronate treatment significantly depletes macrophages in chickens. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire, 78(4):274-282

Kamel, A.A., Geronikaki, A., and Abdou, W.M., 2012. Inhibitory effect of novel S,N-bisphosphonates on some carcinoma cell lines, osteoarthritis, and chronic inflammation. European journal of medicinal chemistry, 51(239-249)

Kawata, T., Tenjou, K., Tokimasa, C., Fujita, T., Kaku, M., Matsuki, A., Kohno, S., Tsutsui, K., Ohtani, J., Motokawa, M., Shigekawa, M., Tohma, Y., and Tanne, K., 2004. Effect of alendronate on osteoclast differentiation and bone volume in transplanted bone. Experimental animals / Japanese Association for Laboratory Animal Science, 53(1):47-51

Khojasteh, A., Dehghan, M.M., and Nazeman, P., 2019. Immediate implant placement following 1-year treatment with oral versus intravenous bisphosphonates: a histomorphometric canine study on peri-implant bone. Clin Oral Investig, 23(4):1803-1809

Kim, M.K., Hong, J.R., Kim, S.G., and Lee, S.K., 2015. Fatal Progression of Gorham Disease: A Case Report and Review of the Literature. J Oral Maxillofac Surg, 73(12):2352-2360

Kim, M.K., Yoon, C.S., G., K.S., Park, Y.W., Lee, S.S., and Lee, S.K., 2019. Effects of 4-Hexylresorcinol on Protein Expressions in RAW 264.7 Cells as Determined by Immunoprecipitation High Performance Liquid Chromatography. Scientific reports, 9(3379-3394)

Kim, S.M., Eo, M.Y., Cho, Y.J., Kim, Y.S., and Lee, S.K., 2017a. Wound healing protein profiles in the postoperative exudate of bisphosphonate-related osteonecrosis of mandible. Eur Arch Otorhinolaryngol, 274(9):3485-3495

Kim, S.M., Eo, M.Y., Cho, Y.J., Kim, Y.S., and Lee, S.K., 2017b. Differential protein expression in the secretory fluids of maxillary sinusitis and maxillary retention cyst. Eur Arch Otorhinolaryngol, 274(1):215-
Kim, S.M., Eo, M.Y., Kim, Y.S., and Lee, S.K., 2017c. Histochemical observation of bony reversal lines in bisphosphonate-related osteonecrosis of the jaw. *Oral surgery, oral medicine, oral pathology and oral radiology, 123*(2):220-228

Kim, S.M., Jeong, D., Kim, M.K., Lee, S.S., and Lee, S.K., 2017d. Two different protein expression profiles of oral squamous cell carcinoma analyzed by immunoprecipitation high-performance liquid chromatography. *World journal of surgical oncology, 15*(1):151

Kim, S.M., Eo, M.Y., Cho, Y.J., Kim, Y.S., and Lee, S.K., 2018. Immunoprecipitation high performance liquid chromatographic analysis of healing process in chronic suppurative osteomyelitis of the jaw. *J Craniomaxillofac Surg, 46*(1):119-127

Kim, Y.S., and Lee, S.K., 2015. IP-HPLC Analysis of Human Salivary Protein Complexes. *Korean Journal of Oral and Maxillofacial Pathology, 39*(5):615-622

Kravets, I., 2018. Paget's Disease of Bone: Diagnosis and Treatment. *Am J Med, 131*(11):1298-1303

Kun-Darbois, J.D., Libouban, H., Mabilleau, G., Pascaretti-Grizon, F., and Chappard, D., 2018. Bone mineralization and vascularization in bisphosphonate-related osteonecrosis of the jaw: an experimental study in the rat. *Clin Oral Investig, 22*(9):2997-3006

Lee, S.K., 2013. Ultrastructural Changes of Bone in Bisphosphonate-related Osteonecrosis of Jaws. *Kor J Oral Maxillofac Pathol, 37*(5):183-192

Luo, L., Shen, L., Sun, F., and Ma, Z., 2013. Immunoprecipitation coupled with HPLC-MS/MS to discover the aromatase ligands from Glycyrrhiza uralensis. *Food chemistry, 138*(1):315-320

Majoor, B.C., Appelman-Dijkstra, N.M., Fiocco, M., van de Sande, M.A., Dijkstra, P.S., and Hamdy, N.A., 2017. Outcome of Long-Term Bisphosphonate Therapy in McCune-Albright Syndrome and Polyostotic Fibrous Dysplasia. *J Bone Miner Res, 32*(2):264-276
Marx, R.E., Sawatari, Y., Fortin, M., and Broumand, V., 2005. Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. *J Oral Maxillofac Surg, 63*(11):1567-1575

Marx, R.E., and Tursun, R., 2012. Suppurative osteomyelitis, bisphosphonate induced osteonecrosis, osteoradionecrosis: a blinded histopathologic comparison and its implications for the mechanism of each disease. *Int J Oral Maxillofac Surg, 41*(3):283-289

Mayahara, M., and Sasaki, T., 2003. Cellular mechanism of inhibition of osteoclastic resorption of bone and calcified cartilage by long-term pamidronate administration in ovariectomized mature rats. *The anatomical record Part A, Discoveries in molecular, cellular, and evolutionary biology, 274*(1):817-826

Mian, M., Benetti, D., Aloisi, R., Rosini, S., and Fantozzi, R., 1994. Effects of bisphosphonate derivatives on macrophage function. *Pharmacology, 49*(5):336-342

Miyazaki, T., Miyauchi, S., Anada, T., Imaizumi, H., and Suzuki, O., 2011. Evaluation of osteoclastic resorption activity using calcium phosphate coating combined with labeled polyanion. *Anal Biochem, 410*(1):7-12

Nishida, S., Fujii, Y., Yoshioka, S., Kikuichi, S., Tsubaki, M., and Irimajiri, K., 2003. A new bisphosphonate, YM529 induces apoptosis in HL60 cells by decreasing phosphorylation of single survival signal ERK. *Life Sci, 73*(21):2655-2664

Ohlrich, E.J., Coates, D.E., Cullinan, M.P., Milne, T.J., Zafar, S., Zhao, Y., Duncan, W.D., and Seymour, G.J., 2016. The bisphosphonate zoledronic acid regulates key angiogenesis-related genes in primary human gingival fibroblasts. *Arch Oral Biol, 63*(7-14)

Park, J.M., Lee, S.W., Kim, J.H., Park, Y.W., Song, J.Y., Lee, S.S., Kim, Y.S., and Lee, S.K., 2009. Recurrent Osteomyelitis Caused by Bisphosphonate Intake Showed Abnormal Osteophytes Lack of Harversian System. *Kor J Oral Maxillofac Pathol, 33*(4):181-190

Polyzos, S.A., Anastasilakis, A.D., Makras, P., and Terpos, E., 2011. Paget’s disease of bone and
calcium homeostasis: focus on bisphosphonate treatment. *Exp Clin Endocrinol Diabetes, 119*(9):519-524

Ribatti, D., Maruotti, N., Nico, B., Longo, V., Mangieri, D., Vacca, A., and Cantatore, F.P., 2008. Clodronate inhibits angiogenesis in vitro and in vivo. *Oncology reports, 19*(5):1109-1112

Ruggiero, S.L., Mehrotra, B., Rosenberg, T.J., and Engroff, S.L., 2004. Osteonecrosis of the jaws associated with the use of bisphosphonates: a review of 63 cases. *J Oral Maxillofac Surg, 62*(5):527-534

Sansom, L.N., Necciari, J., and Thiercelin, J.F., 1995. Human pharmacokinetics of tiludronate. *Bone, 17*(5 Suppl):479S-483S

Saraff, V., Sahota, J., Crabtree, N., Sakka, S., Shaw, N.J., and Hogler, W., 2018. Efficacy and treatment costs of zoledronate versus pamidronate in paediatric osteoporosis. *Arch Dis Child, 103*(1):92-94

Sarasquete, M.E., Gonzalez, M., San Miguel, J.F., and Garcia-Sanz, R., 2009. Bisphosphonate-related osteonecrosis: genetic and acquired risk factors. *Oral Dis, 15*(6):382-387

Sato, M., Grasser, W., Endo, N., Akins, R., Simmons, H., Thompson, D.D., Golub, E., and Rodan, G.A., 1991. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *J Clin Invest, 88*(6):2095-2105

Seo, M.H., Myoung, H., Lee, J.H., Kim, S.M., and Lee, S.K., 2019. Changes in oncogenic protein levels in peri-implant oral malignancy: a case report. *Maxillofacial plastic and reconstructive surgery, 41*(1):46

Sharma, D., Hamlet, S.M., Petcu, E.B., and Ivanovski, S., 2016. The effect of bisphosphonates on the endothelial differentiation of mesenchymal stem cells. *Scientific reports, 6*(20580)

Singer, F.R., Bone, H.G., 3rd, Hosking, D.J., Lyles, K.W., Murad, M.H., Reid, I.R., and Siris, E.S., 2014. Paget's disease of bone: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab, 99*(12):4408-4422
Stefanucci, A., Marrone, A., and Agamennone, M., 2015. Investigation of the N-BP Binding at FPPS by Combined Computational Approaches. *Med Chem, 11*(5):417-431

Tatsuda, Y., Iguchi, K., Usui, S., Suzui, M., and Hirano, K., 2010. Protein kinase C is inhibited by bisphosphonates in prostate cancer PC-3 cells. *Eur J Pharmacol, 627*(1-3):348-353

Wat, W.Z., 2014. Current perspectives on bisphosphonate treatment in Paget’s disease of bone. *Ther Clin Risk Manag, 10*(977-983)

Yoon, C.S., Kim, M.K., Kim, Y.S., and Lee, S.K., 2018a. In vivo protein expression changes in mouse livers treated with dialyzed coffee extract as determined by IP-HPLC. *Maxillofacial plastic and reconstructive surgery, 40*(1):44

Yoon, C.S., Kim, M.K., Kim, Y.S., and Lee, S.K., 2018b. *In vitro* protein expression changes in RAW 264.7 cells and HUVECs treated with dialyzed coffee extract by immunoprecipitation high performance liquid chromatography. *Scientific reports, 8*(1):13841

Zhang, Q., Atsuta, I., Liu, S., Chen, C., Shi, S., and Le, A.D., 2013. IL-17-Mediated M1/M2 Macrophage Alteration Contributes to Pathogenesis of Bisphosphonate-Related Osteonecrosis of the Jaws. *Clin Cancer Res, 19*(12):3176-3188

Zhang, X., Han, J., Man, K., Li, X., Du, J., Chu, E.S., Go, M.Y., Sung, J.J., and Yu, J., 2016. CXC chemokine receptor 3 promotes steatohepatitis in mice through mediating inflammatory cytokines, macrophages and autophagy. *J Hepatol, 64*(1):160-170

Ziebart, T., Pabst, A., Klein, M.O., Kammerer, P., Gauss, L., Brullmann, D., Al-Nawas, B., and Walter, C., 2011. Bisphosphonates: restrictions for vasculogenesis and angiogenesis: inhibition of cell function of endothelial progenitor cells and mature endothelial cells in vitro. *Clin Oral Investig, 15*(1):105-111
Figure 1. *In situ* proliferation assay of RAW 264.7 cells. Increases in cell numbers were determined by counting on Petri dishes (A-F), and proliferation indices (%) were calculated by expressing cell growths (final-initial cell counts) as percentages of initial cells counts. Pamidronate-treated (6.5 μM) RAW 264.7 cells had a slightly higher mean proliferation index (73.1 ± 2.32% at 24 h and 74.7 ± 2.8% at 48 h) than non-treated controls (69.9 ± 2.46%) (G).
Figure 2

Expressions of proliferation-related proteins, cMyc/MAX/MAD network proteins, p53/Rb/E2F signaling proteins, and Wnt/β-catenin signaling proteins

Figure 2. Expressions of proliferation-related proteins (A and B), cMyc/MAX/MAD network proteins (C or D), p53/Rb/E2F signaling proteins (E and F), and Wnt/β-catenin signaling proteins (F or H) in pamidronate-treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A, C, E, and G show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, F, and H) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).
Figure 3

Expressions of epigenetic modification-related proteins, protein translation-related proteins, growth factors, and RAS signaling proteins

Figure 3. Expressions of epigenetic modification-related proteins (A and B), protein translation-related proteins (C or D), growth factors (E and F), and RAS signaling proteins (G or H) in pamidronate-treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A, C, E, and G show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, F, and H) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).
Expressions of NFκB signaling proteins, inflammatory proteins were upregulated, and inflammatory proteins downregulated.

Figure 4. Expressions of NFκB signaling proteins (A and B), inflammatory proteins were upregulated (C or D), and inflammatory proteins downregulated (E and F) in pamidronate treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A, C, and E show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, and F) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).
Figure 5

Expressions of p53-mediated apoptosis-related proteins, FAS-mediated apoptosis-related proteins, and cell survival-related proteins

Figure 5. Expressions of p53-mediated apoptosis-related proteins (A and B), FAS-mediated apoptosis-related proteins (C or D), and cell survival-related proteins (E and F) in RAW 264.7 cells treated with pamidronate for different times as determined by IP-HPLC. Line graphs, A, C, and E show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, and F) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).
Figure 6

Expressions of cell protection-related proteins, differentiation-related proteins, and oncogenesis-related proteins

Figure 6. Expressions of cell protection-related proteins (A and B), differentiation-related proteins (C or D), and oncogenesis-related proteins (E and F) in pamidronate-treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A, C, and E show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, and F) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).
Figure 7

Expressions of angiogenesis-related proteins and of osteogenesis-related proteins

Figure 7. Expressions of angiogenesis-related proteins (A and B) and of osteogenesis-related proteins (C or D) in pamidronate-treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A and C show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B and D) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).
Figure 8

Star plot of global protein expression in pamidronate-treated RAW 264.7 cells

Figure 8. Star plot of global protein expression in pamidronate-treated RAW 264.7 cells. Representative proteins (n=73) of each signaling pathway are plotted in a circular manner. The expressions of proliferation, some growth factors, cellular apoptosis, protection, and differentiation-related proteins were upregulated, while the expressions of protein translation-, cell survival-, angiogenesis-, and osteogenesis-related proteins were downregulated. RAS signaling and NFkB signaling were suppressed by the up-regulations of the downstream effector proteins, ERK-1 (p-ERK-1) and p38 (p-p38), respectively. The expressions of inflammatory proteins and oncogenesis-related proteins in RAW 264.7 cells were variably altered, but epigenetic methylation was increased by pamidronate treatment. Blue, yellow, and red spots indicate after 12, 24, and 48 hours of pamidronate treatment, respectively.
Figure 9

Highly up- and down-regulated proteins by pamidronate in RAW 264.7 cells

Figure 9. Highly up- and down-regulated proteins by pamidronate in RAW 264.7 cells. The cells were reactive to pamidronate by marked upregulation of some proteins for cellular proliferation, protection, differentiation, RAS signaling, NFkB signaling, and oncogenic proteins, but gradually degenerated by marked downregulation of M2 macrophage differentiation-related inflammatory proteins and survival-related proteins and by marked upregulation of apoptosis-related proteins. The major protein expressions for angiogenesis and osteoclastogenesis were dramatically suppressed (A-C). Blue, yellow, and red spots indicate after 12, 24, and 48 hours of pamidronate treatment, respectively.
Up- and down-regulated proteins by pamidronate in RAW 264.7 cells

A

140%

130%

120%

110%

100%

90%

80%

70%

Proliferation
p53/Wnt/β-catenin signaling
Epigenetic modification
Protein translation
Growth factors
RAS signaling
NFκB signaling

12H 24H 48H
S = ± 2.61%

B

140%

130%

120%

110%

100%

90%

80%

70%

Inflammatory proteins
p53-mediated apoptosis
FAK-mediated apoptosis
Cell survival

12H 24H 48H
S = ± 2.77%

C

140%

130%

120%

110%

100%

90%

80%

70%

Protection
Differentiation
Oncogenesis
Angiogenesis
Osteogenesis

12H 24H 48H
S = ± 2.29%
Table 1 (on next page)

Antibodies used in the study

Antibodies used in the study.
Table 1. Antibodies used in the study.

| Proteins                              | No. | Antibodies                                                                 |
|---------------------------------------|-----|-----------------------------------------------------------------------------|
| Proliferation-related                 | 11  | Ki-67®, PCNA®, CDK4®, MPM2®, PLK4®, cyclin D2, p14®, p16®, p21®, p27®, lamin A/C |
| eMyc/MAX/MAD network                  | 3 (1)| eMyc®, MAX®, MAD-1®, (p27)                                                 |
| p53/Rb/E2F signaling                  | 4 (2)| p53, Rb-1®, E2F-1®, MDM2, (p21), (CDK4)                                    |
| Wnt/β-catenin signaling               | 6   | Wnt1®, β-catenin®, APC®, snail®, TCF-1®, E-cadherin                        |
| Epigenetic modification               | 7   | histone H1®, DMAP1®, KDM4D®, HDAC-1®®, MB24®, DNMT1®, PCAF®               |
| Protein translation                   | 5   | DOHH®, DSH®, elF5A-1®, elF5A-2®, elF2AK3®                                 |
| Growth factor                         | 18  | FGF-1®, FGF-2®, FGF-7®, CTGF, HGFA®, TGF-β1®, TGF-β2®, TGF-β3®, SMAD4®, PDGF-A®, IGF-1®, IGFIIR®, GH®, GHRIH®, HER1®, HER2®, ERα®, Met® |
| RAS signaling                         | 22  | NRAS®, KRA®, HRAS®, STAT3®, PI3K, pAKT1/2/3, RAF-B®, JAK2®, JNK-1®, ERK-1®, p-ERK-1®, Rab 1®, mTOR, PTEN, NF-1, AKAP, caveolin-1, AMPK®, SOS-1®, SOS-2®, PKC®, p-PKC® |
| NFκB signaling                        | 16 (6)| NFκB®, IKK®, TNFa, GADD45®, GADD153®, MDR, p38®, p-p38®, PGC-1α, ATF6, NRF-2®, SRC-1®, (pAKT1/2/3, PTEN, ERK1®, p-ERK®, AMPK, mTOR®) |
| Upregulated inflammatory proteins     | 17  | CD3, CD4, NCAM (CD56), CD80 (B7-1), Pdcd-1/1, (CD279), IL-8, IL-12, MMP-3®, IL-6, IL-10, IL-28®, cathepsin G®, cathepsin K®, COX1, lipoxygen, versican, kininogen |
| Downregulated inflammatory proteins   | 27 (1)| CD3, CD4, NCAM (CD56), CD80 (B7-1), Pdcd-1/1, (CD279), IL-8, IL-12, MMP-3®, IL-6, IL-10, IL-28®, cathepsin G®, cathepsin K®, COX1, lipoxygen, versican, kininogen |
| p53-mediated apoptosis                | 15 (2)| PUMA®, NOXA®, BCL2®, BAX®, BAD®, BAK®, BID®, AIF®, APAF-1®, caspase 9®, c-caspase 9®, caspase 3®, c-caspase 3®, PARP-1®, c-PARP-1®, (MDM2®, p53®) |
| FAS-mediated apoptosis                | 5 (3) | FASL®, FAS®, FADD®, FLIP®, caspase 8®, (BID®, caspase 3®, c-caspase 3®) |
| Cell survival-related                 | 5 (11)| TERT®, survivin®, SP-1®, SP-3®, FAK, (pAKT1/2/3, PTEN, AMPK, BCL2, NFR2, ATF6, PGC-1α, PKC, p-PKC, p38, p-p38) |
| Protection-related                    | 12 (13)| HSP-27®, HSP-70®, HSP-90®, TGFα 2®, LC3, mucin 1, mucin 4, HO-1®, SOD-1®, GSTO1®, SVCT2®, NOS-1®, (PLC-β2, PI3K®), p-PKC®, p-PKC®, PKC®-1, AMPK®, JNK-1, PLC-β2, PI3K, PKC, p-PKC® |
| Differentiation-related               | 11 (11)| HCAM (CD44), (caveolin-1, SP-1, SP-3, PLC-β2, PI3K®), p-PKC®, PKC®, FAK, APIM1, ICAM-1 (CD54), NCAM (CD56), PECAM (CD31)) |
| Oncogenesis-related                   | 10 (10)| BRCAl®, BRCAl®2, NF-1®, ATM®, CEA®, 14-3-3®, maspin®, DBMTI®, YAP, PIM1, (MBD4, BCL2, SP-1, PTEN®), mucin 1, mucin 4, survivin®, TERT®, pAKT1/2/3®, mTOR) |
| Angiogenesis-related                  | 14 (9)| HIF-1α®, VEGF-A®, VEGF-C®, angiogenin®, LYVE-1®, CMG2®, WIF®, FLT-4®, ET-1®, PAI-1®, VEGFR2®, p-VEGFR2, plasminogen®, leptin®, (CD31, MMP-2, MMP-10, FGF-2, PDGF-A, PECAM-1 (CD31), VCAm (CD106), COX1) |
| Osteogenesis-related                  | 11 (4)| OPG®, RANKL®, BMP-2®, BMP-3®, BMP-4®, ALP®, osteocalcin®, osteopontin®, osteonectin®, RUNX2®, osterix®, (HSP-90, cathepsin K, CTGF, TGF-β1) |
| Control housekeeping proteins         | 3   | α-tubulin®, β-actin®, GAPDH®                                                 |

| Total                                 | 218 | (73) |

Santa Cruz Biotechnology, USA; ¹ DAKO, Denmark; ² Neomarkers, CA, USA; ³ ZYMED, CA, USA; ⁴ Abcam, Cambridge, UK; ⁵ kindly donated from M. H. Park in NIH, USA (Park and Wolff 2018), the number of antibodies overlapped; ( ) .

**Abbreviations:** AIF®; apoptosis inducing factor, AKAP®; A-kinase anchoring proteins, ALP®; alkaline phosphatase, AMPK®; AMP-activated protein kinase, pAKT®; v-akt murine thymoma viral oncogene homolog, p-Akt1/2/3 phosphorylated (p-Akt, Thr 308), APAF-1®; apoptotic protease-activating factor 1, APC®; adenomatous polyposis coli, ATFR6®; activating transcription factor 6, ATM®; ataxia telangiectasia caused by mutations, BAD®; BCL2 associated death promoter, BAK®; BCL2 antagonist/killer, BAX®; BCL2 associated X, BCL-2®; B-cell leukemia/lymphoma-2, BID®; Bcl-2 interacting-domain death agonist, BMP-2®; bone morphogenesis protein.
2, BRCA1; breast cancer type 1 susceptibility protein, c-caspase 3; cleaved-caspase 3, CD3; cluster of differentiation 3, CDK4; cyclin dependent kinase 4, CEA; carcinoembryonic antigen, CMG2; capillary morphogenesis protein 2, COX-1; cyclooxygenase-2, CTGF connective tissue growth factor, CXCR4; C-C chemokine receptor type 4, CyRP-1; cystein rich protein, DHH; deoxyhypusine synthase, DMAP1; DNA methyltransferase 1 associated protein, DMBT1; deleted in malignant brain tumors 1, DNM1; DNA 5-cytosine methyltransferase 1, DOHH; deoxyhypusine hydroxylase, E2F-1; transcription factor, eIF2AK3 (PERK); eukaryotic translation initiation factor 2 (protein kinase R (PKR)-like endoplasmic reticulum kinase), eIF5A-1; eukaryotic translation initiation factor 5A-1, ERK-1; extracellular signal-regulated protein kinase 1, ERβ; estrogen receptor beta, ET-1; endothelin-1, FADD; FAS associated via death domain, FAK; focal adhesion kinase, FASL; FAS ligand, FGF-1; fibroblast growth factor-1, FLIP; FLICE-like inhibitory protein, FLT-4; Fms-related tyrosine kinase 4, GADD45; growth arrest and DNA-damage-inducible 45, GAPDH; glyceraldehyde 3-phosphate dehydrogenase, GH; growth hormone, GHRH; growth hormone-releasing hormone, GSTO1; glutathione S-transferase ω1, HCAM (CD44); homing cell adhesion molecule, HDAC-10; histone deacetylase 10, HER1; human epidermal growth factor receptor 1, HGF-α; hepatocyte growth factor α, HIF-1α; hypoxia inducible factor-1α, HO-1; heme oxygenase 1, HRAS; GTPase HRas, HSP-70; heat shock protein-70, ICAM (CD54); intercellular adhesion molecule 1, IGF-1; insulin-like growth factor 1, IGFIIR; insulin-like growth factor 2 receptor, IKK; ikappaB kinase, IL-1; interleukin-1, JNK-1; Jun N-terminal protein kinase, KDM4D; Lysine-specific demethylase 4D, KRAS; V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, LC3; microtubule-associated protein 1A/1B-light chain 3, LTA4H; leukotriene A4 hydrolase, LYVE-1; lymphatic vessel endothelial hyaluronan receptor 1, MAD-1; mitotic arrest deficient 1, MAX; myc-associated factor X, MBD4; methyl-CpG-binding domain protein 4, MCP-1; monocyte chemotactic protein 1, M-CSF; macrophage colony-stimulating factor, MDM2; mouse double minute 2 homolog, MDR; multiple drug resistance, MMP-1; matrix metalloprotease-1, MPM2; mitotic protein monoclonal 2, mTOR; mammalian target of rapamycin, eMyC; V-myc myelocytomatosis viral oncogene homolog, NFR2; nuclear factor kappa-light-chain-enhancer of activated B cells, NCAM (CD56); neural cell adhesion molecule 1, NF-1; neurofibromin 1, NFκB; nuclear factor kappa-light-chain-enhancer of activated B cells, NOS-1; nitric oxide synthase 1, NOXA; Phorbol-12-myristate-13-acetate-induced protein 1, NRAS; neuroblastoma RAS Viral Oncogene homolog, PPP1R3; nuclear factor (erythroid-derived)-like 2, POG; osteoprotegerin, PAI-1; plasminogen activator inhibitor-1, PARP-1; poly-ADP ribose polymerase 1, c-PARP-1; cleaved-PARP-1, PCNA; proliferating cell nuclear antigen, Pdcd-1/1 (CD279); programmed cell death 1, PDGF-A; platelet-derived growth factor-A, PECAM-1 (CD31); platelet endothelial cell adhesion molecule-1, PECAM-1; peroxisome proliferator-activated receptor gamma coactivator 1-α, PI3K; phosphatidylinositol-3-kinase, PIM-1; Proto-oncogene serine/threonine-protein kinase 1, PKC; protein kinase C, PLCβ-2; phosphatidylinositol-4,5-bisphosphate phosphodiesterase β-2, PLK4; polo like kinase 4 or serine/threonine-protein kinase, PTEN; phosphatase and tensin homolog, PUMA; p53 upregulated modulator of apoptosis, Rab 1; Rab GTPases, RAF-B; v-Raf murine sarcoma viral oncogene homolog B, RANKL; receptor activator of nuclear factor kappa-B ligand, RB-1; retinoblastoma-1, RUNX2; Runt-related transcription factor-2, SHH; sonic hedgehog, SMAD4; mothers against decapentaplegic, drosophilia homolog 4, SOD-1; superoxide dismutase-1, SOS-1; son of sevenless homolog 1, SP-1; specificity protein 1, SRE1; steroid receptor coactivator-1, STAT3; signal transducer and activator of transcription-3, SVCT2; sodium-dependent vitamin C transporter 2, TERT; human telomerase reverse transcriptase, TGF-β1; transforming growth factor-β1, TGF-β; transforming growth factor-β1, TNFa; tumor necrosis factor-α, VCAM; vascular cell adhesion molecule-1, VEGF-A vascular endothelial growth factor A, VEGFR2: vascular endothelial growth factor receptor 2, p-VEGFR2: vascular endothelial growth factor receptor 2 (Y951), vWF: von Willebrand factor, Wnt1; proto-oncogene protein Wnt-1, YAP; Yes-associated protein.