Clodronate alleviates cachexia and prolongs survival in nude mice xenografted with an anaplastic thyroid carcinoma cell line

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Abstract

Cancer cachexia is one of the most common manifestations of advanced malignant disease and is frequently associated with decreased survival. Previously, we reported the establishment of a new anaplastic thyroid carcinoma cell line, Thena, and its mouse xenograft, Thena-Nu, which induced cachexia in athymic nude mice. Subsequent studies showed that the addition of clodronate to Thena-Nu cultures reduced cell proliferation as well as cytokine production in a dose- and time-dependent manner. Weekly administration of clodronate induced tumor cytostasis, attenuation of cachexia, as well as prolongation of survival in Thena-Nu-bearing mice. Reduced

serum interleukin 6, tumor necrosis factor- α , and granulocyte colony stimulating factor levels were detected, whereas, serum leukemia inhibitory factor levels were not reduced. Liver necrosis, observed in tumor-bearing mice, was also improved following clodronate treatment. Discontinuation of clodronate treatment, however, resulted in progressive tumor growth and weight loss. Our results demonstrated that clodronate could exert therapeutic efficacy on amelioration of cancer cachexia in the hosts. Nevertheless, this study also points out that a longer period of treatment is required to maintain these effects.

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Introduction

Cachexia is a paraneoplastic syndrome manifested as progressive weight loss with depletion of adipose tissue and skeletal muscle, and contributes to a poor quality of life, which is observed in about half of all the cancer patients after the onset of disease (Tisdale 2002). Proinflammatory cytokines, including tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, leukemia inhibitory factor (LIF), and interferon γ , have been proposed as mediators of cancer cachexia (Noguchi *et al.* 1996). Various agents demonstrated to suppress cytokine secretion, such as corticosteroid, megestrol acetate, medroxyprogesterone acetate, and thalidomide (Loprinzi *et al.* 1993, Mantovani *et al.* 1998), have been administered in attempts to retard or to halt progressive cachexia in cancer patients. Positive outcome in the reverse of anorexia and weight loss was observed in some randomized studies; however, no survival advantage was reported.

It was further identified that ATP-ubiquitin-dependent proteolysis was responsible for muscle wasting (Llovera *et al.* 1995), which has been shown to be upregulated by TNF- α and IL-6 (Tsujioka *et al.* 1996, Llovera *et al.* 1997). Recently, TNF- α -induced activation of nuclear factor- κ B (NF- κ B), a transcription factor regulating the expression of a variety of cytokines (Yamamoto & Gaynor 2001), was implicated as

playing a major role in muscle protein loss *in vitro* and *in vivo* by destabilizing *MyoD*, leading to the inhibition of myogenesis (Langen *et al.* 2004). The complexity of cancer cachexia has thus made the treatment regimen even more complex. In this regard, drugs able to intervene in the above-mentioned pathways should be considered as potential candidates for treating cancer cachexia.

Clodronate is a non-nitrogen containing bisphosphonate and has been widely used in the treatment of bone complications arising from malignancy. Adjuvant clodronate was proven to reduce bone as well as non-osseous metastases in a randomized clinical trial on breast cancer (Diel *et al.* 1998), suggesting that clodronate not only elicits effects on osteoclasts, but also on tumor cells. *In vitro* studies showed that clodronate was metabolized by mammalian cells to a non-hydrolyzable ATP analog, adenosine 5'-(β , γ -dichloromethylene) triphosphate (AppCCl₂p), a process catalyzed by several aminoacyl-tRNA synthetases. This incorporation may result in inhibition of tRNA aminoacylation by these enzymes, leading to inhibition of protein synthesis (Rogers *et al.* 1996, Frith *et al.* 1997). Clodronate was demonstrated to downregulate the production of IL-6 and TNF- α (Monkkonen *et al.* 1994), as well as NF- κ B activation (Makkonen *et al.* 1999) in mouse macrophage-like RAW264 cells stimulated with lipopolysaccharides; inhibition of IL-6 production by osteosarcoma MG-63 cells was also

documented (Giuliani *et al.* 1998). Cancer patients receiving clodronate treatment showed reduction in serum IL-6 levels, which was not associated with cytotoxic effects (Sauty *et al.* 1996). These results suggest that clodronate may possess therapeutic potential for cancer cachexia.

Recently, we reported the establishment of a new anaplastic thyroid carcinoma cell line, Thena, and its nude mouse xenograft, Thena-Nu, producing high levels of proinflammatory cytokines, including IL-6, LIF, and TNF- α , and inducing cachexia in athymic nude mice (Chang *et al.* 2003). In the present study, we would like to evaluate the effects of clodronate in Thena-Nu cells *in vitro* as well as in Thena-Nu-bearing nude mice.

Materials and Methods

In vitro cell growth

Cells were seeded to six-well plates (Nunc, Roskilde, Denmark) at 1×10^5 per well in RPMI medium (Invitrogen) containing 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 units/ml penicillin–50 μ g/ml streptomycin (Invitrogen). Culture supernatants were discarded following overnight incubation and replaced by fresh medium containing 2% FBS and various concentrations of clodronate (disodium clodronate; Schering, Jena, Germany). Triplicate wells were harvested for 3 consecutive days. Culture supernatants were collected for cytokine assay. Cell number and viability were determined by the Trypan Blue exclusion method. Cell proliferation assay was performed by dimethylthiazolyl and thymidine incorporation methods.

Cell-cycle analysis

Following incubation with various concentrations (100–400 μ g/ml or 400–1600 μ M) of clodronate for 72 h, cells were harvested, washed with PBS, and fixed in 75% ethanol for 2 h at -20°C . After incubation, cells were treated with 100 μ g/ml propidium iodide (Sigma) in PBS containing 1% glucose and 20 μ g/ml RNase for 30 min at room temperature. DNA content of stained cells was analyzed by flow cytometry.

Tumor inoculation and clodronate treatment

Athymic nude mice (Balb/c nu/nu, 8 weeks old), purchased from the Animal Facility of the National Science Council, Taiwan, were injected s.c. with 5×10^6 cells in 0.1 ml PBS in the right hind leg. Tumor growth was monitored every other day. Tumor size was measured with a caliper and tumor volume was calculated by the formula: $(ab^2)/2$; where a represents the longer diameter and b is the shorter diameter of tumors (Tamura *et al.* 1995). Body weight of mice was measured once a week with a digital scale. Signs of s.c. fat or muscle wasting were grossly observed as well. Treatment was started at tumor size 6–8 mm in diameter, which is comparable to advanced stage in human (Kelland 2004). Tumor-bearing mice were either

injected with 200 μ g (0.816 μ mol) clodronate in 0.1 ml normal saline (clodronate, $n=5$) or with 0.1 ml normal saline (untreated, $n=6$) around tumors intradermally; non-tumor-bearing mice (control, $n=4$) received 0.1 ml normal saline weekly. Treatment was given for 8 consecutive weeks followed by 2-week observation. Mice presenting with poor activity and severe cachexia were presumed dead and were killed; others were killed at day 90 after tumor inoculation. Serum was collected for cytokine assay; livers and spleens were fixed in formaldehyde for histopathological check using hematoxylin and eosin (H&E) stain. All procedures used in this animal study were performed in accordance with institutional guidelines for animal care at Chang Gung Memorial Hospital.

ELISA

Levels of IL-6, LIF, and TNF- α in culture supernatants or mouse serum were determined by the ELISA method following the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). Since massive neutrophil infiltration was observed in primary tumor sections, levels of granulocyte colony stimulating factor were also evaluated. In brief, samples were loaded to 96-well plates precoated with anti-cytokine antibody and incubated at room temperature for 2 h. After three washes, horseradish peroxidase (HRP)-conjugated secondary antibody was added and the plate was further incubated for 2 h. A chromogen (tetramethylbenzidine and hydrogen peroxide mixed at 1:1) was then added for color formation. The reaction was stopped by the addition of 2 M H_2SO_4 . Results were read with a Dynex MRXII ELISA reader (ThermoLabsystems, Chantilly, VA, USA) at 450 nm with correction at 570 nm. The sensitivities of the kits are as follows: IL-6, 0.7 pg/ml; LIF, 8 pg/ml; TNF- α , 1.6 pg/ml; and G-CSF, 20 pg/ml.

In situ apoptosis stain

To determine whether clodronate induced tumor apoptosis *in vivo*, paraffin sections (5 μ m) of mouse xenograft tumors were stained by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling method (TUNEL) according to the recommendation of the manufacturer (R&D Systems). In brief, following fixation and rehydration, the cells were incubated with proteinase K, quenched with methanol containing 3% hydrogen peroxide, and labeled with a mixture containing TdT and brominated dNTPs at 37°C for 1 h in a humidified chamber. Reaction was stopped by immersing in 0.01 M EDTA. Samples were then incubated with anti-bromodeoxyuridine antibody followed by streptavidin–HRP. Sections were stained with 3,3'-diaminobenzidine tetrachloride and counterstained with Methyl Green. Results were evaluated with a light microscopy.

Statistical analysis

All values are expressed as means $\pm$ s.d. Differences in cytokine levels in culture supernatants or mouse serum, tumor size, and body weight were analyzed using Student's *t*-test. Differences

in survival were analyzed by Kaplan–Meier's method. *P* values less than 0.05 were considered statistically significant.

Results

Clodronate inhibited cell proliferation and reduced cytokine secretion in vitro

In our previous report, we demonstrated that cells derived from Thena nude mouse xenograft (Thena-Nu) produced

even higher levels of cytokines in the culture supernatants than their parental counterparts (Chang *et al.* 2003). Therefore, Thena-Nu cells were used in this study. *In vitro* cell growth assay demonstrated that clodronate had a dose- and time-dependent effect on the inhibition of cell proliferation. As illustrated in Fig. 1A, 100 µg/ml clodronate did not inhibit growth of Thena-Nu cells at all time points tested. Clodronate at 200 µg/ml did not inhibit cell growth until drug exposure for 72 h ($P < 0.05$); whereas a significant reduction in total cell number was observed in cells

Figure 1 Inhibition of Thena-Nu cell growth by clodronate treatment. Cells were seeded to six-well plates at 1×10^5 /well. Following overnight incubation the medium was discarded and fresh medium containing different concentrations of clodronate (100, 200, or 400 µg/ml) was added. Medium alone served as control. Triplicate wells of each set were harvested. (A) Total viable cell number was counted by Trypan Blue dye exclusion. (B) Flow cytometric analysis demonstrated a slight accumulation in G₀/G₁ phase with a slight reduction in both S phase and G₂/M phase in a dose-response manner. a, Medium alone; b, 100 µg/ml; c, 200 µg/ml; and d, 400 µg/ml. * $P < 0.05$ and † $P < 0.01$ compared with control.

incubated with 400 µg/ml clodronate for 48 h ($P<0.01$). Cell-cycle analysis showed that treatment with 200 and 400 µg/ml clodronate for 72 h induced a significant accumulation in G₀/G₁ phase accompanied with a significant decrease in both S and G₂/M phases, whereas 100 µg/ml generated a slight decrease in G₂/M phase (Fig. 1B). Proliferation assay showed comparable results to those obtained from the growth curve assay (data not shown), indicating that clodronate at these doses did not induce significant cell death.

Incubation of Thena-Nu cells in the presence of clodronate also exhibited a dose- and time-dependent effect in the reduction of IL-6, LIF, TNF- α , and G-CSF production (Fig. 2). Treatment of Thena-Nu cells with 200 µg/ml clodronate induced a >40% reduction in cytokine production following a 72-h incubation accompanied with only a slight decrease in cell number (~12%). Interestingly, 100 µg/ml clodronate did not induce growth inhibition in Thena-Nu cells, but significantly decreased their cytokine production after a 48- or 72-h exposure. These results suggest that growth inhibitory effects of clodronate may ascribe to its ability to reduce autocrine/paracrine factors for cell growth. To better clarify the capability of clodronate to downregulate production of proinflammatory cytokines in the subsequent animal experiments, 200 µg is chosen due to its ability to induce a ~50% cytokine inhibitory effect following a 72-h exposure.

Clodronate induced tumor cytostasis, alleviated body weight loss, and prolonged survival of tumor-bearing mice

We investigated the effects of clodronate on thyroid cancer *in vivo*. Eighteen days following Thena-Nu cells inoculation tumors grew to a total volume between 205 and 320 mm³, mimicking the advanced stage of human cancer. Clodronate treatment was then started on day 19. The tumor stopped growing in the clodronate-treated group for more than a month, whereas rapid tumor growth was observed in untreated mice (Fig. 3A). Discontinuation of clodronate treatment resulted in a rapid tumor re-growth.

Subcutaneous fat wasting around the injection site was observed in tumor-bearing mice 15 days following inoculation. Significant difference on body weight between non-tumor-bearing (18.73 ± 0.64 g) and tumor-bearing (17.55 ± 0.20 g) mice was seen on day 18 ($P<0.05$, Fig. 3B). Clodronate induced a slight but not significant body weight gain in tumor-bearing mice 2 weeks after treatment, whereas untreated mice showed a gradually decreased body weight. On day 32, a significant difference in body weight between clodronate-treated (18.18 ± 0.32 g) and untreated (17.14 ± 0.42 g) groups was seen ($P<0.05$). Compared with the untreated group, clodronate-treated mice disclosed a sustained body weight and a reduced severity of s.c. fat wasting around the injection site for up to 5 weeks (data not shown). However, progressive weight loss was demonstrated after clodronate discontinuation. An inverse

Figure 2 Reduction of cytokine secretion in Thena-Nu cells by clodronate treatment (100, 200, 400 µg/ml). Culture supernatants collected from the growth curve assay (Fig. 1) were assayed for content of cytokines, including (A) IL-6, (B) LIF, (C) TNF- α , and (D) G-CSF. Results are expressed as nanogram per 10⁶ cells. * $P<0.05$, † $P<0.01$, and ‡ $P<0.001$ compared with control.

relationship between tumor size and body weight in untreated mice was obtained ($r^2=0.6201$, data not shown).

A significantly longer survival was seen in the clodronate-treated group than the untreated group (Fig. 4, $P<0.01$).

Figure 3 (A) Inhibition of tumor growth and (B) preservation of body weight in Thera-Nu-bearing mice with clodronate treatment. Tumor-injected mice were treated with clodronate (clodronate, 200 µg in 0.1 ml normal saline) or with normal saline (untreated, 0.1 ml), starting from day 19, once a week for 8 consecutive weeks, followed by 2 weeks of observation. Tumor volume and body weight of mice were measured once per week. Non-tumor-bearing mice (control) were included for comparison. Arrowhead denotes date of starting treatment and arrow, final treatment. * $P < 0.05$ between untreated and clodronate and † $P < 0.01$ between untreated and clodronate.

Figure 4 Prolongation of survival in Thera-Nu-bearing mice by clodronate treatment Clodronate (—), $n = 5$; untreated (---), $n = 6$ ($P < 0.05$).

All the untreated mice became moribund before day 54, whereas all the clodronate-treated mice survived to the end of this study (day 90).

Clodronate reduced liver necrosis in tumor-bearing mice

H&E staining showed that degenerated hepatocytes, accompanied with profound neutrophil infiltration were presented in liver sections of both groups. Neither tumor metastasis nor hepatomegaly was observed. Massive liver necrosis was found in 6/6 untreated tumor-bearing mice (Fig. 5A), whereas only very few small foci of necrotic areas were observed in livers of 2/5 clodronate-treated mice. Survival of tumor-bearing mice seemed to be correlated with the severity of liver necrosis. However, liver sections of clodronate-treated mice showed more intensive neutrophil infiltration, prominently around portal veins, than untreated ones (Fig. 5B). Marked splenomegaly (>sevenfold) was found in tumor-bearing mice, despite clodronate treatment, and no difference in spleen pathology was observed (data not shown). Sections of tumor tissues from both groups were evaluated for apoptosis by TUNEL stain and no tumor apoptosis was seen (data not shown), indicating that attenuation of cancer cachexia was not due to induction of tumor apoptosis by clodronate.

Clodronate treatment reduced serum levels of human cytokines in cachectic nude mice

High levels of human IL-6, LIF, and G-CSF, as well as low levels of TNF- α were detected in the sera of tumor-bearing mice. A significant decrease in serum IL-6, TNF- α , and G-CSF was obtained in mice treated with clodronate, whereas there was no difference in levels of LIF for both groups, as depicted in Table 1. None of these four human cytokines were detectable in sera of non-tumor-bearing mice.

To investigate the levels of cytokines in tumor tissue extracts, weighed tissues were immersed in 1 ml PBS, homogenized, centrifuged, and supernatants were collected and assayed for cytokine contents. Levels of human IL-6 were greatly reduced in tumors of clodronate-treated mice ($P < 0.01$). Although slightly decreased, there was no significant difference in the levels of G-CSF and TNF- α between both groups. Again, no decrease in levels of LIF for both groups was obtained (Table 1).

Discussion

Various cytokines have been postulated to be responsible for the metabolic changes in cachexia of mouse xenograft models (Noguchi *et al.* 1996). So far, only a few anti-cachectic drugs were applied to these animal models to evaluate their therapeutic efficacy (Tamura *et al.* 1995, Kurebayashi *et al.* 1999). However, no anti-tumor effect and survival impact were discussed. Furthermore, most experiments were conducted shortly after tumor implantation, which might be less clinically relevant.

Figure 5 Improvement of liver necrosis in tumor-bearing mice following clodronate treatment. (A) H&E stain revealed patchy necrosis (arrow) in the liver of untreated mice. (B) Necrosis was greatly improved in clodronate-treated mice. Increased neutrophil sequestration and transmigration in the hepatic vasculature (asterisk) was seen in clodronate-treated mice (original magnification, $\times 200$).

Clodronate was reported to exert growth and cytokine inhibitory effects through impairment of intracellular ATP levels by its non-hydrolyzable metabolite, AppCCl₂p, following cellular metabolism (Monkkonen *et al.* 2001). Although cellular uptake of clodronate was low ($\sim 0.04\%$), the capacity of epithelial cells to metabolize to AppCCl₂p was comparable to that of macrophages (Monkkonen *et al.* 2003). In our study, clodronate suppressed cell growth and production of proinflammatory cytokines in Thena-Nu cells in a dose- and time-dependent manner. Dose-dependent reduction of NF- κ B activity in Thena-Nu cells following clodronate treatment was demonstrated by western blotting (data not shown), suggesting that

suppression of NF- κ B activity may also be involved in clodronate-induced growth and cytokine inhibitory effects (Makkonen *et al.* 1999).

The anti-tumor effect of clodronate was associated with dose and time of exposure (Shipman *et al.* 1997, Senaratne *et al.* 2000, Sonnemann *et al.* 2001). Previous studies, complimented by our results, suggest that the anti-tumor effect of clodronate is cell-type specific. Study on melanoma cells showed that treating with clodronate at 1000 μ M did not significantly change cell-cycle distribution, but tended to accumulate in G₀/G₁ phase (Forsea *et al.* 2004). Consistently, Thena-Nu cells treated with clodronate induced cytostasis in Thena-Nu cells.

Table 1 Levels of human cytokines in sera and tumor extracts of Thena-Nu-bearing mice. Mice were treated with clodronate or left untreated and serum was collected at the time of death. Levels of human cytokines in mouse sera were assayed by the ELISA method. Results are expressed as picogram per milliliter. Note that all human cytokines were undetectable in sera of non-tumor-bearing mice

	Serum (pg/ml)	Tumor (ng/g tissue)
<i>IL-6</i>		
Untreated	1156.4 ± 128.7	134.38 ± 23.76
Clodronate	622.2 ± 73.8 [†]	72.07 ± 8.22 [†]
<i>TNF-α</i>		
Untreated	37.7 ± 4.7	1.24 ± 0.25
Clodronate	20.3 ± 3.2*	0.97 ± 0.13
<i>LIF</i>		
Untreated	1156.7 ± 114.7	2.72 ± 0.76
Clodronate	1138.8 ± 264.6	2.85 ± 0.42
<i>G-CSF</i>		
Untreated	2881.3 ± 547.1	221.43 ± 19.68
Clodronate	1130.2 ± 379.2*	198.21 ± 25.83

* $P < 0.05$; [†] $P < 0.01$. Serum (pg/ml) and tumor (ng/g tissue).

Our subsequent animal studies demonstrated that treatment with clodronate reduced tumor progression, attenuated cachexia, and prolonged survival in Thena-Nu-bearing mice. However, the cessation of drug treatment resulted in a gradual disease progression, suggesting that a longer period of treatment is required. The dosage we used in animal studies is clinically obtainable (~10 mg/kg), as compared with that used in treating hypercalcemia in human subjects (~25 mg/kg for a 60 kg individual). Two tumor-bearing mice receiving clodronate treatment 5 weeks after inoculation did not show any improvement, suggesting that early treatment is required to obtain better outcome.

Significant reduction in serum levels of human cytokines, including IL-6, TNF-α, and G-CSF, was detected in clodronate-treated mice; however, we did not detect a significant difference in serum LIF levels between these two groups. While redundancy of the cytokine network was observed, the cytokine profile following clodronate discontinuation may suggest the major role of LIF in this animal model of cancer cachexia. We did not detect levels of mouse cytokines due to insufficient serum. Nevertheless, all these four human cytokines have been reported to react with mouse cells by binding to their respective receptors (Demetri & Griffin 1991, Tartaglia *et al.* 1991, Peters *et al.* 1996).

TNF-α was reported to play the major role of cachexia in numerous human diseases and animal models (Tisdale 2002). Prolonged exposure to TNF-α has been shown to stimulate protein loss in skeletal muscle cells directly by NF-κB activation (Ladner *et al.* 2003). TNF-α may also act indirectly to increase expression of other cachectic factors (Billingsley *et al.* 1996) or work in corporation with other factors to induce muscle protein loss (Acharyya *et al.* 2004). Therefore, continuous production of TNF-α, IL-6, and LIF in our model may activate NF-κB to release many of the same cytokines in a positive feedback response and further

aggravate the disease. Clodronate treatment significantly reduced serum levels of TNF-α and IL-6, but not LIF, and attenuated cachexia in Thena-Nu-bearing mice; tumor cytostasis and reduced s.c. fat wasting were also observed. The observation of reduced fat wasting in tumor-bearing mice is supposed to be ascribed to reduction in serum IL-6 levels following clodronate treatment, as IL-6 has been reported to deplete fat tissues in mice (Greenberg *et al.* 1992). Studies to elucidate the mechanisms of action of clodronate in this cachectic animal model are underway.

Histopathologically, Thena-Nu-injected mice demonstrated severe liver pathology, characterized by massive liver necrosis surrounded by degenerated hepatocytes with heavy neutrophil infiltration (Fig. 5A). Proinflammatory cytokine-mediated neutrophil-induced organ damage has been well documented in several situations (Jaeschke & Smith 1997); nevertheless, it was seldom reported in tumor-bearing hosts. The pathological changes in Thena-Nu-injected mice were similar to those observed in nude mice xenotransplanted with human lung cancer cell lines producing high levels of G-CSF (Suzuki *et al.* 1993). Mice injected with murine marrow cells containing G-CSF cDNA insert showed high serum G-CSF levels and heavy neutrophil infiltration in the liver; however, no liver damage was observed (Chang *et al.* 1989), suggesting that other factors participated in the process of liver necrosis seen in Thena-Nu-bearing mice. Reactive oxygen species and proteases, generated by infiltrating neutrophils after stimulation with TNF-α, were demonstrated to induce hepatocyte necrosis directly (Jaeschke *et al.* 2002). Furthermore, IL-6 was shown to augment neutrophil cytotoxic potential by enhancing release of esterase and superoxide (Johnson *et al.* 1998). LIF was also reported to induce neutrophil leukocytosis (Metcalf & Gearing 1989a) and liver necrosis in mice (Metcalf & Gearing 1989b). In this context, the reduction of liver necrosis might be due to a reduction in serum cytokine levels following the clodronate treatment. Interestingly, profound neutrophil extravasation and transmigration in the hepatic vasculature were seen in clodronate-treated mice, suggesting that neutrophil-induced liver necrosis should be found if clodronate-treated mice were not killed. Liver necrosis may compromise anti-cancer therapy; accordingly, clodronate-induced improvement of liver pathology may enhance chemotherapeutic efficacy, which is worthy of further study.

In conclusion, we demonstrated that clodronate significantly reduced cell proliferation and cytokine production of Thena-Nu cells *in vitro*, and effectively alleviated cachexia and prolonged survival in tumor-bearing nude mice. Clinical trials utilizing clodronate on cancer cachexia are warranted.

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