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Running Title: Incadronate Inhibits Mevalonate Pathway and ATL Cell Growth

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Summary

Anti-resorptive bisphosphonates are used for the treatment of hypercalcemia and bone complications associated with malignancies and osteoporosis, but have also been shown to have anti-tumour effects in various cancers. Adult T-cell leukaemia (ATL) is a fatal T-cell malignancy caused by infection with human T-cell leukaemia virus type I (HTLV-I), and remains incurable. ATL is associated with osteolytic bone lesions and hypercalcemia, which are major factors in the morbidity of ATL. Thus, the search for anti-ATL agents that have both anti-tumour and anti-resorptive activity is warranted. The bisphosphonate agent, incadronate prevented cell growth of HTLV-I-infected T-cell lines and primary ATL cells, but not of non-infected T-cell lines or normal peripheral blood mononuclear cells. Incadronate induced S-phase cell cycle arrest and apoptosis in HTLV-I-infected T-cell lines, and treatment of these cells with substrates of the mevalonate pathway blocked the incadronate-mediated growth suppression. Incadronate also prevented the prenylation of Rap1A protein. These results demonstrated that incadronate-induced growth suppression occurs by interfering with the mevalonate pathway. Importantly, treatment with incadronate reduced tumour formation from an HTLV-I-infected T-cell line, when these cells were inoculated subcutaneously into severe combined immunodeficient mice. These findings suggest that incadronate could be potentially useful for the treatment of ATL.

Keywords: bisphosphonate, incadronate, human T-cell leukaemia virus type I, adult T-cell leukaemia, mevalonate

Introduction

Adult T-cell leukaemia (ATL) is a unique malignancy of mature $CD4^+$ T cells caused by human T-cell leukaemia virus type I (HTLV-I) (Poiesz *et al,* 1980; Hinuma *et al,* 1981; Yoshida *et al,* 1982). ATL arises after a long latent period of ≥50 years by a presumed multistep mechanism of tumourigenesis (Okamoto *et al,* 1989). ATL is classified into four subtypes: acute, lymphoma, chronic and smoldering. In the relatively indolent smoldering and chronic types, the median survival time is ≥2 years. No curative therapy for ATL exists and the condition often progresses to death with a median survival time of 13 months in aggressive cases (Yamada *et al,* 2001). Death is usually due to severe infection or hypercalcemia, often associated with resistance to intensive, combined chemotherapy. Clinical trials using the novel combination of interferon and arsenic trioxide or zidovudine exhibited better therapeutic responses, although efficacies were limited and few patients achieved long-term remission (Hermine *et al,* 2004). Therefore, new therapeutic strategies for ATL need to be established.

The high frequency of tumour-induced osteolysis and hypercalcemia is the most striking feature of ATL; about 70% of ATL patients have high serum Ca^{2+} levels during the clinical course of the disease, particularly during the aggressive stages (Kiyokawa *et al,* 1987). The frequency and severity of the hypercalcemia is in fact the highest among haematological malignancies (Roodman, 1997). A striking feature of ATL-induced hypercalcemia is that the bone lesions are predominantly osteolytic with little associated osteoblastic activity. A variety of ATL cell-expressing factors that

directly and/or indirectly stimulate osteoclast differentiation and activity, such as interleukin-1, tumour necrosis factor-β, parathyroid hormone-related peptide and receptor activator of nuclear factor-κB ligand, have been associated with hypercalcemia in these patients (Wano *et al,* 1987; Watanabe *et al,* 1990; Ishibashi *et al,* 1991; Nosaka *et al,* 2002).

Bisphosphonates are the standard therapy for tumour-associated osteolysis and hypercalcemia, including for multiple myeloma and ATL. Clinical trials have shown that bisphosphonates can improve overall survival in patients with multiple myeloma and breast cancer, when administered during the early stages of cancer prior to the development of overt bone metastasis (Berenson *et al,* 1998; Cameron, 2003), but the mechanism of this effect on survival remains to be fully understood. Whether bisphosphonates have direct effects on tumour cells or indirect effects mediated by changes in the bone microenvironment has long been debated. A growing body of evidence *in vitro* suggests that bisphosphonates act directly on tumour cells (Santini *et al,* 2003).

Bisphosphonates can be divided into two groups based on their N-terminal structure. Low-potency nitrogen-free bisphosphonates are metabolized to potentially cytotoxic analogues of ATP, whereas high-potency nitrogen-containing bisphosphonates are not metabolized, but can inhibit the mevalonate pathway (Benford *et al,* 1999). With the objective of finding new agents for the treatment of ATL, the present study investigated the anti-tumour potential of a nitrogen-containing bisphosphonate, incadronate, on HTLV-I-infected T-cell lines and primary ATL cells

in vitro and *in vivo*, as well as possible mechanisms underlying the anti-tumour activities.

Materials and methods

Cell lines

The HTLV-I-negative human T-cell leukaemia cell lines, Jurkat and MOLT-4, and HTLV-I-infected T-cell lines, MT-4 (Yamamoto *et al,* 1982), C5/MJ (Popovic *et al,* 1983), SLB-1 (Koeffler *et al,* 1984) and HUT-102 (Poiesz *et al,* 1980) were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 100 U/ml penicillin and 100 μg/ml streptomycin.

Clinical samples

The diagnosis of ATL was based on clinical features, haematological findings and the presence of anti-HTLV-I antibodies in sera. Monoclonal HTLV-I provirus integration into the DNA of leukaemic cells was confirmed by Southern blot hybridization in all patients (data not shown). Peripheral blood mononuclear cells (PBMC) from three healthy volunteers and patients with ATL, consisting of four acute (ATL 1, 3, 4 and 7) and three chronic types (ATL 2, 5 and 6), were analysed. Two male and five female patients were between 51-70 years of age (mean 63 years). The leukocyte count was between 21,500-116,900/μl. Mononuclear cells were isolated by Ficoll-Paque density

gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and washed with phosphate-buffered saline. All samples were obtained after informed consent.

Reagents

Incadronate (cycloheptylaminomethylene-1,1-bisphosphonate) was kindly provided by Astellas Pharma Inc. (Tokyo, Japan) (Nagao *et al*, 1991). Geranylgeraniol (GGOH) and farnesol (FOH) were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal antibody to survivin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rabbit polyclonal antibody to Bcl-xL was purchased from BD Transduction Laboratories (San Jose, CA, USA). Mouse monoclonal antibodies to Bcl-2, Bax and actin were purchased from NeoMarkers (Fremont, CA, USA). Mouse monoclonal antibody to Tax, Lt-4, was described previously (Tanaka *et al*, 1990).

Cell viability and assays of apoptosis

The effects of incadronate on cell growth were assessed using the cell proliferation reagent, water-soluble tetrazolium-8 (Wako Chemicals, Osaka, Japan). Briefly, 1×10^5/ml (cell lines) or 1×10^6/ml (PBMC) were incubated in a 96-well microculture plate in the absence or presence of various concentrations of incadronate. After 72 h of culture, water-soluble tetrazolium-8 (5 μl) was added for the last 4 h of incubation and the absorbance at 450 nm was measured using an automated microplate reader. Mitochondrial dehydrogenase cleavage of the water-soluble tetrazolium-8 to formazan dye provided a measure of cell proliferation. Fifty % inhibitory concentration (IC_{50}) was extrapolated from trend line data. Apoptotic events in cells were detected by

staining with phycoerythrin-conjugated Apo2.7 monoclonal antibody (Beckman Coulter, Miami, FL, USA) (Zhang *et al*, 1996) and analysed by flow cytometry (FACSCaliber, Becton Dickinson, San Jose, CA, USA).

Cell cycle analysis

Cell cycle analysis was performed with the CycleTEST PLUS DNA reagent kit (Becton Dickinson). In brief, 1×10^6 cells were washed with a buffer solution containing sodium citrate, sucrose and dimetylsulphoxide, suspended in a solution containing RNase A, and then stained with 125 μg/ml propidium iodide for 10 min. After passing the cells through a nylon mesh, cell suspensions were analysed on a FACSCaliber using CellQuest. The population of cells in each cell cycle phase was determined using ModFit software.

In vitro measurement of caspase activity

Measurement of caspase activity was performed with the Colorimetric caspase assay kits (Medical & Biological Laboratories, Nagoya, Japan). Cell extracts were prepared using Cell Lysis buffer and assessed for caspase-3, -8 and -9 activities by means of colorimetric probes. Colorimetric caspase assay kits are based on detection of the chromophore *p*-nitroanilide after cleavage from caspase-specific-labeled substrates. Colorimetric readings were performed in an automated microplate reader at an optical density of 400 nm.

Western blot analysis

Cells were lysed in a buffer containing 62.5 mmol/l Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (20 μg) determined by the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA) were subjected to electrophoresis on sodium dodecyl sulphate-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with the specific antibodies. The bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

In vivo administration of incadronate

Five-week-old female C.B-17/Icr-scid [severe combined immunodeficient (SCID)] mice obtained from Ryukyu Biotec Co. (Urasoe, Japan) were maintained in containment level 2 cabinets and provided with autoclaved food and water *ad libitum*. Mice were engrafted with 5×10^6 HUT-102 cells by subcutaneous injection in the post-auricular region, and then randomly placed into two cohorts of five mice each that were injected subcutaneously with vehicle and incadronate, respectively. Treatment was initiated on the next day of cell injection. Incadronate was dissolved in distilled water at a concentration of 0.12 mg/ml, and incadronate (0.6 mg/kg body weight) was administered subcutaneously every day for 21 days. Control mice were injected subcutaneously with the same volume of the vehicle (distilled water) only. Tumour size was monitored once weekly. All mice were sacrificed on day 21, and the tumours were dissected out and weighed. This experiment was performed according to the guidelines for Animal Experimentation of the University of the Ryukyus and approved by the Animal Care and Use Committee of the University of the Ryukyus.

Statistical analysis

Data are expressed as mean ± SD. Volumes and weights of tumours from incadronate-treated mice were compared with those of the vehicle-treated controls by the Mann-Whitney *U*-test. A P value < 0.05 was considered statistically significant.

Results

Incadronate inhibits growth of HTLV-I-infected T-cell lines and primary ATL cells

We first examined the effects of incadronate on the growth of HTLV-I-infected T-cell lines. Culture of cells in the presence of various concentrations (0-125 μmol/l) of incadronate for 72 h resulted in the suppression of cell growth in a dose-dependent manner in all four HTLV-I-infected T-cell lines tested, as assessed by the water-soluble tetrazolium-8 assay (Fig 1A). Although the sensitivity to incadronate varied among the cell lines, HTLV-I-non-infected acute lymphoblastic T-cell leukaemia cell lines (Jurkat and MOLT-4) were less susceptible to incadronate than the HTLV-I-infected T-cell lines (Fig 1A). We also examined the effects of incadronate on freshly isolated ATL cells from seven patients. All ATL cell populations treated with incadronate showed reduced cell survival compared with cells from three normal healthy controls (Fig 1B). The concentrations of incadronate required to inhibit growth of cells by 50% (IC_{50}) were shown in Table I. The appearance of suppression of cell growth could also be detected in HTLV-I-infected

T-cell lines and primary ATL cells after treatment *in vitro* with incadronate for 24 h, but this suppression was most marked at 72 h incubation.

Incadronate induces apoptosis of HTLV-I-infected T-cell lines

We next examined whether induction of apoptosis accounted for the suppressed cell growth observed in all HTLV-I-infected T-cell lines. Cells were treated with incadronate then probed with the Apo2.7 monoclonal antibody. Incadronate increased the proportion of apoptotic cells in all HTLV-I-infected T-cell lines, and this effect was dose dependent (Fig 2). Incadronate did not alter the proportion of apoptotic cells in the HTLV-I-non-infected T-cell lines, Jurkat and MOLT-4 (Fig 2). These results implicated induction of apoptosis as a mechanism by which incadronate inhibits proliferation of HTLV-I-infected T-cell lines. The appearance of apoptosis could also be detected in HTLV-I-infected T-cell lines after treatment with incadronate for 24 h, but this induction of apoptosis was most marked at 72 h incubation.

Incadronate-induced apoptosis is caspase dependent

We next examined the extent of caspase activation during the incadronate-induced apoptosis. Incadronate treatment resulted in activation of caspase-3, -8 and -9 in MT-4 and HUT-102 cells (Fig 3). Caspase activation was most marked at 48-72 h incubation. These results demonstrate that caspase activation plays a role in the incadronate-induced apoptosis observed in HTLV-I-infected T-cell lines.

Incadronate induces accumulation of cells in the S phase of the cell cycle

Since incadronate clearly inhibits proliferation of HTLV-I-infected T-cell lines, we analysed cell cycle progression by flow cytometry after incubation with incadronate (Fig 4). The cells were incubated with incadronate for 24 h, since incubation for 48 h induced cell death. Incadronate inhibited cell cycle progression, as evidenced by an increased proportion of cells in the S phase, with a corresponding decreased number of cells in the G2/M phase in all HTLV-I-infected T-cell lines. However, incadronate had no effect on cell cycle progression in HTLV-I-non-infected T-cell lines. Thus, incadronate reduced proliferation of HTLV-I-infected T-cell lines by arresting cells in the S phase of the cell cycle.

Incadronate inhibited proliferation of HTLV-I-infected T-cell lines via the mevalonate pathway

Nitrogen-containing bisphosphonates inhibit cell proliferation via inhibiting the mevalonate pathway (Shipman *et al,* 1998; Benford *et al,* 1999). Mevalonate is synthesized from 3-hydroxy-3-methylglutaryl coenzyme A and can be targeted by statin derivatives to reduce cholesterol synthesis in hypercholesterolemic patients. Nitrogen-containing bisphosphonates inhibit the synthesis of farnesyl diphosphate (FPP) by inhibiting FPP synthase. FPP and its derivative, geranylgeranyl diphosphate (GGPP), are essential for the post-translational prenylation and thus proper functioning of small GTP-binding proteins (Ras, Rho, Cdc42, Rac and Rap1A) (Benford *et al,* 1999). Therefore, the incadronate-induced effects on our HTLV-I-infected T-cell lines were examined with respect to mevalonate signaling.

FOH and GGOH are cell-permeable precursors of FPP and GGPP, respectively. Addition of FOH and GGOH to HTLV-I-infected T-cell lines reduced the inhibitory effects of incadronate on the proliferation of HUT-102 and SLB-1 cells (Fig 5), implicating the mevalonate pathway in the incadronate-induced cell growth suppression. GGOH had a more marked effect on the inhibition than FOH, therefore GGPP synthesis might be the rate-limiting step in these cell lines. The appearance of reversal of incadronate effect by FOH and GGOH could also be detected in HTLV-I-infected T-cell lines after treatment for 24 h and 72 h, but this effect was most marked at 48 h incubation.

Intracellular signaling induced by incadronate in HTLV-I-infected T-cell lines

Since FOH and GGOH prevented the incadronate-induced cell growth inhibition (Fig 5), we postulated that prenylation is critical for this inhibition. We used an antibody that specifically recognizes unprenylated Rap1A, a substrate of geranylgeranyl transferase, for Western blotting (Fig 6A). Unprenylated Rap1A was absent in untreated HTLV-I-infected T-cell lines, but accumulated markedly after treatment for 48 h with 62.5 or 125 μmol/l incadronate. The appearance of unprenylated Rap1A could also be detected in HTLV-I-infected T-cell lines after treatment with incadronate for 24 h, but this inhibition of prenylation was most marked at 48 h incubation. In contrast, unprenylated Rap1A was undetectable in Jurkat cells after treatment with incadronate. Unprenylated Rap1A was also absent in untreated primary ATL cells, whereas it accumulated after incadronate treatment (Fig 6B).

To clarify the molecular mechanisms of incadronate-induced inhibition of cell growth and apoptosis in HTLV-I-infected T-cell lines, we examined the expression of several intracellular regulators of apoptosis, including Bax, Bcl-2, Bcl-xL and survivin, by Western blot analysis. As shown in Fig 7A, incadronate did not alter Bax, Bcl-2 or Bcl-xL levels. In contrast, incadronate significantly decreased the expression of survivin in a dose-dependent manner in SLB-1 and HUT-102 cells. The modulation and deregulation of cellular signaling by viral regulatory protein Tax results in the upregulation of expression of a large number of cellular genes involved in cell proliferation and survival. Although survivin is a Tax-responsive gene (Kawakami *et al,* 2005), incadronate did not change the protein level of Tax. Equivalent protein loadings were confirmed with a specific antibody for the housekeeping gene product, actin (Fig 7A). To elucidate the relationship of survivin levels with the apoptotic effect of incadronate, we examined the expression of unprenylated Rap1A and survivin in HUT-102 cells treated with incadronate in the absence or presence of FOH or GGOH. Addition of FOH and GGOH to HUT-102 cells reversed incadronate-induced unprenylation of Rap1A and inhibition of survivin expression (Fig 7B).

Anti-tumour effects of incadronate on subcutaneous HUT-102 tumours

Finally, we examined the effects of incadronate against ATL *in vivo*. SCID mice (n = 10) were inoculated with HUT-102, and then divided into two groups: untreated mice (n = 5) and incadronate (0.6 mg/kg every day)-treated mice (n = 5). Treatment commenced on the next day after inoculation. At day 21 post-treatment, the mean

tumour volumes (Fig 8A) and weights (Fig 8B) were significantly lower than those of vehicle-treated mice ($P < 0.05$, Mann-Whitney *U*-test; Fig 8). During the period from day 0 to 21, the control mice showed signs of severe disease, including piloerection. In contrast, mice treated with incadronate showed no significant adverse effects and tolerated this dose well. These results suggest that incadronate also has an anti-ATL effect *in vivo*.

Discussion

ATL invariably follows a fatal clinical course despite the introduction of various chemotherapeutic agents. Although many ATL patients initially respond to chemotherapy, drug-resistance eventually develops, preventing a curative treatment. Although allogenic haematopoietic stem cell transplantation has produced promising results in ATL patients, it often causes serious clinical side effects and introduces the risk of graft-versus-host disease (Utsunomiya *et al*, 2001). Therefore, a novel therapeutic approach based on new insights into the pathogenesis of ATL is of paramount importance for these patients.

Bisphosphonates have been used for the treatment of hypercalcemia and bone complications associated with malignancies including multiple myeloma and ATL, and osteoporosis. Bisphosphonates bind avidly to hydroxyapatite bone mineral surfaces and are selectively internalized by osteoclasts where they inhibit their activity (Russel & Rogers, 1999). Nitrogen-containing bisphosphonates induce apoptosis of osteoclasts by blocking the mevalonate pathway (Benford *et al*, 1999). Recent work

has focused on the direct and/or indirect effects of bisphosphonates on tumour cells, particularly within the bone microenvironment. However, bisphosphonate efficacy for ATL has not yet been addressed.

This study showed for the first time that incadronate inhibits cell proliferation and induces apoptosis of HTLV-I-infected T-cell lines and primary ATL cells. Incadronate induced apoptosis through the activation of caspase-3, -8 and -9, and significantly attenuated the anti-apoptotic protein, survivin. The cell-permeable precursor of GGPP, GGOH, prevented the inhibitory effect of incadronate in HTLV-I-infected T-cell lines. By Western blot analysis, we found that the small GTP-binding protein, Rap1A, was not prenylated, when the cells were treated with incadronate in HTLV-I-infected T-cell lines and primary ATL cells. Furthermore, addition of FOH and GGOH to HUT-102 cells reversed incadronate-induced unprenylation of Rap1A and inhibition of survivin expression. These results indicate that protein prenylation is important for incadronate-induced cell growth suppression. We also studied several intracellular signaling molecules involved in cell proliferation and survival, and found nuclear factor-κB and activating protein-1 to be unaffected by incadronate (data not shown).

HTLV-I-infected T-cell lines treated with incadronate were arrested in the S phase. Thus, the anti-proliferative activity of incadronate is due to apoptotic cell death and/or cell cycle arrest in S phase. Interestingly, incadronate did not inhibit the proliferation of non-infected T-cell lines and normal PBMC, demonstrating that incadronate effectively induces cytotoxicity in ATL cells without toxicity to normal PBMC. Furthermore, incadronate did not prevent prenylation in non-infected T-cell

lines, suggesting prevention of protein prenylation as an attractive molecular target in the treatment of ATL. Although the Ras/mitogen-activated protein kinase pathway is crucial for cellular growth and proliferation of human leukaemias, this pathway has not yet been investigated in ATL, where it might play an important role in the leukemogenesis.

There are some reports of reduced growth in melanomas and cervical carcinomas upon treatment of animals with bisphosphonates (Giraudo *et al,* 2004; Yamagishi *et al,* 2004). Our study implicates incadronate as an effective anti-ATL agent *in vivo* due to the demonstrated inhibition of cell growth in an HTLV-I-infected T-cell line, HUT-102, in SCID mice. In our model, SCID mice bearing HUT-102 cells did not develop hypercalcemia. HTLV-I-encoded Tax oncoprotein transgenic mice show large granular lymphocytic leukaemia/lymphomas, and these mice spontaneously develop hypercalcemia, high-frequency osteolytic bone metastases and enhanced osteoclast activity (Gao *et al,* 2005). After the completion of this work, another nitrogen-containing bisphosphonate, zoledronic acid, was reported to protect Tax transgenic mice from bone and soft-tissue tumours and to prolong their survival (Gao *et al,* 2005). These data indicate that the bisphosphonate-mediated osteoclast inhibition does not only block bone metastasis but also prevents tumour progression, thus suggesting a direct anti-tumour effect of the bisphosphonates on tumour cells.

Therapeutically effective serum concentrations of incadronate may be difficult to achieve *in vivo*. Incadronate has high affinity for mineralized bone and rapidly localizes to bone. In the present study, at least 15.6 μmol/l incadronate was required *in vitro* over 48 to 72 h to induce apoptosis in ATL cells. According to a

previous study evaluating incadronate efficacy for the treatment of hypercalcemia, peak serum concentrations after treatment at a dose of 10 mg, were less than 1.2 μmol/l, indicating that sufficient serum concentrations for anti-ATL activity may not be readily obtained. However, the concentration of bisphosphonates in bone tissue is known to be high in osteoclast bone (Sato *et al,* 1991). Moreover, it is known to that bisphosphonates concentrations in bone marrow are much higher than those in other organs, because bisphosphonates incorporated in bone marrow osteoclasts disrupt osteoclasts and release bisphosphonates (Hiraga *et al,* 2001). Thus, incadronate may directly promote apoptosis in bone marrow ATL cells.

In conclusion, incadronate elicits anti-ATL activity via the mevalonate pathway both *in vitro* and *in vivo,* suggesting intervention with bisphosphonates as a promising therapeutic strategy for ATL. The efficacy and safety of bisphosphonates for ATL treatment should be verified in early-phase clinical trials.

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Table I. IC_{50} for inhibition of cell growth of incadronate.

Cells	HTLV-I	IC_{50} for inhibition of cell growth (μmol/l)
MT-4	+	34.4
C5/MJ	+	79.0
SLB-1	+	56.3
HUT-102	+	27.1
Jurkat	-	>125.0
MOLT-4	-	>125.0
ATL 1	+	5.6
ATL 2	+	78.0
ATL 3	+	8.3
ATL 4	+	52.3
ATL 5	+	90.3
ATL 6	+	84.8
ATL 7	+	66.8
Normal 1	-	>125.0
Normal 2	-	>125.0
Normal 3	-	>125.0

Figure legends

Fig 1. Inhibitory effects of incadronate on cell growth of HTLV-I-infected T-cell lines and primary ATL cells. Cells were incubated in the presence of various concentrations of incadronate (15.6, 31.3, 62.5 and 125 μmol/l) for 72 h, and *in vitro* growth of the cultured cells was measured by water-soluble tetrazolium-8 assay. Relative growth of the cultured cells is presented as the mean determined on human T-cell lines (A), and PBMC from ATL patients and healthy controls (B) from triplicate cultures. A relative growth of 100% was designated as the total number of cells that grew in the 72-h culture in the absence of incadronate. Data are mean ± SD.

Fig 2. Incadronate induces apoptosis in HTLV-I-infected T-cell lines. Human T-cell lines were incubated with various concentrations of incadronate (15.6, 31.3, 62.5 and 125 μmol/l) for 72 h. Cells were harvested, then stained with the Apo2.7 monoclonal antibody, and analysed by flow cytometry. Data represent the percentages of apoptotic cells (mean ± SD; n = 3 experiments).

Fig 3. Incadronate-induced apoptosis is caspase-dependent. Indicated cells were incubated with incadronate (125 μmol/l) for 24-72 h. Caspase activity was assayed as described in Materials and methods and expressed relative to untreated cells, which were assigned a value of 1. Values represent the mean ± SD of three experiments.

Fig 4. Incadronate induces accumulation of cells in the S phase of the cell cycle in HTLV-I-infected T-cell lines. Human T-cell lines were incubated in the absence or presence of incadronate (125 μmol/l) for 24 h. Then, the cells were washed, fixed, stained with propidium iodide, and analysed for DNA content by flow cytometry. Each experiment was repeated three times and similar results were obtained. The variation between three experiments was less than 10%.

Fig 5. Incadronate inhibits the proliferation of HTLV-I-infected T-cell lines by inhibiting the mevalonate pathway. HUT-102 and SLB-1 cells were pretreated with the indicated concentrations of FOH or GGOH for 1 h. Then, cells were incubated with and without incadronate (125 μmol/l) for 48 h, and then analysed by water-soluble tetrazolium-8 assay. A relative growth of 100% was designated as the total number of cells that grew in the 48-h culture in the absence of incadronate. Data are mean ± SD of triplicate assays.

Fig 6. Incadronate prevents the prenylation of Rap1A. Cell lysates (20 μg per lane) from the indicated T-cell lines (A) and primary ATL cells (B) treated with 62.5 or 125 μmol/l incadronate for 48 h were subjected to Western blotting using antibodies specific for the unprenylated form of Rap1A and for actin.

Fig 7. Western blot analysis of apoptosis-associated proteins and the viral protein Tax. (A) The indicated cells were treated with 62.5 or 125 μmol/l incadronate for 48 h. (B) HUT-102 cells were pretreated with GGOH or FOH (50 μmol/l) for 1 h. Then, cells

were incubated with and without incadronate (125 μmol/l) for 48 h, and then lysed. Protein levels were detected by Western blotting with antibodies directed against each protein.

Fig 8. Incadronate inhibits growth of HUT-102 cells in SCID mice. (A) HUT-102 cells (5×10^6 per mouse) were inoculated subcutaneously into SCID mice. The mice (five per group) received a single subcutaneous injection of either vehicle or incadronate (0.6 mg/kg) every day. Treatment was initiated on the next day of inoculation. The mice were monitored for tumour volumes at 7, 14 and 21 days after cell inoculation. Incadronate suppressed the growth of HUT-102 cells in contrast to the significant increase in the tumour burden generated in vehicle-treated control mice. (B) Weight of tumours removed from incadronate-treated mice and untreated mice on day 21 after cell inoculation. Data are mean ± SD of five mice.