Identification of a vesicular ATP release inhibitor for the treatment of neuropathic and inflammatory pain

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Abstract

Although acute nociception is an important biological warning system, chronic neuropathic and inflammatory pain may result from nerve injury, inflammation, or other pathological processes (1–4). Although the incidence of chronic pain is estimated at 20–25% worldwide (1, 4), optimal drug treatment regimens with few side effects are currently unavailable for the treatment of chronic pain. Recently, researchers have proposed that inhibitors of purinergic chemical transmission, which plays a key role in the pathological pain response, may allow for targeted treatment of pathological neuropathic and inflammatory pain. However, such therapeutic analgesic agents have yet to be developed. In the present study, we demonstrated that clodronate, a first-generation bisphosphonate with comparatively fewer side effects than traditional treatments, significantly attenuates neuropathic and inflammatory pain unrelated to bone abnormalities, which plays a key role in the pathological pain response, may allow for targeted treatment of pathological neuropathic and inflammatory pain. However, such therapeutic analgesic agents have yet to be developed.

Despite the high incidence of neuropathic and inflammatory pain worldwide, effective drugs with few side effects are currently unavailable for the treatment of chronic pain. Recently, researchers have proposed that inhibitors of purinergic chemical transmission, which plays a key role in the pathological pain response, may allow for targeted treatment of pathological neuropathic and inflammatory pain. However, such therapeutic analgesic agents have yet to be developed. In the present study, we demonstrated that clodronate, a first-generation bisphosphonate with comparatively fewer side effects than traditional treatments, significantly attenuates neuropathic and inflammatory pain unrelated to bone abnormalities, which plays a key role in the pathological pain response, may allow for targeted treatment of pathological neuropathic and inflammatory pain. However, such therapeutic analgesic agents have yet to be developed.

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which produce effects similar to the beneficial phenotypes observed in VNUT–/– mice, are candidate therapeutic drugs for chronic pain that may act via VNUT inhibition.

In the present study, we demonstrate that clodronate, a non-nitrogen-containing bisphosphonate, is a potent and selective inhibitor of VNUT in vivo. In addition, our findings indicate that clodronate-evoked inhibition of vesicular ATP release is important for the treatment of chronic neuropathic and inflammatory pain, as well as the accompanying inflammation.

**Results**

**Clodronate Is a Selective and Potent Inhibitor of VNUT.** To identify the targets of the first-generation bisphosphonates clodronate and etidronate, we used a quantitative transport assay system involving proteoliposomes containing only purified protein (22, 23). The cDNAs of vesicular neurotransmitter transporters or SLC17 transporters were cloned into Escherichia coli or baculovirus expression vectors, and overexpressed in E. coli or insect cells. The membrane fractions were solubilized, and the transporters were purified via nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography. The final fractions contained major protein bands of the expected apparent molecular masses, as determined via staining with Coomassie Brilliant Blue (Fig. 1A).

These purified proteins were incorporated into proteoliposomes, following which the inhibitory effects of the first-generation bisphosphonates on these transporters were examined. We observed that at half-maximal inhibitory concentration of 15.6 nM, clodronate exerted a stronger inhibitory effect on VNUT-mediated ATP uptake than etidronate (Fig. 1B). Neither clodronate nor etidronate exhibited strong inhibitory effects on other vesicular neurotransmitter transporters or SLC17 family transporters (Fig. 1B). Our cis-inhibition analysis also indicated that clodronate was the strongest VNUT inhibitor among the bisphosphonates examined (Fig. 2).

**Clodronate Is an Allosteric Modulator of VNUT Cl− Dependence.** We further examined the mechanism underlying the inhibitory effect of clodronate on VNUT-mediated ATP uptake. Exposure to a high concentration of clodronate had no effect on the Δψ, based on oxonol-V fluorescence quenching (Fig. 3A). An analysis of Cl− dependency for ATP uptake revealed no change in ATP transport following exposure to up to 2 mM Cl−: ATP uptake markedly increased following treatment with 3–7 mM Cl− and plateaued beyond 8 mM Cl− (Fig. 3B). Notably, Cl−-dependent VNUT activation exhibited strong positive cooperativity for ATP transport, with a Hill coefficient of −3 for Cl− (Fig. 3C). Clodronate shifted Cl− concentration toward a higher activation level, suggesting a competitive interaction (Fig. 3B and C). Photoaffinity labeling for ATP binding showed results almost identical to the results obtained for ATP transport-mediated substrate specificity (Fig. S2), and was not inhibited by either clodronate or etidronate (Fig. 3D). In addition, keto acids, such as acetoacetate or glyoxylate, which are known to inhibit SLC17 transporters as a Cl−-dependent manner (23, 24), did not inhibit ATP binding (Fig. 3D). These effects of clodronate were completely reversible (Fig. 3E).

**Clodronate Modulates Vesicular ATP Release.** ATP is primarily released from neurons, astrocytes, and microglia via VNUT-mediated exocytosis (12, 25–28). Previous studies have demonstrated that depolarization-dependent ATP release from neurons is inhibited by tetanus neurotoxin or the cell-permeable Ca2+-chelator EGTA-tetraacetoxyethyl ester (AM), both of which are known inhibitors of exocytosis (25). In the present study, we observed complete inhibition of such ATP release following treatment with a low concentration (100 nM) of clodronate, compared with the concentration described for bone resorption inhibition (29) (Fig. 4A). In a parallel experiment, 1 μM clodronate did not inhibit glutamate release, suggesting that clodronate selectively inhibits vesicular ATP release (Fig. 4B).

The effects of clodronate on ATP release were also completely reversible (Fig. 4A). Previous researchers have proposed various mechanisms of astrocytic ATP release, such as those mechanisms involving vesicular transporter or plasma membrane channel-mediated pathways (9, 26). In the present study, depolarization-dependent ATP release from astrocytes was inhibited by tetanus neurotoxin, the extracellular Ca2+-chelator EGTA, and EGTA-AM, supporting the notion that astrocytic ATP release occurs via a vesicular mechanism (26) (Fig. 4C). However, our findings indicate that neither ATP nor glutamate release from astrocytes was inhibited by clodronate, even at 1 μM (Fig. 4C and D). In microglia, ATP release is known to be inhibited by botulinum neurotoxin A or the cell-permeable Ca2+-chelator O,O′-bis(2-aminoophenyl)ethylendeglycol-N,N,N′,N′-tetraacetic acid-AM (27). In the present study, we also observed that a low concentration of clodronate resulted in complete inhibition of vesicular ATP release from microglia, similar to the effect observed in neurons (Fig. 4E).

To examine the accessibility of bisphosphonate to neurons and astrocytes, we measured the uptake of a commercially available radiolabeled bisphosphonate. Na+-dependent bisphosphonate transport activity was detected in neurons but not astrocytes, and this activity was completely inhibited by clodronate and inorganic
Transport involves bisphosphonate

D mice, carrageenan- or CFA-evoked hind-paw

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Fig. S4

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VNUT

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Fig. 5 B and

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VNUT-Mediated Immune Control by Clodronate. Immune cells contribute to chronic inflammatory pain through the release of inflammatory mediators and infiltration of inflammatory cells (31). In the human monocyte cell line THP-1 (32), clodronate inhibited vesicular ATP release in a manner similar to the vesicular ATP release observed in neurons, suggesting that clodronate exerts an anti-inflammatory effect (Fig. 6 A and B). In vivo, carrageenan-evoked or CFA-evoked hind-paw edema was attenuated following exposure to clodronate, but not to etidronate (Fig. 6 C and D and Fig. S5 A and B). The anti-inflammatory effect of clodronate was stronger than the effect of diclofenac in the therapeutic range, comparable to the maximal effect of the moderate steroid hydrocortisone and to the effect of the strong steroid prednisolone in the therapeutic range (Fig. 6D and Fig. S5B). Glucocorticoids exert a strong anti-inflammatory effect but can cause a wide range of severe side effects, such as metabolic dysfunction (diabetes, hyperlipidemia, and osteoporosis) and increased susceptibility to infections (33). In VNUT−/− mice, carrageenan- or CFA-evoked hind-paw edema was also attenuated to ∼70% of the edema observed in wild-type controls (Fig. 6 C and D and Fig. S5 A and B). Moreover, the inflammatory response was decreased in VNUT−/− mice compared with the inflammation observed in wild-type controls (Fig. S5C). Notably, TNF-α and IL-6 were detected in the blood after carrageenan injection, and the release of these cytokines was strongly inhibited by clodronate (Fig. 6 E and F). Similarly, VNUT−/− mice exhibited markedly decreased blood cytokine levels compared with wild-type controls (Fig. 6 E and F). Release of inflammatory mediators from THP-1 cells was inhibited by apyrase (ATP diprophosphohydrolase) or suramin (nonselective inhibitor of purinoceptors), suggesting that cytokine release requires autocrine ATP release (Fig. S6).

VNUT-Mediated Chronic Neuropathic Pain Control by Clodronate. Approximately 60% attenuation of neuropathic pain was observed after a prior clodronate injection at a dose lower than the dose used for inflammatory pain (Fig. 7A). VNUT−/− mice also exhibited reduced hyperalgesia compared with wild-type controls, and this analgesic effect of clodronate was lost in VNUT−/− mice (Fig. 7A). The analgesic effect of clodronate was stronger than analgesia induced by pregabalin and gabapentin (Fig. 7 B and C), both of which are in widespread clinical use. Some bisphosphonates attenuate the neuropathic pain associated with complex regional pain syndrome 1 in conditions involving bone abnormalities (16). In the present study, bisphosphonate compounds other than clodronate exerted weak or no analgesic effects, similar to their inhibitory effects on VNUT, suggesting that the analgesic effect of other bisphosphonates depends on the inhibition of bone resorption (Fig. 7B). The analgesic effect of clodronate has both fast- and long-acting properties compared with the effect of pregabalin and gabapentin and was completely reversible, suggesting that clodronate at this dose is not toxic and is without side effects (Fig. 7C). Notably, pregabalin at the effective dose (0.1–10 mg/kg) seemingly induced drowsiness and reduced exploratory behavior, although these effects were not observed for clodronate, even at a dose of 10 mg/kg (Movie S1). Although pregabalin did not induce drowsiness at a dose of 0.001 mg/kg, administration at this dose did not attenuate neuropathic pain (Fig. 7C).

Moreover, previous studies have indicated that clodronate-containing liposomes induce macrophage apoptosis by selective delivery of a high concentration of clodronate into macrophages (34), which may be involved in the clodronate analgesic effect. However, as expected, the low concentration of clodronate

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>IC50 of VNUT (µM)</th>
<th>Bone resorption inhibitory effect</th>
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<tr>
<td>Clodronate</td>
<td>0.0156</td>
<td>+</td>
</tr>
<tr>
<td>Etidronate</td>
<td>20.8</td>
<td>+</td>
</tr>
<tr>
<td>Tiludronate</td>
<td>&gt;100</td>
<td>+</td>
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<tr>
<td>Medronate</td>
<td>7.52</td>
<td>+</td>
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<tr>
<td>Difluoromethylene diphosphonic acid</td>
<td>1.51</td>
<td>NT</td>
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<tr>
<td>Pamidronate</td>
<td>&gt;100</td>
<td>++</td>
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<tr>
<td>Alendronate</td>
<td>&gt;100</td>
<td>+++</td>
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<tr>
<td>Neridronate</td>
<td>&gt;100</td>
<td>++++</td>
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<tr>
<td>Ibandronate</td>
<td>22.7</td>
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<tr>
<td>Risedronate</td>
<td>&gt;100</td>
<td>++++</td>
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<tr>
<td>Minodronate</td>
<td>0.267</td>
<td>++++</td>
</tr>
<tr>
<td>Zoledronate</td>
<td>&gt;100</td>
<td>++++</td>
</tr>
<tr>
<td>Methylene bisphosphonic dichloride</td>
<td>1.81</td>
<td>NT</td>
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<tr>
<td>Pyrophosphate</td>
<td>&gt;100</td>
<td>−</td>
</tr>
<tr>
<td>Chloromethylphosphonate</td>
<td>&gt;100</td>
<td>NT</td>
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Fig. 2. Clodronate is the strongest inhibitor of VNUT. The inhibitory potencies of bisphosphonates toward ΔΨ-dependent ATP uptake by proteoliposomes containing purified VNUT were assayed in the presence of 10 mM Cl− at 2 min and are shown as IC50 (n = 3–13). The effect of pyrophosphate on ATP uptake was examined in ref. 10. The degrees of bone resorption inhibitory effect are indicated as follows: −, none; +, weak; ++, moderate; ++++, strong; ++++, very strong (18, 43). NT, not tested.

Phosphate (P), suggesting that P transport involves bisphosphonate uptake into neurons (Fig. 4F). We further examined the gene expression pattern of Na+−dependent phosphate transporters, which physiologically consist of the SLC20 and SLC34 families, between neurons and astrocytes (30). However, no neuron-specific signals were detected, suggesting that cellular uptake of bisphosphonate occurs via a novel phosphate transporter or other transport mechanisms (Fig. S3).

Clodronate Controls Chronic Inflammatory Pain Through VNUT. We analyzed the effects of clodronate on inflammatory and neuropathic pain unrelated to bone abnormalities. Approximately 40% attenuation of carrageenan- or complete Freund’s adjuvant (CFA)-evoked inflammatory pain was observed following injection of 10 mg/kg clodronate (Fig. 5 A and B and Fig. S4 A and B). In addition, VNUT−/− mice exhibited reduced hyperalgesia relative to wild-type controls, and the analgesic effect of clodronate was lost in VNUT−/− mice (Fig. 5 A and B and Fig. S4 A and B). Notably, clodronate did not alter baseline sensory thresholds (Fig. 5 C and D). Clodronate-mediated analgesia was stronger than the analgesic effect induced by acetaminophen and diclofenac (first-choice drugs), and comparable to analgesia of the nonnarcotic opioid tramadol in the therapeutic range (Fig. 5 E and F and Fig. S4 C and D). Etidronate, which exerted a lower inhibitory effect of VNUT, had no analgesic effect (Fig. 5 E and F and Fig. S4 C and D).
and mice
Fig. S9
S5
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binding site. In vivo, -Kato et al.
Lower
n
Clodronate (Clo) reversibly inhibits VNUT through Cl
= 7
www.pnas.org/cgi/doi/10.1073/pnas.1704847114
test). NS, not significant. NS,
D
SEM (** = 0.01; two-tailed paired Student
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formation was measured as a 2-min quench of oxonol V fluorescence
in the absence or presence of 100 μM Clo. Control activity was subtracted from the quench in the presence of carbonyl cyanide m-chlorophenyl hydrazone (n = 7–8). (B) ATP uptake for 1 min at various [Cl−] values in the presence (△) or absence (●) of 100 nM Clo, or in the absence of valinomycin (○) (n = 3–12). (C) Hill plot of ATP uptake in the presence or absence of 100 nM clodronate. Data were taken from Fig. 3B. (D, Upper) Photoaffinity labeling of VNUT protein (4 μg) upon UV illumination with biotin-11-ATP in the presence or absence of the indicated concentrations of various compounds. (D, Lower) Each protein was analyzed using 10% SDS/PAGE and visualized using Coomassie Brilliant Blue staining. The arrowhead indicates the position of VNUT protein. (E) Inhibition due to 1 μM clodronate was fully reversed after washing the proteoliposomes (n = 4–8). In all cases, data are mean ± SEM (**p < 0.01; two-tailed paired Student’s t test). NS, not significant. NS, not significant.

Fig. 3. Clodronate (Clo) reversibly inhibits VNUT through Cl− competition. (A) Val-evoked Δψ formation was measured as a 2-min quench of oxonol V fluorescence in the absence or presence of 100 μM Clo. Control activity was subtracted from the quench in the presence of carbonyl cyanide m-chlorophenyl hydrazone (n = 7–8). (B) ATP uptake for 1 min at various [Cl−] values in the presence (△) or absence (●) of 100 nM Clo, or in the absence of valinomycin (○) (n = 3–12). (C) Hill plot of ATP uptake in the presence or absence of 100 nM clodronate. Data were taken from Fig. 3B. (D, Upper) Photoaffinity labeling of VNUT protein (4 μg) upon UV illumination with biotin-11-ATP in the presence or absence of the indicated concentrations of various compounds. (D, Lower) Each protein was analyzed using 10% SDS/PAGE and visualized using Coomassie Brilliant Blue staining. The arrowhead indicates the position of VNUT protein. (E) Inhibition due to 1 μM clodronate was fully reversed after washing the proteoliposomes (n = 4–8). In all cases, data are mean ± SEM (**p < 0.01; two-tailed paired Student’s t test). NS, not significant. NS, not significant.

(10 mg/kg) did not induce apoptosis of blood cells, including macrophages (Fig. 7D and E). Similarly, low concentrations of clodronate, which resulted in complete inhibition of vesicular ATP release, did not induce apoptosis in neurons, astrocytes, microglia, or THP-1 cells. As expected, clodronate-containing liposomes induced complete apoptosis in phagocytic microglia and THP-1 cells, but not in neurons or astrocytes (34, 35) (Fig. S7). Recent reports have suggested that ATP is also stored in microglial lysosomes in a VNUT-mediated manner, and that inhibition of this process leads to cell death (36). Although lysosomal storage of ATP in microglia was inhibited by clodronate, clodronate did not induce microglial apoptosis, suggesting the existence of other functions of lysosomal ATP release (Fig. S8).

Finally, the pathogenesis of neuropathic pain, but not inflammatory pain, is thought to involve VNUT (13). Our analysis revealed that VNUT is involved in pathological inflammatory pain in two well-characterized models. Consistent with the findings of a previous study (13), we observed that basal nociception and weak inflammation with less chronicity were not affected by clodronate in an inflammatory pain model (one-fourth volume of CFA compared with Figs. S4 and S5) (Fig. S9). These observations strongly suggest that VNUT inhibition improves chronic pathogenesis more extensively than pain under physiological conditions.

Discussion
Previous attempts to develop new therapeutic drugs for the treatment of chronic neuropathic and inflammatory pain with reduced side effects have been unsuccessful. In the present study, we observed that clodronate is a potent and selective inhibitor of vesicular storage and release of ATP, which is mediated by allosteric modulation at the VNUT Cl− binding site. In vivo, clodronate was more effective than other agents in attenuating chronic neuropathic and inflammatory pain and the accompanying inflammation without affecting basal nociception in wild-type mice. Consistent with these observations, VNUT−/− mice exhibited reductions in chronic pain and inflammation, for which clodronate was ineffective. These observations indicated that clodronate-evoked inhibition of purinergic chemical transmission is important for the treatment of neuropathic and inflammatory pain with reduced side effects. Furthermore, the present study identifies a transporter-targeted analgesic and antiinflammatory drug.

Cl− dependency is a unique feature of SLC17 transporters (23). VNUT-mediated ATP transport is activated by Cl−, and this activation is inhibited competitively and reversibly by keto acids, such as acetoacetate and glyoxylate (23, 24). Regulation of the metabolic anion switch between Cl− and keto acids safely controls purinergic chemical transmission in response to changes in metabolic state. However, keto acids are metabolized in the body, and they do not show high specificity among SLC17 transporters.
Low K dependence, because VNUT is highly conserved in both mammals and plants (60% of the total). Consistent with these observations, a recent study has reported that VNUT gene expression in the spinal cord is significantly up-regulated in pathological conditions (13). The study further reported that VNUT in spinal dorsal horn neurons, but not in astrocytes and microglia, is responsible for the pathogenesis of neuropathic pain, suggesting that VNUT may be an important target for the treatment of neuropathic pain (13). In the present study, clodronate was consistently taken up by neurons, inhibiting neuronal vesicular ATP release and thereby attenuating neuropathic pain on treatment of neuropathic pain (13). In the present study, clodronate was consistently taken up by neurons, inhibiting neuronal vesicular ATP release and thereby attenuating neuropathic pain (13).

We elucidated two significant phenotypes associated with the loss of vesicular ATP release using clodronate and VNUT$^{−/−}$ mice. First, VNUT was involved in pathological neuropathic and inflammatory pain in vivo (Figs. 5 and 7). Although VNUT gene defects were not associated with basal nociception and weak inflammation with low chronicity, VNUT contributed to chronic hyperalgesia (~40–60% of the total). Consistent with these observations, a recent study has reported that VNUT gene expression in the spinal cord is significantly up-regulated in pathological conditions (13). The study further reported that VNUT in spinal dorsal horn neurons, but not in astrocytes and microglia, is responsible for the pathogenesis of neuropathic pain, suggesting that VNUT may be an important target for the treatment of neuropathic pain (13). In the present study, clodronate was consistently taken up by neurons, inhibiting neuronal vesicular ATP release and thereby attenuating neuropathic pain (Figs. 4 and 7). Our results strongly support the notion that VNUT is involved in the pathogenesis of not only neuropathic pain but also inflammatory pain, and that a specific inhibitor may serve as an effective and safe analgesic drug in this patient population. Further studies are required to clarify in vivo VNUT function in primary afferent nerve terminals of the spinal dorsal horn, which may be involved in inflammatory pain.

Second, our study demonstrates that VNUT is involved in the immune response in vivo (Fig. 6). We propose that the following mechanism underlies the antiinflammatory effect of VNUT inhibition:
VNUT is also localized in secretory vesicles in immune cells (e.g., monocytes, macrophages, T cells) and is responsible for vesicular storage and release of ATP (32, 38). Released ATP or degraded ADP and adenosine bind to various purinoceptors in an autocrine or paracrine manner, stimulating the release of inflammatory mediators and thus leading to inflammation (39). We observed that clodronate completely inhibited the release of ATP from immune cells and reduced blood levels of inflammatory mediators, such as TNF-α and IL-6, which are released mainly from macrophages and T cells (Fig. 6). These observations indicated that VNUT is a key molecule for the induction of pathological pain and inflammation, and that VNUT inhibition is therefore essential for improving pathological symptoms.

Because purinergic chemical transmission is involved in disease pathogenesis (9), clodronate-evoked purinergic chemical transmission blockade may be effective in the treatment of several chronic diseases, including chronic auto-inflammatory diseases, diabetes, and neurological disorders, among others. It should be stressed that the therapeutic effects of clodronate were stronger than the effects of widespread drugs for neuropathic pain, such as pregabalin or gabapentin (Fig. 7C). In addition, these effects were comparable to the effects of widespread drugs for inflammatory pain or inflammation, such as tramadol or prednisolone, in the therapeutic range (Figs. 5 and 6). However, tramadol and prednisolone are associated with severe side effects, strongly suggesting that clodronate is promising for the treatment of intractable diseases associated with abnormalities in purinergic transmission, with few side effects. Notably, VNUT−/− mice exhibit an improvement in blood glucose homeostasis, insulin sensitivity, and other pathological conditions, with no significant changes in phenotype (12). These observations suggest that clodronate may improve a wide range of diabetic symptoms, such as neuropathic pain, inflammation, hyperglycemia, and insulin sensitivity. Because no effective drugs or therapies for these diabetic symptoms have yet been developed (40), further studies regarding the wide range of applications of clodronate are currently in progress in our laboratories.

In summary, our findings indicate that clodronate selectively and robustly inhibits VNUT and can safely regulate purinergic chemical transmission in vivo, thereby attenuating pathological neuropathic and inflammatory pain and the accompanying inflammation in conditions without bone abnormalities. Notably, clodronate is approved for clinical use in the treatment of osteoporosis in many countries, and its clinical safety in humans is well established (21). Therefore, it is important to evaluate analgesic effects of clodronate for painful diseases independent of bone abnormalities in humans. Given its potency and the side effects of existing analgesics, clodronate, a nonopioid and nonsteroidal drug, might serve well as a novel analgesic or antiinflammatory drug.

Materials and Methods

Animal experiments were performed in accordance with the guidelines set by the Animal Care and Use Committees of Okayama University and Ajinomoto Co., Inc. All experiments were carried out in accordance with the approved institutional guidelines. Additional information on experimental methods is included in SI Materials and Methods.

Expression and Purification of Transporters in E. coli. Vesicular neurotransmitter transporters were expressed and purified as previously described (22). Briefly, E. coli C43 (DE3) cells were transformed with the expression vectors and grown in Terrific Broth medium containing 30 μg/mL kanamycin sulfate at 37 °C. E. coli cells were grown until A600 reached 0.6–0.8, following which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, followed by incubation for a further 16 h at 18 °C. The cells were then harvested by centrifugation and suspended in buffer consisting of 70 mM
Tris HCl (pH 8.0), 100 mM NaCl, 10 mM KCl, 15% glycerol, and 2 mM PMSF.

The cell suspension was then disrupted by sonication with a TOMY UD200 tip sonifier (OUTPUT4) and centrifuged at 5,856 × g at 4 °C for 10 min to remove large inclusion bodies and cell debris. The supernatant was carefully sonicated until clear in a bath-type sonicator and stored at 250 mM imidazole, 100 mM NaCl, 10 mM KCl, 20% glycerol, and 0.1% DTM, without loss of activity for at least a few months.

Reconstitution. Aliquots of 20 μg of purified protein were mixed with liposomes (550 μg of lipid), frozen at −80 °C, and left at this temperature for at least 15 min. The mixture was diluted 60-fold with reconstitution buffer containing 40 mM MES-Tris (pH 5.7), 150 mM potassium acetate, and 5 mM magnesium acetate. Reconstituted proteoliposomes were pelleted after centrifugation at 200,000 × g for 1 h at 4 °C and then suspended in 0.2 mL of reconstitution buffer.

Transport Assay. The reaction mixture (130 μL) consisting of 0.3 μg of protein incorporated into proteoliposomes, 20 mM MOPS-Tris (pH 7.0), 140 mM potassium acetate, 5 mM magnesium acetate, 10 mM KCl, and 2 μM valinomycin, as well as 100 μM [3H]ATP (0.5 MBq/μmol; PerkinElmer), 100 μM [2,3-3H]-glutamate (0.5 MBq/μmol; PerkinElmer), 100 μM [2,3,4-3H]-l-aspartate (0.5 MBq/μmol; PerkinElmer), 100 μM [2,3,4-3H]-GABA (0.5 MBq/μmol; PerkinElmer), or 100 μM p-[glucy-l-cystyl-2-H]-p-aminohippuric acid (0.5 MBq/μmol; PerkinElmer), was incubated at 27 °C. At the indicated time points, the proteoliposomes were separated from the external medium using centrifuge columns containing Sephax G-50 (fine) to terminate transport. The radioactivity in the eluate was measured via liquid scintillation counting (PerkinElmer).

For serotonin transport by VMAT2, proteoliposomes containing VMAT2 (0.3 μg of protein) were incubated in 20 mM MOPS-Tris (pH 7.5), 140 mM potassium acetate, 5 mM magnesium acetate, 10 mM KCl, and 10 μM [2-3H] serotonin (0.5 MBq/μmol; PerkinElmer) at 27 °C.

ATP and Glutamate Release from Neurons, Astrocytes, and Microglia. Primary cultured neurons or astrocytes (2.0 × 105 cells per 3.5-cm dish) were washed three times with Krebs-Ringer bicarbonate buffer composed of 128 mM NaCl, 1.9 mM KCl, 1.2 mM KH2PO4, 1.3 mM MgSO4, 26 mM NaHCO3, 10 mM D-glucose, 10 mM Hepes-NaOH (pH 7.4), 2.4 mM CaCl2, and 0.2% (wt/vol) BSA. After the cells had been incubated in Krebs-Ringer bicarbonate buffer at 37 °C for 3 h, 55 mM KCl was added to stimulate ATP and glutamate release.
Clodronate (Clo) attenuates neuropathic pain via VNUT inhibition. (www.pnas.org/cgi/doi/10.1073/pnas.1704847114)

release. After incubation at 37 °C for 20 min, aliquots were collected and the amount of ATP was measured using an ATP bioluminescent assay kit (Sigma-Aldrich), whereas the amount of glutamate was measured via HPLC on a COSMOSIL C18-ARII column (4.6 × 150 mm; Nacalai Tesque) and fluorescence detection, as previously described (12). In primary cultured microglia (1.0 × 10^6 cells per 96-well plate), 5 μM Ca^{2+} ionophore A23187 was added to stimulate ATP release. Aliquots were collected after 5 min, and the amount of ATP was measured. The addition of clodronate at our experimental concentration exerted no impact on the ATP bioluminescence assay. The slopes of the standard curves in the absence and presence of clodronate at 10 μM were as follows: 96.4 ± 9.0 and 92.8 ± 5.7 relative luminescence unit/mL of ATP, respectively (n = 3, not significant in two-tailed Student’s t test).

**Plantar Test.** The plantar test was performed as previously described (41). C57BL/6 mice (male, weighing 22–30 g at the time of the test) were acclimatized to an elevated metal mesh floor chamber (10.0 × 16.0 × 9.0 cm) for 60 min before the von Frey test. Mechanical hyperalgesia was assessed by measuring the left hind-paw withdrawal response to stimulation with a series of von Frey filaments (0.04–2.0 g; Aesthesio) presented perpendicular to the plantar surface. We determined the 50% paw withdrawal threshold using Dixon’s up-down method (42). Clodronate was injected i.v. via the tail vein 60 min before the von Frey test in a volume of 100 μL per 10 g of body weight.

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**Fig. 7.** Clodronate (Clo) attenuates neuropathic pain via VNUT inhibition. (A) The von Frey test was performed 60 min after an i.v. injection of saline (open bar) or Clo at the indicated concentrations (gray bars) in WT mice (Left) and VNUT−/− mice (Right) at 10 d after nerve injury (filled bars) (n = 7 mice). (B) Various compounds at the indicated concentration were assayed, and this dataset is the same as the dataset in A. The injection of compounds was performed at the time of maximal effect (n = 7 mice). (C) The von Frey test was performed the indicated time after i.v. injection of saline, Clo, pregabalin (Pre), or gabapentin (Gab) at the indicated concentrations in WT mice (n = 6–10 mice). Blood cells (D) or macrophages (E) were prepared 60 min after an i.v. injection of saline (open bars) or 10 mg/kg Clo (gray bars) in WT mice, and the apoptosis assay was performed (n = 3–5 mice). In all cases, data are mean ± SEM (**P < 0.01; one-way ANOVA followed by Dunnett’s test or two-tailed paired Student’s t test). NS, not significant.