Discovery of Orally Bioavailable Cathepsin S Inhibitors for the Reversal of Neuropathic Pain

Osamu Irie,*[,†] Takatoshi Kosaka,[†] Takeru Ehara,[†] Fumiaki Yokokawa,[†] Takanori Kanazawa,[†] Hajime Hirao,[†] Astuko Iwasaki,[†] Junichi Sakaki,[†] Naoki Teno,[†] Yuko Hitomi,[†] Genji Iwasaki,[†] Hiroaki Fukaya,[†] Kazuhiko Nonomura,[†] Keiko Tanabe,[†] Shinichi Koizumi,[†] Noriko Uchiyama,[†] Stuart J. Bevan,[†] Marzia Malcangio,[§,‡] Clive Gentry,[§,‡] Alyson J. Fox,[§,‡] Mohammed Yaqoob,[§,‡] Andrew J. Culshaw,[§,‡] and Allan Hallett[‡]

Novartis Institutes for BioMedical Research, Ohkubo 8, Tsukuba, Ibaraki 300-2611, Japan, 5 Gower Place, London WC1E 6BS, United Kingdom

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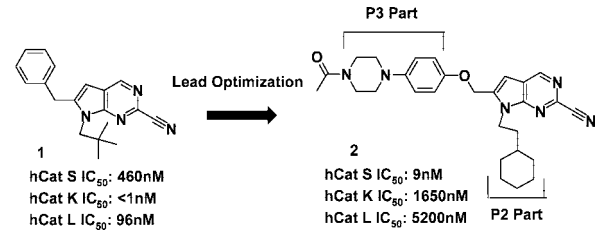
Abstract: Cathepsin S inhibitors are well-known to be an attractive target as immunological therapeutic agents. Recently, our gene expression analysis identified that cathepsin S inhibitors could also be effective for neuropathic pain. Herein, we describe the efficacy of selective cathepsin S inhibitors as antihyperalgesics in a model of neuropathic pain in rats after oral administration.

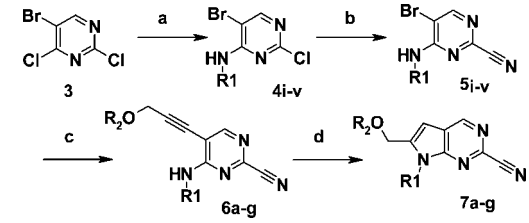
There is a clear medical need for new classes of effective analgesics, particularly in the treatment of chronic pain states. Current marketed therapies are based largely on two well-established classes of analgesics opiates and the nonsteroidal anti-inflammatory drugs (NSAIDs). Both classes of drugs have limited efficacy and evoke undesirable side effects. The lack of suitable therapies has stimulated our research to identify novel targets for neuropathic pain. We have recently discovered that the mRNA encoding cathepsin S was up-regulated in rat dorsal root ganglia following peripheral nerve injury by using a gene expression analysis approach.[1] We also published that LHVS[a], a potent nonspecific cathepsin S inhibitor, preferentially reversed hyperalgesia in a rat model after subcutaneous injection.[2] These findings suggest that cathepsin S inhibitors would be applicable for the reversal of neuropathic hyperalgesia. We thus sought to discover reversible, nonpeptidic, selective, and orally active cathepsin S inhibitors. Herein, we describe the efficacy of selective cathepsin S inhibitors as antihyperalgesics in a model of neuropathic pain in rats after oral administration.

Cathepsin S is a lysosomal cysteine protease belonging to the papain superfamily, and it is expressed in professional antigen presentation cells (APC) such as dendritic cells, B lymphocytes, and macrophages. The major role of cathepsin S in these cells is in the important proteolytic events that lead to antigen presentation. Cathepsin S inhibitors are thus being developed as immunological therapeutic agents.[3] Eleven members of the cysteine cathepsin family have been identified in the human gene (cathepsins B, C, H, F, K, L, O, S, V, W, and X).[4] Recent cathepsin gene knock out studies in mice have revealed that these lysosomal cysteine proteases have specific and individual functions, which are important for the normal functioning of an organism.[5]

Chart 1

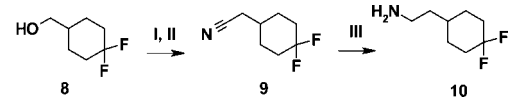


Scheme 1[a]



[a] Reagents and conditions: R_1NH_2, MeOH, rt, 18 h; (b) NaCN, DABCO, DMSO-H_2O, 60 °C, 4 h; (c) R_2-$OCH_2C'CH$, $Pd(PPh_3)_2Cl_2$, CuI, NEt_3, DMF, 80 °C, 4 h; (d) DBU, DMF, 100 °C, 4 h.

Scheme 2[a]



[a] Reagents and conditions: (I) TsCl, NEt_3, CH_2Cl_2, 0 °C rt, 15 h; (II) NaCN, DMSO, rt, 15 h; (III) $LiAlH_4$, H_2SO_4, Et_2O, 0 °C−rt, 15 h.

We recently reported the discovery of the selective and orally bioavailable cathepsin S inhibitor **2** after optimization of the P2 and P3 substituents of the potent cathepsin K inhibitor **1**[6] by using computer-assisted modeling studies[7] (Chart 1). Compound **2** exhibited potent inhibitory activity and selectivity toward human cathepsin S. To avoid off-target effects due to other cathepsins and to assess the efficacy of the cathepsin S inhibition for the reversal of neuropathic pain, we initially attempted to improve the selectivity of the compound **2** against cathepsin K and L.

The inhibitor syntheses are outlined in Schemes 1−3. Treatment of 5-bromo-2,4-dichloropyrimidine **3** with a variety of amines followed by addition of sodium cyanide, gave compounds **5i**−**v** (Scheme 1, Table 1). Sonogashira coupling reaction of **5i**−**v** with alkynes using a catalytic amount of $Pd(PPh_3)_2Cl_2$ and CuI followed by cyclization under basic conditions provided 2-cyano-pyrropyrimidines **2** and **7a**−**g**.

Preparation of the P2 amine for **7g** is shown in Scheme 2. Treatment of 4,4-difluorocyclohexylmethanol **8** with TsCl in the presence of triethylamine followed by the addition of sodium cyanide provided nitrile **9**. Reduction of **9** with $LiAlH_4$ in the

* To whom correspondence should be addressed. Phone: +81 29 865 2384. Fax: +81 29 865 2308. E-mail: osamu.irie@novartis.com.
† Novartis Institutes for BioMedical Research, Ohkubo 8, Tsukuba, Ibaraki 300-2611, Japan.
‡ Novartis Institutes for BioMedical Research, 5 Gower Place, London WC1E 6BS, United Kingdom.
§ Present address: Novartis Horsham Research Center, Wimblehurst Road, Horsham, RH12 5AB, United Kingdom.

[a] Abbreviations: LHVS, morpholineurea-leucine-homophenylalanine-vinyl phenyl sulfone; hCat, human cathepsin; DABCO, 1,4-diazabicyclo[2.2.2]octane; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; TsCl, *p*-toluenesulfonyl chloride; MCA, 4-methylcoumaryl-7-amide; MHC II, major histocompatability complex II; lIP10, leupeptin-induced polypeptide 10; CLIP, class II associated leupeptin induced peptide.

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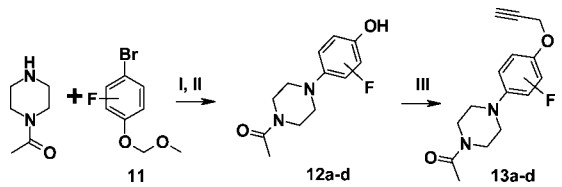
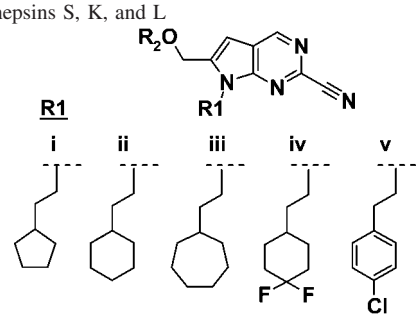
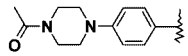
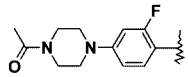
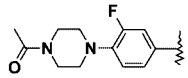
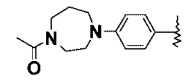
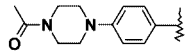
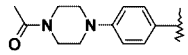
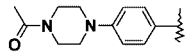
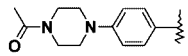
Scheme 3[a]



[a] Reagents and conditions: (I) $Pd_2(dba)_3$, NaOtBu, 2-(di-*tert*-butylphosphino)biphenyl, toluene, 80 °C, 4 h; (II) 4N HCl in EtOAc, MeOH, rt, 0.5 h; (III) $BrCH_2C{\equiv}CH$, K_2CO_3, DMF, 70 °C, 12 h.

Table 1. Inhibitory Activity of Pyrropyrimidine Compounds **2**, **7a**−**g** against Cathepsins S, K, and L



Compound	R1	R2	IC50 (nM)[a] Cat S	Cat K	Cat L
2	ii		9	1650	5200
7a	ii		18	5900	6800
7b	ii		9	>1000	>1000
7c	ii		11	290	>1000
7d	i		5	79	2300
7e	v		17	>1000	>1000
7f	iii		50	>2000	>2000
7g	iv		10	5400	6200

[a] Inhibition of recombinant human cathepsins K, L, and S in a fluorescence assay, employing Z-Phe-Arg-MCA (Cat K and L) and Z-Leu-Leu-Arg-MCA (Cat S) as synthetic substrates. Data represent means of two experiments performed in duplicate. Individual data points in each experiment were within a 2-fold range with each other.[9]

presence of sulfuric acid afforded the desired 4,4-difluorocyclohexylethylamine **10**.

Acetylene derivatives for **2** and **7c**−**g** were synthesized by alkyation of commercially available phenol derivatives under basic conditions (Scheme 3, conditions (III)). Buchwald−Hartwig cross-coupling reactions[8] with *O*-protected fluoro-substituted bromophenol derivatives followed by deprotection and alkylation with propargyl-bromide gave the fluoro-substituted alkynes for **7a** and **b**.

The IC_{50} values of the compounds are shown in Table 1. Substitutions by fluorine on the phenyl ring of the P3 did not significantly affect the selectivity while the potencies for cathepsin S were maintained (compare **2**, **7a**, **7b**). Expansion of the piperidine ring led to a loss of selectivity toward cathepsin K due to the structural differences of the S3 subsite between cathepsins S and K (compare **2**, **7c**). The X-ray crystal structures of human cathepsin S,[10] K,[6] and L[11] suggest that the S2 subsite is critical for both the potency and selectivity toward cathepsin S because the S2 pocket in cathepsin S is larger than that of cathepsin K and L (compare **2**, **7d**, **7e**, **7f**, **7g**). Indeed, the 4,4-difluorocyclohexylethyl group **7g** is the optimal size for the P2 substituent based on the affinity to cathepsin S (IC_{50} = 10 nM, K_i = 4.0 ± 0.3 nM), and selectivity against other cysteine proteases, with >100-fold selectivity against human cathepsins B (IC_{50} > 30 μM), C (K_i > 10 μM), H (K_i > 10 μM), and X (K_i = 0.65 μM).

Cathepsin S is responsible for the final proteolytic cleavage of the MHC II associated invariant chain (Ii) mediating the cleavage of lip10 to CLIP in B cells and dendritic cells.[12] We then evaluated the cellular activities of the specific cathepsin S inhibitors by measuring Ii processing in mouse splenocytes. Compounds **7a** and **7g** induced the accumulation of the lip10 fragment in a concentration dependent manner beginning at 10 μM and 1 μM, respectively. For the comparison, in the same assay, LHVS showed the cellular activity at 10 nM.

The pharmacokinetic profiles of compounds **2**, **7a**, and **7g** were determined after oral (3 mg/kg, suspension) and intravenous (1 mg/kg, bolus) administration to Sprague−Dawley rats by LC/MS quantification. The results are shown in Table 2. The absolute bioavailability of **7g** was 21% with modest exposure level (AUCinf; 394 ± 3 nM·h). After intravenous administration, the CLp value was 3.1 ± 0.4 L/h/kg and the terminal elimination half-life was 0.7 ± 0.1 h.

With promising compounds for in vivo studies in our rodent disease models in hand, we then evaluated our cathepsin S specific compounds for their inhibition of rat and mouse cathepsins. The inhibitory potencies to mouse cathepsin S enzyme are similar to that of the human enzyme (Table 3).[13] On the other hand, these selective cathepsin S inhibitors did not show any efficacy against in-house recombinant rat cathepsin S enzyme at 1 μM due to the ^{137}Gly → Cys mutation (papain numbering), which was confirmed by comparison of the cDNA sequence compared to the Mason's reported sequence.[14] The mutated sequence has already been reported in the rat EST database (ACC nos. BQ196525 and BQ200106). Our modeling studies suggest that the mutated Cys at 137 position is located in the bottom of the S2 subsite, which is a key residue for potency to the cathepsin S enzyme (Figure 1), thus causing species specificity (Table 4). We next prepared the reported rat ^{137}Gly cathepsin S enzyme, and then we remeasured the IC_{50} values to this cathepsin S (Table 3).[14] All compounds maintained inhibitory potencies to the rat ^{137}Gly cathepsin S enzyme as well as the selectivity to rat cathepsins K, L, and B (>100-fold selectivity).

Before in vivo experiments with compounds **7a** and **7g** were started, genotyping[15] of the Wister rat strain commonly used for our neuropathic models[2,3] was carried out. After separation of the animals having ^{137}Gly or ^{137}Cys cathepsin S enzymes, the ^{137}Gly cathepsin S animals were subsequently used in the chronic neuropathic pain studies.

Table 2. Pharmacokinetic Parameters of **2**, **7a**, and **7g** in Male Sprague−Dawley Rats (iv 1 mg/kg; po 3 mg/kg), Values are the Means of Three Individual Experiments

compd	CLp (L/h/kg)	iv $t_{1/2}$ (h)	F (%)	po AUC[a] (nM·h)	po C_{max}[a] (nM)	Vd (L /kg)
2	3.9 ± 0.7	1.1 ± 0.1	53	285 ± 139	61 ± 34	5.6 ± 0.8
7a	2.7 ± 0.3	1.7 ± 0.1	24	174 ± 42	30 ± 6	5.7 ± 0.4
7g	3.1 ± 0.4	0.7 ± 0.1	21	131 ± 1	37 ± 2	3.7 ± 0.2

[a] Dose normalized to 1 mg/kg, means ± SD.

Table 3. Inhibitory Activity of Compounds, **2**, **7a**, and **7g** against Rat Cathepsins S, K, L, B and Mouse Cathepsin

	rat cathepsin IC_{50} (nM)[a]				
compd	^{137}Gly S	^{137}Cys S	K	L	mouse S IC_{50} (nM)[a]
2	10 ± 1	>10000	>10000	>10000	10 ± 1
7a	12 ± 1	>10000	>10000	>10000	14 ± 1
7g	28 ± 3	>10000	>10000	>10000	20 ± 2

[a] Inhibition of recombinant rat cathepsins K, L, S and mouse cathepsin S[14] in a fluorescence assay, employing Z-Phe-Arg-MCA (Cat K and L), Z-Leu-Leu-Arg-MCA (Cat S) and Z-Arg-Arg-MCA (Cat B) as synthetic substrates. Values represent means ± SD of three experiments.[9]

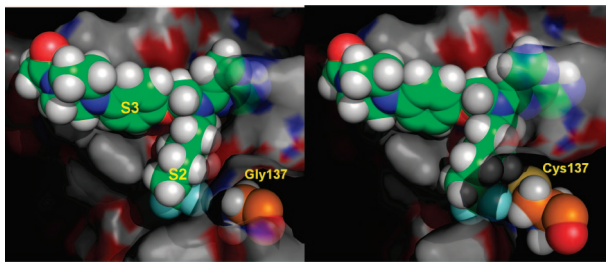


Figure 1. Compound **7g** docked into the rat 137Gly (left) and rat 137Cys (right) cathepsin S. The compound covalently binds to the catalytic residue, Cys25, which is located on the right side of the figures (not shown for the sake of clarity). The P2 4,4-difluorocyclohexylethyl group snugly fits in the S2 pocket with a number of hydrophobic contacts when residue 137 is Gly, while the bulkier Cys residue rejects the P2 group, which renders the compound basically inactive.

Table 4. Binding Site Residues for Human, Rat, and Mouse Cathepsin S

	S3			S2		S1	
residue number[a]	64	70	**137**	162	211	25	67
human cathepsin S	Lys	Phe	**Gly**	Val	Phe	Cys	Asn
rat ^{137}Cys cathepsin S	Lys	Phe	**Cys**	Met	Tyr	Cys	Gly
rat ^{137}Gly cathepsin S	Lys	Phe	**Gly**	Met	Tyr	Cys	Gly
mouse cathepsin S	Lys	Tyr	**Gly**	Val	Tyr	Cys	Gly

[a] Papain numbering.

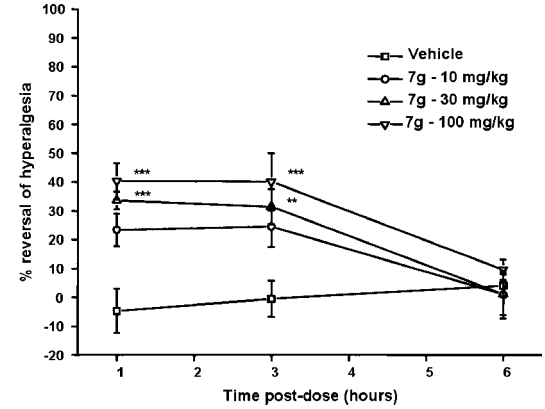


Figure 2. Oral activity of compound **7g** against neuropathic mechanical hyperalgesia in ^{137}Gly-cathepsin S rats. Graph shows mean ± SEM reversal of hyperalgesia from 6 animals per treatment group. ***p < 0.001, **p < 0.01, *p < 0.05 compared to vehicle by ANOVA followed by Tukey's HSD test carried out on withdrawal threshold data.

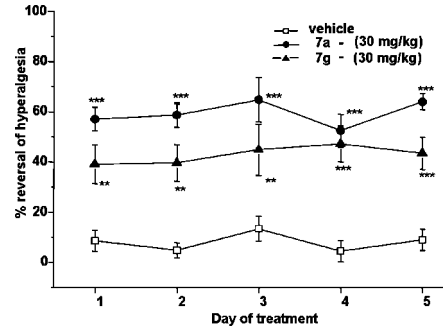


Figure 3. Effect of repeated administration of **7a** and **7g** on neuropathic pain in rats. **7a** and **7g** were administered orally in 0.5% methylcellulose/water twice daily for 5 days. Paw withdrawal thresholds were measured 3 h following administration. Each point represents mean ± SEM from 6 animals/group. ***p < 0.001, **p < 0.01, *p < 0.05 compared to vehicle by ANOVA followed by Tukey's HSD test.

The effect of the specific cathepsin S inhibitor **7g** was examined in a disease model of chronic neuropathic pain in rats following oral administration as a solution in 0.5% methylcellulose/water. In this model, partial ligation of the sciatic nerve induces a peripheral neuropathy that results in mechanical hyperalgesia lasting for several weeks. Compound **7g** reversed the established mechanical hyperalgesia in a dose dependent fashion (Figure 2). On the other hand, **7g** was not active in rats having ^{137}Cys cathepsin S, as **7g** showed no inhibition of ^{137}Cys cathepsin S at 1 μM (Table 3). These findings demonstrate that an inhibition of the cathepsin S enzyme is efficacious in the reversal of neuropathic pain. Compound **7a** was also active in the rat having ^{137}Gly cathepsin S enzyme, producing a dose-related, maximum 54% reversal of hyperalgesia (100 mg/kg po), with an effect that was rapid in onset (1 h).

Following twice daily administration for 5 days (30 mg/kg po), the antihyperalgesic activity of compounds **7a** and **7g** against neuropathic hyperalgesia was maintained (Figure 2). The first dose of **7a** produced 57% reversal of hyperalgesia, which increased to 63% following the final dose on day 5 (Figure 3). Compound **7g** was slightly lower, producing approximately 40−45% reversal throughout the study, because of lower in vitro potency to rat cathepsin S. The results suggested that there was evidence for the development of tolerance to the antihyperalgesic effect of **7a** and **7g** and no obvious side effect.

Compounds **7a** and **7g** were inactive at the maximum concentration tested of 10 μM against a panel of cytochrome P450 enzymes, including CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. They did not possess genotoxic potential, as evidenced by the negative results obtained in the standard Ames test by using liver S9 enzyme. Neither compound induced increased numbers of cells containing in the in vitro micronucleus test.

In conclusion, we demonstrate that cathepsin S specific inhibitors, which have greater than 1000-fold selectivity against rat cathepsin K, L and B, showed an antihyperalgesic effect in a rat model of neuropathic pain after oral administration. These

results suggest that cathepsin S inhibition has great promise as a new treatment for neuropathic pain. The cathepsin S inhibitors discovered possess excellent inhibitory activity to the cathepsin S enzyme, high selectivity against other cathepsins K, L, B, H, X, and C, and show cellular activity and oral bioavailablity without significant toxicological properties. We therefore believe that they are useful compounds in the search for the mechanism of neuropathic pain involving cathepsin S and constitute a novel therapeutic approach for the treatment of chronic pain.

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Supporting Information Available: Experimental procedures for the synthesis of compounds and characterization (^{1}H NMR and MS) of compounds **2** and **7a**−**g**. Description of in vitro and in vivo tests including pharmacokinetic analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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