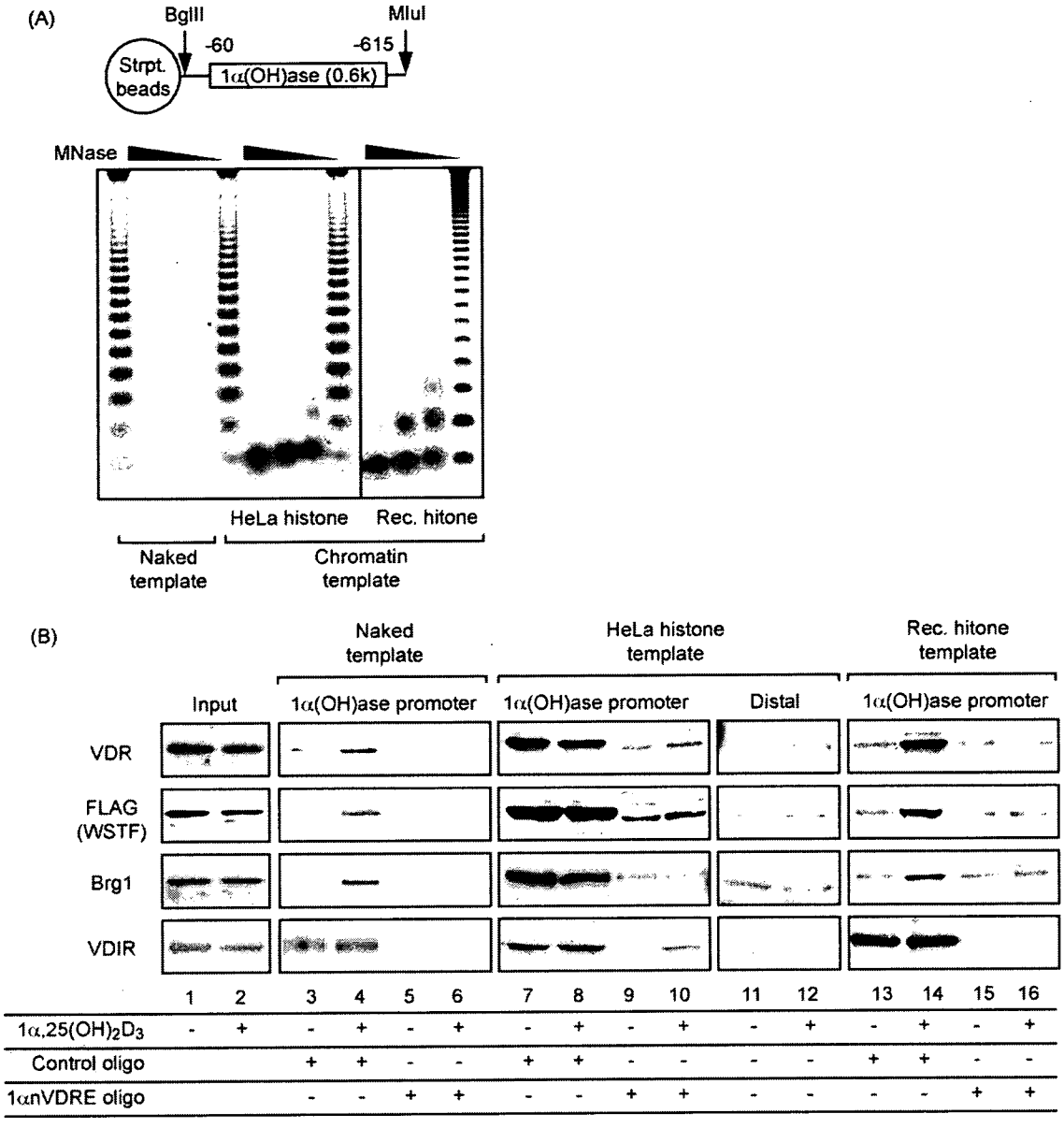


Fig. 5. Chromatin structures are required to target unliganded VDR to the 1α(OH)ase promoter. (A) Chromatin template containing the 1α(OH)ase gene promoter immobilized to streptavidin beads. Schematic representation of the DNA template containing the 1α(OH)ase gene promoter is illustrated above. Chromatinized template reconstituted *in vitro* was confirmed using the standard *Micrococcal* nuclease (MNase) digestion assay. The 123 bp ladder DNA was used as a size marker. (B) Stabilization of the ligand-free VDR/WSTF complex on the 1α(OH)ase promoter required chromatin structure *in vitro*. Whole cell extracts from MCF7 cells stably expressing FLAG-WSTF treated with or without 1α,25(OH)$_2$D$_3$ (10^{-8} M) were mixed with immobilized templates. The template beads were then concentrated using a magnet and analyzed by Western blotting using the indicated antibodies.

with or without 1α,25(OH)$_2$D$_3$ were incubated with either naked or chromatin DNA templates containing 1αnVDRE. Proteins bound to the DNA templates were then analyzed by immunoblotting (Fig. 5B). WSTF and VDR bound to naked DNA templates only in the presence of ligand, while VDIR stably associated with naked DNA templates even in the absence of ligand (Fig. 5B). The specific recruitment of WSTF, together with VDR and VDIR, to 1αnVDRE was confirmed by the finding that addition of excessive synthetic 1αnVDRE–oligonucleotides blocked recruitment (Fig. 5B). In contrast, for the chromatin templates with HeLa histone octamers, recruitment of WSTF and VDR was ligand-independent (Fig. 5B). However, WSTF and VDR were removed to significant extents from chromatinized templates by the addition of excess 1αnVDRE–oligonucleotides (Fig. 5B), indicating a role for DNA-bound VDIR in the stable association of VDR/WSTF with chromatin. This supported the significance of WSTF function in unliganded VDR recruitment to the native 1α(OH)ase promoter.

5. WSTF associates with acetylated histones for ligand-induced transrepression by VDR

We next examined the role of WSTF in binding to acetylated histones during VDR-mediated gene repression. A WSTFΔC construct was first assessed in MCF7 cells by immunoprecipitation using anti-FLAG antibodies and Western blotting with anti-acetylated lysine 14 of histone H3 (AcH3K14), anti-acetylated histone H3 (AcH3), anti-Brg1 and anti-VDR antibodies. Both wild-type WSTF and the

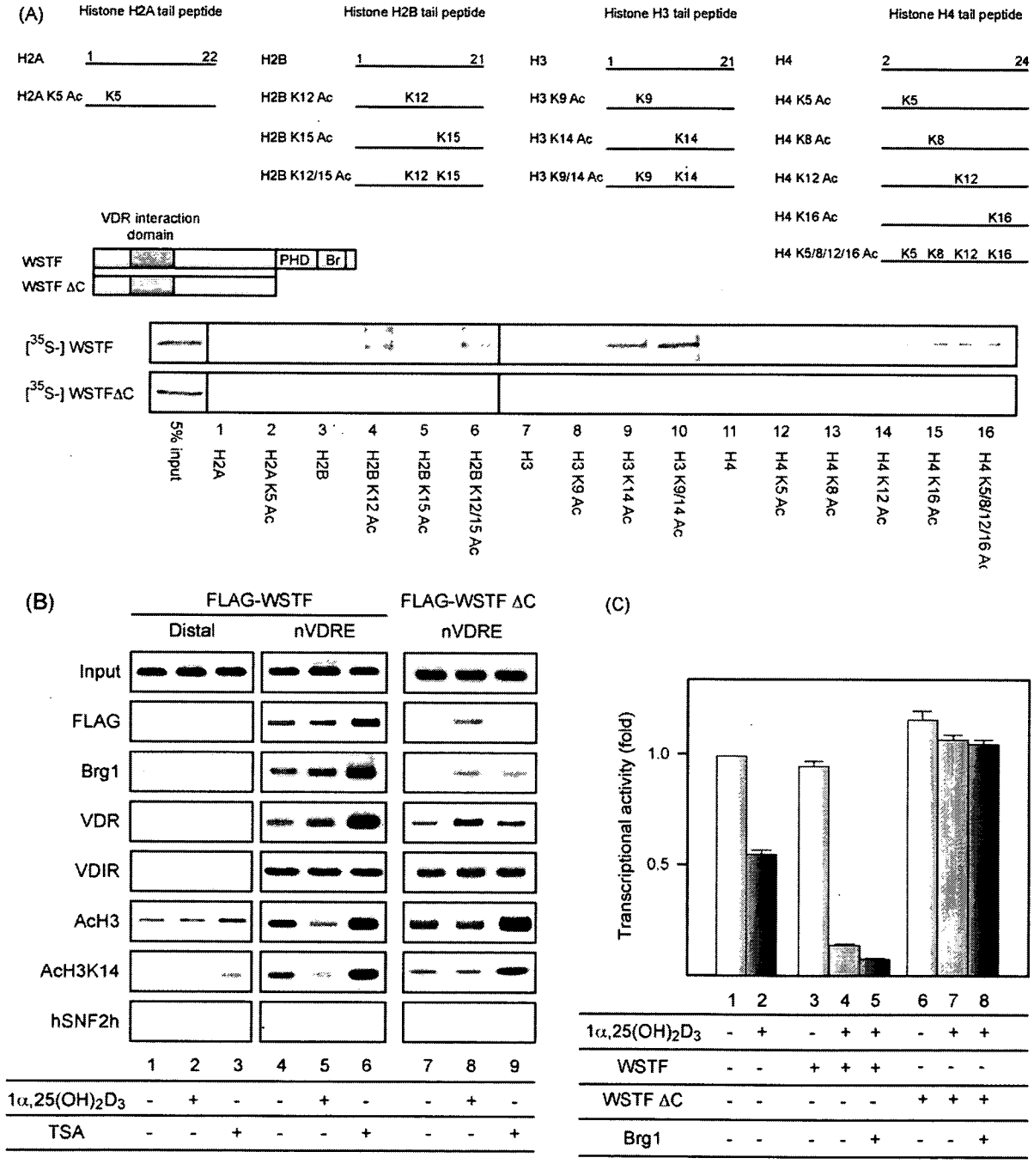


Fig. 6. The WSTF C-terminal region is indispensable for the promoter targeting of ligand-unbound VDR and for VDR-mediated transrepression of the 1α(OH)ase gene. (A) Site-specific recognition between the WSTF bromodomain and histones with tail modification. Schematic diagrams of the WSTF deletion mutants used are illustrated (upper panel). [^{35}S]-labeled WSTF and a WSTFΔC mutant translated *in vitro* were incubated with a series of acetylated N-terminal histone tails immobilized onto streptavidin beads. Histone tail peptides were tested for WSTF binding (middle panel). Bound WSTF were resolved by SDS-PAGE, followed by autoradiography (lower panel). (B) Histone acetylation-dependent recruitment of WSTF to the 1αnVDRE region. MCF7 cells transfected with FLAG-tagged WSTF or FLAG-tagged WSTFΔC were treated with either 1α,25(OH)$_2$D$_3$ (10^{-8} M) for 45 min, or TSA (10^{-7} M) for 120 min, and then subjected to ChIP analysis. (C) A WSTF mutant with a deleted C-terminal bromodomain and PHD finger (WSTFΔC) exerted a partial dominant-negative effect on the ligand-induced transrepression function of VDR. The amounts of each transfected plasmid are described in Fig. 1A.

WSTFΔC mutant appeared to associate with VDR and Brg1, that is an ATPase subunit of WINAC, as well as the other SWI/SNF chromatin remodeling complex subtypes. However, the WSTFΔC mutant exhibited no clear association with AcH3 and AcH3K14 (Fig. 6A). These findings imply that the highly acetylated state of histones induces a stable association between WSTF and chromatin *in vivo*. The WSTFΔC mutant was again unable to associate with the 1αnVDRE region, regardless of the hyperacetylated state of histone H3 (right panel, Fig. 6B). A reporter assay using the luciferase gene driven by the 1α(OH)ase promoter showed that WSTF expression potentiated the 1α,25(OH)$_2$D$_3$-induced transrepression along with Brg1 (Fig. 6C), whereas the WSTFΔC mutant acted as a dominant-negative factor in terms of the ligand-induced transrepression (Fig. 6C, black bars). Thus, it appears that WSTF interacts with acetylated histones via the bromodomain *in vivo*, and that this interaction is indispensable for VDR-mediated transrepression through the 1αnVDR.

6. Discussion

6.1. WINAC supports ligand-induced transrepression by VDR on the human 1α(OH)ase gene promoter region

A large number of co-regulator complexes appear to support transcription control by NRs at multiple but sequential steps [2,24,25]. ATP-dependent chromatin remodeling complexes are considered to facilitate the promoter-specific recruitment of other co-regulator complexes [13,26]. We have previously reported that the WINAC disfunction resulted in a failure of proper transcriptional regulation by VDR, possibly because of impairment of co-regulator recruitment to VDR-target gene promoter [11]. These findings strongly suggested that ATP-dependent chromatin remodeling activity is indispensable for subsequent co-regulator recruitment in responding to ligand binding.

In the present study, we have challenged to define the function of WSTF in the ligand-induced transrepression by VDR. From our previous findings [22], it has been considered that ligand-unbound VDR/RXR on the VDRE mainly associates with HDAC complex to actively repress target genes. Reflecting this model, ligand binding led to co-repressor dissociation from VDR, and WINAC assisted promoter recruitment of HDAC co-repressor complex in VDIR-mediated transrepression on a negative VDRE. These results imply a difference in the set of associating factors/complexes with unliganded VDR on negative VDREs from positive VDREs on the VDR target gene promoters. Indeed, ligand binding significantly increased the interaction of VDR/WINAC with HDAC complex (Fig. 4). Hence, in addition to ligand-induced transactivation by VDR, WINAC has also important role of VDR-mediated transrepression mechanism. The proposed mechanism of the ligand-induced

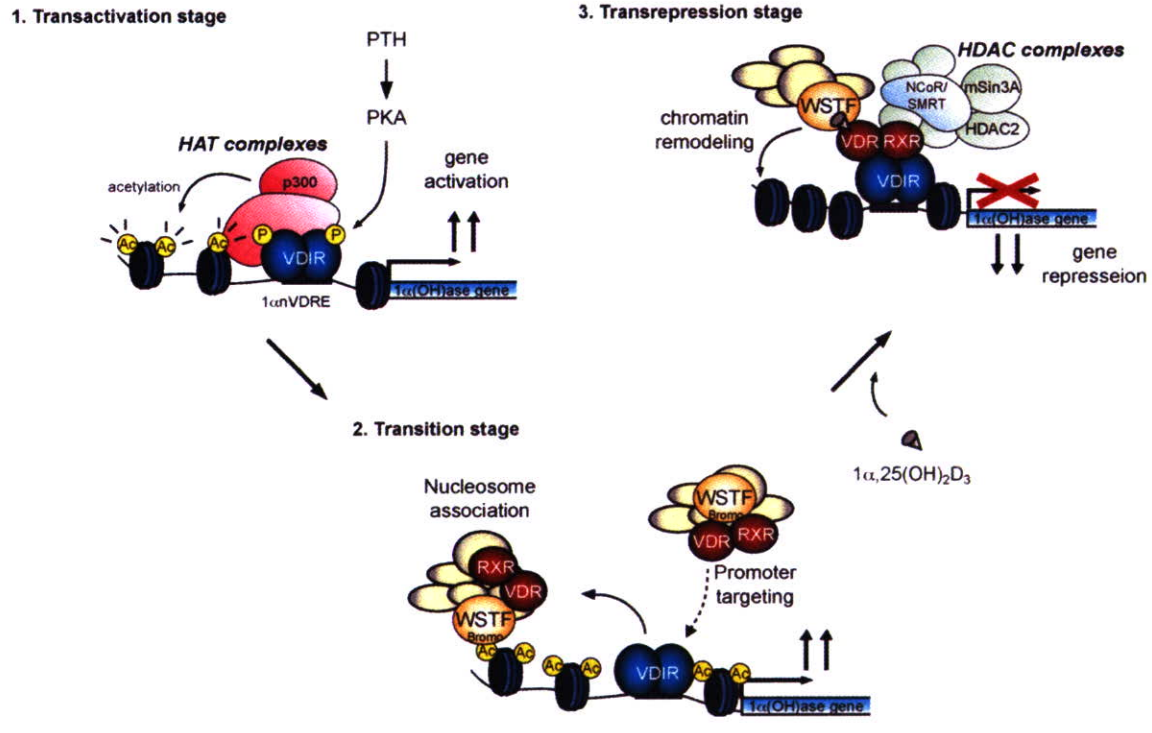


Fig. 7. Model demonstrating the role of WINAC in the ligand-induced transrepression function of VDR at the 1α(OH)ase gene promoter. p300 is recruited to VDIR, which was phosphorylated via PKA signaling, and it acetylates the nucleosomes around the 1α(OH)ase gene promoter region (transactivation stage). WINAC, along with VDR, sequentially targets VDIR through interaction between unliganded VDR and VDIR, and is retained on the acetylated promoter via the WSTF bromodomain. VDR becomes receptive to 1α,25(OH)$_2$D$_3$ binding (transition stage). Upon 1α,25(OH)$_2$D$_3$ binding, HDAC co-repressor complexes are recruited to the ligand-bound VDR/VDIR complex, and they then deacetylate the nucleosomes. WINAC then exerts its ATP-dependent chromatin remodeling activity (transrepression stage).

transrepression by VDR in the present study appears to be dependent on the promoter-content, since it is unlikely that all of the VDR target gene promoters for Vitamin D-induced transrepression harbor VDIR binding sites. The other mode and mechanism of ligand-induced transrepression might be unrevealed in the other promoters for VDR and the other NRs, like recently reported in the transrepressive function of PPARγ [27].

6.2. WSTF associates with acetylated histones to anchor VDR/WINAC complex upon VDIR on the 1α(OH)ase gene promoter region

Physical interaction of acetylated histones with the ATP-dependent chromatin remodeling complex components is a critical step in activation of chromatin, modulating its architecture by rearrangement of nucleosome arrays [14,18]. Hassan et al. found that both the SAGA and SWI/SNF complexes are capable of anchoring to promoter nucleosomes through direct contact of acetylated histones with the bromodomains [16]. However, it remained unclear how these chromatin remodeling complexes selectively discriminate their target chromosomal areas from others through their chromatin recognition modules. To address this point, we have examined two aspects of WINAC promoter targeting. One is the mechanism by which specific chromatin areas are recognized by WSTF through association with sequence-specific regulators, and the other is the preference of WSTF for a specific chromatin condition.

Thus, considering all of these results, we conclude that WINAC facilitates VDR-mediated transrepression of the 1α(OH)ase gene through a physical interaction between the WSTF bromodomain and an acetylated nucleosomal array (Fig. 7).

References

[1] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, The nuclear receptor superfamily: the second decade, Cell 83 (1995) 835–839.

[2] C.K. Glass, M.G. Rosenfeld, The co-regulator exchange in transcriptional functions of nuclear receptors, Genes Dev. 14 (2000) 121–141.

[3] W. Gu, S. Malik, M. Ito, C.X. Yuan, J.D. Fondell, X. Zhang, E. Martinez, J. Qin, R.G. Roeder, A novel human SRB/MED-containing cofactor complex, SMCC, involved in transcription regulation, Mol. Cell 3 (1999) 97–108.

[4] C. Rachez, Z. Suldan, J. Ward, C.P. Chang, D. Burakov, H. Erdjument-Bromage, P. Tempst, L.P. Freedman, A novel protein complex that interacts with the Vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system, Genes Dev. 12 (1998) 1787–1800.

[5] T. Heinzel, R.M. Lavinsky, T.M. Mullen, M. Soderstrom, C.D. Laherty, J. Torchia, W.M. Yang, G. Brard, S.D. Ngo, J.R. Davie, E. Seto, R.N. Eisenman, D.W. Rose, C.K. Glass, M.G. Rosenfeld, A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression, Nature 387 (1997) 43–48.

[6] Y. Kamei, L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.C. Lin, R.A. Heyman, D.W. Rose, C.K. Glass, M.G. Rosenfeld, A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors, Cell 85 (1996) 403–414.

[7] S.A. Onate, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Sequence and characterization of a co-activator for the steroid hormone receptor superfamily, Science 270 (1995) 1354–1357.

[8] J. Yanagisawa, H. Kitagawa, M. Yanagida, O. Wada, S. Ogawa, M. Nakagomi, H. Oishi, Y. Yamamoto, H. Nagasawa, S.B. McMahon, M.D. Cole, L. Tora, N. Takahashi, S. Kato, Nuclear receptor function requires a TFTC-type histone acetyl transferase complex, Mol. Cell 9 (2002) 553–562.

[9] D.V. Fyodorov, J.T. Kadonaga, The many faces of chromatin remodeling: switching beyond transcription, Cell 106 (2001) 523–525.

[10] T. Ito, M. Bulger, M.J. Pazin, R. Kobayashi, J.T. Kadonaga, ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor, Cell 90 (1997) 145–155.

[11] H. Kitagawa, R. Fujiki, K. Yoshimura, Y. Mezaki, Y. Uematsu, D. Matsui, S. Ogawa, K. Unno, M. Okubo, A. Tokita, T. Nakagawa, T. Ito, Y. Ishimi, H. Nagasawa, T. Matsumoto, J. Yanagisawa, S. Kato, The chromatin remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome, Cell 113 (2003) 905–917.

[12] B. Lemon, C. Inouye, D.S. King, R. Tjian, Selectivity of chromatin-remodelling cofactors for ligand-activated transcription, Nature 414 (2001) 924–928.

[13] G.J. Narlikar, H.Y. Fan, R.E. Kingston, Cooperation between complexes that regulate chromatin structure and transcription, Cell 108 (2002) 475–487.

[14] M.H. Jones, N. Hamana, J. Nezu, M. Shimane, A novel family of bromodomain genes, Genomics 63 (2000) 40–45.

[15] C. Dhalluin, J.E. Carlson, L. Zeng, C. He, A.K. Aggarwal, M.M. Zhou, Structure and ligand of a histone acetyltransferase bromodomain, Nature 399 (1999) 491–496.

[16] A.H. Hassan, P. Prochasson, K.E. Neely, S.C. Galasinski, M. Chandy, M.J. Carrozza, J.L. Workman, Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes, Cell 111 (2002) 369–379.

[17] R.H. Jacobson, A.G. Ladurner, D.S. King, R. Tjian, Structure and function of a human TAFII250 double bromodomain module, Science 288 (2000) 1422–1425.

[18] F. Winston, C.D. Allis, The bromodomain: a chromatin-targeting module? Nat. Struct. Biol. 6 (1999) 601–604.

[19] A. Murayama, K. Takeyama, S. Kitanaka, Y. Kodera, T. Hosoya, S. Kato, The promoter of the human 25-hydroxyvitamin D3 1-alpha-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1-alpha,25$(OH)_2D_3$, Biochem. Biophys. Res. Commun. 249 (1998) 11–16.

[20] K. Takeyama, S. Kitanaka, T. Sato, M. Kobori, J. Yanagisawa, S. Kato, 25-Hydroxyvitamin D3 1-alpha-hydroxylase and Vitamin D synthesis, Science 277 (1997) 1827–1830.

[21] T. Yoshizawa, Y. Handa, Y. Uematsu, S. Takeda, K. Sekine, Y. Yoshihara, T. Kawakami, K. Arioka, H. Sato, Y. Uchiyama, S. Masushige, A. Fukamizu, T. Matsumoto, S. Kato, Mice lacking the Vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning, Nat. Genet. 16 (1997) 391–396.

[22] A. Murayama, M.S. Kim, J. Yanagisawa, K.I. Takeyama, S. Kato, Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching, EMBO J. 23 (2004) 1598–1608.

[23] S. Kato, R. Fujiki, H. Kitagawa, Vitamin D receptor (VDR) promoter targeting through a novel chromatin remodeling complex, J. Steroid Biochem. Mol. Biol. 89/90 (2004) 173–178.

[24] N.J. McKenna, B.W. O'Malley, Combinatorial control of gene expression by nuclear receptors and coregulators, Cell 108 (2002) 465–474.

[25] V. Perissi, M.G. Rosenfeld, Controlling nuclear receptors: the circular logic of cofactor cycles, Nat. Rev. Mol. Cell. Biol. 6 (2005) 542–554.
[26] B.M. Emerson, Specificity of gene regulation, Cell 109 (2002) 267–270.
[27] G. Pascual, A.L. Fong, S. Ogawa, A. Gamliel, A.C. Li, V. Perissi, D.W. Rose, T.M. Willson, M.G. Rosenfeld, C.K. Glass, A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma, Nature 437 (2005) 759–763.

Available online at www.sciencedirect.com
ScienceDirect
Journal of Steroid Biochemistry & Molecular Biology 103 (2007) 269–276
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Creative synthesis of novel vitamin D analogs for health and disease

Atsushi Kittaka [a,*], Nozomi Saito [a], Shinobu Honzawa [a], Kazuya Takenouchi [b], Seiichi Ishizuka [b], Tai C. Chen [c], Sara Peleg [d], Shigeaki Kato [e], Midori A. Arai [a]

[a] *Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Sagamihara, Kanagawa 199-0195, Japan*
[b] *Teijin Pharma Ltd., Tokyo 191-8512, Japan*
[c] *Boston University School of Medicine, Boston, MA 02118, USA*
[d] *The University of Texas, Houston, TX 77030, USA*
[e] *The University of Tokyo, Tokyo 113-0032, Japan*

Abstract

We report new analogs of 1α,25-dihydroxyvitamin D_3 (**1**) in three categories. First, design and synthesis of ligands for a mutant vitamin D receptor (VDR)(Arg274Leu), which possess proper functional groups at both C1α and C2α positions of **1** to study the biological activity of the mutant VDR. Among our synthetic analogs, 1α-methyl-2α-(3-hydroxypropyl)-25-hydroxyvitamin D_3 (**8**) showed 7.3-fold greater transcriptional activity for the VDR(Arg274Leu) than that of **1**. Next, we examined the antiproliferative activity of 2-substituted 19-norvitamin D_3 analogs on an immortalized normal prostate cell line, PZ-HPV-7, and we found MART 10 (**14**) showed the activity even at very low concentration of 10^{-10} to 10^{-11} M. We also synthesized 25-hydroxy-19-norvitamin D_3 (**13**) using Julia-type olefination to connect between the C5 and C6 positions, effectively, to test it as a prohormone type agent for antiprostate diseases. Synthesized compound **13** showed potent antiproliferative activity in PZ-HPV-7, which has high 1α-hydroxylase activity. Finally, we describe design and synthesis of a new TEI-9647 analog, 2α-(3-hydroxypropoxy)-24-propyl-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactone (**17**), which showed the strongest VDR antagonism. Its IC_{50} value is 7.4 pM to inhibit differentiation of HL-60 cells induced by 10 nM of **1**.

Keywords: Vitamin D analogs; Chemical synthesis; Structure–activity relationships; Vitamin D receptor; Antagonist

1. Introduction

1α,25-Dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$, **1**), the physiologically active hormonal form of vitamin D_3, regulates cellular proliferation and differentiation in addition to its classical role in calcium and phosphorus homeostasis and bone mineralization [1–4]. The cellular and physiological actions of 1α,25$(OH)_2D_3$ are mediated primarily through the specific receptor, vitamin D receptor (VDR), which belongs to the nuclear receptor superfamily acting as a ligand-dependant transcription factor [5,6]. The VDR–[1α,25$(OH)_2D_3$] signaling works to stimulate intestinal calcium and phosphate absorption to prevent rickets, enhance of bone remodeling, differentiation of skin cells, potential anticancer actions, and so on. Therefore, 1α,25$(OH)_2D_3$ (**1**) and some synthetic analogs of **1** are clinically used in the treatment of bone diseases, secondary hyperparathyroidism, and psoriasis [1].

In order to investigate the structure–activity relationships of the natural hormone, we systematically synthesized the A-ring modified analogs, such as 2α-alkyl-, and 2α-(ω-hydroxyalkyl)-1α,25$(OH)_2D_3$ (Fig. 1) [7–11]. Elongation of the 2α-alkyl chain as in **2b–d**, however, decreased the binding affinity and agonistic activity for the VDR [9]. On the other hand, in regard to modification with the 2α-(ω-hydroxyalkyl) group, it was found that **3c** with the 2α-(3-hydroxypropyl) group on **1** had a three-fold higher binding activity for the VDR than **1** [9,10]. We also synthesized 2α-(ω-hydroxyalkoxy)-1α,25$(OH)_2D_3$ (**4a–c**), and **4b** showed 1.8-fold stronger binding affinity for the VDR than **1** [11–13]. Posner and Johnson also synthesized ED-71 [14] related analogs starting with the [4 + 2] cycloaddition of pyrone derivatives [15]. We report here the design and synthesis of the new analogs of **1** based on our accumulated knowledge of the 2α-functional group in three categories: for

* Corresponding author. Tel.: +81 42 685 3713; fax: +81 42 685 3713.
E-mail address: akittaka@pharm.teikyo-u.ac.jp (A. Kittaka).

0960-0760/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2006.12.002

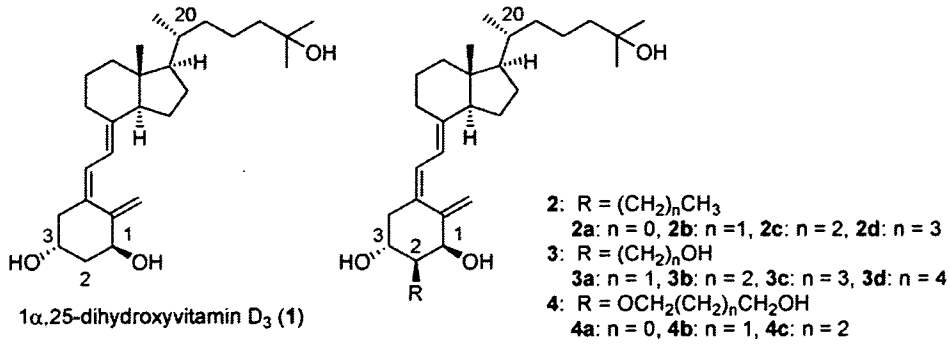


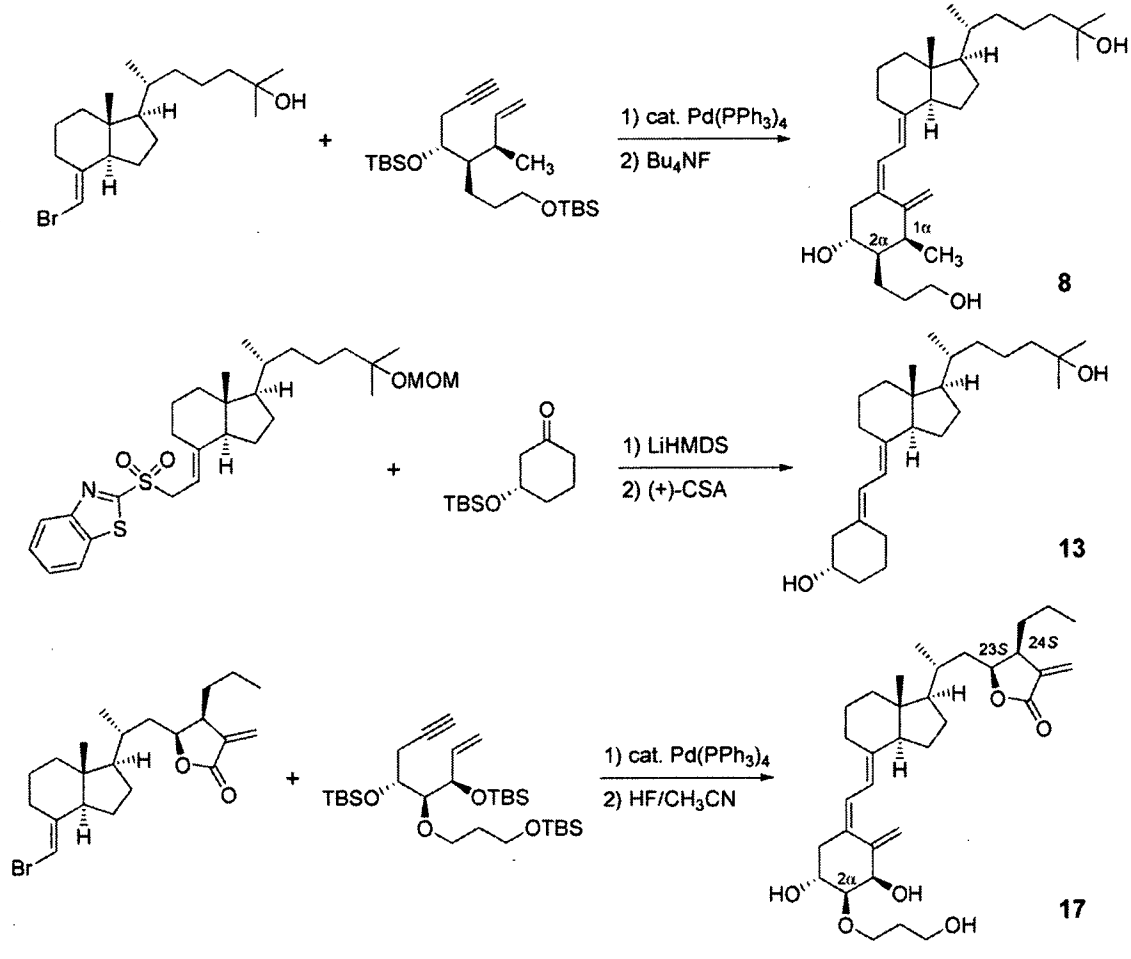
Fig. 1. Structures of 1α,25-dihydroxyvitamin D_3 (**1**) and its 2α-substituted analogs **2–4** [7–13].

hereditary vitamin D-resistant rickets (HVDRR), for prostate cancer, and for Paget's disease.

2. Material and methods

2.1. Preparation of vitamin D analogs

All vitamin D analogs in this report were synthesized at the Faculty of Pharmaceutical Sciences, Teikyo University according to the representative synthetic route presented in Scheme 1. Construction of the vitamin D_3 triene skeleton, for example, compounds **8** and **17**, was accomplished by Pd-catalyzed alkenylative cyclization of enyne as the A-ring precursor with the CD-ring bromoolefin [16]. To connect the C5–C6 double bond of 19-norvitamin D_3 analogs, for example, compound **13**, Julia-type olefination was utilized. Full synthetic details and physiological data of the synthetic compounds will be reported elsewhere.



Scheme 1. Synthesis of 1α-alkyl vitamin D_3 analog **8**, 19-norvitamin D_3 analog **13**, and TEI-9647 analog **17**.

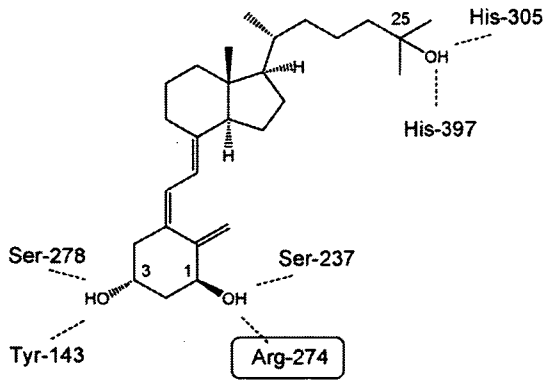


Fig. 2. Amino acid residues forming hydrogen bonds with the hydroxyl groups of **1**.

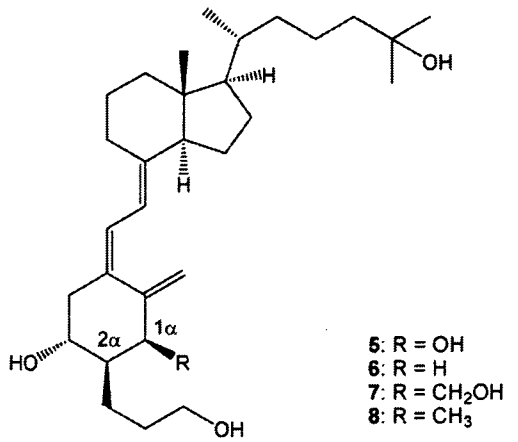


Fig. 3. Structures of 2α-(3-hydroxypropyl)-1α-modified 25(OH)D_3 analogs (**5–8**).

3. Results and discussion

3.1. Designed ligands for HVDRR

Hydrogen bonds between three hydroxyl groups of **1** and hydrophilic amino acid residues in the ligand-binding domain (LBD) of VDR are essential for its actions. For example, Arg-274 is well known to form a hydrogen bond with 1α-OH of **1** [17], and its mutation would lead to loss of the affinity for **1** and its actions (Fig. 2) [18].

In fact, substitution of the amino acid to a hydrophobic leucine is shown to cause hereditary Vitamin D-resistant rickets, a rare genetic disease caused by a generalized resistance to **1** [19]. The mutation causes a 1000-fold decrease in the affinity for **1**, thus the vitamin D action is disrupted. Vitamin D derivatives that bind specifically to a mutated VDR of this type would be a candidate for therapeutic agent against HVDRR [20]. A few research groups have reported such ligand designs for the mutant VDR. Posner and co-workers reported that 1α-hydroxymethyl-16-ene-26a,27a-bishomo-25-hydroxyvitamin D_3 was an effective ligand for the mutant VDR [20]. Koh and co-workers developed derivatives whose 1α-hydroxy group is protected by a substituted benzyl group [21,22]. On the other hand, we have approached the issue by using 2α-hydroxypropylated **1** (**5**) [23]. In this case, we have shown that **5** would form a hydrogen bond with the Asp-144 of the mutant VDR through the terminal hydroxy group of the 2α-substituent, thus could restore its complex formation ability with the mutant VDR. We were interested in whether these motifs could increase biological activity of Posner and co-workers 1-hydroxymethylated analogs, and we decided to synthesize the analog (**7**), which possesses the 2α-(3-hydroxypropyl) group on the A-ring (Fig. 3). Furthermore, introduction of a methyl group at 1α-position of 25(OH)D_3 would be also expected to recover the affinity for the mutant VDR by interacting with the hydrophobic pocket formed by the mutation. We designed and synthesized the analog of 1α-methyl-2α-(3-hydroxypropyl)-25(OH)D_3 (**8**) as the new ligand for the mutant VDR. It was found that the transcriptional activity of **5–8**, in which compound **6** was also synthesized as the standard compound for the mutant VDR(Arg274Leu), was 5.6–7.3-times greater than that of the natural hormone **1**, when tested by Dual-Luciferase® reporter assay [24].

Originally, we considered that the affinity of the 2α-methyl analog (**2a**) for the normal VDR would be greater because of hydrophobic interaction between 2α-methyl group and the hydrophobic pocket of the LBD [11]. We examined the hypothesis that the affinity for VDR would be greater if a larger hydrophobic group, for example, the phenyl group, could interact with the hydrophobic pocket of the mutant VDR(Arg274Leu) and restore ligand-mediated VDR action. In order to test this hypothesis, we synthesized novel analogs that have aromatic groups at the 2α-position. That is, we synthesized 2α-phenyl- (**9**), 2α-benzyl- (**10**), and 2α-phenethyl-1α,25-$(OH)_2D_3$ (**11**) (Fig. 4).

Contrary to our expectation, these analogs showed a poor ability to stabilize the conformation of the mutant VDR as shown by protease sensitivity assay [20], and only the 2α-benzyl analog induced detectable stabilization of the mutant VDR at the almost same level as the natural hormone **1** [25].

On the other hand, in all cases where biological activities via the normal VDR were assayed, the 2α-benzyl analog (**10**) was the most potent in the aromatic analog series. Data from biological assays showed that these analogs induce HL-60 cell differentiation and transcriptional activities as potently as **1**, whereas the affinity for the normal VDR was less than 10% relative to the natural hormone [25]. Further studies are now on the way to elucidate the discrepancies between binding affinity of the analogs for VDR in vitro and their potencies in the cellular assays, including studies on metabolism by CYP27A1 and CYP24A1 [26].

3.2. Designed ligands for prostate cancer

It has been shown that prostate cells possess 1α-hydroxylase (1α(OH)ase), and can convert 25-hydroxyvitamin D_3, 25(OH)D_3 (**12**), to **1** intracellularly to inhibit their prolifera-

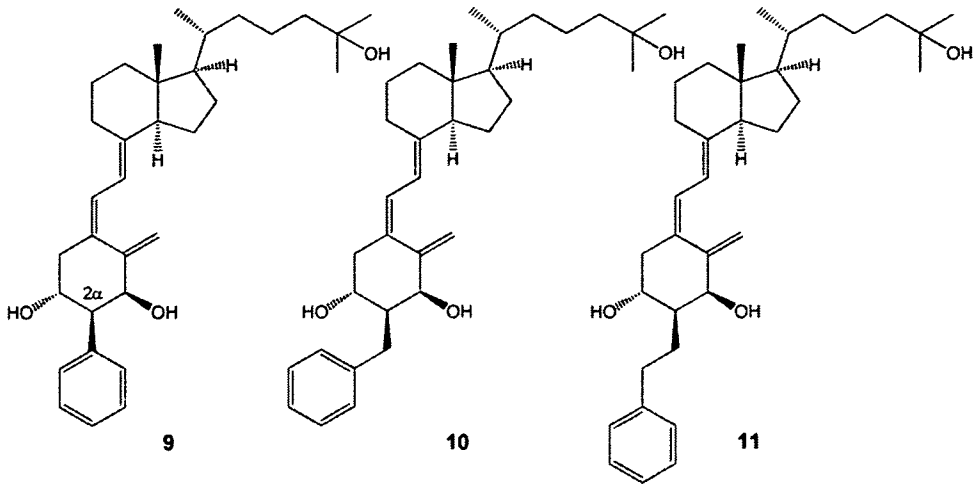
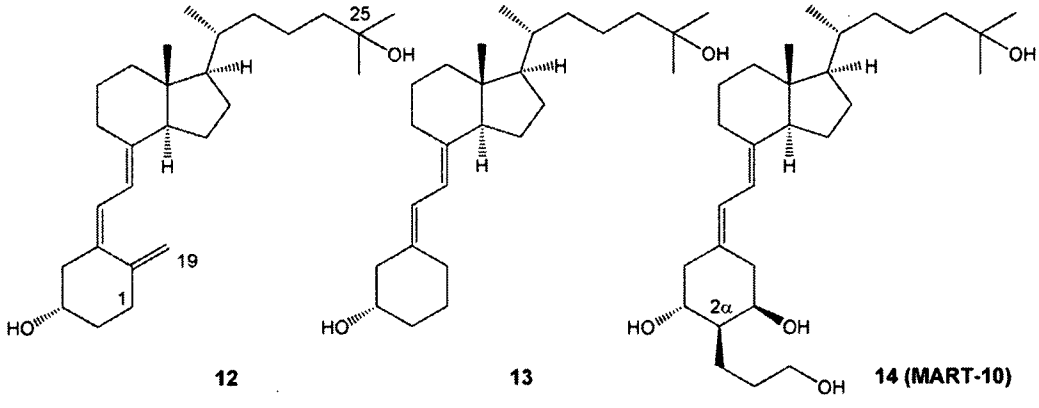


Fig. 4. Structures of 2α-phenyl-, 2α-benzyl-, and 2α-phenethyl-1α,25(OH)$_2$D$_3$ (**9–11**).

tion [27]. It is also known that a 19-demethylenated analog of **1**, i.e., 1α,25-dihydroxy-19-norvitamin D_2, possesses pro-differentiation and antiproliferative activities similar to **1** [28]. Since 19-norvitamin D derivatives are known to be less calcemic than **1** when administered systemically [29], we are interested in knowing whether 25-hydroxy-19-norvitamin D_3, 25(OH)-19-norD_3 (**13**) (Fig. 5), can exert potent antiproliferative activity toward prostate cells which possess 1α(OH)ase activity and therefore can be used as chemopreventive agents without causing hypercalcemic side



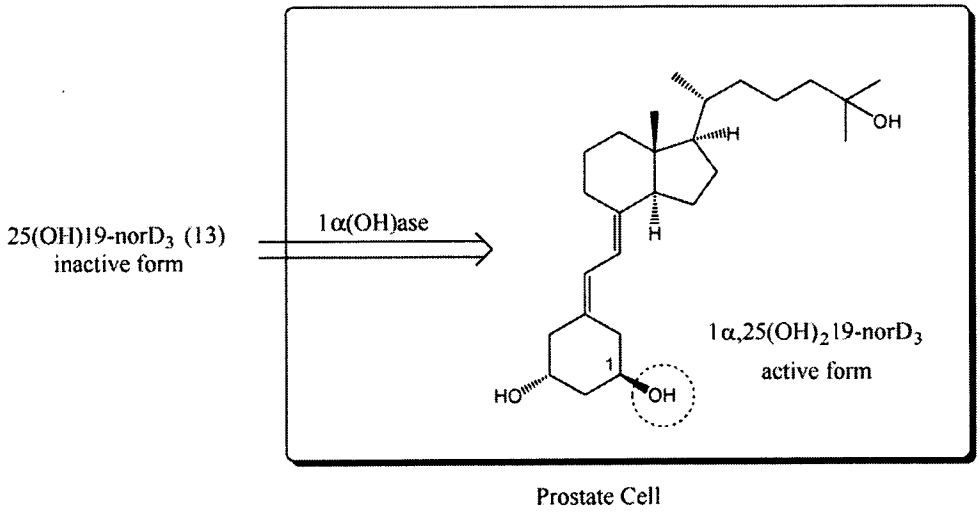


Fig. 5. Structures of **12** and 19-norvitamin D_3 analogs (**13**,**14**), and concept of antiagent for prostate disease on 25-hydroxy-19-norvitamin D_3 (**13**) as a prohormone analog.

effects. We synthesized **13** by using Julia-type olefination [30] to test its antiproliferative activity in PZ-HPV-7 normal prostate cells [31].

25-Hydroxy-19-norvitamin D_3 (**13**) showed high antiproliferative activity in PZ-HPV-7 cells, almost at the same level as that of the natural hormone (**1**) [31]. When the cells were treated with both **13** and the natural hormone (**1**), synergistic effect was observed. It is interesting that **13** showed such high antiproliferative activity, even though the binding affinity of **13** for the vitamin D receptor is low [32]. To evaluate a more precise molecular mechanism, we also studied the metabolism of **13** by 1α(OH)ase (CYP27B1) in an in vitro system. After incubating **13** with the *E. coli* extract containing the recombinant 1α(OH)ase (CYP27B1), the resulted mixture was analyzed by HPLC. It was shown that **13** was hydroxylated at the 1α-position to form 1α,25(OH)$_2$-19-norvitamin D_3, which is known to be active [31]. It seems that the antiproliferative activity of **13** in prostate cells is primarily the result of its hydroxylation by endogenous 1α(OH)ase (CYP27B1) at the 1α-position leading to the formation of the active 1α,25(OH)$_2$-19-norvitamin D_3 compound [31].

We also synthesized 2α-(3-hydroxypropyl)-1α,25-dihydroxy-19-norvitamin D_3 (MART 10, **14**), which showed strong HL-60 cell differentiation inducing effect [30], and tested antiproliferative activity in the prostate cells, PZ-HPV-7. The results are summarized in Fig. 6.

We can see the highly potent antiproliferative activity of MART 10 (**14**), which showed the activity in very low concentration, even from 10^{-10} to 10^{-11} M (Fig. 6). The cancer cell antiinvasion activity of MART 10 has also been examined and will be reported elsewhere.

3.3. Designed ligands for Paget's disease

Paget's disease [33–35], which is known as the most flagrant example of disordered bone remodeling and the second most common bone disease after osteoporosis in Anglo-Saxons [35]. Recent studies on Paget's disease suggested a specific increase in osteoclast sensitivity to the differentiation activity of **1** as the principal mechanism for abnormal bone formation [33–35]. The VDR antagonists are expected to be potent therapeutic agents for some diseases caused by hypersensitivity to 1α,25-dihydroxyvitamin D_3, such as Paget's disease.

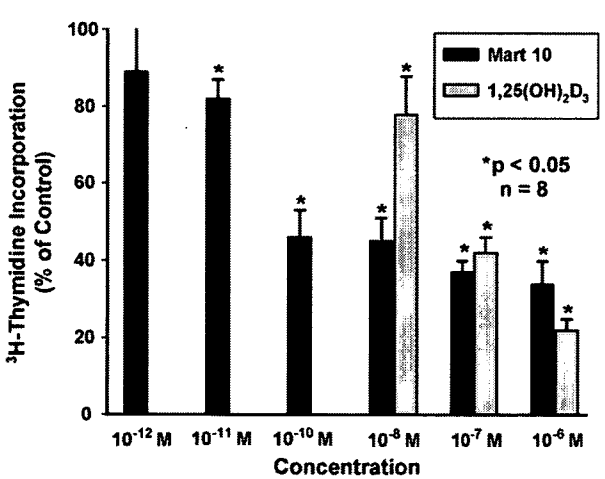


Fig. 6. Antiproliferative activity of MART 10 (**14**) on immortalized normal prostate cells (PZ-HPV-7).

In 1999, studies on the modification of the side-chain structure based on the 1α,25-dihydroxyvitamin D_3-26,23-lactone metabolite derived from **1** led to the discovery of the TEI-9647 (**15**) and TEI-9648 (**16**) (Fig. 7) [36,37]. Both vitamin D_3 analogs **15** and **16**, which have an α-methylene-γ-butyrolactone part on the side-chain, are the first specific antagonists of VDR-mediated genomic action of **1**, and **15** with 23*S*-configration has stronger antagonism (IC_{50} 9.4 nM) than **16** with 23*R*-configration (IC_{50} 134.3 nM). After our synthetic studies on the C2α and C24-double functionalization of **15** and **16** to investigate the structure–activity relationships, we found that 2α-(3-hydroxypropoxy)-24-propyl-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactone (**17**) showed the strongest VDR antagonism, as far as we know, and its IC_{50} value was 7.4 pM to inhibit differentiation of human leukemia cells (HL-60 cells) induced by 10 nM of **1** [51].

Structures of representative C2α and C24 modified TEI-9647 analogs and biological activities are shown in Fig. 8 and Table 1 [38–42].

When the VDR antagonist TEI-9647 (**15**) binds to the ligand-binding domain of VDR, the complex may change into an unusual transcriptionally inactive form. We speculate that some amino acid residues in the LBD, which participate in the conformational change of the VDR, react with the *exo*-methylene moiety on the lactone ring of **15**. Namely, there are two cysteine residues, Cys-403 on helix 11 and Cys-410 in the hinge region between helices 11 and 12, in the LBD of the human VDR. Recently, it was revealed that the two cysteines, Cys-403 and Cys-410, play an important role in the VDR antagonism of TEI-9647 (**15**) [46]. Furthermore, the *exo*-methylene lactone structure is indispensable for the antagonistic action of the vitamin D_3 lactones [47]. Based on these results, we consider that the nucleophilic thiol groups of the cysteines could attack the α-methylene-γ-lactone of TEI-analogs via 1,4-addition to give the corresponding cysteine adduct [48]. Such interaction between the ligand

Table 1
Biological activity of C2α and C24 modified TEI-9647 analogs **17–21**

Compound	Binding affinity for VDR[a]	Antagonistic activity (IC_{50}, nM)[b]	Reference
TEI-9647 (**15**)	12	9.4	
17	37	0.0074	[51]
18	23	0.13	[39]
19	34	0.23	[40]
20	67	0.093	[41]
21	111	0.49	[42]

[a] Chick intestinal VDR [43,44]. The potency of **1** is normalized to 100.
[b] Antagonistic activity was assessed in terms of IC_{50} for the differentiation of HL-60 cells induced by 10 nM of **1** [45].

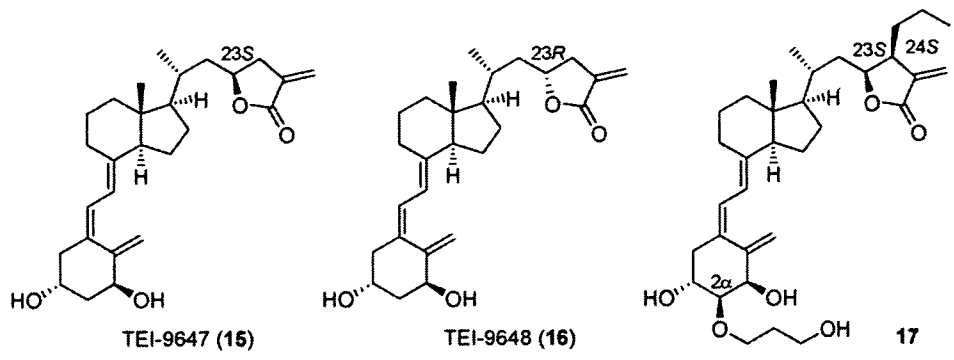


Fig. 7. Structures of TEI-9647 (**15**), TEI-9648 (**16**), and 2α-(3-hydroxypropoxy)-24-propyl-TEI-9647 (**17**).

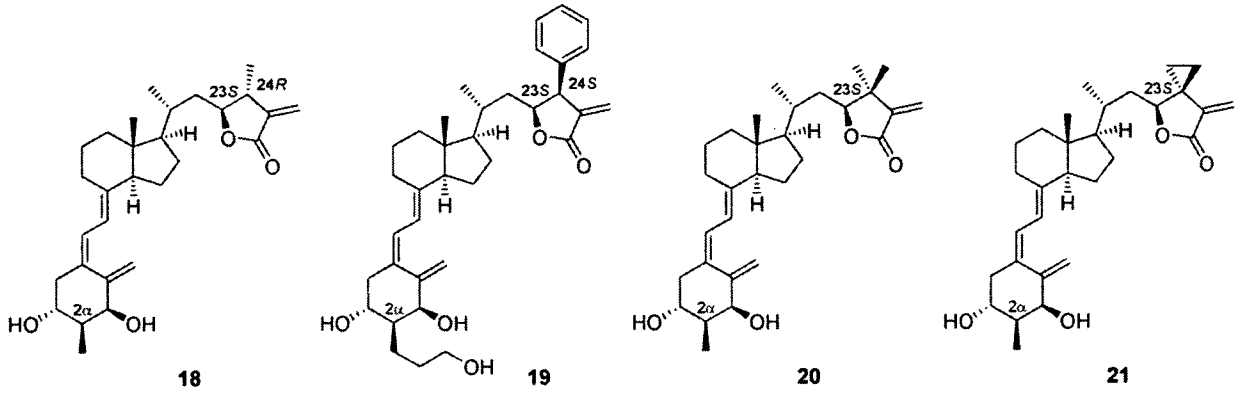


Fig. 8. Structures of C2α and C24 modified TEI-9647 analogs **18–21**.

and the LBD might prevent correct positioning of helix 12 to activate the target genes. Therefore, it is conceivable that the VDR antagonists, whose *exo*-methylene moiety is located at a more favorable position to react with Cys-403 and/or Cys-410 after binding, show stronger VDR antagonistic activity. The novel vitamin D_3 lactones we synthesized, which showed more potent antagonistic activity, might be situated in a preferable position of the *exo*-methylene group toward the cysteine residues after binding to the LBD of the VDR, and vice versa. The other VDR antagonists ZK-159222 [49] and DLAMs [50] would have presumably different mechanism as antagonists on VDR from TEI-lactone analogs.

4. Conclusions

We synthesized A-ring modified analogs of the natural hormone, which have hydrophobic group at the 1α-position and the 3-hydroxypropyl group at the 2α-position, as the new ligand for the mutant VDR(Arg274Leu). We found that 1α-methyl-2α-(3-hydroxypropyl)-25(OH)D_3 (**8**) showed 7.3-fold greater transcriptional activity through the mutant VDR than **1**. Next, we synthesized 25-hydroxy-19-norvitamin D_3 (**13**) and MART-10 (**14**) by using Julia-type olefination, which we have reported as the new coupling method for the synthesis of 19-norvitamin D derivatives [30]. Both compounds exhibited high antiproliferative activity in the immortalized normal prostate cells. Finally, we synthesized 2α-substituted 24-alkyl-TEI-lactone analogs, such as **17** to investigate structure–activity relationships of VDR antagonists. It was found that antagonistic activity of 2α-(3-hydroxypropoxy)-24-propyl-TEI-9647 (**17**) increased about three orders over the original TEI-9647. We expect that these highly potent vitamin D_3 analogs would contribute to understanding the mechanisms involved in the expression of agonistic and antagonistic activity on VDR, for example, based on X-ray crystallography data if available, as well as to finding the seeds of new medicines for treating VDR related diseases [52].

Acknowledgements

We thank Ms. Junko Shimode and Ms. Akiko Tonoki (Teikyo University) for spectroscopic measurements. This study has been supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (#15790015 and 17790019 to N.S., #17790095 to S.H., and #16790027 to M.A.A.), and by Grants-in-Aid from Japan Society for the Promotion of Science (#15590021 and 17590012 to A.K.). N.S. deeply thanks Takeda Science Foundation, and A.K. gratefully acknowledges Uehara Memorial Foundation.

References

[1] D. Feldman, J.W. Pike, F.H. Glorieux (Eds.), Vitamin D, second ed., Elsevier Academic Press, New York, 2005.

[2] R. Bouillon, W.H. Okamura, A.W. Norman, Structure–function relationships in the vitamin D endocrine system, Endocrinol. Rev. 16 (2) (1995) 200–257.

[3] G.D. Zhu, W.H. Okamura, Synthesis of vitamin D (calciferol), Chem. Rev. 95 (6) (1995) 1877–1952.

[4] R.A. Ettinger, H.F. DeLuca, The vitamin D endocrine system and its therapeutic potential, Adv. Drug Res. 28 (1996) 269–312.

[5] R.M. Evans, The steroid and thyroid hormone receptor superfamily, Science 240 (4854) (1988) 889–895.

[6] P. Chambon, The nuclear receptor superfamily: a personal retrospect on the first two decades, Mol. Endocrinol. 19 (6) (2005) 1418–1428.

[7] K. Konno, T. Fujishima, S. Maki, Z.-P. Liu, D. Miura, M. Chokki, S. Ishizuka, K. Yamaguchi, Y. Kan, M. Kurihara, N. Miyata, C. Smith, H.F. DeLuca, H. Takayama, Synthesis, biological evaluation, and conformational analysis of A-ring diastereomers of 2-methyl-1,25-dihydroxyvitamin D_3 and their 20-epimers: unique activity profiles depending on the stereochemistry of the A-ring and at C-20, J. Med. Chem. 43 (22) (2000) 4247–4265.

[8] K. Konno, S. Maki, T. Fujishima, Z.-P. Liu, D. Miura, M. Chokki, H. Takayama, A novel and practical route to A-ring enyne synthon for 1α,25-dihydroxyvitamin D_3 analogs: synthesis of A-ring diastereomers of 1α,25-dihydroxyvitamin D_3 and 2-methyl-1,25-dihydroxyvitamin D_3, Bioorg. Med. Chem. Lett. 8 (2) (1998) 151–156.

[9] Y. Suhara, K. Nihei, H. Tanigawa, T. Fujishima, K. Konno, K. Nakagawa, T. Okano, H. Takayama, Syntheses and biological evaluation of novel 2α-substituted 1α,25-dihydroxyvitamin D_3 analogues, Bioorg. Med. Chem. Lett. 10 (10) (2000) 1129–1132.

[10] Y. Suhara, K. Nihei, M. Kurihara, A. Kittaka, K. Yamaguchi, T. Fujishima, K. Konno, N. Miyata, H. Takayama, Efficient and versatile synthesis of novel 2α-substituted 1α,25-dihydroxyvitamin D_3 analogues and their docking to vitamin D receptors, J. Org. Chem. 66 (26) (2001) 8760–8771.

[11] For our review article of 2-substituted 1, 25-dihydroxyvitamin D_3, see: H. Takayama, A. Kittaka, T. Fujishima, Y. Suhara, Design, synthesis, and biological studies of the A-ring-modified 1,25-dihydroxyvitamin D_3 analogs, in: J. Reichrath, M. Friedrich, W. Tilgen (Eds.), Vitamin D Analogs in Cancer Prevention and Therapy, Recent Results in Cancer Research, vol. 164, Springer-Verlag, Berlin Heidelberg, 2003, pp. 289–317.

[12] A. Kittaka, Y. Suhara, H. Takayanagi, T. Fujishima, M. Kurihara, H. Takayama, A concise and efficient route to 2α-(ω-hydroxyalkoxy)-1α,25-dihydroxyvitamin D_3: remarkably high affinity to vitamin D receptor, Org. Lett. 2 (17) (2000) 2619–2622.

[13] N. Saito, Y. Suhara, M. Kurihara, T. Fujishima, S. Honzawa, H. Takayanagi, T. Kozono, M. Matsumoto, M. Ohmori, N. Miyata, H. Takayama, A. Kittaka, Design and efficient synthesis of 2α-(ω-hydroxyalkoxy)-1α,25-dihydroxyvitamin D_3 including 2-*epi*-ED-71 and its 20-epimers with HL-60 cell differentiation activity, J. Org. Chem. 69 (22) (2004) 7463–7471.

[14] K. Miyamoto, E. Murayama, K. Ochi, H. Watanabe, N. Kubodera, Synthetic studies of vitamin D analogs. XIV. Synthesis and calcium regulating activity of vitamin D_3 analogs bearing a hydroxyalkyl group at the 2β-position, Chem. Pharm. Bull. 41 (6) (1993) 1111–1113.

[15] G.H. Posner, N. Johnson, Stereocontrolled total synthesis of calcitriol derivatives: 1,25-dihydroxy-2-(4′-hydroxybutyl)vitamin D_3 analogs of an osteoporosis drug, J. Org. Chem. 59 (25) (1994) 7855–7861.

[16] B.M. Trost, J. Dumas, M. Villa, New strategies for the synthesis of vitamin D metabolites via palladium-catalyzed reactions, J. Am. Chem. Soc. 114 (25) (1992) 9836–9845.

[17] N. Rochel, J.M. Wurtz, A. Mitschler, B. Klaholz, D. Moras, The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand, Mol. Cell 5 (1) (2000) 173–179.

[18] K. Kristjansson, A.R. Rut, M. Hewison, J.L. O'Riordan, M.R. Hughes, Two mutations in the hormone binding domain of the vitamin D receptor cause tissue resistance to 1,25-dihydroxyvitamin D_3, J. Clin. Invest. 92 (1) (1993) 12–16.

[19] J.P. Malloy, J.W. Pike, D. Feldman, The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D-resistant rickets, Endocrinol. Rev. 20 (2) (1999) 156–188.

[20] S.A. Gardezi, C. Nguyen, J.P. Malloy, G.H. Posner, D. Feldman, S. Peleg, A rationale for treatment of hereditary vitamin D-resistant rickets with analogs of 1α,25-dihydroxyvitamin D_3, J. Biol. Chem. 276 (31) (2001) 29148–29156 (A-ring stereochemistry of (1α,3β): M.A. Hatcher, S. Peleg, P. Dolan, T.W. Kensler, A. Sarjeant, G.H. Posner, A-ring hydroxymethyl 19-nor analogs of the natural hormone 1α,25-dihydroxyvitamin D_3: synthesis and preliminary biological evaluation, Bioorg. Med. Chem. 13 (12) (2005) 3964–3976).

[21] S.L. Swann, J.J. Bergh, M.C. Farach-Carson, J.T. Koh, Rational design of vitamin D_3 analogues which selectively restore activity to a vitamin D receptor mutant associated with rickets, Org. Lett. 4 (22) (2002) 3863–3866.

[22] S.L. Swann, J.J. Bergh, M.C. Farach-Carson, C.A. Ocasio, J.T. Koh, Structure-based design of selective agonists for a rickets-associated mutant of the vitamin D receptor, J. Am. Chem. Soc. 124 (46) (2002) 13795–13805.

[23] A. Kittaka, M. Kurihara, S. Peleg, Y. Suhara, H. Takayama, 2α-(3-Hydroxypropyl)- and 2α-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 accessible to vitamin D receptor mutant related to hereditary vitamin D-resistant rickets, Chem. Pharm. Bull. 51 (3) (2003) 357–358.

[24] N. Takahashi, Design for mutant receptor: synthesis of 1-methylvitamin D_3 derivatives, dissertation, Teikyo University, 2005 (in Japanese).

[25] S. Honzawa, K. Hirasaka, Y. Yamamoto, S. Peleg, T. Fujishima, M. Kurihara, N. Saito, S. Kishimoto, T. Sugiura, K. Waku, H. Takayama, A. Kittaka, Design, synthesis and biological evaluation of novel 1α,25-dihydroxyvitamin D_3 analogues possessing aromatic ring on 2α-position, Tetrahedron 61 (47) (2005) 11253–11263.

[26] D. Abe, T. Sakaki, T. Kusudo, A. Kittaka, N. Saito, Y. Suhara, T. Fujishima, H. Takayama, H. Hamamoto, M. Kamakura, M. Ohta, K. Inouye, Drug Metab. Dispos. 33 (6) (2005) 778–784.

[27] G.G. Schwartz, T.C. Chen, Vitamin D, sunlight, and the natural history of prostate cancer, in: D. Feldman, J.W. Pike, F.H. Glorieux (Eds.), Vitamin D, Elsevier Academic Press, New York, 2005, pp. 1599–1615.

[28] T.C. Chen, G.G. Schwartz, K.L. Burnstein, B.L. Lokeshwar, M.F. Holick, The in vitro evaluation of 25-hydroxyvitamin D_3 and 19-nor-1α,25-dihydroxyvitamin D_2 as therapeutic agents for prostate cancer, Clin. Cancer Res. 6 (3) (2000) 901–908.

[29] R.R. Sicinski, P. Rotkiewics, A. Kolinski, W. Sicinska, J.M. Prahl, C.M. Smith, H.F. DeLuca, 2-Ethyl and 2-ethylidene analogues of 1α,25-dihydroxy-19-norvitamin D_3: synthesis, conformational analysis, biological activities, and docking to the modeled rVDR ligand binding domain, J. Med. Chem. 45 (16) (2002) 3366–3380.

[30] K. Ono, A. Yoshida, N. Saito, T. Fujishima, S. Honzawa, Y. Suhara, S. Kishimoto, T. Sugiura, K. Waku, H. Takayama, A. Kittaka, Efficient synthesis of 2-modified 1α,25-dihydroxy-19-norvitamin D_3 with Julia olefination: high potency in induction of differentiation on HL-60 cells, J. Org. Chem. 83 (19) (2003) 7407–7415.

[31] M.A. Arai, R. Tsutsumi, H. Hara, T.C. Chen, T. Sakaki, N. Urushino, K. Inouye, A. Kittaka, Synthesis of 25-hydroxy-19-norvitamin D_3 analogs and their antiproliferative activities on prostate cells, Heterocycles 66 (2005) 469–479.

[32] T. Okano, K. Nakagawa, N. Kubodera, K. Ozono, A. Isaka, A. Osawa, M. Terada, K. Mikami, Catalytic asymmetric syntheses and biological activities of singly dehydroxylated 19-nor-1α,25-dihydroxyvitamin D_3 A-ring analogs in cancer cell differentiation and apoptosis, Chem. Biol. 7 (3) (2000) 173–184.

[33] Recent reviews on Paget's disease, see: S.V. Reddy, Etiology of Paget's disease and osteoclast abnormalities, J. Cell Biochem. 93 (4) (2004) 688–696.

[34] G.D. Roodman, J.J. Windle, Paget disease of bone, J. Clin. Invest. 115 (2) (2005) 200–208.
[35] S.V. Reddy, N. Kurihara, C. Menaa, G.D. Roodman, Paget's disease of bone: a disease of the osteoclast, Rev. Endocrinol. Metab. Disord. 2 (2) (2001) 195–201.
[36] D. Miura, K. Manabe, K. Ozono, M. Saito, Q. Gao, A.W. Norman, S. Ishizuka, Antagonistic action of novel 1α,25-dihydroxyvitamin D_3-26,23-lctone analogs on differentiation of human leukemia cells (HL-60) induced by 1α,25-dihydroxyvitamin D_3, J. Biol. Chem. 274 (23) (1999) 16392–16399.
[37] K. Ozono, M. Saito, D. Miura, T. Michigami, S. Nakajima, S. Ishizuka, Analysis of the molecular mechanism for the antagonistic action of a novel 1α,25-dihydroxyvitamin D_3 analogue toward vitamin D receptor function, J. Biol. Chem. 274 (45) (1999) 32376–32381.
[38] N. Saito, T. Matsunaga, T. Fujishima, M. Anzai, H. Saito, K. Takenouchi, D. Miura, S. Ishizuka, H. Takayama, A. Kittaka, Remarkable effect of 2α-modification on the VDR antagonistic activity of 1α-hydroxyvitamin D_3-26,23-lactones, Org. Biomol. Chem. 1 (24) (2003) 4396–4402.
[39] N. Saito, H. Saito, M. Anzai, A. Yoshida, T. Fujishima, K. Takenouchi, D. Miura, S. Ishizuka, H. Takayama, A. Kittaka, Dramatic enhancement of antagonistic activity on vitamin D receptor: a double functionalization of 1α-hydroxyvitamin D_3-26,23-lactones, Org. Lett. 5 (25) (2003) 4859–4862.
[40] N. Saito, T. Matsunaga, H. Saito, M. Anzai, K. Takenouchi, D. Miura, S. Ishizuka, H. Takayama, A. Kittaka, Synthesis and 2α-modification of 24-phenylvitamin D_3 lactones: effects on VDR antagonistic activity, Heterocycles 67 (1) (2006) 311–336.
[41] N. Saito, M. Masuda, M. Matsunaga, H. Saito, M. Anzai, K. Takenouchi, D. Miura, S. Ishizuka, M. Takimoto-Kamimura, A. Kittaka, 24,24-Dimethylvitamin D_3-26,23-lactones and their 2α-functionalized analogues as highly potent VDR antagonists, Tetrahedron 60 (36) (2004) 7951–7961.
[42] N. Saito, M. Masuda, H. Saito, K. Takenouchi, S. Ishizuka, J.-I. Namekawa, M. Takimoto-Kamimura, A. Kittaka, Synthesis of 24,24-ethanovitamin D_3 lactones using ruthenium-catalyzed intermolecular enyne metathesis: potent vitamin D receptor antagonists, Synthesis 15 (2005) 2533–2543.
[43] J.A. Eisman, A.J. Hamstra, B.E. Kream, H.F. DeLuca, A sensitive, precise, and convenient method for determination of 1,25-dihydroxyvitamin D in human plasma, Arch. Biochem. Biophys. 176 (1) (1976) 235–243.
[44] M. Inaba, H.F. DeLuca, Stabilization of 1,25-dihydroxyvitamin D_3 receptor in the human leukemia cell line, HL-60, with diisopropylfluorophosphate, Biochim. Biophys. Acta 1010 (1) (1989) 20–27.
[45] S.J. Collins, F.W. Ruscetti, R.E. Gallagher, R.C. Gallo, Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide, J. Exp. Med. 149 (4) (1979) 969–974.
[46] E. Ochiai, D. Miura, H. Eguchi, S. Ohara, K. Takenouchi, Y. Harada, Y. Azuma, T. Kamimura, A.W. Norman, S. Ishizuka, Molecular mechanism of the vitamin D antagonistic actions of (23*S*)-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactone depends on the primary structure of the carboxyl-terminal region of the vitamin D receptor, Mol. Endocrinol. 19 (5) (2005) 1147–1157.
[47] K. Takenouchi, R. Sogawa, K. Manabe, H. Saitoh, Q. Gao, D. Miura, S. Ishizuka, Synthesis and structure–activity relationships of TEI-9647 derivatives as vitamin D_3 antagonists, J. Steroid Biochem. Mol. Biol. 89–90 (1–5) (2004) 31–34.
[48] Some biologically active natural products having an α-methylene-γ-lactone structure react with the thiol group of cysteine to give the corresponding cysteine adduct S.M. Kupchan, D.C. Fessler, M.A. Eakin, T.J. Giacobbe, Reactions of alpha methylene lactone tumor inhibitors with model biological nucloephile, Science 168 (929) (1970) 376–377.
[49] S. Väisänen, M. Peräkylä, J.I. Kärkkäinen, A. Steinmeyer, C. Carlberg, Critical role of helix 12 of the vitamin D_3 receptor for the partial agonism of the carboxylic ester antagonists, J. Mol. Biol. 315 (2) (2002) 229–238.
[50] Y. Kato, Y. Nakano, H. Sano, A. Tanatani, H. Kobayashi, R. Shimazawa, H. Koshino, Y. Hashimoto, K. Nagasawa, Synthesis of 1α,25-dihydroxyvitamin D_3-26,23-lactams (DLAMs), a novel series of 1α,25-dihydroxyvitamin D_3 antagonist, Bioorg. Med. Chem. Lett. 14 (10) (2004) 2579–2583.
[51] N. Saito, T. Matsunaga, H. Saito, M. Anzai, K. Takenouchi, D. Miura, J.-i. Namekawa, S. Ishizuka, A. Kittaka, Further synthetic and biological studies on vitamin D hormone antagonists based on C24-alkylation and C2α-functionalization of 25-dehydro-1α-hydroxyvitamin D_3-26,23-lactones, J. Med. Chem. 49 (24) (2006) 7063–7075.
[52] S. Hourai, T. Fujishima, A. Kittaka, Y. Suhara, H. Takayama, N. Rochel, D. Moras, Probing a water channel near the A-ring of receptor-bound 1α,25-dihydroxyvitamin D_3 with selected 2α-substituted analogues, J. Med. Chem. 49 (17) (2006) 5199–5205.

8. K. Berns *et al.*, *Nature* **428**, 431 (2004).
9. T. R. Brummelkamp *et al.*, *Nat. Chem. Biol.* **2**, 202 (2006).
10. T. Sjoblom *et al.*, *Science* **314**, 268 (2006).
11. L. D. Wood *et al.*, *Science* **318**, 1108 (2007).
12. R. A. Dickins *et al.*, *Nat. Genet.* **37**, 1289 (2005).
13. G. K. Smyth, T. Speed, *Methods* **31**, 265 (2003).
14. V. M. Draviam *et al.*, *Nat. Cell Biol.* **9**, 556 (2007).
15. M. Ito, T. Nakano, F. Erdodi, D. J. Hartshorne, *Mol. Cell. Biochem.* **259**, 197 (2004).
16. H. Jin, T. Sperka, P. Herrlich, H. Morrison, *Nature* **442**, 576 (2006).
17. L. T. Vassilev *et al.*, *Science* **303**, 844 (2004).
18. N. L. Solimini, J. Luo, S. J. Elledge, *Cell* **130**, 986 (2007).
19. J. M. Silva *et al.*, *Science* **319**, 617 (2008).
20. We thank A. L. Brass for the pMSCV-PM, pMSCV-PM-FF, and pMSCV-PM-mir30 vectors and for scientific advice; M. J. Solimini for help with data analysis; E. R. McDonald for scientific advice; T. Waldman and B. Vogelstein for the HCT116 and DLD-1 cell lines; and T. Moore from Open Biosystems for help with assembling library pools. G.H. is a fellow of the Helen Hay Whitney Foundation. X.L.A. is supported by a National Research Service Award fellowship, M.E.S. is supported by an American Cancer Society fellowship, and A.S. is supported by grant T32CA09216 to the MGH Pathology Department. T.F.W. is a fellow of the Susan G. Komen Foundation and is supported by grant PDF0403175. This work is supported by grants from NIH and the U.S. Department of Defense to G.J.H., J.W.H., and S.J.E. G.J.H. has a paid consulting relationship with Open Biosystems.

Supporting Online Material

www.sciencemag.org/cgi/content/full/319/5863/620/DC1
Materials and Methods
SOM Text
Figs. S1 to S3
Tables S1 to S6
References
Data Sets S1 to S9

14 August 2007; accepted 20 December 2007
10.1126/science.1149200

Cathepsin K–Dependent Toll-Like Receptor 9 Signaling Revealed in Experimental Arthritis

Masataka Asagiri,[1,2] Toshitake Hirai,[1,4] Toshihiro Kunigami,[1,4] Shunya Kamano,[1,5] Hans-Jürgen Gober,[1] Kazuo Okamoto,[1] Keizo Nishikawa,[1] Eicke Latz,[6] Douglas T. Golenbock,[6] Kazuhiro Aoki,[3] Keiichi Ohya,[3] Yuuki Imai,[7,9] Yasuyuki Morishita,[8] Kohei Miyazono,[8] Shigeaki Kato,[7,9] Paul Saftig,[10] Hiroshi Takayanagi[1,2]*

Cathepsin K was originally identified as an osteoclast-specific lysosomal protease, the inhibitor of which has been considered might have therapeutic potential. We show that inhibition of cathepsin K could potently suppress autoimmune inflammation of the joints as well as osteoclastic bone resorption in autoimmune arthritis. Furthermore, *cathepsin K*$^{-/-}$ mice were resistant to experimental autoimmune encephalomyelitis. Pharmacological inhibition or targeted disruption of cathepsin K resulted in defective Toll-like receptor 9 signaling in dendritic cells in response to unmethylated CpG DNA, which in turn led to attenuated induction of T helper 17 cells, without affecting the antigen-presenting ability of dendritic cells. These results suggest that cathepsin K plays an important role in the immune system and may serve as a valid therapeutic target in autoimmune diseases.

Both innate and adaptive immune systems contribute to the inflammation seen in autoimmune diseases, but the molecular mechanism underlying this process is not completely understood (*1*, *2*). The cathepsins constitute a family of lysosomal cysteine proteases that were initially recognized as nonspecific scavengers of cellular proteins and that were also found to display cell type–specific functions (*3*, *4*). Cathepsins L and S are fundamental in processing of major histocompatibility complex (MHC) class II antigens and MHC class II trafficking and maturation (*3*, *4*). In contrast, cathepsin K is highly expressed in osteoclasts and is involved in degradation of bone matrices such as type I collagen (*5*). The loss-of-function mutation in the *cathepsin K* gene in humans causes pycnodysostosis, a rare genetic disorder characterized by impaired osteoclastic bone resorption (*6*). In mice, the targeted disruption of *cathepsin K* similarly results in the pycnodysostotic phenotype (*4*, *5*, *7*). Among matrix-degrading enzymes expressed in osteoclasts, cathepsin K is the only one for which an essential role in bone resorption has been clearly demonstrated in both mice and humans (*8*). Thus, cathepsin K remains a potential therapeutic target for the treatment of bone diseases such as osteoporosis and autoimmune arthritis, in which osteoclast activity is increased (*9*, *10*).

Through screening, we obtained a potent orally active cathepsin K inhibitor named NC-2300 (Fig. 1A and figs. S1 to S3), which suppresses osteoclastic bone resorption both in vivo and in vitro (figs. S1 and S4). Computer-assisted simulation of the cathepsin K/NC-2300 complex indicated that NC-2300 blocks the active-site cleft where Cys[25] and His[162] of cathepsin K form the catalytic site (Fig. 1B and fig. S5). To test the effects of the inhibitor on disease models, we treated adjuvant-induced arthritis (AIA) in rats with oral administration of NC-2300 and compared the results with the effects of alendronate, which is one of the bisphosphonate compounds used clinically as an inhibitor of osteoclastic bone resorption. Bone loss in arthritis occurs mainly in two forms: bone erosion at the inflamed joints and periarticular osteoporosis (*11*). Radiological analysis revealed that NC-2300, but not alendronate, markedly suppressed bone erosion (Fig. 1C), although bone mineral density analysis showed that both compounds had a comparable inhibitory effect on periarticular osteoporosis (fig. S6A). NC-2300 also ameliorated paw swelling (Fig. 1D) and improved locomotive activity (fig. S6B) without affecting the onset rate of arthritis. NC-2300 reduced inflammation even when administered after the onset of disease (fig. S7). These results indicate that cathepsin K also functions in cells other than osteoclasts, allowing it to participate in autoimmune inflammation.

In AIA, local injection of adjuvant stimulates antigen presentation by dendritic cells (DCs), leading to T cell autoimmunity, the production of inflammatory cytokines by macrophages, and osteoclast-mediated bone destruction (*9*, *12*, *13*). The adjuvant effects are mainly dependent on the pathogen-associated molecular patterns (PAMPs)–induced activation of Toll-like receptor (TLR) signaling (*14*, *15*). Therefore, we next analyzed the expression and function of cathepsin K in T cells, macrophages, and DCs. Cathepsin K mRNA was barely detected in nonadherent bone marrow (BM) cells or splenic T cells (Fig. 2A), and NC-2300 showed no effects on T cell activation (fig. S8A). Although macrophages have been reported to express cathepsin K (*4*), NC-2300 had no effects on the activation of BM-derived macrophages stimulated by PAMPs (fig. S8B). BM-derived DCs (BM-DCs) did express a detectable level of cathepsin K mRNA, although this was much lower than expression in osteoclasts (Fig. 2A). Nevertheless, cathepsin K activity was confirmed in DCs and was inhibited by NC-2300 (Fig. 2B).

To investigate whether cathepsin K has a role in antigen presentation in DCs, DCs were cultured with fluorescein isothiocyanate (FITC)–labeled ovalbumin. The uptake of ovalbumin-FITC was observed by flow cytometry in NC-2300–treated DCs as well as in nontreated cells (Fig. 2C). In addition, NC-2300–treated DCs stimulated proliferation of splenic T cells from ovalbumin-specific DO11.10 TCR transgenic mice to an extent similar to that of nontreated DCs (Fig. 2D). These results suggest that cathepsin K activity is not required for the antigen uptake, processing, or presentation by

[1]Department of Cell Signaling, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8549, Japan. [2]Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Tokyo 113-8549, Japan. [3]Department of Hard Tissue Engineering, Section of Pharmacology, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8549, Japan. [4]Nippon Chemiphar Co., Ltd., Saitama 341-0005, Japan. [5]Department of Orthopaedic Surgery, Juntendo University School of Medicine, Tokyo 113-8421, Japan. [6]Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA 01605, USA. [7]Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0032, Japan. [8]Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan. [9]Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan. [10]Biochemical Institute, Christian-Albrechts-University Kiel, D-24098 Kiel, Germany.

*To whom correspondence should be addressed. E-mail: taka.csi@tmd.ac.jp

DCs. Considering the crucial role of cathepsins L and S in antigen presentation (*3*, *4*, *16*), these results also indicate that the effects of NC-2300 on these cathepsins are negligible.

Complete Freund's adjuvant (CFA) is composed of killed mycobacteria, which contain various PAMPs including TLR2, TLR4, and TLR9 agonists (*14*, *17*). Therefore, we tested the responses of DCs to these PAMPs with or without cathepsin K inactivation. Enzyme-linked immunosorbent assay revealed that the production of cytokines such as interleukin-12 (IL-12) and IL-23 by DCs was significantly inhibited by NC-2300 when stimulated with oligodeoxynucleotides containing unmethylated CpG motif (CpG: the TLR9 ligand), but not with Malp2 and peptidoglycan (PGN) (the TLR2 ligands) or lipopolysaccharide (LPS: the TLR4 ligand) (Fig. 2E). CpG-induced expression of IL-6, IL-12, and IL-23 in BM-DCs was down-regulated at the mRNA level (Fig. 2F), and IFN-β production by Flt3L-induced BM-DCs in response to CpG was suppressed by NC-2300 (Fig. 2F). These results suggest that cathepsin K plays an important role in the gene induction program regulated by TLR9 signaling.

Deoxyribonuclease treatment of the mycobacterial components in CFA greatly reduces the severity of AIA without affecting the induction efficiency, and this reduction is recovered by supplementation with CpG DNA (*18*, *19*), indicating that TLR9-mediated immune responses determine the severity of autoimmune inflammation. Because mycobacterial DNA can be detected weeks after CFA inoculation (*19*), CpG DNA may augment autoimmune inflammation throughout the course of arthritis. These observations lend support to the notion that cathepsin K–dependent TLR9 signaling contributes to autoimmune inflammation.

How does cathepsin K regulate TLR9 signaling? After being taken up into the cells, CpG DNA locates within the endosomal compartment, where CpG DNA binds to TLR9 (*14*, *18*). The binding of CpG with TLR9 results in the conformational change of TLR9 (*20*), leading to activation of MyD88 and downstream signaling such as the mitogen-activated protein kinase, interferon regulatory factor (IRF), and nuclear factor kappa B (NF-κB) pathways (*14*). Activation of these pathways leads to the production of inflammatory cytokines and up-regulation of cell-

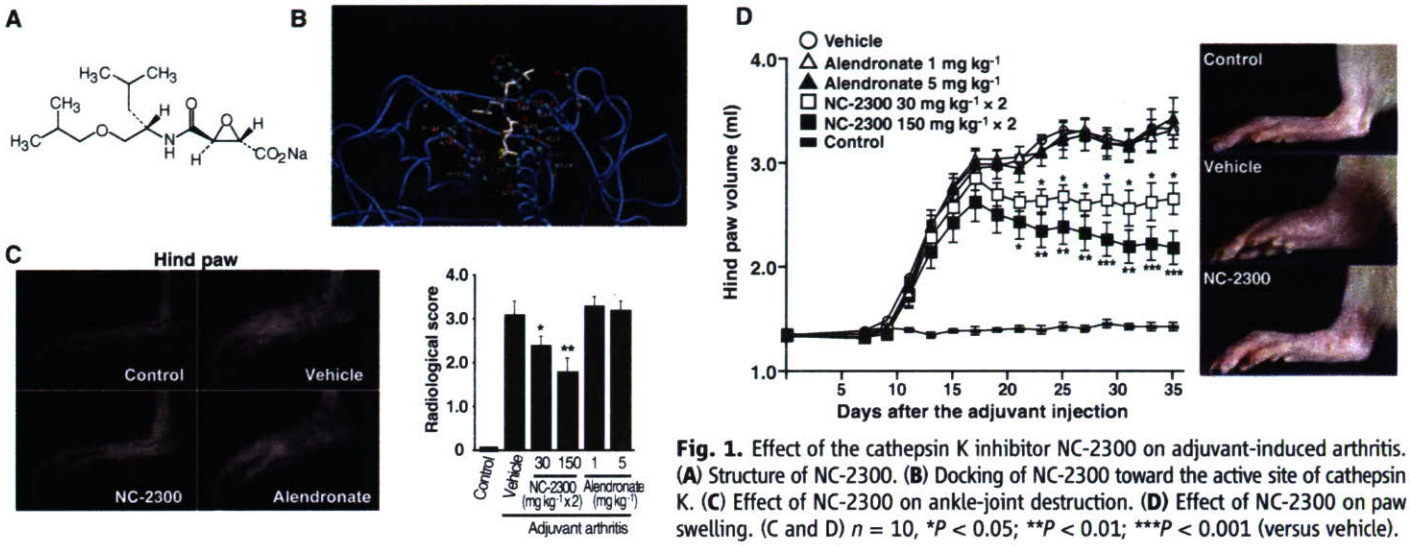


Fig. 1. Effect of the cathepsin K inhibitor NC-2300 on adjuvant-induced arthritis. **(A)** Structure of NC-2300. **(B)** Docking of NC-2300 toward the active site of cathepsin K. **(C)** Effect of NC-2300 on ankle-joint destruction. **(D)** Effect of NC-2300 on paw swelling. (C and D) $n = 10$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (versus vehicle).

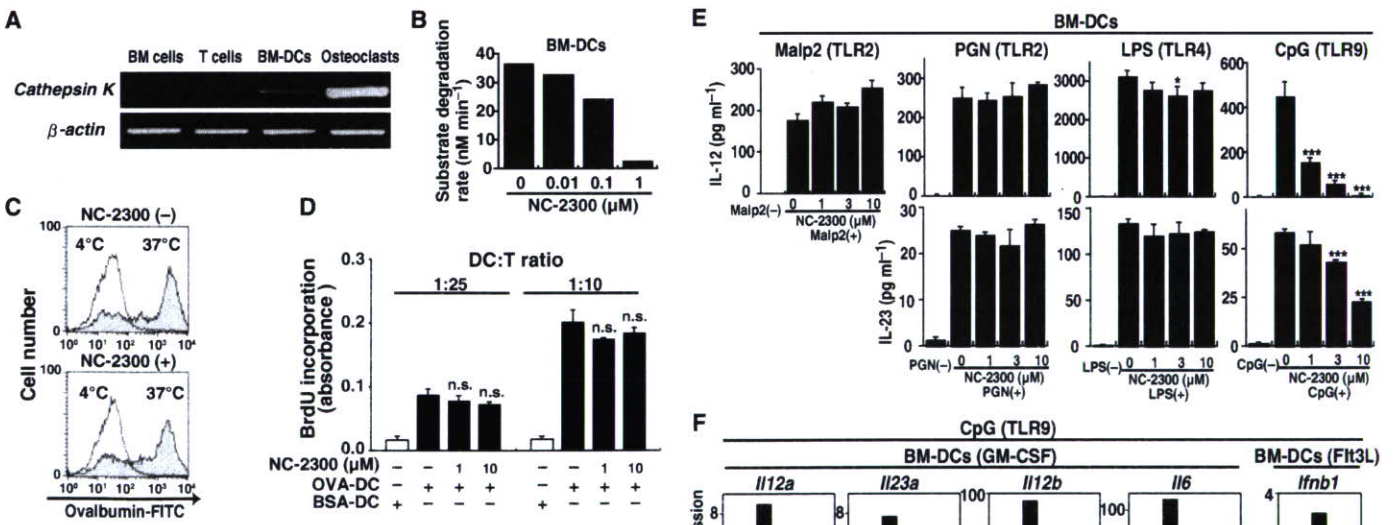


Fig. 2. Selective suppression of TLR9 response in dendritic cells (DCs) by cathepsin K inhibition. **(A)** Reverse transcription–polymerase chain reaction analysis of the cathepsin K mRNA. **(B)** Enzymatic activity of cathepsin K in BM-DCs. **(C)** Effect of NC-2300 on antigen uptake. **(D)** Effect of cathepsin K inhibition on the presentation of exogenous antigens by DCs. BrdU, bromodeoxyuridine; OVA, ovalbumin; BSA, bovine serum albumin; n.s., not significant. **(E)** Effects of cathepsin K inhibition on TLR-stimulated cytokine production. $^{*}P < 0.05$; $^{***}P < 0.001$. **(F)** Effect of NC-2300 on the cytokine mRNA expression in CpG-treated BM-DCs.

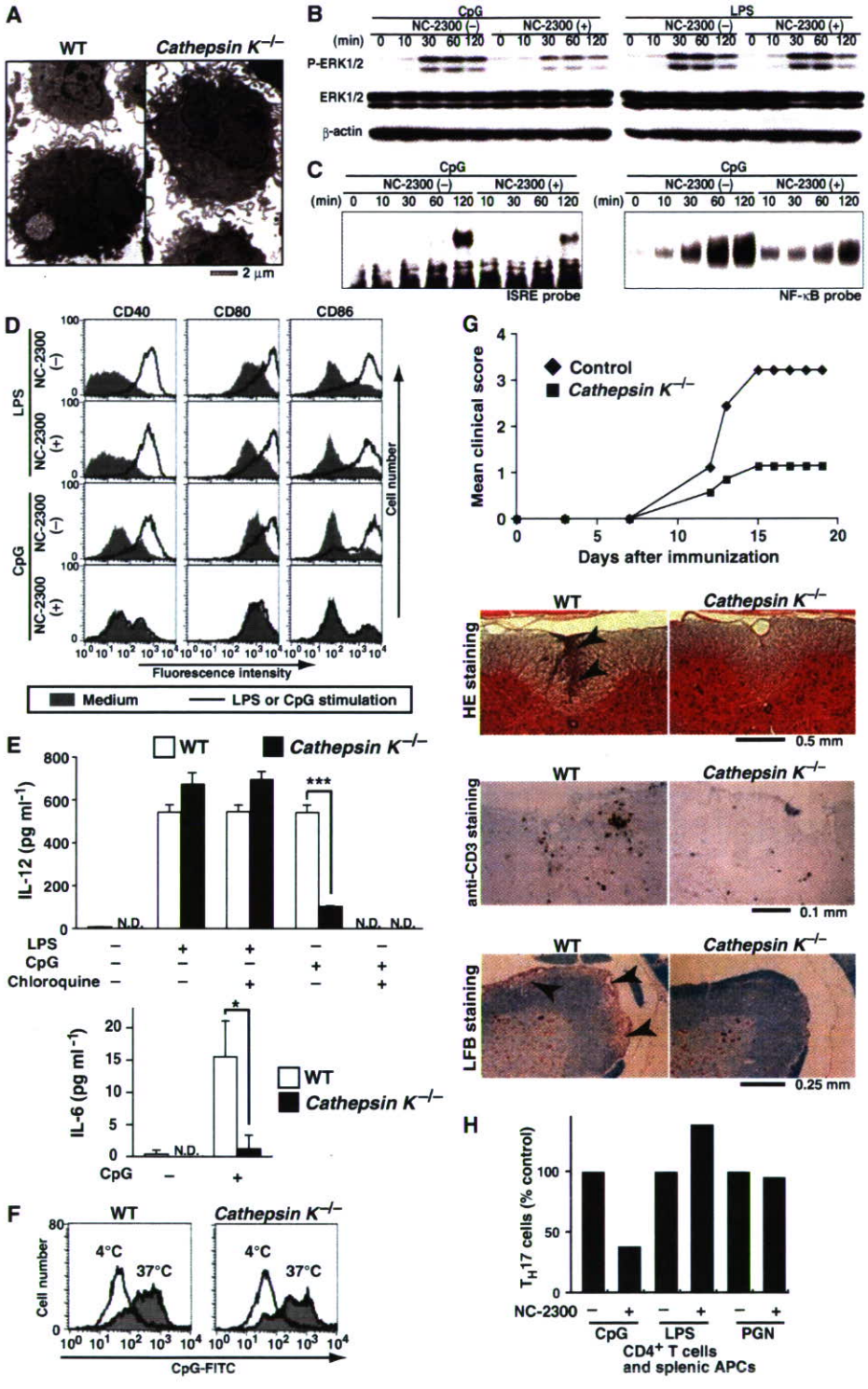


Fig. 3. Cathepsin K regulation of TLR9-mediated immune responses and autoimmune inflammation. (**A**) Electron micrographs of WT and *cathepsin* $K^{-/-}$ BM-DCs. (**B**) Suppression of ERK phosphorylation by NC-2300 in CpG-stimulated DCs. (**C**) Effect of NC-2300 on CpG-induced activation of transcription factors, IRF (left) and NF-κB (right) (electrophoretic mobility shift assay). ISRE, IFN-stimulated response element. (**D**) Impaired TLR9-mediated expression of cell-surface molecules in NC-2300–treated DCs. (**E**) Cytokine production in WT or *cathepsin* $K^{-/-}$ DCs in response to LPS or CpG. N.D., not detected. *$P < 0.05$; ***$P < 0.001$. (**F**) Uptake of CpG in *cathepsin* $K^{-/-}$ DCs. (**G**) Severity of EAE in *cathepsin* $K^{-/-}$ mice. Clinical scores (top). Lumbar spinal cord sections stained with hematoxylin and eosin (HE), immunostained for CD3, and stained with luxol fast blue (LFB) (bottom). Arrowheads indicate inflammatory cellular infiltrates (HE) and demyelinated areas (LFB). (**H**) Cathepsin K inactivation results in defective T_H17 polarization in response to CpG, but not to LPS or PGN.

surface molecules such as CD40, CD80, and CD86 in DCs (*2*, *21*). Compounds that block endosomal acidification such as chloroquine inhibit CpG-driven signaling (*14*, *18*). A normal number of BM-DCs were generated in BM cells derived from *cathepsin* $K^{-/-}$ mice (*5*, *22*), and they exhibited a normal morphology (Fig. 3A), suggesting that cathepsin K is dispensable for the DC differentiation. CpG-induced, but not LPS-induced, phosphorylation of extracellular-signal regulated kinases 1 and 2 (ERK 1/2) in DCs was suppressed by cathepsin K inactivation (Fig. 3B). CpG-induced activation of IRF and NF-κB was also suppressed by cathepsin K inactivation in DCs (Fig. 3C). In addition, NC-2300 reduced expression of CD40, CD80, and CD86 in DCs (Fig. 3D). CpG-induced, but not LPS-induced, production of cytokines such as IL-12 and IL-6 in *cathepsin* $K^{-/-}$ DCs was significantly suppressed (Fig. 3E). Thus, cathepsin K inactivation leads to the blockade of essentially all the downstream pathways of TLR9 signaling in DCs, suggesting that cathepsin K plays a critical role in the signaling events proximal to TLR9. However, the endocytosis of CpG was not affected in *cathepsin* $K^{-/-}$ DCs (Fig. 3F) and the endosomal acidification was not inhibited by NC-2300 (fig. S9), whereas chloroquine suppressed the cathepsin K activity in DCs (fig. S10).

It remains to be elucidated how cathepsin K regulates CpG-TLR9 signaling in the endosome, but the results of pharmacological inhibition indicate that the proteolytic activity of this enzyme is crucial for the mechanism. It is conceivable that cathepsin K may be involved in the degradation of proteins that inhibit the interaction between CpG and TLR9, or cathepsin K–mediated proteolysis may result in the conformational change of TLR9 that augments its signal transduction (*20*). Because fluorescence resonance energy transfer analysis showed that the CpG-induced conformational change of TLR9 was not affected in human embryonic kidney (HEK) 293 cells by NC-2300 (fig. S11), we cannot rule out the possibility that cathepsin K degrades a cytoplasmic protein that modifies the proximal TLR9 signaling. The observation that *cathepsin* $K^{-/-}$ DCs responded normally to TLR3 or TLR7/8 stimulation suggests that the role of cathepsin K is TLR9-specific in DCs (fig. S12).

To clearly demonstrate that cathepsin K plays a critical role in autoimmune inflammation in an osteoclast-independent manner, we subjected *cathepsin* $K^{-/-}$ mice to experimental autoimmune encephalomyelitis (EAE), in which TLR9 signaling plays an important role (*23*, *24*). The frequency of the onset of EAE was not different between control and *cathepsin* $K^{-/-}$ mice, but the severity of the paralytic symptoms was much lower in the *cathepsin* $K^{-/-}$ mice than in control mice (Fig. 3G, top). Histological analysis of spinal cords demonstrated a marked decrease in inflammation (Fig. 3G, HE staining), T cell infiltration (Fig. 3G, anti-CD3 staining), and demyelination (Fig. 3G, LFB staining) in *cathepsin* $K^{-/-}$ mice. Because T helper 17 (T_H17) cells play an essential role in the autoimmune inflammation in EAE (*25*), we examined the effect of cathepsin K inactivation on the ability of DCs to induce

T_H17 cells. The ability of DCs to induce T_H17 cells was markedly inhibited by cathepsin K inactivation when stimulated with CpG, but not with LPS or PGN (Fig. 3H). Taken together with the results on the role of cathepsin K in CpG-induced cytokine expression in DCs, the impaired induction of T_H17 cells by cathepsin K inactivation was caused, at least in part, by the reduced DC expression of cytokines that are involved in the induction and expansion of T_H17 cells such as IL-6 and IL-23 (*25*, *26*).

Our results show that cathepsin K, which was thought to be an osteoclast-specific enzyme, plays a critical role in the immune system. Cathepsin K functions under the acidified conditions in the endosome, where engagement of CpG by TLR9 occurs, and plays an important role in the signaling events proximal to TLR9. Thus, careful attention should be paid to the side effects of cathepsin K inhibitors on the immune system in the treatment of osteoporosis, whereas they may have dual benefits in the treatment of autoimmune arthritis, the pathogenesis of which is dependent on both DCs and osteoclasts (*9*).

References and Notes

1. J. Banchereau, V. Pascual, A. K. Palucka, *Immunity* **20**, 539 (2004).
2. R. M. Steinman, D. Hawiger, M. C. Nussenzweig, *Annu. Rev. Immunol.* **21**, 685 (2003).
3. V. Turk, B. Turk, D. Turk, *EMBO J.* **20**, 4629 (2001).
4. K. Honey, A. Y. Rudensky, *Nat. Rev. Immunol.* **3**, 472 (2003).
5. P. Saftig *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13453 (1998).
6. B. D. Gelb, G. P. Shi, H. A. Chapman, R. J. Desnick, *Science* **273**, 1236 (1996).
7. W. Chen *et al.*, *Hum. Mol. Genet.* **16**, 410 (2007).
8. M. Asagiri, H. Takayanagi, *Bone* **40**, 251 (2007).
9. H. Takayanagi, *Nat. Rev. Immunol.* **7**, 292 (2007).
10. Y. Yasuda, J. Kaleta, D. Brömme, *Adv. Drug Deliv. Rev.* **57**, 973 (2005).
11. S. Ochi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 11394 (2007).
12. R. Holmdahl *et al.*, *Immunol. Rev.* **184**, 184 (2001).
13. A. Marshak-Rothstein, *Nat. Rev. Immunol.* **6**, 823 (2006).
14. S. Akira, S. Uematsu, O. Takeuchi, *Cell* **124**, 783 (2006).
15. A. Iwasaki, R. Medzhitov, *Nat. Immunol.* **5**, 987 (2004).
16. R. J. Riese *et al.*, *J. Clin. Invest.* **101**, 2351 (1998).
17. S. B. Su *et al.*, *J. Immunol.* **175**, 6303 (2005).
18. A. M. Krieg, *Annu. Rev. Immunol.* **20**, 709 (2002).
19. A. Ronaghy *et al.*, *J. Immunol.* **168**, 51 (2002).
20. E. Latz *et al.*, *Nat. Immunol.* **8**, 772 (2007).
21. I. Mellman, R. M. Steinman, *Cell* **106**, 255 (2001).
22. T. Nakamura *et al.*, *Cell* **130**, 811 (2007).
23. M. Prinz *et al.*, *J. Clin. Invest.* **116**, 456 (2006).
24. B. M. Segal, J. T. Chang, E. M. Shevach, *J. Immunol.* **164**, 5683 (2000).
25. H. Park *et al.*, *Nat. Immunol.* **6**, 1133 (2005).
26. E. Bettelli *et al.*, *Nature* **441**, 235 (2006).

27. We thank K. Takeda, M. Yamamoto, H. Oda, S. Imajoh-Ohmi, M. Matsuura, K. Nishioka, T. Kanda, K. Sato, S. Hida, Y. Iwai, H. Hemmi, H. Yanai, S. Kano, Y. Omatsu, T. Onodera, T. Honda, and A. Suematsu for discussion and assistance. This work was supported in part by a Grant-in-Aid for Creative Scientific Research from the Japan Society for the Promotion of Science (JSPS); grants for the Genome Network Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT); Grants-in-Aid for Scientific Research from JSPS and MEXT; Health Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan; and grants from the Deutsche Forschungsgemeinschaft and the NIH.

Supporting Online Material

www.sciencemag.org/cgi/content/full/319/5863/624/DC1
Materials and Methods
Figs. S1 to S12
References

5 September 2007; accepted 14 December 2007
10.1126/science.1150110

Systemic Leukocyte-Directed siRNA Delivery Revealing Cyclin D1 as an Anti-Inflammatory Target

Dan Peer,[1] Eun Jeong Park,[1] Yoshiyuki Morishita,[1] Christopher V. Carman,[2] Motomu Shimaoka[1]*

Cyclin D1 (CyD1) is a pivotal cell cycle–regulatory molecule and a well-studied therapeutic target for cancer. Although CyD1 is also strongly up-regulated at sites of inflammation, its exact roles in this context remain uncharacterized. To address this question, we developed a strategy for selectively silencing CyD1 in leukocytes in vivo. Targeted stabilized nanoparticles (tsNPs) were loaded with CyD1–small interfering RNA (siRNA). Antibodies to β_7 integrin (β_7 I) were then used to target specific leukocyte subsets involved in gut inflammation. Systemic application of β_7 I-tsNPs silenced CyD1 in leukocytes and reversed experimentally induced colitis in mice by suppressing leukocyte proliferation and T helper cell 1 cytokine expression. This study reveals CyD1 to be a potential anti-inflammatory target, and suggests that the application of similar modes of targeting by siRNA may be feasible in other therapeutic settings.

RNA interference (RNAi) has emerged as a powerful strategy for suppressing gene expression, offering the potential to dramatically accelerate in vivo drug target validation, as well as the promise to create novel therapeutic approaches if it can be effectively applied in vivo (*1*). Cyclin D1 (CyD1) is a key cell cycle–regulating molecule that governs the proliferation of normal and malignant cells (*2*, *3*). In inflammatory bowel diseases, colon-expressed CyD1 is aberrantly up-regulated in both epithelial and immune cells (*4*, *5*). Although CyD1 has also been implicated in promoting epithelial colorectal dysplasia and carcinogenesis, it is not clear whether leukocyte-expressed CyD1 contributes directly to the pathogenesis of inflammation and whether it might serve as a therapeutic target.

To address these questions, we used RNAi silencing of CyD1 in an experimental model of intestinal inflammation. A major limitation to the use of RNAi in vivo is the effective delivery of siRNAs to the target cells (*6*, *7*). RNAi in leukocytes, a prime target for anti-inflammatory therapeutics, has remained particularly challenging, as these cells are difficult to transduce by conventional transfection methods and are often disseminated throughout the body, thus requiring systemic delivery approaches (*8*). One possibility is to use integrins, which are an important family of cell-surface adhesion molecules, as targets for siRNA delivery (*8*). Specifically, we have shown that antibody-protamine fusion proteins directed to the lymphocyte function-associated antigen–1 (LFA-1) integrin selectively delivered siRNAs to leukocytes, both in vitro and in vivo (*8*). However, whether an integrin-directed siRNA delivery approach can induce

[1]Immune Disease Institute and Department of Anesthesia, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA. [2]Department of Medicine, Beth Israel Deaconess Medical Center, and Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA.

*To whom correspondence should be addressed. E-mail: shimaoka@cbrinstitute.org

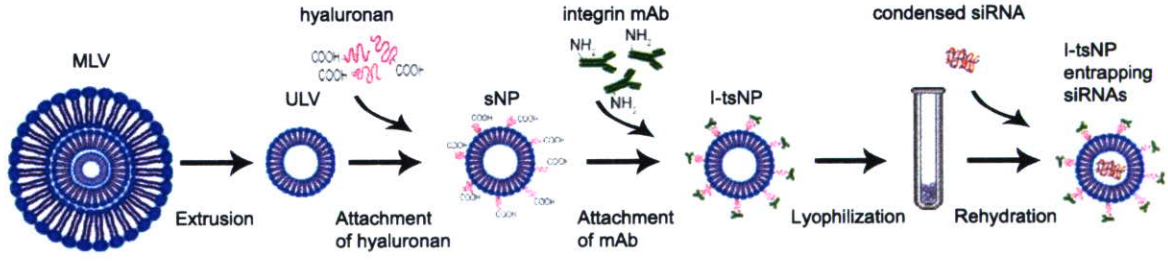


Fig. 1. The processes involved in generating I-tsNPs. Multilamellar vesicle (MLV) [prepared as described in (*9*)] is extruded to form a unilamellar vesicle (ULV) with a diameter of ~100 nm. Hyaluronan is covalently attached to DPPE in the ULV. A monoclonal antibody (mAb) to the integrin is covalently attached to hyaluronan, generating I-tsNP. siRNAs are entrapped by rehydrating lyophilized β_7 I-tsNP with water containing protamine-condensed siRNAs.

The C-terminus of ephrin-B1 regulates metalloproteinase secretion and invasion of cancer cells

Masamitsu Tanaka[1], Kazuki Sasaki[1,2], Reiko Kamata[1] and Ryuichi Sakai[1,*]

[1]Department of Growth Factor Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Tokyo 104-0045, Japan
[2]Department of Pharmacology, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan
*Author for correspondence (e-mail: rsakai@gan2.res.ncc.go.jp)

Accepted 28 April 2007
Journal of Cell Science 120, 2179-2189 Published by The Company of Biologists 2007
doi:10.1242/jcs.008607

Summary

Interaction of the Eph family of receptor protein tyrosine kinases and their ligands, ephrin family members, induces bi-directional signaling via cell-cell contacts. High expression of B-type ephrin is associated with high invasion potential of tumors, however, the mechanism by which ephrin-B promotes cancer cell invasion is poorly understood. We show that interaction of ephrin-B1 with the Eph receptor B2 (EphB2) significantly enhances processing of the extracellular domain of ephrin-B1, which is regulated by the C-terminus. Matrix metalloproteinase-8 (MMP-8) is the key protease that cleaves ephrin-B1, and the C-terminus of ephrin-B1 regulates activation of the extracellular release of MMP-8 without requirement of de novo protein synthesis. One possible mechanism by which ephrin-B1 regulates the exocytosis of MMP-8 is the activation of Arf1 GTPase, a critical regulator of membrane trafficking. In support of this hypothesis, activation of ephrin-B1 increased GTP-bound Arf1, and the secretion of MMP-8 was reduced by expression of a dominant-negative mutant of Arf1. Expression of ephrin-B1 promoted the invasion of cancer cells in vivo, which required the C-terminus of ephrin-B1. Our results suggest a novel function of the C-terminus of ephrin-B1 in activating MMP-8 secretion, which promotes the invasion of cancer cells.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/13/2179/DC1

Key words: Eph, Ephrin, Metalloproteinase, Secretion

Introduction

The members of the Eph receptor family can be classified into two groups based on their sequence similarity and their preferential binding to ligands tethered to the cell surface either by a glycosylphosphatidyl inositol anchor (ephrin-A) or a transmembrane domain (ephrin-B) (Murai and Pasquale, 2003; Blits-Huizinga et al., 2004; Poliakov et al., 2004). The interaction of Eph receptor B2 (EphB2) protein tyrosine kinases and their ephrin-B ligands induces bi-directional signaling via the resultant cell-cell contacts. Ephrin-B has an intracellular domain, which includes sites for tyrosine phosphorylation via Src family kinases and a docking site for proteins with a PDZ domain (Lin et al., 1999; Cowan and Henkemeyer, 2001; Bong et al., 2004). These sites give ephrin-B ligands at least two ways of being involved in intracellular signaling. Although investigation of the functions of Eph receptors and ephrins have focused on the development of the vascular and nervous systems, the roles of Eph-ephrin pathways in epithelial cells and cancers have also attracted interest (Batlle et al., 2002; Klein, 2004; Batlle et al., 2005; Tanaka et al., 2005; Holmberg et al., 2006). Overexpression of B-type ephrin in cancer cells is reported to correlate with high invasion and high vascularity of tumors (Meyer et al., 2005; Castellvi et al., 2006; Nakada et al., 2006), and elevated expression of ephrin-B1 is observed in poorly differentiated invasive tumor cells and other tumors with poor clinical prognosis (Kataoka et al., 2002; Varelias et al., 2002). However, whether ephrin-B actually modifies tumor invasion in vivo has not been established, and the mechanism by which ephrin-B is involved in the invasion of cancer cells is unknown.

Some ephrins are cleaved by a protease. When ephrin-A5 binds with EphA3, their complex creates a recognition motif for a disintegrin and metalloproteinase (ADAM)10 membrane metalloproteinase, which allows effective cleavage of the extracellular domain of ephrin-A5 (Hattori et al., 2000; Janes et al., 2005). Protease-mediated cleavage also occurs for B-type ephrins; a rhomboid transmembrane serine protease RHBDL2 cleaves the transmembrane domain of ephrin-B3 (Pascall and Brown, 2004), and ephrin-B1 and ephrin-B2 are processed within the transmembrane region by the presenilin 1 (PS1)-γ-secretase system to release an intracellular peptide (Georgakopoulos et al., 2006; Tomita et al., 2006).

During the process of screening peptides secreted from pancreas cancer cell lines, we found evidence that the extracellular portion (ectodomain) of ephrin-B1 is secreted. This finding led us to study the mechanism of ephrin-B1 shedding within the ectodomain in cancer cells. In the present study, we show that the C-terminus of ephrin-B1 regulates the exocytosis of MMP-8, a key protease of ephrin-B1 cleavage, in response to the interaction with its receptor EphB2. An accumulating number of reports have shown that increased secretion of metalloproteinases often depends on their transcriptional activation (Chinni et al., 2006; Raymond et al., 2006; Reuben and Cheung, 2006). However, increase of MMP-8 secretion

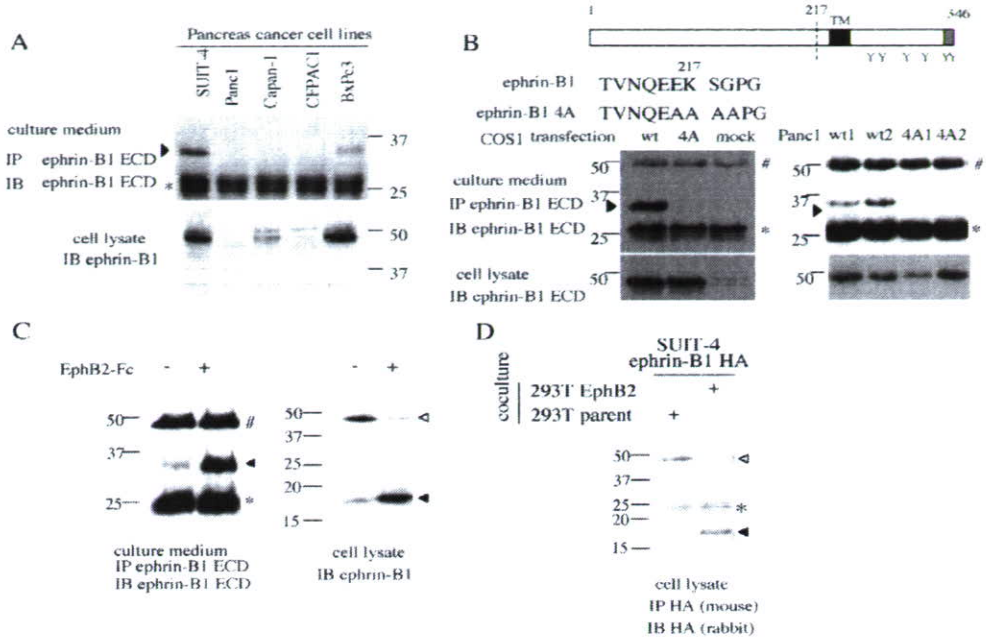


Fig. 1. The ectodomain of ephrin-B1 is secreted into the culture medium of human pancreas cancer cells. (A) Various pancreas cancer cell lines were cultured in medium containing 0.5% FBS. After 6 hours, conditioned medium was collected and subjected to immunoprecipitation (IP) and immunoblotting (IB) with polyclonal antibodies against the extracellular domain of ephrin-B1 (ephrin-B1 ECD). The immunoprecipitated 35 kDa ephrin-B1 fragment is indicated by an arrowhead. The expression of ephrin-B1 in each cell lysate was confirmed by immunoblotting (bottom). (B) A diagram of ephrin-B1 is shown at the top. TM, transmembrane domain; Y, tyrosine phosphorylation sites. Dotted line indicates the cleavage site of ephrin-B1, and the PDZ domain-binding motif is indicated as a gray box at the C-terminus. Four aa residues around the cleavage site were changed to alanine (ephrin-B1 4A) destroying the MMP-8 cleavage site. The conditioned medium of COS-1 cells transfected with wild-type (wt) or mutant ephrin-B1 (4A), or independent PANC-1 clones stably expressing ephrin-B1 were collected and subjected to immunoprecipitation and immunoblotting as described in A. (C) SUIT-4 cells were either treated with EphB2-Fc (4 μg/ml) for 2 hours (+) or left untreated (–). The ephrin-B1 fragment in the medium was detected as in A (left) or the cell lysates were subjected to immunoblotting with anti ephrin-B1 C18 (right panel). Open and filled arrowheads indicate uncleaved ephrin-B1 and its processed fragment (p17), respectively. (D) SUIT-4 cells were transiently transfected with ephrin-B1 tagged with HA at the C-terminus. Transfected SUIT-4 cells were overlayed on a monolayer of parent HEK293T cells or HEK293T cells stably expressing EphB2 for 2 hours. Cell lysates were prepared from co-cultured cells to detect HA-tagged p17 fragment derived from exogenously expressed ephrin-B1 in SUIT-4 cells by immunoprecipitation. # and * indicate the IgG heavy chain and light chain, respectively. Open and filled arrowheads indicate uncleaved ephrin-B1 and its processed fragment (p17), respectively.

induced by stimulation of ephrin-B1 did not depend on the elevation of MMP-8 expression level, but rather it was suggested to depend on the intracellular signaling mediated by ephrin-B1.

MMP-8, also known as neutrophil collagenase, is not only expressed in neutrophils, but it is also expressed in wide variety of cells, including chondrocytes, endothelial cells, synovial fibroblast and various cancer cells (Siller-Lopez et al., 2000; Stadlemann et al., 2003; Lint and Libert, 2006). MMP-8 cleaves all three α-chains of type I, II and III collagen and also a wide range of non-collagenous substrates, and plays important roles in inflammation and in cancer progression (Lint and Libert, 2006). Like other secretory proteins, proenzymes of soluble-type MMPs are secreted after the process of vesicle transport from Golgi to the plasma membrane, and then, extracellularly activated by removal of the propeptide domain (Sternlicht and Werb, 2001). In neutrophils, MMP-8 is stored in specific granules after being transported from Golgi, and released following activation by inflammatory mediators (Sternlicht and Werb, 2001), however, whether it is also the case in various cancer cells has yet to be fully investigated. Our analysis of the interaction between MMP-8 activity and EphB-ephrin-B1 signal transduction revealed a novel function of the C-terminus of ephrin-B1 in the secretion of MMP-8, which leads to the cleavage of ephrin-B1 and involves the negative regulation of the EphB-ephrin-B1 complex. Moreover, regulation of this MMP family member through the ephrin-B1 C-terminus may contribute to the highly invasive phenotype of ephrin-B1-expressing cancer cells by degradation of the extracellular matrix.

Results

The Ephrin-B1 ectodomain is secreted into the culture medium of pancreatic cancer cell lines

During screening of peptides secreted by the pancreatic cancer cell line SUIT-4, we identified,using MALDI-MS/MS analysis, three different peptides derived from ephrin-B1 sharing a common N-terminus. Analysis of these peptides indicated that ephrin-B1 undergoes limited cleavage between aa residues 217 and 218. Immunoprecipitation with antibodies against the extracellular domain of ephrin-B1 revealed a 35 kDa band from the conditioned media of two out of five pancreatic cancer cell lines. The 35 kDa fragment was also detected in COS-1 and PANC-1 cells when ephrin-B1 was expressed (Fig. 1A,B). These results indicate that the 35 kDa ectodomain fragment is released by cleavage of ephrin-B1 in these cell lines. The concentration of serum in the medium did not affect the cleavage of ephrin-B1 (data not shown). The cleavage site in ephrin-B1 estimated by MALDI-MS/MS was confirmed by the observation that substitution of the four amino acids (aa) at positions 216-219 (ephrin-B1$^{216\text{-}219}$) by alanine (ephrin-B1 4A), blocked the production of the 35 kDa ectodomain fragment in COS-1 and PANC-1 cells (Fig. 1B).

Cleavage of ephrin-B1 ectodomain is enhanced by interaction with its receptor EphB2, which is regulated by the C-terminus of ephrin-B1

Next, we examined whether the interaction of ephrin-B1 with its receptor EphB2 modifies the cleavage of ephrin-B1.

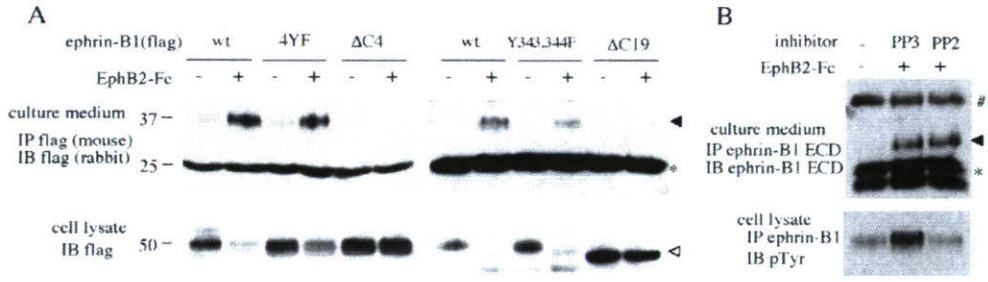


Fig. 2. Activation of ephrin-B1 cleavage requires its C-terminus. (A) Wild-type and various mutants of ephrin-B1 tagged with Flag at the N-terminus were expressed in Capan-1 cells by retrovirus-mediated gene transfer. The cells were treated with EphB2-Fc (2 μg/ml) for 1.5 hours (+) or left untreated (–) and conditioned medium was assayed for the presence of ephrin-B1 fragments by immunoprecipitation (IP) and immunoblotting (IB) with anti-flag antibody. The filled arrowhead indicates the ephrin-B1 ectodomain fragment. (Bottom) Expression of wild-type or mutated ephrin-B1 in cell lysates after treatment with EphB2-Fc. The open arrowhead indicates ephrin-B1 ΔC19. (B) SUIT-4 cells were treated with EphB2-Fc and PP2 or control PP3or left untreated, as indicated. Conditioned medium was collected after 2 hours and subjected to immunoprecipitation and immunoblotting. In the bottom panel, suppression of tyrosine phosphorylation of ephrin-B1 by PP2 treatment is shown. # and * indicate the IgG heavy chain and light chain, respectively.

Incubation of SUIT-4 cells with purified EphB2-Fc, a fusion protein of the extracellular domain of EphB2 with the Fc fragment of mouse IgG2b, significantly increased the amount of the 35 kDa fragment of ephrin-B1 in the conditioned medium (Fig. 1C, left). In EphB2-Fc-treated cell extract, a cellular fragment of ephrin-B1, produced by ectodomain shedding, was detected by immunoblotting with an antibody reacting to the C-terminal of ephrin-B1, as a band at 17 kDa (p17; Fig. 1C, right). The accumulation of p17 was also detected in ephrin-B1-expressing cells after contact with cells expressing EphB2. When ephrin-B1-expressing cells were overlayed on EphB2-expressing cells and co-cultured, significant reduction of uncleaved ephrin-B1 together with production of the p17 fragment was observed (Fig. 1D). This result indicates that cleavage of ephrin-B1 is also enhanced when ephrin-B1-expressing cells contact with heterologous cells expressing the EphB2.

In order to examine the processing mechanism of the ephrin-B1 ectodomain, several mutants of ephrin-B1 were analyzed. The cleavage of ephrin-B1 was also increased by EphB2-Fc treatment of Capan-1 cells expressing wild-type ephrin-B1 (Fig. 2A). However, expression of ephrin-B1 lacking the C-terminus (ΔC4 and ΔC19; the MMP-8 cleavage site at aa 217-218 is intact in these mutants), did not produce the 35 kDa ectodomain fragment in the culture medium upon treatment with EphB2-Fc (Fig. 2A). However, mutation of any of the four tyrosine residues in the cytoplasmic region (4YF) and tyrosines located at the C-terminus of ephrin-B1 (Y343, 344F) did not affect ephrin-B1 cleavage (Fig. 2A). In contrast to the significant reduction of full length wild-type or YF mutants of ephrin-B1 in cell lysates after EphB2-Fc treatment, level of C-terminally truncated ephrin-B1 mutants remained almost unchanged (Fig. 2A, bottom panels). We also observed similar results using PANC-1 cells, and confirmed that expression of ΔC mutants were localized on cell membrane (data not shown). In addition, treatment with PP2, an inhibitor of Src family kinases, significantly blocked tyrosine phosphorylation of ephrin-B1, but it did not affect cleavage of ephrin-B1 (Fig. 2B). Thus, the C-terminus of ephrin-B1, but not tyrosine phosphorylation of ephrin-B1, is required for induction of the proteolysis of ephrin-B1.

The Ephrin-B1 ectodomain is processed by MMP-8

The cleavage of ephrin-B1 was inhibited in PANC-1 cells expressing ephrin-B1, by incubation with the pan-matrix metalloproteinase (MMP) inhibitor GM6001, but not by inhibitors of other proteases including a cysteine protease, a serine protease, an aspartic protease or calpain (Fig. 3A). The amount of cleaved ephrin-B1 ectodomain was increased by treatment of DCI (3,4-dichloroisocoumarin), a serine protease inhibitor and TPCK (N^{α}-tosyl-phe chloromethyl ketone), a chymotrypsin inhibitor of unknown function. As the inhibition of ephrin-B1 cleavage by GM6001 was also confirmed in SUIT-4 cells (data not shown), we further attempted to identify the metalloproteinase responsible. When SUIT-4 cells were treated with natural MMP inhibitors, TIMPs, at low concentration (100 nM), cleavage of ephrin-B1 was most effectively inhibited by TIMP-1 compared with TIMP-2 and TIMP-3 (Fig. 3B). TIMP-3, which is known to inhibit tumor necrosis factor-α converting enzyme (TACE) did not inhibit the cleavage of ephrin-B1 at all at concentrations from 5-250 nM (data not shown), whereas it completely inhibited the cleavage of TNF-α expressed in THP-1 cells at 100 nM (Fig. 3B, right panel). Among inhibitors of several MMPs expressed in SUIT-4 cells, including MMP-1, MMP-2 or 3, MMP-8 and MMP-9, only the inhibitor of MMP-8 blocked the cleavage of ephrin-B1 (Fig. 3C), and this effect was also seen in COS-1 cells and PANC-1 cells expressing ephrin-B1 (data not shown).

In order to examine whether MMP-8 cleaves ephrin-B1, ephrin-B1-Fc fusion protein, which consists of the entire extracellular region of ephrin-B1 and the Fc fragment of mouse IgG2b, was incubated with purified activated MMPs in vitro. Incubation of ephrin-B1-Fc with activated MMP-8 produced two fragments of ephrin-B1-Fc corresponding to the predicted sizes (Fig. 3D). However, other membrane-type metalloproteinases such as MT1-MMP and ADAM10, which are also expressed in SUIT-4 cells, did not cleave ephrin-B1-Fc in vitro (Fig. 3D).

Expression of MMP-8 protein was detected at approximately the same levels in all of the cell lines we examined (Fig. 4A, left), although it was detected at high level when the cells were replated and decreased after the cells reached confluence (Fig. 4A, right). When expression of