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Review Article

Novel dipeptidyl peptidase IV inhibitors with antiarthritic effects

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Introduction

Dipeptidyl peptidase IV (DP IV , EC 3.4.14.5) is a serine protease which cleaves NH_2-terminal dipeptides from polypeptides with proline, and less effectively, alanine at the penultimate position (1, 2). DP IV is a highly glycosylated transmembrane protein (ectoenzyme) consisting of two identical subunits with approximately 110 kDa molecular mass (1, 3). cDNAs encoding DP IV have been cloned from human, mouse and rat species (4-6). Human DP IV was deduced to be a polypeptide of 766 amino acid residues composed of three parts: a cytoplasmic NH_2-terminal tail of 6 amino acid residues, a hydrophobic transmembrane region of 22 amino acid residues and an extracellular domain of 738 amino acid residues with 9 potential glycosylation sites (7).

DP IV shares a COOH-terminal stretch of approximately 200 amino acid residues with nonclassical serine hydrolases, including prolyl endopeptidase and acylamino acid hydrolase (5). This conserved region contains the putative catalytic triad residues (Ser^{630}-Asp^{708}-His^{740} in humans) of DP IV which are organized in an order different from those of classical serine proteases (His^{57}-Asp^{102}-Ser^{195} in chymotrypsin and Asp^{32}-His^{64}-Ser^{221} in subtilisin) (8, 9). Nonclassical serine hydrolases have molecular masses much larger than those of the chymotrypsin and subtilisin families.

Physiological and pathological roles of DP IV

DP IV is expressed in many tissues such as liver, kidney, small intestine, salivary gland, blood cells and plasma. The highest activity was found in the kidney proximal tubules and the intestinal brush-border membrane, suggesting that DP IV might participate in the metabolism and uptake of proline-containing peptides in these tissue (10, 11). On the basis of its unique specificity toward proline, DP IV has also been postulated to be important in the maturation and degradation of biologically active peptides such as substance P, tumor necrosis factor-α, growth hormone-releasing factor, RANTES and glucagon-like peptide-1, although the *in viv o*natural substrates of this enzyme have not been clearly identified (12-14).

In the immune system, DP IV has been identified as CD26, a surface differentiation marker in the transduction of mitogenic signals in thymocytes and T cells (5, 15). The expression of CD26 rapidly increased with mitogenic or antigenic stimulation. DP IV/CD26 not only marks the activated state but is itself involved in the signal transducing process as a costimulatory factor. Cross-linking of CD26 and CD3 with solid-phase immobilized monoclonal antibodies elicited T-cell activation in the absence of antigen-presenting cells (16). Modulation of CD26 on human T cells with an anti-CD26 monoclonal antibody led to an enhanced phosphorylation of CD3ξ chain and increased the activity of the CD4-associated tyrosine kinase $p56^{lck}$ (17). However, it is unlikely that CD26 directly participates in transducing the activation signal across the T cell membrane since this molecule has a very short cytoplasmic region. Torimoto *et al.* reported that protein tyrosine phosphatase (CD45RO) is associated with CD26 and provides a putative mechanism for the costimulation (18). Another association includes the strong binding of adenosine deaminase (ADA) type I to CD26 (19). This may be of particular importance since ADA activity participates in regulation of the early stages of signal transduction in T cells. Recently, DP IV has been shown to be expressed not only by T cells but also by B cells, NK cells and monocytes (20-22).

An important question is whether the costimulatory potential depends on the enzymatic activity of DP IV. Specific inhibitors of DP IV were able to suppress mitogen-induced and antigen-induced DNA synthesis of T cells, cytokine production and T cell-dependent immunoglobulin production *in vitro* (23-25). This issue was investigated by Tanaka *et al*. using Jurkat cells (CD26-) transfected with the wild-type gene ($CD26^+$/DP IV^+) or the mutant gene ($CD26^+$/DP IV-) lacking enzyme activity by the substitution of residue 630 from serine to alanine (2). After stimulation with a combination of anti-CD26 and anti-CD3 antibodies, the wild-type-transfected cells produced substantially more IL-2 than the mutant or CD26- control transfectants. Nevertheless, the mutant transfected cells still produced significantly more IL-2 than CD26- control transfectants. These results suggested that DP IV activity would play an important but not an absolute role in the costimulatory activity of CD26. In contrast, von Bonin *et al*. argued that the dipeptidyl peptidase activity would not be necessary for costimulation, based on the results of experiments using the mutant CD26 (27). They transfected BWδζ cells, mouse TCR-positive T-cell hybridoma, with the human $CD26^+$/DP IV-. Some transfectants of this mutant CD26 responded as well as or even better than the wild-type CD26-transfected cells in terms of IL-2 production after stimulation with anti-CD26 and anti-CD3. Whether the enzymatic activity of CD26 is required for its costimulatory function is still debated.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by marked infiltration and accumulation of T lymphocytes in the sublayer of the synovium. In patients with RA, serum DP IV activity was reduced, although the number of peripheral blood T lymphocytes expressing CD26 was increased (28, 29). The proportion of CD26-positive cells in blood correlated with severity of the disease (30). Increments of CD26-positive cells have also been detected in patients with multiple sclerosis and Grave's disease in both peripheral blood and inflamed tissues (31, 32). Although these results infer involvement of DP IV in the development of immunological disorders, its exact role has not been examined.

Antiarthritic effects of known DP IV inhibitors

In order to assess the relevance of DP IV in autoimmune diseases, we evaluated the *in vivo* effects of several known DP IV inhibitors by using two arthritis models, one induced by collagen and one by alkyldiamine (33). These animal models share several pathological features associated with RA. A transition analog of DP IV, (*S*)-alanylpyrrolidine-boronic acid (Ala-boroPro) (34), suppressed hind paw swelling associated with collagen- and alkyldiamine-induced arthritis. A competitive inhibitor of DP IV, Lys(Z[NO_2])-thiazolide (35) and an irreversible inhibitor, Ala-Pro-nitrobenzoylhydroxylamine (36), also suppressed alkyldiamine-induced arthritis dose-dependently. Based on these results, we concluded that DP IV inhibitors had antiarthritic effects, although the precise mechanisms of their therapeutic effects have yet to be elucidated.

Discovery of new DP IV inhibitors from microbial metabolites

The accumulating data indicating the physiological and/or pathological importance of DP IV/CD26 in the immune system encouraged us to search for a new chemotype with DP IV inhibitory activity. We used DP IV purified from rat kidney and microbial metabolites as a screening source (37, 38). Among approximately 20,000 buthanol-extracts of fermentation broths, one extract was found to contain novel DP IV inhibitors referred to as TMC-2A, TMC-2B and TMC-2C. A producer strain was identified as *Aspergillus oryzae* A374 from its culture and morphological characteristics. A typical fermentation of TMC-2 using a 30 liter jar fermentor gave approximately 160 μg total of TMC-2 compounds per ml on day 3, as assessed by the DP IV inhibitory activity of culture filtrate. TMC-2 compounds were isolated in pure forms from the fermentation broth by several steps, including ion-exchange column chromatography, silica gel column chromatography and crystallization.

The molecular formulas of TMC-2A and the deoxy derivatives TMC-2B and TMC-2C were determined to be $C_{28}H_{34}N_4O_9$, $C_{28}H_{34}N_4O_8$ and $C_{28}H_{34}N_4O_8$, respectively, on the basis of elemental analysis, HR-FAB-MS and NMR spectral data. Detailed analyses of ^1H- and ^{13}C-NMR data including ^1H-^1H COSY, ^1H-^{13}C long range COSY, selective INEPT and NOESY revealed the unique planar structures consisting of three moieties, tryptophan, highly substituted isoquinoline and dihydroxy- or hydroxyleucine (Fig. 1). The three-dimensional structure of TMC-2A was determined by X-ray analysis (Fig. 2) and acid hydrolysis of TMC-2A gave L-tryptophan. Taken together, the absolute structure of TMC-2A was established to be (2*S*,2'*S*,2''*S*)2-[2'-[2''-amino-3''-(indol-3''-yl)-1''-oxopropyl]-1',2',3',4'-tetrahydro-2',8'-dihydroxy-7'-methoxyisoquinol-3-yl-carbonylamino]-4-hydroxymethyl-5-hydroxypentanoic acid.

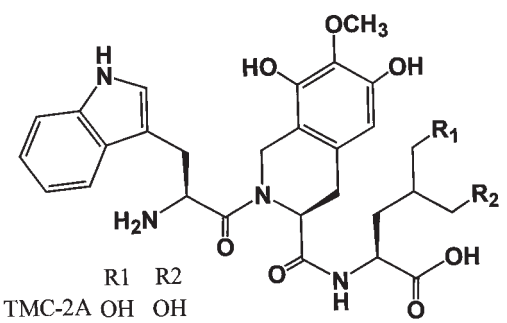

Fig. 1. Structure of TMC-2A, TMC-2B and TMC-2C.

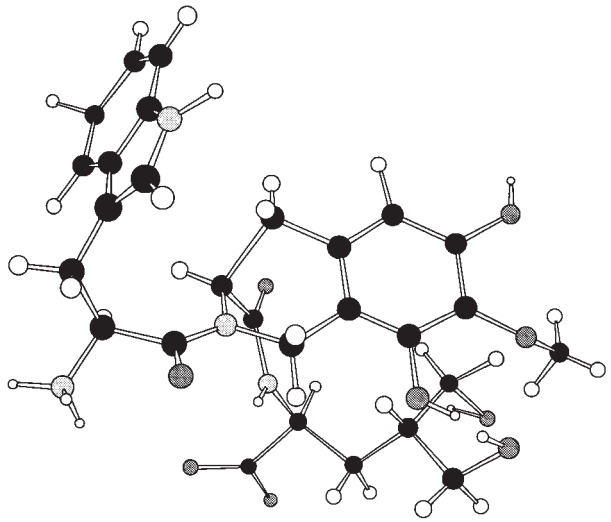

Fig. 2. Molecular structure of TMC-2A. Open, lightly dotted, densely dotted and closed circles represent H, N, O and C atoms, respectively. (Reproduced with permission from Y. Asai et al. *TMC-2A, -2B and -2C, new dipeptidyl peptidase IV inhibitors produced by Aspergillus oryzae A374. II. Isolation and structure determination.* J Antibiotics 1997, 50: 653-8. Copyright © Japan Antibiotics Research Association.)

We performed detailed kinetic analysis to elucidate the inhibitory mechanism of TMC-2A. The K_m value of DP IV from rat kidney was 260 μM in our assay system. A reciprocal plot analysis revealed that TMC-2A behaved as a noncompetitive inhibitor. The K_i value of TMC-2A was calculated to be 5.3 μM. To investigate enzyme selectivity, we tested the inhibitory activity of TMC-2A, TMC-2B and TMC-2C against other serine proteases (proryl endopeptidase, subtilisin and trypsin), cysteine proteases (cathepsin C and proline aminopeptidase) and a metalloprotease (leucine aminopeptidase). None of the TMC-2 compounds inhibited these proteases, demonstrating their high selectivity to DP IV. It was confirmed that TMC-2A inhibited human DP IV (prepared from mononuclear cells and adenocarcinoma Caco-2 cells) as well as rat DP IV (from kidney and spleen) at essentially the same potency.

Chemical synthesis of TMC-2A derivatives

In order to find the critical structure of TMC-2 essential for the inhibition of DP IV, we utilized solid-phase combinatorial chemistry. TMC-2A was divided into three amino acid components: **A** (tryptophan), **B** (highly substituted isoquinoline) and **C** (dihydroxy-leucine), and 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid (Tic) was used instead of the highly substituted Tic of TMC-2A. At first, part **A** was substituted with other natural amino acids by synthesizing H-**A**aa-Tic-Xaa-OH where Xaa denotes the equimolar mixture of 19 natural amino acids except Cys. The Trp derivative exhibited potent inhibitory activity, whereas the other derivatives did not. Next, in order to examine the effects of part **C**, derivatives of H-Trp-Tic-**C**aa-OH were synthesized. The Glu and Ser derivatives showed strong inhibition of DP IV, while others only displayed moderate inhibition. Interestingly, the compound that lacked part **C**, H-Trp-Tic-OH, still exhibited similar potent inhibitory activity as the Glu and Ser derivatives. Finally, Tic was replaced by several natural amino acids (Glu, Phe, Pro, Trp, Tyr and Val) and the derivatives of H-Trp-**B**aa-OH were synthesized. None of these derivatives showed any significant inhibitory activity.

In conclusion, the critical structure for DP IV inhibition was identified as H-Trp-Tic-OH and referred to as TSL-225 (Fig. 3) (39). TSL-225, like TMC-2A, inhibited rat DP IV noncompetitively, with a K_i value of 3.6 μM (40).

Antiarthritic effects of TMC-2A and TSL-225

We evaluated the immunopharmacological effects of TMC-2A on alkyldiamine-induced arthritis (40). Immunization of rats with alkyldiamine resulted in development of chronic arthritis, and continuous administration of TMC-2A dose-dependently suppressed paw swelling (Fig. 4a). The body weights of the arthritic rats were reduced as compared to control rats, although treatment with TMC-2A restored the decreased body weights to a certain extent (Fig. 4b). TMC-2A treatment also showed a tendency to suppress the splenomegaly associated with the development of arthritis (Fig. 4c). A high dose of TMC-2A (30 mg/kg) suppressed the increase of serum mucoprotein which reflects the acute phase protein synthesis (Fig. 4d).

We further evaluated the pharmacological effects of TSL-225 and TMC-2A on adjuvant-induced arthritis, demonstrating that TSL-225 also dose-dependently suppressed arthritis (Fig. 5).

Mechanism of action of DP IV inhibitors

In order to investigate the mechanism of action of DP IV inhibitors, we examined their effects on mitogen (concanavalin A and phytohemagglutinin)- and antigen (purified protein derivative of tuberculin)-induced T-cell

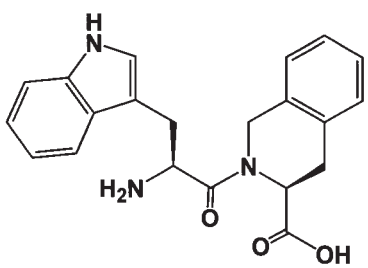

Fig. 3. Structure of TSL-225.

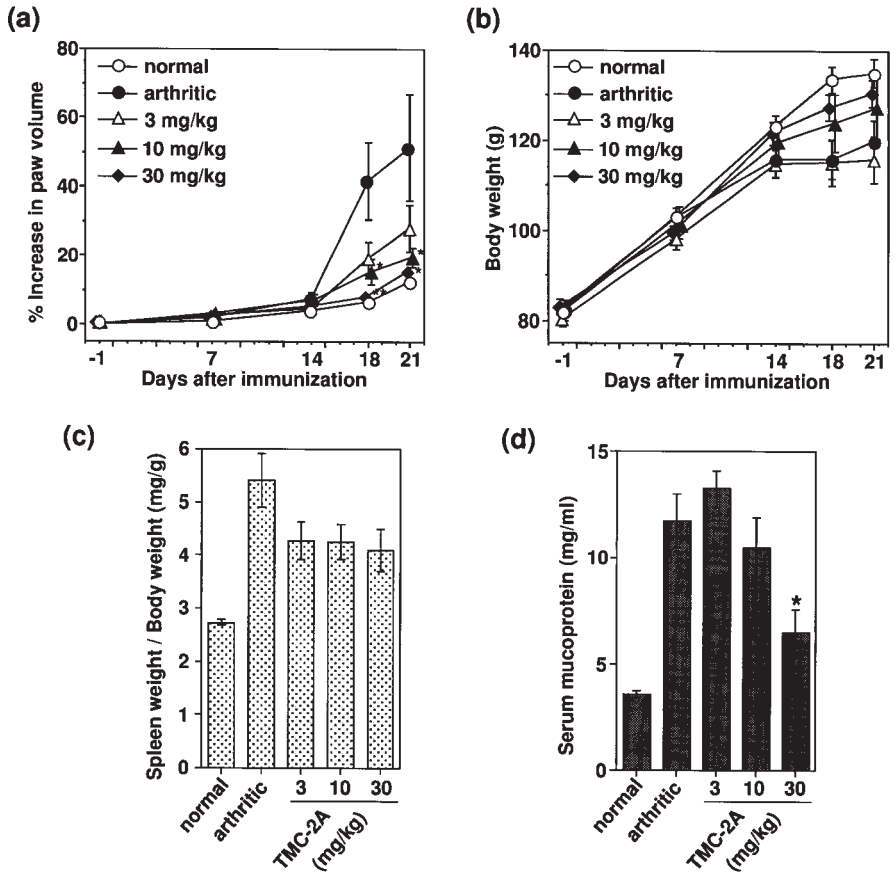

Fig. 4. Effect of TMC-2A on alkyldiamine-induced arthritis in F344 rats. Values represent mean ± SE of 10 rats for the arthritic group and 5 rats for other groups; $^{**}p$ <0.01, $^{*}p$ <0.05 compared with saline-treated arthritic rats (Bonferroni/Dunn-test). (Reproduced with permission from S. Tanaka et al. *Anti-arthritic effects of the novel dipeptidyl peptide IV inhibitors TMC-2A and TSL-225.* Immunopharmacology 1998, 40: 21-6. Copyright © Elsevier Science.)

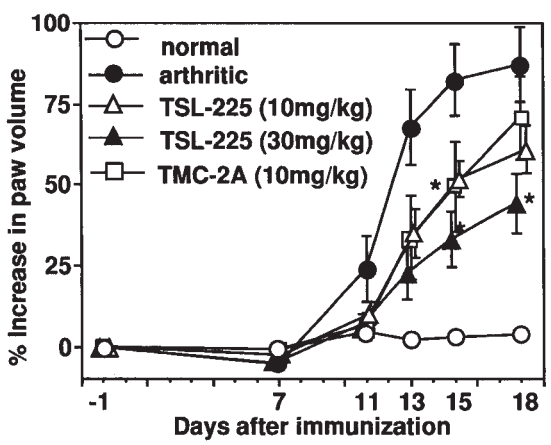

Fig. 5. Effect of TMC-2A and TSL-225 on adjuvant-induced arthritis in Lewis rats. Values represent mean ± SE of 4 rats; $^{*}p$ <0.05 compared with saline-treated arthritic rats (Bonferroni/Dunn-test). (Reproduced with permission from S. Tanaka et al. *Anti-arthritic effects of the novel dipeptidyl peptide IV inhibitors TMC-2A and TSL-225.* Immunopharmacology 1998, 40: 21-6. Copyright © Elsevier Science.)

activation and proliferation (33, 40). The competitive inhibitor, Lys(Z[NO_2])-thiazolidide suppressed mitogen- and antigen-induced proliferation of T cells without adversely affecting cell viability. However, the inhibitor also suppressed the proliferative response of T lymphocytes from DP IV deficient rats to a similar extent as that observed in normal rats. These data suggest that the suppression of T-cell activation by this inhibitor could not be due to the inhibition of DP IV activity and that DP IV might not be an essential molecule for T-cell activation in our system. However, there is the possibility that, in the DP IV deficient rats, a costimulatory role of DP IV might be compensated by other molecules.

The irreversible inhibitor, Ala-Pro-nitrobenzoylhydroxylamine, did not inhibit proliferation of normal or mutant splenic cells stimulated with concanavalin A, phytohemagglutinin or a purified protein derivative of tuberculin. The noncompetitive inhibitors, TMC-2A and TSL-225, did not inhibit the antigen-induced proliferation of T lymphocytes at 30 μM. These data suggest that the *in vivo* antiarthritic effects of the DP IV inhibitors may not be exerted through the suppression of T-cell activation.

Other possible targets of DP IV inhibitors include B cells, NK cells and/or monocytes, as they have been shown to suppress proliferation of these cells (20-22). DP IV is also considered an auxiliary adhesion factor (22, 41, 42). DP IV may act as a functional collagen receptor that assists in the homing of T cells to inflammatory sites. Indeed, the percentage of $CD3^+CD26^+$ cells in synovial fluid of patients with RA was found to be higher than in healthy control subjects (29). DP IV inhibitors may suppress the migration of activated T cells to inflamed tissues. DP IV was also suggested to be a component of an RA-specific plasminogen receptor complex on the surface of synovial fibroblasts (43). Plasminogen activation at the surface of synovial fibroblasts from RA patients by the urinary-type plasminogen activator induced a significant increase in cytosolic free Ca^{2+} concentration (43). Therefore, DP IV may have the potential to control the activation of synovial fibroblasts in RA.

Further studies are currently under way to clarify the antiarthritic actions of DP IV inhibitors.

Conclusions

We have discovered the novel DP IV inhibitors, TMC-2A, TMC-2B and TMC-2C, from the fermentation broth of *Aspergillus orizae* A374. TMC-2A inhibited DP IV in a noncompetitive manner (K_i = 5.3 μM). An approach using combinational synthesis of the derivatives resulted in the identification of TSL-225, which had a slightly more potent inhibitory activity than TMC-2A. *In vivo* evaluation revealed that TMC-2A and TSL-225 had antiarthritic effects, although their mechanism of action remains to be clarified. DP IV inhibitors with antiarthritic effects may be useful for evaluating the physiological and pathological roles of DP IV in the immune system. Discovery of the lead compounds warrants the development of therapeutically useful DP IV inhibitors for the treatment of immunological diseases.

Acknowledgements

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