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Structure-Based Drug Discovery

Structure-Based Approach To Improve a Small-Molecule Inhibitor by the Use of a Competitive Peptide Ligand**

Katsuki Ono, Koh Takeuchi, Hiroshi Ueda, Yasuhiro Morita, Ryuji Tanimura, Ichio Shimada, and Hideo Takahashi**

Abstract: *Structural information about the target–compound complex is invaluable in the early stage of drug discovery. In particular, it is important to know into which part of the initial compound additional interaction sites could be introduced to improve its affinity. Herein, we demonstrate that the affinity of a small-molecule inhibitor for its target protein could be successfully improved by the constructive introduction of the interaction mode of a competitive peptide. The strategy involved the discrimination of overlapping and non-overlapping peptide–compound pharmacophores by the use of a ligand-based NMR spectroscopic approach, INPHARMA. The obtained results enabled the design of a new compound with improved affinity for the platelet receptor glycoprotein VI (GPVI). The approach proposed herein efficiently combines the advantages of compounds and peptides for the development of higher-affinity druglike ligands.*

The design of high-affinity compounds from initially screened "hits" is essential in the early stage of drug discovery. At this stage, the original compounds are modified by chemical approaches to pick up additional interactions in the binding site. Structural information on the target–compound complex provided by NMR spectroscopy and/or X-ray crystallography is critical to guiding strategic improvement of the small-molecule inhibitor.[1]

NMR spectroscopy has been widely used for hit improvement, as key early developments in this field were made on the basis of the technique "SAR by NMR" (the use of NMR spectroscopy to determine structure–activity relationships) in the late 1990s.[2] NMR spectroscopic techniques hold a firm position, even after recent expansions in strategies based on crystal-structure determination,[3] especially when the crystallization of target–compound complexes is difficult. Protein-based NMR spectroscopic approaches, exemplified by "SAR by NMR", provide information on compound-binding sites in the target protein. This information enables the modeling of target–compound complex structures in combination with computational approaches. However, protein-based NMR spectroscopic approaches require ^{15}N and/or ^{13}C isotopic labeling, and more costly deuterium labeling should be considered when the molecular weight of the target protein is large (>30 K).[4] On the other hand, ligand-based NMR spectroscopic approaches, such as saturation transfer difference (STD) and WaterLOGSY experiments,[5] have no restrictions on the size of the target protein, and there is no requirement for isotopic labeling. However, it is often difficult to obtain the relative position and orientation of fragments with a ligand-based NMR spectroscopic approach. The only exception is the INPHARMA method (interligand NOEs for pharmacophore mapping), which allows the identification of a common pharmacophore between two competing compounds.[6] Although INPHARMA may infer common epitopes, the information from overlapping small-molecular ligands may often be insufficient to link or grow compounds to improve their affinity. Instead, information on additional non-overlapping interaction sites and/or the relative position of compounds that bind distinct sites in the target protein is more important for improving the affinity of a compound.

To overcome this weakness in the ligand-based NMR spectroscopic approach, we propose a new strategy, in which information on target protein–peptide interactions is used constructively. A protein–peptide interface is often larger than low-molecular-weight compounds and consists of multiple key interactions. Furthermore, peptide ligands that directly bind to a biologically relevant site in the target

[*] Dr. K. Ono, Dr. K. Takeuchi, Prof. I. Shimada, Prof. H. Takahashi
Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST)
2-3-26 Aomi, Koto-ku, Tokyo 135-0064 (Japan)
E-mail: hid@tsurumi.yokohama-cu.ac.jp
Dr. K. Ono
Japan Biological Informatics Consortium (JBIC)
2-3-26 Aomi, Koto-ku, Tokyo 135-0064 (Japan)
H. Ueda, Y. Morita, Dr. R. Tanimura
Toray Branch Office of JBIC
6-10-1 Tebiro, Kamakura-shi, Kanagawa 248-8555 (Japan)
Prof. I. Shimada
Graduate School of Pharmaceutical Sciences
The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 133-0033 (Japan)
E-mail: shimada@iw-nmr.f.u-tokyo.ac.jp
Prof. H. Takahashi
Graduate School of Medical Life Science, Yokohama City University
1-7-29 Suehirocho, Tsurumi-ku, Yokohama, Kanagawa 230-0045 (Japan)

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protein can be obtained relatively easily by phage display[7] or other approaches for in vitro evolution.[8] Although peptides have limited utility as therapeutic agents, their efficacy, selectivity, and specificity are often better than those of small fragments.[9] Thus, the introduction of information from target-protein–peptide interactions in the design of high-affinity ligands would be an effective way to compensate for the problem originating from the small interaction surface of compounds.

Herein, we demonstrate that the affinity of a small-molecule inhibitor for its target protein could be successfully improved by the generation of hybrid molecules in which non-overlapping moieties from a competitive peptide were introduced. In this strategy, overlapping and non-overlapping peptide–compound pharmacophores were discriminated by using INPHARMA. This novel approach combines the advantages of peptides and small molecules for the development of higher-affinity ligands by the ligand-based NMR spectroscopic approach.

Our approach was applied to the interaction between the platelet receptor glycoprotein VI (GPVI) and collagen. GPVI is primarily responsible for initial collagen-dependent platelet adhesion and aggregation; deficiency of GPVI causes a loss of collagen-stimulated platelet activation.[10] Interestingly, the absence of GPVI does not significantly prolong bleeding time, and a recent phase I clinical study demonstrated that GPVI–Fc dimers (Fc is the fragment crystallizable region of an antibody) inhibited collagen-induced platelet aggregation without affecting general hemostasis.[11] Therefore, unlike other antiplatelet agents, which are often associated with adverse bleeding events, an inhibitor of the GPVI–collagen interaction would be an interesting target for a safer anti-aggregation drug. In fact, it has been shown that the nonpeptide angiotensin II type 1 (AT1) receptor antagonist losartan and its metabolite also inhibit collagen-dependent platelet activation by GPVI at the clinical dose.[12]

Recently, we used an approach based on a phage-displayed peptide library to create a 12 amino acid peptide, pep-10L (H2N-YSDTDWLYFSTS-COOH), which inhibits the GPVI–collagen interaction.[13] The structure of pep-10L in the GPVI-bound state was determined by structural calculation by using transferred NOE (TrNOE) information.[13] The structure revealed that the central part of pep-10L (Asp5-Phe9) adopts a helical conformation (Figure 1 A), in which the side chains of Trp6, Leu7, and Phe9 form a hydrophobic cluster upon binding to GPVI.[13] By the use of STD experiments and site-directed mutagenesis, the hydrophobic cluster was identified as the key interaction site of pep-10L.[13] The chemical-shift perturbation (CSP) of GPVI resonances upon binding to pep-10L indicated that the peptide binds to the proposed collagen-binding site[14] with a K_D value of 5.7×10^{-5} M (see Figure S1 in the Supporting Information).

Interestingly, the site seems to overlap with the binding site of losartan, which we proposed recently on the basis of NMR spectroscopy.[15] By using NMR spectroscopic methods in combination with in silico tools, we found that losartan specifically interacts with extracellular immunoglobulin (Ig)-like domain 1 of GPVI with a K_D value of 1.7×10^{-4} M, and that the phenyltetrazole (PTZ) moiety in losartan is the key chemical structure for the interaction with GPVI (Figure 1 B).[15]

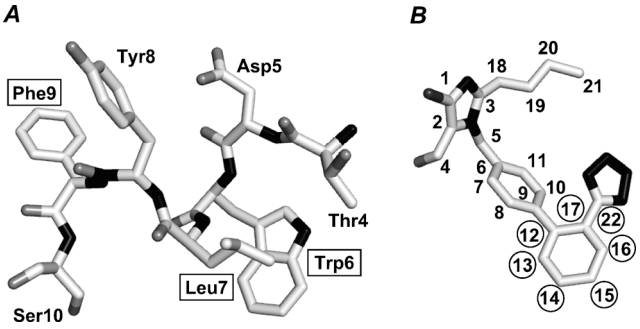


Figure 1. Structural representation of pep-10L peptide and losartan. A) The GPVI-bound structure of pep-10L, as determined by structural calculation by the use of transferred NOE (TrNOE) information.[13] Only residues 4–10, which represent the minimal binding sequence for binding to GPVI, are shown. Residue numbers and three-letter codes are indicated. The residues important for GPVI binding are indicated by black boxes. B) The GPVI-bound structure of losartan.[15] All carbon atoms in losartan are numbered. The carbon atoms in the phenyltetrazole (PTZ) moiety, which is important for GPVI binding, are circled.

To determine whether there was direct competition between losartan and pep-10L on the GPVI surface, we performed NMR spectroscopic competition experiments. We recorded ^1H–^{15}N correlated spectra of ^{15}N-labeled pep-10L alone (see Figure S2A), in the presence of unlabeled GPVI–Fc (see Figure S2B), and after adding losartan to the mixture (see Figure S2C). When the pep-10L/GPVI-Fc ratio was set to 1:0.25, the signal intensity of pep-10L significantly decreased in comparison to the free state (see Figure S2B). This decrease in intensity reflects accelerated relaxation owing to interaction with a larger molecule, GPVI–Fc. Upon the subsequent addition of losartan (25-fold concentration relative to pep-10L) to the mixture of pep-10L and GPVI–Fc, the intensity of the pep-10L resonances were restored to up to 80 % of their original intensity (see Figure S2C). These results indicated that pep-10L and losartan compete for the same binding site in the GPVI molecule.

With the competing low-molecular-weight compound losartan and the peptide pep-10L, we started to investigate which parts of the compound overlap with pep-10L by using the INPHARMA method (Figure 2). Two-dimensional ^1H–^1H NOESY experiments were performed with various mixing times (60, 100, 200, and 300 ms) with losartan (1.4 mM), pep-10L (0.9 mM), and GPVI–Fc (25 μM) in D_2O buffer. At a mixing time of 200 ms, interligand NOE peaks between the PTZ moiety in losartan and pep-10L were clearly observed in the NOESY spectrum (Figure 2 C). Interligand NOE peaks were not detected in the absence of GPVI–Fc or with the Fc portion alone, thus excluding the possibility of a direct interaction between losartan and pep-10L or of indirect magnetization transfer via the Fc fragment (data not shown). In view of the competition between pep-10L and losartan for the GPVI–Fc binding site, these NOE peaks could be identified as protein (GPVI)-mediated INPHARMA peaks.

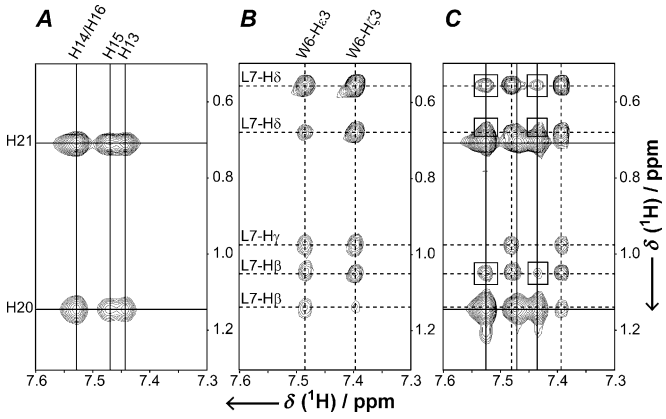


Figure 2. Expanded region of the ^{1}H–^{1}H NOESY spectra recorded for mixtures of A) losartan (1.4 mM) and GPVI–Fc (25 μM), B) pep-10L (0.9 mM) and 25 μM GPVI–Fc, and C) losartan (1.4 mM), pep-10L (0.9 mM), and GPVI-Fc (25 μM). The chemical shifts of losartan and pep-10L are indicated by orthogonally crossed solid and broken lines, respectively. Peaks in (A) and (B) indicate intramolecular TrNOE peaks, whereas signals in black boxes in (C) are the INPHARMA peaks mediated by the GPVI–Fc hydrogen atoms. NMR spectroscopic data were collected on an Avance 800 MHz spectrometer equipped with a cryogenically cooled probe head. The mixing time (τ_m) was set to 200 ms. All spectra were recorded in 20 mM sodium phosphate buffer (NaPi; pH 6.5) in D_2O. The procedures used for the expression and purification of GPVI–Fc were as previously described.[13]

Twenty-four INPHARMA peaks were observed in the experiment (Table 1). The strongest sets of INPHARMA peaks were observed between the H14/H16 hydrogen atoms in the PTZ moiety and the side-chain hydrogen atoms of Trp6

Table 1: Observed INPHARMA peaks between the hydrogen atoms of losartan and pep-10L.

Losartan[a]	Pep-10L
H7/H11 or H8/H10	T4 (Hα), W6 (Hβ), L7 (Hβ, Hδ), Y8 (Hβ), F9 (Hα, Hβ), S10 (Hβ)
H13	W6 (Hβ), L7 (Hβ, Hδ), S10 (Hβ)
H14/H16	T4 (Hα), D5 (Hα), W6 (Hβ, Hη2, Hζ2, Hζ3), L7 (Hβ, Hδ), S10 (Hβ)
H15	T4 (Hα), D5 (Hα), W6 (Hζ2)

[a] The numbering of losartan hydrogen atoms corresponds to the carbon-atom numbering in Figure 1 B.

(Hη2 and Hζ3) and Leu7 (Hβ and Hδ). These results indicate that the PTZ moiety in losartan is positioned at a similar site to that occupied by the side chains of Trp6 and Leu7 in pep-10L. The phenyl group (H7/H11 and H8/H10) in losartan showed INPHARMA peaks with Trp6 (Hβ), Leu7 (Hβ and Hδ), Tyr8 (Hβ), Phe9 (Hα and Hβ), and Ser10 (Hβ) of pep-10L at a mixing time of 200 ms. Thus, the phenyl group in losartan is located in the position corresponding to the center of the hydrophobic cluster formed by Trp6, Leu7, and Phe9 on the surface of GPVI. No INPHARMA peaks from the imidazole group and the alkyl chain of losartan were observed, even at a longer mixing time of 300 ms. The absence of such peaks may indicate that the imidazole group and alkyl chain do not stably bind to the GPVI surface, as suggested by molecular-dynamics simulations of the GPVI–losartan complex.[15] The AT1-receptor antagonists valsartan and olmesartan showed affinity comparable to that of losartan (see Figure S3); thus, the substitution of the imidazole group has only a marginal effect on the affinity of AT1-receptor antagonists. On the other hand, telmisartan, which does not have a PTZ moiety, did not bind to GPVI (see Figure S3). In line with this observation, 2-biphenylcarboxylic acid has fourfold lower affinity as compared to 5-biphenyl-2-yl-2*H*-tetrazole. Thus, the lack of a tetrazole ring in telmisartan at least partially explains its lack of affinity for GPVI. Interestingly, although the side chain of Phe9 is important for the interaction of GPVI and pep-10L, there were no INPHARMA peaks between the aromatic ring of Phe9 in pep-10L and losartan. Thus, pep-10L has an additional interaction site (the phenyl portion of Phe9) that does not overlap with losartan, and the compound-binding affinity of GPVI might be improved by introducing a similar interaction to that of Phe9 of pep-10L.

As discussed above, the phenyl ring in the PTZ moiety can be positioned between the side chains of Trp6 and Leu7 (Figure 3). The orientation of the PTZ moiety was estimated from INPHARMA peaks between H7/H8 in losartan and

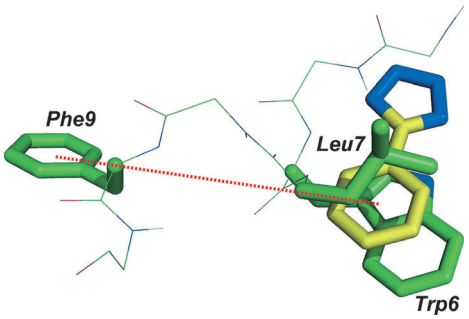
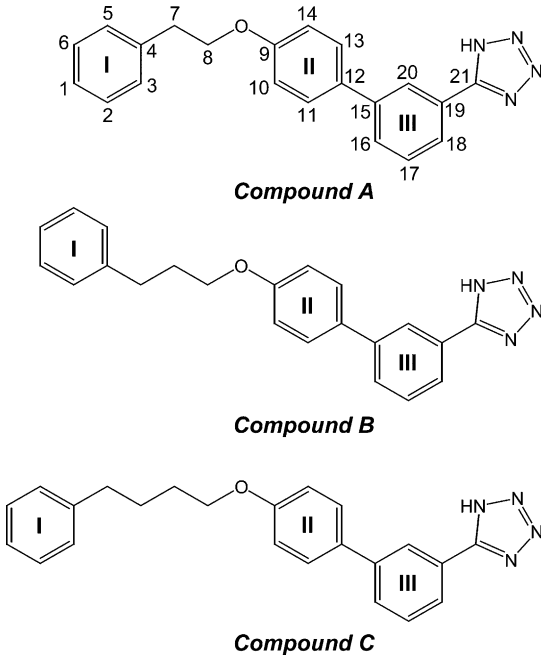


Figure 3. Superimposition of pep-10L (green) and the PTZ moiety in losartan (yellow) on the basis of INPHARMA information. The main chain of pep-10L (Thr4-Ser10) is rendered as a line model, whereas the side chains of key residues in the GPVI interaction, Trp6, Leu7, and Phe9, are shown as a stick model. Carbon atoms in pep-10L, the carbon atoms of the PTZ moiety, nitrogen atoms, and oxygen atoms are colored green, yellow, blue, and red, respectively. The red broken line indicates the linker region between two fragments.

residues forming a hydrophobic cluster in pep-10L as well as the other INPHARMA peaks from H13 and H15 of the PTZ ring (Table 1). The estimated distance between the PTZ moiety in losartan and the phenyl ring of Phe9 in pep-10L was expected to be approximately 9 Å, with Phe9 oriented in the direction corresponding to the *meta* position of the phenyl ring of the PTZ moiety.

On the basis of this observation, we then constructed a new compound that linked the important binding fragment in losartan, the PTZ ring, and the phenyl ring of pep-10L (Figure 3). First, we reconsidered the core structure to be used in the hybrid compounds. Whereas 5-biphenyl-2-yl-2*H*-tetrazole retained an affinity ($K_D = 3.6 \pm 0.5 \times 10^{-4}$ M) comparable to that of losartan, the methylated analogue of PTZ, 5-(2-methylphenyl)-2*H*-tetrazole, showed only limited affinity

toward GPVI ($K_D = 1.1 \pm 0.1$ mM). Thus, biphenyl tetrazole was selected as the mother scaffold. We also tried attaching the second phenyl moiety to the *meta* position of the phenyl ring of PTZ instead of the *ortho* position, since the peptide pharmacophore was positioned in the direction corresponding to the *meta* position. Interestingly, the resulting 5-biphenyl-3-yl-2*H*-tetrazole showed stronger affinity ($K_D = 8.3 \pm 0.4 \times 10^{-5}$ M) than that of 5-biphenyl-2-yl-2*H*-tetrazole (see Figure S3). Thus, we used 5-biphenyl-3-yl-2*H*-tetrazole as the core fragment for affinity improvement. Then, considering the spatial accuracy of NOE information and the flexibility of the Phe9 side chain in the pep-10L structure, we designed a linker to connect the phenyl ring corresponding to Phe9 in the peptide in such a way as to allow a certain ambiguity in length. Furthermore, the following desired characteristics were taken into account: 1) a simple chemical structure; 2) rigidity to maintain the spatial arrangement; and 3) ease of chemical synthesis. Compounds A–C with different linker lengths were synthesized (Scheme 1; see the Supporting Information for details), whereby the linker length of compound B is most consistent with our estimate.



Scheme 1. Chemical structure of the synthesized compounds. The carbon atoms of aliphatic and aromatic groups are labeled with numbers for compound A.

We performed CSP experiments to investigate the binding affinity of compounds A, B, and C for GPVI. Two-dimensional ^{1}H–^{15}N TROSY-HSQC spectra of the Ig-like domains 1 and 2 in GPVI were recorded with increasing concentrations of each compound. The maximum concentration was set to 0.8 mM. Except for compound B, which did not show chemical-shift perturbation, each cross-peak in the TROSY-HSQC spectra of the GPVI/compound mixtures appeared as a single average resonance that reflected the free/bound ratio and thus the fast exchange between the free and bound states on the chemical-shift time scale. From the concentration dependence of the chemical-shift values, the K_D value of compound A for GPVI was estimated to be $5.2 \pm 0.4 \times 10^{-5}$ M, which is comparable to the binding affinity of pep-10L ($K_D = 5.7 \times 10^{-5}$ M) and stronger than that of both losartan and the core fragment, 5-biphenyl-3-yl-2*H*-tetrazole. The K_D value of compound C was much weaker at $8.5 \pm 0.5 \times 10^{-4}$ M.

To determine the binding mode of the best compound, compound A, the INPHARMA experiments were performed with a mixture of compound A (0.5 mM), pep-10L (0.9 mM), and GPVI–Fc (25 μM) in D_2O at 288 K. The mixing time was set to 60 and 300 ms. A total of 25 INPHARMA peaks were observed in the experiment (Table 2). As expected, the PTZ moiety in compound A provided INPHARMA peaks with the side chain of Trp6 and Leu7, which indicates that the binding mode found for the PTZ moiety of losartan and GPVI is preserved in compound A (see Figure S4). Although the INPHARMA peaks between phenyl ring I in compound A and the phenyl ring of Phe9 in pep-10L cannot be discriminated from the strong intraligand NOE peaks owing to signal overlap, the INPHARMA peak between phenyl ring I in compound A and the Phe9 Hα atom was clearly observed (see Figure S3). This result suggests that phenyl ring I in compound A is positioned near the side chain of Phe9 in pep-10L.

Table 2: Observed INPHARMA peaks between the hydrogen atoms of compound A and pep-10L.

Compound A[a]	Pep-10L
H2/H6 or H3/H5	W6 (Hα), L7 (Hα, Hδ), Y8 (Hα, Hβ), F9 (Hα), S10 (Hα, Hβ)
H10/H14	W6 (Hα, Hζ3), L7 (Hβ, Hδ), S10 (Hα, Hβ)
H11/H13 or H16	L7 (Hδ), S10 (Hα, Hβ)
H17	S8 (Hα)
H18	L7 (Hδ), S10 (Hα)
H20	T4 (Hγ2), W6 (Hε3), L7 (Hδ), S10 (Hα, Hβ)

[a] The numbering of the hydrogen atoms in compound A corresponds to the carbon-atom numbering in Scheme 1.

A ligand-based NMR spectroscopic approach has the advantage of nonrestricted molecular size and no requirement for isotopic labeling of target proteins. However, determination of the relative position of distinct compounds is often difficult by such an approach. Herein, we have proposed a novel ligand-based strategy for improving the affinity of small-molecule compounds in which information gained from a competitive peptide ligand is used to overcome this problem. The strategy takes advantage of the larger interaction surface of a peptide ligand and enables the introduction of additional interaction sites that do not overlap with a small compound. The INPHARMA information enables the discrimination of overlapping sites from non-overlapping sites and would most effectively be used to design a new compound by a linking or growing approach, as shown herein. In theory, this approach would be most appropriate for systems with K_D values higher than 10 μM, and the relative fragment/peptide K_D value should be between 0.1 and 10.[6b] It

would be applicable to a system with a K_D value of 1 μM; however, the sensitivity would be lower. A lower-affinity fragment would be less problematic; thus, the strategy may be particularly be useful for the improvement of low-molecular-weight hit compounds from fragment-based screening into medium-sized compounds with higher activity. By this strategy it is also possible to define the active moiety in compounds to replace nonfunctional groups with new functional groups that may be introduced in a different part of the original compound. The strategy preserves the advantages of a ligand-based drug-discovery strategy by NMR spectroscopy while overcoming the lack of information for the improvement of the affinity of compounds.

In most cases, immature compounds in the drug-discovery stage have affinities for nontarget proteins. This off-target effect often causes side effects and is one of the major reasons that bioactive compounds fail to pass clinical trials. Such failures could be avoided by identifying the fragment necessary for target binding and excluding unnecessary moieties as much as possible during compound improvement, thus improving specificity. The method proposed herein could be used to efficiently develop such compounds by evaluating the feasibility of linking or growing strategies in a structure-guided manner.

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