Review Article

Screening Techniques Using the Periplasmic Expression of Peptide Libraries and Target Molecules

Tadashi Kimura[1,2*]

[1]Cellular Glycome-targeted Technology Research Group, Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), AIST Tsukuba Central, Umezono, Tsukuba, Ibaraki, Japan

[2]United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Yanagido, Gifu, Gifu Prefecture, Japan

Abstract

Middle molecular weight pharmaceuticals and peptide drugs are now becoming attractive strategy for developing new medicines. Several peptide display techniques, such as phage display and mRNA display, are used for drug discovery. Membrane proteins, such as G-protein coupled receptors, ion channels and transporters are important pharmaceutical targets. Animal peptide toxins contain many type of naturally occurring ligands which effect on several types of membrane proteins. Animal peptide toxins are suitable for a template of peptide display technique against membrane proteins. Along with several peptide display techniques, cost-effective peptide display technique against membrane protein has been developed, named intra periplasm secretion and selection (PERISS). In the PERISS technique, *Escherichia coli* periplasmic space is used for peptide display, and target membrane proteins are actively expressed inner membrane of *Escherichia coli*. In this review, several peptide templates for peptide display and application of PERISS technique are discussed.

Keywords: Peptide display; ICK peptide; Membrane protein; PERISS; Spheroplast; Electrophysiology

Introduction

As the population continues to age in developed country, the increasing number of patients and treatment costs are of great concern in the treatment of many different illnesses. In order to sustain healthy lifestyles and combat these illnesses, a current major focus in pharmaceutical research is the development of innovative new drugs that demonstrate increased therapeutic efficacy.

Pharmaceutical antibodies have started replacing low-molecular weight pharmaceuticals, and now make up a large portion of the pharmaceutical market. Recently, middle molecular weight pharmaceuticals and peptide drugs are having attracted a lot of interest. Low-molecular weight pharmaceuticals are able to permeate cell membranes; hence, they can target intracellular markers. However, they also have low target specificity, making side-effects a concern. Pharmaceutical antibodies are highly specific to their targets, but it is currently difficult to design pharmaceutical antibodies that are able to permeate cell membranes. In contrast to low-molecular weight pharmaceuticals, which can be chemically synthesized, making production inexpensive, antibody drugs are produced in CHO cell cultures, making them more expensive. Peptide drugs have high target specificity, and drug delivery technologies allow them to permeate cell membranes. However, peptides are easily degraded by proteinases; therefore, pharmaceuticals need to be made degradation-resistant by chemical modification or other means. In spite of some difficulties, middle molecular weight pharmaceuticals and peptide drugs are world's hope for the future. In this paper, several peptide display and related techniques are reviewed.

Peptide Display Techniques

Peptide display techniques fall roughly into three categories: cell-free, cell surface, or *E. coli* periplasm (Table 1) [1-9].

Cell-free synthesis peptide display techniques

Cell-free synthesis peptide display techniques include the ribosome and mRNA display [10], cDNA display [11], and bead display [12]. Cell-free peptide display technologies by definition do not use living cells; hence, molecules that exhibit cytotoxicity can be displayed, which holds promise for increasing library size.

Cell surface peptide display techniques

Cell surface peptide display techniques include phage display [13], *Lactobacillus* display [14], yeast display [15], *E. coli* display [16], and retrovirus display [17]. These techniques use living cells, and display peptides on the surface of the phages/cells.

E. coli periplasm peptide display techniques

Two *E. coli* periplasm peptide display techniques are the intra periplasm secretion and selection (PERISS) technique, explained in this article, and the anchored periplasmic expression (APEx) technique [9,18]. In the APEx technique, a peptide library is expressed in the periplasm and immobilized by *E. coli* inner membrane proteins. The *E. coli* outer membrane is then removed, and fluorescently labeled target molecules are added. *E. coli* cells with peptides bound to target molecules become fluorescent; hence, they can be detected via fluorescence-activated cell sorting. In this way, peptides that bind to the target molecule can be identified.

This method is different from the PERISS technique, which is described in detail below. One benefit of *E. coli* periplasm peptide display systems is that the periplasmic space has optimal conditions for

the formation of disulfide bridges; thus, peptides with multiple disulfide bonds can be expressed in their active state. For example, the camel antibody VHH, a so-called nanobody, can be expressed [19]. Using this technique, peptides from spiders and scorpions having 2 to 6 disulfide bonds within 17 to 76 amino acid residues showed a 60% success rate in the expression of the single correct isoform [20]. Chemical synthesis of peptides that contain three or more disulfide bonds is difficult; thus, a 60% success rate is considerable. Peptides obtained using *E. coli* periplasm peptide display techniques can be produced using the same periplasmic expression system. Usually, *E. coli* cannot perform secretory expression, but this has been achieved using a surfactant in the culture medium [JPN patent 5808529]. This technology holds potential for the industrialization of peptides with multiple disulfide bonds by periplasmic production.

Suitable Peptide Templates for Peptide Display

Many different peptides are used as templates for peptide display. For example, Fujii designs molecules with helix-loop-helix structures in a peptide library using artificial peptides as templates, and builds a peptide library by adding mutations to amino acids on the outer side, which do not affect the protein's three-dimensional structure [21]. This technology, which screens target molecules against a microantibody library presented by phage display, has been commercialized by Interprotein Inc. Meanwhile, Suga has developed a technique using a peptide library that includes special amino acids. In the RAPID system, which is based on the flexizyme system, a ring-shaped peptide library consisting of peptides approximately 15 amino acids long prepared with special amino acids has been created *in vitro* [22]. This technology has been commercialized by Peptidream Inc. Other peptide libraries are explained in detail by Kubo [23].

Another library different from the two peptide libraries stated above can be used—one that uses peptides found in toxins from various poisonous animals as templates. Animal toxins are promising pharmaceutical candidates because of their high target specificity. As an example, ziconotide, an inhibitor cysteine knot (ICK) peptide found in cone snails, is sold as a peptide drug that decreases pain experienced by patients with late-stage cancer. A peptide included in the three-finger scaffold of snake venom was used as a peptide library template because of its high specificity. An attempt was also made to create an anti-IL-6 peptide [11]. Over 30 new peptide candidates, including ICK peptides (GTx1,2), a peptide (GTx3) that is homologous with MIT1 from snake venom and Bv8 (a peptide discovered in frog skin), peptides homologous with peptides of unknown physiological functions (GTx4,5,6), a novel peptide (GTx7), and peptides homologous with translationally controlled tumor protein, cysteine-rich secretory protein, and venom allergen (GTx-TCTP, GTx-CRISP, GTx-VA) are discovered [24,25]. A common trait among these peptides is that they all have multiple cysteines in their peptide sequence, and form disulfide bonds. It has shown that the peptide GTx1-15 is voltage-dependent T-type calcium channel inhibitor [26]. GTx7-1 has an inhibitory effect on guinea pig isolated right atrial preparation [JPN patent 5019442]. Using these animal toxin peptides as templates can increase the likelihood of creating new peptide drugs. Companies such as Israel's Alamone Labs, France's Smartox Biotechnology, and Britain's Venomtech are all engaged in the research and development of pharmaceuticals based on natural toxins. The research and development of pharmaceuticals using animal toxin peptides is summarized well in the book Toxins and Drug Discovery [27].

The target molecules of ICK peptides, which possess three intra-peptide disulfide bonds, are mainly membrane proteins (such as ion channels); they are generally considered to be suitable as templates for the development of peptide drugs [28,29].

GTx1-15, which used as an ICK peptide template, is stable in animal blood for multiple hours after intravenous administration without degradation by proteinases [30]. GTx1-15 is expected to have low immunogenicity like ziconotide which shows low immunogenic potential at animal models [31]. Optimization of the activity of ICK peptides is being conducted by the American companies Pfizer Inc. [32] and Amgen Inc. [33]. It is believed that ICK peptides can be valuable template peptides.

Target Molecules for Screening Periplasmic Expression

In this paper, membrane proteins used to refer to proteins that anchor in the cell membrane, and proteins in the cytoplasm are referred to as soluble proteins.

Membrane proteins

G-protein coupled receptors (GPCRs), ion channels, and transport proteins in the cell membrane are important pharmaceutical targets. The development of pharmaceuticals that act on GPCRs and similar proteins is ongoing [34], but that is not to say that pharmaceuticals that act on ion channels are necessarily being developed, especially for their ion channel characteristics. In order to shed light on this, PERISS method was performed, specifically targeting ion channels.

In general, it is thought that the expression of membrane proteins on *E. coli* inner membranes is difficult. Since only a small quantity of membrane protein can be expressed on *E. coli* inner membranes, techniques such as Western blot cannot detect the proteins. Thus, techniques for the direct measurement of the activity of the expressed membrane proteins on the *E. coli* inner membrane have been developed to allow the observation of the expression of small protein quantities that could not otherwise be detected [35]. When the *E. coli* cells are proliferating, inhibition of cell division by antibiotics results in long, rod-shaped *E. coli*, called "snake". By removing the outer membrane of these snake bacteria through techniques such as enzymatic treatment, giant spheroplasts with diameters of over 5 µm can be obtained. Through the application of patch-clamp electrophysiological techniques to these giant spheroplasts, the activity of ion channels expressed in the *E. coli* inner membrane can be directly measured. Successful measurement of expression using the patch clamp technique serves to confirm expression of the target ion channel on *E. coli* inner membranes. In addition to ion channels, some GPCRs are expressed on *E. coli* inner membranes. As detailed below, human type 2 muscarinic acetylcholine receptor has been used for the PERISS technique.

If there is a high level of membrane protein expression, there will be many target molecules in the *E. coli* inner membrane; thus, screening with the PERISS technique will definitely be favorable. For increasing membrane protein expression on *E. coli* inner membranes, a chimaera membrane protein, which is fusion protein of membrane protein and maltose binding protein which is known as expression enhancer [36], or other expression enhancing protein, is synthesized. The chimaera membrane protein can achieve considerably higher expression of the target protein.

Soluble proteins

If the target molecule is a soluble protein, it will diffuse into the *E. coli* periplasmic space if it is expressed in the periplasm. However, the

target molecule needs to be immobilized in the *E. coli* inner membrane for the PERISS screening technique. By immobilizing a soluble protein on the *E. coli* inner membrane as a membrane protein, it can then be treated as a target molecule. There are methods for immobilizing soluble proteins on the *E. coli* inner membrane that are similar to the APEx techniques described in Section E. coli periplasm peptide display techniques; however, instead of using these methods, a method is developing, wherein the soluble protein is fused onto the side of a membrane protein facing the periplasmic space. With this technique, either membrane proteins or soluble proteins can be used as target molecules for the PERISS technique.

Intra Periplasm Secretion and Selection (PERISS) Technique

Basic principles of the PERISS technique

E. coli has both an outer membrane and an inner membrane, and the space between them is called the periplasm. In the PERISS technique, the target molecule DNA and peptide library DNA are integrated in tandem into a plasmid (Figure 1(1)). This allows both the target molecule and the peptide library to be expressed on a plasmid. The plasmid is transfected into *E. coli*, which expresses the peptide library in the periplasmic space, and expresses the target molecule on the *E. coli* inner membrane (Figure 1(2)). If the expressed target molecule is a membrane protein, it will be expressed on the *E. coli* inner membrane. In the case of a soluble protein, it is expressed as a fusion protein of a membrane protein and the soluble protein, so that the soluble protein is secured in the inner membrane, as explained in Section Soluble proteins. *E. coli* exhibits some incompatibility with plasmids; one *E. coli* cell can only retain one type of plasmid; thus, peptide expression in the periplasmic space is limited to one peptide from the library. Peptides expressed in the periplasmic space will bind to the target molecule if they have a strong binding affinity for it; if not, the target molecule will not bind to the peptide and will instead diffuse throughout the periplasmic space. The *E. coli* outer membrane is then removed enzymatically to form spheroplasts (Figure 1(3)). The peptide is expressed as a fusion protein with a tag sequence; hence, when the peptides that contain the tag sequence are concentrated using magnetic

Peptide display configuration	Display method	Pros	Cons	References
Cell-free peptide display	Ribosome & mRNA display	Large library size, Use of non-natural amino acids, speedy	Degradation of mRNA	[1]
	cDNA display	Large library size, Stability of cDNA	Degradation of mRNA	[2]
	Bead display	Large library size	Degradation of mRNA	[3]
Cell surface peptide display	Phage display	Many applications	Small library size	[4]
	Lactobacillus display	Established safety of *Lactobacillus*	Small library size	[5]
	Yeast display	Many applications	Small library size	[6]
	E. coli display	Many applications, Low cost, easy to use	Small library size	[7]
	Retrovirus display	Clinical use	Small library size	[8]
E. coli periplasmic peptide display	Anchored periplasmic expression (APEx)	Low cost, easy to use	Small library size	[9]
	Intra periplasm secretion and selection (PERISS)	High through put screening against membrane proteins, Use of disulfide rich peptides	Small library size	This review

Table 1: Comparison of peptide display techniques.
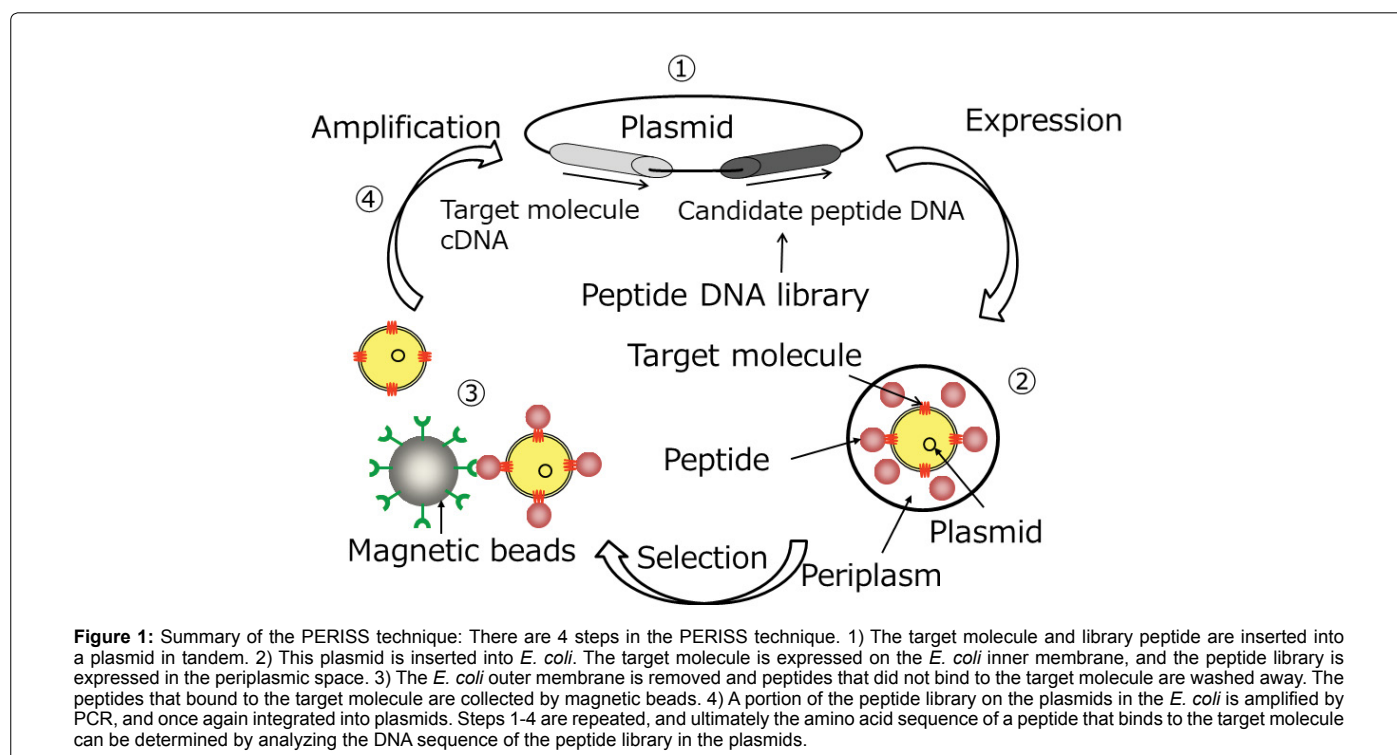
Figure 1: Summary of the PERISS technique: There are 4 steps in the PERISS technique. 1) The target molecule and library peptide are inserted into a plasmid in tandem. 2) This plasmid is inserted into *E. coli*. The target molecule is expressed on the *E. coli* inner membrane, and the peptide library is expressed in the periplasmic space. 3) The *E. coli* outer membrane is removed and peptides that did not bind to the target molecule are washed away. The peptides that bound to the target molecule are collected by magnetic beads. 4) A portion of the peptide library on the plasmids in the *E. coli* is amplified by PCR, and once again integrated into plasmids. Steps 1-4 are repeated, and ultimately the amino acid sequence of a peptide that binds to the target molecule can be determined by analyzing the DNA sequence of the peptide library in the plasmids.

beads, the target molecule is collected along with the *E. coli*. A part of the peptide library from the plasmid in *E. coli* is amplified by PCR, then integrated into the plasmid once again (Figure 1(4)); this process is repeated several times. Finally, I can analyze the DNA sequence from that part of the peptide library in the plasmid in *E. coli*, so that the amino acid sequence of peptides that bind to the target molecule can be determined. It is possible to monitor changes in the contents of the peptide library using next generation sequencing.

The process of spheroplast formation should be undertaken with particular care. Papers on the APEx technique make only brief mention of spheroplast formation, but in order to maintain favorable conditions for *E. coli* during spheroplast formation, one needs specialized knowledge regarding optical conditions for cell wall-degrading enzymes, such as buffer composition, temperature, reaction time, etc. If the reaction deviates from ideal conditions, it often results in the formation of what is called a "ghost," where although it may appear that spheroplasts are formed, and the cytoplasm leaks out through a hole in the inner membrane. As discussed below, in the PERISS technique, plasmids in the *E. coli* cytoplasm are assumed to reproduce the information contained in DNA from a peptide library; ghost spheroplast formation reduces the effectiveness of the technique, and hence must be avoided.
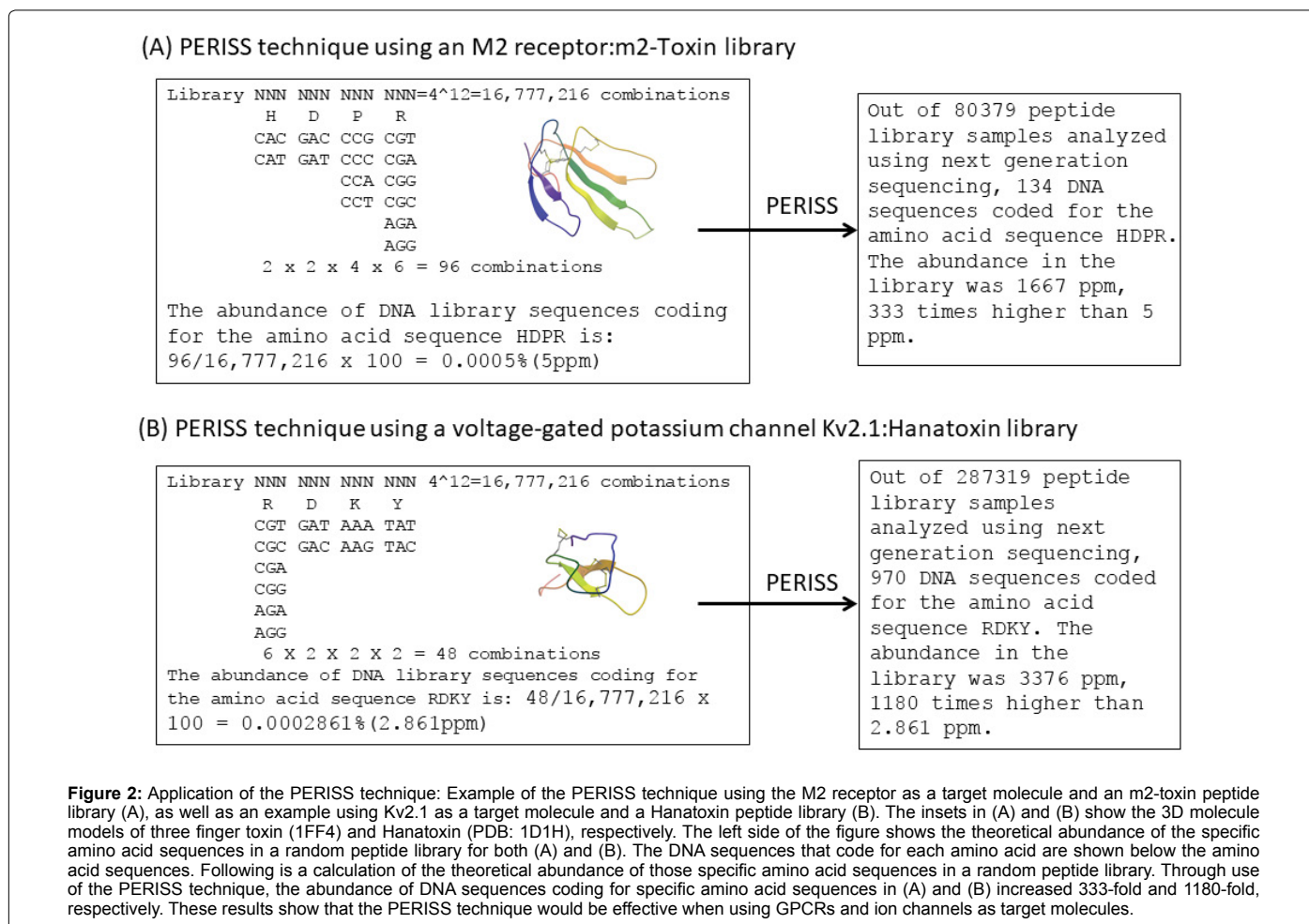
Application of the PERISS technique

PERISS was conducted using a peptide library with tarantula venom

GTx1-15 as a template, and human type 2 muscarinic acetylcholine receptor (M2 receptor) as a target molecule. A new peptide with a high specificity for the M2 receptor was discovered [37, JPN patents 5717143 and 5787298]. The PERISS technique was also performed in the same manner using M2 receptor as the target molecule and the M2 receptor specific snake venom M2 toxin instead of spider venom. M2 toxin is a three-finger scaffold venom peptide. Four amino acid residues in the middle finger structure, which were thought to play an important role in binding to the M2 receptor, were converted into a library, and the PERISS technique was conducted. As a result, the DNA sequence coding the original amino acid sequence was concentrated 333-fold in the peptide library (Figure 2A) (manuscript in preparation). The PERISS technique was also conducted with the tarantula venom, hanatoxin, using human potassium channel Kv2.1 as the target molecule. Hanatoxin binds to the voltage sensor domain on Kv2.1, which inhibits Kv2.1 activity. The four amino acid residues important in binding to the Kv2.1 sensor of membrane potential in hanatoxin were converted into a library, and the PERISS technique was conducted. As a result, the DNA sequence coding the original amino acid sequence was concentrated 1180-fold in the peptide library (Figure 2B).

Pros and cons of the PERISS technique

In peptide display techniques other than PERISS, the peptide library and the target molecule must be prepared separately. When creating a peptide library for cell-free peptide display techniques, a separately prepared target molecule is added to a library and allowed

(A) PERISS technique using an M2 receptor:m2-Toxin library

```
Library NNN NNN NNN NNN=4^12=16,777,216 combinations
        H    D    P    R
       CAC  GAC  CCG  CGT
       CAT  GAT  CCC  CGA
                 CCA  CGG
                 CCT  CGC
                      AGA
                      AGG
       2 x 2 x 4 x 6 = 96 combinations

The abundance of DNA library sequences coding
for the amino acid sequence HDPR is:
96/16,777,216 x 100 = 0.0005%(5ppm)
```

```
Out of 80379 peptide
library samples analyzed
using next generation
sequencing, 134 DNA
sequences coded for the
amino acid sequence HDPR.
The abundance in the
library was 1667 ppm,
333 times higher than 5
ppm.
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(B) PERISS technique using a voltage-gated potassium channel Kv2.1:Hanatoxin library

```
Library NNN NNN NNN NNN 4^12=16,777,216 combinations
        R    D    K    Y
       CGT  GAT  AAA  TAT
       CGC  GAC  AAG  TAC
       CGA
       CGG
       AGA
       AGG
        6 X 2 X 2 X 2 = 48 combinations
The abundance of DNA library sequences coding for
the amino acid sequence RDKY is: 48/16,777,216 x
100 = 0.0002861%(2.861ppm)
```

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Out of 287319 peptide
library samples
analyzed using next
generation sequencing,
970 DNA sequences coded
for the amino acid
sequence RDKY. The
abundance in the
library was 3376 ppm,
1180 times higher than
2.861 ppm.
```

Figure 2: Application of the PERISS technique: Example of the PERISS technique using the M2 receptor as a target molecule and an m2-toxin peptide library (A), as well as an example using Kv2.1 as a target molecule and a Hanatoxin peptide library (B). The insets in (A) and (B) show the 3D molecule models of three finger toxin (1FF4) and Hanatoxin (PDB: 1D1H), respectively. The left side of the figure shows the theoretical abundance of the specific amino acid sequences in a random peptide library for both (A) and (B). The DNA sequences that code for each amino acid are shown below the amino acid sequences. Following is a calculation of the theoretical abundance of those specific amino acid sequences in a random peptide library. Through use of the PERISS technique, the abundance of DNA sequences coding for specific amino acid sequences in (A) and (B) increased 333-fold and 1180-fold, respectively. These results show that the PERISS technique would be effective when using GPCRs and ion channels as target molecules.

to react. These techniques, therefore, have the advantage of being immediately available for use in the screening of low-molecular weight compounds. If the target molecule is a membrane protein, there is a benefit to screening cells expressing the membrane protein in culture, but the beneficial three-dimensional structure cannot be maintained when extracting the membrane protein from the cell membrane of the cultured cells, and many membrane proteins therefore lose their activity in the process. The surfactants needed to successfully extract a membrane protein from the cell membrane while preserving its activity differ based on the specific membrane protein; setting the conditions for this requires specialized knowledge. New technologies such as nanodiscs can be used, but if the target molecule is a membrane protein *in vitro*, these methods pose various anticipated difficulties. In the PERISS technique, membrane proteins are expressed on the *E. coli* inner membrane for screening; hence, there is no need to extract the membrane protein. Proteins such as ion channels that are expressed on the *E. coli* inner membrane can be directly observed electrophysiologically. There are no major differences compared to the previously reported basic characteristics of the expressed ion channels, but since the lipid composition of the inner membrane of *E. coli*, which is a prokaryote, differs from that of mammalian cells, the activities of peptides determined by the PERISS technique must then be re-evaluated in a eukaryotic system, such as by the patch-clamp technique using culture cells, and by *Xenopus* oocyte expression system using two electrode voltage clamp method. When creating a peptide library in *E. coli* by the PERISS technique, the library size is smaller than that of a cell-free system. By creating a library using an ICK peptide, which acts on ion channels, it is expect that there is a higher chance of including peptides that act on ion channels than in a random peptide library with no designated target molecule. This may allow the technique to overcome the disadvantage of a small library size. Furthermore, the small library size is made up for by creating multiple libraries, which can be done relatively inexpensively since the libraries are created using *E. coli*.

The PERISS technique has advantages and disadvantages, but it can complement other peptide display techniques and contribute to the field of peptide drug development.

Future Steps

The intra periplasm secretion and selection (PERISS) technique, a periplasmic peptide display technique in *E. coli*, can use various membrane proteins and soluble proteins as target molecules. Leveraging the characteristics of the periplasmic space and using animal toxin peptides with multiple disulfide bonds as peptide library templates, both increase the probability of peptides binding to the target molecule. Furthermore, the activity of the expressed membrane proteins in *E. coli* using PERISS can be measured electrophysiologically. Based on these three techniques and insights, membrane protein effective middle molecular weight pharmaceuticals and peptide drug research and development will go on.

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