รายงานวิจัยฉบับสมบูรณ์

โครงการ
การออกแบบและสร้างสารประเภท Peptidomimetics เพื่อพัฒนาเป็นยารักษาโรคที่เกี่ยวข้องกับ Formyl Peptide Receptor

โดย

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สิ้นสุดโครงการ 30 ตุลาคม พ.ศ. 2555

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Abstract:

G-protein coupled receptors (GPCRs) control and regulate a variety of cellular and physiological processes as well as implicated in numerous diseases, thereby major targets of therapeutic agents. While the GPCR therapeutic developments have focused heavily on non-peptide-activated GPCRs, the effective therapeutics targeting peptide-activated GPCRs, which associated in many diseases, are longed for. The study was proposed to design and to develop non-natural peptide mimics or peptidomimetics that effectively binds to one of the peptide-activated GPCRs, named *N*-formyl peptide receptor (FPR). We focused on β-peptide as this class of peptidomimetic by mimicking the primary structure of the natural ligand, *N*-formyl-methionine-leucine-phenylalanine (fMLF). We synthesized β-peptide based peptidomimetics and investigated their ability to activate FPR through FPR-mediated transient elevation in the intracellular calcium ion concentration. In addition, the compounds were also tested for their ability to elicit FPR responses such as cell migration. We discovered the peptidomimetic FPR agonist and FPR antagonist. These peptidomimetics have potential to be drug leads, as they should resist to the degradation by cellular proteases and peptidases, thereby increase the bioavailability over the natural peptides, though the further experiments shall be conducted to confirm.

Keywords: G-protein coupled receptors (GPCRs), peptidomimetics, β-peptide

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บทคัดย่อ:

G-protein coupled receptors (GPCRs) โปรตีนทำหน้าที่ควบคุมกลไกการทำงานทางชีวภาพและกลไกการทำงานของร่างกายในหลายๆ ด้าน ดังนั้น โรคที่เกิดขึ้นหลายๆชนิด จึงเป็นผลมาจากการทำงานที่ผิดปกติของโปรตีนชนิดนี้ ด้วยเหตุนี้เอง G-protein coupled receptors (GPCRs) จึงนับว่าเป็นเป้าหมายหลักของอุตสาหกรรมยาในปัจจุบันที่จะพัฒนาหาสารที่สามารถควบคุมการทำงานของโปรตีนชนิดนี้ให้ได้ ซึ่งการพัฒนายาที่เกี่ยวข้องกับ GPCRs โดยมากจะมุ่งไปที่ GPCRs ที่กระตุ้นโดยสารประกอบที่ไม่ใช่สารกลุ่มเปปไตด์ ส่วนกลุ่มโปรตีน GPCRs ที่ถูกกระตุ้นโดยสารกลุ่มเปปไตด์นั้นนับว่าเป็นกลุ่มใหญ่และมีส่วนสำคัญในการเกิดโรคต่างๆ อันหลากหลาย ดังนั้นสารที่มีความสามารถในการควบคุมโปรตีนในกลุ่มนี้จึงเป็นที่ต้องการยิ่ง ในงานวิจัยที่เสนอนี้มีจุดมุ่งหมายในการออกแบบและสังเคราะห์สารประเภท Peptidomimetics ซึ่งจะมีลักษณะทางโครงสร้างที่คล้ายคลึงกับสารประกอบเปปไตด์ที่มีความสามารถเกาะจับและกระตุ้นการทำงานของ โปรตีน GPCRs ได้อยู่แล้วในธรรมชาติ โปรตีนในกลุ่ม GPCRs ที่เลือกนำมาศึกษาวิจัยในโครงการนี้คือ *N*-formyl peptide receptor (FPR) โดยได้เริ่มศึกษาจากการออกแบบสารประกอบ **β**-peptide ที่มีลักษณะทางโครงสร้างคล้ายคลึงกับ the primary structure ของเปปไตด์ธรรมชาติที่สามารถจับและกระตุ้นการทำงานของ FPR ได้เป็นอย่างดียิ่ง ซึ่งโครงสร้างของ the primary structure คือ *N*-formyl-methionine-leucine-phenylalanine (fMLF). สารประกอบ **β**-peptide ที่ออกแบบนี้ได้ถูกสังเคราะห์ขึ้นโดยอาศัยวิธี solid phase synthesis และถูกนำไปทดสอบความสามารถในการกระตุ้นการทำงานของ FPR โดยการวัดการเปลี่ยนแปลงของปริมาณแคลเซียมในเซลล์ ซึ่งเป็นกลไกทางชีวภาพภายในเซลล์ที่ควบคุมโดยการทำงานของ FPR นอกจากนี้แล้ว ยังได้ทดสอบการแสดงออกของเซลล์ที่ควบคุมโดยการทำงานของ FPR ด้วยเช่นกัน อาทิ เช่น การเคลื่อนที่ของเซลล์ที่ควบคุมโดยสารเคมี (chemotaxis) พบว่า สารประกอบ **β**-peptide นี้สามารถควบคุมการเคลื่อนที่ของเซลล์ได้โดยผ่านทางการควบคุมของ FPR สารประกอบ **β**-peptide นับว่ามีศักยภาพสูงในการนำไปพัฒนาต่อเป็นยารักษาโรคที่เกี่ยวข้องกับ FPR เนื่องจาก งานวิจัยที่ผ่านมา พบว่ามีความสามารถในการต้านการย่อยสลายโดยโปรตีนในเซลล์ได้ดีกว่าสารประกอบเปปไตด์ทั่วไป ดังนั้นจึงสามารถออกฤทธิ์ในการควบคุมได้นาน หากว่ายังต้องมีการนำไปพัฒนาต่อเพิ่มเติมในด้านนี้ต่อไปในอนาคต

Keywords: G-protein coupled receptors (GPCRs), peptidomimetics, **β**-peptide

1. ความสำคัญและที่มาของปัญหาที่ทำการวิจัย

Cells monitor and respond to extracellular signals via cell-surface receptors. These receptors bind to a variety of environmental stimuli, transduce a variety of signals across the membrane surface, and coordinate consequential intracellular responses. G-protein coupled receptors (GPCRs) constitute one of the largest families of mammalian cell surface receptors known. It has been suggested that the human genome may encode more than 1000 different GPCRs. These receptors recognize a wide range of structurally diverse molecules including ions, photons, small molecules, peptides, hormones, and proteins. They control and regulate a variety of cellular and physiological processes, including perception, neurotransmission, and immune regulation. GPCRs are also implicated in numerous diseases including cancer, human immunodeficiency virus (HIV) infection, hypertension, sensory defected, and various neuronal disorders. Given their roles, it is not surprising that they are major targets of therapeutic agents. It has been estimated that more than 50% of current drugs on the market modulate GPCR activity.

Controlling GPCR activities are proposed to be an excellent strategy to treat diseases that mediated by GPCRs. More than half of the current therapeutics modulate GPCRs activities. However, these therapeutics target only about 30 GPCRs, and more surprisingly only a few of those are peptide-activated GPCRs. The GPCR therapeutic developments have focused heavily on designing small molecules to exert therapeutic effects to non-peptide-activated GPCRs. Peptide-activated GPCRs are not very attractive therapeutic targets partially due to a common perception that protein-protein interactions are complex and required a large surface area for mimicry. Thus, the compounds that could mimic the protein-protein interactions is believed to be larger than traditional pharmaceuticals and perceived to be a poor drug lead. However, recent knowledge about protein-protein interactions has led to a realization that a compound can be effectively designed and developed to mimic these interactions, leading to a control of the target receptor activities.

Almost all of the peptide-activated GPCRs are implicated in diseases, of which effective therapeutics are longed for. These diseases, including cancer, HIV-1 infection neuronal disorders, raise therapeutic concern not only in the developing countries but also worldwide. Specifically, in Thailand, these diseases are listed as the most common causes of death. Hence, there is impetus to develop potent agonists or antagonists to control the activities of these GPCRs. Effective GPCRs agonists or antagonists that possess the drugability are highly desired. Unfortunately, the natural peptide ligands are not good drug candidates due to their fast degradations by cellular proteases and peptidases, resulting in their poor bioavailability. However, we envisioned that these natural peptide ligands are useful for ligand-based drug design. Especially, the non-natural peptide mimics or peptidomimetics have started to find their important applications in therapeutic use.

The project we proposed here was aiming to design and to develop non-natural peptide mimics or peptidomimetics that effectively binds to one of the peptide-activated GPCRs named *N*-formyl peptide receptor (FPR). FPR is one of the essential GPCR therapeutic targets. They are expressed on the surface of neutrophils and leukocytes, involving in host defense against invading pathogens as well as being implicated in HIV-1 infection and Alzheimer's disease. Therefore, FPR ligand that possesses drugability is being sought. We choose to focus on β-peptide, specifically β_2 and β_3-peptides. This class of peptidomimetic can be designed to mimic the primary structure as well as the secondary and tertiary structures of the natural peptide, providing flexibility for our design and for the optimization of the peptidomimetic structure. We hypothesized that our designed peptidomimetics can bind and activate FPR. Our results could potentially be beneficial for the FPR therapeutic development. Furthermore, this research would expand the scope of peptidomimetic application in therapeutic use, specifically in peptide-activated GPCR therapeutics.

2. วัตถุประสงค์ของโครงการ

This investigation was aimed to design and to develop the peptidomimetic agonists for formyl peptide receptor (FPR), one of the peptide-activated GPCRs. The proposed peptidomimetics was designed to mimic the natural peptide ligand of FPR, N-formyl-methionine-leucine-phenylalanine (fMLF). These peptidomimetics have potential to be drug leads, as they resist to the degradation by proteases and peptidases, thereby increase the bioavailability over the natural peptides.

(1) To design and generate peptidomimetics mimic FPR ligand.

(2) To determine the ability of the compounds synthesized in (a) to bind to FPR.

(3) To test the compounds synthesized in (a) for their ability to elicit FPR signaling responses.

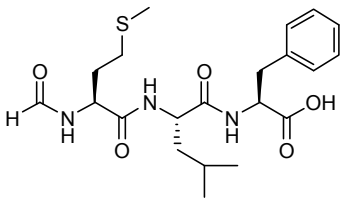
(4) To test of the resistance of compounds against degradation by proteases or peptidases (if necessary).

3. ผลงานวิจัยที่เกี่ยวข้อง **(Literature review)**

Cells respond to extracellular stimuli through cell surface receptors. The receptors transmit information from the extracellular environment and transduce signals into cellular responses. G-protein coupled receptors (GPCRs) are the largest protein family of cell surface receptors, accounting for approximately 2% of the human genome.[1] They are implicated in many diseases, and more than half of current pharmaceuticals exert therapeutic effects by binding GPCRs and controlling their signaling responses.[2] Surprisingly, the current pharmaceuticals target approximately 30 GPCRs, and only a few of those are peptide-activated GPCRs.[2,3] The focus has been concentrated on the development of small molecule with drugability agonist and antagonists of GPCRs that designed to bind within the hydrophobic transmembrane regions of GPCRs. However, most of peptide ligands are believed to bind mainly to the extracellular loops or the N-terminal domain of the GPCRs. The development of peptide-based drugs targeting

GPCRs are impeded by a perception that such peptide ligand-protein receptor interactions or protein ligand-protein receptor interactions are complex and involved binding of large surface areas; therefore, the compounds would have to be much larger than the traditional therapeutics. Here, we propose to investigate the design and synthesis of peptidomimetic to mimic peptide ligand-GPCR interactions. We envision that these types of compounds will be beneficial to the development of peptide activated GPCRs therapeutics.

N-formylated peptide receptor (FPR) is one of the best-studied GPCRs.[4] It has been extensively exploited as a model system to investigate GPCR signaling response, specifically GPCR-mediated leukocyte chemotaxis. Human FPR was defined biochemically in 1976 as a high-affinity receptor for *N*-formyl-methionine-leucine-phenylalanine (fMLF) on the surface of neutrophils.[5,6] The structure of *N*-formyl-methionine-leucine-phenylalanine (fMLF) is shown in **Figure 1**. *N*-formyl peptides can be formed from either an endogenous source such as the mitochondrial proteins of ruptured host cells or an exogenous source such as the proteins of invading pathogens. Since bacterial and mitochondrial proteins are the only sources of *N*-formyl peptides in nature, it has been generally thought that the FPR evolved to mediate the trafficking of phagocytes to sites of bacterial invasion or tissue damage.



formyl-Met-Leu-Phe-OH (fMLF)

Figure 1. Structure of endogenous α-peptide ligand, *N*-formyl-methionine-leucine-phenylalanine (fMLF)

For almost 25 years, speculation about the biological roles of FPR had been limited to anti-bacterial host defense. Nevertheless, mounting evidences have suggested that FPR may function in a more complex manner. A large number of non-formylated peptidic ligands have been identified. Interestingly, these ligands include HIV-1 envelope proteins, suggesting that FPR may play a role in HIV-1 infection (**Table 1**).[7,8] It has been suggested that FPR can potentially play several roles in HIV-1 infection. Specifically, activation of FPR by HIV-1 envelope proteins may promote host immune responses during initial HIV-1 infection. In addition, activation of FPR by agonist has shown to induce desensitization of chemokine receptors CCR5 and CXCR4, which function as HIV-1 co-receptors.[9] The down-regulation of CCR5 and CXCR4 potentially interferes with the propagation of HIV-1 infection.[10] Hence, FPR induced HIV-1 co-receptor desensitization provides a strategy for the development of anti-HIV-1 therapeutics. In addition, FPRs are also implicated in Alzheimer's disease. Interest in the potential role of FPRs in Alzheimer's disease came from the discovery that $A\beta_{42}$ is a FPR agonist (**Table 1**).[11] $A\beta_{42}$ is a cleavage fragment of the amyloid precursor protein and its aggregated form is a major component of plaques seen in the brain tissue of patients suffering from Alzheimer's disease. It has been demonstrated that $A\beta_{42}$ recruits microglia chemotaxis. Microglia are the immune cells of the brain.[12] Recruitment of these cells has been shown to potentially eliminate amyloid deposits by phagocytotic mechanisms. Because the recruitment of microglia is possibly mediated by FPR activation, FPR has potential as a target for Alzheimer's drug development. Hence, FPRs not only play an important role in the regulation of immune response to microbial invasion, but also potentially in HIV-infections and Alzheimer's disease. Controlling FPR activities are proclaimed to be an excellent novel strategy to restrain the infection of invading pathogens as well as the infections of these diseases. Hence, FPR constitute an attractive pharmacological target, and the development of potent FPR therapeutics is particularly desired.

Table 1: Agonist ligands that can activate FPR receptor.

Agonist	Origin	Receptor	EC_{50}
Bacterial peptides			
fMLF and analogs	Escherichia coli	FPR	0.1 – 1 nM
HIV-1 envelope peptides			
T20 (DP178)	$HIV\text{-}1_{LAV}$ gp41	FPR	0.5 μM
T21 (DP107)	$HIV\text{-}1_{LAV}$ gp41	FPR	0.1 μM
Peptide library derived agonists			
W peptide (WKYMVm)	random peptide library	FPR	1 nM
Host-derived agonists			
MHC binding peptide	NADH dehydrogenase subunit I	FPRL1	0.5 nM
LL-37	cleaved from anti-microbial protein	FPRL1	1.0 μM
SAA	acute phase protein	FPRL1	0.1 μM
$A\beta_{42}$	APP	FPRL1	1.0 μM

The canonical ligand for FPR is *N*-formylated peptide, *N*-formyl-methionine-leucine-phenylalanine (fMLF). The structure-activity relationship (SAR) data on FPR indicated that the formyl moiety on the *N*-terminus was extremely significant for binding affinity and signaling responses.[13] However, recent studies have shown that the formyl moiety may be less essential than the previous studies gave reason to believe. The finding revealed that FPR also recognizes non-formylated peptide with high affinity as well as peptides with modifications.[14] Notably, these non-formylated peptides are also able to activate the receptor, resulting in proper FPR-mediated signaling responses. In addition, FPR also recognizes and is activated by peptides that completely lack of sequence similarity to the peptides originally isolated from bacteria. For example, WKYMVm, a synthetic D-hexapeptide modified based on a sequence isolated from a random peptide library, was reported as one of the most potent chemoattractant of several leukocyte cell lines as well as neutrophils.[15] This particular peptide binds to FPRs with high affinity. Besides the *N*-formylated peptides originated from endogenous sources, and the non-formylated peptides isolated from the peptide libraries, there are some peptides that emanated from pathogens and associated with human diseases, including HIV and Alzheimer's disease. Specifically, HIV-1 envelope proteins, gp41 and gp120, have been found to contain domains that interact with FPR and its homologue FPRL1.[7,8] FPR receptors also bind to host-derived ligands such as the acute phase protein serum amyloid A (SAA), the 42 amino acid peptide from amyloid β ($A\beta_{42}$).[11] SAA and $A\beta_{42}$ are endogenous proteins that when aggregated tend to precipitate and result in the amyloid deposition associated with Alzheimer's disease.

As these formylated and non-formylated peptides bind and activate FPR and its homologues with high affinity, they appear to be ideal foundation for the FPR-mediated therapeutic development. Unfortunately, these peptides themselves provide inferior drug candidates. The use of peptides as drugs is severely hampered, specifically by their rapid proteolytic degradation that resulting in their poor bioavailability.[16] Numerous researches

have been focused on the design and synthesis of non-natural peptide mimics with an improved stability toward the degradation by proteases and peptidases. These "peptidomimetics" can be based on any oligomer that can mimics peptide primary structure through the use of amide bond isosteres or through the use of modification of the native peptide backbone, including the extension of the peptide chain or the incorporation of the heteroatom. Examples of these peptidomimetics are β-peptides, peptoids, azapeptides, oligocarbamates, and oligoureas. These peptidomimetics have been shown to resist the degradation from proteases and peptidases. In addition, they may have reduced immunogenicity and improved bioavailability relative to the peptide analogues. Besides the mimicry of the natural peptide primary structure, some sequence specific peptidomimetics can exhibits well-defined secondary structures such as helices, turns, and sheet like structures as well (**Figure 2**). Such peptidomimetics are termed "foldamers", and their examples include β-peptides, γ-peptides, poly-N-substituted glycines or peptoids.[17] The Sequence specific peptidomimetics begin to be very useful in the mimicry and optimization of natural peptide products for therapeutic development. For example,

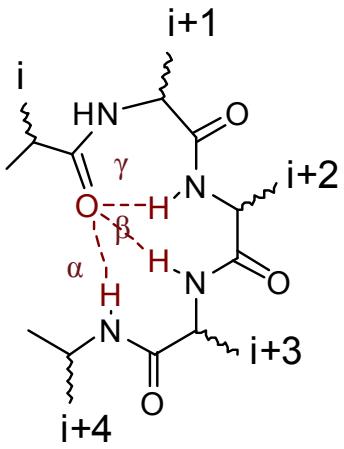


Figure 2. α-turn, β-turn, and γ-turn structures of α-peptides.

the recent developments include the anti-bacterial peptides which mimic natural peptide-based antibiotics such as magianins, anti-cancer therapeutics which mimic pro-apoptotic proteins thereby inducing or activating apoptotic pathway in specific cells, and also inhibitors of HIV proteases for treatment of HIV-1 infection.[18]

The goal of this proposed research specifically is to design and to develop the peptidomimetic mimicking the natural peptide ligand fMLF of FPR. We envision that our proposed investigation will lead to the development of new types of FPR ligands. Given the role of FPR in inflammation, immune cell trafficking, angiogenesis, differentiation and development, as well as their implication in several diseases, including HIV-1 infection and Alzheimer's diseases, these peptidomimetics can potentially be drug leads for further development of therapeutics target FPR.

In addition, FPR belongs to the G-protein coupled receptor family. GPCRs are members of a transmembrane proteins family. However, since most of the peptide-activated GPCRs are implicated in many diseases, there is impetus to investigate the rational design of effective compounds that can mimic the peptide ligand-receptor interactions. We envision that our proposed investigation will gain useful information about the mimicry of peptide-activated GPCR ligand. Such information will essentially lead to a realization that peptidomimetics can be designed to effectively bind to peptide-activated GPCRs and to modulate target GPCR activity. In addition, the results may expand the scope of peptidomimetic applications in therapeutic use.

4. ระเบียบวิธีวิจัย

Aim 1: To design and to develop the peptidomimetic agonists for formyl peptide receptor (FPR).

FPR is displayed at high levels on the surface of neutrophils and monocytes where it mediates responses to *N*-formylated peptides, such as *N*-formyl-methionine-leucine-phenylalanine. (fMLF). The structure of FPR has not yet been elucidated. However, to design of effective peptidomimetic to mimic FPR-ligand interactions, the information about structure-activity relationship (SAR) of the natural ligand is essential.

The three-dimensional structure of these transmembrane GPCRs is difficult to obtain. Indeed, there are only three X-ray crystal structures reported, yet none of which is FPR. In addition, neither of these X-ray crystal structures is for their active states. Nevertheless, plenty information of structure-activity of formyl peptides is available. The seven transmembrane α-helices of FPR have been mapped based on the amino acid sequence and homology to bacterial rhodopsin. The residues involved in ligand binding and the receptor-ligand binding interaction have been investigated.

Similar to other peptide-activated GPCRs, the formyl peptides bind mainly to the extracellular loops of the FPR. With regard to the binding of canonical formylated peptide fMLF, it has been suggested that there are several interactions that stabilize the conformation of the receptor and facilitate ligand binding. Specifically, these interactions include the hydrogen bonding interactions of the formyl moiety on the N-terminus of the peptide with Arg884 and Lys85 of the first extracellular loop, hydrogen bonding interactions of the peptide backbone with the first and the second extracellular loops which are known to be responsible for the binding of formyl peptides, formation of the disulfide bridges between the formyl-methionine with Cys residues, as well as the interaction of the peptide with Arg163. These interactions are thought to be essential for formyl peptide to bind and activate FPR.

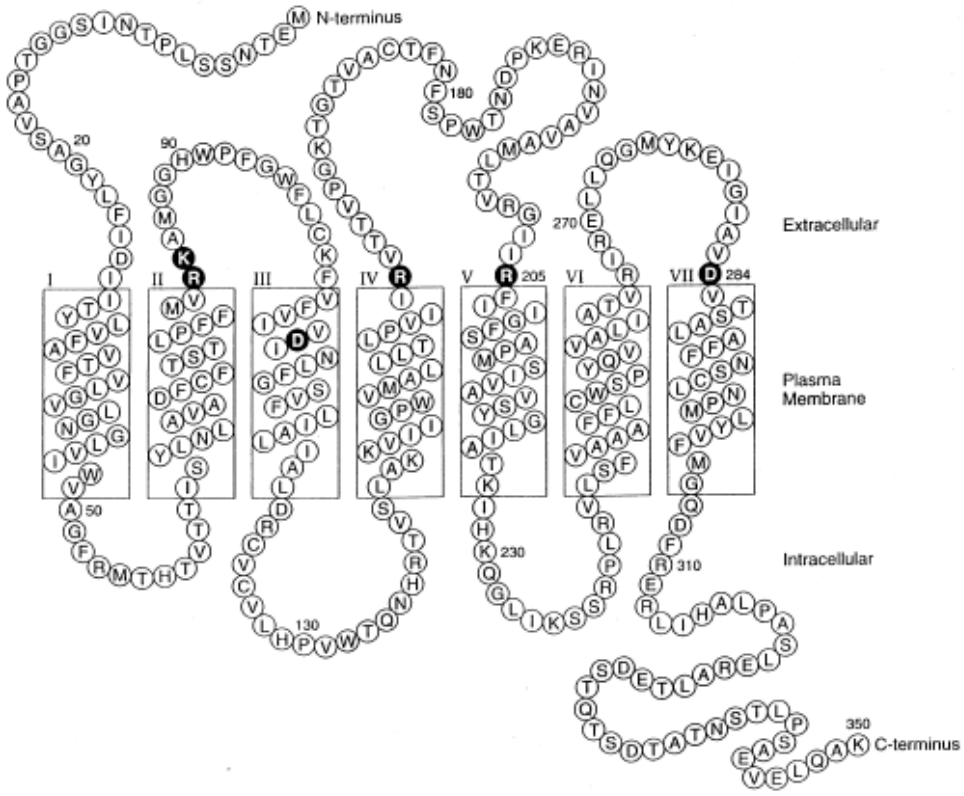
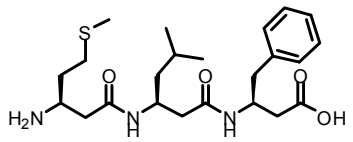


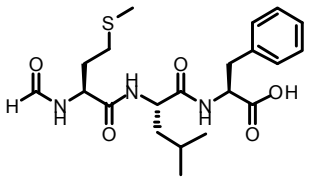
Figure 2. Schematic representation of human neutrophil FPR.
(*Eur. J. Biochem.* 1999, 264, 495-499.)

The initial design focused on the primary structural mimicry, which included all essential interactions between the ligand and receptor. The β-amino acids are commercially available, therefore, easing the synthesis of the peptidomimetics. The natural agonist ligand of the FMR is *N*-formyl-methionine-leucine-phenylalanine. (fMLF), of which the structure is shown in **Figure 3**. Based on the previously reported structure-activity relationships

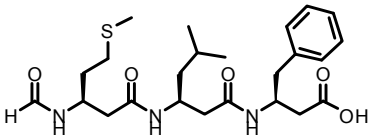
discussed prior in the section of literature review, we synthesized two β-peptide based peptidomimetics, anticipating to be an agonist and antagonist for FPRs. The peptidomimetic **(1)** is aimed to show agonistic activity similar to the natural agonistic α-peptide, fMLF. The peptidomimetic **(2)**, on the other hand, possesses the bulky *tert*-butyl group in place of the essential formyl moiety at the N-terminus. Based on the previous investigation, in case of α-peptide, the steric hindrance at the N-terminus may completely alter the ligand activity, specifically from agonist to antagonist. We, therefore, incorporate the investigation of this effect in the case of β-peptide based peptidomimetic.



Peptidomimetic (**1**)



formyl-Met-Leu-Phe-OH (fMLF)



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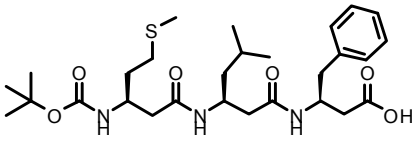
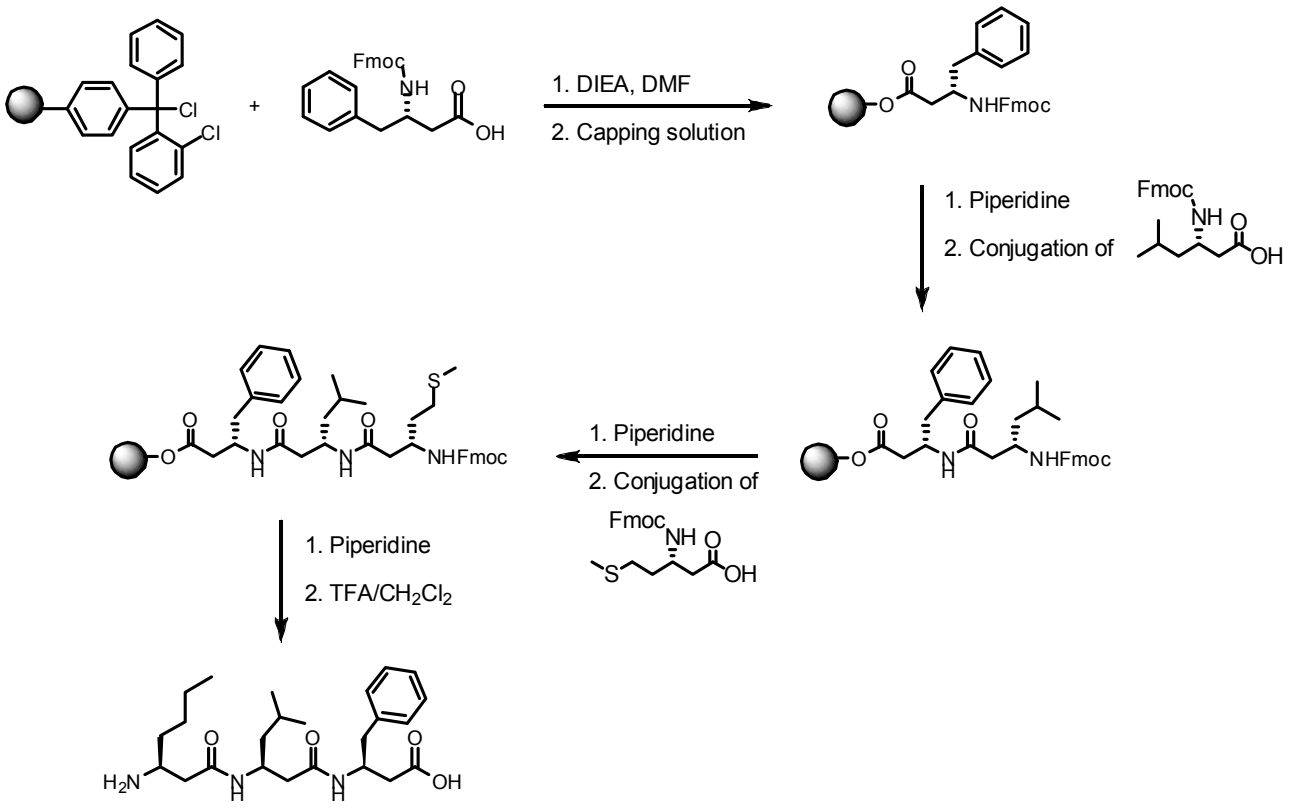


Peptidomimetic (**3**)

Figure 3: Designed agonists (**1-2**) and antagonist (**3**).

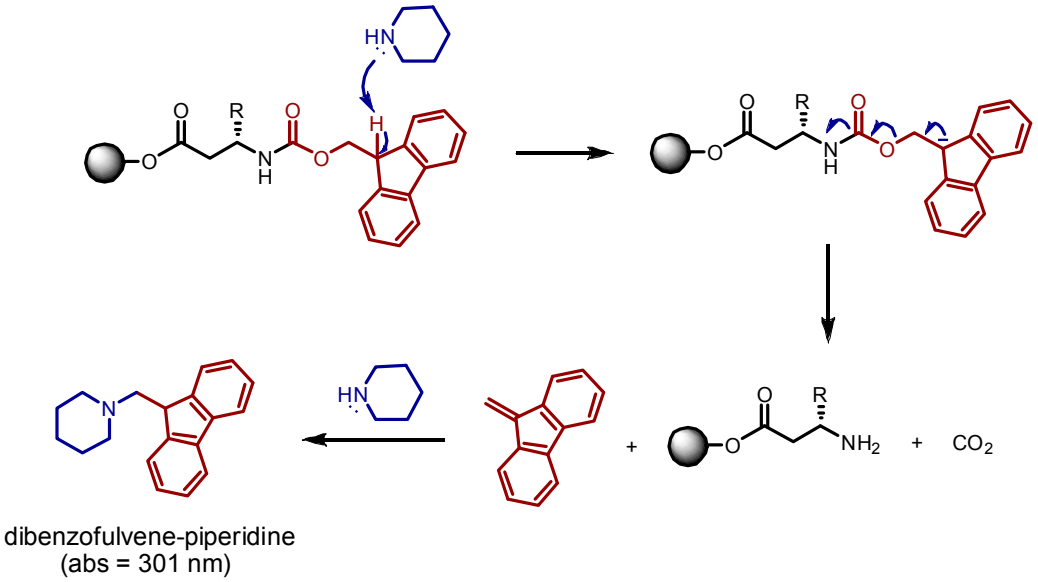
Our original plan was to synthesize the β-peptide based peptidomimetics in solution. However, due to the high cost of β-amino acids, we alternated to solid phase synthesis. The solid phased synthesis is more suitable, particularly for dealing with such small scale. We chose acid labile 2-chlorotritylchloride resin as the solid support due to its characteristic hindered structure of the 2-chlorotrityl group that can suppress the side reactions such as racemization and diketopiperazine formation.



Peptidomimetic (**1**)

Scheme 1. Synthetic route to compound **1**

The synthesis of compound **1** was shown in **Scheme 1**. First, the Fmoc-L-β-homo-phenylalanine was loaded to the activated 2-chlorotrityl chloride resin in the presence of a base. Then, the unreacted 2-chlorotrityl group was capped by adding a CH_2Cl_2/MeOH/DIEA solution. The loading level was determined by the UV absorbent measurement of the Fmoc cleavage (as shown in **Scheme 2**).



Scheme 2. Fmoc-Cleavage by piperidine.

The loading condition of the first amino acid was also optimized. We have found that the reaction time allowed for the capping step is crucial. Only 20 minutes is necessary for capping the unreacted 2-chlorotrityl chloride (**Table 2**). If allowed extended period, the loading level would decrease.

Table 2. Conjugation of Fmoc-beta-Phe-OH to resin.

Conjugation Step	Capping period	Average Absorbance (301 nm)	mmol	% yield
Fmoc-beta-Phe-OH to resin	4 hours	0.8	0.022	44%
Fmoc-beta-Phe-OH to resin	20 mins	1.412	0.039	77.7%

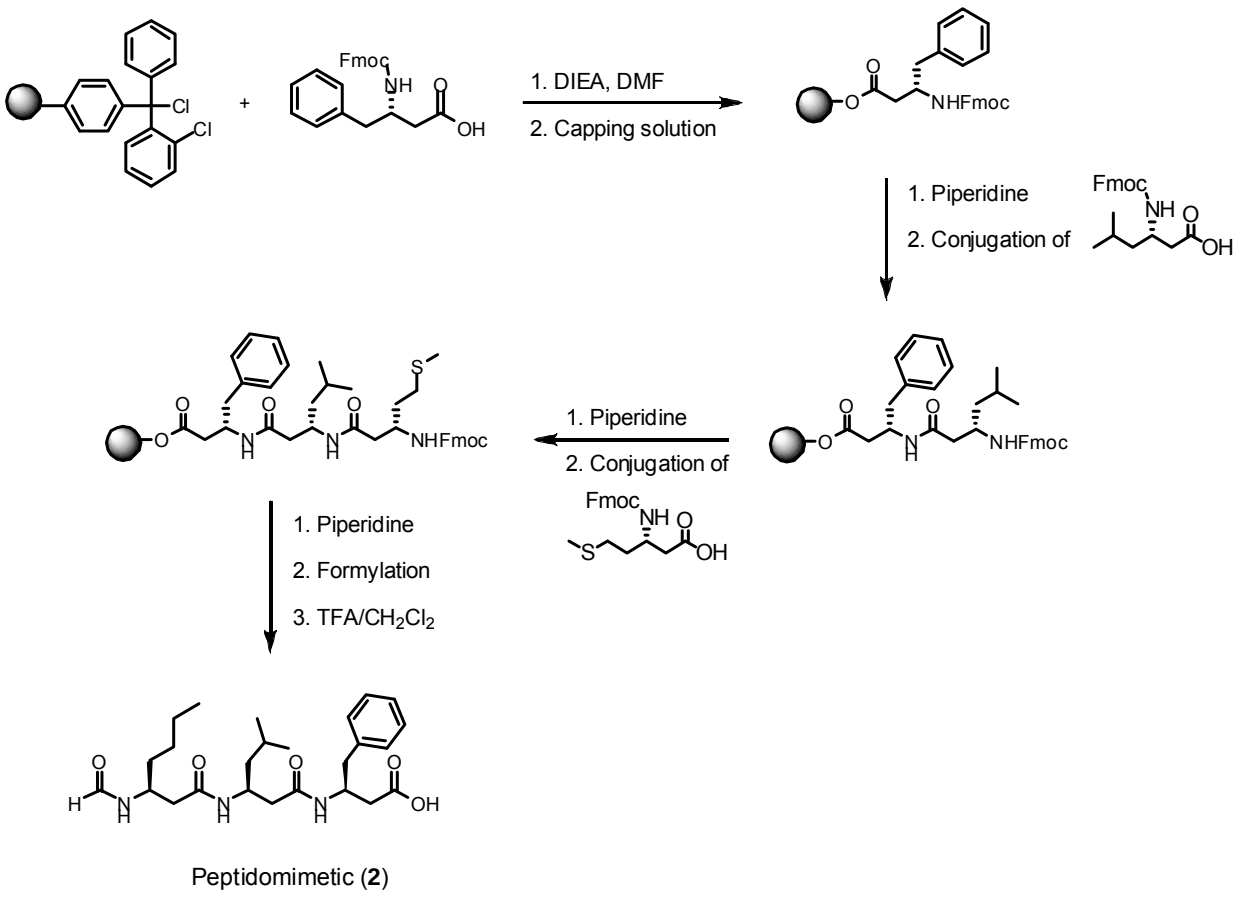
To the L-β-homo-phenylalanine loaded 2-chlorotrityl chloride resin, the Fmoc- L-β-homo-leucine was subsequently conjugated via amide bond, which was formed utilizing the standard peptide coupling reagents in the presence of a base. Fmoc cleavage was performed for further conjugation as well as for determining the coupling efficiency. The resulting unprotected dipeptide bound to the solid support was then coupled to the last amino acid, Fmoc- L-β-homo-methionine. Next, the Fmoc moiety was cleaved by a solution of piperidine in DMF, leaving the terminal amine as the N-terminus **(Scheme 1)**. The yield of each step was summarized in **Table 3**.

Table 3. Percent yield of each synthetic step required for the synthesis of compound **1**

Conjugation Step	Capping period	Average Absorbance (301 nm)	mmol	% yield
Fmoc-beta-Phe-OH to resin	20 mins	1.412	0.039	77.7%
Fmoc-beta-Leu-OH to Fmoc-beta-Phe-resin	-	1.1431	0.032	80.9%
Fmoc-beta-Met-OH to Fmoc-beta-Leu-beta-Phe-resin	-	1.040	0.029	90.6%

Structural activity relationship analysis of the endogenous fMLF peptide ligand suggested that the fomyl group is required for the potency. Therefore, we envisioned that compound **2**, which is the formylated N-terminal amine adduct of the peptidomimetic **1**, should be able to active FPR more efficiently. The synthesis of peptidomimetic 2 could be achieved from the peptidomimetic **1 (Scheme 3)**. The formylation was planned to achieve via the previously reported condition, formic acid in DCC. However, the condition raises a concern as the formylation condition may lead to the cleavage of the peptidomimetic from the extremely acid labile of linkage to the solid support. Nevertheless, we decided to take risk with the reported reaction condition. The peptidomimetic conjugated to the solid supported was treated with the solution of formic acid and the standard peptide coupling reagent in DMF. The reaction mixture was stirred at 4°C to avoid the decomposition of formic acid. The filtrate was collected to explore whether the acidic condition of the reaction could potentially cleave the peptidomimetic from the solid support. After characterization the filtrate, no peptidomimetic was recovered, suggesting that the compound was still attached to the resin after treating with the condition mentioned above. The resin was then treated

with 1% TFA/ CH_2Cl_2 followed by washing with CH_2Cl_2 and MeOH multiple times. The filtrate and washes were combined and concentrated. The crude peptidomimetic was then purified by reverse-phase HPLC. The characterization of the synthetic peptidomimetic was performed using Mass Spectroscopy. The ESI-MS calculated for the starting material of the reaction, peptidomimetic **(1),** is $C_{24}H_{37}N_3O_5S$ [M] is 479.25, but was found 452.25 and 474.24, which was respectively corresponding to [M+H+] and [M+Na+] of the non-formylated peptidomimetics ($C_{23}H_{37}N_3O_4S$: [M] = 451.25). The result suggested that the formylation did not occur and the starting material **(1)** was recovered. We attempted the formylation of the peptidomimetics by varying reaction conditions. However, we could not obtain the desired compound **2**.



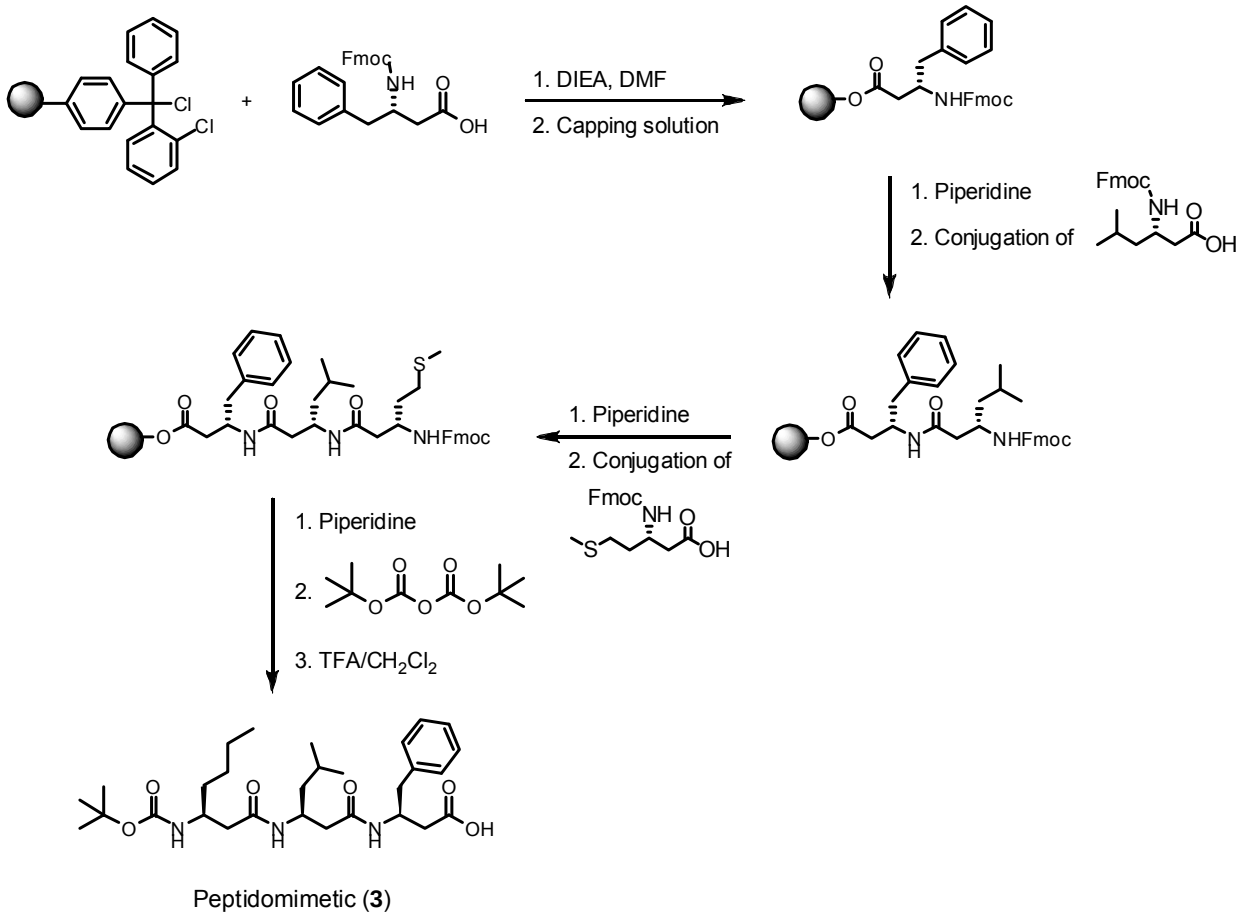
Scheme 3. Synthetic route to achieve peptidomimetic **2**.

The peptidomimetic **(3)** was designed, on the other hand, possesses the bulky *tert*-butyl group in place of the essential formyl moiety at the N-terminus. Based on the previous investigation, in case of α-peptide, the steric hindrance at the N-terminus may completely alter the ligand activity, specifically from agonist to antagonist. We, therefore, incorporate the investigation of this effect in the case of β-peptide based peptidomimetic.

For the synthesis of the anticipated peptidomimetic antagonist, the N-terminal amine was reacted with Boc_2O in order to form the steric hindered *tert*-butyl carbamate at the N-terminus **(Scheme 4)**. The resulting peptidomimetic was cleaved from the solid support, using extremely mild acidic condition (1% TFA/ CH_2Cl_2). The mild acidic solution would only cleave the desired *tert*-butyl carbamate peptidomimetic **(2)** from the resin, and not cleave the *tert* -butyl carbamate from the peptidomimetic since the cleavage of the *tert* -butyl carbamate requires stronger acidic condition. Therefore, with extremely acid labile 2-chlorotrityl chloride resins, we could control the synthesis of the anticipated peptidomimetic antagonist. The crude peptidomimetic was then purified by reverse-phase HPLC. The characterization of the synthetic peptidomimetic was performed using Mass Spectroscopy (ESI-MS calculated for $C_{28}H_{45}N_3O_6S$ [M+Na^+] is 574.29 found 574.2926). The experimental yield is summarized in **Table 4**.

Table 4. Percent yield of each synthetic step required for the synthesis of compound **3**

Conjugation Step	Capping period	Average Absorbance (301 nm)	mmol	% yield
Fmoc-beta-Phe-OH to resin		0.8	0.022	44%
Fmoc-beta-Leu-OH to Fmoc-beta-Phe-resin		0.6642	0.018	83.0%
Fmoc-beta-Met-OH to Fmoc-beta-Leu-beta-Phe-resin		0.6364	0.018	95.8%



Scheme 4. Synthetic route to achieve peptidomimetic **3.**

Aim 2 and 3: To test the compounds synthesized in (a) for their ability to elicit FPR signaling responses.

We, next, tested the ability of our synthesized peptidomimetics for their ability to activate the formyl peptide receptor (FPR). In case of the natural α-peptide, N-formyl-methionine-leucine-phenylalanine (fMLF) binds to the surface of neutrophils and leukocytes via the receptor, called N-formyl peptide receptor (FPR), with high-affinity. FPR serves as the chemoreceptor, functioning in the transduction of the cellular signaling such as directing migration to the site of inflammation in response to a gradient of chemoattractant peptide, fMLF, that are locally produced by either an endogenous source such as the mitrocondrial proteins of ruptured host cells or an exogenous source such as the proteins of invading pathogens.

The human FPR belongs to the heptahelical transmembrane GPCR family. Spectrofluorometric experiments using fluorescein labeled formyl peptide indicates that the assembly of the receptor with the G-protein complex is rapid, occurring within a fraction of a second. Upon binding of ligand, the heterotrimeric G protein complex rapidly dissociates into the α and $\beta\gamma$ subunits, leading to the activation of phospholipase C (PLC), and phosphoinositide 3-kinase (PI3K). The phosphoinositide 3-kinase phosphorylates the membrane phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP2 is also cleaved by PLC into two secondary messengers, inositol triphosphate (IP3) and diacylglycerol (DAG)

IP3 binds to IP3-sensitive calcium channels, initiating the intracellular calcium release, which is the first major response in its signaling cascade. Meanwhile, diacylglycerol activates protein kinase C (PKC), resulting in multiple cellular responses such as actin polymerization, activation of MAPK, chemotaxis, phagocytosis, and superoxide anion release **(see Figure 3)**.

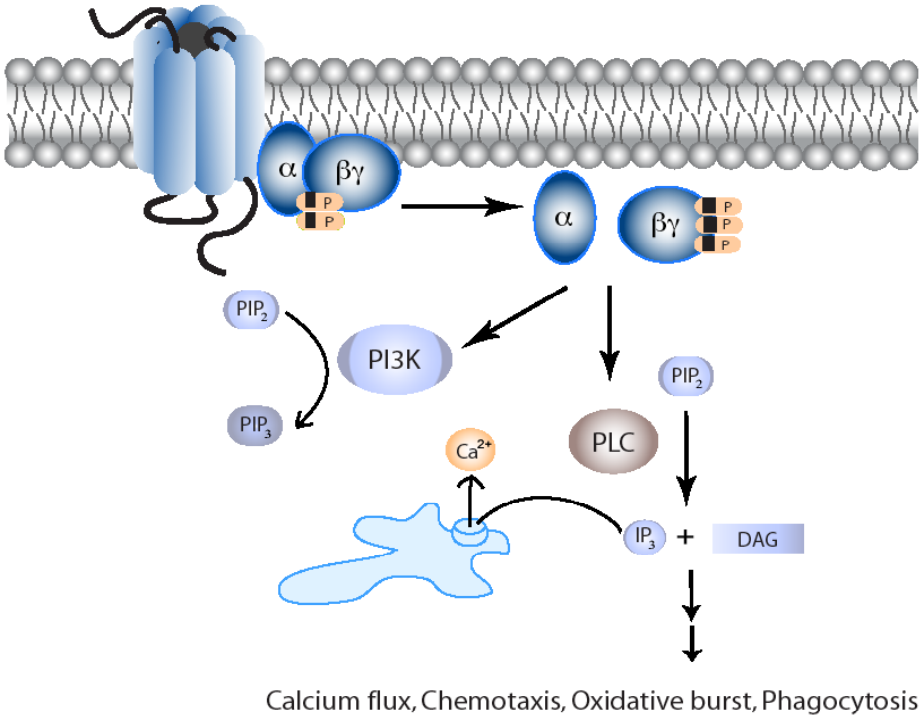


Figure 3. FPR receptor signaling.

To test ability of the designed compounds to activate or to inhibit the N-formyl peptide receptor signaling cascade, we aimed to perform several cell based assays. A monocytic cell line U937 and FPR-transfected U937 were received as general gifts from Professor Laura L. Kiessling (Department of Chemistry and Biochemistry, University of Wisconsin, Madison, USA) and Professor Eric R. Prossnitz (Department of Cell Biology and Physiology, University of New Mexico, New Mexico, USA). These two cell lines would be used as the model cell lines for the investigation.

The monocytic U937 cells were grown in RPMI 1640 supplemented with fetal bovine serum, L-glutamine, penicillin, and streptomycin. Meanwhile, the human stably FPR-transfected U937 cells, (transfection was done in E. Prossnitz Laboratory), were grown in RPMI 1640 supplemented with fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin, HEPES buffer (pH 7.0), and active geneticin.

First, we aimed to determine the ability of both FPR-transfected U937 cells and the non-transfected U937 cells in response to the canonical N-formylated peptide, N-formyl-methionine-leucine-phenylalanine. We expected the stably FPR-transfected U937 cells line to be able to transduce FPR signaling in response to the N-formylated peptide since it expressed the receptor FPR. With the non-transfected U937, however, no response should be observed.

To monitor FPR activation, we chose to investigate the early step in the FPR-mediated signaling pathway, namely the transient elevation in the intracellular calcium ion concentration. FPR-transfected U937 cells and U937 cells lacking the receptor were separately loaded with the ratiometric calcium dye Indo-1 to monitor the changes in intracellular calcium ion concentration that occur when cells are stimulated with formylated peptide fMLF. Indo-1 is UV light-excitable and is excited at a wavelength of 338 nm. The emission maximum of indo-1 shifts from 480 nm when dye is not bound to calcium ion to 401 nm when the dye is bound. The emission ratio can be measured using a fluorometer or flow cytometry **(Figure 4)**.

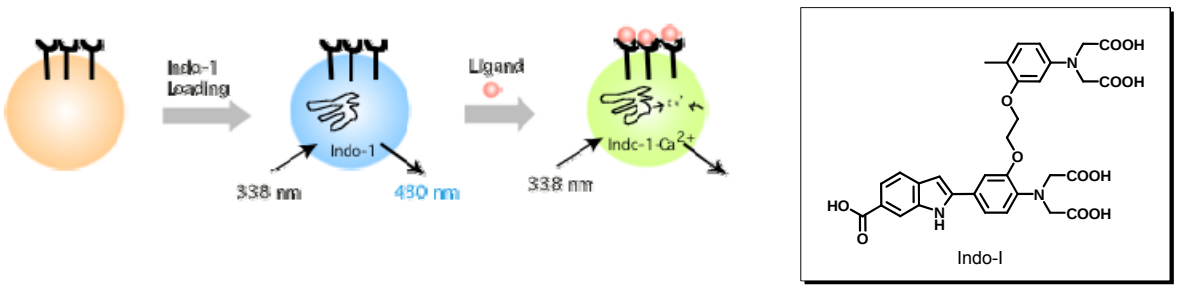


Figure 4. Monitoring intracellular calcium ion concentration inside the cells in real time using Indo-1 AM

Upon addition of 10 μM fMLF to the Indo-1 loaded FPR-transfected cells, abrupt increase in intracellular calcium ion concentration was observed. The signal is gradually declined to the basal concentrations as the result of calcium ion clearance from the cytosol **(Figure 5)**. Analysis of the curve of emission ratios versus time provides insights into the ability of the compound to elicit changes in intracellular calcium ion concentration. The results observed suggested FPR-transfected U937 cells can be activated in response to fMLF. The U937 cells, lacking the receptor, did not appear to transduce any signal in response to fMLF at the same concentration, therefore serving as the negative control.

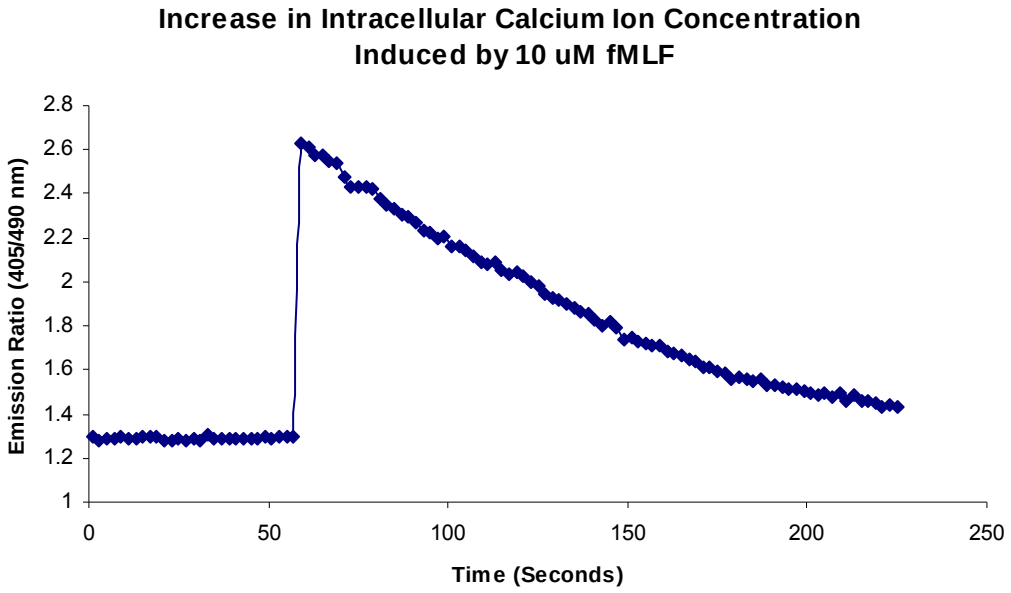


Figure 5. Increase in intracellular calcium ion concentration induced by 10 μM of natural α–peptide

Subsequently, to obtain the median effective concentration value, we performed the calcium flux assay at various concentration of fMLF: 10 μM, 1 μM, 100 nM, 10 nM, and 1 nM. The transient elevations in the intracellular calcium ion concentration at these particular concentrations were shown in **Figure 6**. The results indicated that the median effective concentration is roughly in the interval of 1-10 nM, which is corresponded to the previously reported value.

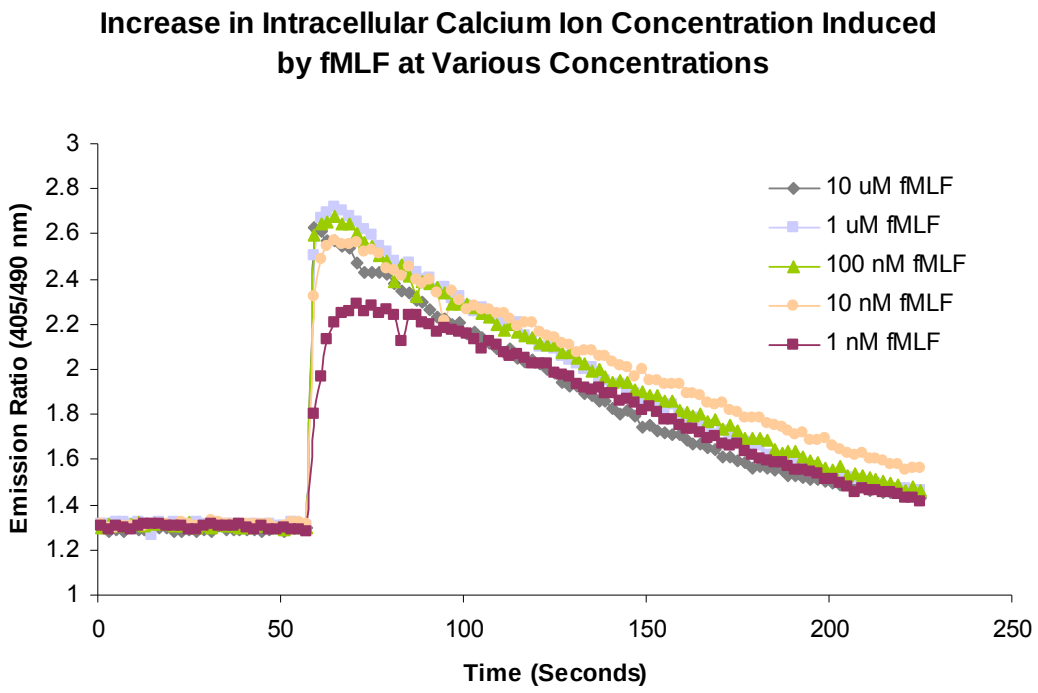


Figure 6. Increase in intracellular calcium ion concentration induced by natural α–peptide fMLF at various concentrations.

Subsequently, we investigated the ability of the peptidomimetic **1**, which lacking formyl group at its N-terminus, to activate the FPR-mediated signaling response. FPR-transfected U937 cells were loaded with indo-1 dye, followed by the treatment of peptidomimetic **1** at various concentration. We found that the peptidomimetic **1**, similar to the endogenous ligand fMLF, could induce FPR-mediated signaling response such as an increase in intracellular calcium ion concentration. Similar calcium flux profile of an abrupt

increase in intracellular calcium ion concentration followed by the gradually decline of signal to the basal concentrations as the result of calcium ion clearance from the cytosol was observed. However, it is important to mention that the peptidomimetic 1 is less potent than the endogenous peptide fMLF, as it required much higher concentration to activate the signaling response. (Figure **7**) The significantly less in potency may perhaps due to the lack of formyl group at the N-terminus. However, due to difficulty in the formylation step of the peptidomimetic **2** synthesis, we could not investigate any further.

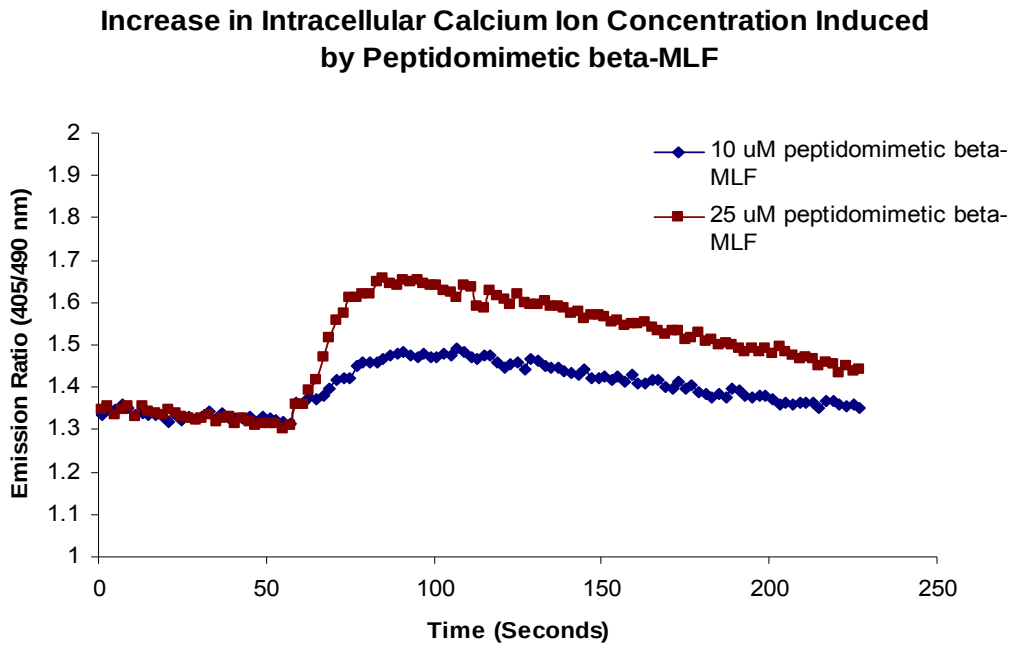


Figure 7. Increase in intracellular calcium ion concentration induced by peptidomimetic **1** at various concentrations.

Then, we evaluated our designed peptidomimetics for their ability to activate or to inhibit FPR signaling. We achieved the synthesis compound **3**, which possesses the steric moiety, tert-butyl group, thereby expected not to activate the FPR receptor. To monitor its biological response in the transient elevation in intracellular calcium ion concentration assay, the peptidomimetic **3** at 10 μM was added to the Indo-1 loaded FPR-transfected U937 cells. We observed no elevation in intracellular calcium concentration. This result obtained from this experiment indicated that our compound could not activate the FPR signaling **(Figure 8**).

However, the result did not indicate that the peptidomimetic **3** is the inhibitor of FPR. There is a possibility that the compound could not bind to the receptor, thereby not activate or inhibit, giving no response as the result.

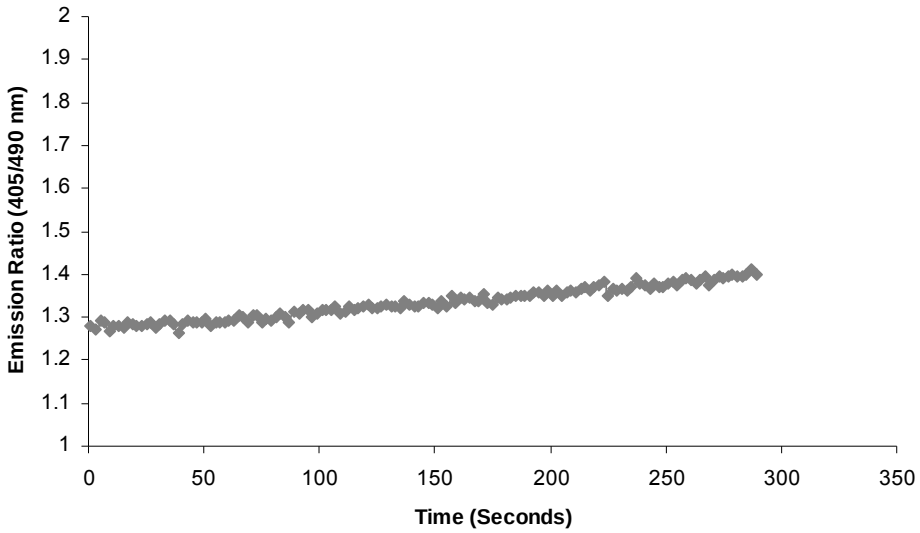


Figure 8. Increase in intracellular calcium ion concentration induced by 10 μM of peptiomimetic **3**.

To determine whether or not the peptidomimetic **3** functions as the inhibitor, we performed the competitive assay between the synthesized peptidomimetic **3** and the canonical peptide fMLF. Based on the concentration response curve, fMLF at 1 nM was used. In the competition assay, cells were treated with the mixture of peptidomimetic **3** at 10 μM and fMLF at 1 nM, the abrupt increase in intracellular calcium ion concentration was observed. However, in comparison to the fMLF at 1 nM alone, the level of transient elevation was decreased, suggesting that the addition of the peptidomimetic **3** resulted in the decrease in total signal as well as the signal intensity **(Figure 9)**. Even though the data would have to be repeated at least couple times to get the error intervals, the preliminary results suggested that the peptidomimetic **3** competed with the canonical peptide fMLF for the FPR receptor.

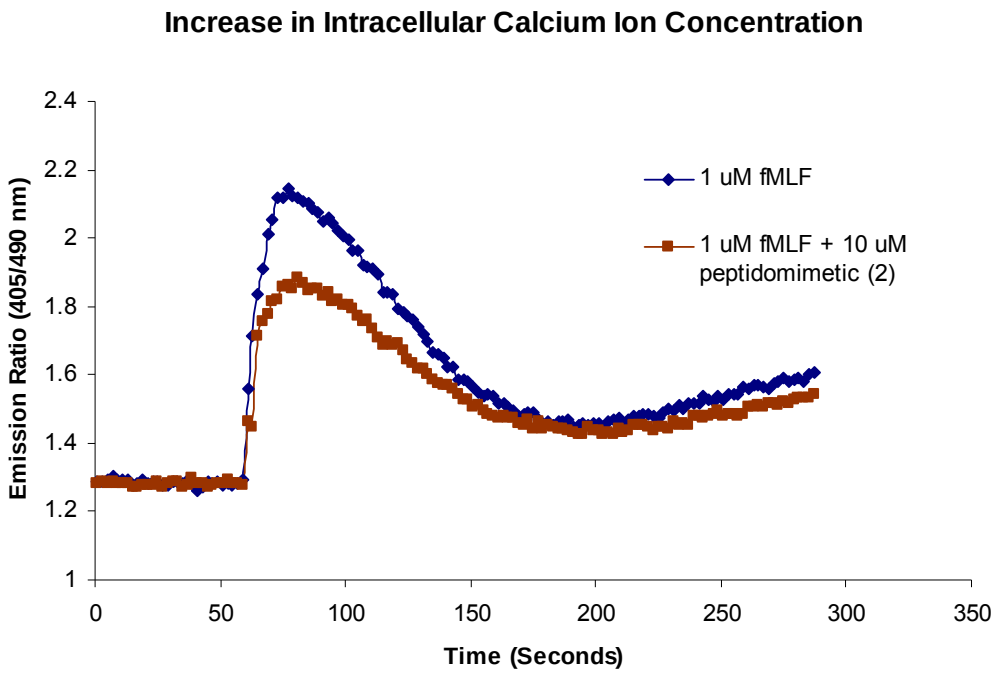
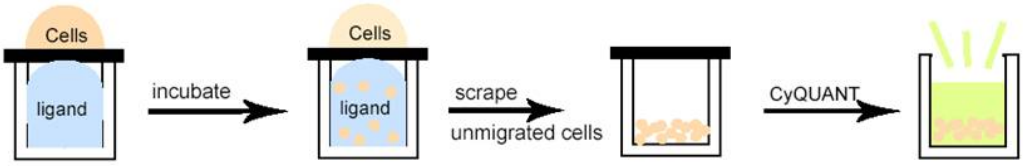


Figure 9: Increase in intracellular calcium ion concentration induced by 10 μM of peptidomimetic **3** in addition to 1 μM fMLF.

Based on our investigation of the synthesized peptidomimetic **1** and **3**, we found that the peptidomimetic **1** functioned as the agonist of FPR receptor while peptidomimetic **3** functioned as antagonist of FPR receptor. For the agonist **1**, we then evaluated whether the compound 1 could induce FPR-mediated cellular response such as cell migration. To investigate cell migration or chemotaxis, we utilized the commercially available assay called ChemoTx by NeuroProbe. In brief, FPR-transfected U937 was loaded on the top of the membrane with the compound of interest at the bottom of the well. If the compound could induce cell migration, the cell would migrate down to the bottom well. The number of cells could be quantitatively determined using CyQuant dye. As shown in **Figure 10**, peptidomimetic **1** could induce cell migration, though less potent than fMLF.



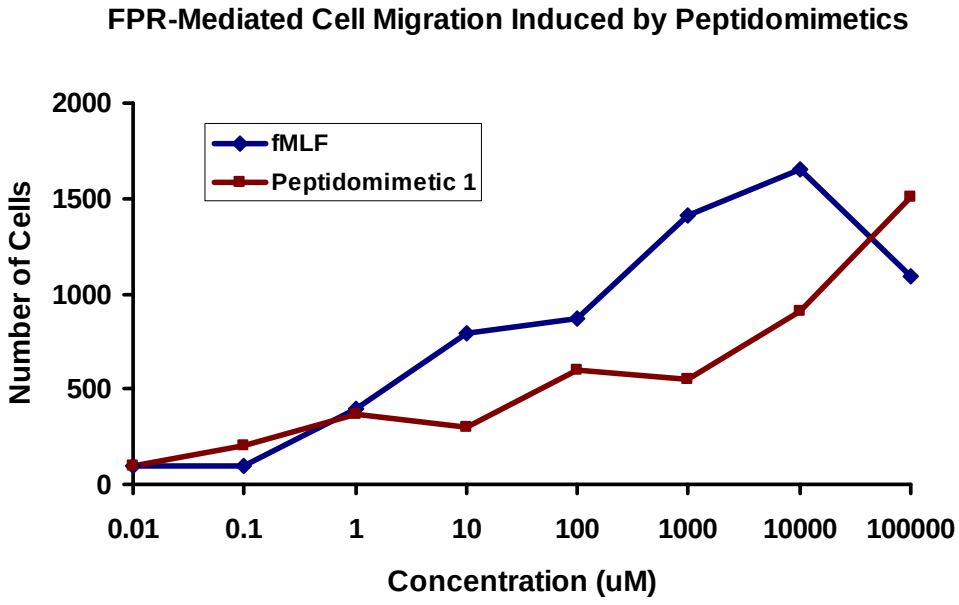


Figure 10. Chemotaxis profile of fMLF and peptdiomimetic **1**

In conclusion, the peptidomimetics mimicking the primary structure of the FPR natural peptide ligand, fMLF, were designed and synthesized. Our preliminary results showed that the peptidomimetic (**1**) could elicit FPR signaling. Conjugation of the bulky group (**3**) altered the biological activity from agonistic to antagonistic.

5. เอกสารอ้างอิง

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   Phetsang, W., Chaturongakul, S., Jiarpinitnun, C. “Electron-Withdrawing Substituted Benzenesulfonamides against the Predominant Community-Associated Methicillin-Resistant Staphylococcus aureus Strain USA300” (submitted to European Journal of Medicinal Chemistry)

   Titiwat Sungkaworn,[a] Chutima Jiarpinitnun,[b] Pongkorn Chaiyakunvat,[b] and Varanuj Chatsudthipong[a,*] “The bivalent display of angiotensin II peptides suppresses hyper-responsiveness of AT1R under oxidative stress condition” (submitted to European Journal of Medicinal Chemistry)

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

   ผลงานวิจัยที่ดำเนินการภายใต้ทุนวิจัยนี้ได้มีส่วนในการสร้างนักวิจัยใหม่ โดยมีส่วนในการนำไปประยุกต์สอนวิธีการดำเนินการวิจัยที่ใช้ศาสตร์จากหลากหลายสาขาวิชา เป็นตัวอย่างใช้ในการกระตุ้นความสนใจให้นักศึกษามองเห็นปัญหาวิจัยในภาพรวม และวิธีการนำเคมีสังเคราะห์มาใช้ในการศึกษาปัญหาวิจัยทางชีวภาพ

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
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