DEVELOPMENT OF CELL – BASED MODEL FOR NFAT (NUCLEAR FACTOR OF ACTIVATED T-CELL) NUCLEAR – CYTOPLASM TRANSLOCATION AND ITS APPLICATION FOR IMMUNOMODULATOR SCREENING

AKACHAI KHUEKANKAEW

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Pharmacology
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By Akachai Khuekankaew

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Title DEVELOPMENT OF CELL – BASED MODEL FOR NFAT (NUCLEAR FACTOR OF ACTIVATED T-CELL) NUCLEAR – CYTOPLASM TRANSLOCATION AND ITS APPLICATION FOR IMMUNOMODULATOR SCREENING

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ABSTRACT

Nuclear factor of activated T-cells (NFATs) together with calcium ion play an important role in immune reactions. The NFAT family encodes four distinct classes of proteins including NFAT1 (NFATp), NFAT2 (NFATc), NFAT3 and NFAT4 (NFATx). The objective of this study is to develop and validate the standard procedure for investigation NFAT4 and NFAT3 nuclear-cytoplasm translocation for bioactive immuno-modulator screening.

NFAT4 (N) and NFAT3 (N) genes were isolated from the library, GFP-fusion recombined, pcDNA 3.1 vector-ligated and propagated in DH5α strain E.coli. Human Embryonic Kidney (HEK) cells and primary porcine skin fibroblasts were cultured in modified Eagle's medium containing 5% fetal bovine serum and supplemented with 100U/ml penicillin and 100µg/ml streptomycin. The vectors containing GFP as a negative control, GFP-NFAT3(N) as a dominant negative control, and GFP-NFAT4(N) were transfected into HEK cells and primary porcine skin fibroblasts using Lipofectamine 2000 (Invitrogen). The transfected cells were cultured for 24 h allowing protein expression. The GFP and NFAT fusion proteins nuclear-cytoplasm translocation patterns were studied by adding 1µM of calcium ionophore (A23187) with/without 1 and 5µM cyclosporine A (CsA). The fluorescence images from GFP were captured and analyzed with an inverted fluorescent microscope (Carl Zeiss). The kinetic of A23187 (stimulation) and CsA (inhibition) were intensively studied.

Furthermore, this cell-base model was applied for determining the NFAT pathway inhibitory effect of curcumin (Curcuma longa) and andrographolide (Andrographis paniculata) extracts. The previous studies reported that both extracts could inhibit interleukin 2 production induced by T-cell activation.

The fluorescent signal from the GFP transfected cells is diffused throughout the cells both with and without cytosolic calcium induction by A23187, whereas the GFP-NFAT4(N) transfected cells significantly showed the translocation of the signal from cytoplasm to nucleus. The GFP-NFAT4(N) nuclear translocation, however, was significantly inhibited by 1 and 5μM CsA. As a dominant negative, the fluorescent signal from the GFP-NFAT3(N) transfected cells remained predominantly in the cytosol even after A23187 stimulation. NFAT nuclear translocation kinetic studies of A23187 and CsA were performed. The results suggested that the stimulation of NFAT nuclear translocation by A23187 was transient and reversible, whereas the inhibitory effect of NFAT nuclear translocation by CsA was last longer than 24 hours and irreversible. Using competitive kinetic study, the results confirmed that A23187 and CsA did not compete at the same effective site of calcineurin, and CsA was the non-competitive irreversible inhibitor for calcineurin enzyme. Our study confirmed that both curcumin and anndrographolide extracts cold not inhibit or stimulate NFAT nuclear translocation, indicating both substances may exert their pharmacologic action by other pathways, but not the NFAT pathway, such as c-jun transcription factor and NF- $\varkappa$B activation.

By induction of intracellular calcium concentrations in HEK cells and primary porcine skin fibroblasts transfected with either NFAT4 (N) or NFAT3 (N), demonstrated a specific patterns of cytoplasm-nuclear NFATs-protein translocation. Potentially, the findings from this study can be applied for the development of a standard cell-based method for screening of bioactive immuno-modulators signaling through NFATs pathway.

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ABBREVIATIONS

A23187	=	calcium ionophore
AIDS	=	human Immunodeficiency virus
AP1	=	Activator protein 1
bps	=	base pairs
BTP	=	3, 5-bistriflouromethyl pyrazole
Ca^{2+}	=	calcium ion
CaN	=	Calcineurin
CK1	=	casien kinase1
CO_2	=	carbon dioxide
COX-2	=	Cyclooxygenase-2
CpN	=	Cyclophylin
CsA	=	cyclosporin A
CTLs	=	cytotoxic T lymphocyte
DAG	=	diacylglycerol
DMEM	=	Dulbecco modified eagle's medium
DMSO	=	dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
E coli	=	Escherichia coli
EDTA	=	ethylene diamine tetra acetic acid
ER	=	endoplasmic reticulum
FBS	=	Fetal bovine serum
FK506	=	tacolimus
FKBP	=	FK506-binding protein
FOXP	=	Forkhead box P-family proteins
g	=	gram
GFP	=	green florescent protein
GSK	=	Glycogen synthase kinase
h	=	hour
HEK293	=	human embryonic kidney 293

HIV	=	human immunodeficiency virus
HLA	=	human leukocyte antigens
IC50	=	50% Inhibitory Concentration
IFN-γ	=	Interferon gamma
IL-2	=	Interleukin-2
IL-3	=	interleukin-3
IL-4	=	interleukin-4
iNOS	=	Inducible nitric oxide synthase
IP3	=	inositol 1,4,5-triphosphate
IP3R	=	inositol 1,4,5-triphosphate receptor
JNK	=	c- jun-N-terminal kinase
Kb	=	kilobase
kg	=	kilogram
L	=	litre
MAPK	=	mitogen-activated protein kinase
mg	=	milligram
MHC	=	major histocompatibility complex
min	=	minute
ml	=	milliliter
mM	=	millimolar
NFAT	=	nuclear factor activated T cell
NF-kB	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHR	=	NFAT-homology region
NK	=	Natural killer
NLS	=	nuclear-localization signal
NO	=	Nitric oxide
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
PIP2	=	phosphatidylinositol 4,5-bisphosphate
PKA	=	protein kinase A
RBCs	=	red blood cells

RHR	=	REL-homology domain
SLE	=	systemic lupus erythematosus
SPs	=	SPXX-repeat motifs
SRR	=	serine-rich regions
STAT3	=	signal transducer and activator of transcription-3
TAD	=	transactivation domain
TCR	=	T cells receptor
TNF-alpha	=	Tumour Necrosis Factor alpha
TNF-α	=	tumor necrosis factor α
µM	=	micromolar
µl	=	microliter
°C	=	degree celsius

CHAPTER I

INTRODUCTION

Nuclear Factor of Activated T cells family or NFATs is an important group of transcription factor proteins which stimulate the immune system in T cells. The expression of NFAT proteins can be found in many white blood cells such as T cells, B cells, natural killer (NK) cell, monocytes and other cells that are not related to immunity (non-immune related cells) such as muscle cell, cardiac cell, and neuronal cell [1, 2]. The mechanism of T cells stimulation via NFAT proteins is mainly controlled by the calcium ion concentrations in cytoplasm (cytoplasmic Ca^{2+} ion) [3, 4]. Once increased Ca^{2+} ion facilitates calcineurin enzyme function in the cytoplasm (Calcineurin is a Ca^{2+} ion dependent phosphatase enzyme), leads to NFAT protein dephosphorylation, exposes of NFAT NLS motif, allows the re-localization of NFAT protein from the cytoplasm into the nucleus (NFAT import), and finally promotes the NFAT protein – DNA binding. Likewise the other transcription factors such as AP1, Forkhead box P-family proteins (FOXP2, FOXP3), and GATA family proteins, the NFAT protein is able to turn on gene transcription of specific proteins depending on cell types, for example, interleukin-2 in T-cell for NFAT protein.

In T cells, it has been known that the NFAT proteins and other transcription factors play a role in many cytokines gene expression, affecting on T cell proliferation, survival and differentiation [5]. Therefore, by inhibiting or decrease of NFAT proteins activation is the obvious target for new drug development for immune suppression for tissue or organ transplant patients, in order to prevent or reduce graft rejection and autoimmunity. To date, there are four types of protein inhibitors to prevent NFAT nuclear translocation, which are AKAP79 (scaffold protein preventing calcineurin substrates form interactions, CABIN protein (blocks calcineurin activity), calcineurin B homolog, and MCIP1,2,3 proteins which prevent NFAT2 phosphorylation and nuclear import [4]. For the NFAT small molecule inhibitors, which are to date being used as immune-suppressive drugs, are Cyclosporine A (CsA) and FK506. Both Cyclosporine A and FK506 repress the function of the NFAT proteins by inhibition of

the Calcineurin enzyme. Because these molecules are highly specific inhibitors for NFAT pathway, so as mentioned above, both CsA and FK506 are used as therapeutic application in immune suppression (via T-cell suppression) to prevent graft reject [2] and prevention of joint erosion. The major side effects may include nephrotoxicity, neurotoxicity, diabetogenicity, and gastrointestinal toxicity [6]. Other newer substances such as 3, 5-bistriflouromethyl pyrazole (BTP) derivatives are able to reduce the cytokine levels in Th1 and Th2 T-cells without Calcineurin enzyme inhibition [6]. From these reasons, the discovery of new lead compounds is a need especially from natural sources (both Cyclosporine and Tacrolimus (FK506) are discovered and isolated form natural source form earth fungi *Tolypocladium inflatum* and bacteria *Streptomyces tsukubaensis* respectively). There are many reports showed that some of plant extracts may be used as therapeutic immunomodulatory. Besides, there are several studies reported that the Ca^{2+}-NFAT signaling may control cell differentiation and cell development in many cell types, and tissues, in case of malfunctioning of the Ca^{2+}-NFAT signaling may causes cell and tissue abnormal growth, leading to cancer [7].

The development of cell-based model for NFAT nuclear-cytoplasm translocation of this study will be useful for screening herbal bioactive immunomodulators based on inhibitory or stimulatory responses of NFAT nuclear-cytoplasm translocation. The cells used in this model are porcine skin fibroblast and HEK293 cell lines. The system is convenient, high throughput, and low cost, since the model does not need actual T cells which is time consuming process for extraction, low yields, and high cost. Isolated primary porcine fibroblasts from pig skin tissue is easy to isolated and cultured with high yields, and is similar human fibroblasts. Moreover, in the model the RHR (Rel homology region) of NFAT proteins are deleted in order to prohibit functional NFAT proteins which may cause any negative or positive effects on cultured cells such differentiation, abnormal cellular functions. Still, the model maintains its ability to maintain cytoplasm-nucleus transport of the NFAT proteins. The modified NFAT proteins also fused with green fluorescent protein (GFP), this will allow easily tracking cytoplasm-nucleus transportation of NFAT proteins by fluorescent microscope. Furthermore, this system provides real time cytoplasm-nucleus transport of the NFAT proteins via fluorescent microscope.

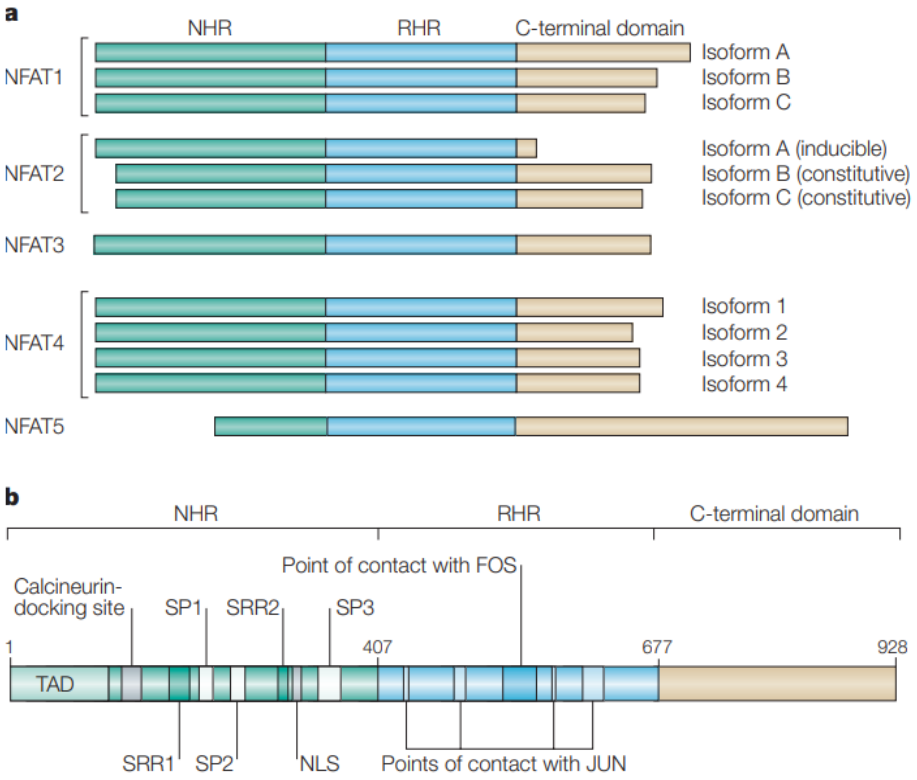
The application of the cell-based model in this study will be used for screening bio-active compounds that contain stimulatory or inhibitory effect on NFAT Nuclear-cytoplasm translocation (immunomodulator). In the study, as an example for this model application, we use the extracts from plants commonly found in Thailand and supportive therapeutic information, of which led compounds can be developed to be future therapeutic drugs. From literature reviews by [8] there were some herbal extracts reported to have the effect on interleukin-2 and other cytokines production such as the extracts form *Pinus maritima*, *Silybum marianum* (Milk Thistle) , *Tripterygium wilfordii*, *Withania somnifera.* Several studies In Thailand, also reported the similar effects by the extracts from *Allium sativum* (กระเทียม), *Caesalpinia sappan* (แก่นฝาง) *Murdannia loriformis* (หญ้าปักกิ่ง), *Centella asiatica* (ใบบัวบก), etc. [9]. However, the mechanisms at the molecular level of those extracts are still uncertain. Therefore, the application of this study model would be able to confirm the activity of those extracts with a timely fashion and high output. After the activity of the chosen extracts is positively identified, purification of the lead compounds can be further explored.

CHAPTER II

LITERATURE REVIEW AND RELATED RESEARCH

NFAT (Nuclear Factor of Activated T cells)

NFAT is a family of transcription activators that binds to specific DNA sequences and activates the DNA transcription, especially the transcription of cytokine genes in activated T cells. This family consists of NFAT1, NFAT2, NFAT3 (a dominant negative NFAT), NFAT4, and NFAT5. The molecular structure of NFAT protein can be divided into three main regions (domain) (Figure 1), regulatory domain or NFAT homology region (NHR), highly conserved DNA binding domain or Rel-homology region (RHR) and carboxy-terminal domain. The NFAT homology region (NHR) contains N-terminal transactivation domain (TAD), binding sites for Casein kinase1 (CK1) enzyme and Calcineurin (phosphatase) enzyme, multiple serine-rich region - SRR and SP motifs (targets of phosphorylation by kinase enzymes and dephosphorylation by phosphatase enzymes), and nuclear localization sequence (NLS) regulatory domain, which controls the NFAT proteins localization between nucleus and cytoplasm of the cell. The RHR region is found to be a highly conserved amino acid sequence among NFAT proteins in the family, this domain functions as a DNA binding site to initiate various gene expression in T cells [5].



Motif	Amino-acid sequence	Maintenance kinase	Export kinase
SRR1	SPASSGSSASFISD	CK1	CK1
SP2	SPRTSPIMSPRT	ND	GSK3
SP3	SPQRSRSPSPQPSP	ND	ND (GSK3 for NFAT2)

Figure 1 The drawing represents the molecular structure domains of NFAT proteins in the NFAT family of transcription factor.NHR (NFAT homology region) contains N-terminal transactivation domain (TAD), Casein kinase1 (CK1) and Calcineurin phosphatase binding sites, multiple serine-rich region - SRR and SP motif (phosphorylation site for kinase enzymes) and dephosphorylation site by phosphatase enzymes), and Nuclear Localization Sequence (NLS) regulatory domain RHR (Rel-homology region) contains DNA binding site (highly conserved DNA binding domain) C-terminal domain (carboxy-terminal domain) [5]

NFATs play a significant role as the promoter of the expression of cytokines such as interleukin-2 (IL-2), IL-3, IL-4, and tumor necrosis factor α (TNF-α) which are related to T cell activation and immune response [7]. The NFAT3 is known to be a dominant negative [3, 10, 11]. The mechanism of T cells stimulation of NFAT proteins is demonstrated in Figure 2, defined by its translocation from its inactive form in the cytoplasm to its active form in the nucleus, where it can bind to promoter regions on cytokine genes and activate their transcription [12]. Its activity is aided by calcineurin, a Ca^{2+}/calmodulin-dependent phosphoserine/threonine phosphatase, which plays a key role in NFAT's ability to translocate into the nucleus and act as a transcription factor [10]. Calcium signaling is critical to NFAT activation because calmodulin, a well-known calcium sensor protein, activates calcineurin [1, 4, 13, 14]. Once activated, calcineurin rapidly dephosphorylates the serine-rich regions (SRR) and SP-repeats in the amino termini of NFAT proteins [15, 16], resulting in a conformational change that exposes a nuclear localization signal [17], and leading to NFAT nuclear import [5, 10, 18]. Nuclear import of NFAT proteins is opposed by maintenance kinases in the cytoplasm and export kinases in the nucleus. Export kinases, such as PKA and GSK, must be inactivated for NFAT nuclear retention [7, 19].

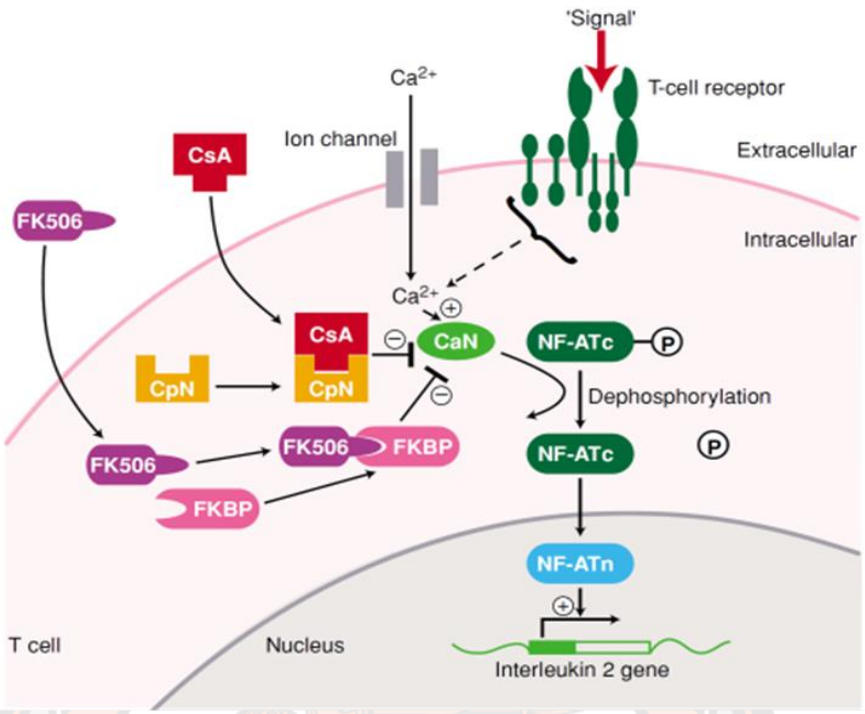


Figure 2 The drawing demonstrates the mechanism of NFAT proteins (Nuclear Factor of Activated T cells pathway). After T-cell receptor (TCR) activation by "ligands" or signaling molecules, the calcium ion concentration in cytoplasm is increased. The increased concentration of calcium ion activates Calcineurin enzyme (phosphatase), leading to NFAT dephosphorylation, resulting to NFAT translocation (NFAT Import) to the nucleus, NFAT-DNA binding, and finally expression of Interleukin-2 gene. The Casein kinase1 (CK1) and Glycogen-synthase kinase3 (GSK3), which localize inside the nucleus, deactivate NFAT protein by NFAT phosphorylation, leading to NFAT-DNA detachment and NFAT protein relocalization to cytoplasm (NFAT Export). Cyclosporine A (CsA) which is able to interact with Cyclophylin (CpN) protein, also Tacrolimus (FK506) which is able to interact with FK506-binding protein (FKBP), these complexes inhibit the function of Calcineurin (CaN) enzyme, so the phosphatase activity is decreased. Finally, the NFAT proteins nucleus translocation is inhibited. (Expert Reviews in Molecular Medicine 2000 Cambridge University Press)

Calcineurin

Calcineurin is a protein phosphatase also known as protein phosphatase, and calcium-dependent serine-threonine phosphatase [12, 20]. It activates the T cells of the immune system and can be blocked by specific drugs. Calcineurin activates nuclear factor of activated T cell (NFAT) by dephosphorylating it (Figure 4). The dephosphorylated NFAT then translocates into the nucleus, where it up-regulates the expression of interleukin 2 (IL-2), which, in turn, stimulates the proliferation and differentiation of T cells. Calcineurin is therefore the target of a class of immuno-suppressing drugs called calcineurin inhibitors, such as cyclosporin A (CsA) and Tacolimus (FK506). Calcineurin and NFAT protein have been shown to participate in signaling cascade that governs the development and function of the immune, nervous, cardio-vascular and musculo-skeletal systems [15].

Cyclosporin A (CsA) and Tacolimus (FK506)

CsA and FK506 are originally the substances isolated from fungus and bacteria respectively. Both have been clinically used as immune-suppressant in tissue-transplanted patients to prevent graft rejection and patients with autoimmune diseases. CsA and FK506 bind to their intracellular receptors, immunophilins, creating composite surface that block the activity of specific targets. For CsA/cyclophilin and FK506/FKBP (FK binding protein) the target is Calcineurin [21]. Because of the large surface area of interaction of the drug-immunophilin complex with calcineurin, FK506 and CsA have a specificity for their biologic targets that is equivalent to growth factor receptor interaction. Inhibition of the activity of calcineurin results in a complete block in the translocation of the cytosolic component of the NFAT, resulting in a failure to activate the gene regulated by the NFAT transcription factor. These genes include those required for B-cell help such as interleukin and CD40 ligand as well as those necessary for T-cell proliferation such as IL-2 [22, 23]. The immunophilin–drug complex (cyclophilin A-cyclosporin A) is suggested as a noncompetitive inhibitor of calcineurin phosphatase activity [24]. Due to, this immunophilin–drug complexes bind to the active site of catalytic and regulatory subunits of calcineurin by forming the multiple hydrogen bonding. Particularly, H-bonding between immunophilin complex and Arg-122 residue

of calcineurin is involved in forcing reorientation of the Arg-122 side chain, which affects the calcineurin catalytic activity [25].

CHAPTER III

RESEARCH METHODOLOGY

Part 1 Cloning and modification of NFAT genes, and cell cultures (Development of cell-based model of NFAT nuclear-cytoplasm translocation)

1. Cloning NFAT4 (full length) and NFAT3 (full length) from available cDNA library, then inserting NFAT4 and NFAT3 (as dominant negative NFAT) gene into a plasmid, which contains gene of fluorescent protein (GFP- green fluorescent protein or YFP- yellow fluorescent protein).

2. Deleting of RHR domain out of NFAT4 (full length) and NFAT3 (full length) whereas NHR domain still intact to get "NFAT4(N)" and "NFAT3(N)" respectively, in order to avoid the functional (active) proteins once the these gene are transcripted and translated into proteins to prevent the side effects on NFAT4 and NFAT3 proteins on cell cultures. By using specific primers with PCR technique, the modified NFAT4(N) and NFAT3(N) will be cloned and inserted into a plasmid in frame with fluorescent protein (GFP) to make GFP- NFAT4(N) and GFP- NFAT3(N) fusion gene.

3. GFP-NFAT plasmids in 2 will be propagated in *E. Coli* host and examined the right size and proper insertion of the modified genes, which will be further used in the further experiments in Part 2.

4. HEK cell line (Human Embryonic Kidney 293 cell line) which proven to be no NFAT protein expression and other cells such as primary fibroblasts (isolated form porcine skin) will be cultured. Primary porcine skin fibroblasts is used in this model because of its similarity to human origin, availability. Comparison to immortalized cell line, primary cells is more genetic instability, reducing un-expected results from genetic effect.

5. Experiment analysis and conclusion.

Part 2 Study the effect of modified GFP-NFATs on NFAT nuclear-cytoplasm translocation in cell cultures (Testing the Cell-based model of NFAT nuclear-cytoplasm translocation)

1. Introduce plasmid from Part 1 into cell cultures by transient transfection method.

2. Adding calcium ionophore A23187 to stimulate in increase of Ca^{2+} ion concentration in the cytoplasm. Calcium ionophore A23187 mimics the binding effect of T-cell receptor and its ligand to generate cellular Ca^{2+} ion influx and activate Calcineurin enzyme, which dephosphorylated NFAT protein (from plasmid gene expression via transient transfection), allowing NFAT nuclear import translocation.

3. Observing cultured cells with NFAT nuclear translocation by following the fluorescent protein – GFP, GFP-NFAT4(N), and GFP-NFAT3(N). Fluorescent patterns will be photographed and then counting for the numbers of positive and negative green fluorescent cells. The conclusion from the experiment can be drawn by comparison the result in experimental groups to the results from positive and negative control groups (calcium ionophore A23187 and Cyclosporine + calcium ionophore A23187), and form GFP-NFAT3(N) group (dominant negative NFAT, internal control).

4. Analysis by descriptive statistic (mean, SD.), and ANOVA and/or t-test statistic.

Part 3 Application of Cell-Based Model of NFAT nuclear-cytoplasm translocation for bio-active screening of herbal extracts by testing stimulatory or inhibitory effect on NFAT nuclear translocation (Screening of immunomodulators)

1. Obtaining the plant extracts from of interest and available in Thailand with supportive evidence pharmacologic effect as immunomodulators approx. 2-5 extracts such as from *Allium sativum* (กระเทียม), *Caesalpinia sappan* (แก่นฝาง) *Murdannia loriformis* (หญ้าปักกิ่ง), *Centella asiatica* (ใบบัวบก), *Curcuma longa (*ขมิ้นชัน*)*, *Andrographis paniculata (*ฟ้าทะลายโจร*)*[9].

2. Testing the plant extracts mentioned above by using developed cell model to investigate the effect on NFAT nuclear translocation. The cultured cell

group treated with calcium ionophore A23187 is used as a positive control. The standard NFAT suppressor (Cyclosporine) treated group is used as a negative control. The GFP-NFAT3(N) transfected group which is a dominant negative NFAT is used as an internal control.

3. If the experiment in 2 has a positive result, the extract will be selected for further purification until pure active compound is isolated.

4. Re-testing the purified compound from 3 using the same cell-based for NFAT nuclear translocation model to confirm pharmacologic response and its potency (lead active compounds).

5. Analysis, conclusion by statistic tool such as ANOVA, t-test for statistical significant level.

Culture of HEK cell line and primary porcine skin fibroblasts

The culture of HEK cell line and primary porcine skin fibroblasts will be in DMEM medium (Gibco) supplemented with 10% FBS (Gibco) and penicillin 100U/ml and streptomycin 100µg/ml (Gibco) the volume of 500µl medium solution will be used in each 24-well plate. The density of cells will be 15,000 cells/cm^2, culture for 18-24 hours at 37°C, CO_2 at 5%. Then the plasmid will be transfected into the cultured cells by transient transfection method using Lipofectamine™ 2000 (Invitrogen).

Transient transfection of the plasmid in to the cultured cells

The plasmid will be introduced into the cultured cells by the method of transient transfection. The GFP plasmid, GFP-NFAT3 plasmid, and GFP-NFAT4 plasmid will be transfected into HEK293 and primary porcine skin fibroblasts cells by Lipofectamine as follows.

1. Dilute 300ng. of plasmid pcDNA3.1_GFP-NFAT4(N) vector, pcDNA3.1_GFP-NFAT3(N) vector or pcDNA3.1_GFP vector in 25µl of basal cell culture media (no fetal bovine serum and Pen/Strep).

2. Dilute 1µl Lipofectamine™2000 in 25µl basal cell culture media (no fetal bovine serum and Pen/Strep), incubate the solution in 1 and 2 for 5 minutes in room temperature.

3. Mix 25µl Lipofectamine™2000 (in 2) and 25µl diluted DNA (in 1), then incubate the mixture 20 minutes for complete of DNA-Lipofectamine™2000 complex.

4. Pipet 50µl of DNA-Lipofectamine™2000 complex in 3 to each well of 24-well plate containing 18-24 hours cultured cells in 200µl of media (supplemented with 5%FBS without Pen/Strep).

5. Incubate plasmid transfected cells at 37°C, 5% CO_2 for 6 hours.

6. Then, add 750µl of complete media (10% FBS, Pen/Strep). Culture the cells further at 37°C, 5% CO_2 for another 18 hours to allow the cells to make protein corresponding to the transfected plasmid.

7. Finally the cells are ready for the planed experiments.

Study of nuclear translocation of NFATs

In each experiment group (control group, GFP group, GFP-NFAT4(N) group, and GFP-NFAT3(N) group) will get stimulation of cytosolic calcium ion increase by the compound calcium ionophore (A23187) (SIGMA-ALDRICH) 1µM for 30 minutes (mimicking the stimulation by ligand-T-cell receptor stimulation). By doing so, the calcium ion concentration in the cytoplasm will be increased and high enough to activate calcineurin enzyme in the cytoplasm. The activated enzyme will downstream activate NFAT protein (form plasmid expression, de-phosphorylated form) allowing the NLS motif (nuclear localization signal) to be exposed and translocate itself from the cytoplasm into the nucleus (nuclear-cytoplasm translocation). As a transcription factor, activated NFAT is able to turn on related gene expression involved in immune function such as interleukin-2 gene (Figure 2). In order to confirmed that activation of nuclear translocation of the NFAT protein is through the calcineurin enzyme, in the experiment, cyclosporine A (CsA) (Merck), a natural calcineurin inhibitor isolated from the fungus *Tolypocladium inflatum* at two concentration levels (1 and 5µM 45 minutes incubation) will be used. The fluorescent microscope (CARL ZEISS-AXIO OBSERVER Z1) will be used for NFAT translocation patterns and image will be captured for further analysis.

Statistical analysis

The number of positive cells (nucleus-GFP signal positive cells) and the number of negative cells (cytoplasm-GFP signal positive cells) in four random fields will be individually counted (4 fields per 1 coverslip) the results will report as % Mean±SD. (duplicate results). The data obtained from the experiments will be analyzed by repeated measurements of one-way analysis of variance - ANOVA at significance of $p \leq 0.05$.

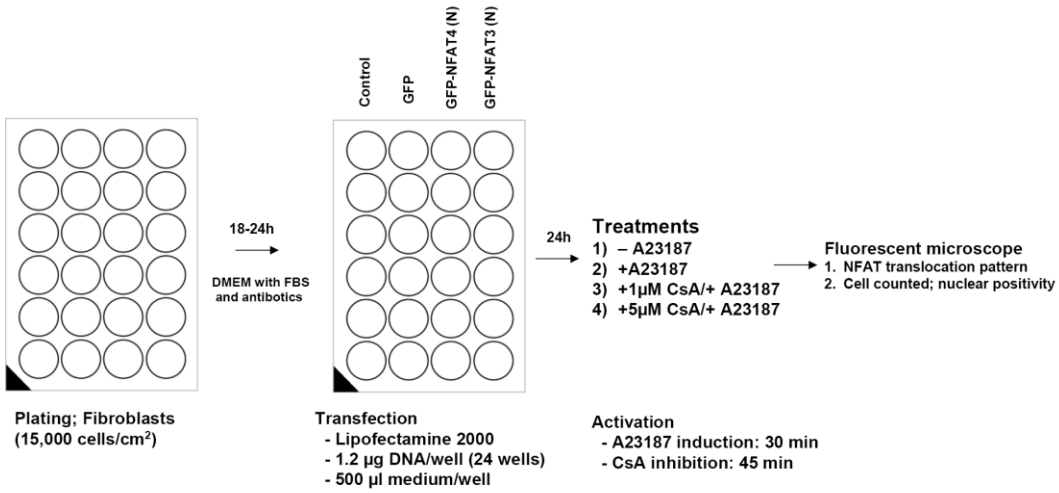


Figure 3 Time flow of experiment for study NFAT-nuclear translocation

CHAPTER IV

RESULTS

Part 1 Cloning and modification of NFAT genes, and cell cultures (Development of cell-based model of NFAT nuclear-cytoplasm translocation)

1. Using PCR (polymerase chain reaction) method, NFAT4 (full length) and NFAT3 (full length) (a dominant negative NFAT) from human cDNA library can be cloned. Amino acid sequence of NFAT3 (full length) and NFAT4 (full length) is shown in Figure 4 below.

Figure 4 The image demonstrates an amino acid sequence of NFAT4 and NFAT3 full length protein, the underlined sequence represents a regulatory domain (NHR) where the Nuclear localization signal domain and calcineurin docking domain located, other parts are DNA binding domain (RHR) and C-terminal domain

2. After Regulatory region (NHR), which contains Nuclear Localization Signal domain and calcineurin docking domain of NFAT4 and NFAT3 gene, is cloned and designated as NFAT4(N) and NFAT3(N) to avoid un-wanted effects of wild type proteins (Figure 4), the construct NFAT4(N) and NFAT3(N) gene is ligated into a vector, plasmid pcDNA3.1 (Figure 5) which contains green fluorescent protein (GFP) gene at the ligation site. The size of NFAT4(N) is 1,053 bps (351 amino acids), ligated at XhoI and ApaI endonuclease sites. The size of NFAT3(N) is 1,023 bps (341 amino acids), ligated at BamHI and XbaI endonuclease sites. Finally, GFP fusion proteins, GFP-NFAT4(N) and GFP-NFAT3(N) are generated as shown in Figure 6

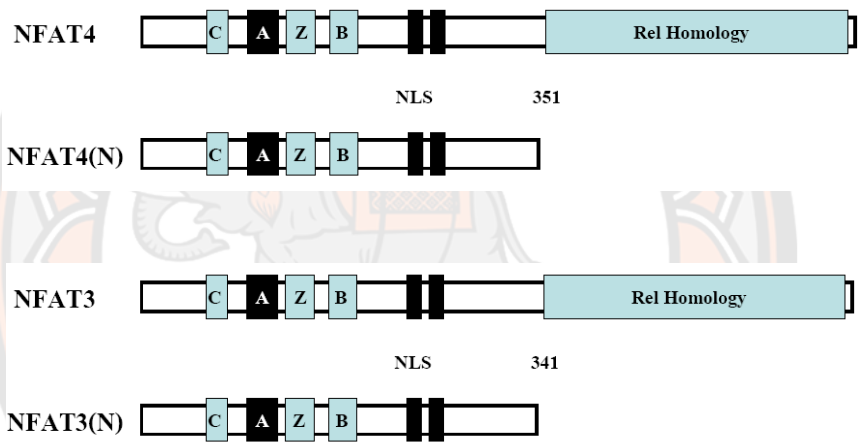


Figure 5 The drawing demonstrates essential domains of human NFAT4, NFAT4(N), NFAT3, and NFAT3(N) constructs. The C-terminal (Rel homology domain) contains DNA binding domain, The N-terminal contains calcium-depending nuclear shuttling regulatory domain. The C domain represents Calcineurin interacting amino acid sequence (putative calcineurin binding site), the A domain represents serine rich amino acid sequence (serine rich domain), the Z domain contains NLS signal amino acid sequence (NLS masking domain), and the B domain represents the linker region. NLS = Nuclear Location Signal

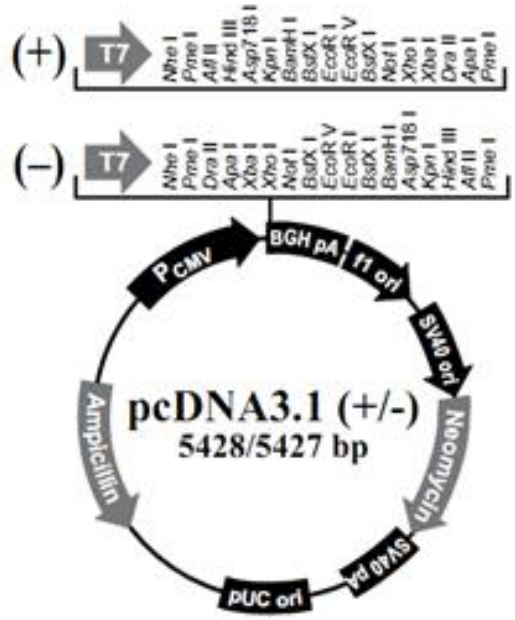


Figure 6 The shutter and expressing vector, Plasmid pcDNA3.1 (+) with endonuclease enzyme restriction sites for fashioned gene constructs, GFP-NFAT4(N) and GFP-NFAT3(N)

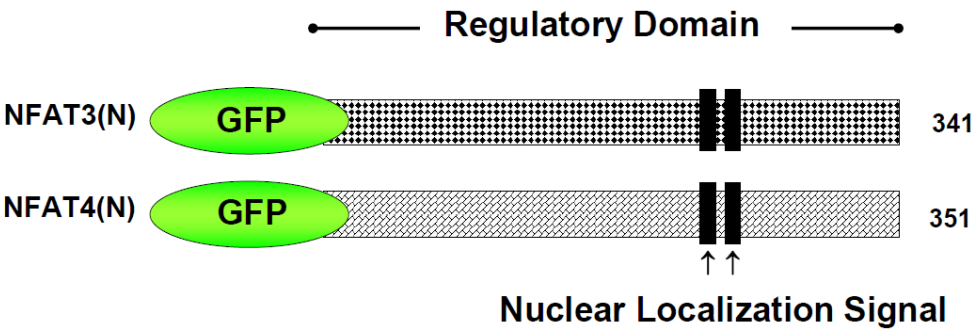


Figure 7 Diagram shows the final construct of GFP-NFAT fusion protein GFP-NFAT3(N) and GFP-NFAT4(N), after GFP gene (Green fluorescent protein) is placed at the N-terminal of NFAT3(N) and NFAT4(N) gene

3. All plasmids of pcDNA3.1_GFP-NFAT3(N), pcDNA3.1_GFP, pcDNA3.1_GFP-NFAT4(N) are propagated in the host, *E. Coli* (DH5α). All plasmids are purified by plasmid isolation kit (PureLink®). The proper sizes of each genes and plasmids are checked by agarose gel electrophoresis. The PCR technique, using T7 and SP6 specific primers and restriction endonucleases technique are applied. (Figure 8, 9). All plasmids are ready for transient transfection into the cell –based model in the next Part 2.

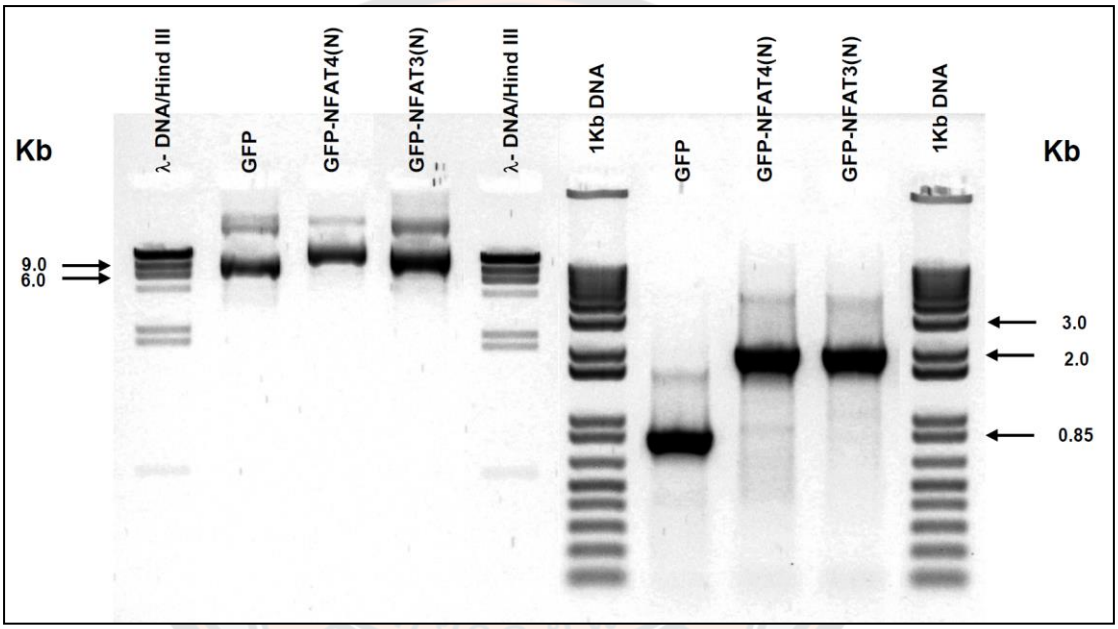


Figure 8 Agarose gel electrophoresis demonstrates the sizes of plasmid pcDNA3.1_GFP ~ 6 Kb plasmid (lane 2) pcDNA3.1_GFP-NFAT4(N) ~ 8 Kb (lane 3) and pcDNA3.1_GFP-NFAT3(N) ~ 8 Kb (lane 4) respectively. The sizes of (gene fragments) GFP gene ~ 0.8 Kb (lane 7), GFP-NFAT4(N) gene ~ 2 Kb (lane 8) and GFP-NFAT4(N) gene ~ 2 Kb (lane 9)

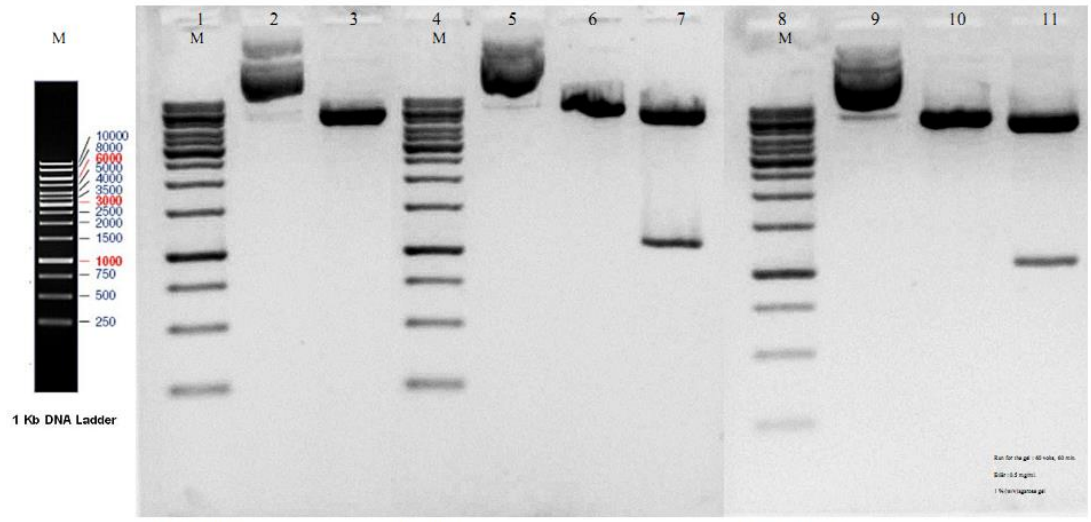


Figure 9 Agarose gel electrophoresis demonstrates the proper sizes of NFAT4(N) gene which is 1,053 bps, NFAT3(N) gene which is 1,023 bps. All genes are cloned into plasmid pcDNA3.1, which contains green fluorescent protein (GFP) gene. The cloning sites XhoI and ApaI is for NFAT4(N) gene and cloning sites BamHI and XbaI is for NFAT3(N) gene

4. Culturing of HEK cell line (Human Embryonic Kidney 293 cell line) and fibroblasts. The HEK cells and primary fibroblasts is reported that no NFAT protein expression in the cells.

We have successfully cloned NFAT4(N) gene (DNA 1,053 bps or 351 amino acids), and NFAT3(N) dominant negative gene (DNA 1,023 bps or 341 amino acids) from human cDNA library. To avoid the molecular side effects of intact NFAT proteins, NFAT4 and NFAT3 on the cultured cells only N-terminal domain of NFAT genes NFAT4(N) and NFAT3(N) are cloned. The N-terminal of NFATs are still able to provide docking site for calcineurin and NLS domain (nuclear localization signal domain). Once NFAT protein is dephosphorylated by calcineurin (phosphatase enzyme), the NFAT protein gets activated by exposing NLS and translocated into the nucleus. Difference from full length NFAT both NFAT4(N) and NFAT3(N) contains

no DNA binding domain and C-terminal domain, so it can not bind to promoter sequence on DNA sequence of the chromosome and so no gene product expression occurs. The GFP-NFAT fusion proteins of NFAT4(N) and NFAT3(N) are constructed in order to generate the observable trekking signal for NFAT protein cytoplasm-nucleus translocation pattern of NFAT4(N) and NFAT3(N) protein that is convenient to observe by fluorescent microscope. Furthermore, we are successful find the suitable conditions to culture HEK cell lines and primary cells (porcine fibroblasts). After the experiments in Part 1 is finished, the cells and all created plasmids (mentioned above) are ready for the next experiments in Part 2.

Part 2 Study the effect of modified GFP-NFATs on NFAT nuclear-cytoplasm translocation in cell cultures (Testing Cell-based model of NFAT nuclear-cytoplasm translocation)

Transient transfection method is used to introduce the plasmids (from Part 1) into the cultured cells, the cells are able to produce protein corresponded to gene encoded in the plasmid. After calcium ionophore A23187 is added to the culture medium, the Ca^{2+} ion in cytoplasm of the cells will be increased, mimicking the stimulation of T-cell receptor by its ligand (Figure 2). The increase in calcium ion in the cytoplasm activates calcineurin enzyme, and then calcineurin enzyme de-phosphorylates the phosphorylated form of NFAT to dephosphorylated form, allowing NFAT protein to move into the nucleus (nuclear import translocation). We are able to follow this NFAT cytoplasm-nuclear translocation by following green fluorescent protein- GFP (GFP, GFP-NFAT4(N) and GFP-NFAT3(N)) signals. Accumulation of GFP fluorescent signal within nucleus is increased once calcineurin enzyme is activated (in case of NFAT4(N)), and the signal can be capture and photographed by the microscope software. By counting the number of positive cells (GFP signal accumulation in nucleus) or negative (GFP signal accumulation in cytoplasm), compared with positive control group (calcium ionophore A23187 alone) and negative control group (cyclosporine + calcium ionophore A23187) and GFP-NFAT3(N) group (a dominant negative NFAT as internal control)**,** meaningful results can be achieved.

1. Testing the effect of calcium ionophore (A23187) on cytoplasm-nuclear translocation of NFAT4(N), NFAT3(N) and control plasmid (GFP vector)

As shown in Figure 10, this figure demonstrate the theoretical (expected) results which are true when the actual experiments are conducted. After calcium ionophore is added the NFAT4(N) translocates into the nucleus, but as a dominant negative the NFAT3(N) does not respond to calcium ionophore stimulation. The NFAT3(N) protein still locate in the cytoplasm. For GFP protein, which used for GFP-NFAT fusion (NFAT3(N) and NFAT4(N)) protein trekking, the signal of GFP protein distribute throughout the cells (fuzzy signal) in both with or without calcium ionophore stimulation. The results are consistent in both HEK293 cell lines (Figure 10) and primary porcine fibroblasts (Figure 11)**.** Since the HEK293 cell line is low in cost, and easy and cheap maintenance when compared to the primary fibroblasts isolated from pig skin tissue, so the rest of the experiments the HEK293 cell-base will be used.

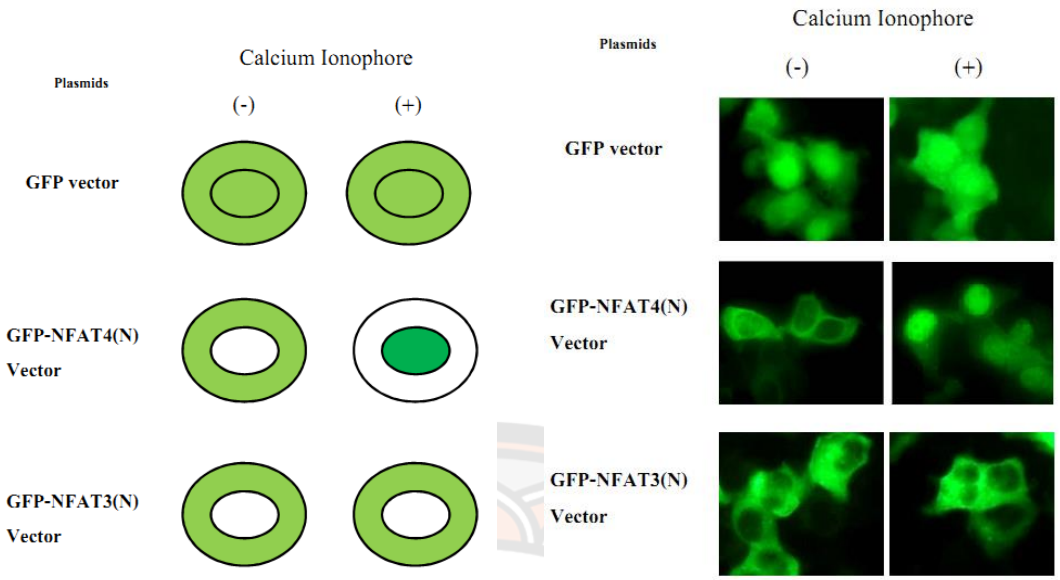


Figure 10 Demonstrates the theoretical results of NFAT nuclear translocation patterns (left panel) and actual results from the experiments (right panel) of GFP-NFAT4(N), GFP-NFAT3(N) and GFP proteins in the HEK293 cell line before and after calcium ionophore stimulation. Before calcium ionophore stimulation, NFAT4(N) distributes in the cytoplasm, after calcium ionophore stimulation, NFAT4(N) translocates and to the nucleus. In a case of NFAT3(N), which is a dominant negative, does not respond to calcium ionophore stimulation, no nuclear signal accumulation observed. In a case of GFP protein, the signals distribute in both in the cytoplasm and nucleus in both with and without calcium ionophore stimulation. (20X, HEK293 cell line with/without 1μM A23187 30 min.)

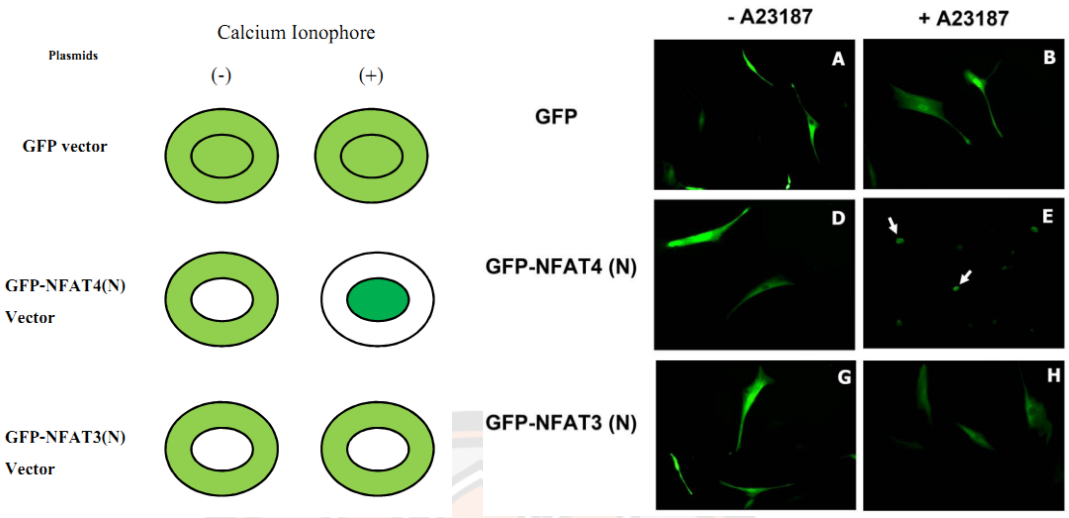


Figure 11 Demonstrates the theoretical results of NFAT nuclear translocation patterns (left panel) and actual results from the experiments (right panel) of GFP-NFAT4(N), GFP-NFAT3(N) and GFP proteins in the primary porcine fibroblasts before and after calcium ionophore stimulation. Before calcium ionophore stimulation, NFAT4(N) distributes in the cytoplasm, after calcium ionophore stimulation, NFAT4(N) gets activated and translocated into the nucleus. In a case of NFAT3(N), which is a dominant negative, does not respond to calcium ionophore stimulation, no nuclear signal accumulation observed. In a case of GFP protein, the signals distribute in both in the cytoplasm and nucleus in both with and without calcium ionophore stimulation. (20X, primary porcine fibroblasts with/without 1μM A23187 30 min.)

2. Testing the effect of cyclosporine A (CsA) on cytoplasm-nuclear translocation of NFAT4(N), NFAT3(N) and control plasmid (GFP vector)

As shown in the Figure 12 the effect of cyclosporine A (CsA) on cytoplasm-nuclear translocation of NFAT4(N), NFAT3(N) and control plasmid (GFP vector) has clearly demonstrated. The upper panel shows the diagram representing theoretical NFAT nuclear translocation results and the lower panel shows the photograph representing actual NFAT nuclear translocation results taken from the cells with GFP-NFAT4(N), GFP-NFAT3(N) and GFP plasmid transient transfection. The actual results are correlated well with the theoretical results. Without activation (dephosphorylation), NFAT4(N) protein normally locates in the cytoplasm, but once calcium ionophore is added, NFAT4(N) gets activated and transported into the nucleus. When cyclosporine

A – CsA which is a inhibitor of calcineurin phosphatase enzyme is added, the nuclear translocation of NFAT4(N) protein is prohibited. In contrast, NFAT3(N) which is a dominant negative does not respond to calcium ionophore stimulation, no NFAT3(N) nuclear transportation significantly detected. In case of GFP protein which is used as a tracker for GFP-NFAT fusion (NFAT3(N) and NFAT4(N)), the result has shown that GFP protein is still distributed throughout the cells, with or without calcium ionophore stimulation. CsA does not have any effect on NFAT3(N) and GFP in both two concentrations (1 and 5μM).

Figure 13 is the conclusion results of the experiment. The positive cells (nuclear translocation) are individually counted, after A23187 treatment alone, and with/without calcineurin inhibitor CsA (1 or 5 μM). This has been done by after all slides (culture wells) are fixed, then the positive cells in each group (N = 8, 2X4 fields) are expressed as % positive nuclear NFAT-localize cells. ANOVA has been applied for statistic significance. It is clearly shown the stimulator calcineurin A23187 in 1μM for 30 minutes is able to activate NFAT4(N) protein, and drive the protein into the nucleus with more than 80% positive cells, that is statistically significant (ANOVA, $p < 0.01$). However, this effect can be inhibited by calcineurin inhibitor, cyclosporine A in both concentrations (1 and 5μM, 45 minutes before 1μM A23187 30 minutes incubation) (Image 20X, HEK293 cell line)

In conclusion, we have sucessfully cloned NFAT4(N) and NFAT3(N) gene. The experiments in this part have been demonstrated that the proteins expressed from the cloned plasmids are functionally intact and the results from the experiments are well correlated to the theory. Therefore, this cell-based model is ready for further applications such as the screening of bioactive compounds that affect on NFAT Nuclear-cytoplasm translocation (immunomodulator) especially the extracts form plants available in Thailand.

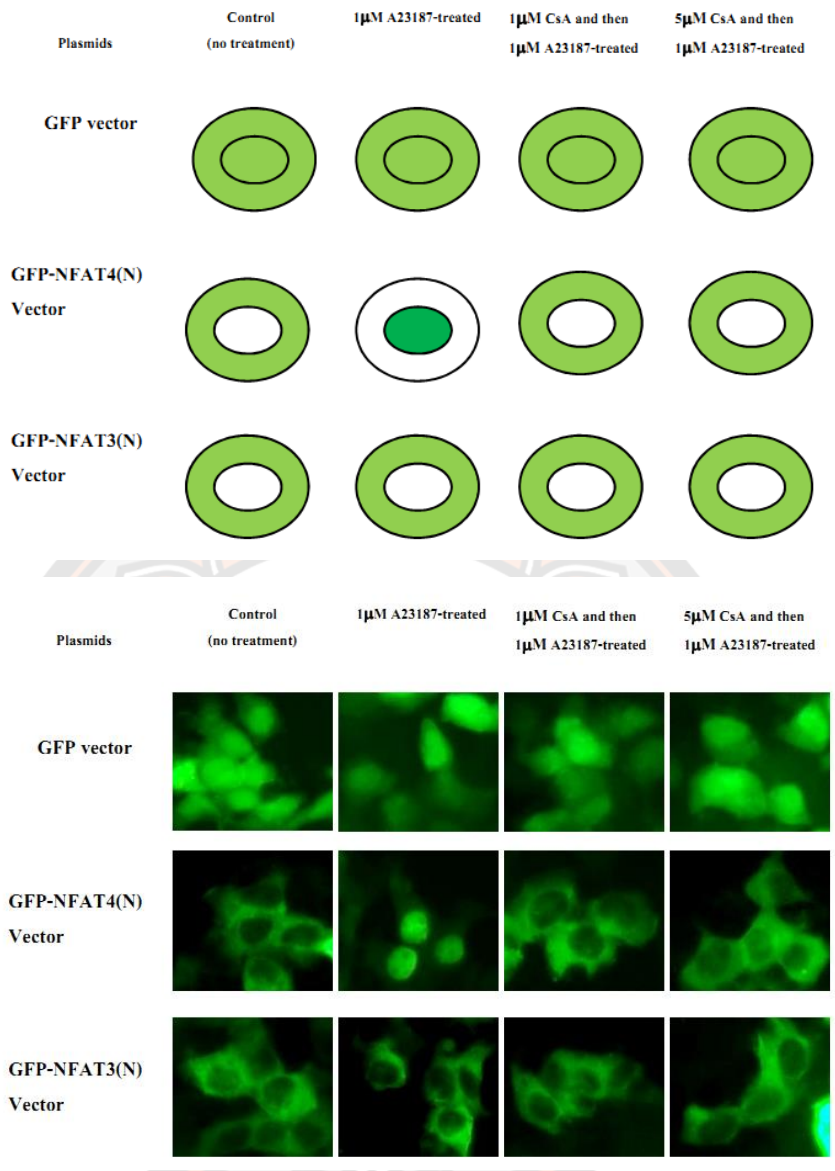


Figure 12 Demonstrates NFAT nuclear translocation in theory (upper panel) and from actual experiment (lower panel) of GFP-NFAT4(N), GFP-NFAT3(N) and GFP protein . The cultured cells are transient transfected by GFP-NFAT4(N), GFP-NFAT3(N) and plasmids. The NFAT4(N) protein normally locates the cytoplasm, once gets activated by calcium ionophore, the NFAT4(N) activated and moves to the nucleus. In the presence of cyclosporine A – CsA, an inhibitor of calcineurin phosphatase enzyme, the nuclear translocation of NFAT4(N) protein is inhibited Whereas, the NFAT3(N) protein (a dominant negative) does not respond to calcium ionophore

stimulation, no nuclear translocation of the NFAT3(N) protein detected. In case of GFP, a trekker for GFP-NFAT fusion (NFAT3(N) and NFAT4(N)) translocation, the GFP protein diffuses throughout the cells with or without calcium ionophore. The CsA (calcineurin inhibitor) does not have the effect on NFAT3(N) and GFP nuclear translocation from both concentrations (20X, HEK293 cell line treated with/without 1 or 5μM CsA 45 min. Then followed by 1μM A23187 30 min. treatment)

Table 1 CsA inhibits GFP-NFAT4 and GFP–NFAT3 plasmid activation by the calcineurin inducer A23187

Treatments	Percentage of positive cells for nuclear translocation (% Mean ± SD, N = 8 fields randomized)		
	GFP vector	NFAT4(N)	NFAT3(N)
Control	2.98±2.74	2.82±0.75	2.35±1.46
A23187 1μM	2.05±2.59	84.97±2.63	2.95±1.63
CsA 1μM then A23187	2.24±7.20	1.57±0.97	1.37±0.51
CsA 5μM then A23187	1.63±8.47	2.39±2.13	1.48±0.89

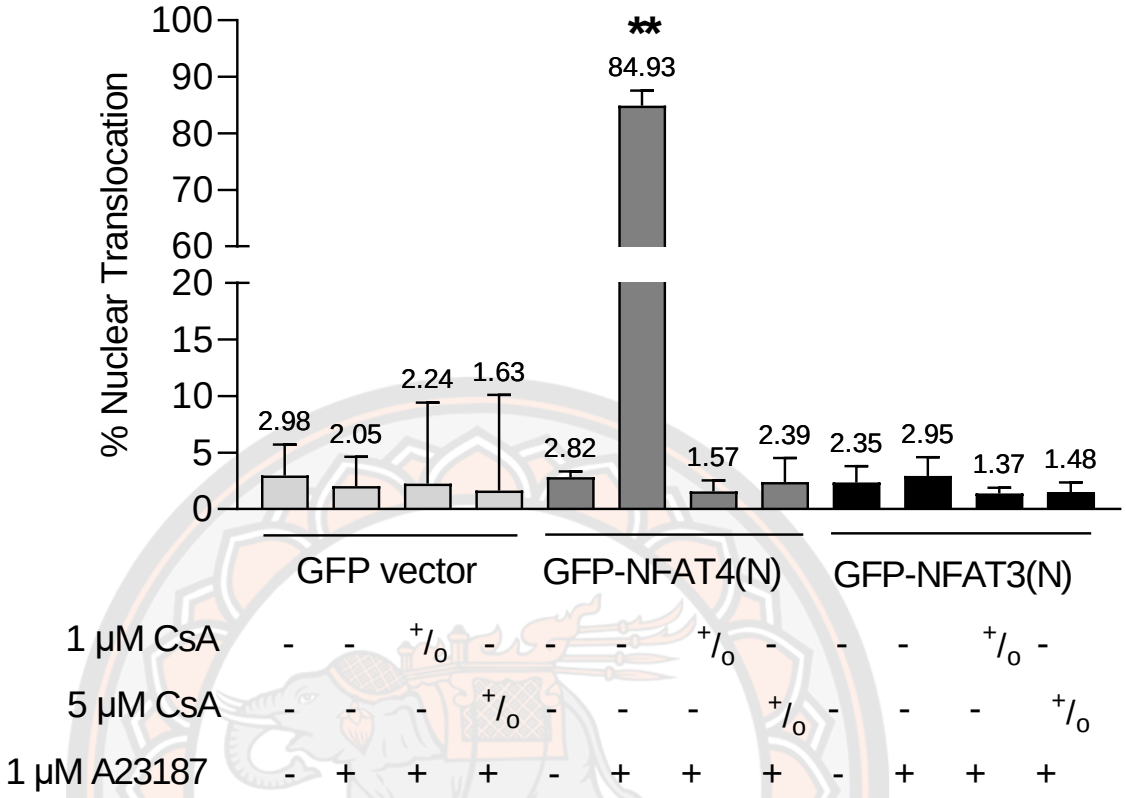


Figure 13 CsA inhibits GFP-NFAT4 and GFP–NFAT3 plasmid activation by the calcineurin inducer A23187.The percentage of NFAT nuclear translocation was determined from 8-randomized fields of cells after cells were pretreated with 1 µM or 5 µM of CsA for 45 minutes, then withdrawn and treated with 1 µM of A23187 for 30 minutes, respectively. Bar graphs are represented as mean ± SD. +/o; treat then withdrawn, - ; non-treat, + ; treat, ** p<0.01.

3. Testing the effect of calcium ionophore (A23187) concentrations on the cytoplasm-nuclear translocation of NFAT4(N)

Figure 14 demonstrates statistical results, in this experiment, each experiment group is exposed to A23187 in various concentrations 0.1, 0.2, 0.3, 0.4, 0.5, or 1µM for 30 minutes. Then, the slide (well) is fixed, the positive (nuclear translocation NFAT) cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** p <0.01 ANOVA). The result

has demonstrated that only treatment group of A23187 1μM for 30 minutes without calcineurin inhibitor CsA is able to generate sifgnificantly NFAT4(N) nuclear translocation (positive result) This clearly demonstrates "all or none effect" of A23187 stumulation, at least calcium ionophore (A23187) 1μM is required. However, this effect is effectively prohibited by CsA 1μM concentration.

Table 2 Validation of A23187 concentration activating NFAT4-nuclear translocation

Treatments	**Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± SD, N = 8 fields randomized)**
1μM CsA then 1μM A23187	1.18±1.34
0.1μM A23187	1.14±0.96
0.2μM A23187	1.18±1.28
0.3μM A23187	1.24 ±1.12
0.4μM A23187	1.32±1.43
0.5μM A23187	2.10±1.33
1μM A23187	78.21±6.85

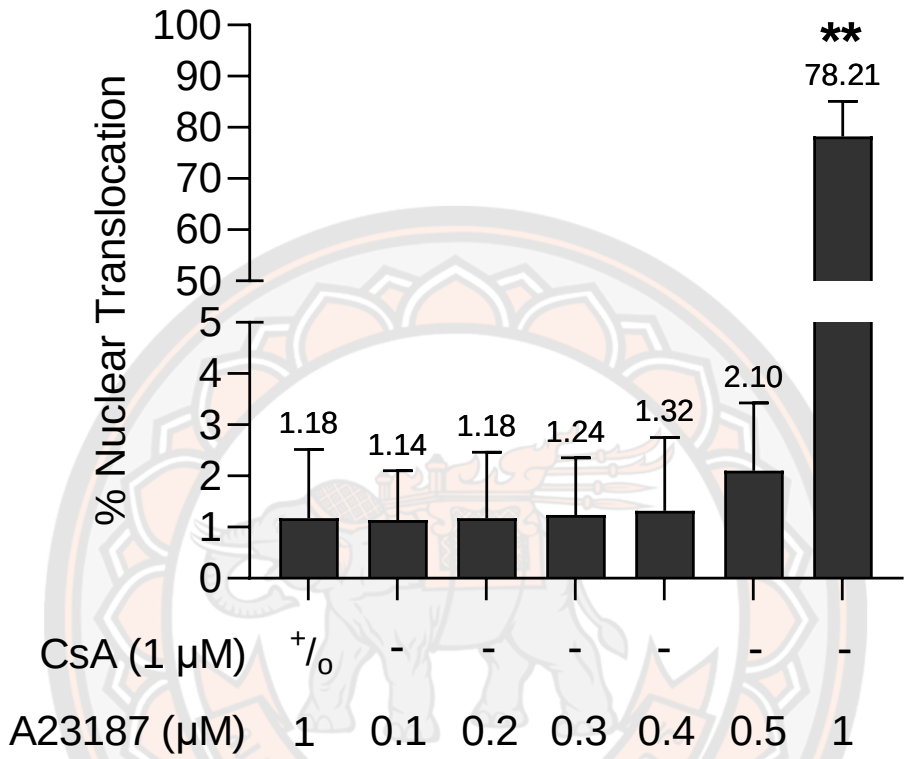


Figure 14 Validation of A23187 concentration activating NFAT4-nuclear translocation.The negative control, HEK293 cell line transfected GFP-NFAT4 plasmid were pretreated with 1 µM of CsA for 45 minutes then treat with 1 µM of A23187 for 30 minutes. An experimental group, cells were only treated with varying concentration of A23187 (0.1, 0.2, 0.3, 0.4, 0.5, and 1 µM) for 30 minutes. Then, 8-randomized fields of cell exhibiting –NFAT4 nuclear translocation were counted. The data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD.
+/o ; treat then withdrawn, - ; non-treat, ** p<0.01

4. Testing the effect of incubation time of calcium ionophore (A23187) 1μM on the cytoplasm-nuclear translocation of NFAT4(N)

Figure 15 demonstrates statistical results, in this experiment, each group is treated with A23187 1μM with different incubation times (5, 10, 15, 30, 60, or 120 minutes), before the slide is fixed and the positive cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** $p < 0.01$ ANOVA). It clearly shows that A23187 at 1μM incubation time vary form 5-120 minutes, all is able to activate NFAT4(N) nuclear translocation (positive result) in the same significance. Therefore, we can draw a conclusion that the incubation time of A23187 required for further experiment is at 30 minutes incubation.

However, as demonstrated in Figure 16, the number of passage of cell lines used in the experiment is important, the older cells (high passage number, 60 passage in this case) are less respond to A23187 stimulation, the maximum effect (response) is reduced significantly (from 80% to 35-60%) even longer incubation time, when compared to the younger cells (15 passage in Figure 15).

Table 3 Validation of the optimal timing of A23187 induces NFAT4-nuclear translocation

Treatments	**Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)**					
	5 min.	10 min.	15 min.	30 min.	60 min.	120 min.
1μM A23187	78.68± 6.27	80.20± 8.23	81.32± 7.28	76.06± 7.23	76.26± 4.82	74.51± 4.44

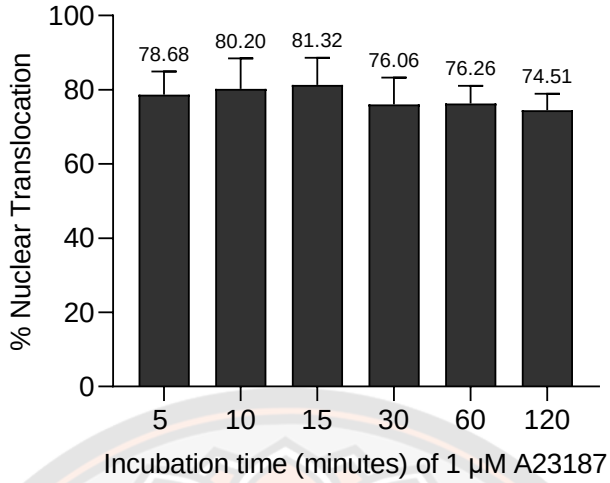


Figure 15 Validation of the optimal timing of A23187 induces NFAT4-nuclear translocation (passage #15)HEK293 cells passaging number fifteen transfected GFP-NFAT4 plasmid were treated with 1 µM of A23187 then further incubated for 5, 10, 15, 30, 60, and 120 minutes. After indicated time point, cells were fixed and counted GFP nuclear –positive cells from 8-randomized fields of cells. Data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD.

Table 4 Validation of the optimal timing of A23187 induces NFAT4-nuclear translocation in HEK293 cells late passage

Treatments	Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)					
	15 min.	30 min.	45 min.	60 min.	90 min.	120 min.
1μM A23187	34.51± 12.45	62.36± 4.61	66.73± 11.81	58.06± 10.61	49.77± 7.59	45.31± 7.03

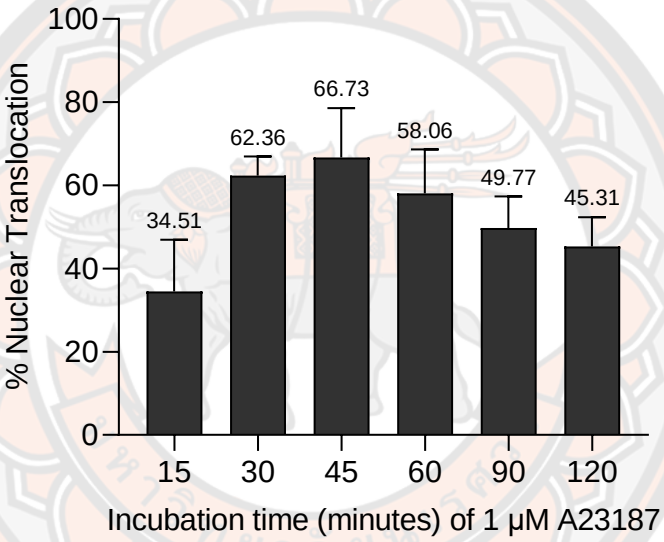


Figure 16 Validation of the optimal timing of A23187 induces NFAT4-nuclear translocation in HEK293 cells late passage (passage#60)HEK293 cells passaging number sixty transfected GFP-NFAT4 plasmid were treated with 1 μM of A23187 then further incubated for 15, 30, 45, 60, 90, and 120 minutes. After indicated time point, cells were fixed and counted GFP nuclear –positive cells from 8-randomized fields of cells. Data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD.

5. Testing the effect of withdrawal time of calcium ionophore (A23187) 1μM on cytoplasm-nuclear translocation of NFAT4(N)

Figure 17 demonstrates results from the experiment. After the cells is exposed to A23187 1μM for 30 minutes, then the medium is changed to new medium without A23187 (A23187 withdrawal) at different time points (15, 30, 45, 60, 120, or 180 minutes) before the slide is fixed and the positive cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** $p < 0.01$ ANOVA). The results clearly demonstrates that after A23187 1μM is withdrawn just only 15 minutes, the NFAT4(N) protein is significantly translocated back form the nucleus to the cytoplasm. The number of positive NFAT4(N) nuclear translocation decreased from 80% to 2% within 15 minutes and further decreased to less than 1% within 30 minutes. Therefore, the effect of calcium ionophore (A23187) is considered as transient and reversible.

Table 5 Validation of the retention time of A23187 induces NFAT4-nuclear translocation

Treatments	**Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)**						
	0 min	15 min	30 min	45 min	60 min	120 min	180 min
1μM A23187 (30min.)	80.59± 4.28	-	-	-	-	-	-
1μM A23187 (30min.) then complete media	-	1.98± 2.05	0.83± 1.11	0.32± 0.65	0.23± 0.66	0.32± 0.46	0.32± 0.45

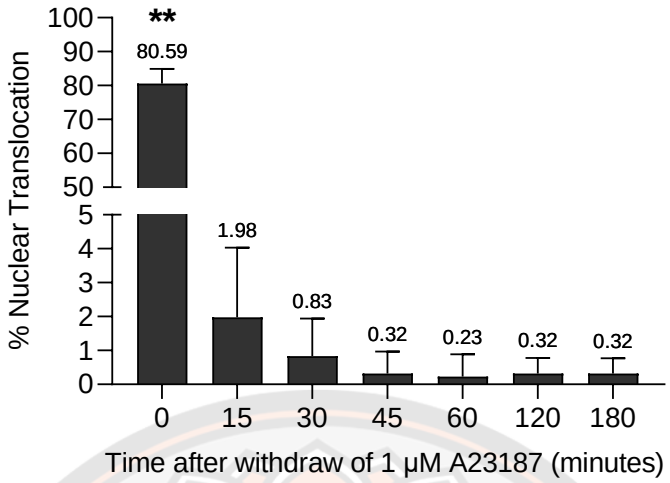


Figure 17 Validation of the retention time of A23187 induces NFAT4-nuclear translocation.HEK293 cells transfected GFP-NFAT4 plasmid were pretreated with 1 µM of A23187 for 30 minutes then withdrawn and further incubated in A23187 free medium for 15, 30, 45, 60, 120, and 180 minutes. After indicated time point of incubation, cells were fixed and counted GFP nuclear –positive cells from 8-randomized fields of cells. Data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD. ** p<0.01.

6. Testing of the effect of calcineurin enzyme inhibitor - CsA (cyclosporine A) on the cytoplasm-nuclear translocation of NFAT4(N)

Figure 18 demonstrate the results from the experiment to investigate the effect of CsA at various concentrations on NFAT4(N) nuclear translocation. After the cultured cells are incubated with CsA at different concentrations (0, 0.001, 0.01, 0.1, 0.5, 1, 2µM) for 45 minutes to inhibit NFAT4(N) nuclear translocation, the CsA are withdrawn, then A23187 1µM is added for 30 minutes, the slides are fixed and the positive cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** p <0.01 ANOVA). The results clearly demonstrates that the NFAT4(N) nuclear translocation can be significantly inhibited by CsA at only 0.1µM, % positive nuclear NFAT-localized cells dropped from nearly 80% to approx. 3%, and to approx. 1% at CsA 0.5µM and beyond. The inhibitory effect by CsA is very strong, that A23187 1µM 30 minutes exposure can not reverse the effect.

The older culture cell lines (high number of passage) are also used in this experiment, even though these high passage number cell lines have a lower response to A23187 stimulation, the cell older culture cell lines (in this case at 60 passage number) are still effectively inhibited by CsA at the same CsA concentration, at least 0.1 μM CsA (Figure 19).

Table 6 Validation of the optimal concentration of CsA inhibits A23187-induced NFAT4-nuclear translocation

Treatments	Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)
1μM A23187	79.03±3.45
0.001μM CsA then 1μM A23187	82.09±4.13
0.01μM CsA then 1μM A23187	78.02±5.40
0.1μM CsA then 1μM A23187	3.41±4.65
0.5μM CsA then 1μM A23187	0.43±0.49
1μM CsA then 1μM A23187	1.10±1.19
2μM CsA then 1μM A23187	0.50±0.39
Medium (control)	0.62±1.03

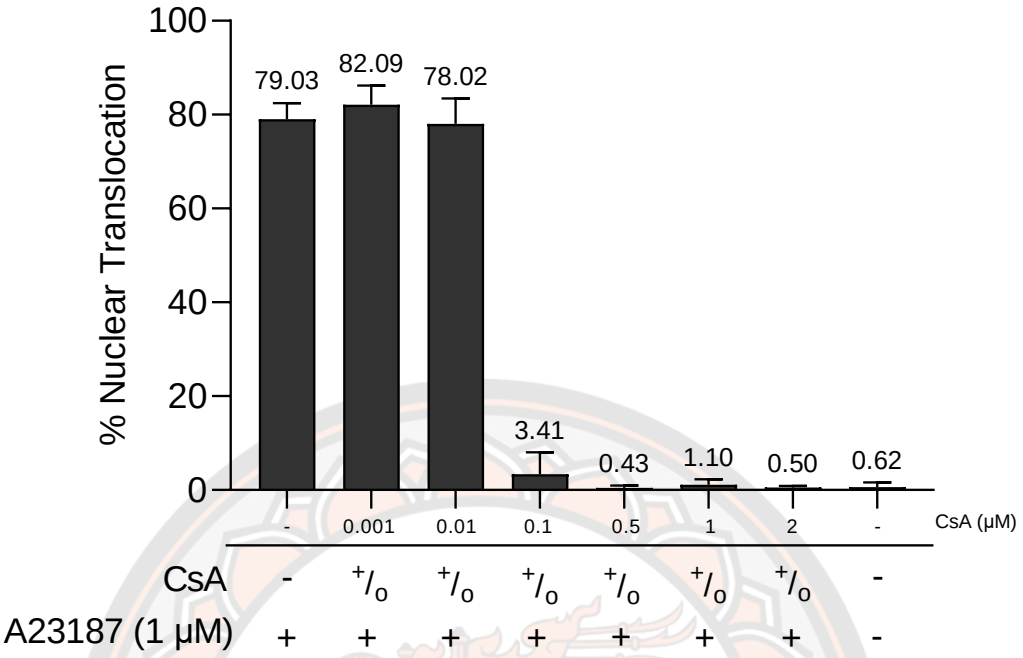


Figure 18 Validation of the optimal concentration of CsA inhibits A23187 induced NFAT4-nuclear translocation (passage #15)HEK293 cells passaging number fifteen transfected GFP-NFAT4 plasmid were pretreated with varying concentration of CsA for 45 minutes then withdrawn and further incubated in culture medium containing 1 μM of A23187 for 30 minutes. After indicated time, cells were fixed and counted GFP nuclear –positive cells from 8-randomized fields of cells. Data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD.

$^{+}/_{o}$; treat then withdrawn, -; non-treat, +; treat.

Table 7 Validation of the optimal concentration of CsA inhibits A23187-induced NFAT4-nuclear translocation in HEK293 late cell passage

Treatments	Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)
1μM A23187	64.30±8.72
0.01μM CsA then 1μM A23187	59.70±2.49
0.1μM CsA then 1μM A23187	1.10±0.74
0.5μM CsA then 1μM A23187	0.80±0.79
1μM CsA then 1μM A23187	0.90±0.98
2μM CsA then 1μM A23187	1.00±1.01
5μM CsA then 1μM A23187	0.80±0.58
Medium (control)	1.30±0.95

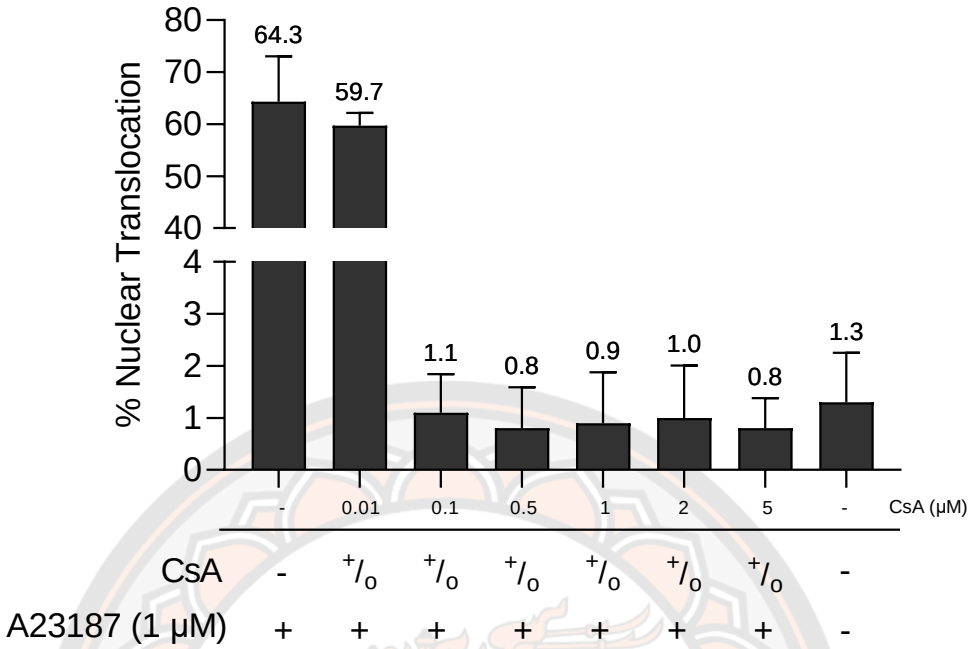


Figure 19 Validation of the optimal concentration of CsA inhibits A23187-induced NFAT4-nuclear translocation in HEK293 late cell passage (passage #60)HEK293 cells passaging number sixty transfected GFP-NFAT4 plasmid were pretreated with varying concentration of CsA for 45 minutes then withdrawn and further incubated in culture medium containing 1 μM of A23187 for 30 minutes. After indicated time, cells were fixed and counted GFP nuclear – positive cells from 8-randomized fields of cells. Data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD. $^{+}/_{o}$; treat then withdrawn, -; non-treat, +; treat

7. Testing of the effect of withdrawal time of calcineurin enzyme inhibitor – CsA (cyclosporine A) 1μM on cytoplasm-nuclear translocation NFAT4(N)

Figure 20 shows the results from the effect of withdrawal time of 1μM CsA. After the cultured cell lines are incubated with 1μM CsA to inhibit the NFAT4(N) nuclear translocation for 45 minutes, the CsA is withdrawn in each experimental group at different time points (30, 45, 60, 90 min., 2, 3, 6, 12, or 24 hours). Afterwards, A23187 1μM is added for 30 minutes to stimulate the NFAT4(N) nuclear translocation. The slides are fixed and the positive cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** p <0.01 ANOVA). The results demonstrates that CsA is a very potent inhibitor for the NFAT4(N) nuclear translocation. The inhibitory effect significantly lasts more than 24 hours after 1μM CsA withdrawal. Since the CsA inhibitory effect is still intact even after A23187 1μM is added, this suggests that the inhibition of CsA is likely non-competitive to A23187.

Table 8 Validation of the retention time of CsA inhibits A23187-induced NFAT4-nuclear translocation

Treatments	**Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)**									
	0min	30 min	45 min	60 min	90 min	2h	3h	6h	12h	24h
1μM A23187 for 30min.	79.93 ± 3.55	-	-	-	-	-	-	-	-	-
1μM CsA for 45 min. then 1μM A23187	-	0.81 ± 0.91	0.57 ± 0.68	0.50 ± 0.59	0.48 ± 0.52	0.16 ± 0.30	0.71 ± 0.65	0.24 ± 0.47	0.20 ± 0.38	0.49 ± 0.61

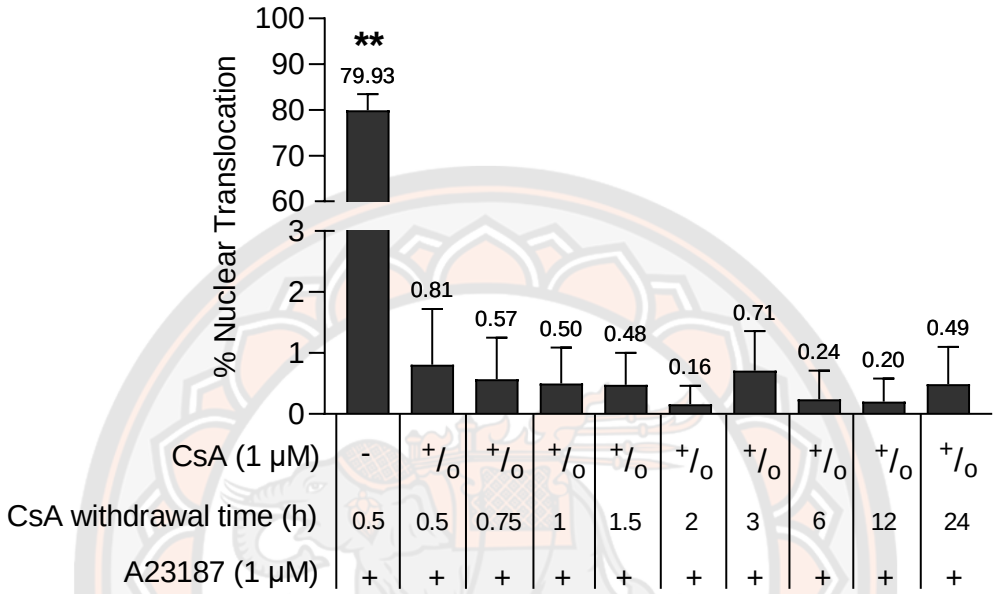


Figure 20 Validation of the retention time of CsA inhibits A23187-induced NFAT4-nuclear translocation.HEK293 cells transfected GFP-NFAT4 plasmid were pretreated with 1 μM of CsA for 45 minutes then withdrawn and further incubated in CsA free medium for different time point until 24 h. After indicated time point of incubation, cells were subsequently treated with 1 μM of A23187 and incubated for 30 minutes. The fixed GFP nuclear –positive cells from 8-randomized fields of cells were counted. Data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD. $^{+}/_{o}$; treat then withdrawn, -; non-treat, +; treat, ** $p<0.01$.

8. Testing the effect of the incubation time of A23187 1μM and calcineurin enzyme inhibitor – CsA (cyclosporine A) 1μM on the cytoplasm-nuclear translocation of NFAT4(N)

Figure 21 demonstrates effect of the incubation time of A23187 and CsA on the NFAT4(N) nuclear translocation. The cell lines in each experimental group is incubated with CsA 1μM and A23187 1μM for various times (5, 10, 15, 30, or 60 minutes). The slides are then fixed and the positive cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** $p < 0.01$ ANOVA). The results reveal that CsA, an inhibitor for NFAT4(N) nuclear translocation, is very potent inhibitor and significantly able to neutralize the effect of A23187 1μM, a stimulator for NFAT4(N) nuclear translocation. Longer period of CsA and A 23187 incubation times (such as 60 minutes) does not reverse the effect of CsA by A 23187. This suggests that the inhibitory effect of CsA is non-competitive inhibitor for A23187.

Table 9 Validation of the efficacy of CsA-competitor inhibits A23187-induced NFAT4-nuclear translocation

Treatments	Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)
Positive control	80.56±3.13
5 min.	1.28±1.17
10 min.	0.50±0.81
15 min.	0.56±0.690
30 min.	0.71±0.74
60 min.	0.59±1.14

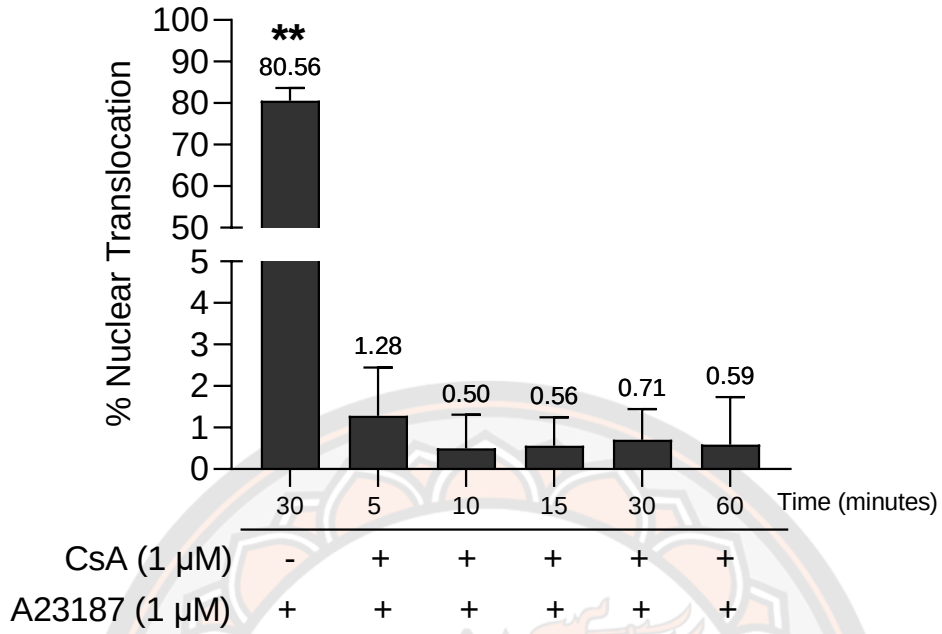


Figure 21 Validation of the efficacy of CsA-competitor inhibits A23187-induced NFAT4-nuclear translocation. HEK293 cells transfected GFP-NFAT4 plasmid were treated with the combination of 1 μM of CsA and 1 μM of A23187 and further incubated for 5, 10, 15, 30, and 60 minutes. After indicated time point of incubation, cells were subsequently fixed and counted of the GFP nuclear – positive cells from 8-randomized fields of cells. Data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD. ** p<0.01.

9. Testing of effect of A23187 concentrations on the cytoplasm-nuclear translocation of NFAT4(N)

Figure 22 demonstrates effect of A23187 concentrations on NFAT4(N) nuclear translocation. The cell lines in each experimental group is treated with CsA 1μM for 45 minutes. Then, A23187 is added at different concentrations (1, 2, 5, or 10μM) in each group for 30 minutes. The slides are fixed and the positive cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** p <0.01 ANOVA). The results demonstrates that CsA is a very potent inhibitor for inhibiting the NFAT4(N) nuclear translocation,

at 1μM it is able to significantly and nearly completely inhibit NFAT4(N) nuclear translocation, even in the presence of high dose of A23187 up to 10μM. this experiment also confirm that CsA is non-competitive inhibitor for A23187. As shown in Fig.22 CsA and A23187 expresses their action in different targets. The A23187 increases calcium ion level in the cytosol, the increased cytosolic calcium ion in turn activates calcineurin enzyme at calcium binding site, CsA binds to cytosolic cyclophylin (CpN) protein and then this complex inhibits calcineurin enzyme.

Table 10 CsA acts as non-competitive irreversible inhibitor

Treatments	**Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)**
Positive control	78.75±7.42
Medium control	0.40±0.59
1μM CsA then 1μM A23187	0.72±0.91
1μM CsA then 2μM A23187	0.61±0.68
1μM CsA then 5μM A23187	0.65±0.89
1μM CsA then 10μM A23187	0.35±0.52

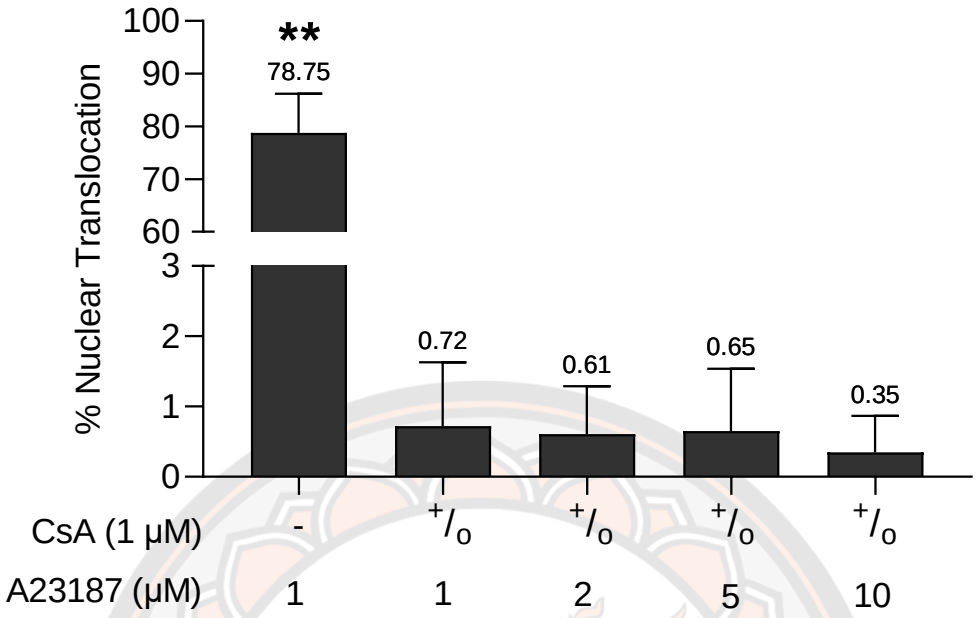


Figure 22 CsA acts as non-competitive irreversible inhibitor. HEK293 cells transfected GFP-NFAT4 plasmid were pretreated with 1 μM of CsA for 45 minutes then withdrawn and further treated with varying concentrations of A23187 (1, 2, 5, and 10 μM) for 30 minutes. After indicated time, fixed GFP nuclear –positive cells from 8-randomized fields of cells were counted. Data are shown as the percentage of NFAT4 nuclear translocation, and bar graphs are represented as mean ± SD. $^{+}/_{o}$; treat then withdrawn, - ; non-treat. ** $p<0.01$.

Part 3 Application of Cell-Based Model of NFAT nuclear-cytoplasm translocation for bio-active screening of herbal extracts by testing the stimulatory or inhibitory effects on NFAT nuclear translocation (Screening for immunomodulators)

To demonstrate the application of cell-based model for screening of bioactive herbal extracts that may have the potential to be used as immunomodulators. From the previous studies, there are some herbal extracts, available from plants commonly found in Thailand, with documented or tested pharmacologic actions as immunomodulators such as *Allium sativum* (กระเทียม), *Caesalpinia sappan* (แก่นฝาง), *Murdannia loriformis* (หญ้าปักกิ่ง), *Centella asiatica* (ใบบัวบก), *Curcuma longa* (ขมิ้นชัน), and *Andrographis paniculata* (ฟ้าทะลายโจร), and others [9]. In this study, we are interested in curcumin *(*from *Curcuma longa)* and andrographolide (from *Andrographis paniculata*) since the extracts from both herbs are widely studied and reported that the extracts inhibit interleukin 2 (IL-2) production and T-cell signaling [26, 27]. However, the mechanism of action (pathway) at molecular levels of those extracts is still unclear and under-studied. To avoid a variation in the constituents in curcumin, and andrographolide extracts, we have purchased both extracts from the commercial available source (SIGMA-ALDRICH).

Both curcumin, and andrographolide extracts are tested for their inhibitory effect against the NFAT nuclear translocation by using our cell-based for NFAT nuclear translocation model. The ionophore A23187 is used as a positive control (stimulator), CsA (Cyclosporine), inhibitor for NFAT suppressor as a negative control (inhibitor), and GFP-NFAT3(N) as a dominant negative NFAT as internal control. If the results from this experiment is promisive, the extracts will be subjected to further purification and testing to achieve pure active compound. The statistic tool such as ANOVA, t-test will be applied for testing significant levels among the treatment groups.

Curcumin the extract from Tumeric rhizome *(Curcuma longa)* has long been reported to have many pharmacologic actions such as antioxidant, anti-microbial, anti-inflammatory, and anti-carcinogenic properties, etc. The anti-inflammatory effects of curcumin is purposed that curcumin inhibits NF-κB activity via redox regulation. Kliem and colleagues reported that the immunosuppressive phytochemical effect of curcumin may from T cell-activation-induced Ca^{2+} mobilization, IC50 is approx. 12.5 μM [26].

As mentioned earlier, the inhibition of NFAT activation down regulates cytokine production (NFAT-regulated cytokine expression). The study also suggested curcumin may enhance the action of CsA with a different pathway. Shazaan and colleagues reported that extracted curcumin (1, 3 and 9 μM) inhibited T – cell proliferation and interleukin 2 (IL-2) production by human T lymphocyte. The results suggest that acts as T-lymphocyte immunosuppressants[28].

Andrographolide the extract is from plant, *Andrographis paniculata*. Carretta M.D. and colleagues reported that andrographolide extract contains anti-inflammatory and immunomodulatory effects [27]. From many previous studies suggested that andrographolide may impair immune response via inhibition pathways of NF-κB and mitogen-activated protein kinases (MAPK-ERK1/2) pathway. Moreover, andrographolide can decrease IFN-gamma and IL-2 production by stimulator, concanavalin A in murine T-cell. However, the pathway for cytokine production reduction is not elucidated. In this study, reported that andrographolide reduces NFAT luciferase activity by inhibiting nuclear translocation of c-jun transcription factor due to c-jun-N-terminal kinase (JNK) phosphorylation. Furthermore, andrographolide is able to promote NF-κB activation in Jurkat cells, decrease ERK1 and ERK5 phosphorylation induced by anti-CD3 or PMA/Ionomycin. Even though andrographolide has no effect on cell viability at 10- 50 μM, it increases apoptosis at 100 μM. the researcher proposed that andrographolide has the immunomodulatory effects via NFAT activation, ERK1/ERK5 phosphorylation in T-cells. Andrographolide reduces IL-2 production in Jurkat cells stimulated by phorbol myristate acetate and ionomycin (PMA/Ionomycin)

1. Testing the effect of partial purified curcumin at different concentrations on the cytoplasm-nuclear translocation of NFAT4(N), using in house developed cell-based model.

Figure 23 demonstrates effect of the commercial partial purified curcumin on cytoplasm-nuclear translocation using cell-based model developed by our team. Curcumin extract (65% purity from SIGMA-ALDRICH) at different concentrations (1, 5, or 10μM) is used in each of experimental group to test its inhibitory effect on the NFAT4(N) nuclear translocation. The cell lines in each group is exposed to curcumin extract for 45 minutes, then A23187 1μM, a stimulator for the NFAT4(N) nuclear

translocation for 30 minutes. The slides are then fixed and the positive cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** p <0.01 ANOVA). The result demonstrates that in every concentrations of curcumin extract (1-10μM) in the presence of activator A23187, there is no inhibitory effcet on the NFAT4(N) nuclear translocation (compared to the control group which is treated with CsA 1μM).

Table 11 The effect of partial purified curcumin on NFAT4- nuclear translocation

Treatments	Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)
1μM CsA then 1μM A23187	0.75 ± 0.69
1μM A23187	79.74 ± 4.18
0.05% DMSO then 1μM A23187	83.62 ± 3.00
1μM Curcumin then 1μM A23187	82.91 ± 4.13
5μM Curcumin then 1μM A23187	86.87 ± 4.97
10μM Curcumin then 1μM A23187	82.24 ± 4.24

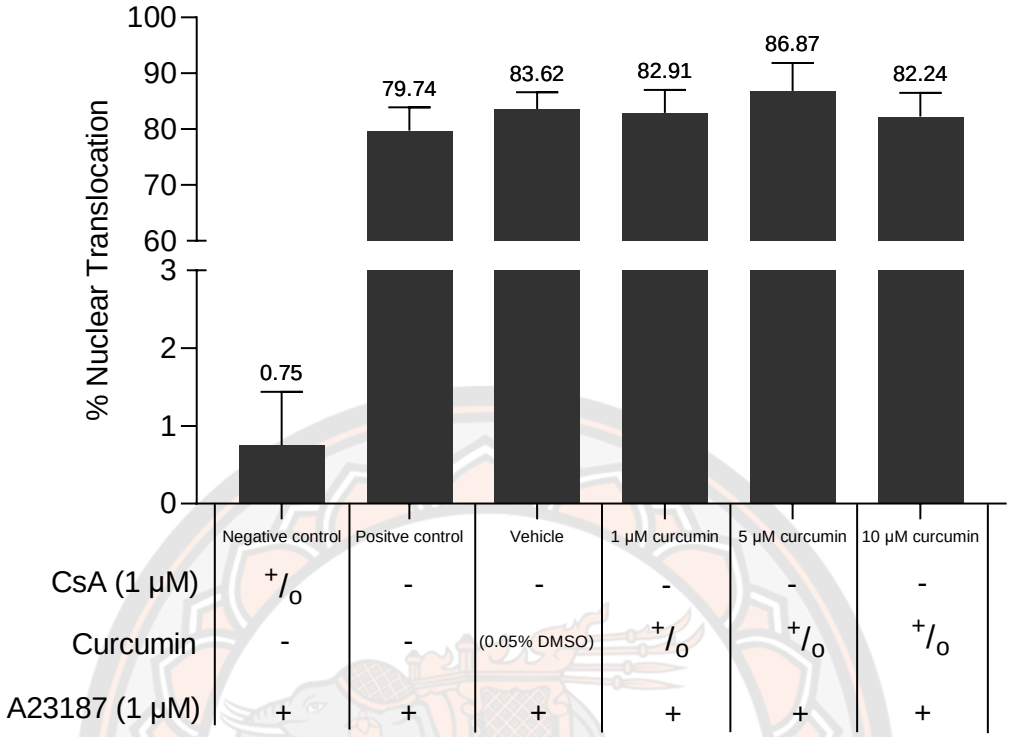


Figure 23 The effect of partial purified curcumin on NFAT4- nuclear translocation.HEK293 cells transfected GFP-NFAT4 plasmid were pretreated with 1, 5, and 10 µM of curcumin for 45 minutes and then withdrawn. After that, cells were subsequently treated with 1 µM of A23187 for 30 minutes. After indicated time, fixed GFP nuclear – positive cells from 8-randomized fields of cells were counted. Data are shown as the percentage of NFAT4 nuclear translocation. Bar graphs are represented as mean ± SD. 0.05% DMSO-medium was used as a vehicle control of curcumin treatment. CsA treatment was used as a negative control, inhibited NFAT4-nuclear translocation.

$^{+}/_{o}$; treat then withdrawn, -; non-treat. +; treat

2. Testing the effect of partial purified) andrographolide at different concentrations on the cytoplasm-nuclear translocation of NFAT4(N), using in house developed cell-based model.

Figure 24 demonstrates the effect of the commercial partial purified andrographolide on cytoplasm-nuclear translocation using cell-based model developed by our team. Andrographolide extract (98% purity from SIGMA-ALDRICH) at different concentrations (1, 10, 50 and 100 μM) is used in each of experimental group to test its inhibitory effect on the NFAT4(N) nuclear translocation. The cell lines in each group is exposed to andrographolide extract for 45 minutes, then A23187 1μM, a stimulator for the NFAT4(N) nuclear translocation for 30 minutes. The slides are then fixed and the positive cells (N = 8, 2X4 fields) are counted and expressed as % positive nuclear NFAT-localized cells (20X, HEK293 cell line, ** p <0.01 ANOVA). The result demonstrates that in every concentrations of andrographolide extract (1-100μM) in the presence of activator A23187, there is no inhibitory effcet on the NFAT4(N) nuclear translocation (compared to the control group which is treated with CsA 1μM).

Table 12 The effect of purified andrographolide on NFAT4- nuclear translocation

Treatments	Percentage of nuclear translocation of GFP-NFAT4(N) signal in HEK293 cell (% Mean ± S.D.)
1μM CsA then 1μM A23187	0.89 ± 0.72
1μM A23187	79.14 ± 4.04
0.05% DMSO then 1μM A23187	78.03 ± 8.77
1μM Andrographolide then 1μM A23187	83.74 ± 3.96
10μM Andrographolide then 1μM A23187	81.66 ± 6.36
50μM Andrographolide then 1μM A23187	77.94 ± 4.46
100μM Andrographolide then 1μM A23187	72.81 ± 8.41

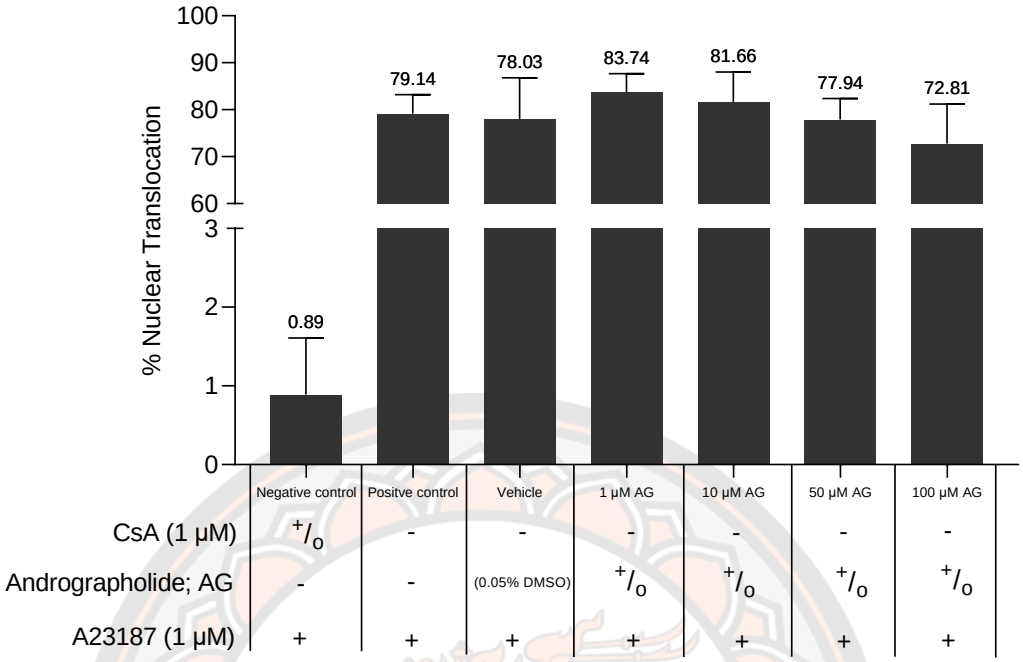


Figure 24 The effect of purified andrographolide on NFAT4- nuclear translocation.HEK293 cells transfected GFP-NFAT4 plasmid were pretreated with 1, 10, 50, and 100 µM of andrographolide for 45 minutes and then withdrawn. After that, cells were subsequently treated with 1 µM of A23187 for 30 minutes. After indicated time, fixed GFP nuclear –positive cells from 8-randomized fields of cells were counted. Data are shown as the percentage of NFAT4 nuclear translocation. Bar graphs are represented as mean ± SD. 0.05% DMSO-medium was used as a vehicle control of andrographolide treatment. CsA treatment was used as a negative control, inhibited NFAT4-nuclear translocation. $^{+}/_{o}$; treat then withdrawn, -; non-treat. +; treat.

CHAPTER V

CONCLUSION

The main objective of this study is to develop the Cell-based model for the NFAT nuclear-cytoplasm translocation study, which is easy to prepare, high throughput, and low cost. This model can be applied for screening or testing bio-active compounds especially from local herbal extracts (Thailand) that may contain pharmacological effects on the NFAT signaling pathway, leading to the discovery of new potential nuclear-cytoplasm translocation stimulators or inhibitors (immunomodulators).

In Part 1. Development of cell-based model of NFAT nuclear-cytoplasm translocation. The NFAT4 (full length) and NFAT3 (full length) from the human cDNA library were cloned, NFAT4 and NFAT3 (a dominant negative NFAT) were incorperated into a cloning vector (plasmid pcDNA3.1) containig fluorescent protein (GFP) gene. Then, the RHR domian of NFAT4 (full length) and NFAT3 (full length) were deleted, only the NHR domain (containing calcineurine ezyme docking domain and NLS motif) still intact, so the final genes called "NFAT4(N)" and "NFAT3(N)" were obtained). Without RHR domain (containing DNA binding motif – Figure 1), NFAT4(N) and NFAT3(N) proteins are not able to turn on down stream gene expression, so this can prevent side effects to the culured cell lines and enhence this cell-based model more robust. By using the PCR technique (polymerase chain reaction) and specific primers NFAT4 and NFAT3 modification, NFAT4(N) and NFAT3(N) can be acheived (Figure 4) and ligated into the plasmid (Figure 6). The green fluorescent protein (GFP) was pre-inserted in the plasmid which be placed at the N-terminal of NFAT4(N) and NFAT3(N) gene, so GFP-NFAT4(N) and GFP-NFAT3(N) were obtained (Figure 7). The *E.coli* host (strain DH5α) was selected for palsmid propagation. All plasmids were intensively tested for right gene sequences and sizes (Figure 4, 8 and 9) intact protein expression (Figure 10, 11 and 12). The HEK293 cell line and primary porcine skin fibroblasts were selected for this entire study since there is no expression of NFAT proteins in those cells.

For this Part 1. We have successfully isolated, cloned, modified NFAT4(N) and NFAT3(N) gene from the human cDNA library. The NFAT4(N) and NFAT3(N) proteins still have the N-terminal domain that are able to interact with calcineurin enzyme and NLS motif (nuclear localization signal) that allow proteins reversal cytoplasm - nucleus translocation. To test the function of the cell-based model, the constructed plasmids were transient transfection into cell lines. The GFP potein, a tracking signal protein, did not affect the proteins translocation patterns of GFP-NFAT4(N) and GFP-NFAT3(N) proteins. No obvious side effects of GFP-NFAT(N) proteins on culture cells was observed (cell morphology, vitality) in both HEK293 cell line and primary porcine skin fibroblasts. The result confirmed that our cell–based model generate the same result patterns as expected in theory. Finally the cell-based model the NFAT nuclear-cytoplasm translocation study is sucessfully established and ready for Part 2 experiemnts.

In Part 2 Testing Cell-based model of NFAT nuclear-cytoplasm translocation. The aim of this part is to study the effect and robustness of the modified GFP-NFATs on NFAT nuclear-cytoplasm translocation in cell cultures. The calcium ionophore A23187 was used in the study to increase cytosolic Ca^{2+} ion (mimicking T-cell and its ligand interaction that generates the increase of cytosolic Ca^{2+} ion concentration). The increase in cytosolic Ca^{2+} ion concentration stimulates and activates the cytosolic calcineurin enzyme, which further activates the NFAT proteins (by dephosphorylation), allowing NFAT proteins tannslocate from the cytoplasm into the nucleus (nuclear import translocation). To make the cell-based model to be easy tracking GFP gene was inserted at N-terminal of NFAT(N) genes, so the GFP, GFP-NFAT3(N), and GFP-NAT4(N) were easy to be tracked by using fluorescent microscope, and obteained photographs from the experiments. The positive cells (nucleus localization) or the negative green fluorescent cells (cytoplasmic localization) were counted and expressed as % Mean of the positive cells (Figure 13). The positive control and negative control groups were conducted throughout the study. The GFP-NFAT3(N) which is a dominant negative NFAT was used as an internal control. To prove the robustness of this developed cell-based model, kinetic and concentration (dose) – response studies of cyclosporine (CsA – an inhibitor) and A23187 (a stimulator) were intensively conducted (Figure 13-22).

Testing the effect of A23187 on cytoplasm-nuclear translocation of the NFAT4(N), NFAT3(N) and control plasmid (GFP vector) (Figure 12, 13). A23187, a calcium ionophore, is used to stimulate the increase of cytosolic calcium ion. An increase of available cytosolic calcium ion activates calcineurin phosphatase enzyme via calcium-calmodulin, calcineurin then de-phosphorylates serine-rich motifs of the NFAT4 protein, exposing NLS signal and allowing NFAT4 protein nuclear tanslocation. The NFAT3 protein, a dominant negative NFAT, does not respond to calcium ionophore, so no nuclear translocation observed in NFAT3(N) protein. With or without calcium ionophore stimulation, the GFP protein signal was diffused in both cytoplasm and nucleus because the lack of NLS (short sequences of positively charged lysines or arginines) in GFP protein. We found that both cell-base model HEK293 cell lines and primary porcine fibroblasts generate the same patterns of cytoplasm-nuclear translocation; therefore, HEK293 cell line was selected for the rest of the study sine this cell lines is cheap, available, and easy to prepare.

Testing the effect of cyclosporine A (CsA) on cytoplasm-nuclear translocation of the NFAT4(N), NFAT3(N) and control plasmid (GFP vector) (Figure 12, 13). From the experiment, once the culture cells were stimulated with calcium ionophore in the presence of CsA, NFAT4(N) nuclear localization was disappeared. So this means that CsA is an effective inhibitor (both 1 and 5μM) for calcineurin enzyme function (see Figure 2). CsA binds to cyclophylin protein (CpN) forming the complex protein (CsA-CpN) that inhibits calcineurin enzyme to interact with NFAT4(N) proteins. However, NFAT3(N) and GFP protein was not affected by CsA.

Testing the effect of A23187 concentrations on cytoplasm-nuclear translocation of the NFAT4(N) (Figure 14). In the experiment, the cell lines were exposed to A23187 at concentrations from 0.1 - 1μM for 30 minutes, we found that the cell-based model requires A23187 concentration at least 1 μM to generate cytoplasm-nuclear translocation of the NFAT4(N). The dose - response relation suggests the stimulation by A23187 is likely to be “all or none effect”.

Testing the effect of incubation time of calcium ionophore on cytoplasm-nuclear translocation of the NFAT4(N) (Figure 15). In this experiment, the cell-based model were incubated with A23187 1 μM for 5-120 minutes. The results from all experiment groups were positive and statistically insignificant difference of % positive

cells response. Therefore, incubation time required for A23187 incubation period to cell-base model stimulation is 5 minutes and optimal time for 30 minutes. Furthermore, we found that numbers of passage of the cell lines was also crucial, to get the better cytoplasm-nuclear translocation of the NFAT4(N), the lower number of cell lines passage is required (Figure 15 – #15 passage, Figure 16 – #60 passage).

Testing the withdrawal time effect of calcium ionophore on cytoplasm-nuclear translocation of NFAT4(N) (Figure 17). In this experiment, the cell-based model were incubated with A23187 1μM for 30 minutes to stimulate nuclear translocation of NFAT4(N), and afterwards A23187 was withdrawn at different time points. We found that after A23187 was withdrawn only 15 minutes, the NFAT4(N) protein resided in the nucleus was translocated back to the cytoplasm significantly. This is because in the nucleus, there are some kinases enzymes such as CK1, GSK3 enzymes that can change dephosphorylated form NFAT to phosphorylated form. So the NFAT4(N) protein was translocated back to the cytoplasm. Therefore, the result in this experiment suggested that the stimulatory effect of A23187 is reversible, and the developed cell-based model is robust.

Testing the concentrations effect of calcineurin inhibitor (CsA) on the cytoplasm-nuclear translocation of NFAT4(N) (Figure 18, Figure 19). In this experiment, the cell-based model were incubated with CsA at various concentrations (0.01 - 5μM) and followed by stimulation with A23187 for 30 minutes The results showed that CsA is a strong inhibitor for calcineurin enzyme, required only 0.01μM to observe near complete inhibition of the NFAT4(N) nuclear translocation. The inhibitory effect of CsA is potent, as even in a very low concentration 0.1μM, there is nearly no NFAT4(N) nuclear translocation observed. Notably, the cell lines with high passage (Figure 19, #60 passage) was still responsive to CsA at 0.1μM.

Testing the withdrawal time effect of calcineurin inhibitor on cytoplasm-nuclear translocation of NFAT4(N) (Figure 20). In this experiment, the cell-based model were incubated with CsA 1μM for 45 minutes, and followed by CsA withdrawal periods (30 minutes - 24 hours), then stimulated by A23187 stimulation. The results confirmed that CsA is very potent and able to inhibit A23187 stimulation significantly

even 24 hours withdrawal of CsA. This may be CsA can form a very stable complex with cyclophylin providing the longer duration with calcineurin enzyme interaction.

Testing the effect of A23187 exposure times on cytoplasm-nuclear translocation of the NFAT4(N) (Figure 21). In the experiment, the cell-based model was exposed to CsA 1μM for 45 minutes and fallowed by A23187 1μM incubation at different durations. The results suggested that CsA 1μM could inhibit and tolerate the stimulatory effect of A23187 1μM more than 60 minutes incubation time. This results also confirmed that may be CsA can form a very stable complex with cyclophylin providing the longer duration with calcineurin enzyme interaction. Moreover, the inhibitory effect of CsA is non-competitive with A23187, and exert their actions at the different sites of calcineurin enzyme (see Figure 2).

Testing the concentrations effect of A23187 in the presence of CsA on cytoplasm-nuclear translocation of the NFAT4(N) (Figure 22). In this experiment, the cell-based model was exposed to CsA (1μM) for 45 minutes, followed by different concentrations of A23187 (1-10μM) stimulation for 30 minutes. The results clearly demonstrated that CsA is a very potent inhibitor against A23187, as A23187 10μM could not significantly reverse the inhibitory effect of CsA 1μM.

In Part 3. Screening for immunomodulators. The aim of this part is to apply the Cell-Based Model of NFAT nuclear-cytoplasm translocation for bio-active screening of herbal extracts by testing the stimulatory or inhibitory effects on NFAT nuclear translocation (immunomodulators). The commercial available extracts from *Curcuma longa* and from *Andrographis paniculata* were selected as example extracts for the developed cell-based model since the previuos studies have reported the pharmacologic effects of anti-inflammatory, immunomodulatory effects, interleukin-2 (IL-2) production suppression [26, 27], 2009), and these two extracts are from plants that are commonly found in Thailand. Unfortunately, the actual mechanism action (pathway) of those extracts still inconclusive. The calcinuerine-NFAT pathway is one of the possible pathways to describe the effects, so by using this cell-based model, the clues for pharmacologic effects of these extracts may be obtained. Statistic tool, ANOVA and t-test was applied to all experiments.

Curcumin is an extract form plant rhizome of *Curcuma longa.* From the study by Kliem C. et al. (2012), curcumin extract contains immunosuppressive properties, inhibitory effect on T cell-activation-induced Ca^{2+} mobilization with the IC50 approximately 12.5μM[26]. it may inhibit NFAT activation, leading to suppression of cytokine production. Furthermore, curcumin may enhance the effect of CsA possibly by NF-κB activation.

Andrographolide is an extract form herb *Andrographis paniculata* . From the study performed by Carretta MD and colleagues andrographolide extract contains anti-inflammatory and immunomodulatory effects. They suggested that andrographolide may express its action through the NF-κB or mitogen-activated protein kinases (MAPK) pathway. Furthermore, andrographolide reduces IFN-gamma and IL-2 production in T-cell of murine model; however, the repression mechanism of cytokine production is not clear [27].

Testing the effect of (partial purified) curcumin on cytoplasm-nuclear translocation of the NFAT4(N) (Figure 23). In this experiment, the cell-based model was incubated with curcumin extract at various concentrations (1-10μM) for 45 minutes and followed by A23187 1μM 30 minutes. The results revealed that all concentrations of curcumin extract (1-10 μM) do not show any significant inhibitory effect on cytoplasm-nuclear translocation of the NFAT4(N). The higher concentrations of curcumin extract may be required for the test o observe the positive inhibitory effect.

Testing the effect of (purified) andrographolide on cytoplasm-nuclear translocation of the NFAT4(N) (Figure 24). In this experiment, the cell-based model was incubated with andrographolide extract at various concentrations (1-100μM) for 45 minutes and followed by A23187 1μM 30 minutes. The results revealed that all concentrations of andrographolide extract (1-100μM) do not show any significant inhibitory effect on cytoplasm-nuclear translocation of the NFAT4(N). The higher concentrations of andrographolide extract may be required for the test of observe the positive inhibitory effect.

Even though In the previous studies reported that curcumin and andrographolide extracts contain inhibitory effect on cytokine (such as IL-2) production, using our developed cell-base model, the results from our study have

clearly demonstrated that those extracts do not express their inhibitory effect in cytokine production via NFAT pathway. Still, there are other pathways that may modulate cytokine transcription (expression) such as c-jun transcription factor and NF-κB activation as shown in Figure 25. Thus, future experiments are needed to exploring the pharmacologic activity of curcumin and andrographolide extracts.

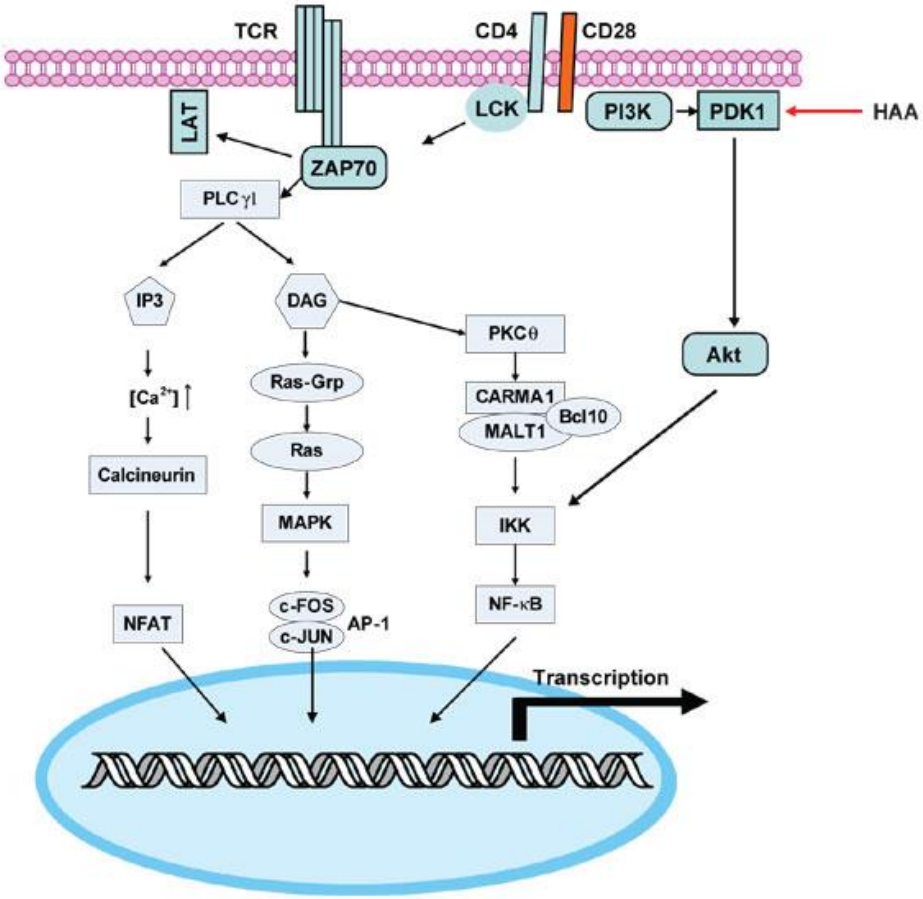


Figure 25 Schematic illustration of TCR signaling pathways.Upon TCR stimulation, PLC-g1 is activated generating inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 induces calcium release, thus activating the NFAT pathway, and DAG phosphorylates PKCq, thus activating the NF-kB pathway. HAA acts on PDK1, which mediates NF-κB activation via CD28 signaling [29]

In summary, in this study, we have successfully cloned and modified genes GFP-NFAT4(N) and GFP-NFAT3(N). We also proved that HEK293 cell lines and porcine skin fibroblasts can be used as a cell-based model. We also clearly demonstrate the robustness of our cell-based model. Moreover, we demonstrate the application of our developed cell-base model for screening potential bio-active herbal extracts curcumin and andrographolide. This cell-base model is a useful tool for bio-active screening of NFAT signaling pathway, and this model application is considered as a high throughput, cost-effective, time efficient method.

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