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นางสาวปาจรีย์ สอนสังเสน

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BOOLEAN MODEL FOR BIOCHEMICAL PROCESSES RELATED TO EFFECT OF CHA IN CANCER CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Applied Mathematics and Computational Science

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มะเร็งเป็นกลุ่มของโรคที่เกี่ยวกับการเจริญเติบโตของเซลล์ที่ผิดปกติ ในปัจจุบันมะเร็งเป็นหนึ่งในโรคที่สำคัญที่เป็นสาเหตุทำให้เสียชีวิตเป็นประจำทุกปี เพื่อที่จะยับยั้งเซลล์มะเร็งไม่ให้เจริญเติบโต จึงได้มีการศึกษา วิจัยและนำเสนอสารเคมีหลายชนิด ตัวอย่างเช่น สารประกอบคูมารินได้รับการศึกษาว่าสามารถยับยั้งเซลล์มะเร็งได้ อย่างไรก็ตาม รายละเอียดของกระบวนการทางชีวเคมีซึ่งช่วยอธิบายถึงผลกระทบของสารนี้ค่อนข้างน้อยและยาก ดังนั้น จึงเป็นสิ่งสำคัญที่ต้องทำความเข้าใจกับผลของสารประกอบคูมารินในการเปลี่ยนวิถีทางชีวเคมีที่มีความละเอียดมากขึ้นด้วยวิธีทางคณิตศาสตร์

ในการศึกษาครั้งนี้เราได้สร้างแบบจำลองบูลีน ซึ่งรวมกระบวนการตายของเซลล์ การส่งสัญญาณของแคลเซียมและการอักเสบ เพื่ออธิบายถึงผลของสารประกอบคูมารินในเซลล์มะเร็ง แบบจำลองทางคณิตศาสตร์นี้จะถูกใช้เพื่ออธิบายกระบวนการส่งสัญญาณภายในเซลล์และเพื่อระบุเป้าหมายใหม่ที่เป็นไปได้ในการรักษาโรคมะเร็ง

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Cancer is a group of diseases involving abnormal cell growth. In the present, cancer is one of the major diseases causing death annually. To inhibit cancer cells, several substances have been proposed and studied; for example, a compound namely a coumarin compound has been studied since it can inhibit cancer cells. However, the detailed biochemical processes explaining the effect of this substance is quite rare. Therefore, it is an important task to understand this effect in changing the biochemical pathways in more detail by mathematical methods.

In this study, we construct Boolean model which includes apoptosis, calcium signaling pathway, and inflammation to explain the effect of a coumarin compound in cancer cells. Finally, the model will be used to explain the signaling processes and identify more new potential targets in treating cancers.

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Student's Signature .....
Advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Motivation and Literature Surveys

Cancer is a disease caused by abnormal cell growth to become malignant tumor cells. Cancer cells are hyperplasia and unruliness. In addition, these cells can invade to normal cells or organs in the body. At present, cancer disease is one of the leading causes of death in the world [17]. According to the Bureau of Policy and Strategy, Ministry of Public Health, cancer diseases are the first deaths in Thailand and the number of deaths is increasing as shown in Table 1.1 and the number of cancer patients is increasing every year (Figure 1.1) [5].

Table 1.1**:** The number of deaths from 2012 to 2014 in Thailand.

Diseases	**2012**	**2013**	**2014**
Coronary artery disease	24996	28022	29689
Cancer	43829	45892	47086
Diabetes	4705	5480	6114
Emphysema	1421	1597	1619

In 2015, Ferlay estimated the number of new cancer cases and cancer death is 14.1 million and 8.2 million, respectively, which occurred in 2012 worldwide by using the data from population-based cancer registries (PBCR) [35]. Leukemia accounted for some 352,000 new cases (2.5% of all new cancer cases) and for 265,000 deaths (3.2% of all deaths) [15]. Since the number of deaths is concerned, then therapy options need to apply into the patient. In present, there are many types of cancer treatment such as surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy. Some cancer patients will have only one treatment. But most people have a combination of treatments.

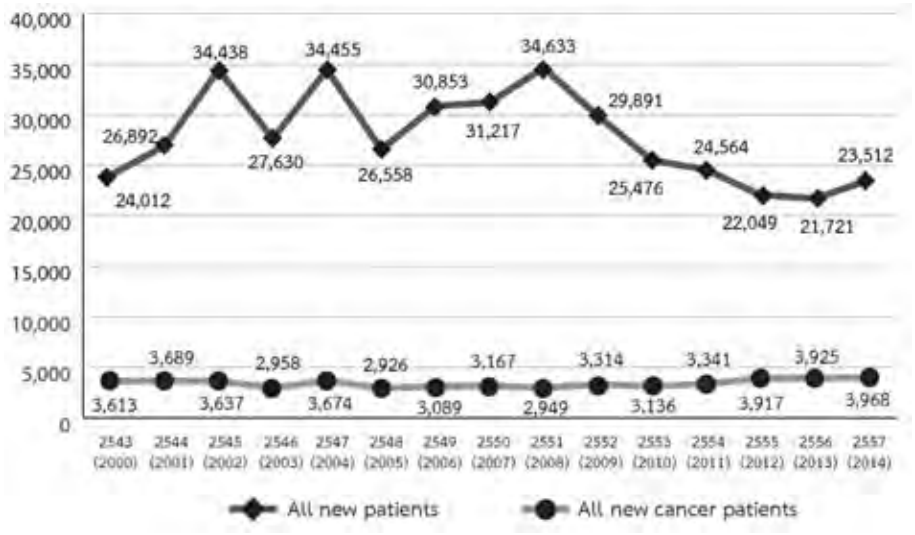

Figure 1.1**:** Trend of number of all new patients 2000-2014 [23].

Now, treatments can relieve suffering from cancer but the performance of treatment is not enough to treat cancer. Moreover, cancer treatments can cause side effects such as mouth and throat problems, hair loss or thinning. And some chemotherapy can include an increased risk of developing other cancer. Then, the performance of treatment and drugs need to be developed to reduce the cancer deaths and decrease the side effects. Therefore, to inhibit cancer cells, several substances have been proposed and studied; especially, substances from herbs to treat cancer in the traditional medicine. For example, a coumarin compound from *Clausena harmandiana* which is herb in Thailand as shown in Figure 1.2. This compound has been studied recently and found that it inhibits Jurkat T cell line and induces cell death via apoptosis process [46].

Figure 1.2**:** *Clausena harmandiana.*
(`https://www.flickr.com/photos/adaduitokla/11278705123/in/photostream/`)

However, the detailed process explaining the effect of this a coumarin compound is complexity and still unclear. Thus, it is a challenging task to understand the effect of this compound both in mechanical and biological details. Understanding the mechanism of a signaling pathway gives the contribution to the drug development for the cancer treatment. Additionally, this mechanism can be captured as a mathematical formula [41]. Dynamically changing systems in biology are often modeled to understand the complex behavior of cells and organisms. Mathematical modeling of biological processes provides deep insights into complex cellular systems. To understand biological systems, mathematical methods in modeling is used to predict the dynamics of this complex systems, and the behaviors of computational modeling are compared with experimental observation [26]. The molecular level data on individual components and interactions between components can be obtained from the experimental literature or research such as Boolean network modeling. The Boolean network model is one of the most commonly used methods which represents each gene/protein as a node of Boolean functions and interprets the activities of proteins as Boolean signals.

Boolean model is a simplest discrete model which is an "on/off" model. This model is applied to a protein interaction network as a Boolean network that each node represents a protein and each edge represents an interaction of two proteins. Each protein has one of two binary states. Therefore, its value could be either 1 ("on") or 0 ("off") which means either protein is activated or inhibited, respectively. Boolean model is widely applied in biological systems as gene regulatory. For example, in 2008, Zhang and his team translated the T-LGL survival signaling network into a Boolean model to explain the behavior of signaling abnormalities in T-LGL leukemia. Researchers were able to identify the key mediators of CTL survival, potential therapeutic targets for T-LGL leukemia and offering insights into CTL manipulation [55]. In 2009, Schlatter and his coworker constructed a model of intrinsic and extrinsic apoptosis pathway at different timescales to describe the numerous interactions and signal that control apoptosis. They found that Fas activates several proteins in the signaling network and if XIAP is a knockout scenario, it leads to the process of apoptosis via inhibition of caspase3 [42]. Recently in 2013, Fumiã and Martins integrated the main cancer signaling pathways by constructing a Boolean

model to explain the effects of several targeted inhibitors colorectal cancer at distinct environmental conditions that Boolean network is controlled by attractors associated to apoptotic, proliferative, and quiescent phenotypes [18].

1.2 Research Objectives

In this study, we constructed a Boolean network model to explain the effect of the coumarin compound in Jurkat T cells and to study the behavior of related proteins in cancer cells by integrating the calcium signaling pathway and the apoptosis pathway from published databases and manually correcting the process with literature searches. To investigate the accuracy of the model, our model is compared with real-time PCR experiment.

The scope of this study, it to study the behavior of our selected protiens in Jurkat T cells by collecting the data from database and literature searches. Then, we compare our results with the experimental data. In the experiment, we treat a coumarin compound in Jurkat T cells at 24 hours and measure gene expressions of each protein in our selected protiens at 0 and 24 hours to predict how each protein acts with the other protiens in Jurkat T cells by comparing with the wildtype cells before treating a coumarin compound. We focused on activation or inhibition of each protein before and after treating the cell with a coumarin compound.

1.3 Thesis overview

This thesis is separated into 5 chapters organized as follows: Chapter 1 is an introduction to this study including the motivation and literature surveys, the research objectives, and thesis overview. Chapter 2 is about the background knowledge used in this thesis, which includes two branches as follows: one is the biological overview; signaling pathway, apoptosis, calcium signaling pathway, and protien-protien interaction(PPI). The other one is mathematical background includes basic graph theory and Boolean network model. Chapter 3 involves methods to construct a Boolean model to study the effect

of a coumarin compound in cancer cells. There are four topics to build the model; network construction, activating, and inhibiting processes, Boolean model, and simulation of signaling flow. Chapter 4 presents the obtained model which construct in Chapter 3. The accuracy of the model by experimental data and analyze the behavior of a coumarin compound in cancer cells are also in this chapter. The last Chapter, Chapter 5, discussion and conclusion of our model and method are explained and the suggestion of the possible research in the future is also discussed.

CHAPTER II

PRELIMINARIES

In this chapter, we present the background knowledge to use in this thesis. We give an overview of systems biology related with signaling pathway focused on apoptosis and calcium signaling pathway. Moreover, we give mathematical background including basic graph theory, basic logic gate, and Boolean network model as the following.

2.1 Signaling pathway

Cells in organisms communicate with each other during the process of cell proliferation, differentiation into specific cell types, and program cell death. The communication between cells is called cell signaling which uses chemical signals-proteins or other molecules. Figure 2.1 show the process of cell signaling which has 3 processes as follows [8]:

1. Reception: in this process, the receptor on the cell membrane bind with a signal form external cells such as hormones, chemical or enzyme. After that, the receptor changes its activities to send messengers inside the cell.

2. Signal transduction: after the signal through the cell, the signal is relayed through proteins or molecules to other until the signal is sent to the target.

3. Response: this last process, the target cell exhibit activities to respond the signal. There are cell a change in the behavior for responding to the signal such as alteration in the activity of a gene or even the induction of a whole process such as cell division or apoptosis [28].

Signaling pathway is a series of intracellular reactions after the receptor bind chemical signal. This is the process that describes a group of molecules in a cell when a chemical

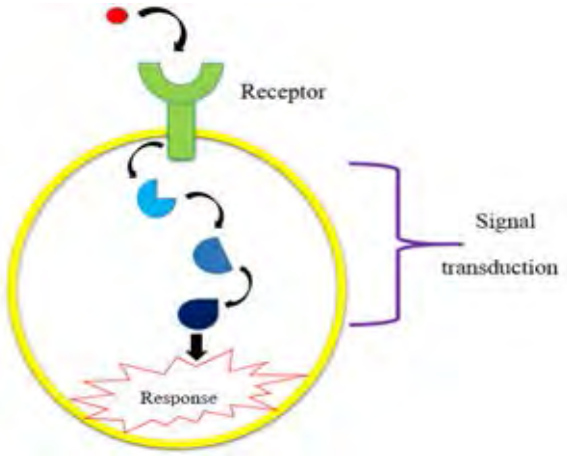

Figure 2.1**:** The processes of cell signaling.

or physical signal is transmitted through a cell as a series of molecular events. After the receptor binds a signal, it brings the signal into the cell and sends to the first molecule in a pathway. Then, the signal sent to activates another molecule until the target molecule is activated and the cell function is activated to response the signal. Abnormal activation of signaling pathways can lead to many diseases such as cancer. In addition, drugs are being developed to prevent these pathways. These drugs may help to stop cancer cell growth and treat cancer cells [49].

Understanding the cell signaling, we get how cells take advantage this large signaling tool to collect the specific signaling pathways that they require to communicate with other cells. The relationship between cell signaling and biology is providing deep insights into the underlying complex biological system which cause many of the major human diseases [8, 28].

2.2 Apoptosis

Apoptosis is a word from Greek. It means “dropping off” or “falling off” as in leaves from a tree [31]. Apoptosis is a process of programmed cell death that occurs in developing and adult animal tissues, cell death during embryonic development: they can separate the cells between them die, or cells that are no longer needed or are a threat to the organism are destroyed [1]. Cell death helps to control cell numbers and balances cell

division. If this were not so, the tissue would uncontrollably grow or is less than normal. Moreover, apoptosis kill specific abnormal cells, this mean apoptosis damage the cells without damaging its neighbors [31]. Apoptosis occurs by enzymes called caspases, which induce cell death by cleaving specific proteins in the cytoplasm and nucleus. Caspases are synthesized in the cell as procaspases. Procaspase activation can occur from outside the cell by the activation of death receptors on the cell surface [1].

Apoptosis pathway is initiated by either extracellular or intracellular death signal as shown in Figure 2.2. The intrinsic pathway of caspase activation, the cell kills itself because it senses cell stress such as DNA damage, growth factor or calcium iron whereas in the extrinsic pathway of caspase activation, the cell kills itself because of signals from other cells. The process is initiated by ligand binding to cell surface death receptors, such as TNF-α, hormone or drug [52].

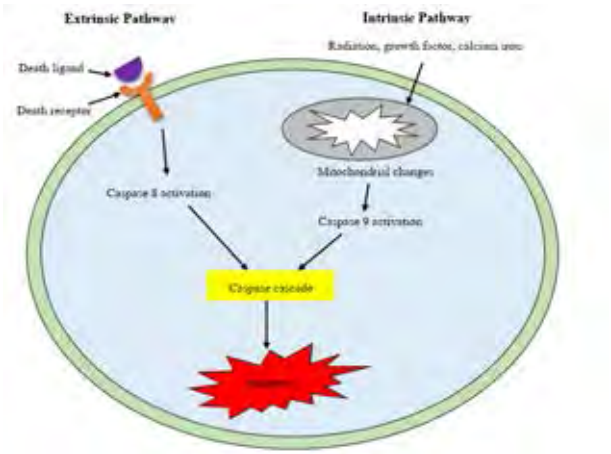

Figure 2.2**:** This figure represents the two main pathways of apoptosis which are extrinsic and intrinsic pathways.

In 2002, the Nobel Prize in medicine was awarded to the Sydney Brenner, Horvitz and John E Suston found the first gene (Nuc-1) related with apoptosis. Now apoptosis has been found in the tumor development, and the alteration of apoptotic pathways is a common character of tumors, so molecules involving apoptosis signaling pathway is targets for cancer therapy [22].

2.3 Calcium signaling pathway

Calcium signaling pathway is a second major messenger involved in a variety of intracellular signaling pathways. Calcium ion (Ca^{2+}) has a multitude of functions that has essential signal transduction element involved in cell growth and survival including cell cycle, differentiation, proliferation, and cell death. Moreover, the intracellular calcium ions act as the second messenger to migration and death [11]. In 2008, Pinton review that severe Ca^{2+} dysregulation can promote cell death through necrosis, and Ca^{2+} increases are induced by milder insults promote cell death through apoptosis [38].

When Ca^{2+} signaling triggers deep inside the cell, one of three genes which encode an identical calcium binding protein for Ca^{2+} is a protein Calmodulin (CaM). Calmodulin (CaM) is a central regulator of cellular Ca^{2+} responses which has a vital role in mediates processes such as inflammation, metabolism, apoptosis, and the immune response. Calcium-bound calmodulin associates with and activates serine/threonine phosphatase Calcineurin (CaN) [38].

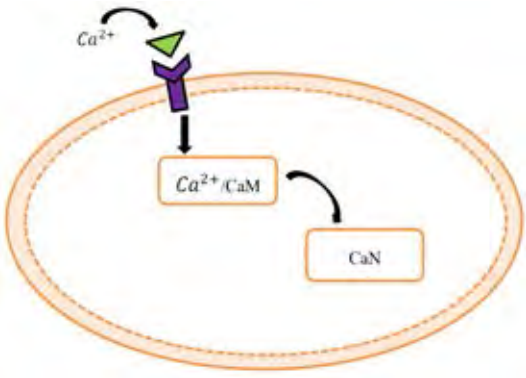

Figure 2.3**:** In brief, calcium signaling pathway.

In present, pharmacological treatment is focused on drugs that regulate the activity of the Ca^{2+} signals because the Ca^{2+} signals play key roles in the regulation of diverse cellular processes. In addition, the drug which can inhibit the Ca^{2+} signals can be detected in a highly specific manner which is a support to the drug development [43] for example, calcium signaling is a target of the drug in treating cancers. Signaling from calmodulin is involved in various types of pathways involving calcineurin, including apoptotic responses. Olofsson et al investigate the potential involvement of differently

calmodulin regulated pathways by several drugs prompted and found that calmodulin induced signaling pathways linked to apoptosis signaling [33].

2.4 Inflammation

The functional relationship between inflammation and cancer have been proposed for a long time. Many researcher reviews that inflammation can cause cancer and cancer also cause inflammation [10]. Moreover, the relation between inflammation and cancer helps to suggest that sites of inflammation are ideal microenvironments for cancer to develop and treatment of chronic inflammation should lead to inhibit the cancer [9].

To understand the role of inflammation that it lead to cancer, it is important to understand what inflammation is and how it work in cells. In this study, we focused on TNF-α signaling pathway because TNF-α is also a key in inflammation and is important in early events in tumors regulating a cascade of cytokines [50]. Thus TNF-α may be one of the ways in which inflammation perform as a tumor promoter.

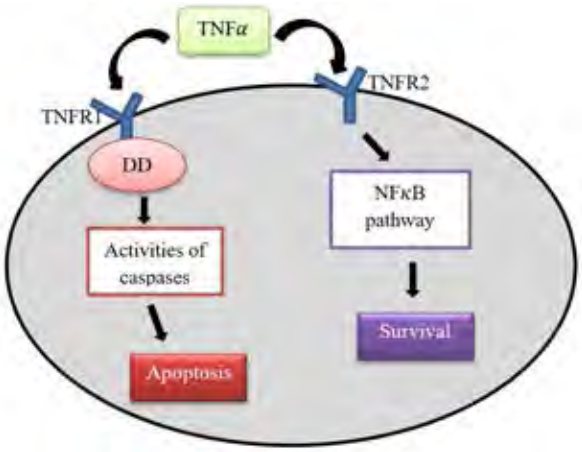

Figure 2.4**:** TNF-α signaling pathway.

Tumor necrosis factor alpha (TNF-α) is a cytokine, was initially considered as a widely applicable intracellular signal pathways including apoptosis and cell survival as well as inflammation and immunity. TNF-α is involved in various pathological processes and physiological, including differentiation, cell proliferation, apoptosis, and modulation of immune responses and induction of inflammation. In inflammation pathway, TNF-

α binds to its receptors (TNFR1, TNFR2). TNFR1 is expressed in all human tissues whereas TNFR2 is mostly expressed in immune cells, mediates limited biological responses and limited cells such as T lymphocytes (T cell). TNFR1 contains a protein–protein interaction domain called death domain. The death domain can recruit other death domain-containing proteins and couples the death receptors to caspase activation and apoptosis. While TNFR2 signaling activates NFκB pathway leading to survival [50]. In conclusion, TNF is an anti-cancer agent.

2.5 Graph

Definition 2.1**.** A graph G is defined by $G(V, E)$, where V is a non-empty finite set of vertices and E is a finite set of edges which connect between two vertices in the graph.

Definition 2.2**.** A walk in the graph $G = (V, E)$ is a finite sequence of the form

$$V_{i_0}, e_{j_1}, v_{i_1}, e_{j_2}, ..., e_{j_k}, v_{i_k},$$

which consists of alternating vertices and edges of G. The walk start at a vertex v_{i_0} and the terminal vertex is v_{i_k}. When k is the length of the walk.

Definition 2.3**.** A walk is a trail if any edge is traversed at most once. Then, the number of times that the vertex pair (u, v) can appear as consecutive vertices in a trail is at most the number of parallel edges connecting u and v.

Definition 2.4**.** A trail is a path if any vertex is visited at most once except possibly the initial and terminal vertices when they are the same. A closed path is a circuit. For simplicity, we will assume in the future that a circuit is not empty, i.e. its length greater than or equal 1. We identify the paths and circuits with the subgraphs induced by their edges.

Definition 2.5**.** The graph $G_1 = (V_1, E_1)$ is a subgraph of $G = (V, E)$ if

1. $V_1 \subset V$ and
2. Every edge of G_1 is also an edge of G.

2.6 Basic Logic gate

Definition 2.6**.** A mathematical statement is a declarative sentence that is true or false, but not both.

For example, five is less than eight is a mathematical statement but $3x - 5 = 21$ is not a mathematical statement, because it involves the variable x and its truth value depends on the value that x assumes. In Mathematics, mathematical statements are often indicated using capital letters. For an arbitrary mathematical statement P, we can indicate the possible truth values for P and the negation of P, symbolized by $\sim P$ in the table below, called a truth table.

P	$\sim P$
T	F
F	T

Definition 2.7**.** Given two statements P and Q, the compound statement, P and Q, called the conjunction, is denoted by $P \wedge Q$ and is defined by the following truth table.

P	Q	$P \wedge Q$
T	T	T
T	F	F
F	T	F
F	F	F

Definition 2.8**.** Given two statements P and Q, the compound statement, P or Q, called the disjunction, is denoted by $P \vee Q$ and is defined by the following truth table.

P	Q	$P \vee Q$
T	T	T
T	F	T
F	T	T
F	F	F

LogicGate

Logic gates are the basic building blocks of any digital system. Most logic gates having one or more than one input and only one output. The relationship between the input and the output is based on logic operations or gates such as AND, OR, or NOT. The value of each input has either 0 or 1 which represents "off" or "on" state, respectively.

Gate AND is an electronic circuit that gives output "on" (or 1) only if all its inputs are "on".

P	Q	$P \wedge Q$
1	1	1
1	0	0
0	1	0
0	0	0

Figure 2.5**:** AND gate diagram.

Gate OR is an electronic circuit that gives output "on" (or 1) if one or more of its inputs are "on".

P	Q	$P \vee Q$
1	1	1
1	0	1
0	1	1
0	0	0

.

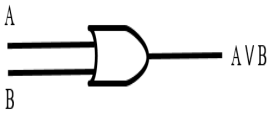

Figure 2.6**:** *OR* gate diagram.

Gate *NOT* is an electronic circuit that produces an inverted version of the input at its output. If the input variable is P, the inverted output is known as *NOT* P.

P	$\sim P$
1	0
0	1

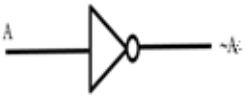

Figure 2.7**:** *NOT* gate diagram.

2.7 Boolean model

Dynamically changing systems in biology and chemistry are often modeled to understand the complex behavior of cells and organisms. In Biology, Mathematical and Computational modeling of gene regulatory networks is necessary because their complex behavior is difficult to understand. The model provides deep insights to explian the complex interactions between genes into complex cellular systems. Moreover, the roles of mathematical models for describing gene regulatory networks at a system level, simulation the behavior of the network, predicting new structures, and relationships of elements. If we can use the knowledge of mathematics and computer programming to build a gene network models which has effective and apply it to control the genes in the experiment, we may find new treatment or develop the potential of treatment for diseases, such as cancer [54]. The quantitative and continuous models such as differential equations have been widely used. They have many free parameters which are hard to constrain from

experimental data. On the other hand, a qualitative data of molecular level on individual components and its interactions can be obtained from the experimental literature and describe the behavior by discrete model such as Boolean network modeling extremely useful [51]. Boolean model is commonly used to explain the dynamical behavior of the network by the interactions between protein; for instance, Song Li and his co-worker integrate the multitude of recent experimental findings concerning the molecular signaling network for study the response of individual guard cell pairs to the local ABA signal in plat [30]. Next year, Thakar J et al. integrated the known temporal information with the interaction network and developed dynamic models for bacteria and the components of the immune system [47]. Hao Ge and Min Qian built the Boolean model for understanding the main function of the negative feedback in the p53 pathways at a low steady state level, and each sequence of protein states in the negative feedback loops [19].

In computer science, a Boolean model is an information retrieval model that we can assign any query which is a set of words in the form of a Boolean expression of terms. The model just searches the query on each document. Boolean models are applied to describe real gene regulatory relations as a Boolean network model which consists of a set of nodes whose represent protein and is determined by other nodes in the network through Boolean functions. We assume that a Boolean network model consists of a set of Boolean variables $\{p_1, p_2, ..., p_n\}$ whose value is determined by other variables in the network through a set of Boolean functions $F = \{f_1, f_2, ..., f_n\}$ which are an edge in the model, one assigned to each variable. Each node p_i is a binary variable, its value can be either 0 or 1 which represents inactive and active, respectively. The value of output nodes-node has only edge come to them are determined by the current or prior values of its regulators inputs-node without edge come into them.

A Boolean function describes how to determine a Boolean value output based on some logical calculations (AND, OR, and NOT) from its Boolean inputs. These logical gates are used to perform logical operations on one or more logical inputs and produce a single logical output. In Figure 2.8, the model has two input nodes A and B and has

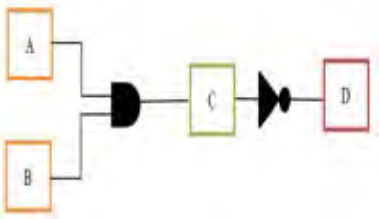

Figure 2.8**:** Example of Boolean Model.

only output node D. Boolean functions are f_C =A AND B and $f_D = NOT$ C.

2.8 Real time PCR

Polymerase chain reaction (PCR) is a technique used in molecular biology to DNA synthesis by copies of a segment of DNA sequence. PCR is widely used to make many the quantity of DNA by PCR machine. The method of PCR has 3 step as the following [2]:

1. Denaturing: In this method, the double-stranded template DNA is heated to separate it into two single strands.

2. Annealing: Next step, the temperature is lowered to enable the DNA primers to attach to the template DNA.

3. Extension: Last step, the temperature is raised and the new strand of DNA is made by the Taq polymerase enzyme.

Quantitative PCR or real-time PCR (qPCR) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). qPCR is the linearity of DNA amplification to determine absolute or relative quantities of a known sequence in a sample. By using a fluorescent reporter in the reaction, it is possible to measure DNA generation in the qPCR assay. In qPCR experiments, reference genes, such as GADPH, are used as controls to normalize the data by correcting for differences in quantities of cDNA. GADPH is a perfect reference gene if it does not exhibit changes in expression between samples from various experimental conditions or time points [6]. DNA amplification is monitored at each cycle of PCR(Cq). After that the Cq values are measured, different

methods can be used to determine the expression level of the target gene relative to the control. In this study, we used ΔΔCq method to calculate relative gene expression from Cq values.

The ΔΔCq method is used to calculate relative gene expression-the phenotypic manifestation of a gene or genes by the processes of genetic transcription and genetic translation from quantification cycle(Cq) values obtained by quantitative real-time PCR(qPCR). This method assumes that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other. We can determine the relative expression level of our target gene in samples using the steps below [6, 37].

1. Normalize the Cq of the target gene to that of the non-targeted GAPDH gene(ref) expression levels within the same sample to determine ΔCq by

$$\Delta\text{Cq} = \Delta\text{Cq(target)} - \Delta\text{Cq(ref)}.$$

2. Transformed the ΔCq for each target gene to the ΔCq Expression by

$$\Delta\text{Cq Expression} = 2^{-\Delta C_q}.$$

3. Determine the standard deviation.

4. Normalize to treatment control by

$$\Delta\Delta\text{Cq} = \frac{2^{-\Delta C_q(target)}}{2^{-\Delta C_q(ref)}}.$$

The relative expression level of the target gene was expressed as the ratio of the targeted ΔCq Expression to the non-targeted ΔCq Expression. We show how to calculate the ratio of the relative expression in the table below:

Table 2.1**:** Example of ΔΔCq calculations.

Gene	Cq(ref)	Cq(target)	ΔCq	ΔCq Expression	ΔΔCq
Non-target gene	21.9	23.1	1.2	0.43	1.00
target gene	27.5	34.3	6.7	0.01	0.02

In Table 2.1, we can see that the target gene has $\Delta\Delta$Cq=0.02 and this mean the target gene is inhibited when cells were treated with some compounds comparing with the non-target gene.

CHAPTER III

METHOD TO CONSTRUCT THE MODEL

In this chapter, we construct the Boolean network model which focused on how proteins exhibit in Jurkat T cells after we teart a coumarin compound at 24 hours.

3.1 Network construction

In 2015, Suauam et al. investigated the mechanism of coumarin compound in yeast and Jurkat T-cells and found that a coumarin compound inhibits the calcineurin pathway and inhibit NFAT dephosphorylation [46]. Moreover, the researchers from Department of Microbiology, Faculty of Science, Chulalongkorn University found that a coumarin compound treats Jurkat T cell arrested in S phase and induce the cell death by apoptosis [36]. Then in this study, we focused on a set of proteins involved in the apoptosis, calcium signaling, and inflammation pathway.

In this method, we construct the network of protein-protein interactions using interactions data from STRING database (http://string-db.org) by using R programming. STRING database is a search tool for the retrieval of interacting genes or proteins. In STRING, each protein-protein interaction is annotated with one or more "scores". These scores do not indicate the strength or the specificity of the interaction. But, they are indicators of confidence. All scores rank from 0 to 1, with 1 being the highest possible confidence. A score of 0.5 would indicate that roughly every second interaction might be erroneous [32]. In this work, we are interested in the interaction score ≥ 0.800. Then the network consists 10573 nodes and 203319 interactions. Next step, we find subnetwork which has the set of proteins consisted of calmodulin, calcineurin, Caspases, and NFAT transcription factor. We find all possible paths between our selected proteins in the network as a result in a subnetwork of 235 interactions of 43 proteins. Since the subnetwork is still complicated, the proteins involving with apoptosis calcium signaling, and inflammation pathway were filtered. We consider the nodes which has hight degree

because it may be importent nodes that it connect to another nodes and found it in many pathway. Finally, our subnetwork contains 15 proteins and 40 interactions. The set of protein in the subnetwork consists CASP3, CASP7, CASP8, CASP9, CALM1, XIAP, BIRC2, PARP1, AKT1, NFATC2, PPP3CA, NFKB, TNF-α, PTGS2, and NOS2.

Table 3.1**:** Proteins in the subnetwork.

Gene Name	Full Gene Name
AKT1	V-akt murine thymoma viral oncogene homolog 1
BIRC2	Baculoviral IAP repeat containing 2
CALM1	Calmodulin
CASP3	Caspase 3
CASP7	Caspase 7
CASP8	Caspase 8
CASP9	Caspase 9
NFATC2	Nuclear factor of activated T-cells 2,
NFKB	Nuclear factor of kappa light polypeptide gene enhancer
NOS2	Nitric oxide synthase 2, inducible
PARP1	Poly (ADP-ribose) polymerase 1
PPP3CA	Calcineurin
PTGS2	Cyclooxygenase 2
TNF-α	tumor necrosis factor
XIAP	X-linked inhibitor of apoptosis

The subnetwork consists of gene and interaction as shown in Figure 3.1.

Figure 3.1**:** Our subnetwork contains 15 proteins and 40 interactions.

3.2 Activiting and inhibiting processes

It was known that calmodulin is an important part of the calcium signaling pathway. When calmodulin binds calcium ions, it causes activation of calcineurin. Activated calcineurin rapidly causes the dephosphorylation of NFAT transcription factor [14].

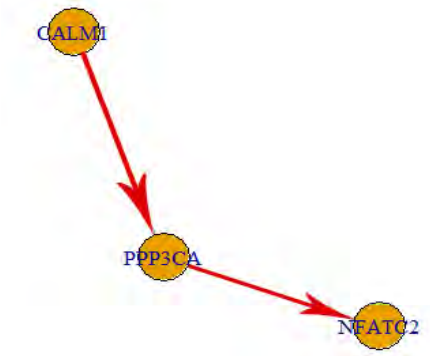

Figure 3.2**:** The interaction of the set of proteins in calcium signaling pathway. An arrowed line represents activating process.

Caspase activity is necessary for the apoptotic cell. Initiations of the caspase families of proteins are responsible for apoptosis. Activation of CASP9 is the initial caspase in apoptotic cascade [56]. AKT1 is an anti-apoptosis in cellular survival pathway and inhibits the apoptosis through the inhibition of CASP9 [12]. In this study, all interactions

of caspases including x-linked inhibitor cooperatively via BIRC2 and PARP1 in apoptosis pathway were retrieved from KEGG database (http://www.kegg.jp).

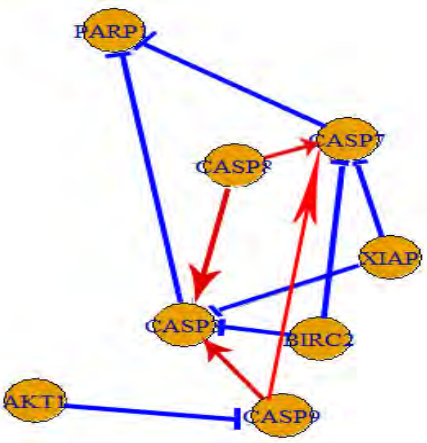

Figure 3.3**:** The interaction of the set of proteins in apoptosis pathway. An arrowed line represents activating process and a line with bar at the end represents inhibiting process.

The tumor necrosis factor (TNF-α) is a cytokine produced by many cell types, such as lymphocytes. The function of TNF is involved in the regulation of biological processes including cell proliferation, differentiation, and apoptosis. This cytokine has been implicated in apoptosis and cell survival as well as in inflammation and immunity. This protein involving a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. TNF trigger either survival or apoptosis by different two receptors. For survival, TNF binds the death domain of TNFR1 receptor that induces the activation of nuclear factor kappa B (NFκB). Then NFκB induces the expression of antiapoptotic factor. Another way, when NFκB is blocked, TNF induce apoptosis by TNF bind TNFR2 receptor leading to the activation of CASP8 [50]. However, TNF induces NFκB and apoptosis can occur together when simultaneously with cellular inhibitors of apoptosis such as XIAP and BIRC2 [20].

Prostaglandin-endoperoxide synthase 2(PTGS2), also known as cyclooxygenase(COX2), is the key enzyme in responsible for inflammation and pain. COX2 enzyme has an important function on cancer cell to inhibit apoptosis and enhance cell migration [10]. So, literatures suggest that COX2 inhibitors are responsible for an inhibition of apoptosis. Then the analyze the effects of COX2 inhibitors are used to improve a good strategy in

cancer therapy [44]. In 2004, Liang-Liang Yu test the expression of COX2 and NFκB in normal and tumor colorectal tissues and found COX2 expression is regulated by NFκB.

Inducible nitric oxide synthase (iNOS or NOS2) is one of three key enzymes generating nitric oxide (NO) from the amino acid l-arginine. NOS2 have important functions in inflammation. There are several studies suggesting that TNF-α activates iNOS expression through NFκB in a variety of cell types [34]. In the most normal cell, NOS2 protein does not appear to be present. The activation of NOS2 associated with several human malignant tumors as well as an anti-infectious and anti-tumor mechanism of innate immunity [3]. Many research data have clearly the expression of NOS2 associate the proliferation of tumor cells [4, 24]. This mean inhibition of the expression of NOS2 may be to lead to apoptosis.

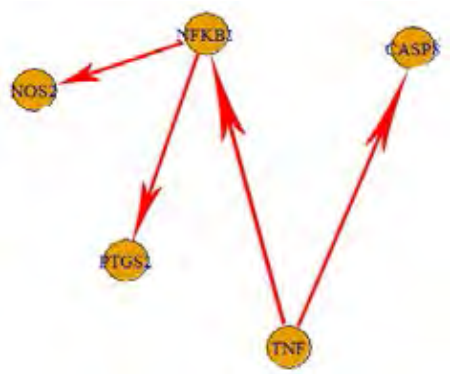

Figure 3.4**:** The interaction of the set of proteins in inflammation pathway. An arrowed line represents activating process.

3.3 Boolean model

From the activating and inhibiting method, we got an interaction of all proteins that we are interested in. And then we constructed the function of each edge in the subnetwork. In a Boolean network, a set of nodes (proteins) is $V = \{v_1, v_2, ..., v_{16}\}$ as the following:

Table 3.2**:** The number of proteins in the model.

Gene Name	nodes (i)	Gene Name	nodes (i)
AKT1	v_1	NFATC2	v_8
BIRC2/XIAP	v_2	NFκB	v_9
CALM1	v_3	NOS2	v_{10}
CASP3	v_4	PARP1	v_{11}
CASP7	v_5	PPP3CA	v_{12}
CASP8	v_6	PTGS2	v_{13}
CASP9	v_7	TNF-α	v_{14}

Since BIRC2 and XIAP have the same function in the pathway, we assume that BIRC2 and XIAP have the same nodes and we used BIRC2/XIAP to represent BIRC2 and XIAP. In this model, AKT1, XIAP/BIRC2, CALM1, TNF-α are input nodes. Because they have only edged out from them.

A set of edges E representing Boolean functions is the set $F = \{f_5, f_6, ..., f_{13}, f_{15}, f_{16}\}$. From all the interaction of the set of proteins in pathway, we got the function of the model as the following: in calcium signaling pathway, calmodulin binds calcium ions, it causes activation of calcineurin, and activated calcineurin causes activation of NFAT transcription factor. So, we got $f_{12} = v_3$ and $f_8 = v_{12}$. In caspases activation, AKT1 inhibits the apoptosis through the inhibition of CASP9, so we got $f_7 = NOT\ v_1$. From inflammation, CASP8 is activated by TNF-α. Hence $f_6 = v_{14}$. Caspases activation is initiated by either CASP8 or CASP9 [7] and CASP3 and CASP7 are inhibited from BIRC2/XIAP. So, we got $f_5 = (v_6\ OR\ v_7)\ AND\ NOT\ v_2$. Since NFATC2 can activate CASP3, so we got $f_4 = v_8\ OR\ (v_6\ OR\ v_7)\ AND\ NOT\ v_2$. Finally, the apoptosis can occur in caspases activation when PARP1 is inhibited from CASP3 and CASP7. This is $f_{11} = NOT\ v_4\ AND\ NOT\ v_5$.

In inflammation, TNF binds the death domain of TNFR1 receptor that induces the activation of nuclear factor kappa B (NFκB). NFκB activates the expression of COX2 and NOS2. Therefore, we got $f_9 = v_{14}$, $f_{10} = v_9$ and $f_{13} = v_9$.

Table 3.3**:** All Boolean function in the model.

Gene Name	Node (i)	function
AKT1	v_1	input nodes
BIRC2/XIAP	v_2	input nodes
CALM1	v_3	input nodes
CASP3	v_4	$f_4 = v_8\ OR\ (v_6\ OR\ v_7)\ AND\ NOT\ v_2$
CASP7	v_5	$f_5 = (v_6\ OR\ v_7)\ AND\ NOT\ v_2$
CASP8	v_6	$f_6 = v_{14}$
CASP9	v_7	$f_7 = NOT\ v_1$
NFATC2	v_8	, $f_8 = v_{12}$
NFKB	v_9	$f_9 = v_{14}$
NOS2	v_{10}	$f_{10} = v_9$
PARP1	v_{11}	$f_{11} = NOT\ v_4\ AND\ NOT\ v_5$
PPP3CA	v_{12}	$f_{12} = v_3$
PTGS2	v_{13}	$f_{13} = v_9$
TNF-α	v_{14}	input nodes

3.4 **Simulation of the network flows**

With the Boolean network model, we could simulate the network behavior and analyze proteins in the network through signal processing method. The dynamic behavior of the network can be fully captured by the possible cases of input proteins [54]. In the Boolean model with n input proteins, a protein could be either activated or inhibited by other proteins. Therefore, all possible cases of a set of n proteins are 2^n. To obtain the possible cases of a Boolean network model, assuming n input proteins(nodes) in the network, we evaluate the values of each protein to be 0 if it is inhibited or 1 if it is activated. The Boolean function for a set of proteins was designed from literature. Then, the logical result for a set of proteins is calculated through the logical function. For example, in the model has 3 input proteins. Therefore, all possible cases of signaling flow simulation with $n = 3$ are $2^3 = 8$ possible cases as shown in Table 3.4. We used the values

of input proteins in the Boolean function f_i, then we obtained the value of output protein.

Table 3.4**:** All possible cases of signaling flow simulation with $n = 3$.

Inputnodes	$\mathbf{f_1}$	...	$\mathbf{f_k}$	**Outputnode**
(0, 0, 0)				0/1
(0, 0, 1)				0/1
(0, 1, 0)				0/1
(0, 1, 1)				0/1
(1, 0, 0)				0/1
(1, 0, 1)				0/1
(1, 1, 0)				0/1
(1, 1, 1)				0/1

3.5 **Experimental data**

The experimental data is supported by National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok, Thailand. In the experiment, we treat a coumarin compound in Jurkat T cells and measure gene expression of our selected genes at 0 and 24 hours using realtime PCR experiment. Then we used $\Delta\Delta$Cq method to calculate the relative of gene expression between our selected gene and reference gene (GAPDH) in control (DMSO) and cells with treating a coumarin compound (CHA) as a result as shown in Table 3.5.

Table 3.5**:** The experimental data.

Treatment	**DMSO at 24 hr**	**CHA at 24 hr**	**ΔΔCq**	**Comparison with GAPDH**
GAPDH	24.56	24.10	-	-
CASP3	27.21	28.44	0.31	inhibit
CASP7	31.22	31.67	0.53	inhibit
CASP8	26.11	26.48	0.57	inhibit
CASP9	34.55	38.64	0.04	inhibit
NFκB	30.24	31.05	0.42	inhibit
TNF-α	31.45	35.04	0.06	inhibit
COX2	32.09	32.84	0.43	inhibit
NOS2	32.53	32.43	0.78	inhibit
BIRC2	31.08	32.79	0.22	inhibit
CALM1	27.49	27.31	0.83	inhibit
NFATC2	31.20	31.47	0.60	inhibit
XIAP	30.92	32.21	0.30	inhibit
AKT1	16.11	23.45	0.01	inhibit
PARP1	28.56	31.40	0.10	inhibit
PPP3CA	24.68	27.27	0.12	inhibit

CHAPTER IV

RESULTS

4.1 Boolean network model of a coumarin compound in Jurkat T-cell line

From the set of filtered proteins and interactions, our model comprises 14 proteins and 17 interactions as shown in Figure 4.1. Four inputs are calmodulin1 (CALM1), tumor necrosis factor (TNF-α), v-akt murine thymoma viral oncogene homolog 1 (AKT1), and X-linked inhibitor of apoptosis/baculoviral IAP repeat containing 2 (XIAP/BIRC2). The output of the model is apoptosis.

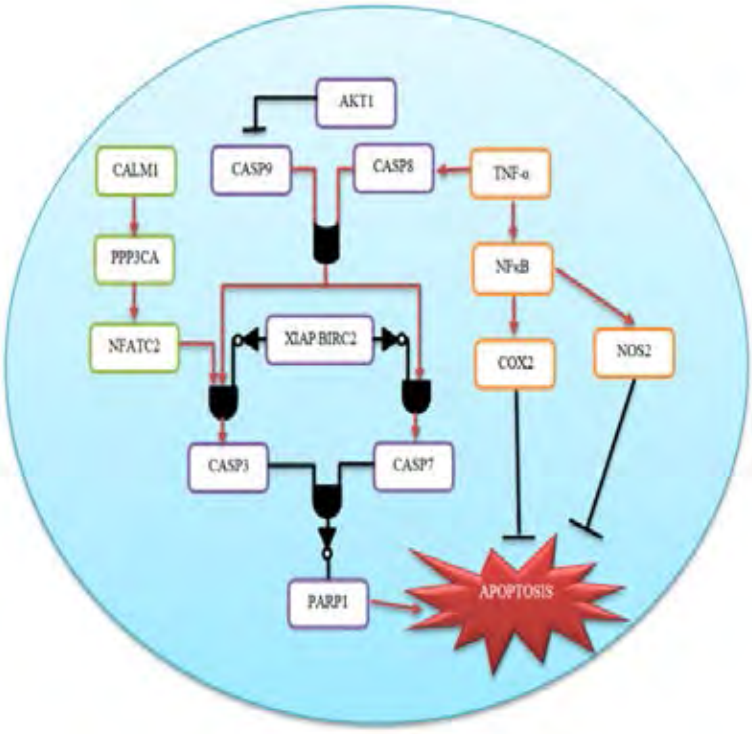

Figure 4.1**:** The proposed mechanism of inflammation, calcium signaling, and effected of caspases by a coumarin compound with a Boolean network model. An arrowed line represents activating process while a line with bar at the end represents inhibiting process.

With above Boolean network model, we simulated the all possible activities of four input proteins to understand the activation of the apoptosis. Each input protein (CALM1, TNF-α, AKT1, XIAP/BIRC2) was set to be either 0 or 1. There were 16 of all possible cases as shown in Table 2. From the model, the apoptosis can occur in 3 way; the inhibition of PARP1, the inhibition of COX2, or NOS2. For first way, the behaviors were as the following: (i) if CALM1 is off (first 8 cases), all proteins in calcium signaling were not activated. This lead to the inhibition of CASP3. However, the apoptosis could arise with the activation of CASP7 due to PARP1 inhibited. In other, the apoptosis was activated from the inhibition of COX2 or NOS2 through inflammation pathway. (ii) If CALM1 is on (last 8 cases), the apoptosis could arise when CASP3 and CASP7 were activated when inhibiting XIAP/BIRC2, and leading to an inhibition of PARP1 results in apoptosis. In another way, the inhibition of COX2 or NOS2 leads to apoptosis.

4.2 Simulation of the network

From our model, there are 4 input proteins. Thus we have the simulation results of 16 all possible cases of the input nodes as shown in Table 4.1.

If proteins CALM1, TNF-α, AKT1, and XIAP/BIRC2 were all inactivated, protein PPP3CA would be also inactivated because of the inactive CALM1. It then led to NFATC2 also inactive. The inhibition of TNF-α lead to CASP8 inhibited while CASP9 was activated because there were no AKT1 to inhibit its' activity. Active (or "on") state of protein XIAP/BIRC2 activated protein CASP3 and CASP7. Therefore, CASP3 was "on" and CASP7 was "on". Then, the PARP1 was inhibited from CASP3 and CASP7 resulting in apoptosis process was activated.

In turns, notice that if proteins CALM1, TNF-α, AKT1, XIAP/BIRC2 were all active, PPP3CA and NFATC2 would be activated. CASP9 was inhibited by active AKT1, CASP3, and CASP7 were inhibited by XIAP/BIRC2. Therefore, PAPR1 was activated and the apoptosis was inactive. For inflammation pathway, the activation of TNF-α lead to NFκB activated. Then COX2 and NOS2 are active, so the apoptosis is not active.

This means if all input active then the apoptosis cannot occur.

Table 4.1**:** All possible cases of the model.

Input nodes	Intermidiate nodes										Output Nodes
(v_1, v_2, v_3, v_{14})	v_{12}	v_8	v_7	v_6	v_4	v_5	v_{11}	v_9	v_{13}	v_{10}	
(0, 0, 0, 0)	0	0	1	0	1	1	0	0	0	0	Activated
(0, 0, 0, 1)	0	0	1	0	0	0	0	0	0	0	Activated
(0, 0, 1, 0)	0	0	1	1	1	1	1	1	1	1	Activated
(0, 0, 1, 1)	0	0	1	1	0	0	0	1	1	1	-
(0, 1, 0, 0)	0	0	0	0	0	0	0	0	0	0	Activated
(0, 1, 0, 1)	0	0	0	0	0	0	0	0	0	0	Activated
(0, 1, 1, 0)	0	0	0	1	1	1	1	1	1	1	Activated
(0, 1, 1, 1)	0	0	0	1	0	0	0	1	1	1	-
(1, 0, 0, 0)	1	1	1	0	1	1	1	0	0	0	Activated
(1, 0, 0, 1)	1	1	1	0	0	0	0	0	0	0	Activated
(1, 0, 1, 0)	1	1	1	1	1	1	1	1	1	1	Activated
(1, 0, 1, 1)	1	1	1	1	0	0	0	1	1	1	-
(1, 1, 0, 0)	1	1	0	0	0	0	0	0	0	0	Activated
(1, 1, 0, 1)	1	1	0	0	0	0	0	0	0	0	Activated
(1, 1, 1, 0)	1	1	0	1	1	1	1	1	1	1	Activated
(1, 1, 1, 1)	1	1	0	1	0	0	1	1	1	1	-

4.3 **Investigation**

To make our model more accurate, the literature search was performed to support these discrete behaviors among our 14 selected proteins. Notice that all of these 14 proteins in the model are all related to apoptosis either directly or indirectly. However, with the complex communication of cell signaling, the system of calcium signaling pathway or caspase pathway to apoptosis is still unclear. A recent study found that in apoptosis, caspases activation is initiated by either CASP8 or CASP9 [7]. They also reported that the extrinsic pathway is when CASP8 is activated, the downstream effector caspases are also

activated either directly or indirectly while the intrinsic pathway, activation of CASP9 can directly cleave to and activate CASP3 and CASP7 [29], [45]. XIAP is a direct inhibitor of apoptosis through CASP3 and CASP7 [13]. In addition, XIAP binding BIRC2 also inhibited the activity of CASP3 and CASP7 [40]. PARP1 is cleaved by CASP3 and CASP7 [25]. NFATC2 is regulated by the calcium and calcineurin signaling pathways [21]. In Pathway Commons database (http://www.pathwaycommons.org/) NFATC2 is a protein that controls the expression of CASP3. The expression level of NFATC2 is increased in activated T cells and active caspase 3 may restrict the level of increased NFATC2 [53]. Although the order of the processes related to caspase activation is well understood, caspase activation pathways are still unclear and some caspase activation does not result in apoptosis [7]. For protein in inflammation pathway, TNF-α is important cytokine which important in the regulation of immune responses associated with inflammation. TNF-α affect to activation of NF B that inhibition of the NFκB could be an effective treatment approach for many cancers [10]. COX2 plays a pivotal role as a mediator of inflammation. Many literature searches proposes COX2 overexpression prevented apoptosis by increasing proliferation of mutated cells. In 2003, Totzke investigate the activity of COX2 inhibition in tumor cells and found that activity of COX2 is induced by TNF and COX2 inhibitors enhance death receptor-induced apoptosis [48]. Moreover, the inhibition of COX2 has been suggested in cancer treatment as well as combination with Radiotherapy, Chemotherapy, and Photodynamic Therapy [44]. Of course, an experimental validation is needed to further evaluate the activity among these proteins in the model and there is still an open case to study the effect of the coumarin compound in treating cancer. This model was established as a preliminary model for further study with the aim of understanding the complex system of these cellular components.

4.4 Comparison of the model prediction and the experimental data

From the experimental data, all input are inhibited. They correspond to case1 in Boolean model, that is $(v_1, v_2, v_3, v_{14}) = (0, 0, 0, 0)$.

Table 4.2**:** case 1 in Boolean model.

Input nodes:(v_1, v_2, v_3, v_{14})	Intermidiate nodes										Output Nodes
	v_4	v_5	v_6	v_7	v_8	v_9	v_{10}	v_{11}	v_{12}	v_{13}	
(0, 0, 0, 0)	1	1	0	1	0	0	0	0	0	0	Activated

Table 4.3**:** Result from the experimental.

Input nodes:(v_1, v_2, v_3, v_{14})	Intermidiate nodes										Output Nodes
	v_4	v_5	v_6	v_7	v_8	v_9	v_{10}	v_{11}	v_{12}	v_{13}	
(0, 0, 0, 0)	0	0	0	0	0	0	0	0	0	0	Activated

In Table 4.2, the most of the intermediate nodes are inhibited except v_4, v_5, and v_7. But in Table 4.3, v_4, v_5, and v_7 are inhibited. Since v_4, v_5, and v_7 are in apoptosis, so apoptosis is not activated in the model, while, inflammation is activated in this case. Therefore, we may summarize that a coumarin compound induces cell death via inflammation.

The results of all of 14 proteins in our model show that there are 11 (78.57%) proteins that our model gave the some activities while 3 proteins (21.43%) the model provided. These three protiens belong to apoptosis. This has many reasons: 1) in different cell types they may have differenct behaviors. This is because it has such different structures and biochemical functions which are specific to enzymes. 2) there are other proteins which have an effect to our proteins in the model. In this case, we could modify the model by adding some proteins to the model to be more accurate in the future work. 3) the last idea, a coumarin compound may not really effect to Jurkat T cell through apoptosis process.

CHAPTER V

DISCUSSION AND CONCLUSION

Over the last decade, the knowledge of the biological system of humans such as cells, tissues or organs would be to study and develop therapies for treating the disease such as cancer. A biological system is very complex because its components such as genes or other molecules are connected as a complicate network or pathway. To understand this complex system, it is not sufficient to study an individual molecule in such a pathway. Therefore, it is necessary to study the interactions among molecules in a pathways. The understanding of the interaction of molecules is pivoted for understanding human diseases and used to develop the treatment of the diseases [16]. To understand this, the mathematical model is used as a tools to capture how biological systems work for responding to a stimuli. In this study, we used Boolean model to study the interactions among proteins and to understand the effect of a coumarin compound in Jurkat T cells.

Boolean model is used to understand the behavior of the biological system which contains various components and provides us an ability to investigate the functions of each component in the model based on experimental data [39]. In addition, Boolean model is used to predict a consequencing behavior of the large system [27]. Moreover, Boolean model also used to investigate the design of the experiment if it is difficult and expensive to be performed [42].

Here, we investigated a mechanism behind the treatment of cancer with a coumarin compound in Jurkat T cells at 24 hours. A Boolean network model of this mechanism was constructed to explain the activities among involved proteins in calcium signaling pathway, apoptosis pathway, and inflammation pathway as we know that the coumarin compound affected to these pathways. The model contained three proteins involving with calcium signaling pathway; namely, CALM1, PPP3CA, and NFATC2. The other

proteins (CASPEs, AKT1, PARP1, XIAP/BIRC2) were related to apoptosis. TNF-α, NFκB, COX2, NOS2 were related with inflammation. The model was manually curated with the experts and the literature, and could well describe the discrete regulation (active or inactive) of each protein when treated with a coumarin compound in Jurkat T cell line.

We tested the accuracy of the model by fitting with the real-time PCR experimental data. We found that this coumarin compound caused cell death by inflammation. However, this coumarin compound may lead to cell death by apoptosis if the model is improved.

Boolean model is a simplest discrete model and widely used to study the behavior of a biological system. However, it has some disadvantages. Since the model is used to predict activations of the system without the exact timing of cascades of the biochemical reactions, so it may be unrealistic or may contain an error to predict the real behavior of gene or protein activities. However, Boolean model can be used to simplify the behavior patterns of protein activity with much less input.

Finally, our model could be used to further study the complex behavior of each protein and used as a preliminary model for the effect of a coumarin compound to apoptosis pathway.

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APPENDICES

APPENDIX A : Code in R program. This code is used to construct the network of the interection of proteins and find the subnetwork which consists of the set of proteins involving in calcium signaling pathway, apoptosis pathway, and inflammation.

```
1  install.packages("igraph")
2  install.packages("network")
3  install.packages("sna")
4  install.packages("ndtv")
5
6  ##Construct network of PPI
7  mydata <- read.delim("E:/project/dataset1.txt")
8  data <- mydata[mydata$Score>=800,]
9  c3 <- unique(data[,3])
10 c4 <- unique(data[,4])
11 vertex <- union(c3,c4)
12 nodes <- unique(vertex)
13 links <- unique(data[,3:4])
14 typeof(vertex)
15 typeof(nodes)
16 typeof(links)
17 head(nodes)
18 head(links)
19 nrow(nodes); length(unique(nodes))
20 nrow(links); nrow(unique(links[,c("G1", "G2")]))
21 link <- links[order(links$G1, links$G2),]
22 rownames(link) <- NULL
23
24 library(igraph)
25
26 net <- graph.data.frame(link, nodes, directed=T)
27 V(net)
28 E(net)
29 degree(net)
30 plot(net)
```

```
31
32 ##Find path between interest proteins
33 v<-c("CASP3","CASP7","CASP8","CASP9","CALM1","NFATC2","PPP3CA","
       NFKB1","TNF", "PTGS2","NOS2")
34 allshortestpath <- all_shortest_paths(net, from =v, to=v, mode = '
       all',weights = NULL)
35 allshortestpath$res
36
37 ##Plot the subnetwork
38 newv <- c("CASP3","CASP7","CASP8","CASP9","CALM1","XIAP","BIRC2","
       PARP1","AKT1","NFATC2","PPP3CA",
39 "NFKB1", "TNF", "PTGS2", "NOS2")
40 subnetwork <- subgraph(net,newv)
41 newsubnetwork <- as.undirected(subnetwork,mode = c("each"))
42 plot(newsubnetwork)
43 E <- E(subnetwork)
```

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