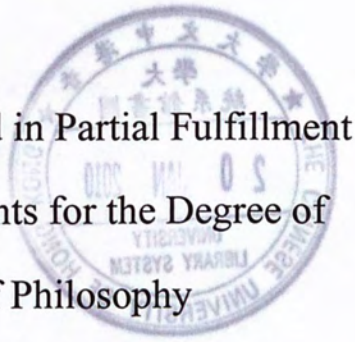
Further Exploration to the Cucurbitacin D (LC978) Signal Transduction Pathway during Fetal Hemoglobin Induction

ZHANG, Siwei



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Thesis/ Assessment Committee

Professor LAM Hon Ming (Chair)

Professor FUNG Ming Chiu (Thesis Supervisor)

Professor GE Wei (Committee Member)

Professor CHOW King Lau (External Examiner)

Statement

All the experimental work reported in this thesis was performed by the author, unless otherwise specified.

Siwei ZHANG

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Abstract:

β-thalassemia has been one of the most prevalent gene defect hemoglobinopathies in Asia-Pacific regions. Its common distribution and severity of symptoms have brought heavy burdens to both patients and public health service. Multiple treatments have been developed to cure this anemia, however, current treatments all fell in either extremely high cost or cytotoxic phenomenon. Here we propose the investigation of a newly developed chemical, cucurbitacin D (CuD), as a candidate drug against β-thalassemia. In the *in vitro* experiments, this compound showed high efficiency in activating γ-hemoglobin expression including human K562 cell lines, human normal bone marrow progenitor cells, and β-thalassemia major patient progenitor cells. All of above resembled a potential way to arise similar process *in vivo* and relieving the acute symptoms of β-thalassemia. Here we propose further investigations on the molecular mechanism and signal transduction pathway of CuD in K562 cells. Recent researches have indicated that CuD and hydroxyurea interacted with p38 MAPK/JAK2-STAT3 pathways and thus interfered the binding activity of STAT3 to regulate γ-globin gene expression level. Here we provided a detailed investigation on the signal transduction pathways that involved during CuD/hydroxyurea-mediated γ-globin gene expression, with the STAT3 rested at the core of the whole regulatory system.

Keywords: cucurbitacin D, LC978, K562 cell line, p38 MAPK pathway, JAK2, STAT3

摘要：

乙型地中海贫血症现今已成为亚洲及泛太平洋地区最为广泛的血红蛋白基因缺损症之一。其广泛的分布与严重的症状为患者与公共医疗体系都带来了严重的负担。至今为止，多样的治疗方法已被开发出来，然而，现今的治疗方法大部分有其局限性，主要表现在高昂的治疗费用与难以控制的副作用两方面。在本部分研究中，我们着重于葫芦素 D 在细胞模型中重新激活丙种血红蛋白基因表达的能力。前述研究已经表明葫芦素 D 可能具有在重症乙型地中海贫血患者体内重新激活胎儿血红蛋白的合成与减轻地中海贫血症状的能力。我们的研究表明葫芦素 D 与羟基脲通过影响 p38-丝裂原途径与 STAT3 途径的激活从而影响转录因子 STAT3 与靶序列的结合能力而控制丙种血红蛋白基因表达水平。在本部研究中，我们提出了葫芦素 D 与羟基脲通过不同细胞信号途径从而控制转录因子 STAT3 活化的模型，并试图以此解释丙种血红蛋白基因的不同调控途径。

List of Abbreviations

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
DTT	Dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde phosphate dehydrogenase
HbF	Fetal hemoglobin
HRP	Horseradish peroxidase
LC978	Lead compound LC978 (Xing, 2003) i.e., Cucurbitacin D
LCR	Locus control region
M-MLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
OBD	Optimal biological dose
OD	Optical density
PCR	Polymerase chain reaction
pd(T)12-18	Polydeoxythymidine, oligomers of 12 to 18 bases
rcf.	Relative centrifugal force
rpm.	Rotation per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription
RTase	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SEAP	Secreted alkaline phosphatase
SEM	Standard error of the mean
TMB	3,3',5,5'-Tetramethyl benzidine
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume

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1. General introduction

1.1. Types, structure and function of human hemoglobin

1.1.1. Structure and functions of human hemoglobin

Hemoglobin is one of the major proteins presented in erythrocytes (red blood cells), and its main function is to provide oxygen carrier in systematic blood circulation between respiratory system (lung) and tissues as an oxygen transportation mediator (Lecks et al. 1950). The hemoglobin is a tetrameric macromolecule, which consists of a tetramer assembly of four globin subunits. Each globin subunit contains a prosthetic heme group located at the center, with a ferrous atom chelated at the very central of heme group. The heme group and central ferrous atom are the main structure for the coupling and uncoupling of oxygen molecule. It consists of a porphyrin ring, which chelates one single iron atom in ferrous state via four nitrogen atoms on four p-orbits. The other two free p-orbit form two σ-bonds, one connects to the histidine residue on globin main chain to hold the ferrous atom in position, and another is used for the binding site of oxygen molecule. Once the central iron atom was oxidized from ferrous state to ferric state, the oxygen-binding ability of globin molecule is completely lost.

Four globin subunits are included in the hemoglobin tetramer. In adult, the four globin subunits are composed by two identical α-chains and two identical β-chains. The α- and β-chains are bonded non-covalently and held in position by means of hydrogen bonds, Van-der-Waals force, and hydrophobic interactions. Though the α and β chains do not possess much similarity on primary structure, their three-dimensional structures are comparable (Choi et al. 1988).

Successful binding of oxygen molecules onto hemoglobin tetramers depends on the relative movements of the globin subunits. This is done by the contacts between the

α1β1 and α2β2 stabilize the hemoglobin molecule. During the oxygenation and deoxygenation process, both β subunits slide relatively to the position of two α subunits, resulted the contact between α1β2 and α2β1 subunits (Lalezari et al. 1990). Two interchangeable conformations of human hemoglobin facilitate the loading and unloading of oxygen molecules, which are represented as T and R states. Hemoglobin molecules that are not coupled with oxygen are called T-state hemoglobin. Once it couples with one oxygen molecule, it triggers a change in conformation to the R-state, causing the heme group located at the center of globin subunit to adapt a more planar conformation. The confirmation change of heme group leads to a shifting of histidine residue approaches to the ferrous atom of heme group, and a more cooperative binding ability of oxygen is presented (Choi and Engel 1988; Chakalova, Carter et al. 2005).

One of the most distinctive characters of hemoglobin and its interaction with oxygen is the cooperative binding curve. As indicated, the transition from T-state to R-state will facilitate the affinity of globin units to the oxygen molecule, and this results a sigmodal oxygen dissociation curve (Chakalova et al. 2005). In other words, the hemoglobin affinity to oxygen climbs faster than the increase of oxygen partial pressure. When the oxygen partial pressure is only 26.8mmHg, the hemoglobin already reaches its 50% saturation level (Chakalova et al. 2005).

Different factors play role in interfering the oxygen dissociation curve of hemoglobin, and two of the most important ones are environment pH value and the partial pressure of carbon dioxide. Under the circumstance of reduced pH value and increased CO2 pressure, the hemoglobin affinity towards oxygen tends to reduce, which favors a more effective unloading of oxygen in tissues with higher concentration of respiratory product.

1.1.2. Types of human hemoglobin

Human erythrocyte progenitor cells synthesize different types of hemoglobin at

different developmental stages according to the places where hematopoiesis take place. To be detailed, during the embryonic stage, the hematopoiesis mainly takes place in the yolk sac, and the most synthesized hemoglobin tetramers are ζ2ε2, ζ2γ2 and α2ε2. In the fetal stage, the hematopoiesis location shifts to liver, spleen, and bone marrow, and predominant synthesized tetramer is fetal hemoglobin (α2γ2). This fetal hemoglobin dominancy persists until the prenatal and perinatal stage, which is around 6 months post birth. After which, adult hemoglobin α2β2 takes place to be the major form of hemoglobin in body circulation, with a small percentage of α2δ2 and α2γ2 tetramer remain to be expressed (Hoffbrand et al., 2001).

1.2. Regulatory mechanism of human hemoglobin expression

1.2.1. The human α and β locus

Two different gene clusters are responsible for the direction of hemoglobin synthesis during development. The human α-locus, which is located on the p (short) arm of chromosome 16, contains seven different genes in a tandem manner. The globin genes sequence located on α-locus is 5'-ζ-ψζ1-ψα2-ψα1-α2-α1-θ-3', with three pseudogenes starting with ψ. Regulation system of the α-locus is not fully clarified, but the order of tandem gene arrangement is known to be critical in regulation the expression manner, and autonomous developmental regulation also has been found in human transgenic mice (Albitar et al. 1991; Sharpe et al. 1993; Tang et al. 2006).

The human β-globin gene cluster is located on the p (short) arm of human chromosome 11, with five functional genes arranged in a tandem manner. Interestingly, the arrangement of globin genes 5'-ε-Gγ-Aγ-δ-β-3'determines their expression sequence. As mentioned, the ε gene is firstly expressed in yolk sac during the developmental process. And it followed by two Gγ and Aγ genes, which are expressed in prenatal and perinatal stage. Finally, the δ and β globin genes are expressed in adult stage. These gene shifts are rigidly controlled during developmental process, and each of the latter globin gene expression will almost

silence the expression of former gene completely (Grosveld et al. 1987; Simon et al. 2001; de Andrade et al. 2006)

1.2.2. Development of globin genes switching concept

The initial idea of globin genes switching mechanism was raised during 1970s. It was considered that the colonial successions of two alternative stem cell lineages, which possess different gene expression programs, were responsible of the globin gene switching manipulation. Initially, two stem cell lineages, one possesses fetal hemoglobin expression program and another possesses adult hemoglobin expression program, coexists in the host body. During prenatal and perinatal stages the fetal stem cell lineages was put into proliferation and led to the production of fetal hemoglobin in erythrocytes and progenitor cells. After birth, the adult stem cell lineage was switched on and replaced fetal stem cell colonies (Weatherall et al. 1976). However, the existence of persisted gene expression was disputed. Immunohistochemical staining revealed a special cell population, which called F cells, was found in all adults, and the expression of fetal hemoglobin was cellular and lineage restricted (Boyer, Belding et al. 1975; Weatherall, Clegg et al. 1976). Other research results argued that the hemoglobin expression pattern in progenitor cells actually under changes, as one single cell lineage would be able to produce more than one kind of hemoglobin during all its development stages (Papayannopoulou et al. 1976). Experimental results indicated that in vitro cultured human erythroid burst forming units (BFUs) was able to secrete both adult hemoglobin and fetal hemoglobin without segregating into colonies with different gene expression pattern(Papayannopoulou et al. 1977). According to which, it has been postulated that the globin gene switching was connected to differentiative events and transcriptional level control. In vivo experiments using baboon model also indicated that rapid regeneration of erythroid stem cells would enable premature terminally-differentiated cells to produce fetal hemoglobin via the perseverance of

primitive globin gene expression scheme(Umemura et al. 1988). And these findings all supported the idea that gene shifting is able to take place in one single cell lineage.

1.2.3. Factors that regulate globin gene expression

1.2.3.1. The locus control region (LCR)

LCR is one of the most significant regulation components that determine gene expression level. It is able to regulate the expression of linked genes to physiological levels in either tissue-specific or copy number dependent manner at the ectopic chromatin sites via the looping model (Gerstein et al. 2007).

The LCR of β-globin gene cluster was situated at 6-22kb upstream at the beginning of the first globin gene, ε, with a length of 16kb in total. This LCR contains five DNAse I Hypersensitive Sites (HSs), which carry core elements for the regulation of globin-gene cluster functions. Among these 5 hypersensitive sites, HS1 and HS2 are considered to possess classical enhancer activity (Jackson et al. 2003; Jia et al. 2003). HS3 and H4 are considered to be able of manipulating and trigger transcription activation via interaction with other chromatin domains (Jackson et al. 2003; Vieira et al. 2004; Harju et al. 2005). HS5 works as an insulator to isolate the core components from other globin gene promoters to prevented unexpected interaction and gene regulation (Li et al. 1994; Li et al. 2002). An additional HS is located at the 3' end of the β locus, which is specific to erythroid cells and enabling the stabilization between globin gene and 5' LCR hypersensitive sites (Fleenor et al. 1993).

1.2.3.2. The cis-regulatory elements

Cis-regulatory elements are regions of nucleic acid sequence that regulate the expression of genes located on their same strand, which mainly include promoters, enhancers, and silencers (Wray 2007).

Each globin gene possesses their own promoter region located upstream of the coding

sequence in order to recruit the binding of RNA polymerase and initiation of gene transcription. On the β locus, the promoter of all globin genes demonstrated significant homology in conserved regulatory elements. The TATA, CCAAT, and CACCC/GGGGTG boxes are three main cis-regulating elements found in the globin gene promoters of β locus (Myers et al. 1986). Each of the globin gene located on the β locus has one or two copies of these indicated elements. However, since developmental stage-specific transcription factors are essential for the shifting of globin gene expression, each of the promoter regions of globin genes also possesses unique sequence for stage-specific regulation and controlled by stage-specific transcription factors (Harju et al. 2002).

Enhancers are DNA sequences that able to increase the transcription level from promoters by binding with proteins in a position-independent manner (Spilianakis et al. 2005). Mainly there are two enhancer elements found within the β locus region. The former one was located at 420bp following the poly-A signal of the Aγ gene, and the latter one was situated at the 3'-end of the β-globin gene (McDonagh et al. 1991; Aladjem et al. 1995; Buzina et al. 2005).

Silencers are DNA sequences that interfere with transcription activities and lead to the down-regulation of gene expression by interacting with binding repressor proteins (Temple et al. 2004). On the human β locus region, the most distinctive silencer region is located at the 5'-end area of HS5 with a tandem repeat containing sever continuous GATA sequence to repress the HS2 enhancer activity (Ramchandran et al. 2000). Another two silencers containing the binding sequence of the repressor protein BP-1 and BP-2 was also found located at the 5'-end of the β-globin gene (Berg et al. 1989).

1.2.3.3. The trans-acting factors

The concept of trans-acting element is usually applied to indicate either a DNA sequence containing a gene or its expression product that will be used for the regulation of another target gene (Chen et al. 1996). Mainly, the trans-acting factors

related to globin gene regulation include GATA family, Krüppel family, STAT family and nuclear factor erythroid family (Foley et al. 2002; Perry et al. 2002).

The GATA family is characterized by the target binding sequence of (T/A)GATA(A/G), which belongs part of the zinc-finger transcription factor family. Different subtypes of GATAs are found in interacting with β-promoters and are not tissue-specific (Zhao et al. 1999). Generally speaking, GATA-1 is the most essential one that involved during erythrogeneisis and megakaryocyte differentiation (Perry et al. 2002). The presence of GATA-1 binding sites are also been found in all globin promoters and HS1-HS5 site. The GATA-1 effect towards transcription activity of different globin genes is diverse, depending ton the transcriptional circumstance (Gong et al. 1991; Raich et al. 1995). It also interacts with other transcription factors such as EKLF and FOG in regulating the globin gene promoter transcriptional activity (Crossley et al. 1994; Letting et al. 2004). GATA-2 mainly is not involved at the late developmental stage of hematopoiesis, though some functional analysis has been performed during the early hematopoiesis and globin gene activation period (Anguita et al. 2004; Brecht et al. 2005).

Two main subfamilies of Krüppel-like factors are connected to the hematopoiesis and globin gene expression. The fetal Krüppel-like factor (FKLF) mainly triggers embryonic and fetal globin gene expression during the early stage of human development (Asano et al. 1999; Asano et al. 2000; Mitsuma et al. 2005). In contrast, the erythroid Krüppel-like factor (EKLF) possesses more affinity to the GT/CACC box and regulates the adult hemoglobin production (Harju et al. 2002).

The Signal Transducers and Activators of Transcription (STATs) are interconnected with membrane-bound receptors for a wide variety of ligands, which including cytokings, hormones, growth factors, and neurotransmitters (Darnell et al. 1994). Researches have indicated that the binding of erythropoietin (EPO) to the specific receptors on cell surface will induce the JAK2 tyrosine kinase activity. Several downstream effectors of the JAK2 kinase, including STAT5 and STAT3, have been validated during EPO-induced hematopoiesis (Iwatsuki et al. 1997; Kirito et al. 2002).

1.3. The human hemoglobinopathies

It is without doubt that the maintenance of functional hemoglobin genes to be essential for human health. Any kinds of disorders or abnormalities on hemoglobin will be considered into the clinical term of hemoglobinopathy, in other words, the diseases on hemoglobins. Different kinds of hemoglobinopathies have been found during clinical trails. And thalassemia symptoms are usually found in these patients with hemoglobin disorders.

The reasons of hemoglobin abnormalities are diverse, but mostly come out from defective globin genes or the regulatory components. Most hemoglobin abnormalities will result in either reduced globin production or sequence alternation, which leads to the structure change of hemoglobin tetramers. The alteration of hemoglobin sequence usually produces structural variants and unstable hemoglobin presence in blood, and resulting in incapable oxygen transportation or changes in fluid property of blood. Incapability of oxygen transportation triggers a series of symptoms, mainly including tissue stress, asphyxiation, and growth retardation. The latter physical fluid property changes of blood leads to more severe life-threatening problem such as hemolytic anemia (Tischler et al. 1979). Different categories of thalassemia will be discussed in detail below.

1.3.1. α-thalassemia

α-thalassemia is characterized by inadequate synthesis of human α-globin. Upon clinical trails, the inadequacy of α-globin synthesis is usually contributed to the segment deletion of coding regions on human α-globin gene, with infrequent mutations or alternations on the promoter or LCR region (Albitar et al. 1991; Tang et al. 2006). Since one normal individual possesses four copies of the same α-globin gene, the thalassemia severity is greatly depending on the ration of mutant/normal genes. Fetus with the loss of all functional α-globin genes cannot survive until prenatal stage; and individuals with only one mutant gene usually do not express

abnormalities but still carriers. Individuals with two or three mutant α-globin genes may demonstrate moderate to severe anemia symptom, and splenomegaly are frequently found in patients (Williams et al. 2005).

1.3.2. β-thalassemia

β-thalassemia presents another pathological and genetic scheme which is very different from the α-thalassemia. The pathological origin of β-thalassemia can be elicited in two ways, either by mutations located at the promoter region or the mutations on coding region of the globin gene itself. Up to now, over 200 different kinds of point mutations have been reported to be responsible of β-thalassemia. Either of these two kinds of mutations lead to reduced production level of normal β-globin, which disrupt regular α to β globin production ratio and leaving the α-chains in an excess (Olivieri 1999). Excessive presence of α-chains may precipitate themselves in erythroids, making erythroid membrane to be fragile and finally lead to hemolytic anemia (Rund et al. 2005). Different from α-thalassemia, the severity of β-thalassemia is not determined by the copy number of normal globin gene, but by the amount of normal β-globin production regarding to different types of mutations. Mutant genes with nil β-globin production are designated as β^0 class mutant; mutant genes with partially functional production of β-globin are designated β^+ class mutant. Patients with homozygous β^0 mutations are called β-thalassemia major, while patients with homozygous β^+ mutations are called β-thalassemia intermedia. Patients who possess only one copy of β^+ mutations are β-thalassemia minor (Weatherall et al. 1976; Olivieri 1999; Rund et al. 2005). Generally speaking, four kinds of main mutations determined the malfunction of β-globin gene in patients, which including defective gene transcription, mutations in coding sequence, mutations producing abnormal mRNA post-processing and mutations of abnormal mRNA translation activity.

Currently, β-hemoglobinopathies is one of the most prevalent autosomal disorders in Southern China (Li et al. 2006). The observed β-thalassemia defective gene carrier

frequency in Southern Asia communities ranges from 1.8% to 5.5% (Kazazian et al. 1986). This genetic disorder adds extra burden to the patients' circulatory and respiration system, and usually result to a systematic of acute symptoms as frequently painful crises of joints, chest consolidation syndrome and organ dysfunctions. As a result, these chronic and acute symptoms may also prevalently reduce the life expectancy of patients (Stuart et al. 2004). Due to the prevalent defect gene carrier ratios, there were also distinctive amounts of patients who suffering from severe anemia that caused by carrying two defective genes (Kazazian et al. 1986). In order to extend the life expectancy of these patients, expensive treatments as blood transfusion and marrow transplantation methods have to be applied. Though the medical and public health administrative department both devote great attentions, it was still unlikely for the severe patients to reach their life expectancy over the age of 25 (Li et al. 2006).

1.3.3. Sickle cell anemia

The sickle cell anemia is another type of β-globin gene disorder, and named after the occurrence of sickle-shaped red blood cell in patients. It was identified by a single amino acid substitution, glutamine to valine, to be responsible for the polymerized filamentous structure of mutant hemoglobin (HbS) appearance under low oxygen environment. The sickle-shaped erythrocytes are fragile and tend to rupture or coagulate while passing small blood vessels, interfering microcirculatory status of patients and thus causing vaso-occlusive crisis. The molecular detail of this vaso-occlusive crisis is complicated and including multiple steps as erythrocytes adhesion to slow-flowing venular side of vessel endothelium or post-capillary venule, leucocyte-endothelial adhesion, and formation of heterocellular aggregates. Finally, local hypoxia and propagation of occlusion results in neutrophil trans-migration and adds inflammation to the microvasculature hence triggering dysregulation and dysfunction of microcirculations (Perrine et al. 1972).

However, the sickle gene also has one genetic advantage that ensures its survival through natural selection. The heterozygous carriers are much less prone to be affected to the infection of *Plasmodium falciparum*, which prevalently causes malaria in tropical area. It is a delicately demonstrated example of balanced genetic polymorphism in humans, with three major distinctive haplotypes in Africans. Besides, the war and slave trade during voyage years are responsible to the gene dissemination and flow to North Africa, Middle Asia and even North America (Stuart et al. 2004).

1.4. Current approaches towards β-thalassemia treatment

1.4.1. Blood transfusion

Blood transfusion is one of the traditional methods in treatment of β-thalassemia major and intermediate. It works by transfusing external-origin from donor into patients to compensate the inadequacy of hemoglobin production. This method greatly alleviates oxygen stress in tissue, and it also allow the patients for a better chance of normal growth and systematic development (Johnson et al. 1987; Castilho et al. 2002).

However, the blood transfusion treatment faces severe problem on excessive iron deposition in tissues. Regarding to the excessive income of hemoglobin, the iron-containing deposition product puts stress in heart, liver, and kidney. Hence, patients with blood transfusion treatment require frequent administration of special iron-chelating agents to expel excessive iron in tissue (Schrier et al. 2005). The most widely applied iron-chelating drugs are deferoxamine (DFO) and deferasirox. The former one was developed early and needs frequent injection. The latter one prevented the injection constraint and it can be administered orally for more convenience (Borgna-Pignatti et al. 2006; Neufeld 2006).

1.4.2. Bone marrow transplantation

The bone marrow transplantation treatment method was originally raised by the idea of replacing whole hematopoiesis system in patients and thus restoring functional production of erythrocytes. Similar to other organ-transplantation treatments, the bone marrow transplantation method greatly relies on the compatibility of HLA antigen isotopes between donor and patient, which is sometimes difficult to achieve. Even so, the great administration fee, low successful transplantation rate, and multiple side effects still lie between patients and administration (Benedetti et al. 1992).

1.4.3. Drug-induced activation of fetal hemoglobin production

1.4.3.1. Hydroxyurea

Hydroxyurea is one common derivative from urea by adding two hydroxyl groups onto the NH_2-residues. It was firstly observed of possessing inhibitory ability on ribonucleotide reductase, which hinders DNA synthesis and cancer cell proliferation. Hence, it was originally used for treatment of chronic myelogenous leukemia and other cancer symptoms (Elford 1968). During the following studies, it was discovered of possessing ability on inducing HbF in patients with anemia (Platt et al. 1984). Its mechanism was considered to be accelerating erythroid regeneration by inducing DNA demethylase ability and promoting S-phase DNA synthesis. As a result, rapid erythropoiesis enhances the chance of HbF^+ cell formation via premature commitment of earlier progenitor cells, and hence causing γ-globin gene activation (Galanello et al. 1988). Besides, increased hydroxyurea dose also inhibits erythroid burst-forming unit (BFU-E) colony proliferation, in a manner of accelerating erythroid regeneration and increasing the HbF production level (Yang et al. 1997; Baliga et al. 2000). Hydroxyurea has also demonstrated its curing effecto towards patients with β-thalassemia by reducing the requirement of blood transfusion,

increasing HbF concentration, and inhibiting extramedullary hematopoiesis (Loukopoulos et al. 1998; Saxon et al. 1998; Bradai et al. 2003). It is an FDA-approved drug currently available on pharmaceutical market.

1.4.3.2. Butyrate and short-chain fatty acids

The HbF induction ability of short-chain fatty acids and their derivatives, such as sodium butyrate, arginine butyrate and sodium phenylbutyrate, has been under investigation in laboratory for long time. It has been discovered that overdose ingestion of butyrate was able to hinder the gene switching from embryonic/fetal stage to adult stage in murine cell lines, chicken, and sheep (Kameji et al. 1977; Ginder et al. 1984; Perrine et al. 1988). It was also benchmarked on patients with sickle cell anemia and β-thalassemia, which demonstrated encouraging effects on enhancing HbF production and inhibiting BFU-E formation (Perrine et al. 1989). The widely accepted theory considers butyrate increases HbF production by acting as an histone deacetylase inhibitor thus promoting the transcription efficiency (Safaya et al. 1994). However, other theories focusing on the interference of p38 MAPK signal transduction pathways and enhancement of γ-globin mRNA stability were also proclaimed during research (Pace et al. 2003; Weinberg et al. 2005).

1.4.3.3. Mutagens, DNA methyltransferase inhibitors and other HbF inducible agents.

Besides hydroxyurea and butyrates, drugs with properties of DNA methyltransferase inhibitors or DNA-binding, were also under laboratory trails for their potentials in eliciting HbF production. 5-azacytidine, which is a strong inhibitor of DNA methyltransferase, has been demonstrated able to enhance HbF production in thalassemia patients (Ley et al. 1982; Clegg et al. 1983). Other DNA-binding chemicals such as mithramycin and chromomycin, which interferes transcription via changing DNA structures, have also been tested for their potential in reactivating γ-globin gene expression (Bianchi et al. 2001; Fibach et al. 2003). However, the

HbF inducting effects of these DNA-related drugs usually accompanied by strong side-effects and potential carcinogenicity, which made them inappropriate for human and clinical trials (Clegg et al. 1983).

1.4.3.4. Cucurbitacin D

Cucurbitacins are one large family of plant secretes belong to the group of plant triterpenoids. It derived from phytosterol cucurbitacin group, a group of plant steroids secreted by *Cucurbitaceae* family. Two of the main compounds in this family, Cucurbitacin B and D, were firstly isoloated from seeds of *Iberis umbellata.* Primitive experiments demonstrated these triterpenoid derivatives have the ability of antagonize the function of 20-hydroxyecdysone (20E)-induced morphological changes in *Drosophila melanogaster* cells *in vitro* (Dinan et al. 1997). Later, the characteristic anti-hydroxyecdysone ability was applied for the integration of Cucurbitacins into pesticides. During our drug-induced HbF production research, it was firstly isolated as a lead compound extraction from Traditional Chinese Medicine *Trichosanthes kirilowii.* Upon isolation, it demonstrated activation ability of γ-globin gene in chronic myelogenous leukemia (CML) K562 cell line with an optimal concentration ED_{50} around 10ng/ml. Comparing with the FDA-approved drug hydroxyurea that has an ED_{50} of 25μg/ml, the Cucurbitacin D showed extraordinary γ-globin gene reactivity. Besides, the Cucurbitacin D also demonstrated much lower ED_{50}/IC_{50} ratio that was only 1:67 comparing with the 1:2.5 ED_{50}/IC_{50} level of hydroxyurea. These discoveries indicating the Cucurbitacin D may be a successful drug candidate in reactivating γ-globin gene expression in patients with β-thalassemia (Xing 2003).

1.4.4. Gene therapy

The idea of correcting defective genotype of patients' hematopoietic system has been raised for a long time. However, the main difficulties of low level of expression and

transient transfection were only been overcome after the induction of lentifiral-based gene vectors. Researches using transgenic mice model with β-thalassemia symptoms indicated the thalassemia symptom could be alleviated by inducing lentiviral vector transfection fused with normal human β-globin gene (May et al. 2002). However, potential danger of uncontrollable gene expression and concerns on virus mutation are still preventing these gene therapies from human trials.

1.5. Research Objectives

Two important questions have been raised based on the acquired data and previous research results. Since the enhancement of fetal hemoglobin production induced by CuD in K562 cells has been verified, it is important to explore whether the CuD increases the production of γ-globin by up-regulating the transcription of γ-globin gene, and investigate any potential signal transduction pathways that have been involved during the induction process. The detailed proposals have been list below:

- Detect the expression scheme of γ-globin gene at different time points of post induction by CuD.
- Compare the HbF induction ability of CuD, hydroxyurea and other potential HbF inducers.
- Analyze the potential signal transduction pathways and key regulating factors triggered by Cucurbitacin D and hydroxyurea during γ-globin gene activation. Investigate any potential cross-talking mechanism.

2. Analysis of CuD, Hydroxyurea and other inducers on the induction of α, β, γ, δ, ε , ζ, BP-1 genes and fetal hemoglobin induction

2.1. Introduction

2.1.1. Properties of human K562 cell line

The human K562 cell line is derived from erythroid progenitor cells from one patient with chronic myelogenous keukemia (CML). It is a multi-potential hematopoietic malignant cell line, carrying Philadelphia chromosome type 1 (Ph1), and has the ability of self-differentiation into different progenitor cell lines including erythrocytes, granulocytes and monocytes (Lozzio et al. 1975; Koeffler et al. 1980). The K562 cell line has been widely used during the γ-globin gene activation research and β-thalassemia mechanisms regarding to its ability of producing fetal and embryonic hemoglobins under proper inducers (Rowley et al. 1981). Previous researches have indicated the responsive inducers of K562 cell line include butyrate, hemim, hydroxyurea, amifostine, and 5-azacytidine, as these drugs were also applied during the followed experiments (Andersson et al. 1979; Rutherford et al. 1979; Erard et al. 1981; Rowley et al. 1981; Gambari et al. 1984; Bianchini et al. 2007). Besides, the human K562 cells also presented a potential unfavorable environment of human β-globin gene expression, though whether there was indeed any β-globin gene inhibition mechanism existing is still being disputed (Young et al. 1985; Mookerjee et al. 1992; Chase et al. 2002).

2.1.2. Induction and measurement of fetal hemoglobin

Two alternative methods have been developed to monitor the fetal hemoglobin production *in vitro*, at different stages of gene expression. The real-time RT-PCR method, which monitors the fluctuations of cytosolic mRNA level, is applied to study

the individual globin-gene expression changes on the transcription level. Firstly the cytosolic mRNAs are extracted from total cell lysates, then a reverse-transcription (RT) method is performed to reverse-transcribe extracted mRNAs into cDNAs on a quantitative basis. Finally fluorescence-based detection method is used to quantify copy numbers of different desired gene in the cDNA pool. Usually an internal-control gene that is not easily affected by changes of external environments, as GAPDH or β-actin gene, will be introduced to compensate the dose differences in the original cell lysate.

An alternative way of monitoring fetal hemoglobin production under drug-induced scheme is ELISA method. Different from the real-time RT-PCR method that monitors the gene expression at the transcription level, the ELISA method directly detects the net amount changes of synthesized fetal hemoglobin using specific antibody and chromogens. This method excludes the possible interference on post-transcriptional modifications, and it has been widely applied during drug-activation of γ-globin gene in progenitor cell assay (Iyamu et al. 2003).

2.1.3. Induction of α, β, γ, δ, ε , ζ and BP-1 gene and Real-time RT-PCR analysis

The time-dependent spectrum of γ-globin gene expression in K562 cells under CuD induction will provide useful information on signal transduction mechanism. Since the induction and expression spectrum varies between different chemicals, it is crucial for us to understand the precise expression timeline of γ-globin gene by adding CuD. If γ-globin gene expression level increases within a short period (e.g. three hours) after CuD addition, it will likely be concluded that a direct response mechanism has been involved during the induction process like the response of *Xenopus vitellogenin* to estrogen induction (Hayward et al. 1982). Otherwise, if longer time is needed before γ-globin gene expression was activated, it might acts through the primary response that may include primary response genes and then trigger the down stream responses which lead to the expression of γ-globin gene. In order to precisely inspect this procedure, real-time RT-PCR should be applied. K562 cells will be

synchronized by starving the cell in RPMI 1640 medium with 5% fetal bovine serum. Then the cells will be split into pairs, one as control and one will be treated with CuD for 0, 4, 8, 12, 24, 36, 48, 72 or 96 h. RNA samples will be prepared from the control and treated cells. Real-time RT-PCR will be performed to analyze the expression of γ-globin gene at different time point (Gibson et al. 1996).

2.2. Materials

2.2.1. Chemicals and reagents

Chemical/ Reagent	Source, Catalog Number
RPMI Medium 1640, powder	Gibco 23400-021
Dulbecco's Phosphate-Buffered Saline (D-PBS), powder	Gibco 21600-010
Sodium Bicarbonate ($NaHCO_3$), Cell Culture Tested	Sigma S5761
Fetal Bovine Serum, Qualified	Gibco 10270-106
Antibiotic-Antimycotic (100X), liquid	Gibco 15240-062
Cucurbitacin D, 10μg/ml in PBS	CK Lifesciences
Hydroxyurea (HU)	Sigma H8627
3, 3', 5, 5'-Tetramethylbenzidine (TMB)	Sigma 527A-1KT
Dimethyl sulfoxide (DMSO)	Sigma D2650
Sulphuric Acid (H_2SO_4), 98%	Ajax Chemicals
Triton X-100	Fluka 93426
MTT	Sigma-Aldrich B5525
Trizol™ reagent	Invitrogen 15596-018
M-MLV reverse transcription kit	Invitrogen 28025-013
iTaq SYBR Green Supermix with ROX	Bio-Rad 172-5852

2.2.2. Kits

Kit	Source, Catalog Number
Human Fetal Hemoglobin (HbF) ELISA Quantitation Kit	Bethyl Laboratories E80-136

2.2.3. Buffers and solutions

Buffer/ Solution	Preparation
RPMI Medium 1640	1 pack of RPMI Medium 1640 powder and 2.0g sodium bicarbonate was added to 950ml Milli-Q H_2O. The pH of the mixture was adjusted with 1M NaOH to pH 7.2. Milli-Q H_2O was added to make up the medium to 1L. The medium was filtered through 0.22μm filter. Complete RPMI was prepared by adding FBS and PSF at final concentrations of 10% and 1% respectively.
1X PBS	1 pack of PBS powder was dissolved in 1L Milli-Q H_2O. The solution was autoclaved, cooled to room temperature and stored at 4℃.
50mg/ml Hydroxyurea	0.15g HU was dissolved in 3ml PBS and filtered through 0.22μm filter under sterile condition.
Coating Buffer, 50mM Carbonate- Bicarbonate, pH 9.6	0.0795g Na_2CO_3 and 0.357g $NaHCO_3$ were dissolved in 100ml Milli-Q H_2O.
Wash Solution	The Wash Buffer was freshly prepared. Tween 20 was added to 1X TBS at final concentration of 0.05% (v/v).

Blocking Solution	BSA was added to 1X TBS at final concentration of 1% (w/v).
Sample/ Conjugate Diluent	Tween 20 was added to Blocking Solution at final concentration of 0.05% (v/v).
Stop Solution	10.7ml H_2SO_4 was added slowly to 70ml Milli-Q H_2O. Milli-Q H_2O was added to make up the solution to 100ml.
20% Triton X-100	Triton X-100 was added to Milli-Q H_2O at a final concentration of 20% (v/v).

2.2.4. Cell lines

K562 erythroleukemia cells were cultured under the following indicated conditions: RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (Hyclone) and 1% PSF antibiotic mixture (Gibco) in a humidified incubator at 37℃ and 5% CO_2. Cells were treated with Cucurbitacin D, hydroxyurea and different inducers as indicated concentration and time, with the cell density between 2×10^4 - 2×10^5 cells/ml. All inhibitors were purchased from Merck Biosciences.

2.3. Experimental procedures

2.3.1. Hemoglobin quantity measurement by HbF ELISA

K562 cells were seeded at an initial density of approximately $2x10^3$ cells/well in 96-well plates. Two-fold serial diluted amifostine, 5-AzaC, rhEPO, HU, SPB or CuD was added to the wells and incubated for 5 days. Each well was seeded in triplicate and additional control groups were also performed to show the baseline expression of HbF. All plates were seeded in duplicate in order to perform separate MTT assay and quantitative ELISA. On the fifth day of incubation, the cell culture

plates were harvested by centrifuge at 1,000rcf at 4 for 10 minutes. Excessive liquid medium was removed by vacuum sucking after centrifugation. Then cell lysate was prepared by adding 50ul of ice-cold CytoBuster cell lysis buffer supplied with 1:400 protease inhibitor (Merck) into each well. The cell lysate was homogenized by repetitive pipetting using 200ul yellow tips until no integrate cells was visible under microscope. The whole plate carrying cell lysate was stored in -80℃ until use.

2.3.1.1. MTT assay

K562 cells, seeded at a density of 1×10^4 cells/well, were treated with different compounds and cultured for 3 days in 96-well V-shaped plates. Another individual strip of wells containing a 2-fold dilution of cell density starting from 2×10^4 cells/well and finished 4 dilutions serially was also applied to establish standard cell density curve for data gathering upon seeding. At the third day of seeding 20 μl of MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 2 hours. Upon finishing, the cell culture plates were harvested by centrifuge at 1,000rcf at 4℃ for 10 minutes. Excessive liquid medium was removed by vacuum sucking after centrifugation. Then 100μl of DMSO was added into each well with vigorous shaking to extract cell ingredients. The plate was then scanned at 570nm wavelength by a microplate reader. Standard curve was automatically generated using in-build arithmetic formula with background subtracted.

2.3.1.2. Preparation of capture-antibody coated ELISA plates

On the third day after application of different inhibitors and inducers, 1μl of capture antibody from Human Fetal Hemoglobin Quantization Kit (Bethyl, A80-136) was diluted in 100μl of coating buffer for each well on NUNC MaxiSorp ELISA plate. Then the plate was incubated at 4℃ overnight to ensure appropriate antibody adsorption on plates, altogether with a sealing membrane covering the whole plate in order to minimize evaporation. After incubation the antibody solution was removed by flipping the plate over sink and tapping gently on paper towel to drain any

excessive liquid. After draining, wells were washed with 200µl of washing solution three times to ensure the removal of any excessive antibodies.

2.3.1.3. Plate blocking

200µl of blocking solution was applied to each coated wells on the ELISA plate, then the plate was incubated at 37℃ for one hour with lid on. After the incubation in warm oven, blocking solution was removed by flipping the plate over sink and tapping gently on paper towel to drain any excessive liquid.

2.3.1.4. Sample and standard preparation

Upon preparation, 90µl of sample/conjugate buffer was applied to each well to be sampled. The plate carrying frozen cell lysate was picked up from -80℃ storage and quickly thawed in a 37℃ water bath. Then 10µl of cell lysate was added into 90ul sample/conjugate buffer and mixed well by shaking. Standard curve was prepared by diluting Human Hemoglobin Calibrator 2-folds serially according to the following table. Blank wells are also prepared by mixing 90µl of sample/conjugate buffer and 10µl CytoBuster lysis buffer together. The ELISA plate containing both sample and standard wells was incubated at 37℃ for 90 minutes. After incubation, residual sample solution was removed and the wells were washed three times of 200µl washing buffer each.

Standard Concentration (ng/ml)	Calibrator RC80-135	Sample/ Conjugate Diluent
500	2µl	2ml
250	0.4ml from Step 1	0.4ml
125	0.4ml from Step 2	0.4ml
62.5	0.4ml from Step 3	0.4ml
31.25	0.4ml from Step 4	0.4ml
15.63	0.4ml from Step 5	0.4ml
7.81	0.4ml from Step 6	0.4ml

2.3.1.5. HRP antibody and colorimetric detection

Bethyl sheep anti-human fetal hemoglobin HRP-conjugated antibody was diluted at the concentration of 1:10000 in sample/conjugate buffer. 100µl of HRP-antibody solution was applied into each wells, and the whole plate was incubated at 37℃ for 90 minutes in dark. After incubation, residual sample solution was removed and the wells were washed three times of 200µl washing buffer each. 100µl of single-component TMB substrate solution (Merck) was applied into each well and incubated at room temperature until medium-dark blue color was observed. 100µl of 2mol/L H2SO4 solution was added into each well to stop the colorimetric reaction. The plate was then scanned at 450nm wavelength by a microplate reader. Standard curve was automatically generated using in-build arithmetic formula with background subtracted.

2.3.1.6. Statistical analysis

All results are presented as means ±SEM. Statistical analyses were performed using t-test method. F-test was also performed before applying T-test to ensure proper data distribution. For more than two groups of data comparison, one-way analysis of variance (ANOVA) was performed to distinguish the significance. The Tukey test was used for multiple comparisons when ANOVA indicated statistically significant different between or within groups by using GraphPad Prism software. Differences were considered to be significant when P-factor was less than 0.05 unless specified.

2.3.2. Preparation of mRNA extract from K562 cells

Total RNA was isolated using Invitrogen TrizolTM Reagent protocol.

K562 cells were seeded at $2x10^4$ cells/ml at the initial and treated with different compounds. Each sample was performed in triplicate. After 48 hours of incubation, 50ml of cultures were collected for each sample and a total number of approximately $1x10^7$ cells were harvested by centrifuge at 500rcf for 5 minutes at 4℃

using 50ml Falcon™ centrifugation tubes. After washing with serum-free RPMI for 2 times and decant, 1 ml of Trizol was added to each tubes to homogenize cell pellets with repetitive pipetting and vortex. Then the homogenized cell lysate was transferred into 1.5ml eppendorf tubes and underwent 12,500rcf for 5 minutes at 4℃ to remove any insoluble precipitant. Clear supernatants were transferred to new clear 1.5ml eppendorf tubes and 0.2ml of chloroform was added to each tube to separate aqueous and organic phase. The Trizol™ mix was centrifuged at 12,500rcf for 10 minutes at 4℃ to ensure complete phase separation. Then the colorless aqueous phase was collected by aspiration and 0.5ml of isopropyl was added to each tube to precipitate RNA pellets.

RNA pellets were collected by centrifuge and washed 1 times by 70% ethanol and 3 times of absolute ethanol. Then the RNA pellets were vacuum-dry and resuspended in 50μl of RNAse-free water. To remove possible genomic DNA contamination in the purified RNA, all the RNA was subjected to DNase digestion (Promega) using the standard protocol provided. The concentration and purity of RNA products were determined by UV spectrometry.

2.3.3. Reverse transcription and Real-time PCR analysis

Reverse transcription and Real-time PCR consisted of the following reagents: M-MLV RT buffer (5x, 4μl), DTT (2ul), RNAseOut Inhibitor (40U/μl, 1μl), RNA (0.1μg/μl, 10μl), oligo dT(0.1μg/μl, 1ul), dNTP (10mM, 1μl), M-MLV reverse transcriptase (1μl). The mixture was incubated at 37℃ for 90 minutes and then stopped by heating at 95℃ for 5 minutes and quickly chilled to 4℃. RT-PCR kit and dNTP set were from Invitrogen and GE Lifescience respectively. The Real-time PCR reaction was set by adding 12.5μl of 2X iQ SYBR Green Supermix with ROX (Bio-Rad), 0.5μl of 25pmole/μl Forward Primer, 0.5μl of 25pmole/μl Reverse Primer, 6.5μl of autoclaved Milli-Q H2O and 5μl of diluted cDNA. The cDNA was diluted 1/10 from the initial concentration for sample preparation, and a 4-fold serial dilution consisting totally 5 different concentrations was also performed

to establish standard curves. NTC (non-template control) was also performed to eliminate any DNA contaminants from reagents. All sample wells were performed in triplicate. The PCR reaction was started with an initial activation step at 95℃ for 3 minutes. 40 cycles of denaturation at 95℃ for 30 seconds, annealing at 60℃ (for α, γ, δ, ε, ζ globin genes) or 56℃ (for GAPDH, β globin and BP-1 genes) for 30 seconds and extension at 72℃ for 40 seconds were performed. Melting curve was used to detect amplification efficiency and to exclude any abnormal amplification. Data were analyzed by the iQ5 software according to manufacture's instruction. The relative quantity of the different globin and BP-1 genes was normalized to the relative quantity of the reference gene GAPDH. GAPDH primers: upper primer: 5'-ACC ACA GTC CAT GCC ATC AC-3'; lower primer: 5'-TCC ACC ACC CTG TTG CTG TA-3'. α-globin primers: upper primer: 5'-TCC TGC CGA CAA GAC CAA CGT-3'; lower primer: 5'-GGC TCC AGC TTA ACG GTA TTT G-3'. β-globin primers: upper primer 5'-AGA AGT CTG CCG TTA CTG CCC-3'; lower primer: 5'-AGT TGG ACT TAG GGA ACA AAG GA-3'. γ-globin primers: upper primer: 5'-CAG ACG CCA TGG GTC ATT TCA C-3'; lower primer: 5'-GGT GAA TTC TTT GCC GAA ATG GAT-3'. δ-globin primers: upper primer: 5'-CTG AGG AGA AGA CTG CTG TCA A-3'; lower primer: 5'-GGC ATT GTG TTC CCA AGT TCA G-3'. ε-primesr: upper primer: 5'-CTA GCA AGC TCT CAG GCC TGG-3'; lower primer: 5'-GGC AAT GGC GAC AGC AGA CAC-3'. ζ-primers: upper primer: 5'-GAC TGA GAG GAC CAT CAT TGT G-3'; lower primer: 5'-ACA GAG GAT ACG ACC GAT AGG A-3'. BP-1 primers: upper primer 5'-GCT GAA AGA GGC TCA GAG AGA-3'; lower primer: 5'-AGG TCT GGG AAG ACA GCT TTG-3.

2.4. Results

2.4.1. CuD significantly upregulates HbF expression in K562 cells

Efficacy of CuD to increase expression of HbF was compared to compounds previously suggested to have activity including amifostine, 5-AzaC, rhEPO,

hydroxyurea and SPB. Each was tested at 9 different concentrations with 2-fold serial dilutions. The fetal hemoglobin ELISA method measured fetal hemoglobin content in all treated cell lysate and absolute concentration was read out using standard curve. The dosage with maximal response of amifostine, 5-AzaC, rhEPO, HU, SPB and CuD were 625ng/mL, 400ng/mL, 100U/mL, 25μg/mL, 100μg/mL and 12.5ng/mL, respectively (Table 1, Figure 1). However at that dose, HU (25 μg/mL) caused apoptotic distorted morphology and cell death according to previous researches conducted in our lab, and the MTT assay also indicated a <70% cell proliferation ability comparing to untreated groups (data not shown). In contrast, the CuD (12.5 ng/mL) treated cells looked normal. The ability of CuD to induce hemoglobin (Hb) expression was significantly higher than amifostine, 5-AzaC, rhEPO and SPB.

Table 1. HbF quantitative data of K562 Cells treated by Different Agents (ng/10μl lysate)

Dilution (from starting dose)	Amifostine (10μg/mL)	5-AzaC (400ng/mL)	rhEPO (100U/mL)	HU (100μg/mL)	SPB (100μg/mL)	CuD (50ng/mL)
1	71±10	293±27	109±3	*	99±15	121±10
1/2	70±8	255±18	73±10	*	85±3	139±5
1/4	67±13	209±19	63±3	103±13	62±1	154±10
1/8	73±9	173±13	76±7	80±18	65±8	151±12
1/16	78±7	132±7	67±5	65±6	67±3	115±9
1/32	60±4	103±16	69±14	53±1	76±5	83±5
1/64	67±8	83±5	65±2	51±6	76±6	64±11
1/128	74±11	89±7	61±4	51±6	83±8	54±5
1/256	83±3	94±8	74±8	53±3	83±9	54±4
Control	53±7					

K562 cells were treated by different agents for 5 days. The fetal hemoglobin quantity is measured by ELISA method. "*", indicates <50% of cell viability measured by MTT assay.

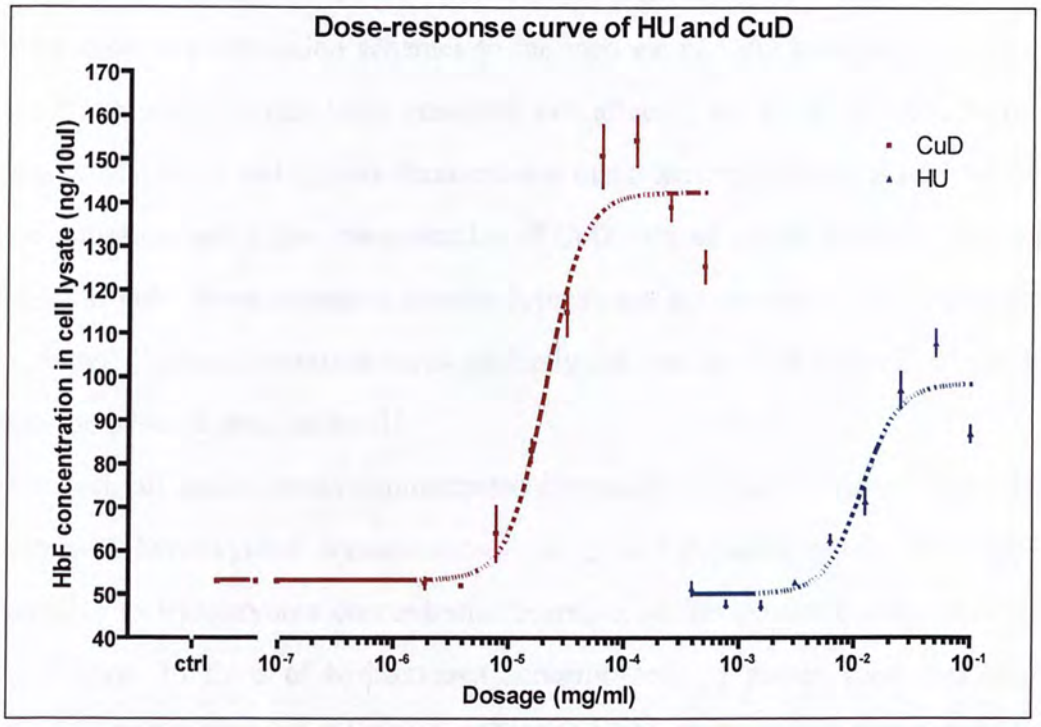


Figure 1. CuD induced dose-response effect in K562 cells. K562 cells were treated with different concentrations of either CuD or HU as indicated for 5 days. The fetal hemoglobin (HbF) level in cell lysate was measured by HbF ELISA kit.

2.4.2. CuD augments α, β, γ, δ, ε , ζ and BP-1 genes at different level in K562 cells

To further confirm the mRNA level changes revealed by RT-PCR, real-time PCR experiments were performed to inspect different globin gene responses in K562 cells under the induction of CuD and HU (Figure 2). All globin genes tested showed different extent of increase under CuD and HU, but their dose-dependent expression scheme was not fully identical and somewhat different. This may imply potential different induction mechanisms between CuD and HU. Moreover, the expression level of BP-1 protein also been down-regulated, which is correspondent to the up-regulation of β-globin gene.

The globin gene responses are different in CuD and HU-treated groups. In

CuD-treated groups, the increasing of drug induction concentration did not result in increased globin gene expression. α, δ, and ζ globin gene demonstrated the least dependence on expression schemes to the increase of CuD induction concentration. Their expression levels were generally not affected by 16 folds increase of CuD concentration. γ and ε genes demonstrated maximum response on 3.12ng/ml of CuD concentration, and higher concentration of CuD reduced globin gene expression level. β-globin gene demonstrated a reverse-dependence scheme upon CuD induction, the increased CuD concentration led to gradually reduced expression level, but still higher than the basal expression level.

However, all globin genes demonstrated increased expression level accompanied by increased hydroxyurea concentration. α, ζ, and β-globin genes were the most sensitive to hydroxyurea concentration increase, as the expression level increased 7 folds upon 16 folds of hydroxyurea concentration. γ globin gene was generally insensitive to hydroxyurea concentration increase, as the expression level remained stable.

The human BP-1 gene expression was inhibited in both CuD and hydroxyurea-induced groups. Previous researches have indicated the inhibition of human BP-1 gene leads to increased globin genes expression, and our experiments revealed similar effect.

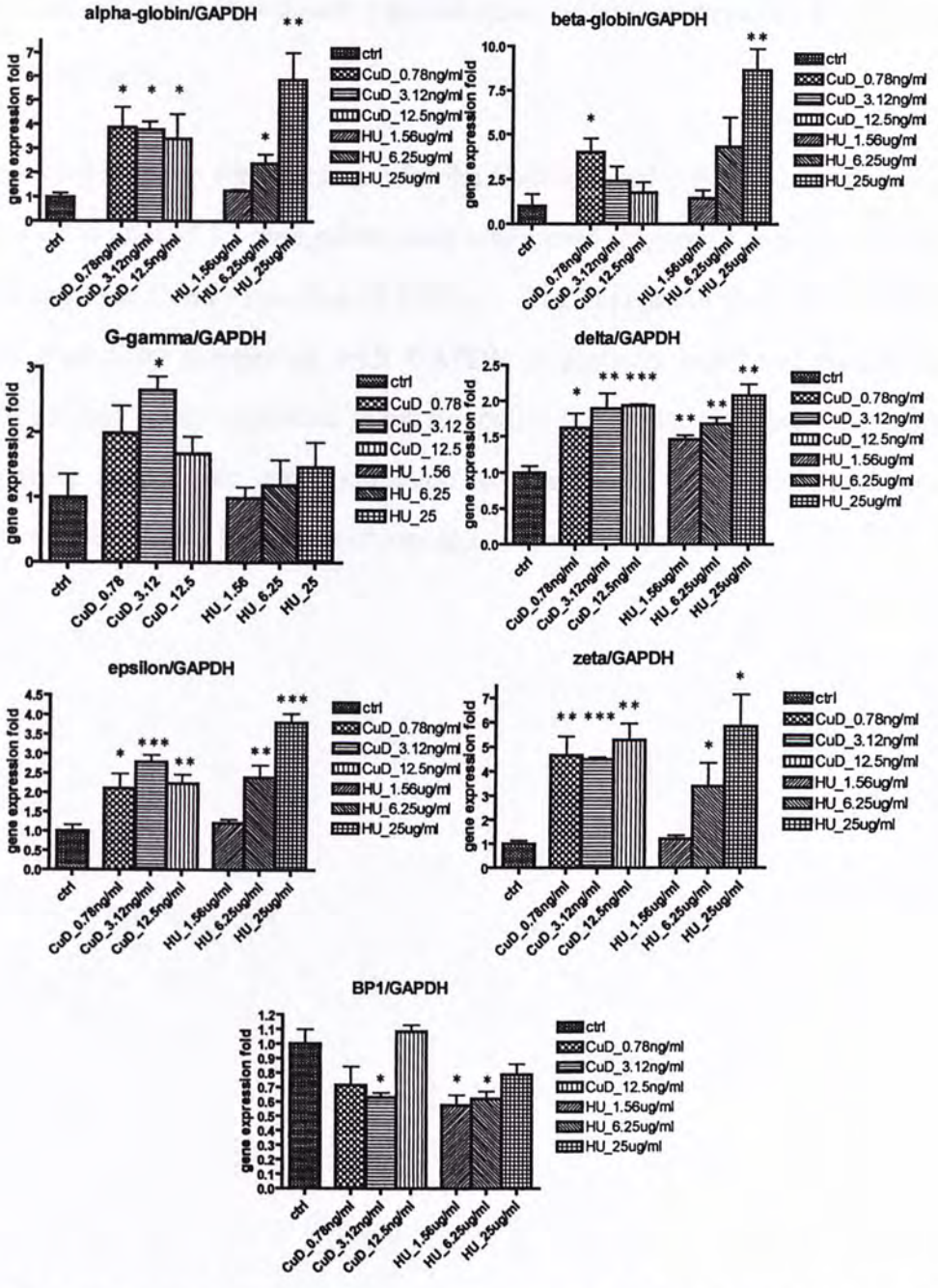


Figure 2. Real-time RT-PCR data showing the response of different globin gene and BP-1 gene expression in K562 cells under induction of CuD or hydroxyurea (HU). K562 cells were treated with different concentrations of CuD and HU as indicated for 48 hours. After treatment, the cells were collected and the expression profile of different globin genes were performed as described in Materials and Methods. Y axis represents the expression level changes of different globin gene comparing with uninduced groups. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$

2.4.3. Cucurbitacin D-induced γ-globin gene activation requires 12-24 hours in K562 cells

In order to determine the exact time for the CuD-induced γ-globin gene activation in K562 cells, a total of 12 time points were established (Figure 3) to make sampling of desired cell lysate and extraction of mRNA. The expression level of γ-globin gene was evaluated by comparing with GAPDH expression level and normalized as expression fold using uninduced group as control. Experiment results demonstrated the increase of γ-globin gene expression occurred at 12 hours post induction, and stabilized after 24 hours by a 2-fold increase.

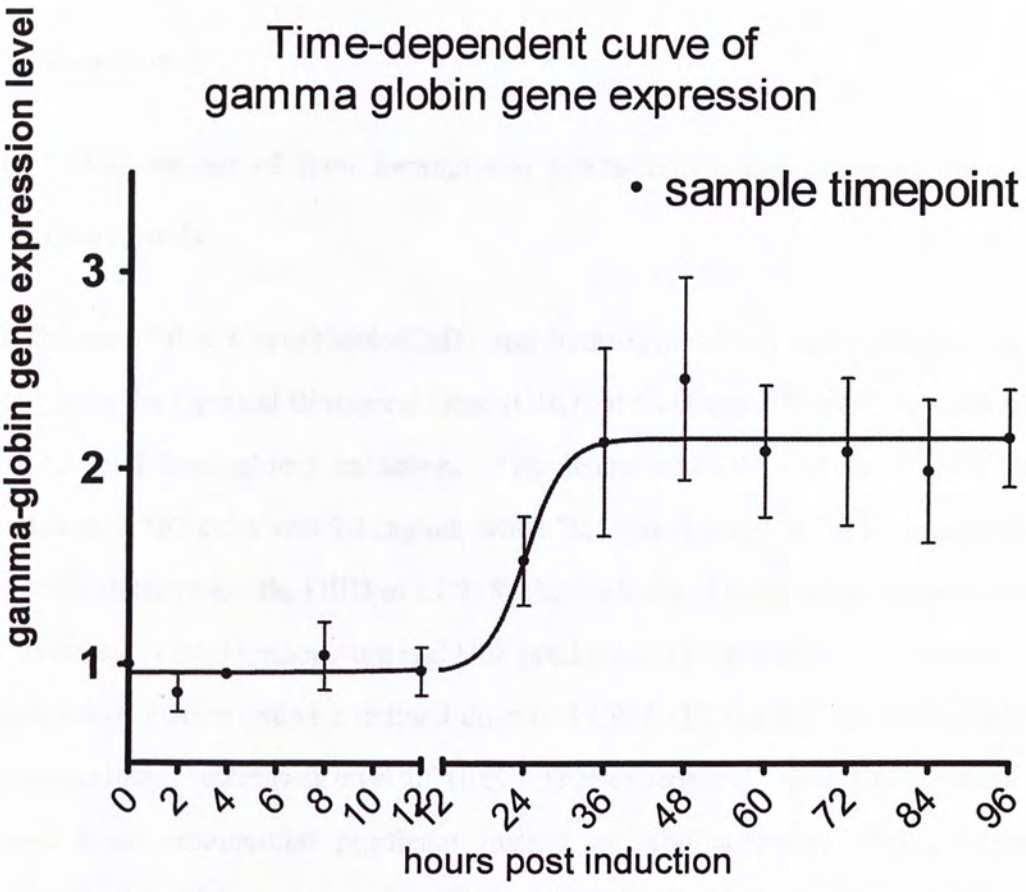


Figure 3. Time-dependent response of γ-globin gene expression in K562 cells under induction of CuD. K562 cells were treated with 3.12ng/ml of CuD and collected at different time points post induction as indicated. The measurement of γ-globin gene expression level was performed by Real-time RT-PCR method.

2.5. Discussion

2.5.1. Enhancement of fetal hemoglobin production using different chemical compounds

Main theme of this Cucurbitacin(CuD) and hydroxyurea(HU) study was aimed in investigating the Optimal Biological Dose (OBD) of CuD and HU on K562 cells with regard to fetal hemoglobin induction. The determined OBD of CuD upon HbF induction in K562 cells was 3.12ng/ml, while HU was 6.25ug/ml. Previous studies has already determined the OBD of LC978 (Cucurbitacin D from HPLC results) by *in vitro* bioassay of total hemoglobin and HbF production (Xing 2003). And the FACS analysis result also revealed a defined dose of LC978 (12.5ng/ml) can induce K562 cells in producing increased level of HbF. This experiment, using Cucurbitacin D obtained from commercial purificant instead of laboratory-mae HPLC extract, revealed similar OBD and response curve, which further validated the LC978 and Cucurbitacin D was the same compound.

Apart from the FDA approved hydroxyurea (HU), a few compounds such as 5-azacytidine, amifostine, recombinant human erythropoietin (rhEPO), and butyrate analogs have shown some capability of HbF activation(Xu et al. 2003; de Andrade et al. 2006). Several have been used clinically to treat thalassemia and sickle cell disease (Ji et al, 2003). Previous studies have been determined the optimum dosage of different HbF inducers, however, the quantitative data of net production of hemoglobin was not determined, as the previous study only gave out the optimum dosage data by indicating the positive percentage of responsive data upon drug induction or the result of TMB staining. Both methods lie in the same defection that the TMB substrate applied in the experiment was not specific towards HbF catalysis and chromogenic. Other cytosolic proteins that possess the ability of oxygen transfer, as α-globin, components of the respiratory chain and hydrogen peroxidase, are also able to catalyze the TMB chromogenic reaction. Hence, a more specific and reliable

proof for the increase of HbF production upon drug effects is definitive essential.

It is important to make a detailed analysis towards data obtained from HbF quantitative data of K562 cells treated by different agents. The basal HbF production level of uninduced K562 cells was 53±7 ng/10μl, which mainly contributed to self-differentiation due to the increased cell density during induction assay. It was distinctive that 5-azacytidine possess the most inductivity among all the treated agents. It has a peak HbF production output of 293±27ng/10μl lysate at 400ng/ml, and this inductivity was still distinctive upon 1/256 of its starting concentration. However, it was also the strongest mutagen among all the inductive agents tested, indicating it might not be appropriate for human or animal use as previously described (Clegg et al. 1983). CuD followed 5-azacytidine as the second strongest inducer with a peak HbF output at 154±10 ng/10μl at 3.12ng/ml induction concentration. Hydroxyurea demonstrated a high cytotoxicity on 100μg/ml and 50μg/ml concentration, and its peak induction concentration was 25μg/ml. This phenomenon is coherence with the previous notion that the HDAC effect of hydroxyurea was paralleled with its cytotoxicity (Mohler 1964; Satyamoorthy et al. 1986). Human rhEPO and sodium phenylbutyrate were also able to increase HbF production level in K562 cells by one fold, with amifostine possessed the lowest inducibility.

For the *in vitro* time course assay, the net production of fetal hemoglobin in K562 cell increased similarly in both the CuD and HU treated cells when using their optimal dosage. However, the absolute amount of Hb was significantly higher in the CuD treated cells. The control, diluent treated cells had a small rise in HbF content which has been noted as the previously reported cellular self-differentiation induced by density increase.

In the HbF ELISA assay, the concentration of fetal hemoglobin in cell lysate both increased in the CuD and HU treated groups, while the CuD treated cells have a significant higher synthesis compared with the HU groups. To be detailed, HU increased the concentration of HbF in K562 cell lysate about 2 fold comparing to its baseline level at the optimal inductionconcentration. In comparison, the CuD

increased HbF concentration in K562 cell lysate to 3 fold comparing with the baseline level. The absolute induction concentration of CuD (3.12ng/ml) was only 1/1000 of that comparing to HU (25μg/ml). The control group remained stable and showed a low level of fetal hemoglobin production. In all, the present study showed that cucurbitacin D (CuD) can reactivate human gamma globin gene and demonstrates a higher potency than the other compounds (Table 1).

2.5.2. CuD increased HbF synthesis by increasing γ-globin mRNA amount

The K562 cell expresses all globin genes included beta-globin (Zoueva et al. 2004)(Fordis *et al*, 1984). The question was answered by the analysis of mRNAs and HbF. The amount of α, β, γ, δ, ε and ζ-globin mRNA have been all increased in both HU-treated and CuD-treated cells, while the production of γ-globin was more significant in the CuD-treated cells (Figure 2). It is suggested that CuD enhances, at least in part, of HbF expression by increasing the amount of γ-globin mRNA in cells. However, it should be clearly noticed that the globin-gene transcription scheme was different in CuD and HU-induced groups. For the CuD-induced group, the increase of mRNA level demonstrated less dependence in changes of CuD concentration, and a stable induction scheme can be clearly observed. In contrast, a strong concentration-dependent increase of mRNA level was observed in all groups treated by HU. The most responsive globin genes toward changes on HU induction were α, β, and ζ genes, where a 7-10 fold changes on mRNA level could be observed. This may implies that CuD and HU works on different induction pathways, one to be dependent on the intensity of signal input, while the other one does not.

Another different trend on CuD/HU-induced globin genes activation should also be noticed. Both CuD and HU are able to induce globin genes located on both α and β locus, regardless of the significant differences on two gene locus and their regulation regions. The globin genes located on α locus (α and ζ genes) are more responsive towards drug induction, which all demonstrated at least 4 fold increase under CuD induction and over 6 folds under HU induction at the maximum. This suggests that

the regulatory region of α locus may be more responsive towards drug induction than those on β locus, and feasible for a future comparative research. The β-globin gene, though also demonstrated great response towards drug induction, should be noticed for its relatively low expression level.

The real-time RT-PCR data also indicated that a 12-hours rest stage is required for CuD-induced γ-globin gene activation. Enhancement of γ-globin mRNA level was observed 24 hours post induction, and come to stabilized level after 36 hours. The delay in mRNA synthesis implies the cytological response towards CuD induction was unlikely to be classified into primary response, in which the inducer directly triggers the activation of signal cascade and transcription initialization. In contrast, it is likely that CuD triggers gene transcription via secondary even tertiary response. However, more investigation and research efforts are required to provide the full scheme of CuD induction mechanism.

2.5.3. CuD and HU down-regulated the BP-1 gene expression

It has been reported that the inhibition of human β-protein 1 (BP-1 gene) will enhance the promoter activity of the β locus (Zoueva et al. 2004). Our real-time PCR data showed similar inhibitory effect on the expression level of BP-1 gene under the induction of CuD at low concentration. However, this inhibitory effect was released at high CuD concentration for unknown reasons. HU-treated cell groups also demonstrated inhibitory effect of BP-1 gene at all concentrations tested. These results indicating that the drug-mediated activation of genes on β locus is likely to be involved with the inhibition of BP-1 expression, with detailed mechanism still under investigation.

3. Determination of potential signal transduction pathways during CuD and HU-mediated fetal hemoglobin production

3.1. Introductions

3.1.1. The p38 MAPK family

The p38 MAPKs are one conserved subfamily of Mitogen-activated Protein Kinases (MAPKs), which mainly in response of cellular stress and cytokine signaling in all eukaryotic cells. The activity of MAPKs are regulated by the phosphorylation of tyrosine and threonine residues mediated by the MAPK upstream activating kinases (MEKs). The signal cascade from the very beginning of membrane-bound receptor to the MAPKs was extremely amplified by the indicated signal cascade (Martin-Blanco 2000). Different classes of MAPKs have been identified, which includes p42-p44 MAPKs (ERKs) that are activated by growth factors; Stress-activated Protein Kinases (SAPKs/JNKs) activated by cytokines, and p38 MAPKs mainly in response to cellular stress. Different subfamilies of MAPKs are highly specified and maintained throughout each cascade and selective enzyme-substrate interactions in each module (Tibbles et al. 1999).

Among the p38 MAPK subfamily, four distinct isoforms of p38 have been identified and named p38α, p38β, p38γ/SAPK3, and p38δ/SAPK4. These isoforms are similar in their molecular size and also share about 60-75% of sequence homology. All these four isoforms are found able to be activated by TNF, IL-1, UV irradiation, and hyper-osmosis. Different p38 isoforms are tissue-specific. The p38α and p38β are expressed ubiquitously in all tissues, p38γ/SAPK3 is mainly found in skeleton and smooth muscle tissue, and p38δ/SAPK4 is expressed in lung and kidney (Han et al. 1994; Raingeaud et al. 1995; Jiang et al. 1996; Lechner et al. 1996; Goedert et al. 1997).

The connection between p38 phosphorylation and γ-globin gene expression was firstly discovered in butyrate-induced HbF production in human K562 cells. Later, direct activation of p38 MAPK using anisomycin or stable vector-based over-expression models revealed similar phenomenon in boosting γ-globin gene expression (Park et al. 2001). It was considered that cellular stress led by increased reactive oxygen or chromatin signaling were responsible for the p38 activation and the followed γ-globin gene expression(Aerbajinai et al. 2007; Wei et al. 2007),

however, the full scheme of induction mechanism is still under investigation.

The STATs are members of transcription factors that activated by JAKs via phosphorylation on tyrosine residues. In the rest state, the STATs usually bind to the membrane-bound JAKs but remain unphosphorylated. After phosphorylation, the activated STATs form dimmers and translocated into nucleus, bind to the target sequence and modulate the expression of downstream genes (Darnell et al. 1994; Zhong et al. 1994). STAT3 is mainly responsible for the cytokine and growth-factor mediated responses and required in the fetal development stage (Heim 1999). It is activated by phosphorylation on Tyr705 site and which triggers dimerization, translocation, and DNA-binding process. However, its transcriptional activity is considered to be regulated by phosphorylation at Ser727 site by upstream signal either from MAPK or mTOR pathway (Ihle 1995; Wen et al. 1995; Yokogami et al. 2000). Two isoforms of STAT3α (86kDs) and STAT3β (79kDs) have been identified (Wen et al. 1995; Biethahn et al. 1999).

3.1.2. The JAK2-STAT3 pathway

The JAK family is one big membrane-bound cytokine receptor family that consists of JAK1, JAK2, JAK3, and Tyk2 with each of the relative molecular weight around 120-130 kDs. This family is characterized by a carboxy-terminal kinase domain followed by another pseudokinase domain, and amino-terminal domains are conserved in all the family members (Ihle et al. 1995; Taniguchi et al. 1995). The JAKs have different patterns in associating with receptors regarding to different receptor structure. The JAK2, one of the mostly-found JAKs responsive to the IL-3 and IL-6 induction, uses single-chain receptors for ligand connection. Excessive binding of ligand presence and receptor aggregation induces concomitant aggregation of JAK2 and facilitating the transphosphorylation of KEYY site in the kinase activation loop, hence dramatically increasing the catalytic activity of JAK2 itself. Activated JAK2 phosphorylates itself and causing the receptor and cellular substrates to be recruited into the receptor complex (Ihle 1995).

The activation of JAK2 is direct upstream event of STAT3 phosphorylation at Tyr705 site and hence leading its dimerization and translocation. However, the role of phosphorylated STAT3 at Tyr705 site during γ-globin gene expression is disputed. Researches using UT7/EPO cells observed induction of EPO was accompanied by activation of JAK2 and phosphorylation of STAT3 at Tyr705 site, and implied a positive effect of STAT3 phosphorylation for erythroid-directed cell differentiation (Kirito et al. 2002). However, the IL-6 induced JAK2 activation and STAT3 phosphorylation demonstrated inhibitory effect of γ-globin gene expression (Foley et al. 2002). Hence, it is still not safe to conclude any certain role of STAT3 phosphorylation during γ-globin gene expression process, and additional detailed investigations on the regulation mechanism of STAT3 need to be performed.

In our investigation of CuD/HU-mediated γ-globin gene expression scheme, we applied sandwich ELISA method to detect if there was any change in CuD/HU-induced K562 cells, and try to make the connection between STAT3 phosphorylation and γ-globin gene expression.

3.1.3. Fundamentals on inhibition assay of p38 MAPK and JAK2-STAT3 pathway

AG490 has been widely applied as a specific inhibitor of JAK2-STAT3 cascade mediated cellular responses. It also inhibits consititutive activation of STAT3 DNA-binding and IL-2/IL-6 induced growth of macrophage tumor cells. Though the AG490 treatment blocks leukemic cell growth and induces apoptosis, no harmful effect on normal hematopoiesis has been found (Kirken et al. 1999; Eriksen et al. 2001; Jaleel et al. 2004).

Gö6976 is a cell-permeable inhibitor of protein kinase C (PKC) activity. It selectively inhibits calcium-dependent PKC pathway and does not affect the calium-independent PKC isotypes (Martiny-Baron et al. 1993; Gschwendt et al. 1996). It has been reported that activation of calcium-dependent PKC pathway was involved

during sodium-butyrate induction and fetal hemoglobin synthesis (Rivero et al. 1998). D4476 is a cell-permeable triaryl-substituted imidazolo compound, which specifically targets the Casein Kinase I pathway. The connection between Casein Kinase I/II and fetal hemoglobin production have been established (Bieker et al. 1998).

Previous research indicated the activity of glycogen synthase kinase-3β was also regulated by JAK2 (Gross et al. 2006). Hence, in order to investigate potential cross-regulation mechanism between STAT3 phosphorylation and GSK3β activation, specified cell-permeable GSK3β inhibitor peptide was applied to block the activity of cytosolic GSK3β and observe the inhibitory effects towards CuD/HU induction.

Previous research done by our laboratory indicated the activation of MAPK family might be involved during CuD-induced fetal hemoglobin production. SB203580, SB203580 sulfone, SB202190, and SKF86002 are different kinds of chemical inhibitors all target the p38 MAPK pathway. SB203580 is a specific p38 MAPK inhibitor that does not significantly inhibit JNK/SAPK and p44/p42 ERK pathway (Powell et al. 2003). SB202190 inhibits p38 MAPK pathway as well as the activation of transcription factor 2 (ATF-2) at its optimum concentration. SB202190 also blocks the interleukin synthesis in specific cell lines (Davies et al. 2000). SKF86002 acts as an anti-inflammatory drug that, by blocking the activation of p38 MAPK pathway, inhibits cytological effects led be osmotic stress and UV-induced apoptosis (Frasch et al. 1998).

SP600125 is a potent cell-permeable inhibitor that blocks activation of c-JUN N-terminal Kinase (JNK). It exhibits 300-fold greater selectivity for the inhibition on JNK pathway comparing with ERK and p38 MAPK pathway, and its effect is reversible in respect to ATP competition (Shin et al. 2002). PD98059 is a selective, cell-permeable inhibitor of MAP kinase kinase 1/2 (MKK1/2, MEK1/2) and mainly prevents the activation of ERK family (Means et al. 2000). U0126 is also a potent and specific inhibitor to MEK1/2 and mainly inhibits the activation of ERK pathway. Different from PD98059-mediated ERK inhibition, its inhibitory effect is noncompetitive with respect to the ATP (DeSilva et al. 1998).

3.1.4. Fundamentals on nuclear translocation of STAT3

Electrophoresis Mobility Shift Assay (EMSA), or called Gel Shift Assay, is a convenient and sensitive method in protein-nucleic acid interaction detections. Based on the concept that the protein-nucleic acid complex possesses reduced electrophoretic mobility comparing with free nucleic acid probes, the EMSA produces retarded assay in the presence of potential protein-nucleic acid interaction (Hellman et al. 2007). It is commonly used in the purpose of qualitative detection, but quantitative results can be produced under appropriate conditions and carefully experiment maintenance (Fried 1989).

The main advantage of the EMSA experiments is simplicity. It only requires very fundamentals of molecular biology, yet still robust enough to adapt with wide ranges of binding conditions. Recently introduced non-radioactive chemiluminescence detection method allows rapid and convenient validation of EMSA results (Berger et al. 1993). The detection threshold of EMSA is also highly sensitive, allowing the assays to be performed with small protein/nucleic acid amounts (Fried et al. 1998). It also adapts with wide range of nucleic acid sizes and structures as well as proteins, even with proteins with different stoicheometry structure or binding site distribution (Hudson et al. 1990; Musso et al. 2000; Tolstonog et al. 2005). These capabilities account the most part for the popularity of EMSA in protein-nucleic acid interaction studies.

3.2. Materials

3.2.1. Chemicals and reagents

Chemical/ Reagent	Source, Catalog Number
RPMI Medium 1640, powder	Gibco 23400-021
Dulbecco's Phosphate-Buffered Saline (D-PBS), powder	Gibco 21600-010
Sodium Bicarbonate ($NaHCO_3$), Cell Culture Tested	Sigma S5761
Fetal Bovine Serum, Qualified	Gibco 10270-106

Antibiotic-Antimycotic (100X), liquid	Gibco 15240-062
Cucurbitacin D, 10μg/ml in PBS	CK Lifesciences
Hydroxyurea (HU)	Sigma H8627
Protease inhibitor cocktail set II	Merck 539132
Protease inhibitor cocktail set III	Merck 539134
Phosphatase inhibitor cocktail set II	Merck 524625
PhosphoSafe™ extraction reagent	Merck 71296
Dimethyl sulfoxide (DMSO)	Sigma D2650
Sulphuric Acid (H_2SO_4), 98%	Ajax Chemicals
Triton X-100	Fluka 93426
Acrylamide	Amersham Pharmacia biotech 17-1304-01
Amido black	Bio-Rad 161-0402
Ammonium persulphate (APS)	Amersham Pharmacia biotech 17-1311-01
Bovine serum albumin (BSA)	Sigma B6066
Bromophenol blue	Bio-Rad 161-0404
Calcium chloride, hydrated form	Sigma C3881
Commassie brilliant blue R-250	Bio-Rad 161-0400
3-[Cyclohexylamino]-1-propane sulfonic acid (CAPS)	Sigma C2632
Diethyl pyrocarbonate (DEPC)	Sigma D5758
N,N-dimethyl formamide (DMF)	Sigma D4551
Dimethyl sulfoxide (DMSO) for cell culture	Sigma D2650
Ethanol, absolute	Ajax 214
Ethylenediamine-tetraacetic acid (EDTA)	Sigma E5134
Glycerol	Sigma G7893
Glycine	Sigma G8898
Hydrocloric acid, 32%	Ajax 265
Imidazole	Sigma I2399

Lauryl sulfate, sodium salt (SDS)	Sigma L5750
Methanol	Tedia MS1922
N, N'-methylenebisacrylamide	Amersham Pharmacia biotech 17-1304-02
beta-mercaptoethanol	Sigma M7154
Nitro blue tetrazolium chloride (NBT)	Roche 1-087-479
Paraformaldehye	Sigma P6148
Sodium acetate	Sigma S8750
Sodium azide	Sigma S2002
Sodium bicarbonate	Sigma S5761
Sodium chloride	Sigma S9625
Sodium hydroxide	Sigma S5881
Sodium phosphate	Sigma S0876
N-N-N'-N'-Tetramethylethylenediamine (TEMED)	Sigma T9281
Tris base	Amersham Pharmacia biotech 17-1321-01
Triton X-100	Sigma T6878
Trizol reagent	Invitrogen 15596-026
Trypan blue solution (0.4%)	Sigma T8154
Tween 20	Sigma P1379
Anti-Jak2 (24B11) rabbit mAb	Cell Signaling 3229
Anti-phospho-Jak2 (Tyr1007/1008) rabbit mAb	Cell Signaling 3771
CytoBuster Extraction Reagent	Merck 71009
SB203580	Merck 559389
SB203580 sulfone	Merck 559399
SB202190	Merck 559388
SKF86002	Merck 567305
SP600125	Merck 420119
U0126	Merck 662005

PD98059	Merck 513000
AG490	Merck 658401
D4476	Merck 218696
Gö6976	Merck 365250
GSK3β inhibitor peptide, cell permeable	Merck 361546
Anti-STAT3 rabbit (124H6) antibody (IHC preferred)	Cell Signaling 9139

3.2.2. Kits

p38 MAP Kinase ELISA Kit	Merck CBA-029
PhosphoDetect p38 MAP Kinase (pThr180/pTyr182) ELISA Kit	Merck CBA-008
SuperSignal West Pico Complete Mouse IgG Detection Kit	Pierce 34081
SuperSignal West Pico Complete Rabbit IgG Detection Kit	Pierce 34084
STAT3 [pY705] ELISA KIT	BioSource KHO0481
Hemoglobin, Plasma Kit	Sigma 527A-1KT
Human Fetal Hemoglobin (HbF) ELISA Quantitation Kit	Bethyl Laboratories E80-136
NE-PER Nuclear and Cytoplasmic Extraction Reagents	Pierce 78833
LightShift Chemiluminescent EMSA Kit	Pierce 20148
Alexa-488 Goat Anti-rabbit SFX kit	Invitrogen A31628
Biotin 3' End Labeling Kit	Pierce 89818

3.2.3. Buffers and solutions

RPMI Medium 1640	1 pack of RPMI Medium 1640 powder and 2.0g sodium bicarbonate was added to 950ml

	Milli-Q H_2O. The pH of the mixture was adjusted with 1M NaOH to pH 7.2. Milli-Q H_2O was added to make up the medium to 1L. The medium was filtered through 0.22μm filter. Complete RPMI was prepared by adding FBS and PSF at final concentrations of 10% and 1% respectively.
1X PBS	1 pack of PBS powder was dissolved in 1L Milli-Q H2O. The solution was autoclaved, cooled to room temperature and stored at 4℃.
Coating Buffer, 50mM Carbonate-Bicarbonate, pH 9.6	0.0795g Na_2CO_3 and 0.357g $NaHCO_3$ were dissolved in 100ml Milli-Q H_2O.
Wash Solution	The Wash Buffer was freshly prepared. Tween 20 was added to 1X TBS at final concentration of 0.05% (v/v).
Blocking Solution	BSA was added to 1X TBS at final concentration of 1% (w/v).
Sample/ Conjugate Diluent	Tween 20 was added to Blocking Solution at final concentration of 0.05% (v/v).
TMB Substrate Solution	Merck CL07
Stop Solution	10.7ml H_2SO_4 was added slowly to 70ml Milli-Q H_2O. Milli-Q H_2O was added to make up the solution to 100ml.

3.3. Experimental procedures

3.3.1. Detection of p38 MAPK phosphorylation status

3.3.1.1. Preparation of cytosolic protein extracts

$1x10^6$ K562 cells were lysed in 150ul of ice-cold PhosphoSafe lysis buffer supplied with 1% protease inhibitor cocktail, protease inhibitor cocktail set II and III (all from Merck Biosciences). Cellular debris was removed by centrifugation method, and equal protein loading amount was determined by the Bradford method (Bio-Rad). Denatured cytosolic protein extracts were stored at -80°C until use.

K562 cells were seeded in 24-well plates on $1x10^6$/well and induced by either 3.12ng/ml Cucurbitacin D or 12.5μg/ml hydroxyurea. Another group treated by 200ng/ml anisomycin was applied as a positive control of p38 phosphorylation. The induction time was as indicated in Figure 4. Upon finishing, $1x10^6$ K562 cells were treated with 150μl of ice-cold PhosphoSafe lysis buffer supplied with 1% protease inhibitor cocktail, protease inhibitor cocktail set II and III (all from Merck Biosciences). Cellular debris was removed by centrifugation method, and equal protein loading amount was determined by the Bradford method (Bio-Rad). Cytosolic protein extracts were stored at -80℃ until use. 10μl of cell lysate was used for each ELISA reaction and all inductions were performed in triplicate to provide statistic results.

3.3.1.2. Quantitative measurement of phospho-p38 and pan-p38 by ELISA method

3.3.1.2.1. Antigen adsorption and establishment of standard curves

100μl of standard diluent buffer was added into zero wells for background correction. The pan/pho-p38 MAPK standards were reconstituted using standard diluent buffer. The initial concentration of pan-p38 standard solution was 2000pg/ml, and phospho-p38 standard solution was 100unit/ml. The reconstituted solution was swirled gently and allowed to sit for 10 minutes to ensure complete reconsititution.

100µl of either pan/pho-p38 MAPK standards was added into the first wells of standard wells, and a 1/2 serial dilution consisted 7 wells was made to establish the standard curve. Unused standard solutions were stored in -80℃.

10µl of cytosolic protein extract and 90µl of standard dilutent was mixed for each reaction for each well to be tested. All samples was made in triplicate to ensure statistical results. After mixing, the wells were covered by special adhesive membrane and incubated 2 hours at room temperature as indicated on kit manual.

3.3.1.2.2. Plate washing and application of detection antibody

The 25x concentrated washing buffer was diluted using Milli-Q water. The plate was decanted, washed 4 times using 250µl of washing buffer each time. 100µl of anti-p38 MAPK or anti-phospho-p38 antibody (detection antibody) solution was directly added into each well except the chromogen blanks. Appropriate mixture was applied by tapping gently on the side of the plate. 10µl of cytosolic protein extract and 90µl of standard dilutent was mixed for each reaction for each well to be tested. All samples were made in triplicate to ensure statistical results. After mixing, the wells were covered by special adhesive membrane and incubated 1 hours at room temperature as indicated on kit manual.

3.3.1.2.3. Plate washing and application of secondary antibody

Incubated plate was decanted and washed 4 times using 250µl of washing buffer each time. The anti-rabbit IgG-HRP working solution was prepared by 1:100 mixing of anti-rabbit IgG-HRP antibody stock and secondary antibody solution together. 100µl of anti-rabbit IgG-HRP working solution (secondary antibody) was added into each well except the chromogen blanks. A plate cover was applied onto the wells to prevent evaporation. The plate was incubated at room temperature for 30 minutes as indicated on kit manual.

3.3.1.2.4. Plate washing and chromogen detection

Incubated plate was decanted and washed 4 times using 250μl of washing buffer each time. 100μl of TMB substrate was quickly added into each well to generate chromogenic results and incubated in dark. The color generation of TMB substrate was monitored until a medium-dark blue color appeared, and 100μl of stop solution was quickly added into each well to stop reaction. The absorbance of each well was read at 450nm using a microplate reader with standard curves automatically generated from in-build program.

3.3.2. Detection of signal cascade on JAK2-STAT3 pathway

3.3.2.1. Preparation of cytosolic protein extracts for Western Blot detection

K562 cells were induced by either 3.12ng/ml Cucurbitacin D or 12.5μg/ml hydroxyurea for the time points indicated prior to cell lysis. Each $5x10^6$ K562 cells were treated with 250μl of ice-cold PhosphoSafe lysis buffer supplied with 1% protease inhibitor cocktail, protease inhibitor cocktail set II and III (all from Merck Biosciences), 1mM PMSF and cellular debris was removed by centrifugation at 4℃. Protein concentrations were determined using Bradford method. 50μl of 5x SDS-loading buffer was added into the cell lysate and boiled for 10 minutes to denature all protein ingredients. Denatured cell lysates were stored in -80℃ until use.

3.3.2.2. Gel running and Western Blot detection

For gel running, a SDS-polyacrymide discontinuous system was used for experiment. The discontinuous system includes 5% of pH 6.8 stacking gel and 10% pH 8.8 resolving gel, which is effective to separate protein molecule weights ranged from 30-150kD. To be detailed, the resolving gel solution was prepared in disposable plastic tube, and acrymide solution was poured into a Bio-Rad miniGel kit. Gel surface was overlaid by isobutanol to prevent evaporation and provide air insulation.

The polymerization was finished by 2 hours at room temperature. After polymerization was complete, the overlay was poured off and stacking gel solution was directly poured onto the surface of polymerized resolving gel. Immediately a clean Teflon comb was inserted into the stacking gel solution to provide wells, and polymerization was finished by 1 hour of incubation at room temperature.

After polymerization is complete, the Teflon comb was carefully removed and wells were washed by running buffer to eliminate any unpolymerized acrymide. Denatured lysates equal to 10μg of total protein was loaded into each lane. An equal volume of 1x SDS gel-loading buffer was loaded into any wells that are unused. A mixture of Invitrogen SeeBlue2 pre-stained protein molecule marker and Cell Signaling biotin-labeled molecule marker was also applied to indicate the molecular weight of desired protein bands during and after electrophoresis. Elecphoresis voltage was applied at 8 V/cm for stacking gel and 15 V/cm for resolving gel.

For western blot, 6 pieces of 3mm filter paper were cut into appropriate size and pre-soaked in transfer buffer. PVDF membrane was cut into appropriate size, activated in 100% methanol and rinsed with transfer buffer. The assembly was set up by stacking 3 pieces of filter papers, the PVDF membrane, the SDS-PAGE gel and another 3 pieces of filter paper at the trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad) to transblot the protein onto PVDF. 15V constant voltage was applied for 45 minutes. The membrane was removed and blocked in blocking buffer for 45 minutes.

Blocked membranes were incubated overnight at 4℃ with 1:1000 dilution of monoclonal anti-pan-p38 and anti-phospho-p38 antibody, anti-pan-STAT3 and anti-phospho-Ser727-STAT3 antibody (Cell Signaling). The primary antibody reaction was followed by three times of washing of TBST and incubation with HRP-linked goat-anti-mouse and goat-anti-rabbit IgG (Amersham) at 1:10000 dilution for 1 hour at room temperature.

Development of membrane was performed by using SuperSignal West Pico chemiluminescent agent (Pierce). PVDF membranes were washed 3 times by TBST, and 3ml of chemiluminescent solution (1:1 mixture of A and B solution) was applied

for each membrane to be detected. The light signal generated was detected by a chemiluminescence detector (Roche), average exposure time was 2 minutes with adjustments on signal intensities.

3.3.3. Quantitative measurement of phospho-STAT3-Tyr705 using ELISA method

3.3.3.1. Preparation of cytosolic protein extracts

K562 cells were seeded in 10ml culture flasks on $1x10^5$/ml and induced by either 3.12ng/ml Cucurbitacin D or 12.5μg/ml hydroxyurea for 24 hours. Upon finishing, $1x10^6$ K562 cells were treated with 50μl of ice-cold PhosphoSafe lysis buffer supplied with 1% protease inhibitor cocktail, protease inhibitor cocktail set II and III (all from Merck Biosciences). Cellular debris was removed by centrifugation method, and equal protein loading amount was determined by the Bradford method (Bio-Rad). Cleared cytosolic protein extracts were stored at -80℃ until use. 10μl of cell lysate was used for each ELISA reaction and all inductions were performed in triplicate to provide statistic results.

3.3.3.2. Reconstitution and Dilution of STAT3 [pY705] Standard

The STAT3 [pY705] standard is prepared using purified, recombinant, phosphorylated STAT3 protein. One Unit of standard is equivalent to the amount of STAT3 [pY705] derived from 20 pg of STAT3 that was phosphorylated by activated JAK.

The STAT3 [pY705] Standard was reconstituted with Standard Diluent Buffer. Each bottle of STAT3 [pY705] standard was reconstituted by adding 1ml of Standard Dilutent Buffer. The bottle was swirled and allowed to sit for 10 minutes to ensure complete reconstitution. Reconstituted standard solution was immediately aliquot into 150μl volumes, labeled as 100 Units/mL STAT3 [pY705]. The standard was used within 1 hour of reconstitution. 0.25 mL of Standard Diluent Buffer was added into to each of 6 tubes and labeled 50, 25, 12.5, 6.25, 3.12 and 1.6 Units/mL STAT3

[pY705] to make serial dilutions. Unused 100U/ml aliquoted standards were stored in -80℃ until use.

The standard diluent was prepared according to the following procedure:

100U/ml	Reconstituted standards		
50U/ml	0.25 mL of the 100 Units/mL std.		0.25 mL of the Dilue
25U/ml	0.25 mL of the 50 Units/mL std.	0.25 mL of the Diluent Buffer	
12.5U/ml	0.25 mL of the 25 Units/mL std.	0.25 mL of the Diluent Buffer	
6.25U/ml	0.25 mL of the 12.5 Units/mL std.	0.25 mL of the Diluent Buffer	
3.12U/ml	0.25 mL of the 6.25 Units/mL std.	0.25 mL of the Diluent Buffer	
1.57U/ml	0.25 mL of the 3.12 Units/mL std.	0.25 mL of the Diluent Buffer	
0U/ml	0.25 mL of the Diluent Buffer	0.25 mL of the Diluent Buffer	

3.3.3.3. Measurement of STAT3 [pY705] concentration in cell lysates

The Anti-rabbit IgG-HRP (100x concentrate) was in 50% glycerol and was viscous. To ensure accurate dilution, the Anti-rabbit IgG-HRP (100x concentrate) was allowed to sit until reach room temperature and gently mixed. Anti-rabbit IgG-HRP (100x concentrate) was slowly pipet out. Excessive concentrate solution was removed from pipette tip by gently wiping with clean absorbent paper. For each 2 strips of wells, 20μl of 100x concentrate Anti-rabbit IgG Horseradish Peroxidase was used to make 2ml of working solution.

For each experiment, 2 strips of 8-well strips was used for the assay. Extra strips and frame were stored in bag in the refrigerator for future use.

100 μL of the Standard Diluent Buffer was added into zero wells. Well reserved for chromogen blank was be left empty. 100 μL of standards was added into the appropriate microtiter wells. 10 μL of cleared cell lysate and 90μL of standard dilutent was mixed to establish experiment reaction system. The plate was then thoroughly mixed using the shaking function from microplate reader. After mixing, wells were covered by adhesive film with plate lid, and incubated for 2 hours at room

temperature.

After incubation, solution in wells was thoroughly decanted. The wells were washed by 250μL of washing buffer for 4 times. Then 100 μL of anti-STAT3 [pY705] (Detection Antibody) solution was filled into each well except the chromogen blank(s). The plate was then thoroughly mixed using the shaking function from microplate reader. After mixing, wells were covered by adhesive film with plate lid, and incubated for 1 hour at room temperature.

After incubation, solution in wells was thoroughly decanted. The wells were washed by 250μL of washing buffer for 4 times. 100 μL anti-rabbit IgG-HRP Working Solution was added into each well except the chromogen blank(s). The plate was then thoroughly mixed using the shaking function from microplate reader. After mixing, wells were covered by adhesive film with plate lid, and incubated for 30 minutes at room temperature.

After incubation, solution in wells was thoroughly decanted. The wells were washed by 250μL of washing buffer for 4 times. 100 μL of Stabilized Chromogen was added into each well. Then the plate was incubate for 30 minutes at room temperature and in the dark until a medium-dark blue color was generated in wells with the most intense signal. After chromogenesis, 100 μL of Stop Solution was added into each well to stop the reaction and solution in the wells should change from blue to yellow. The absorbance of each well was read at 450nm using a microplate reader with standard curves automatically generated from in-build program.

3.3.4. Inhibitor assay of JAK2-STAT3 and p38 MAPK pathway

3.3.4.1. Establishment of inhibitor assay

K562 cells were seeded at an initial density of approximately $2x10^3$ cells/well in V-bottom 96-wells plates (Corning). To be detailed, the K562 cells were initially seeded in 50ml culture flasks to allow cell proliferation until the final density reached $1x10^5$/ml. The cell cultures were centrifuged at 300g for 5 minutes in 50ml flasks to concentrate cells and to separate old medium. After which, the clear supernatant

was discarded and fresh RPMI medium supplied with 10% fetal bovine serum and 1% PSF was used to dilute cell pellets to a density of $4x10^4$/ml. 100µl of diluted cell culture was added into each well to be evaluated.

The diluted cell culture was allowed to equilibrate in wells for 2 hours in 37℃ CO_2 incubator. Then, 50µl of different kinase inhibitors diluted by RPMI-1640 at 4x concentration was added into wells for inhibitor pre-incubation. The pre-incubation procedure was performed by incubating plate in 37℃ CO_2 incubator overnight.

The Cucurbitacin D/hydroxyurea induction was performed in the next morning. Upon induction, 50µl of either 12.5ng/ml Cucurbitacin D or 50µg/ml hydroxyurea solution diluted by RPMI-1640 medium supplied with 10% fetal bovine serum and 1% PSF was added into each well to be evaluated to establish 3.12ng/ml of Cucurbitacin D or 12.5µg/ml hydroxyurea final concentration. The plates were gently shaken using an ELISA shaking bed for 5 minutes to ensure complete mixing. The plate was then incubated in 37℃ CO_2 incubator for 4 days.

Cell harvesting was applied by centrifuge plates at 1,500rcf for 15 minutes. After centrifugation, the supernatant was discarded by vacuum suction. Then, 50µl of CytoBuster extraction reagent was added into each well with gentle pipetting to fully resuspend cells. The plates were incubated at 4℃ for 15 minutes to ensure complete cell lysis, then the plate containing cell lysate were stored in -80℃ until use.

3.3.4.2. HbF ELISA detection

Refer to section 2.3.1

3.3.5. Detection of STAT3 nuclear translocation and DNA binding affinity

3.3.5.1. Preparation of nuclear extract from K562 cells

K562 cells were initially seeded in 50ml culture flasks to allow cell proliferation until the final density reached $1x10^5$/ml. Then cell cultures were induced by either 3.12ng/ml of Cucurbitacin D or 12.5µg/ml hydroxyurea for 24 hours. After

induction, cell cultures were centrifuged in 50ml falcon tube at 300rcf for 5 minutes at 4℃ to separate cell from culture medium. After centrifugation, cell pellets were resuspended by 1.5ml of cold PBS and transferred into 1.5 ml centrifugation tube. A second centrifugation was performed at 500rcf for 5 minutes at 4℃. Excessive PBS was removed by vacuum suction and decanting.

After decant, 20μl of packed cell volume of cells was added with 200μl of ice cold CER I supplied with 1% protease inhibitor, 1% phosphatase inhibitor cocktail and 0.5mM PMSF. The tube was vortexed vigorously on the highest setting for 15 seconds to fully resuspend cell pellet. Resuspended cell pellet was incubated on ice for 10 minutes.

After incubation, 11μl of ice-cold CER II was added into each tube to be extracted. The tubes were vortexed 5 seconds on the highest setting and incubated on ice for 1 minute. Then the tubes were vortexed again for 5 seconds on the highest settings, followed by centrifugation at 14,400rpm for 5 minutes at 4℃. Cleared supernatant containing cytoplasmic extract was quickly transferred into a clean pre-chilled eppendorf tube and stored in -80℃ until use.

The insoluble pellet fraction containing nuclei was resuspended in 100μl of ice-cold NER supplied with 1% protease inhibitor, 1% phosphatase inhibitor cocktail and 0.5mM PMSF. The tube was vortexed vigorously on the highest setting for 15 seconds to fully resuspend cell pellet. After vortexing, samples were place on ice to continue vortexing for 15 seconds every 10 minutes, for a total time of 40 minutes.

After incubation, samples were centrifuged at 14,400rpm for 10 minutes at 4℃. Cleared supernatant containing nuclear protein extract was aliquoted into PCR tubes at 100μl each. All extracts were stored at -80℃ until use.

3.3.5.2. EMSA detection of transcriptional factors binding to γ-promoter region

3.3.5.2.1. 3' end-labeling of EMSA probes

A double-stranded oligonucleotide containing a STAT-3 consensus sequence 5'-ACA CTC GCT TCT GGA ACG TCT GAG GTT ATC AAT AAG-3' and its

complementary sequence was synthesized to make the probe. Equal volume of 2μM oligonucleotide diluted in TE buffer was mixed well at a volume of 100μl in PCR tubes. The probe annealing was performed by using a PCR machine with the following program:

Temperature	Time
95℃	5 minutes
0.1℃/second decrease to 40℃	--
Quickly chill to 4℃	--

Annealed probe was aliquoted and stored in -80℃ until use.

The 3' biotin end labeling of EMSA DNA probe was performed by using Pierce Biotin 3' End Labeling Kit.

Kit components were thawed on ice. Just before use, 4μl of 17U/μl of TdT stock was diluted by 30μl of TdT reaction buffer to achieve 2U/μl final concentration. Reaction system was established by mixing components according to the following chart:

Component	Volume	Final Concentration
Ultrapure water	25μl	--
10μl of 5x TdT reaction buffer	10μl	1x
5μl of 1μM unlabeled oligo	5μl	100nM
5μl of 5μM biotin-11-UTP	5μl	0.5μM
Diluted TdT (2U/μl)	5μl	0.2U/μl
Total Volume	50μl	--

After mixing, the reaction system was incubated at 37℃ for 30 minutes to allow the addition of biotin onto the 3' end of DNA probe. Then 2.5μl of 0.2M EDTA was added into reaction system to quench TdT activity. 50μl of chloroform:isoamyl alcohol was added into each reaction system, mixed well, and incubated 5 minutes at room temperature to extract TdT. Incubated PCR tubes were put into centrifugation for 2 minutes at 16,000rcf to separate organic phase from aqueous phase. separated aqueous phase was stored at -80℃ until use.

3.3.5.2.2. Dot blotting for labeling efficiency estimation

Prior to the EMSA binding experiment, the 3'-biotin labeling efficiency was estimated by using dot blots and developed by Pierce Lightshift Chemiluminescent EMSA Kit. One 6x8cm of positively-charged Nylon membrane was hydrated in TE buffer for 10 minutes to ensure complete equilibration. One sample of Biotin Control Oligo stock and Unlabeled Control Oligo was diluted by TE buffer to make a final concentration of 50nM oligo stocks. DNA probes used for EMSA experiment was also diluted to 50nM concentration using TE buffer.

In microcentrifuge tubes, a series of oligo standards were established using the following table

Component	% Biotin				
	100	75	50	25	0
Biotin Control Oligo (50nM)	12	9	6	3	0
Unlabeled Control Oligo (50nM)	0	3	6	9	12
TE, pH 8.0	48	48	48	48	48
Total Volume	60µl	60µl	60µl	60µl	60µl

50µl of the oligo working stocks was pipette into wells A1-A5 of a 96-well dilution plate. Then a 10-fold dilution of the test TdT labeling reaction in TE buffer was established to achieve a final concentration of 10nM. 50µl of each 10nM test DNA samples was place into unused A wells of the 96-well plate, and also a series of two-fold dilutions of standards and samples were established by removing 25µl aliquots from all A wells and mixing them with 25µl of TE buffer in corresponding B wells. The serial dilution was continuously performed down the plate through H wells.

Equilibrated nylon membrane was placed onto a clean dry paper towel to absorb any excessive residual buffer. 2µl of each samples and standards were blotted onto the membrane. Blotted samples and standards were allowed 30 seconds for full absorption into membrane. After which, nylon membrane containing probes was immediately crosslinked by using UV-crosslinker at 120mJ/cm^2 each side.

Crosslinked membrane was stored at 4℃ until detection.

Development and detection of crosslinked dot-blot membrane was performed using Pierce LightShift Chemiluminescent EMSA Kit.

3.3.5.2.3. EMSA binding reaction and non-denaturing gel electrophoresis

5x EMSA binding buffer was prepared as described before on the following recipe:

HEPES, pH=7.9	100mM
KCl	250mM
DTT	5mM
EDTA	2.5mM
MgCl2	25mM
Glycerol	31.25%

The binding buffer was completely mixed and sterile filtered. After establishment, the binding buffer was stored at -20℃ before use.

The EMSA reaction system was then established. For the binding assay group, 8μl of binding buffer, 4μl of biotin-labeled probe and 24μl of ddH2O was mixed and aliquot into 9μl each. For each 9μl aliquot, 1μl of 10ng/μl nuclear extract from untreated, Cucurbitacin D-treated or hydroxyurea-treated samples was added into and mixed thoroughly. For the cold-probe competitor group, 8μl of binding buffer, 4μl of unlabeled cold probe and 20μl of ddH2O was mixed and aliquot into 8μl each. 1μl of 10ng/μl nuclear extract from untreated, Cucurbitacin D-treated or hydroxyurea-treated samples was also added into buffer system, mixed thoroughly, and incubated 5 minutes at room temperature. Then 1μl of biotin-labeled probe was added into each of the competitor reaction system and thoroughly mixed. Both the binding and competitor reaction system was incubated on ice for 20 minutes before loading to gel.

Tris-boric acid-EDTA buffered non-denaturing gel was prepared using Bio-Rad miniGel electrophoresis apparatus. One gel was prepared according to the following

chart.

30% Acrylamide	1.17ml
5x TBE	0.65ml
70% glycerol	0.51ml
ddH2O	4.23ml
10% AP	36µl
TEMED	4µl

The gel was allowed to polymerize for 2 hours at room temperature. After polymerization, gel electrophoresis unit was filled with 0.5x TBE to the maximum capacity to reduce any heat generated during electrophoresis. A pre-running was applied by using 80V power supply for 60 minutes.

After incubation, 2µl of 6x EMSA loading buffer was added into each of the reaction systems and mixed well to quench binding reaction. 10µl of mixture was carefully loaded into each well to be electrophoresised. The electrophoresis procedure was accomplished by using 80V voltage for 40 minutes until the fast-running bromophenol band reached 1cm above gel bottom.

3.3.5.2.4. Membrane development and chemiluminescence detection

One 6x8cm of positively-charged Nylon membrane was hydrated in TE buffer for 10 minutes to ensure complete equilibration. Equilibrated nylon membrane was placed onto a clean dry paper towel to absorb any excessive residual buffer. 2µl of each samples and standards were blotted onto the membrane. Blotted samples and standards were allowed 30 seconds for full absorption into membrane. After which, nylon membrane containing probes was immediately crosslinked by using UV-crosslinker at 120mJ/cm^2 each side. Crosslinked membrane was stored at 4℃ until detection.

Blocking and 4x washing buffer from LightShift Chemiluminescent EMSA Kit was gently warmed to 37℃ in water bath until all particulate and residual salt has been

dissolved. 10ml of blocking buffer was added into water reservoir with crosslinked nylon membrane. Membrane was blocked by gently shaking at room temperature for 15 minutes.

Conjugate/blocking buffer solution was prepared by adding 50µl of stabilized streptavidin-HRP conjugate to 15ml of blocking buffer. Excessive blocking buffer was decanted from membrane, and the membrane was placed in clean new reservoir containing 15ml of conjugate/blocking solution. The streptavidin-biotin binding reaction was performed by incubation at room temperature for 15 minutes with gentle shaking.

1x washing solution was freshly prepared by mixing 40ml of 4x wash buffer with 120ml ddH2O. After incubation, membrane was decanted on paper towel to absorb any excessive solution, then washed 4 times using 20ml of 1x washing solution for 5 minutes each time with gentle shaking.

After washing, membrane was decanted on paper towel to absorb any excessive solution. Decanted membrane was then applied into a new water reservoir containing 30ml of substrate equilibration buffer for osmosis equilibration. The incubation was performed by gentle shaking for 5 minutes at room temperature.

After washing, membrane was decanted on paper towel to absorb any excessive solution. Decanted membrane was placed in a clean container. Substrate working solution was then prepared by 1:1 mixture of luminol/enhancer solution and stable peroxides solution together. Mixed substrate working solution was poured onto the membrane as it completely covered membrane surface. The membrane was incubated in substrate working solution for 5 minutes at room temperature without shaking.

After incubation, membrane was decanted on paper towel to absorb any excessive solution. Detection of chemiluminescence signal was accomplished by using Roche F1 chemiluminescence detector. The exposure time was 7 minutes. Collected data was gathered and transformed into visible image using in-build software from detector.

3.3.5.3. Preparation of K562 samples for immunofluorescence detection

3.3.5.3.1. Slide coating for cell capture

8-cells chambered slide was coated with poly-l-lysine to ensure appropriate adhesion of K562 cells onto slide. To be detailed, 1ml of 10x concentrated poly-l-lysine solution was mixed with 1ml of PBS and 8ml of ddH2O. The diluted poly-l-lysine solution was applied to the chambers at 1ml/chamber, and the slide was incubated 2 hours at room temperature. After incubation, the chambers were thoroughly washed by ddH2O and allowed to dry at room temperature.

3.3.5.3.2. Preparation of cell slide

K562 cells were initially seeded in 10ml culture flasks to allow cell proliferation until the final density reached $1x10^5$/ml. Then cell cultures were induced by either 3.12ng/ml of Cucurbitacin D or 12.5μg/ml hydroxyurea for 24 hours. After induction, cell cultures were centrifuged in 15ml falcon tube at 300rcf for 5 minutes at 4℃ to separate cell from culture medium. After centrifugation, cell pellets were resuspended by 0.5ml of PBS.

Resuspended cells in PBS were added into cells on the chamber slide respectively to the induction status. The chamber slide containing resuspended K562 cells was then covered with chamber lid, and incubated in 37℃ incubator supplied with 5% CO_2 for 30 minutes to ensure complete cell adhesion to slide.

3.3.5.3.3. Sample fixation and antibody probing treatment

After incubation, excessive PBS in the slide chamber was quickly purged out, and the chamber slide was put onto paper towel with bottom-up to absorb excessive liquid. For each chamber, 1ml of 4% para-formaldehyde in PBS was quickly added into the well to fix cells. Fixed slide was incubated at 4℃ for 1 hour to ensure complete fixation.

After fixation, excessive para-formaldehyde was removed from chamber wells by

vacuum suction. The chamber slide was then put onto paper towel with bottom-up to absorb excessive liquid. The chamber wells were washed three times using 1ml of PBS each for 5 minutes to ensure complete removal of residual para-formaldehyde. Washed chamber slide was permeablized by incubating with 0.05% Triton X-100 in PBS for 5 minutes. Following permeablization, the chamber wells were again washed three times using 1ml of PBS each for 5 times to remove residual Triton X-100. After which, 200μl of highly cross-absorbed blocking reagent was applied into each well to block nonspecific sites. The chamber slide was incubated at room temperature for 1 hour to ensure complete blocking of nonspecific sites.

After blocking, the blocking reagent was removed by flipping chamber slide onto paper towel. Then 200μl of 1:400 mouse anti-STAT3-antibody (124H6) diluted in blocking reagent was applied into each well. The chambers were sealed by parafilm with slide on, and incubated at 4℃ overnight for maximum absorption of primary antibody.

After overnight incubation with primary antibody, he chamber wells were washed three times using 1ml of PBS each for 5 minutes to ensure complete removal of residual antibody. Then 100μl of 1:1000 goat anti-mouse Alexa-488-conjugated detection antibody (highly cross-absorbed) diluted in PBS was applied into each well. The slide was incubated at 37℃ for 1 hour.

After incubation, chamber wells were washed three times using 1ml of PBS each for 5 minutes to ensure complete removal of residual antibody. Then the chamber was removed altogether with rubber sealing ring. 50μl of Invitrogen Antifade mounting reagent was applied onto slide and mounted. Mounted slide with cover glass was wrapped with aluminum foil and stored in 4℃ until detection.

3.3.5.3.4. Sample imaging and immunofluorescence detection

Immunofouorescence detection was performed by using Bio-Rad Radiance 2100 confocal imaging system. The laser-emitting module was pre-heated for 30 minutes before detection. Upon detection, slide containing cell samples was mounted with

Carl-Zeiss Immersol 518N immersion oil to enhance imaging quality. The microscopic detection was then performed by using Argon laser wavelength 488nm, 60x objective lens, 50 lines/second scanning speed and 512x512 image size.

3.4. Results

3.4.1. Activation of p38 MAPK pathway and STAT3 phosphorylation by hydroxyurea

3.4.1.1. The p38 MAPK pathway is activated by hydroxyurea, but not activated by Cucurbitacin D

We examined whether the phosphorylation level of p38 MAPK was altered by the induction of either 3.12ng/ml of Cucurbitacin D or 12.5μg/ml of hydroxyurea during 6 time points in 24 hours of induction period. The indicated concentration of inducing drugs has been verified that they would not interfere with cell viability by MTT assay (data not shown). Fig. 6 demonstrated the phospho-p38/pan-p38 ratio changes at different time points post induction, plus the positive control of p38 activation induced by anisomycin. It can be clearly observed that the phospho-p38/pan-p38 ratio was increased 12-24 hours post induction for 2 folds in hydroxyurea-treated groups, but not in Cucurbitacin D treated groups ($P<0.05$). In all, after 24 hours of induction we observed a 2.3-fold induction of phospho-p38 MAPK under hydroxyurea induction, and direct activation of p38 by anisomycin also confirmed that our K562 cells were responsive to p38 MAPK inducers. This result indicated that p38 MAPK activity is induced in a manner by the γ-globin inducer hydroxyurea. However, the Cucurbitacin D, although it also possesses the ability of inducing γ-globin, cannot elicit similar effects. Hence, it may suggest two different induction mechanisms that lie between Cucurbitacin D and hydroxyurea.

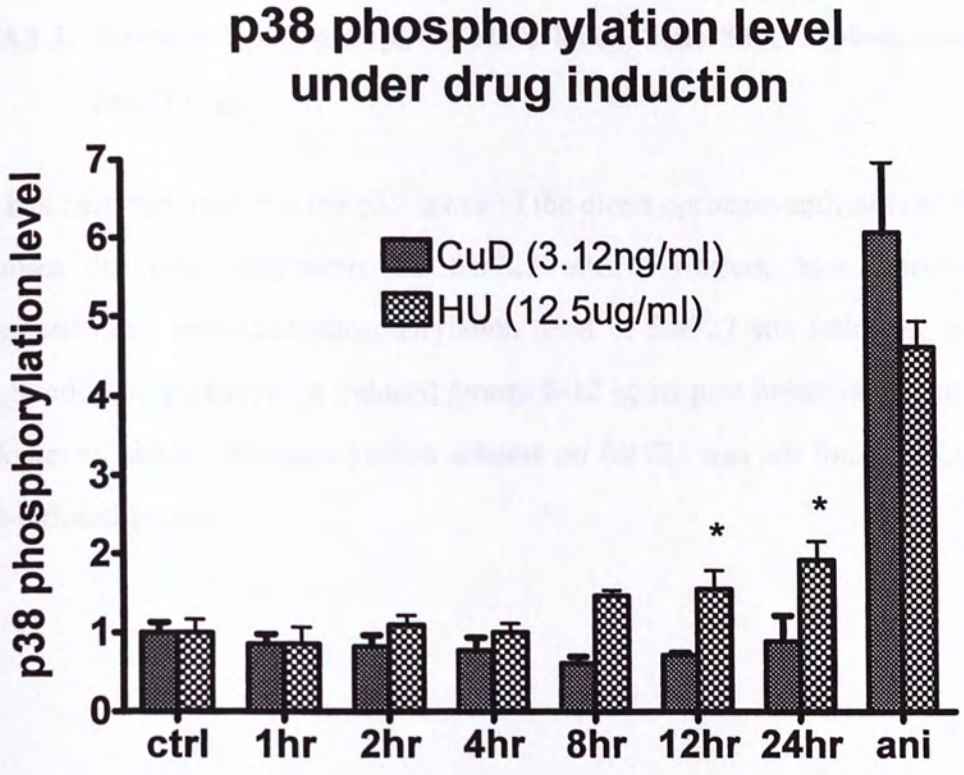
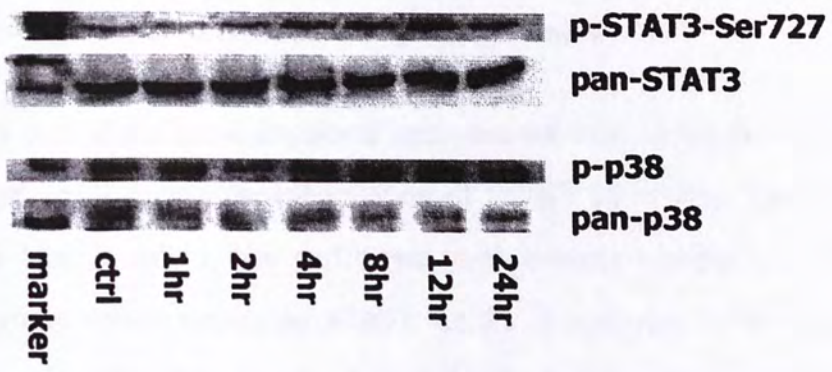


Figure 4. Time-dependent phosphorylation level of p38 MAPK kinase under the induction of CuD or HU in K562 cell line. K562 cells were treated with 3.12ng/ml of CuD or 12.5μg/ml of HU. Cells were collected at different time points as indicated for the measurement of phospho-p38 and pan-p38 using ELISA method. Y axis represents the phospho-p38/pan-p38 ratio after induction. ani: Anisomycin-treated group for positive p38 activation control. *: $P<0.05$

3.4.1.2. Increased p38 phosphorylation level elicits STAT3 phosphorylation at Ser727 site

It has been reported that the p38 is one of the direct upstream activators of STAT3 and causes its phosphorylation on Ser727 site. Western blot detection results demonstrated enhanced phosphorylation level of Ser727 site following p38 MAPK activation in hydroxyurea-induced groups 8-12 hours post induction (Figure 5A, 5B). However, similar phosphorylation scheme on Ser727 was not found in Cucurbitacin D-induced groups.

A



B

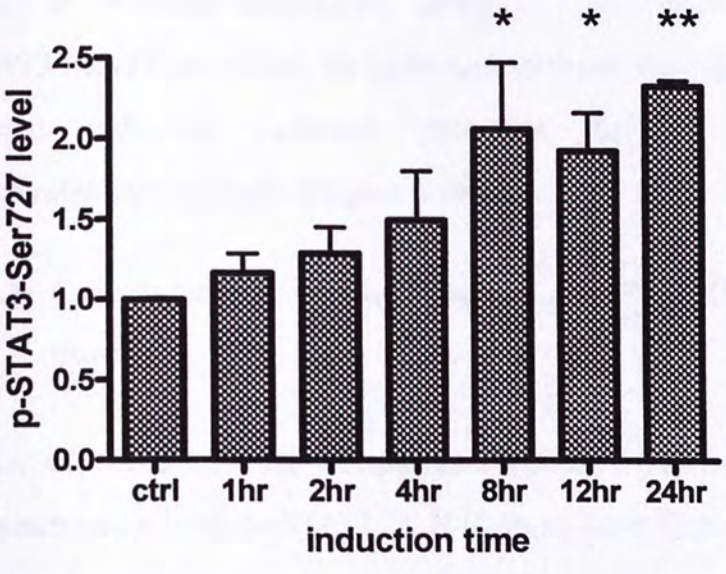


Figure 5. Enhanced p38 phosphorylation and STAT3 phosphorylation level at Ser727 site in K562 cells upon 12.5μg/ml hydroxyurea induction. A: Western blot result of phospho-STAT3-Ser727 and phospho-p38 upon hydroxyurea induction in different time points, total STAT3 and p38 amount were served as an internal control. B: Quantitative analysis result of increased phospho-STAT3-Ser727 level upon hydroxyurea induction. K562 cells were treated with 12.5μg/ml of HU, and collected at different time points as indicated for the measurement of phospho-p38-Ser727. Y axis represents the phospho-p38-Ser727/pan-p38 ratio after induction. *: $P<0.05$; **: $P<0.01$

3.4.2. Activation of JAK2 and STAT3 phosphorylation by Cucurbitacin D

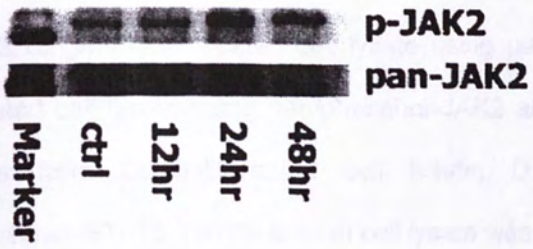
3.4.2.1. Cucurbitacin D promotes JAK2 activation

JAK2 is one of the most important upstream activator molecules of STAT3, and its activation leads to the phosphorylation of STAT3 on residue Tyr705 site. Hence, western blot detection was performed to investigate whether Cucurbitacin D and hydroxyurea would activation STAT3 via JAK2 pathway. The western blot result (Figure 6A, 6B, 6C) indicated enhanced phosphorylation level of JAK2 in Cucurbitacin D-induced groups, while no similar increase of phosphorylation was found in hydroxyurea-induced groups. Altogether with the result of p-STAT3-Ser727 introduced by hydroxyurea, these experiment outputs suggest two different induction pathways may lie between Cucurbitacin D and hydroxyurea-induced γ-globin gene activation.

3.4.2.2. Cucurbitacin D and hydroxyurea promote STAT3 phosphorylation at Tyr705 site

ELISA experiments using anti-phospho-STAT3-Tyr705 kit demonstrated that the phosphorylation level on STAT3 Tyr705 site increased two fold in both Cucurbitacin D and hydroxyurea-induced groups after 24 hours of drug treatment (Figure 7).

A



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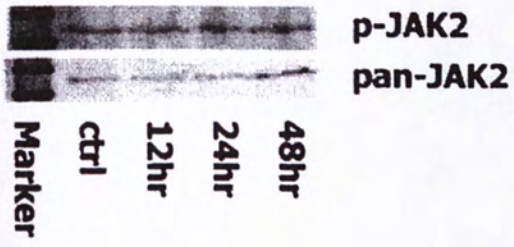

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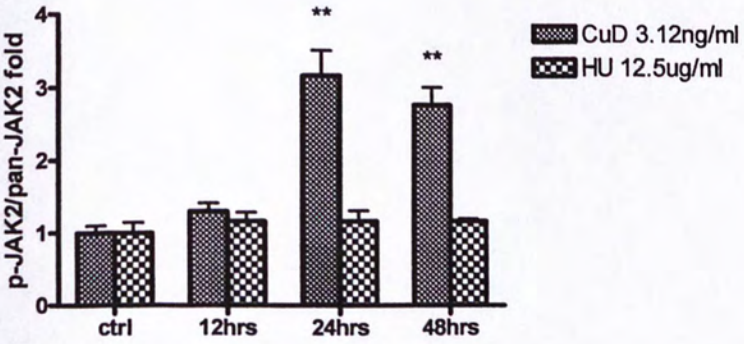
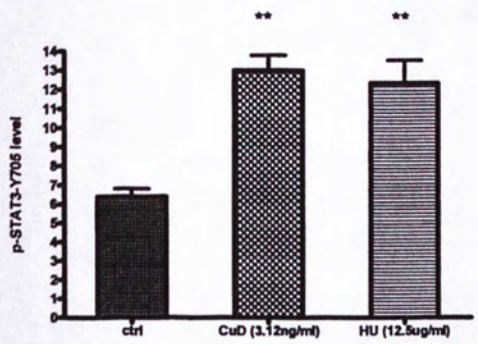


D



(Figure legend on the following page)

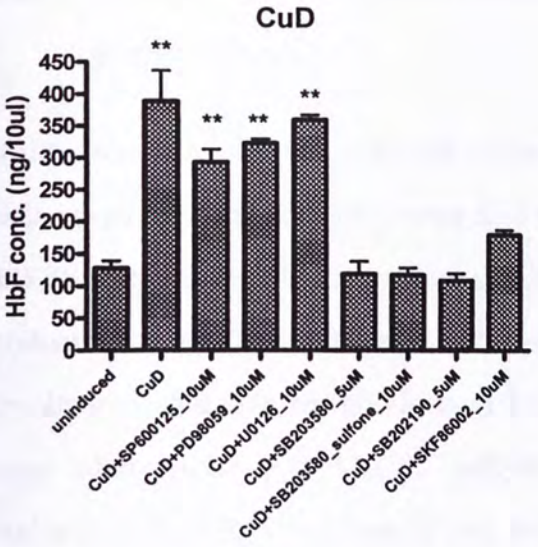
Figure 6. Enhancement of JAK2 and STAT3 phosphorylation in K562 cells under CuD and HU induction. K562 cells were treated by 3.12ng/ml CuD or 12.5μg/ml hydroxyurea for 24 hours. A: 3.12ng/ml CuD treated cell lysate using pan/phosphor-JAK2 antibody; B: 12.5μg/ml HU treated cell lysate using pan/phosphor-JAK2 antibody; C: quantitative JAK2 phosphorylation data from CuD/HU-treated cell lysate; D:STAT3 Tyr705 phosphorylation level, he phosphor-STAT3-Tyr705 level in cell lysate was measured using ELISA method. **: $P<0.01$

3.4.3. Basal activity of signal transduction pathways is essential for HbF induction

3.4.3.1. Activation of γ-globin gene requires presence of basal phosphorylation level of p38 MAPK

In order to validate that whether even basal phosphorylation level of p38 MAPK is required for γ-globin expression, different inhibitors of MAPK pathway and its components, including JNK, ERK and p38 inhibitors, were applied to block specific signal transduction pathway during our drug induction assay. The production efficiency of fetal hemoglobin was observed to evaluation the blocking effect of different inhibitors towards γ-globin gene expression. The representative chart is shown in Figure 7. Both Cucurbitacin D and hydroxyurea significantly increased fetal hemoglobin concentration in cell lysates by 3 fold ($P<0.01$) as previously indicated. By contrast, pretreatment with 10μM of p38 MAPK inhibitors (SB203580, SB203580 sulfone, SB202190 and SK86002) significantly inhibited the fetal hemoglobin production in all experimental groups regardless of different inducing drugs. However, groups pretreated by JNK inhibitor (SP600125) and ERK inhibitors (PD98059 and U0126) did not represent similar inhibitory effects. Hence, we may conclude that the presence of p38 MAPK, even at the basal level, plays an important role during γ-globin gene activation.

A



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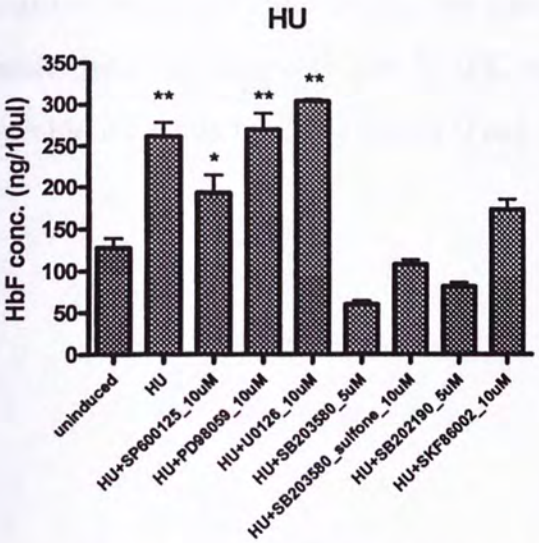

Figure 7. Effects of different MAPK family inhibitors on the fetal hemoglobin (HbF) production in K562 cells upon CuD or HU induction. K562 cells were firstly pre-treated by different inhibitors for 24 hours at the concentration indicated, then induced by either 3.12ng/ml CuD or 12.5μg/ml hydroxyurea for 5 days. The hemoglobin level in total cell lysate was measured by using HbF ELISA kit. JNK inhibitor: SP600125; ERK inhibitors: PD98059, U0126; p38 inhibitors: SB203580, SB203580 sulfone, SB202190, SKF86002. A: 3.12ng/ml Cucurbitacin D-treated groups; B: 12.5μg/ml hydroxyurea-treated groups. *: $P<0.05$; **: $P<0.01$, all significance were achieved by comparing with uninduced group.

3.4.3.2. Inhibition on JAK2-STAT3 pathway results in reduced fetal hemoglobin production

Besides p38 MAPK, other potential pathways that have been reported to be involved during γ-globin gene activation were also studied, which included JAK2-STAT3, CK1, GSK3β and PKC. Blocking effect of different inhibitors towards fetal hemoglobin production under Cucurbitacin D and hydroxyurea induction is shown in Figure 8. Results indicated that the inhibition of JAK2-STAT3 pathway, which was considered cross talking with p38 MAPK pathway, resulted in reduced fetal hemoglobin production in both Cucurbitacin and hydroxyurea-induced groups. Inhibition in PKC pathway also resulted in reduced fetal hemoglobin production in hydroxyurea-induced groups, but not found in Cucurbitacin D-induced groups. The results indicated that both p38 MAPK and JAK2-STAT3 pathways are essential in γ-globin induction by Cucurbitacin D and hydroxyurea.

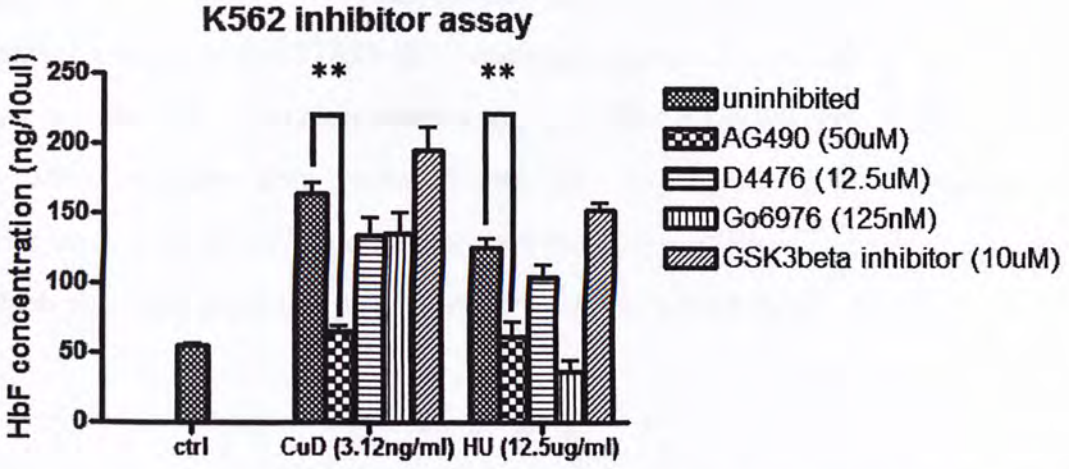

Figure 8. Effects of different inhibitors on the fetal hemoglobin (HbF) production in K562 cell line upon CuD or HU induction. K562 cells were firstly pre-treated by different inhibitors for 24 hours at the concentration indicated, then induced by either 3.12ng/ml CuD or 12.5μg/ml hydroxyurea for 5 days. The hemoglobin level in total cell lysate was measured using HbF ELISA kit. AG490: JAK2 inhibitor; D4476: CK1 pathway inhibitor; Gö6976: PKC inhibitor; inhibition of GSK3beta pathway was performed by using specific cell-permeable inhibitor peptide.

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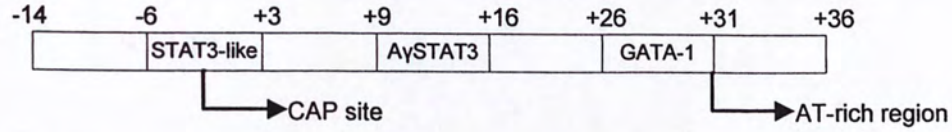
3.4.4. Translocation and DNA binding of STAT under Cucurbitacin D induction

3.4.4.1. Cucurbitacin D and hydroxyurea both enhance binding affinity of transcriptional factors to the Gγ/Aγ promoter

To validate the changes of binding status on the promoter region of γ-globin gene, one consensus sequence containing a STAT3-link binding sequence indicated in previous research (5'-ACA CTC GCT TCT GGA ACG TCT GAG GTT ATC AAT AAG-3')(Foley et al. 2002) was chosen to perform gel retardation assay. This sequence included a STAT3-like binding sequence (5'-TTC TGG AA-3') between +9 to +16 to the location of CAP site, and similar to the STAT3 consensus sequence $TT(N)_{4-5}AA$. The preparation of nuclear extract as described previously in the material and methods section, and a radioisotope-free gel retardation assay was

performed accordingly with equal protein loading. EMSA results revealed enhanced binding affinity to the STAT3-like consensus sequence in both Cucurbitacin D and hydroxyurea-induced nuclear extracts, and this effect could be successfully competed by 100x excessive cold probes (Figure 9). The results further confirmed the translocation of STAT3 into nucleus and their interaction with γ-promoter region, which played an important part in the activation of γ-globin gene.

A



B

Figure 9. The structure of γ-promoter sequence and EMSA result. A: The γ-promoter sequence includes following regulatory elements: STAT3-like sequence, AγSTAT3 sequence, including a stage selector element (SSE)-like element, GATA-1 site and an AT-rich sequence located in +28~+32. The oligo sequence used in EMSA starts from +1 to +36, including the STAT3-like and AγSTAT3 sequences (5'-ACA CTC GCT TCT GGA ACG TCT GAG GTT ATC AAT AAG-3'). B: EMSA result shows enhanced binding affinity to the oligo. K562 cells were induced by either 10ng/ml Cucurbitacin D or 20μg/ml hydroxyurea for 24 hours. The nuclear extracts were collected and binding reaction was performed with the presence of poly dIdC to prevent unspecific binding. The enhanced binding affinity could be successfully competed by 100x cold probe.

3.4.4.2. Cucurbitacin D and hydroxyurea induces nuclear translocation of STAT3

The translocation of transcription factors from cytoplasm to nucleus is essential for the activation of target genes. Hence, we also performed investigation towards the nuclear migration of STAT3 induced by Cucurbitacin D and hydroxyurea. Confocal microscopy results of K562 cells treated with anti-STAT3 antibody also revealed increased presence of STAT3 in nucleus (Figure 10). These results suggested that the activation of γ-globin gene was accompanied with the translocation of STAT3 under drug induction.

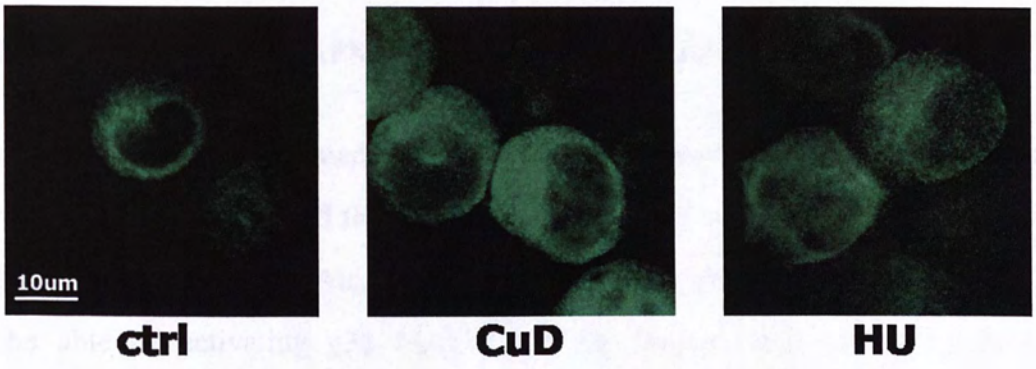


Figure 10. Confocal microscopy images of K562 cells demonstrating the translocation of STAT3. K562 cells were treated by either 10ng/ml Cucurbitacin D or 20μg/ml hydroxyurea for 3 hours before fixation. All images were taken under same magnificent and scanning parameters. Increased presence of STAT3 in nuclear is clearly visible.

3.5. Discussion

3.5.1. The role of p38 MAPK activation during γ-globin gene activation

The original idea of p38-mediated γ-globin gene expression was noted during the investigation of HDAIs and their effects toward globin gene regulations (Pace et al. 2003). Two different HDAIs, sodium butyrate and trichostatin, have been identified to be able of activating p38 MAPK pathway besides their effects on histone hyperacetylation. Experiments using hemin, one compound which is able to induce γ-globin gene expression but cannot activate p38 MAPK pathway, also confirmed the relationship between p38 MAPK and γ-globin gene expression as the pre-treatment of p38 inhibitors did not interfere the expression of γ-globin gene. Up to now, several trans-activators of butyrate responsive elements have been identified (Bohan et al. 1989; Ikuta et al. 1998; Pace et al. 2000).

In this study, a potential relationship between hydroxyurea-induced p38 MAPK activation and γ-globin gene expression has been presented. The hydroxyurea has been reported able to activate p38 MAPK and trigger cell differentiation in human K562 cell line (Park et al. 2001). The effect of hydroxyurea on cellular differentiations also shares some similarities with the effects lead by sodium butyrate. Previous investigations also discovered the inhibition of ERK pathway in K562 cells. In the K562 differentiation process, the enhancement of ERK pathway leads the cells to diffentiate into megakaryocytes, while inhibition of ERK or activation of p38 MAPK pathway leads cells to erythroid phenotype. Hence, the activation of p38 MAPK pathway and the γ-globin gene expression leads by hydroxyurea may caused by the differentiation of K562 cells in erythroid direction.

3.5.2. STAT3 phosphorylation at Ser727 site promotes transcription factor activity and γ-globin gene expression

Recent evidences have demonstrated the importance of signaling pathways in

mediating drug-induced HbF production and the involvement of JAK-STAT pathway. Sodium butyrate has been demonstrated to induce erythroid maturation in mouse erythroleukemia cells associating rapid phosphorylation of JAK2 and STAT5 (Yamashita et al. 1998). Enhanced phosphorylation level of STAT3 at Ser727 was also found accompanied by activation of p38 MAPK pathway in IL-2 or IL-12 induced mouse lymphocytes (Gollob et al. 1999). Regarding to the similar enhancement scheme of p38 MAPK and increased phosphorylation of STAT3 at Ser727 site induced by hydroxyurea in our model, we consider that the increased transcription activity of γ-globin gene was mediated by activated STAT3 under hydroxyurea induction.

However, it should be noticed that neither increased p38 phosphorylation level nor increased STAT3 phosphorylation on Ser727 site was found in Cucurbitacin D-treated groups. Hence, it implies a different induction mechanism of Cucurbitacin D-induced γ-globin gene expression, which does not go through the mediation of p38 MAPK pathway.

3.5.3. The role of JAK2-STAT3 activation during γ-globin gene activation

Recently the investigation of γ-globin gene regulation mechanism mainly focused on the promoter region and organization of different transcription factors, and only limited research paid attention on the aspect of cell signaling and fetal hemoglobin production. The JAK2-STAT3 signaling pathway, which has been implicated in sodium butyrate-induced γ-globin gene activations,(Xie et al. 1999; Foley et al. 2002) is considered helpful for specific gene-based treatments on hematopoiesis deficiencies. Another important pathway that considered important for γ-globin gene regulation is p38 MAPK pathway, along with its direct link between γ-globin gene inducibility and histone deacetylace inhibitors (HDAIs).

In our research, we presented two different but intercrossing signaling pathways that responsible for either Cucurbitacin D or hydroxyurea-induced γ-globin gene activation. Cucurbitacin D, which activates JAK2 and then triggers phosphorylation

of downstream transcription factor STAT3 on Tyr705 site, elicits the enhanced translocation of STAT3 into nucleus thus binding to γ-promoter region and initiates γ-globin gene expression. Similar activation of JAK2-STAT3 cascade has been reported in EPO-induced UT7/EPO cell line. This activation mechanism was considered to be STAT1/STAT3 related mitogenesis-associated molecules and over-expression of c-myc gene (Fukada et al. 1998; Kiuchi et al. 1999). Another rapid activation of JAK2 ant STAT5 induced by sodium butyrate was also reported (Yamashita et al. 1998), and implies the common pathway shared by γ-globin gene inducers.

In contrast, hydroxyurea may play as a multi-role inducer. In one way it possesses a similar behavior as Cucurbitacin D by promoting the phosphorylation of STAT3 on Tyr705 site. In another way, it also activates the p38 MAPK cascade and causes STAT3 phosphorylation on Ser727 site, in a similar manner of sodium butyrate and trichostatin-induced γ-globin gene expression. Regarding there will always be a basal level of phosphorylated STAT3 presence in cytoplasm, either of two alternative phosphorylation sites activate the STAT3 and led to its migration into nucleus. Migrated STAT3 binds to the STAT3 consensus sequence located on the γ-promoter and hence activates gene transcription. Since the STAT3 consensus sequence is also included in the promoter region of ε, δ, and β globin genes,(Foley et al. 2002) the drug induction will also promote transcription of genes indicated above, as demonstrated by real-time RT-PCR results.

However, the idea that hydroxyurea may possess a dual-activator mechanism cannot be excluded. Researches using sodium butyrate as γ-globin gene inducers proposed both the histone-hyperactylation accompanied chromatin structural changes and transcription factor activation by p38 MAPKs or JAK2-STAT3s are both essential for a maximal expression of γ-globin gene.

3.5.4. Inhibitor assay

Our experiment results also suggested that activated STAT3 plays a crucial role in

γ-globin gene activation. The results not only support a direct connection between γ-globin gene inducibility and STAT3 activation, but also imply a total abolishment of STAT3 activity negatively regulates γ-globin gene expression. In our two experiments, one abolished the phosphorylation of Tyr705 site on STAT3 by AG490 treatment, another abolished the phosphorylation of Ser727 site on STAT3 by p38 inhibitors treatment, both resulted in greatly reduced fetal hemoglobin production in K562 cells under drug induction. Hence, the maintenance of basal phosphorylation level on both Tyr705 and Ser727 sites is essential for the γ-globin gene activity.

Results from MAPK kinase inhibitor assay clearly indicated that out of three different subgroups of MAPK family, only p38 MAPK was essential for the successful induction of fetal hemoglobin in K562 cells. Pre-treatment using either JNK/SAPK inhibitors or ERK inhibitors demonstrated no significant results for the reduced fetal hemoglobin production. Hence, we propose the following mechanisms were involved during abolishment of fetal hemoglobin production by p38 inhibitors. In normal cells, the phosphorylation of STAT3 on Ser727 is maintained by the activity of p38 MAPK at its basal level. Regarding the p38 MAPK has the property of autophosphorylation, it ensures the presence of a small portion of activated p38 MAPK in cytoplasm. The activated p38 MAPK thus takes the responsibility to keep the STAT3 Ser727 phosphorylation at a certain level to ensure proper function of gene regulation. However, pre-treatment of inhibitors abolished the kinase function of p38 MAPK and failed in keeping the basal level of STAT3 Ser727 phosphorylation mediated by p38 MAPK. Previous research indicated that an increase of basal level of STAT3 Ser727 phosphorylation may be observed in p38 MAPK inhibitor-treated cells that was led by MKK6-induced activation of unidentified Ser/Thr kinase, however, it is noteworthy that the enhancement of STAT3 Ser727 phosphorylation level was not able to mediate its normal transcription-enhancing function (Xu et al. 2003). In other words, the p38 MAPK inhibitor pre-treatment abolished the normal STAT3 function.

In CuD-trated groups, although CuD induction does not directly affect the STAT3 phosphorylation on Ser727 site, the abolishment of p38 MAPK activity still inhibited

normal STAT3 function thus prevented enhanced fetal hemoglobin production. In HU-treated groups, since the enhanced fetal hemoglobin production was a direct consequence of p38 activation, inhibition of p38 activity leads to reduced drug effect and fetal hemoglobin production.

Results from JAK-STAT kinase assay also indicate that the phosphorylation of STAT3 on Tyr705 site is essential for successful induction of fetal hemoglobin in K562 cells. Pre-treatment of JAK-STAT pathway inhibitor AG490 reduced drug-induced HbF production in both CuD and HU-induced groups. Some members that belong to the Cucurbitacin family, Cucuribitacin A, B, E, I and Q, has demonstrated their effects towards STAT3 phosphorylation on Tyr705 site (Sun et al. 2005; Graness et al. 2006; van Kester et al. 2008). However, the detailed effecting mechanism of Cucurbitacin family members differs from one to another. Treatment using Cucurbitacin B, E, and I towards A549 cells significantly reduced phosphorylated JAK2 and STAT3 Tyr705 in western blot results. Cucurbitacin Q only inhibited the phosphorylation of STAT3 Tyr705 without affecting the JAK2 phosphorylation. In contrast, though Cucurbitacin A inhibited the phosphorylation of JAK2, it enhanced phosphorylation level of STAT3 on Tyr705 site (Sun et al. 2005). Hence, the effects of different Cucurbitacins towards STAT3 phosphorylation cannot be generalized. According to the experiment results, it is assumed that maintenance of STAT3 Tyr705 site is essential for both CuD and HU-induced fetal hemoglobin production.

Previous researches on γ-globin gene expression have indicated the importance of JAK-STAT pathway and p38 MAPK pathway, but failed in establishing reasonable connections and regulation scheme upon these two different regulation system. According to our experiment results, it can be concluded that functional STAT3 activity is essential for the γ-globin gene expression in K562 cell line. Although the two STAT3 phosphorylation sites were regulated by different pathways, inhibition on either of the upstream regulators will results in reduced overall activity of STAT3.

3.5.5. Relations between STAT3 nuclear translocation and enhanced fetal hemoglobin production

In this section, the role of STAT3 during drug-induced γ-globin gene activation is further confirmed using gel retardation assay and confocal imaging. Previous researches have identified one STAT3 motif (TTCTGGAA) located in the 5'-untranslated region of γ-globin gene with structural similarities to the IL-6 responsive element in α_2-macroglobulin gene (Zhang et al. 2001). EMSA results indicated the binding of a heterodimeric STAT1-STAT3 complex to the motif region might contributed to the steady-state expression of γ-globin gene. This STAT3 homolog sequence was observed in the analogous position of ε, δ, and β-globin genes, which suggested further evidence for physiologic role for STAT family proteins during globin gene regulation (Foley et al. 2002). Our EMSA experiments using short DNA probes including STAT3 motif revealed enhanced protein binding activities to the motif region under CuD/HU induction, which suggested the activated STAT3 indeed translocated into nucleus and bind on target sequence to trigger gene transcription.

However, this result is contradictory to previous researches, which suggested an inhibitory role of activated STAT3 towards γ-globin gene expression (Foley et al. 2002). This may be explained by the different activation methods and STAT3 isoforms. The activation of STAT3 by either overexpression of STAT3β or IL-6 induction repressed γ-promoter activity was considered by enhanced binding affinity to AγSTAT3 region and complex formation. In our model, both isoforms of STAT3 were activated, which suggested different components might be involved during transcription complex forming thus leading to the enhanced transcription activity. Besides, inhibition of STAT3 on either of the phosphorylation site resulted in repression of γ-globin gene expression and fetal hemoglobin production, suggested the positive role of STAT3 towards γ-promoter activity.

4. Summery and Prospect

Recently the investigation of γ-globin gene regulation mechanism mainly focused on the promoter region and organization of different transcription factors, and only limited research paid attention on the aspect of cell signaling and fetal hemoglobin production. The JAK2-STAT3 signaling pathway, which has been implicated in sodium butyrate-induced γ-globin gene activations, (Xie et al. 1999; Foley et al. 2002) is considered helpful for specific gene-based treatments on hematopoiesis deficiencies. The activation of JAK2 and STAT5 during erythroid maturation induced by sodium butyrate has been demonstrated in mouse erythroleukemia cells (Yamashita et al. 1998). Researches using UT7/EPO cells observed induction of EPO was accompanied by activation of JAK2 and phosphorylation of STAT3 at Tyr705 site, and implied a positive effect of STAT3 phosphorylation for erythroid-directed cell differentiation (Kirito et al. 2002). Another important pathway that considered important for γ-globin gene regulation is p38 MAPK pathway, along with its direct link between γ-globin gene inducibility and histone deacetylace inhibitors (HDAIs) (Pace et al. 2003). Up to now, several trans-activators of butyrate responsive elements have been identified (Bohan et al. 1989; Ikuta et al. 1998; Pace et al. 2000). Enhanced phosphorylation level of STAT3 at Ser727 was also found accompanied by activation of p38 MAPK pathway in IL-2 or IL-12 induced mouse lymphocytes (Gollob et al. 1999).

In our research, we presented two different but intercrossing signaling pathways that responsible for either Cucurbitacin D or hydroxyurea-induced γ-globin gene activation. Cucurbitacin D, which activates JAK2 and then triggers phosphorylation of downstream transcription factor STAT3 on Tyr705 site, elicits the enhanced binding affinity of γ-promoter region and initiates γ-globin gene expression. Some members that belong to the Cucurbitacin family, Cucuribitacin A, B, E, I and Q, has demonstrated their effects towards STAT3 phosphorylation on Tyr705 site (Sun et al. 2005; Graness et al. 2006; van Kester et al. 2008). However, the detailed effecting

mechanism of Cucurbitacin family members differs from one to another. Treatment using Cucurbitacin B, E, and I towards A549 cells significantly reduced phosphorylated JAK2 and STAT3 Tyr705 in western blot results. Cucurbitacin Q only inhibited the phosphorylation of STAT3 Tyr705 without affecting the JAK2 phosphorylation. In contrast, though Cucurbitacin A inhibited the phosphorylation of JAK2, it enhanced phosphorylation level of STAT3 on Tyr705 site (Sun et al. 2005). Hence, the effects of different Cucurbitacins towards STAT3 phosphorylation cannot be generalized. Hydroxyurea may play as a multi-role inducer. In one way it possesses a similar behavior as Cucurbitacin D by promoting the phosphorylation of STAT3 on Tyr705 site. In another way, it also activates the p38 MAPK cascade and leads STAT3 phosphorylation on Ser727 site, in a similar manner of sodium butyrate and trichostatin-induced γ-globin gene expression. Regarding there will always be a basal level of phosphorylated STAT3 presence in cytoplasm, enhancement on either of two alternative phosphorylation sites activate the STAT3 and lead to its migration into nucleus. Migrated STAT3 binds to the STAT3 consensus sequence located on the γ-promoter and hence activates gene transcription. Since the STAT3 consensus sequence is also included in the promoter region of ε, δ, and β globin genes,(Foley et al. 2002) the drug induction will also promote transcription of genes indicated above, as our previous research data suggested (data not shown).

Our experiment results also suggested that activated STAT3 plays a crucial role in γ-globin gene activation. The results not only support a direct connection between γ-globin gene inducibility and STAT3 activation, but also imply a total abolishment of STAT3 activity negatively regulates γ-globin gene expression. In our two experiments, one abolished the phosphorylation of Tyr705 site on STAT3 by AG490 treatment, another abolished the phosphorylation of Ser727 site on STAT3 by p38 inhibitors treatment, both resulted in greatly reduced fetal hemoglobin production in K562 cells under drug induction. Hence, we propose the following mechanisms were involved during abolishment of fetal hemoglobin production by p38 inhibitors. In normal cells, the phosphorylation of STAT3 on Ser727 is maintained by the activity of p38 MAPK at its basal level. Regarding the p38 MAPK has the property of

autophosphorylation, it ensures the presence of a small portion of activated p38 MAPK in cytoplasm. The activated p38 MAPK thus takes the responsibility to keep the STAT3 Ser727 phosphorylation at a certain level to ensure proper function of gene regulation. However, pre-treatment of inhibitors abolished the kinase function of p38 MAPK and failed in keeping the basal level of STAT3 Ser727 phosphorylation mediated by p38 MAPK. Previous research indicated that an increase of basal level of STAT3 Ser727 phosphorylation may be observed in p38 MAPK inhibitor-treated cells that was led by MKK6-induced activation of unidentified Ser/Thr kinase. However, it is noteworthy that the enhancement of STAT3 Ser727 phosphorylation level was not able to mediate its normal transcription-enhancing function (Xu et al. 2003). Hence, the maintenance of basal phosphorylation level on both Tyr705 and Ser727 sites is essential for the γ-globin gene transcriptional activity.

In general, our data support two alternative pathways for γ-globin gene activation as shown in Figure 11. The activation of JAK2 mediated by Cucurbitacin D is assumed to be elicited by membrane-bound receptors, probably members of GP130 family.(Taga et al. 1997; Xie et al. 1999) Activated JAK2 phosphorylates cytoplasmic STAT3 at Tyr705 site and triggers translocation of STAT3 into nucleus. Migrated STAT3 therefore binds to STAT3 consensus sequence located on the promoter region of γ, ε, δ, and β globin genes and initialize enhanced transcription activity, finally led to increased synthesis of globin products. The mechanism of p38 MAPK pathway initialization mediated by hydroxyurea remains unknown, probably through similar manners as sodium butyrate and trichostatin.(Park et al. 2001) Activated p38 MAPK cascade finally phosphorylates STAT3 at Ser727 site and leads to similar nuclear migration pattern and globin gene activation. Regarding that dual-phosphorylation of STAT3 is required for its translocation and binding to consensus sequences, the maintenance of JAK2 and p38 MAPK at basal level is crucial for γ-globin gene activation induced by Cucurbitacin D and hydroxyurea.

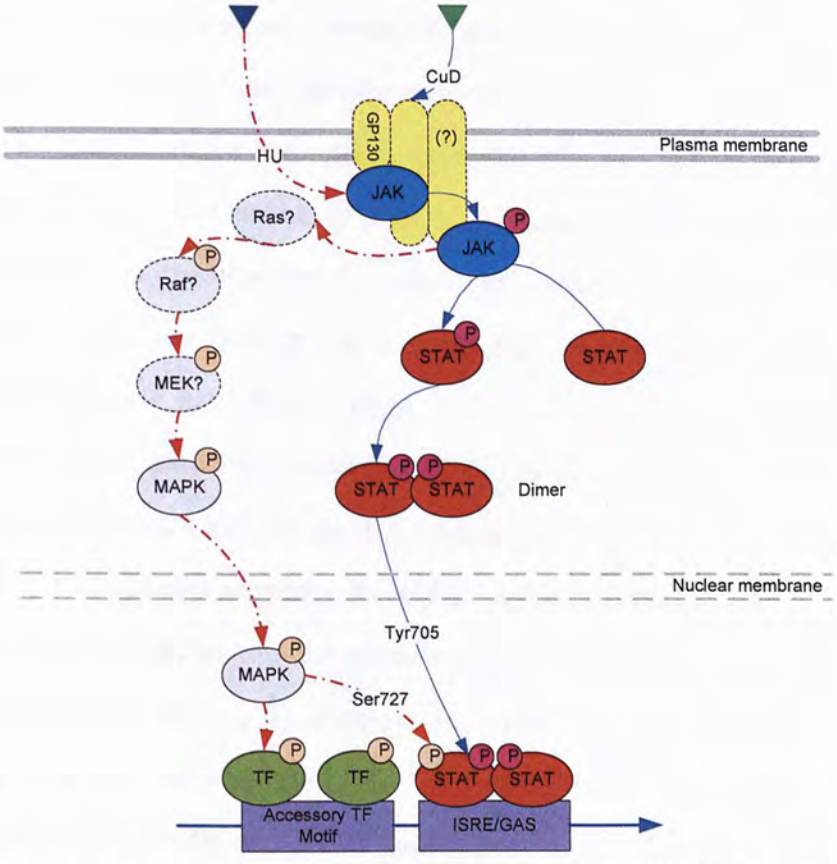


Figure 11. Proposed experimental model for the role of JAK2-STAT3 and p38 MAPK pathways in CuD and HU-induced γ-globin gene activation. Schematic chart is presented for the signal transduction events occurred in CuD and HU-induced γ-globin gene activation, and two alternative ways are shown to be responsible for the initialization of gene transcription. Hydroxyurea, which is represented by blue triangle, initializes the p38 MAPK cascade activation by activating JAK2 kinase. Activated p38 MAPK migrates into nucleus and phosphorylates transcription factors binding to either promoter region (STATs) or accessory TF motif thus promotes transcription activity. Cucurbitacin D, which is represented by green triangle, might interact with unknown membrane-bound receptors of GP130 family thus activate JAK2. Activated JAK2 phosphorylates STAT3 at Tyr705 site, causing its dimerization and translocation into nucleus. Phosphorylated STAT3 finally binds to promoter region of γ-globin gene and promotes transcription activity. Blue solid lines resemble the main γ-globin gene activation pathway mediated by Cucurbitacin D induction; red dotted lines resemble pathway mediated by hydroxyurea.

Further exploration of Cucurbitacin D from *Trichosanthes kirilowii* offered promising ideas on β-thalassemia treatment through gene switch protocol. Previous and ongoing research from our laboratory both indicated the Cucurbitacin D demonstrated to be more effective and reliable comparing the current γ-globin inducer hydroxyurea. The detailed mechanism of signal transduction during drug-induced γ-globin gene activation is essential for the functional analysis of γ-promoter function. Previous researches done by our lab revealed the relations between MAPK pathways and γ-globin gene activation. Here, we provided a more complete scheme of signal transduction pathways towards γ-globin gene activation, with two different starting signal molecules and one central transcription factor STAT3. With the discovery of STAT3 consensus sequence located in different globin promoter region, our research offered key insights into the regulation system of globin gene expression mechanism. Further investigation is required to reveal the detailed regulatory mechanism of transcription factors and their interaction with γ-promoters. Besides STAT3, members of GATA and NF-E family have been reported to play important roles in competition with STAT3 on γ-promoter region thus regulated transcription activity. Transient over-expression of GATA-1 in K562 cell line demonstrated stabilized transcription activity of γ-globin mRNA (Morceau et al. 2000). Mutations on GATA-1 binding site located on γ-promoter also showed hereditary persistence in altered fetal hemoglobin expression scheme in transgenic mice model (Liu et al. 2005). Hence, it would be profitable to explore whether CuD and hydroxyurea interacted with indicated transcription factors thus exerted their effects on γ-globin gene expression.

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