Biological and Pharmacological Studies of a Lead Compound that can Activate the Human Gamma Globin Expression

LIU, Shuk Ming

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Thesis/Assessment Committee

Prof. Peter C.K. Cheung	**(Chair)**
Prof. M. C. Fung	**(Thesis Supervisor)**
Prof. Y. S. Wong	**(Committee Member)**
Prof. N.K.Mak	**(External Examiner)**

STATEMENT

All the experimental study in the thesis was executed by author; otherwise it would be stated specially.

LIU Shuk Ming

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List of Abbreviations

Abbreviation	Description
Prog	Progesterone
ATF	Activating transcription factor
C 18-SPE	Carbon 18- solid phase extraction
C elegans	Caenorhabditis elegans
C.C.	Correlation coefficient
CaCl	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CdCl	Cadmium chloride
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
cGMP	Guanosine 3', 5'-cyclic mono-phosphate
CHOP	CCAAT/enhancer binding protein homologous protein
CREB	cAMP response element binding protein

CREBP	cAMP response element binding protein
Cu	Cucurbitacins
Dex	Dexamethasone
DNMT	DNA methyltransferase
DRED	Death related ced-3/NEDD2-like protein
dUTP	Deoxyuridine triphosphate
ED_{50}	Effective dose (50 %)
EIF2A	Eukaryotic initiation factor-2 alpha
EKLF	Erythroid Kruppel-like factor
ELK1	Member of ets oncogene family
ER	Endoplasmic reticulum
ERK	ExtracellularRegulated Kinase
ETOH	Ethanol
FDA	Food and Drug Administration
GADD	Growth arrest- and DNA damage-inducible gene
GADD 34	Growth arrest and DNA damage-inducible gene 34
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA	Globin transcription factor 1

GCN2	General control nonderepressible-2
	EIF2A kinase 4
Grb2	Growth factor receptor-bound protein 2
GRIM-19	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13
H3 & H4	Histone 3 & Histone 4
Hb A	Hemoglobin F
Hb F	Hemoglobin A
HDAC	Histone deacetylase
HDAC	Histone deacetylase
HPLC	High-pressure liquid chromatography
HRI	heme-regulated inhibitor
	EIF2A kinase 1
HU	Hydroxyurea
HV	Human, in vivo
Hydro	Hydrocortisone
IC_{50}	Inhibitory concentration 50 %
IMiD	Immunomodulatory drugs
JAK	Janus Kinase
KCl	Potassium chloride

KGM	Keratinocyte Growth Medium
KPO_4	Potassium Phosphate
LB	Lysogeny broth
LCR	Locus control region
MAPK	Mitogen-activated protein kinase
MAX	MYCassociated factor X
MEF2	MADS box transcription enhancer factor 2
MEK	Mitogen-activated protein kinase kinase
$MgSO_4$	Magnesium Sulfate
Mife	Mifepristone
MKK	Mitogen-activated protein kinase kinase
mRNA	Messenger RNA
MSK1	Mitogen and stress-activated protein kinase 1
mTOR	Mammalian target of rapamycin
MYC	v-MYC avian myelocytomatosis viral homolog
NaCl	Sodium chloride
NaOAc	Sodium Acetate
NO	Nitric oxide
NR Desorb	Neutral red desorption

NRF2	nuclear factor erythroid 2-like 2
PCR	Polymerase chain reaction
PERK	RNA dependent protein kinase-like ER kinase EIF2A kinase 3
PERK	RNA dependent protein kinase-like
PIAS	Protein inhibitor of activated STAT, 3
PKA	Protein kinase, cAMP-dependent, regulatory, type I a
PKR	RNA-dependent protein kinase (EIF2A kinase 2)
PKR	RNA-dependent protein
PYR complex	SWI/SNF-related protein complex
RAF	V-raf-1 murine leukemia viral oncogene homolog
ROS	Reactive oxygen species
ROS	Reactive oxygen species.
SCFA	Short chain fatty acid
sGC	Soluble guanyl cyclase
SHP2	Protein tyrosine phosphatase, non-receptor type 11
SLS	Sodium Lauryl Sulfate
SOCs	Suppressor of cytokine signaling

SSP	Spongiotrophoblast specific protein
STAT	Signal Transducer and Activator of Transcription
TGF-b	Tumor promoting graft factor-beta
TMB	Tetramethybenzidine
UDP	Uridine diphosphate
UV	Ultraviolet radiation.
UVA	Ultraviolet A
v/v	Volume/volume
w/v	Weight/volume

ABSTRACT

Thalassemia is a global disease. It was report in 2001 that there were 270 million people who carried the severe disease. Most of the cases were found in Africa and south-east Asia. China has a high incidence rate of 0.66% in 2001. In the past, the treatments of the disease were blood transfusion and bone marrow transplantation. However, many defects in such kinds of treatments were reported. The balance of relieving the syndrome of the disease and the adverse effects of the drugs was the consideration to the physician. The drug, hydroxyurea, can activate the gamma globin gene and produce hemoglobin F to replace the beta globin as an oxygen transporter is considered as an better treatment to ameliorate the syndrome. Safety and effectiveness in the long-term treatment using hydroxyurea are questionable. Cucurbatacin D purified from a Chinese herb demonstrates 2000 folds more potent than hydroxyurea. It can activate the gamma globin gene and produce hemoglobin F shown in ELISA and confocal microscopy. The fundamental work for drug development is carrying out through this project. In this project the biological property and toxicity were studied.

Different cucurbitacin derivatives have been compared for the gamma globin induction potential. Cucurbitacin D turned out to be the most potential inducer among the derivatives had been tested. Later I had screened more herbs for the gamma globin induction activities.

One of the herbs showed a higher activity than *Fructus Trichosanthis*, which could be the potential candidate to isolate more potent inducer. In the toxicity study, cucurbitacin D only have a mild toxic effect on the normal cell lines and transgenic mice. Finally, the efficacy of cucurbitacin D was tested on a sickle cell anemia mouse model and demonstrated a significant induction of fetal haemoglobin production. Cucurbitacin D may be a potential drug candidate for treating beta globinopathies.

摘要

地中海貧血是一個全球性的疾病，根據 2001 年的調查顯示，全球大約 27 億患者，大部分是居於非洲和東南亞人士，其中中國也是高患率的地區，發病率為總人口的 0.66%。此前治療此疾病會選用大量輸血、骨髓移殖，惟依臨床考察，此等治療方式有著不少副作用。

如果平衡舒緩病情和減輕治療的副作用，是醫療發展的首要考量。羥基脲 (Hydroxyurea) 是能夠活化珈瑪血紅蛋白基因，而增加胎兒血來代替缺血性的地中海貧血。長期使用羥基脲 (Hydroxyurea) 增加珈瑪血紅蛋白，來傳送氧氣的治療方案，其安全和有效性，仍然受到質疑。因此，業界嘗試在葫蘆科植物中提取葫蘆素 D (cucurbitacin D)，此素材的藥效被證實 2000 倍高於羥基脲 (Hydroxyurea)。同樣地，在單株抗體來偵測珈瑪血紅蛋 的 ELISA 和共焦顯影方法中，證實葫蘆素 D (cucurbitacin D)也有活化珈瑪血紅蛋白的功能。是故，本計劃旨在發展葫蘆素 D 的藥效和毒性基礎研究。

在葫蘆素(cucurbitane)群組中，其成員大部份均能活化珈瑪血紅蛋白基因，尤以葫蘆素 B 及 D(cucurbitacin B & D)的藥效最為顯著。在瓜蔞(*Fructus Trichosanthis*)植物中提取的葫蘆素 D(cucurbitacin D)，已在細胞及線蟲的毒性測試中，證實其毒性較為溫和。本計劃更將以患有鐮刀型貧血的老鼠模型，來測試葫蘆素 D(cucurbitacin D)的藥效。實驗證明葫蘆素 D(cucurbitacin D)，是一種極有潛力治療血病的藥物。

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CHAPTER 1: GENERAL INTRODUCTION

1.1. Medicinal herb of ancient world and hemoglobinopathy

There were several thousands of year's experiences in use of herbal medicine. The first medical material in Chinese history was *Yellow Emperor's Inner Canon*(黃帝內經). The historian estimated the book appear before Han(漢) dynasty 2000 years. During Han (漢)dynasty , the famous edition *Classic of Moxibustion and Acupuncture Preserved in a Pillow* by Huà Tuó (華佗).Toward to middle age, another advance material medical book documented the over eight hundreds material. It documented 833 medicinal substances taken from stones, minerals, metals, plants, herbs, animals, vegetables, fruits, and cereal crops. (*Compendium of Materia Medica*) (本草綱目) by Lǐ Shízhēn(李時珍), the most complete and comprehensive pre-modern herbal book (completed in 1578).

Taoism and Buddhism became as several main streams thinking though out in Chinese culture. It influenced the living style of Chinese in every level. The basic idea of ancient Chinese medicine was also impressed by Taoist and Buddhist thought. Traditional Chinese medicine regarded the body a whole set of system. It is composed of five elements. They were metal, wood, water, fire and earth. They interact with each other. The well balance of each element was the major concern in health body. The weights of the elements are always influenced by environment, weather, emotion and inner condition. The change of element shift to extreme amount, the unbalance condition would cause the diseases. Chinese used the herbal medicine to correct the internal unbalance. Single herb is not always to be prescribed. They formula the herbs were applied to each special condition of the patient.

In western, the herbal medicine is always considered as alternative medical system or protoscience, It was because lack of scientific data to support the pharmacological reaction. Their efficacy and safety were controversy over the western world. Standardization the quality of herbal products was essential for ensure efficacy and consumer safety.

However, development of traditional Chinese medicine was lack of standardization. The herbs were collected from different source, production place, each single herb contained abundance of compounds. Even the same herb grew on different places. It affected the ingredient of the compounds. Traditional Chinese medicine would use the special preservative to make medicine. Heavy metal was the one to prepare the herbal medicine, such as arsenic trioxide (三氧化二砷) and cinnabar (called zhūshā, 朱砂). But the chemicals were toxic and carcinogenic. Thus, traditional Chinese medicine (TCM) might be controlled strictly.

Another problem was the serious contamination of river and soil. The plants absorb many contaminants. It also caused the toxic and carcinogenic compound level rise in the herbal medicine. The people suggested the Good Agricultural Practice (GAP) system would be posed on the planting the herbs. Moreover, identification method for the herbs was not standardizing and reliable. Most recently, the Atlas of Infrared (IR) Spectrum was introduced to identify the different species of Ginsengs (Yap *et al.*, 2007) and also their active ingredients. IR spectrum fingerprint authenticated the composition of ginseng and replaced only morphological identification. The classification of the species were more sophisticated and standardized. It could avoid many morphological fakes. The advantages of scientific methods were applied on industry of tradition Chinese medicine which was significant. Not only for the precious identification, but also rapid and efficiency extraction, the last was high

quality and low cost products. Thus the topic of this study concerned about the biological and pharmacological aspect of an ancient Chinese herb by scientific approach.

There were many documented herbal medicine for increasing the blood level or activating the blood circulation, thought the mechanism was unknown, the efficacy of the medicines were proven by the long period of usage. However an herb contained a wide range of components, the abundant of compound exerted many pharmacological effects. The purification and isolation the specific compound was important for studying the mechanism of the single compound. Cucurbitacin was extracted from *Trichosanthes rosthornii Harms.* The compound exhibited the inducing hemoglobin effect in k562 cell model.

1.2. Thalassemia

Thalassemia is most common blood disease in the world, *Thalassa (θάλασσα)*is Greek. It means sea. *Haema (αἷμα)* means blood. Thalassemia that word indicated the disease geographical spreading. The most common found in Mediterranean region, Africa, and Southeast Asia .i according the data of statistical of Malaysia, it is 40 % of population, and they possess the defective globin gene. China has the high incidence rate 0.66%. Thalasselamia was also a nature protection from malaria. Thalassemia trait-carrier was benefit for natural selection and survival.

Thalassemia syndromes result from inherited defective globin genes, which cause either a total or partial reduction in the hemoglobin chain synthesis. Point mutation or DNA sequence mutation impaired the globin chain synthesis. Unbalance hemoglobin chain develops very severe anemia. The liver and spleen are greatly enlarged, and

cardiomegaly results from severe anemia. Blood transfusion is a common practice in daily life. Because there is no physiologic mechanism for excretion of iron, after 5 to 10 years regular transfusion, the large amount of iron accumulate in the tissue (hemosiderosis), which results in progressive organ dysfunction that is lethal without iron chelating therapy. The endocrine system is aspectially vulnerable to iron damage, growth retardation, diabetes mellitus, hypothyroidism, and hypoparathyroidism are common complicate. In many thalassemia major cases, bone marrow transplantation is most feasible treatment. However the compatible marrow cell do not always available and the patient will suffer from the high-risk operation. In many pathological studies in hemoglobinopathies, many clients who suffer from very severe anemia, they have slightly increasing hemoglobin F than the normal. It leaves a hint to further development in the treatment of the disease. There was a high sound to yield the progression of treatment for the hemoglobinopathies.

1.3. Sickle cell anemia

Sickle cell anemia was high prevalent of human genetic disease. The genetic defect was a glutamate to a valine substitution at position 6 of β globin chain. Formation of hemoglobin tetramer, it become a hemoglobin S ($\alpha_2\beta^s{}_2$). The polymer of hemoglobin S could not reverse when the hemoglobin was oxygenated and deoxygenated. The hemoglobin structure was alter .It was crytstalize and precipitated in cytosol of RBC. When the RBC passed thought the R.E. system in human body, the cell structure was injured. The damaged RBC stimulated the immune and coagulation response. It occluded the micro-circulation. It leaded to an organ damaging. Hemolytic anemia, chest plain, cardio- ischemia, infarction, marrow expansion, jaundice and gallstone were manifested. More shorten RBC life cycle made the anemia serious (15 to 2 days vs. 100 to 120 days).

1.4. The treatment development of sickle cell anemia and thalassemia

The clinical feature of two diseases: polymerization of hemoglobin S and hemoglobin imbalance. First suggestion was bone-marrow transplantation, the high survival rate >70% was, but not feasible. Due to the compatible bone-marrow was not easy to find out. Second suggestion was transfer of β gene into hematopoietic progenitor cell.

There was a big difficult technical problem. Though it was an approved protocol (Bank *et al.*, 2005) applied to patient in 2007, it existed some potential risks. The risks came from the insertion mutation or the danger of inserted vector. The third suggestion was pharmacologic induction of fetal hemoglobin. The feasible method was base on some observations and rationales. The researches studied the patient who carried the β-hemoglobinopathies and mutation of γ-globin. (hereditay persistence of fetal hemoglobin/HPFH). The patients had the higher level of fetal hemoglobin than normal. The patient had the more healthy physical condition than the one who carried the β-globin defect only. They had less severe clinical symptoms. For the thalassemia case, the γ-globin expression would reduce the excess globin chain. For the sickle cell anemia, the γ-globin expression would inhibit the hemoglobin S polymerization. Many drugs were developed to induce the fetal hemoglobin from 1980s.The table showed below as a spectrum of fetal hemoglobin inducers which were discovered within the years.

1.4.1. Agents that induce γ-globin gene expression and/or fetal hemoglobin production

Table 1-1:

The table sourced from journal *Experimental Hematology* 2008 36: 1057- 1072 page 1061

DNA methyltransferase inhibitors (n= 4)		
5-Azacytidine	c, m, p, hc, hv	DeSimone et al. 1982 Ley et al. 1982
Decitabine hv	c, m, p, hc,	Sauntharajah et al. 2003
5,6 dihydro-5-Azacytidine	hv	Carr et al. 1987
S110 p,	hc	Lavelle et al. 2007
Cytotoxic agents (n =23)		
DNA alkylators		
Busulfan	p, hv	Liu et al. 1990
Cisplatin	c	Bianchi et al. 2000
Streptozotosin	c	Lyamu et al. 2000
Nucleoside analogue		
Cytosine arabinoside	c, p, hc, hv	Veith et al. 1985
Inosine monophosphate dehydrogenase inhibitors		
Ribavirin	c	Yu et al. 1989
Mycophenolic acid	c	Yu et al. 1989
Tiazofulin	c	Yu et al. 1989
Ribonucleotide reductase inhibitors		
Didox	c, m	Lyamu et al. 2000 Pace et al. 1994
Hydroxyurea	c, m, hv p,hc	Charache *et al.*,1995
Resveratrol	hc	Rodrigue et al. 2001
Trimidox	c	Lyamu et al. 2000

Agents that induce γ-globin gene expression and/or fetal hemoglobin production

DNA intercalating agents		
Aclarubicin	c	Delgado-Canedo et al. 2005
Chromomycin	c	Bianchi et al. 1999
Distamycin	c	Bianchi et al. 2001
Doxorubicin	c	Delgado-Canedo et al. 2005
Mithramycin	c, hv	Bianchi et al. 1999
Tallimustin	c	Bianchi et al. 2001
Psoralens + UVA irradiation		
Angelecin	c, hc	Lampronti et al. 2003
5-Methoxypsoralen	c, hc	Viola et al. 2008
Trimethyl angelicin	c, hc	Lampronti et al. 2003
Dihydrofolate reductase inhibitor		
Methotrexate	p	Veith et al. 1989
Microtubule inhibitor		
Vinblastine	p	Veith et al. 1985 Letvin et al. 1985
Protein synthesis inhibitor		
Anisomycin	c	Pace et al. 2003
Hormonal agents (n = 2)		
Nomegestrol	hv	Nascimento Mde et al. 1998
Progesterone	hv	da Silva Santos Duarte et al. 2002

(to be continued)

Short chain --fatty acids and derivatives (n =25)		
Butyrate	c, m, p, hc, hv	Atweh et al. 1999
Phenyl butyrate	c, hc, hv	Collins et al. 1995
a-aminobutyric acid	c, p, hc	Constantoulakis et al. 1988
di-methylbutyric acid	c, m	Pace et al. 2002
Tributyrin	c	Witt et al. 2000
Acetate	c, m, p, hc	Stamatoyannopoulos et al. 1994
Phenylacetate	c, hv	Fibach et al. 1993
Phenoxyacetic acid	c, m	Torkelson et al. 1996
Butyryl-hydroxamate	c, m, hc	Cao et al. 2005
a-Methylhydrocinnamic acid	c, m	Torkelson et al. 1996
Caproate	c	Safaya S et al. 1994
Heptanoic acid	hc	Liakopoulou et al. 1995
Hexanoic acid	hc	Liakopoulou et al. 1995
Isobutyramide	m, p, hc, hv	Domenica et al. 2000
Nonanoic acic	hc	Liakopoulou et al. 1995
Octanoic acid	hc	Liakopoulou et al. 1995
Pentanoic acid	p, hc	Liakopoulou et al. 1995
Propionic acid	p, hc	Liakopoulou et al. 1995
Dimethoxyphenyl propionic acid	c	Pace et al. 2002
Propional hydroxamate	c, m, hc	Skarpidi et al. 2003
RB7	c, hc	Mankidy et al. 2006
RB4, RB9, RB29	c	Bohace et al. 2006
Valproic acid and derivatives	c, hc, hv	Kieslich et al. 2003 Collins et al. 2004

Agents that induce γ-globin gene expression and/or fetal hemoglobin production

Histone deacetylase inhibitors (n =11)		
Apicidin	c, hc	Wei et al. 2007 Witt et al. 2003
Compounds "24" and "29"	c, hc	Mai et al. 2007
FK228	c, hc	Cao et al. 2006
Helminthsporium toxin	c, hc	McCaffrey et al. 1997
MS-275	c, hc	Cao et al. 2006
SAHA (Vorinostat)	c, hc	Skarpidi et al. 2003 Witt et al. 2003
SBHA	c, hc	Skarpidi et al. 2003
Scriptaid	c, hc	Cao et al. 2004 Johnsonet al. 2005
Trapoxin	c, hc	McCaffrey et al. 1997 Cao et al. 2004
Trichostatin A	c, hc	McCaffrey et al. 1997
Imunomodulatory drugs (n = 3)		
Thalidomide	hc	Aerbajinai et al. 2007
Revlimid	hc	Moulouh-de Parseval et al. 2008
Pomalidomide	hc	Moulouh-de Parseval et al. 2008

(to be continued)

Agents that induce γ-globin gene expression and/or fetal hemoglobin production

Miscellaneous (n = 4)		
Zileuton (5-lipoxygenase inhibitor)	c	Haynes et al. 2004
Vanadate (phophatase inhibitor)	hc	Amoyal et al. 2007
FG-2216 (HIF-prolyl hydroxylase inhib)	p, hc	Hsieh et al. 2007
CysNO (nitric oxide donor)	c, hc	Cokic et al. 2003
mTOR inhibitors (n = 2)		
Rapamycin	c, hc	Fibach et al. 2006 Mischiati et al. 2004
Everolimus	c, hc	Zuccato et al. 2007
Cytokines (n = 3)		
Erythropoietin	c, m, p, hc, hv	Stamatoyannopoulos et al. 1990
Stem cell factor	p, hc	Miller et al. 1992 Wojda et al. 2003 Gabbianelli et al. 2008
TGF-b	hc	Bohmeret al. 2003

Selected references are included.
c = immortalized erythroid cell line
hc = human, primary cell culture
hv = human, in vivo
m= murine model
mTOR= mammalian target of rapamycin
p = nonhuman primate, in vivo
TGF-b = tumor promoting graft factor-beta
UVA = ultraviolet A.

1.4.2. DNA methyltransferase inhibitor—5-Azacytidine

A remarkable medical achievement was quietly reported in the New England Journal of medicine on 9 December 1982. Ley and his co-worker (1982) treated 3 patients with beta- thalassemia (Ley *et al.*, 1982) and 2 patients with homologus sickle cell anemia with a single drug, 5azacytidine (S Charache *et al.*, 1983),that was the first agent to be used for the hemoglobinopathy. The drug was a hypomethylating agent. When the silencing mechanism was activated on a specify gene, the methyl groups was attached to cytosine residue, “CpG” site by methlytransferase. The gene was methylated which could not be transcribed. By observation, the fetus erythroid was changed to adult. The gamma globin promoter was being hypermethylate in $CD34^{+}$ cells. The fetal progenitor became less methylated in the progress of differentiation in erythroblast under the treatment. (Singh *et al.*, 2007). However, the carcinogenic potential was observed in clinical trial. So the safer derivative was developed Decitabine (5-aza-2’-deoxycytidine).

1.4.3. Histone deacetylase (HDAC) inhibitor

In the non-expression condition, the chromatin was packed around the histone. Altering the chromatin structure by acetylation or deacetylation of histone tails affect activation or repression of the gene. (Kuo & Allis, 1998). Histone deacetylase (HDAC) worked on the histone tail and caused the chromatin condensation. The inhibitor of HDAC bind a central zinc atom in HDAC lead the hyperacetylation of H3 and H4, the conformation change of the chromatin and let it open to the transcription factor. Or hyperacetylation of ε amino groups of lysine molecule of histone reduced the histone and chromatin reaction. (Cao, 2004) The hemoglobin switching from fetus stage to

adult stage, it was dependence of the acetylation level of the specific hemoglobin gene. (Yin *et al.*, 2007) Sodium butyrate, adipicin(Witt *et al.*, 2003), acriptaid(Cao, 2004) and trichosatin A (TSA) (Cao *et al.*, 2004) (Pace *et al.*, 2003) activated the fetal hemoglobin by the mechanism.

1.4.4. Short-chain fatty acids (SCFADs)—Buytrate

Short –chain fatty acids and their derivatives induce the fetal hemoglobin. (Boosalis *et al.*, 2001) They induce the fetal hemoglobin via Stat-5 cell signaling. It prolonged the expression of c-myb and c-myc. Short and prolonged the stat-5 phosphorylation and activation. Butyrate and short- chain fatty acid chain increased fetal hemoglobin expression by duplicated CCAAT box sequence, upstream the transcription site. Alteration the DNA- binding protein on proximal γ-globin promoter would induce gamma globin expression. (Ikut *et al.*, 1998). Butyrate was also a inhibitor of histone deacetylase (HDAC) via p38 MARK cell signaling (Pace *et al.*, 2003) some candidate transcriptional factors were the down stream of p38 MARK cell signaling. They might be the affect of gamma goblin up-regulation. In practical clinical trial, butyrate showed a promising result and low toxicity. The fetal hemoglobin increased rapidly under butyrate exposure. However, it did not show a sustainable up-regulation fetal hemoglobin level in long term user. (Atweh *et al.*, 1999). High dosage and short half-life of butyrate for stimulate the fetal hemoglobin was another limitation. 20g/day administrate to the patient. Tackle the different problem; chemist improved the Arginine butyrate to sodium phenylbutyrate and isobutyramide to achieve the sustainable stimulation of fetal hemoglobin in different hemoglobinpathies.

1.4.5. Ribonucleotide reductase inhibitor---Hydroxyurea (HU)

Hydroxyurea (HU) was famous drug for myeloproliferative disorders over twenty years. The drug mechanism was inhibition of DNA synthesis through inhibition of ribonucleoside diphosphate reductase activities. The enzyme catalyzed the reaction from the UDP (uridine diphosphate) or ribonucleotide to dUTP (deoxyuridine triphosphate), the precursor of DNA. When HU diffused into cell, it became a free radical nitroxide. It quenched with tyrosyl-free radical of R2 subunit of the enzyme, the active site of enzyme. The action inhibited the enzyme activities. HU also showed the inducing fetal hemoglobin effect. Most of studies suggested the stimulation effect through guanosine 3', 5'-cyclic mono-phosphate. (cGMP) signaling pathway. The soluble guanyl cyclase (sGC) reacted with cGMP pathway to produce nitric oxide (NO) in $CD34^+$ human progenitor cell.(Ikuta *et al.*, 2001), (Cokic *et al.*, 2003) In the sickle cell anemia patient's blood was demonstrated the same phenomenon, the NO and cGMP level increased. (Nahavandi *et al.*, 2002)(Conran *et al.*, 2004) The HU could react with doexy-heme by nitroxide pathway (Cpkic *et al.*, 2008)

Most recent study, HU induced fetal hemoglobin via c GMP and cAMP signaling pathway. The former happened in post-transcription level, the latter in transcription level. Keefer *et al.*, 2006)(Baily *et al.*, 2006). HU induced small GTP-binding protein and RAS-related (SAR) protein. Those proteins caused the apoptosis and G1/S phase arrested by decreased thePI3K and ERK phosphorylation and induced the p21 along with GATA-2 expression. (Tang *et al.*, 2007)

Since HU showed the inducing effect of fetal hemoglobin, many of clinical trials were studies. The result seems to be promising; the clinical trial proved HU could reduce the painful crises and episode of acute chest syndrome, transfusion

requirement and hospitalization (Charache *et al*., 1999) (Steinberg *et al*., 1997) (Steinberg *et al*., 2003) the total quality of life was improved. (De Baun *et al*., 2007) if the patient fetal hemoglobin was increased 4% to 20 %, the crises reduced 68% to 84 %(Ballas *et al*., 2006), hospital admission decreased by 18% to 32 %(Lanzkron *et al*., 2008) HU treatment in children studies, the sustainable fetal hemoglobin inducing which improved growth and splenic function.(Kenncy *et al*., 1999) (Haskins *et al*., 2006) Many of improvement increased the survival rate ultimately. (Platt, 2008) However there was about 50% of the case who were non-responders for the HU treatment. Some of the studies reported that single nucleotide polymorphisms (SNP) within the chromosome 6q22.3- 23.2 and 8q11-q12 correlated between HU treatment and gamma globin expression. (Platt, 2008) A current study, the SNPs appeared in promoter of SAR gene which affected the sickle cell anemia patient response to HU. (Ma *et al*., 2007) Some studies reported the patients on treatment up to 6 -8 years that would develop leukemia. Comet assay revealed the reason of the leukemia. Sickle cell patient received HU therapy, which would cause the DNA damage. The damage extent was correlated to the dosage. (Kunrkhaek *et al*., 2008) The reponse of thalassemia patient to hydroxyurea was studied extensively. The moderate fetal hemoglobin increased by hydroxyurea treatment. The dosage range was 7mg/kg/day to 20 mg/kg/day. About 50 % of thalassemia patient showed no response. (Singer *et al*., 2005) The indicator between responder or non-responder was the pre-treatment fetal hemoglobin level. If the higher HbF baseline, there was the better response of HU therapy. Some researches discovered the HU treatment was significant benefit for specific type of β- thalassemia major and intermediate. It was due to the SNPs in β-globin locus.(Bradai *et al*., 2003) (Yavarian *et al*., 2004) The SNPs in HBG2-158 allele affected the HU response. The β- thalassemia intermedia

patients showed the β-globin level increasing, but not the γ-globin under the HU exposure. (Zeng *et al.*, 1995)

1.4.6. The lead compound--Cucurbitacins (Cu)

In many traditional Chinese medicine references, many Chinese herbs were reported to have the stimulation of hemoglobin production. Although we did not know about the inducing mechanism, the Chinese used them for a very long time. Another observation was the gene switching mechanism in many drugs for increasing the hemoglobin production. Based on these two incidences, we suggested some of the Chinese herbs could induce the hemoglobin by gene switching mechanism. Under Prof. Fung's supervision, Dr Xing screened many type of Chinese herbs and discovered a very potency ingredient for inducing fetal hemoglobin, cucurbitacin D, in *Trichosanthes rosthornii Harms* or *Trichosanthes japonica Regal*. The method for isolation, purification the lead compound was applied a patent in USA.
Patent No.:7618657. Natural cucurbitacin was extracted from plant. It constituted a group of highly diverse side chain and oxygenated tetra cyclic triterpenoids. It was divided into glycosylated and non-glycosylated groups. It was well known of their toxicity and bitterness. However, 1980s, the chemists collected cucurbitane glucoside from *Siraitia grosvenori*. It named siamenoside I which exhibited profound sweetness. (Kasai *et al.*, 1989) Cucurbitacin was majorly found in from cucurbitaceae. There are about extant genera in cucurbitaceae, including 960 species.
(Achenbach *et al.*, 1993)Most recently, cucurbitacin had been isolated from several genera of mushroom and shell less marine mollusks. (Kanchanapoom *et al.*, 2002) Cucurbitacin had 12 categories which include A, B, C, D, E, F, (G, H), (I, L), (J, K), (O, P, Q), (R, S, T) and the miscellaneous. The thesis studied B, D, E & I subtype of

cucurbitacins. Since 1960's cucurbitacin was a popular topic for studying its medicinal uses and drug discovery, especially its anti-cancer effect and toxicity behavior. Due to the cytotoxicity effect of Cu, the usages of Cu were controlled by medical restriction strictly. There were many pharmacological effects of Cus.

Cu exhibited as putative, anti-inflammatory, anti-fertility, anti-microbial, anthelmintic, chemopreventive, hepatoprotective effects, moreover, Cus also showed the effects on CNS, cardiovascular and hemoglobin production. (Chen *et al.*, 2005)

Signal Transducer and Activator of Transcription (STAT)

STAT proteins were signal transducers which transduction the extracellular signal from cytokine or growth factor, phosphorylated STAT protein translocated to cell nucleus and bind DNA at STAT-specific binding site. STAT protein regulated the normal cell proliferation, differentiation, survival, apoptosis, mediated the immune response.(Stark *et al.*, 1998)(Horvath *et al.*, 1997) (Ihle *et al.*, 1995)STAT protein was negatively regulated by SOCs, PIAS, proteins, SHP phosphatases, Grb2 and GRIM-19.(Zhang *et al.*, 2003) (Wormald *et al.*, 2004)However, recently STAT3 and STAT5 were reported activate the tumor cell proliferation and anti-apopotosis.(Turkson *et al.*, 2000) (Bowman *et al.*, 1999)Cucurbitacin was a powerful STAT3 inhibitor, the down regulate of STAT3 would be lower the phosphotyrosine level,and inhibit tyrosine kinase activities and activated phosphotyrosine phosphatase which in turn down regulated STAT3, and SHP-2. It affected the MAPK pathway and inhibited ERK reaction. The pathways control the cell proliferation and survival.

Inhibition of STAT3 leaded to death of tumor cell via Janus Kinase (JAK) -STAT pathway, RAF/MEK/ERK ExtracellularRegulated Kinase pathway.

(Turkson *et al.*, 2001) Cucurbitacin was reported to bind to glucocorticosteroid

receptor which altered the prostaglandin and adrenocorticosteroid synthesis and induced the cell morphological changing. (Duncan *et al.*, 1996)

Cucurbitacins and some chemotherapeutic agents, such as doxorubicin and gemcitabine were notified to have the synergistic anti-tumor effect. (Gariboldi *et al.*, 2003) (Gabriela *et al.*, 2010)

2004, a study of 23, 24-dihydrocucurbitacin D was exhibited the anti-inflammation effect. Cu D inhibited nitric oxide (NO) generation and blocking NF-KB activiation, then the condition turn on iNOS gene transcriptionion. (Park *et al.*, 2005)

However, there was a study which reported cucurbitacin D and E to interact with serum albumin. It might be altered the pharmacokinetic profile of cucurbitacin.

1.5. Fetal hemoglobin production mechanism—switching model or normal expression?

1.5.1. Hemoglobin switching model

In the embryonic life, the major hemoglobin transiently expressed was Gower I Gower II and Portland hemoglobin. The stage will held until 24 weeks before birth. Then the embryonic hemoglobin turned off. The next developed hemoglobin was mainly fetal hemoglobin, which included both $\alpha_2\gamma_2^G$ and $\alpha_2\gamma_2^A$. Meanwhile the β gene started to express. 24 weeks after birth, hemoglobin will shift to hemoglobin A which is a tetramer $\alpha_2\beta_2$. A minor type in this stage is hemoglobin A_2 which was $\alpha_2\delta_2$

The δ gene had a number of alterations in its promoter, particularly in the CCAAT box region, which renders it relatively inefficient in globin production. For that reason, the amount of δ mRNA was considerably lower than β mRNA. The hemoglobin concentration of normal adult was approximately 97.5% hemoglobin A, about 2% of hemoglobin A_2, and about 0.5% hemoglobin F.

Gene switch model

The silencing and activated mechanism on hemoglobin expression was through out from embryo to adult. What was the mechanism to control the gene turn on or turn off gene's competition

The first observation was the development control happened in the gene linked together. If the gene was separated, the development control would lose. The experiment was done on the transgenic mice; they carried either the β or γ gene or both of them linked together. The research suggested that the β gene competed with the γ gene to react with LCR (Enver et al. 1990)

1.5.1.1 Cis -element

Positive regulatory element –LCR (locus control region)

The hypothesis was the distance advantage. The length of from LCR to β gene about 25 Kb, it could allow the LCR to form an ach and reacted with each globin gene, under the condition, the gene order and their nearness to LCR determined the order of gene activation order. The nearest gene to LCR was ε, and then was γ, the last was δ & β globin gene. Thus the in the embryonic stage the hemoglobin were Gower1 (ζ2ε2), Gower 2 (α2ε2) and Portland(ζ2γ2), the fetus hemoglobin was switched to $\alpha_2\gamma_2^G$ and $\alpha_2\gamma_2^A$, after birth the hemoglobin was switched to $A(\alpha_2\beta_2)$ & A_2(α2δ2).

Negative regulatory element

One globin gene was activated; the previous globin gene had to be silence. The negative regulatory element was located upstream the promoter between -182 and -467 from initiation site. The experiment was done by Cao's laboratory (Cao *et al.*1989). Transfection assay in the cultured cell showed the event. The length of the element included three transcription factors binding motifs, GATA site at -208, YY1 site at -269 and CACCC site at -379 (Raich *et al.*1995). If removal of that

Region, the embryonic hemoglobin would express through adult stage in a transgenic mouse model. ((Raich *et al.*1992).

1.5.1.2 Trans-element

When the human gamma globin switched to beta globin , the β ach formation depended on the complex of transcription factors, the PYR complex, the other chromatin remodeling complexes and transcription factors interaction leaded the chromatin conformational change. Most recently, the deletion of PYR complex binding site IK-/- in a transgenic mice which carried human γ and β gene. There was no PYR complex activity. It showed the delayed γ to β gene switching. (O'Neill *et al.*2000)

In the fetal stage, the γ globin reacted with βLCR which associated with fetal stage and erythroid specific transcription factors. Moreover, it clustered with embryonic-fetal stage specific chromatin complex and also with SSP, FKLF, NF-E4, factors and repression of DRED. Those entire protein complex showed up-regulated fetal hemoglobin. (Zhou *et al.*2004)(Holmes *et al.*1999).

1.5.2. Post-transcriptional event was a factor to determine the level of fetal hemoglobin

One observation was the patient who carried the heterozygous Corfu deletion , HbA presented, but less HbF accumulated. But the mRNA level of HbF of the case remained unchanged. The fetal hemoglobin level reduced, fetal hemoglobin mRNA level preserved. It indicated the Post-transcriptional event affected the protein amount. (Chakalova *et al.*2005)

1.5.3. Extending the normal expression of gamma globin

Some scientists suggested that the drugs induced fetal hemoglobin by reverse the developmental gene switching. However, there was an experiment mention another point of view of mechanism of fetal hemoglobin induction. 5-Aza treated the human $CD34^+$ progenitor for 20 days, examined the fetal globin mRNA and the beta globin mRNA level change within the cell differentiation. The data compared with the untreated sample. After the treatment, the gamma globin was not only increasing the amount, but also prolonged their production period. (Ring *et al.*1998) Moreover, in adult blood, there was < 1% fetal hemoglobin. Events showed the fetal hemoglobin did not turn off after birth. Thus, the mechanism of drug reaction was extending the normal expression period.

1.5.4. Fetal hemoglobin production mechanism—A cell stress signaling model

There were many compounds to be reported as a fetal hemoglobin inducer. A part of their signaling pathways were worked out. The compounds and part of the pathway were listed out in the table:

The table sourced from journal *Experimental Hematology* 2008 36: 1057- 1072, page 1064

Intracellular signaling pathways implicated fetal-globin gene and /or fetal hemoglobin induction

Table 1-2 :

Agent	Class	Implicated pathways	System	References
5-Azacytidine	DNMT inhibitor	cAMP	hc	Keefer et al. 2006
Hydroxyurea	Cytotoxic	cAMP	hc	Keefer et al. 2006
		NO/cGMP	c, hc	Cokic et al. 2008 Cokic et al. 2003
		P38 MAPK	c	Park et al. 2001
Anisomycin	Cytotoxic	P38 MAPK	c	Pace et al. 2003
Butyrate	SCFA	P38 MAPK	c, hc	Witt et al. 2000 Pace et al. 2003
		ROS/p38 MAPK	c, hc	Hsiao et al. 2006
		cAMP	c	Keefer et al. 2006
Valproate	SCFA	P38 MAPK	c	Witt et al. 2002
Apicidin	HDAC	P38 MAPK	c	Witt et al. 2003
Scriptaid	HDAC	P38 MAPK	c, hc	Johnsonet al. 2005
Trichostatin A	HDAC	ROS/P38 MAPK	c, hc	Hsiao et al. 2006 Pace et al. 2003
Thalidomide	IMiD	ROS/P38 MAPK	hc	Aerbajinai et al. 2007
CysNO	NO donor	NO, cGMP	c, hc	Cokic et al. 2003
Rapamycin	mTOR inhibitor	mTOR	c, hc	Fibachet al. 2006 Mischiati et al. 2004

(to be continued)

c = immortalized erythroid cell line;
cAMP = cyclic adenosine monophosphate;
cGMP =cyclic guanosine monophosphate;
DNMT = DNA methyltransferase;
hc = human primary erythroid cell culture;
HDAC = histone deacetylase;
IMiD = immunomodulatory drugs;
MAPK = mitogen-activated protein kinase;
mTOR = mammalian target of rapamycin;
NO = nitric oxide;
SCFA = short chain fatty acid;

ROS = reactive oxygen species.

The table sourced from journal *Experimental Hematology* 2008 36: 1057- 1072, page 1064

Intracellular signaling pathways implicated fetal-globin gene and

/or fetal hemoglobin induction

There were *three classes* of signaling transduction to induce the fetal hemoglobin.

1.5.4.1 The unfolded protein stimulate the stress response in erythroid cell(chen et al. 2007)

The diversity of stimulation of this class included: Viral infection, heat shock, ultraviolet irradiation, Hypoxia, inadequate heme and nutrient, ROS, endoplasmic reticulum stress, proteosome inhibition. They activated the four kinases,

PERK (RNA dependent protein kinase-like),

PKR (RNA-dependent protein)

GCN2 (general control nonderepressible-2) (EIF2A kinase 4)

HRI (heme-regulated inhibitor) (EIF2A kinase 1)

4 of kinases phosphorylated (EIF2A) eukaryotic initiation factor-2 alpha and caused the global translation inhibition. At the same time, the cell mediated stress response increased ATF4, it increased the secondary transcription. They were growth arrest and DNA damage-inducible gene (GADD) 34, CCAAT/ enhancer binding protein homologous protein. The downstream pathway would lead to apoptosis or the corrective pathway. GADD34 gave the feedback signal to reactivate the protein translation. The experiment of disruption of the ATF4 in mice would lead to severe fetal anemia (Masuokaet al. 2002)

1.5.4.2 p38 mitogen-activated protein kinase (MAPK)pathway

The fetal hemoglobin inducing agents in this class were hydroxyurea, butyrate, trichostatin A, thalidomide and anisomycin, osmotic shock, DNA damage, UV/gamma radiation. HU was converted to nitrogen oxide (NO) it modified the constituent of sGC which in turn increased the cGMP activities and induce the fetal hemoglobin. Inhibition of cGMP which would down-regulate the fetal hemoglobin.

(Cokic et al. 2008)

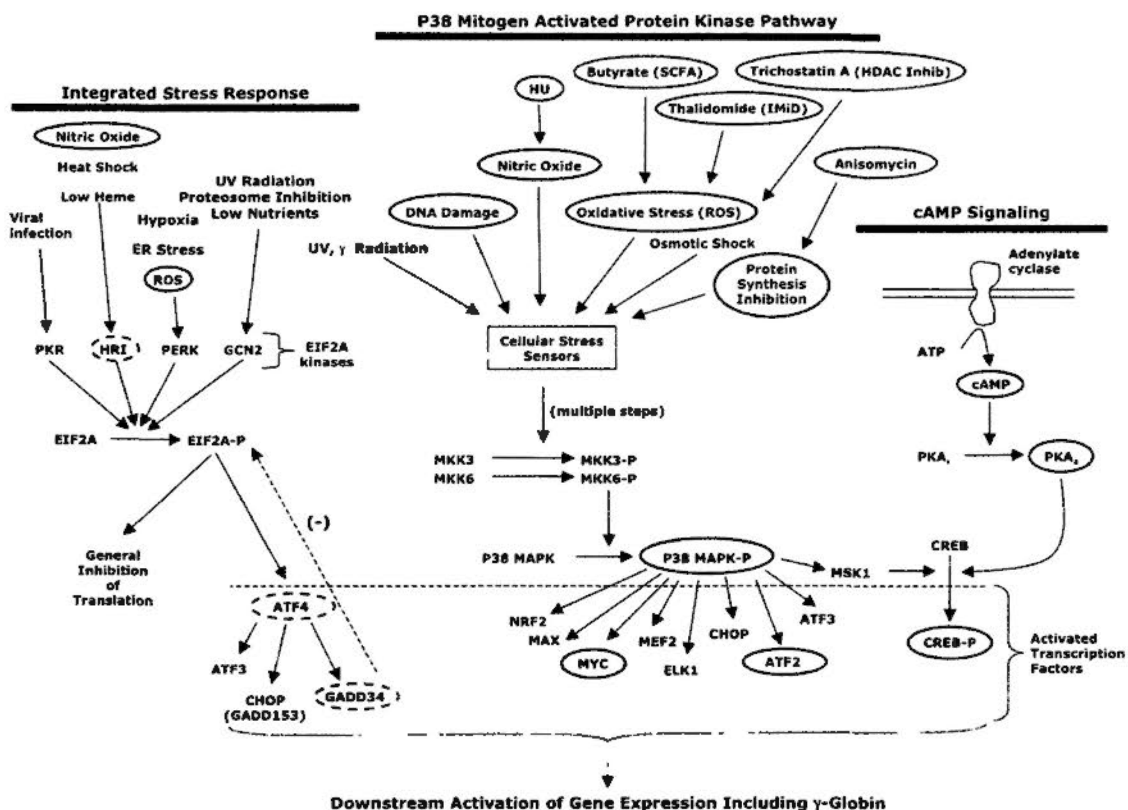
Butyrate, thalidomide and trichostatin A, the three compounds worked as a HDAC inhibitor, they were not only activated the histone hyperacetylation but also produced high O_2 level which in turn induced p38 MAPK phosphorylation. It activated cAMP response element binding protein (CREBP) and activating transcription factor 2 (ATF2) transcription activator protein.(Pace et al. 2006) (Hsiao et al. 2006) Ansiomycin inhibited the protein systhesis strong induce c-fos and c-jun and triggered p38 MAP kinase which in turn to reduce the levels of phosphorylated forms of Cx43 and gap-junctional intercellular communication (GJIC). Phosphorylation of IRS-1 and IRS-2 was activated by ansiomycin which blocked reduced the insulin-induced tyrosine. The mechanism accelerated the protein degradation and inhibited the protein systhesis. (Ogawa *et al.*, 2004)

1.5.4.3 Cyclic adenosine monophosphate (cAMP) signaling pathway

Hydroxyurea(HU), butyrate and 5-Azacytidine could activate the cAMP pathway. For example HU generated the reaction which soluble guanyl cyclase (sGC) reacted with cGMP pathway to produce nitric oxide. cGMP down-regulated the phosphodiesterase 3 and increased the level of cAMP. It activated the protein kinase A which phosphorylated the cAMP response element binding protein (CREBP). CREBP trigger the transcription factors and activated the fetal hemoglobin production.

Three cell stress-response pathway model showed as a scheme below:

The table sourced from journal *Experimental Hematology* 2008 36: 1057- 1072, page 1066



Proposed cell stress signaling model of fetal hemoglobin (HbF) induction. Under this model, many different cellular stresses, including those caused by HbF-inducing drugs activate coordinated stress responses, which include gamma-globin gene activation. Potential pathways involved in these responses include the integrated stress response (also known as unfolded protein response), p38 mitogen-activated protein kinase (MAPK) and cyclic adenosine monophosphate (cAMP) signaling pathways. Blue ovals indicate examples of HbF-inducing agents that have been shown to act, at least in part, through the p38 MAPK stress signaling pathway.

Solid red ovals indicate pathway members that have been experimentally implicated in HbF induction. Dashed red circles indicate factors that are involved in erythropoiesis but have not been directly linked to HbF induction.

ATF	activating transcription factor
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHOP	CCAAT/enhancer binding protein homologous protein
CREB	cAMP response element binding protein
CREBP	cAMP response element binding protein
DNMT	DNA methyltransferase
EIF2A	eukaryotic initiation factor-2 alpha
ELK1	member of ets oncogene family
ER	endoplasmic reticulum
GADD	growth arrest- and DNA damage-inducible gene
GCN2	general control nonderepressible-2 EIF2A kinase 4
HDAC	histone deacetylase
HRI	heme-regulated inhibitor / EIF2A kinase
HRI	heme-regulated inhibitor EIF2A kinase 1
IMiD	immunomodulatory drugs
MAPK	mitogen-activated protein kinase

MAX	MYCassociated factor X
MEF2	MADS box transcription enhancer factor 2
MKK	mitogen-activated protein kinase kinase
MSK1	mitogen and stress-activated protein kinase 1
MYC	v-MYC avian myelocytomatosis viral homolog
NO	nitric oxide
NRF2	nuclear factor erythroid 2-like 2
PERK	RNA dependent protein kinase-like ER kinase EIF2A kinase 3
PERK	RNA dependent protein kinase-like
PKA	protein kinase, cAMP-dependent, regulatory, type I a
PKR	RNA-dependent protein kinase (EIF2A kinase 2)
PKR	RNA-dependent protein
ROS	reactive oxygen species
ROS	reactive oxygen species.
SCFA	short chain fatty acid
UV	ultraviolet radiation.

Chapter 2: Compare the efficacy of fetal hemoglobin induction of different cucurbitacin on k562

2.1 Introduction

There were several decades to develop the drugs for stimulation fetal hemoglobin. The development started at 1960s. The observation from the cancer patients, the high level fetal hemoglobin appeared in their blood picture after hydroxyurea treatment. (Tracewell et al, 1995) later, the similar event happened again after butyrate. The physicians considered used such of medicine to cure the hemoglobinopathies. The promising results were reported the physical condition of the patient under the treatment. The drugs reduced the clinical symptoms and improved the quality of life. However, the negative aspects were reported. The non-responder, carceno risk, many adverse effects appeared. The safety and efficiency medicine was required.

2003, our laboratory extracted cucurbitacin D from *Trichosanthes rosthornii Harms*. We discovered the drug could stimulate fetal hemoglobin in k562 cell model. The compound should be further demonstrated their efficacy preciously. Moreover, we supposed the other iso-form of cucurbitacin might possess the same effect. Thus, in this chapter, we compared the efficacy of iso-form cucurbitacin in inducing total hemoglobin, fetal hemoglobin and globin mRNA level in k562 cell model. Furthermore, we demonstrated the compound could activate the gamma globin in thalassemia major patient's $CD34^+$ stem cell.

K562 cell model was used as an assessment of pharmacoceutic development. There was limitation of k562 cell. It affected the judgment of the data which generated by the model. Could K562 be a cell model for fetal hemoglobin induction?

2.1.1. What is K562?

K562 cell line was isolated from a chronic myelogenous leukaemia patient's pleural fluid. The non-adhence, nuclear cell line was established by Lozzio's laboratory in 1971. (Lozzio & Lozzio, 1975; Drexler, 2000)The early stage cell could develop into erythrocytes, granulocytes and monocytes. (Lozzio & Lozzio, 1981) It was lack of some MHC antigen. K562 was easy to escape from NK cell phagocytosis (Drexler, 2000). K562 cell showed bcr:abl fusion gene and Philadelphia chromosome (Lozzio & Lozzio, 1975). K562 was reported that it could produce the hemoglobin Gower I (ζ2ε2), Hb Gower 2(α2ε2) Portland(ζ2γ2), hemoglobin F(α2γ2) and hemoglobin Bart(γ4)by0.05mM heme for 6 days culture (Rutherford *et al.*,1979) Many agents could activate the gamma globin in k562 cell model, such as hydroxurea (Dover *et al.*,1986)and 5-azacytidine(Charache *et al.*,1983).

2.1.2. Activation of gamma globin

A gene was turn on to produce the protein which needed many levels modification. It was involved loosen the chromosome. Histones were acetylated by acetlase or by adenosine triphosphate. Then LCR loop (ach) formation, it needed many of transcription factors to bind on the chromatin. LCR could interact with structural globins gene by competition. On the contrary, it could repress the gene expression by histones deacetylation via histone deacetylases (HDACs). Moreover, histone N-terminal tails could alter by ubiquitination, methylation and phosphorylation.(Bank A. 2006). The reactions might play a role on remodel of chromatin complex. Butyrate compounds worked as inhibitor of histones deacetylation (HDACs) for gamma gene, (Fathallah *et al.* 2007). 5-azacytidine inhibited DNA methyltransferase,

The induction of gamma globin in cord blood sample and k562 cell model, the same transcription factor was elevated. NF-E4, EKLF and SSP were demonstrated increased when fetal hemoglobin was induced in both normal and leukemic erythroid cell model.(Zhou *et al.*, 2004 and Secchiero *et al.*, 2004) From the data provided, it demonstrated the chromatin epigenetic modification which might be the same.

And the same activated transcription factor indicated the binding site is the same. Thus, we guest the same reaction happened in both cell lines. The k562 cell model could be used for analogue the real situation. And the same activated transcription factors indicated that they acted on similar binding sequences. It was suggested the similar reaction happened in both cell line. The k562 cell model could be used for analogue the real case on certain condition.

However, another experiment they insert the β sickle globin gene in k562 cell. It demonstrated the transcription reaction of the intact β sickle gene sequence, but the k562 could not produce the β sickle globin mRNA, it indicated that the cell could not produce the β globin due to the environmental factors. It was not favor for the β globin production. K562 cell model was not suggested for β globin study or for the β globin switch phenomenon. (Young *et al.*, 1985)

(Creusotor *et al.*, 1986). Didox reduced ribonucletide reductase activities.

The chemicals promoted the gamma globin expression. They could reform the stage-specific transcription factors composition. Remodeling the chromatin complex and stimulation the special transcription factors binding were important for fetal hemoglobin activation within adult stage. (Bank A. 2006)

2.1.3. How does K562 become a cell model?

Compare the similarity between erythroid progenitor cell and k562 cell model.

Cucurbitacin D could activate the fetal hemoglobin in k562 cell line and erythroid progenitor cell. The data was generated by Dr. Xing. (HT Xing.2003).

We compared the two mechanisms which acted on gamma globin production. The differences and similarities of fetal hemoglobin induction were put side by side. We tried to find out the limitation of k562 cell model, and justified the data convincing.

K562 cell model was analogue of the normal human cell. The histone acetylation was activated on expressing gene. Many studies carried out in human erthroleukemic cell line. Recently, the more similar studies worked out on primary erythroid cell. The same result of acetylation of histone was observed in the normal cell line. The LCR acetylation level was the same in all developmental stage. From embryo to adult stage, only the transcription gene will express the high level of acetylation.(Yin *et al.*, 2007) another study for function of histone deacetylation, the cord blood and k562cell model were used to demonstrate the silencing effect of HDAC3 in gamma globin gene expression. The similar results were obtained between k562 cell model and cord blood. (Mankidy *et al.*, 2006)

HbF Calibrator	Bethyl Labs RC80-135-4
Cesium chloride (CsCl)	Invitrogen 15507-023
Guanidine Thiocyanate (GITC)	Sigma G9277
Sodium acetate ($C_2H_3NaO_2$)	Sigma S8750
β-Mercaptoethanol (HOCH 2 CH 2 SH)	Sigma M7154
iQ™ SYBR® Green Supermix	Bio-Rad 170-8880
Tris base NH2C(CH2OH)3	USB 75825
Tween 20	USB 20605
Triton X-100	Fluka 93426
Diethylpyrocarbonate (DEPC)	
Acetic acid, glacial	BDH 100015N
Mouse-anti-human Fetal Hemoglobin Monoclonal Antibody (PE)	BD M076640
Hydroxyurea (HU)	Sigma H8627

2.2 Materials

Chemicals, Kits and Reagents	Company/Cat. No.
RPMI Medium 1640, powder Contains L-glutamine and 25 mM HEPES buffer, but no sodium bicarbonate	Invitrogen 23400-021
Sodium bicarbonate (NaHCO3)	Sigma S5761/S8875
Sodium hydroxide (NaOH)	Sigma S5881
Hydrogen peroxide (H_2O_2, 30% v/v solution)	BDH 101284N
Cucurbitain B,D,E,I	chromadex
Hemoglobin A2, ferrous stabilized human	Sigma H0266
Fetal Bovine Serum (FBS), Certified	Invitrogen 16000-069
Ethanol, absolute	Merck 1.00983.2511
Dulbecco's Phosphate-Buffered Saline (D-PBS), powder	Invitrogen 21600-010
Citric acid monohydrate	UNIVAR 160
Antibiotic-Antimycotic (PSF, 100X), liquid	Invitrogen 15240-062
Acetic acid, glacial	BDH 100015N
31.5-33% Hydrochloric acid (HCl)	BDH 10307
3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma T2885
Trypan blue	
Human Hemoglobin F (HbF) ELISA Quantitation Kit	Bethyl Labs 80-136
Sheep anti-Human HbF Ab, peroxidase-conjugated	Bethyl Labs A80-136P
Sheep anti-Human HbF Ab, affinity purified	Bethyl Labs 80-136A

Equipment	Company/Cat. No.
96 Well Clear Flat Bottom Polystyrene High BindMicroplate	Corning® 9018
96 MicroWell™ Plates Nunclon™Δ, Round Bottom, Polystyrene, Sterile	Nunc 163320
Beckman Ultracentrifuge	Beckman 326819
Hemocytometer	
Microscope.	
SpectraMAX 250 microplate spectrophotometer	
K562 cell line	ATCC® CCL-243™

Reagent preparation

K562 cell line

Erthroleukemic cell line k562 was purchased from ATCC. It was cultured in RPMI 1640 complete medium. The cell density was kept within 3 x 10^5 cells/ml. The cultures was kept in humidified environment with 95% air / 5% CO_2 at 37°C.

Preparation of TMB working buffer

5.11 gm of citric acid and 6.19 gm of sodium phosphorus melts into 450ml milli Q water , adjust the pH to 5.0 with 5M sodium hydroxide, then top up the buffer to 500ml with milli Q water which is the final volume. Stored in 4℃ before use.

Preparation of TMB staining solution

250mg 3,3',5,5'Tetramethyl benzidine (TMB) purchased in sigma company. It is dissolved into 50 ml glacial acetic acid to make a 5mg/ml stock solution.

Preparation of TMB working solution

19.8ml TMB working buffer mixed with 200ul staining solution and 400ul 30%

Hydrogen peroxide.

Freshly prepared before use.

Complete RPMI 1640

2.0gm sodium bicarbonate and one package of RPMI 1640 power melts into 950ml milli Q water, then adjust the pH to 7.0 with 1M sodium hydroxide. Top up the medium to 1 liter with milli Q water. The medium was passed through 0.22 μm filter under a sterile condition. 10 %(v/v) fetal calf serum and 1% (v/v) PSF was added into the medium. Store in 4 ℃.

Dulbecco's Phosphate-Buffered Saline

One package Dulbecco's Phosphate-Buffered Saline was dissolved into 1 liter milli Q water, then sterilized by autoclaved the saline in 121℃15 lbs of pressure for 20 mins.

Dilute the cucurbitacin stock (100 ug/ml)
To make dilution of cucurbitacin stock to 16ug/ml with complete RPMI 1640 medium.

ELISA Blocking Solution
1% BSA (w/v) in 1X TBS, freshly prepared

ELISA Cell Lysis Buffer
1% Triton X-100 in 1X TBS, freshly prepared

ELISA Coating Buffer
50mM carbonate-bicarbonate at pH 9.6. 0.0795g $Na_2CO_3$and 0.357g $NaHCO_3$ were dissolved in 100ml Milli-QH_2O. Solution was stored at 4°C until use.

ELISA Wash Solution
0.05% Tween 20 (v/v) in 1X TBS, freshly prepared

ELISA Sample
Diluent 0.25% Tween 20 (v/v) and 1% BSA (w/v) in 1X TBS, freshly prepared

ELISA TMB StockSolution
5mg/ml TMB in DMSO, stored in airtight glass tube in dark at room temperature

ELISA TMB SubstrateBuffer
2.555g citric acid and 3.095g NaH_2PO_4 were dissolved in 200ml Milli-Q H_2O, pH was adjusted to pH 5.5 with 5M NaOH. Final volume was topped up to 250ml by Milli-Q H_2O. Solution was stored at 4°C until use.

Guanidinium Thiocyanate
Dissolve 4.0 M Guanidinium Thiocyanate in 0.1 M Tris-HCl (pH 7.5) in 100 ml DEPC treated water. Filter the solution with Whatman No.1 filter paper. Add 1%

Beta-mercaptoethanol into yhe GT solution before use.

3M sodium acetate

123g sodium acetate dissolve into 450 ml DEPC treated milli Q water, adjust the pH to 5.2 with glacial acetic acid. Then top up to 500ml of solution, sterile by autoclave.

5.7 M Cesium chloride

87.85g CsCl dissolve in 91.54 ml DEPC treated milli- Q water. Keep the reagent sterile by autoclave.

2.3 Method

2.3.1. Bioassay of k562-Total hemoglobin production

Different cucurbitacin induce total hemoglobin on k562

K562 was multi-potential progenitor cell, which could develop to early-stage, granulocytes monocytes and erythrocytes (Lozzio et al., 1981). Under some condition, k562 would undergo differentiation and produced hemoglobin. The main criterion of the biological assay was preventing the k562 produce hemoglobin autonomously before the cucurbitacin treatment. We could not use the old culture because it would increase the chance the hemoglobin production.

Procedure

1. It took the cell from 3 to 5 passage of cell culture. The cell density should not over3 x10^5 cells/ml.
2. The viable cell determined by trypan blue stain. The auto-differentiated k562 cell could be stain by TMB. Density of cell counted by cell counting chamber which was under an inverted lighted microscope.
3. Round bottom 96 well plate was used for bioassay. Two fold dilution would be performed in series across a row. It was made 1/2048 of final dilution. 200 ul of dilution of cucurbitacin was added into the first well, then transfer 100 ul to second well and mixed with 100 ul pre-deposited complete medium.
4. 20 micro-liter will take from the dilution. It is 320 ng as the intial dosage. Then another 100 ul mixture will be transfer to adjacent well and mixed with 100 ul complete medium until the last well of the row. The last 100 ul mixture will be discarded.
5. The cell initial density for bioassay is 2 x 10^3 cells/ml. The dilution was made

with complete RPMI 1640 medium. Stirrer beaker was used for keeping the cell evenly distribution.. Every single test will be performed triplicate. Add 100 ul cell suspension into every well of 96 well plate.

6. Store the plate in 5% CO_2 at 37°C for 6 days.
7. At the end point of incubation, the plate will be centrifuge at 300 r.c.f. for 10 minutes. Aspirate the supernatant by the peristaltic pump.
8. The cell pellet mixed with 100ul TMB working buffer, then 200ul TMB working solution was added into each well.
9. The plate was kept in the dark for ten minutes, Took the absorbent reading under 600 optical density(OD)by SpectraMAX 250 microplate spectrophotometer.

2.3.2. Method: ELISA assay for different cucurbitacin induce fetal hemoglobin

The last chapter showed the significant result in TMB staining bioassay and the characteristic of k562 cell model was incapable to produce beta-globin. We guessed the hemoglobin produced in TMB staining k562 cell assay. It was the gamma-globin. Thus, further studies on whether cucurbitacin induced fetal hemoglobin in k562, and compared the fetal hemoglobin induction by different cucurbitacins.

2.3.2.1 Prepare the cell lysate

Cell assay set as mention above, different cucurbitacins treated the k562 cell independently, untreated k562 as blank control indicated the fetal hemoglobin produced by auto differentiation. The experiment was performed in 96 well plates. Fetal hemoglobin was induced only by different compound separately. The plates were incubated in humidified incubator for 6 day at 37°C5% CO_2. After incubation period, the plates were centrifuged at 300r.c.f.. Supernatants were aspirated out; the cell pellets were lysed by lysing buffer. The buffer was purchased from New England biolab. 100ul of one tenth diluted lysing buffer was added in each well. Mixed well with cell pellets, then incubated for 15 minutes. The plates were spined at 1000 r.c.f. for 10minutes. 20 ul lysate was taken for ELISA test sample.

2.3.2.2 ELISA Procedures

1. Make the dilution of standard (HbF Calibrator Bethyl Labs RC80-135-4)which was provided by the kit. 5 ul calibrator was added into 6.25 ml sample diluents as the first dilution. The serial two fold dilutions were made. The detection range is from 6ng to 400ng of fetal hemoglobin.

2. Make 100x dilution of anti-fetal hemoglobin with coating buffer, 100ul mixture was added into a well of flat bottom 96 well plate.

3. Incubate the plate for an hour, and then washed it with wash solution thrice.

4. 200ul blocking buffer apply into each, BSA as a blocker fully occupy the left off space after binding the first antibody. Through 30 minutes incubation, rinse the plate with washing buffer thrice.

5. Cell lysate and diluted standard were added into the ELISA plate, incubated it for one hour. It was washed five times with washing buffer.

6. TMB stock solution and TMB substrate solution was mixed before used. 200ul mixture was added into each well the color will developed with 30 minutes, within the time 100ul con sulfuric acid was added and stop the reaction.

7. The reaction absorbances were measured by SpectraMAX 250 microplate spectrophotometer. The optical density is 450 nm (OD450).

2.3.3. Cucurbitacin B, D, E & I induced mRNA of hemoglobin

2.3.3.1 Preparation of the cell lysate

K562 was a leukemia cell line; it imitated a normal cell to produce fetal hemoglobin. High dose of drug would lead the cell undergo apoptosis. Cucurbitacin B, D and I induced fetal hemoglobin in low dosage. ELIZA test results indicated the optimal dosage on inducing fetal hemoglobin effect.

1. In this chapter, we used 3.125ng/ml concentration of cucurbitacin B, D and I. Cucurbitacin E was an exception, which optimal dosage was 80 ng/ml.
2. Fetal hemoglobin could be detected from day -3 co-incubation with cucurbitacin. It reached plateau on day 6. We designed the experiment, and collect the mRNA on day 4.
3. 10 ml cell cultures were harvested into a 15 ml falcon tubes, then the tubes were spined at 300 R.C.F.for 10mins, threw away the supernatants, and then rinse the cell pellets with PBS twice.
4. Aspirated the watery supernatant out; the guanidine thiocyanate was added dropwise and assisted with continuous agitation.
5. Beta-mercaptoethanol had just added into Guanidine thiocyanate freshly at1: 100 (V/V) volume. Guanidine thiocyanate was a strong protein denaturing agent whereas could inhibit the contamination. The cell lysate could store -70 °C until use.

9. 200ul Rnase free milliQ water rinsed off the transparent pellet. Then transfer the mixture in to a 1.5 ml eppendorf.
10. 20ul of 3M sodium acetate and 730 ul absolute ethanol were mixed with RNA solution. The mixture would be span at 14k r.p.m. for 30 minutes in 4°C.
11. The white pellet, at the bottom of the tube, rinsed with 70% ethanol once. Span down the RNA at 14k r.p.m. for 5 minutes. Pour away the supernatant. Dry up the pellet with vaccum pump and suspend the RNA in RNase free milliQ water.
12. RNA quality and quantity were analysis by spectrophotometric method. Store the RNA suspension in -70 °C.

2.3.3.3 First strand cDNA synthesis

1. DNA contamination was greatly influence the quantity of PCR product. Thus the RNA samples were digested with RNase free DNase. The enzyme digested the residual DNA.
2. 1u RNase free DNase was used for 1ug RNA in 10ul 1X Promega RQ1 RNase-free DNase Reaction Buffer. The reaction was taken 30 minutes at 37°C in thermocycler.
3. Then 1ul RQ1 Dnase stop solution was added into the PCR tube. It stopped the reaction. The temperature of the reaction was increased to 72°C continuously which inactivated the Dnase.
4. The DNA free RNA sample was used for cDNA formation. The DNA free RNA sample stored in -70°C.
5. 1ul oligo(dT)12-18 at 0.5ug/ul, 1ul 10mM dNTP and 1ng to 5ug of mRNA were mixed together, the tubes were placed into 65°C.for 10minutes, after span the mixture briefly, then quickly chilled on ice.

2.3.3.2 Isolation of mRNA

1. Prevent RNase and DNA contamination were the major concern in RNA isolation process. Before all the experiment had been worked out, the RNA bench should be assigned for RNA specimen handling. A set of pipette was assigned for handling RNA. The pipette tip contained a filter and RNase free guaranteed.
2. All the glassware and metallic equipment had to baked at 220°C overnight. The plastic wares were treated with 0.1% (v/v)DEPC in milli Q water overnight, then autoclaved them in 121°C for 15 minutes. And were dried in 65°C oven.
3. All the bench and groove had to clean with cleaning agent that inactivated the RNase.
4. The isolation method included the cell lysing, shear the nuclear, spin down the mRNA
5. The cell lysates were thrawed at 65°C for 10 minutes and chilled on ice.
6. 26G syringe needle suck up all the cell lysate. The cell lysates were forced to pass through the needle for 20 times. The action sheared the nuclears of the cells, broken the long sequence of DNA.
7. 2.6ml 5.7M CsCl solutions were added into Rnase free ultracentrifuge tube. The homogenated cell lysates were slowly added into CsCl phase. The formation of CsCl-Gt cushion could isolate the DNA between the two layers. The mRNA would be span down on the bottom of CsCl solution. The centrifugation was at 33.7k r.p.m. for 18 hours in Beckmen SW55Ti rotor in 18°C.
8. After the ultracentrifuge, the GT solution would be aspirated away by a Pasteur pipette which connected with a pump. The CsCl layer removed by inverted the round bottom tube. Then use a surgical blade cut off the round bottom of the tube.

6. Added 4ul 5X First-Strand Buffer, 2ul 0.1M DTT and 1 ul RNaseOUT (Recombinant Ribonuclease Inhibitor) at 40unit/ul into the tubes. They were mixed to together. Incubate at 37°C for 2 minutes.
7. Finally, 1ul (200 unit) M-MLV reverse transcriptase was added into the tubes. After mixing, they were heated up to 37°C for 1 hour. Inactivate the enzyme in 70°C for 15 mins.

2.3.3.4 Measuring the mRNA level by Real-Time Quantitative PCR

The template was ready for PCR reaction.

1. 80 ul DNase-free milli-Q water was added into the tube, then boiled the cDNA for 15 minutes and chilled on ice again.
2. The total PCR volume was 20 ul. 12.5 ul 2xiQ SYBR Green Supermix, 1ul forward primer, 1ul reverse primer (primer concentration at 25 pmole/ul) and 5 ul cDNA were mixed together in an optical PCR tube, the final volume was topped up with DNase-free milli-Q water.
3. The optical PCR tubes were place in BioRad iCycler thermocycler.

2.3.3.5 Real-time PCR program

Heat denaturation was 95°C for 3 mins. The cycles was 95°C for 30 second, 60°C for 30 second, 72°C for 30second and performed 40 cycles. It included denaturation , the primer annealing and elongation.

The signals were detected by laser detector. The mRNA quantity was normalize by the quantity of GAPDH mRNA.

2.3.4. Cucurbitacin D induced fetal hemoglobin in thalassemias major patient's CD 34+ stem cell (Immunofluorescence staining / Confocal Microscropic Examination)

Cucurbitacins could activate the nearly silence gamma globin gene in k562 cell model. Further demonstration cucurbitacin D could activate the fetal hemoglobin in thalassemias major patient's stem cell which was an important implication. Cucubitacin D could have a therapeutic development.

Procedures

1. The sample was provided by Prince Wales Hospital. The bone marrow stem cell was collected from the major thalassemias patient. CD 34^{+} stem cells were sorted by the laboratory in the hospital.
2. The sample was stored in liquid nitrogen. Thawed out the sample in 37°C and washed it 3 times with complete medium.
3. Counted the viable cell. Cucurbitacin D was mixed with cell culture. The compound final concentration was the optimal dosage (3 .125 ng/ml).
4. It was kept in 25 ml culture flasks for 6 days in 37°C/ 5 % CO_2 humidify incubator.
5. After finished incubation period, it was collected in 50 ml falcon, washed it twice with PBS.
6. Fixed the cell with 4 % paraformaldehyde and washed it twice with PBS.
7. The cell pellet was suspensed in 50 ul PBS, 20 ul monoclonal antibodies were added into the cell suspension. It was kept in the dark at 4 °C for 1 hr.

8. The sample was washed twice with the solution which was mixed with PBS and 0.1 % sodium azide.
9. Finally the cell pellet was mixed with 20 ul glycerine and dropped on the glass slide. The glass was covered with a cover slide. Keep it in dark before under the confocal microscope.

2.4 Results

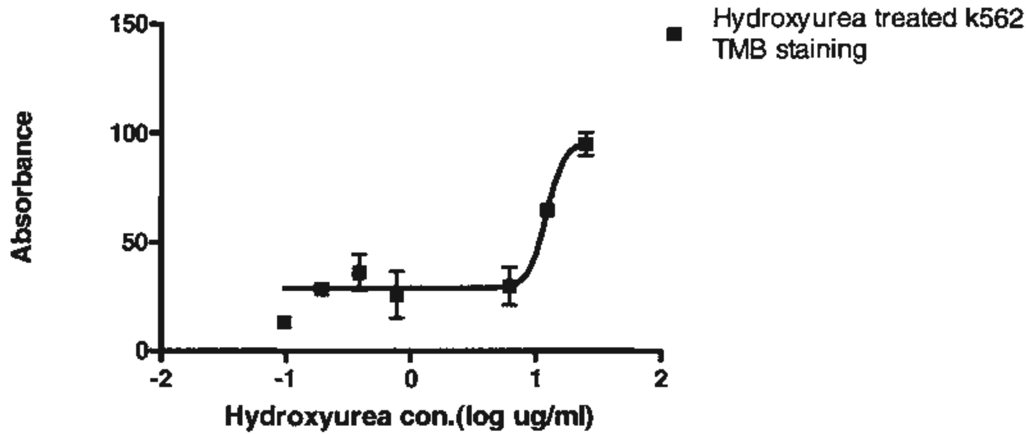
2.4.1. Effect of cucurbitacin B, D, E & I on total hemoglobin induction in k562 cell

We used cucurbitacin B, D, E & I, induced k562 cell with the dosage range from 1600ng /ml to 48.5 pg/ml. Hydroxyurea dosage range was from 1600ug /ml to 48.5 pg/ml. The non-liner regression method drafted the dose-response curves. ED_{50s}were calculated from the dose-response curves. The dose-response curves of the assays showed in figure 2-1 to figure 2-5. Cucurbitacin B is the most potent inducer while hydroxyurea was the least potent one. A comparison of ED_{50} of different cucurbitacins and hydroxyurea was shown in table 2-1.

Table 2-1: Total hemoglobin induction efficiency of cucurbitacin B, D, E, I and hydroxyurea.

	ED_{50} TMB staining
Cucurbitacin B	0.76 ng/ml
Cucurbitacin D	0.60 ng/ml
Cucurbitacin E	13.96 ng/ml
Cucurbitacin I	1.1 ng/ml
Hydroxyurea	12.24 ug/ml

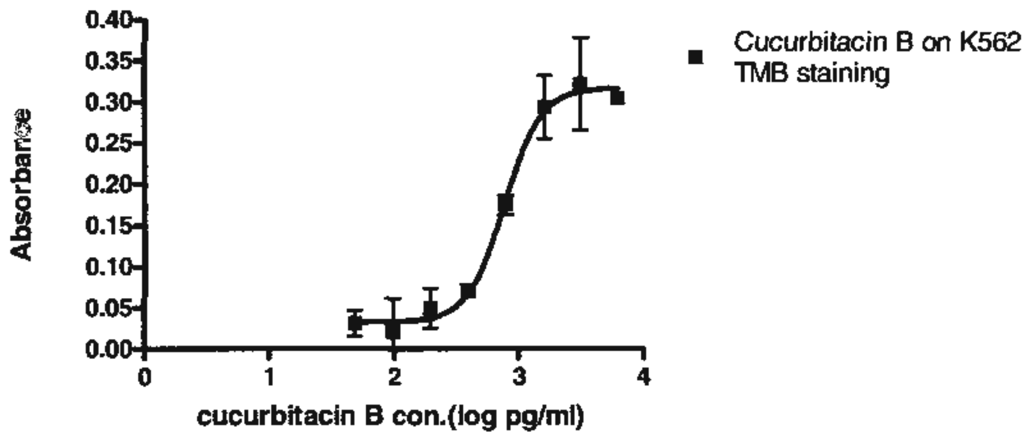
The ED_{50s} of curcubitacin B, D, E, I and hydroxyurea were calculated from their dose-response curves. Statistical program GraphPad Prism® 4 was used to analyze the assay data.Non-liner regression method was used to determine the curves and calculate the ED_{50s}.



	Hydroxyurea treated k562
log(agonist) vs. response -- Variable slope	
Best-fit values	
BOTTOM	28.76
TOP	95.68
LOGEC50	1.088
HILLSLOPE	6.225
EC50	12.24
Span	66.91
Std. Error	
BOTTOM	4.397
TOP	11.52
LOGEC50	0.05786
HILLSLOPE	12.36
Span	13.49
95% Confidence Intervals	
BOTTOM	18.97 to 38.56
TOP	70.01 to 121.3
LOGEC50	0.9589 to 1.217
HILLSLOPE	-21.31 to 33.76
EC50	9.097 to 16.47
Span	36.87 to 96.96
Goodness of Fit	
Degrees of Freedom	10
R2	0.8369
Absolute Sum of Squares	1547
Sy.x	12.44
Number of points	
Analyzed	14

Figure2-1: Dose-response curve of Hydroxyurea on k562 cell line

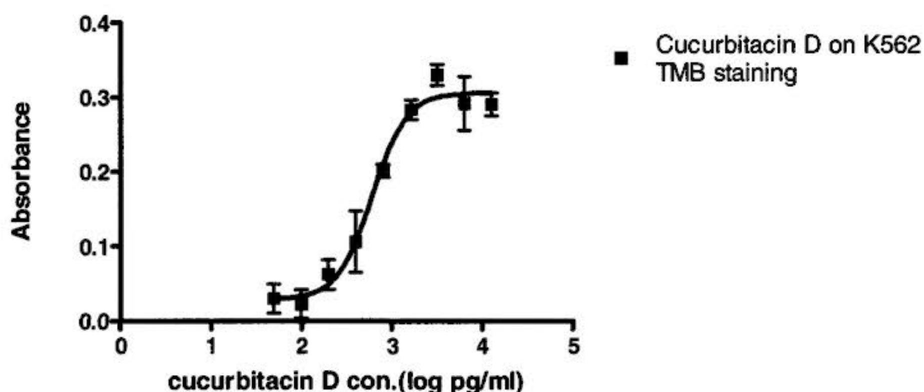
The initial cell no. 2 x 10^3 were treated with different dosage of hydroxyurea for 6 days. The data was plotted as absorbance verse dosage. The triplicate group results plotted as error bar with mean & standard deviation. The EC_{50} of hydroxyurea was estimated as12.24 ug/ml



Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.03217
TOP	0.3164
LOGEC50	2.882
HILLSLOPE	2.941
EC50	762.3
Std. Error	
BOTTOM	0.01733
TOP	0.02124
LOGEC50	0.06284
HILLSLOPE	1.220
95% Confidence Intervals	
BOTTOM	-0.003970 to 0.06832
TOP	0.2721 to 0.3607
LOGEC50	2.751 to 3.013
HILLSLOPE	0.3972 to 5.486
EC50	563.7 to 1031
Goodness of Fit	
Degrees of Freedom	20
R2	0.8870
Absolute Sum of Squares	0.04600
Sy.x	0.04796
Number of points	
Analyzed	24

Figure 2-2: Dose-response curve of cucurbitacin B on k562 cell line

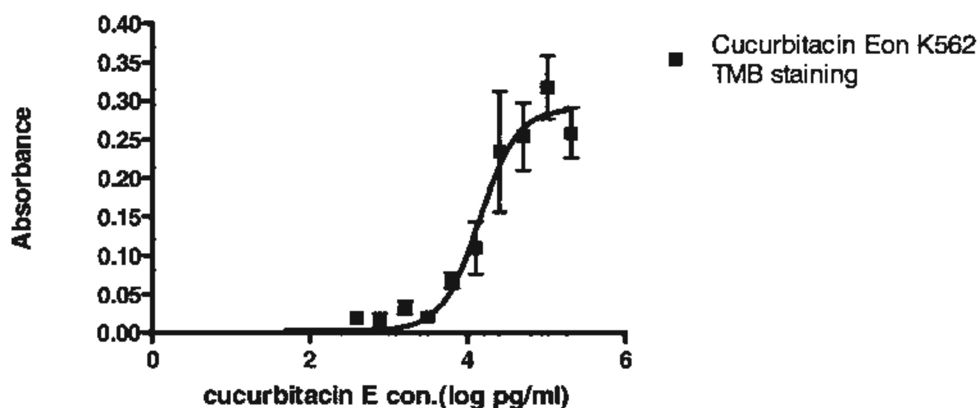
The initial cell no. 2×10^3 were treat with different cucurbitacin B for 6 days. The data was plotted as absorbance verse dosage. The triplicate group results plotted as error bar with mean & standard deviation. The EC50 of cucurbitacin B was 0.76ng/m



Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.02862
TOP	0.3062
LOGEC50	2.776
HILLSLOPE	2.278
EC50	596.5
Std. Error	
BOTTOM	0.01682
TOP	0.01408
LOGEC50	0.06263
HILLSLOPE	0.6696
95% Confidence Intervals	
BOTTOM	-0.006176 to 0.06341
TOP	0.2771 to 0.3354
LOGEC50	2.646 to 2.905
HILLSLOPE	0.8921 to 3.663
EC50	442.7 to 803.9
Goodness of Fit	
Degrees of Freedom	23
R2	0.9152
Absolute Sum of Squares	0.03439
Sy.x	0.03867
Number of points	
Analyzed	27

Figure 2-3: Dose-response curve of cucurbitacin D on k562 cell line

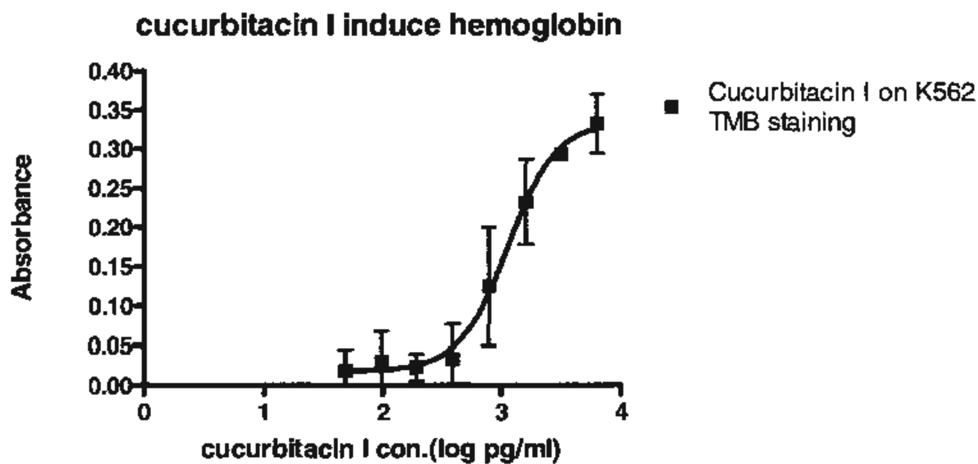
The initial cell no. 2×10^3 were treat with different cucurbitacin D for 6 days. The data was plotted as absorbance verse dosage. The triplicate group results plotted as error bar with mean & standard deviation. The EC50 of cucurbitacin D was estimated as 0.6ng/ml.



Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.002545
TOP	0.2910
LOGEC50	4.145
HILLSLOPE	1.814
EC50	13963
Std. Error	
BOTTOM	0.01253
TOP	0.02414
LOGEC50	0.09207
HILLSLOPE	0.6071
95% Confidence Intervals	
BOTTOM	-0.02292 to 0.02801
TOP	0.2420 to 0.3401
LOGEC50	3.958 to 4.332
HILLSLOPE	0.5804 to 3.047
EC50	9077 to 21479
Goodness of Fit	
Degrees of Freedom	35
R2	0.8507
Absolute Sum of Squares	0.09199
Sy.x	0.05127
Number of points	
Analyzed	39

Figure 2-4: Dose-response curve of cucurbitacin E on k562 cell line

The initial cell no. 2 x 10^3 were treat with different cucurbitacin E for 6 days. The data was plotted as absorbance verse dosage. The triplicate group results plotted as error bar with mean & standard deviation. The EC50 of cucurbitacin E was estimated as 13.96 ng/ml.



Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.01879
TOP	0.3337
LOGEC50	3.054
HILLSLOPE	2.157
EC50	1132
Std. Error	
BOTTOM	0.02450
TOP	0.04591
LOGEC50	0.1150
HILLSLOPE	1.070
95% Confidence Intervals	
BOTTOM	-0.03230 to 0.06989
TOP	0.2380 to 0.4295
LOGEC50	2.814 to 3.294
HILLSLOPE	-0.07380 to 4.389
EC50	651.4 to 1967
Goodness of Fit	
Degrees of Freedom	20
R2	0.8048
Absolute Sum of Squares	0.08765
Sy.x	0.06620
Number of points	
Analyzed	24

Figure 2-5: Dose-response curve of cucurbitacin I on k562 cell line

The initial cell no. 2 x 10^3 were treat with different cucurbitacin B for 6 days. The data was plotted as absorbance verse dosage. The triplicate group results plotted as error bar with mean & standard deviation. The EC50 of cucurbitacin I was estimated as 1.1 ng/ml.

2.4.2. Results of ELISA

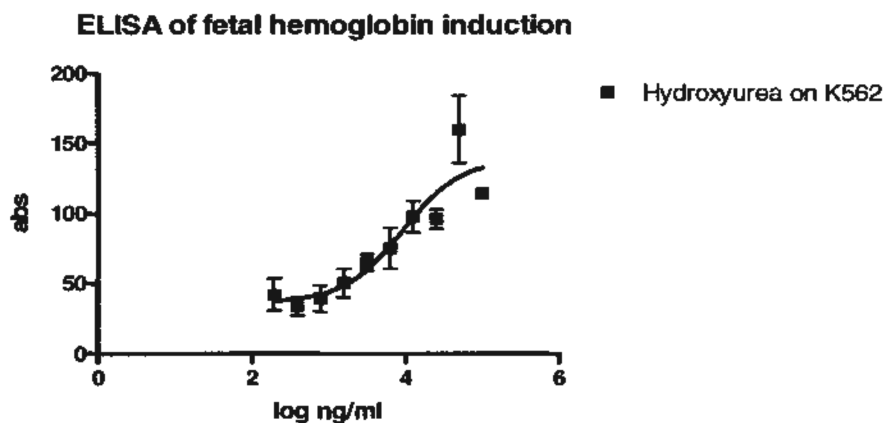
2.4.2.1 Effect of cucurbitacin B, D, E, I & hyroxyurea on fetal hemoglobin induction in k562 cell

Hydroxyurea, cucurbitacin B, D, E & I were about to increase the total hemoglobin production in K562 cells. It was further investigated whether cucurbitacins could induce fetal hemoglobin in K562 cells. Cell lysate samples were prepared as mention above. We used cucurbitacin B, D, E & I, to induce k562 cell with the dosage range from100 ng /ml to 48.8 pg/ml. Hydroxyurea dosage range was from100ug /ml to 48.8 pg/ml. Each test was triplicated. Each group result included error bar with the mean and standard deviations. The ED50s were calculated from the dose-response curves. Non-liner regression method sketched the dose-response curves. The dose-response curves of the assays showed in figure 2-6 to figure 2-10. The most potency compound for inducing fetal hemoglobin in K562 cells was cucurbitacin B while the least potent one was hydroxyurea. Table 2-2 showed the ED50 of different cucurbitacins and hydroxyurea of the fetal hemoglobin induction in K562 cells.

Table 2-2: Fetal hemoglobin induction efficiency of cucurbitacin B, D, E, I and hydroxyurea.

	ED_{50} *ELISA*
Cucurbitacin B	0.36 ng/ml
Cucurbitacin D	1.5 ng/ml
Cucurbitacin E	21.1 ng/ml
Cucurbitacin I	0.9 ng/ml
Hydroxyurea	9.0 ug/ml

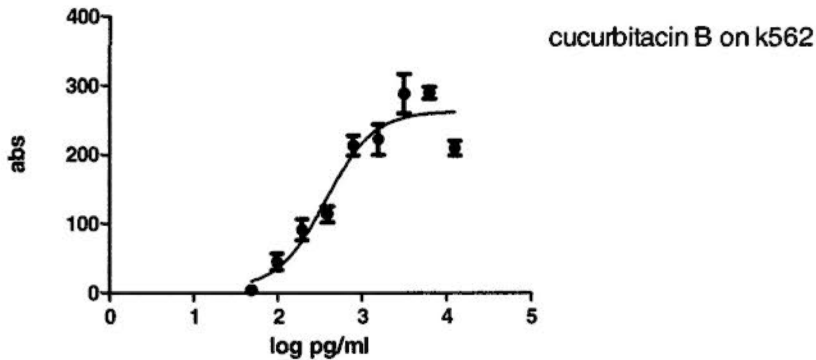
The ED_{50s} of curcubitacin B, D, E, I and hydroxyurea were calculated from their dose-response curves. Statistical program GraphPad Prism® 4 was used to analyze the assay data. Non-liner regression method was used to determine the curves and calculated the ED_{50s}.



	ng/ml
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	36.25
TOP	139.0
LOGEC50	3.954
HILLSLOPE	1.131
EC50	8986
Std. Error	
BOTTOM	11.84
TOP	22.36
LOGEC50	0.2328
HILLSLOPE	0.6686
95% Confidence Intervals	
BOTTOM	11.92 to 60.59
TOP	93.07 to 185.0
LOGEC50	3.475 to 4.432
HILLSLOPE	-0.2433 to 2.506
EC50	2985 to 27055
Goodness of Fit	
Degrees of Freedom	26
R2	0.7292
Absolute Sum of Squares	14053
Sy.x	23.25
Number of points	
Analyzed	30

Figure 2-6: Fetal hemoglobin induced by Hydroxyurea

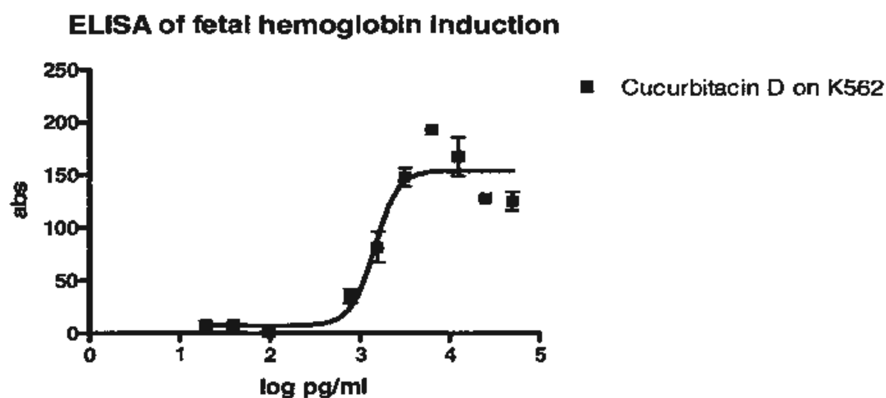
2x 10^3 k562 cell were treated with different concentration cucurbitacin E for 6 days. Fetal hemoglobin were quantified by ELIZA method. Absorbance was detected at 450nm by SpectraMAX 250 microplate spectrophotometer. GraphPad Prism® software was used to plot the sigmoidal dose-response curve. EC50 of Hydroxyurea was estimated as 9.0 ug/ml.



	Cucurbitacin B on K562
log(agonist) vs. response -- Variable slope	
Best-fit values	
BOTTOM	6.875
TOP	262.3
LOGEC50	2.574
HILLSLOPE	1.547
EC50	375.3
Span	255.4
Std. Error	
BOTTOM	30.15
TOP	15.61
LOGEC50	0.1087
HILLSLOPE	0.5522
Span	38.66
95% Confidence Intervals	
BOTTOM	-55.50 to 69.25
TOP	230.0 to 294.6
LOGEC50	2.349 to 2.799
HILLSLOPE	0.4049 to 2.690
EC50	223.6 to 630.0
Span	175.4 to 335.4
Goodness of Fit	
Degrees of Freedom	23
R2	0.8807
Absolute Sum of Squares	32401
Sy.x	37.53
Number of points	
Analyzed	27

Figure 2-7:Fetal hemoglobin induced by cucurbitacin B

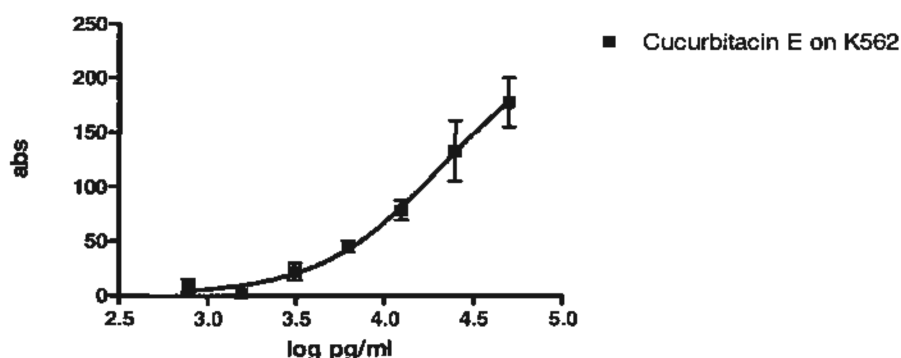
2x 10^3 k562 cell were treated with different concentration cucurbitacin E for 6 days. Fetal hemoglobin were quantified by ELIZA method. GraphPad Prism® software was used to plot the sigmoidal dose-response curve. EC50 of Hydroxyurea was estimated as 0.36 ng/ml.



	pg/ml
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	7.088
TOP	153.7
LOGEC50	3.162
HILLSLOPE	3.284
EC50	1452
Std. Error	
BOTTOM	8.013
TOP	7.049
LOGEC50	0.05301
HILLSLOPE	1.262
95% Confidence Intervals	
BOTTOM	-9.387 to 23.56
TOP	139.2 to 168.2
LOGEC50	3.053 to 3.271
HILLSLOPE	0.6906 to 5.878
EC50	1129 to 1866
Goodness of Fit	
Degrees of Freedom	26
R2	0.8948
Absolute Sum of Squares	15455
Sy.x	24.38
Number of points	
Analyzed	30

Figure 2-8: Fetal hemoglobin induced by cucurbitacin D

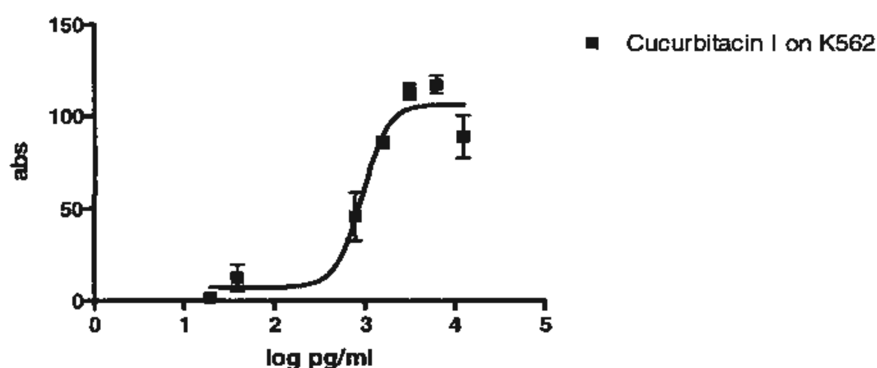
$2x\ 10^3$ k562 cell were treated with different concentration cucurbitacin E for 6 days. Fetal hemoglobin were quantified by ELIZA method. GraphPad Prism® software was used to plot the sigmoidal dose-response curve. EC50 of Hydroxyurea was estimated as 1.5 ng/ml.



	pg/ml
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	1.131
TOP	236.6
LOGEC50	4.324
HILLSLOPE	1.270
EC50	21102
Std. Error	
BOTTOM	15.95
TOP	95.74
LOGEC50	0.3002
HILLSLOPE	0.6718
95% Confidence Intervals	
BOTTOM	-32.53 to 34.79
TOP	34.55 to 438.6
LOGEC50	3.691 to 4.958
HILLSLOPE	-0.1473 to 2.688
EC50	4908 to 90722
Goodness of Fit	
Degrees of Freedom	17
R2	0.8953
Absolute Sum of Squares	9316
Sy.x	23.41
Number of points	
Analyzed	21

Figure 2-9: Fetal hemoglobin induced by cucurbitacin E

2x 10^3 k562 cell were treated with different concentration cucurbitacin E for 6 days. Fetal hemoglobin were quantified by ELIZA method. GraphPad Prism® software was used to plot the sigmoidal dose-response curve. EC50 of Hydroxyurea was estimated as 21.1 ng/ml.



	pg/ml
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	7.345
TOP	106.5
LOGEC50	2.967
HILLSLOPE	3.013
EC50	926.7
Std. Error	
BOTTOM	6.449
TOP	5.987
LOGEC50	0.05832
HILLSLOPE	1.189
95% Confidence Intervals	
BOTTOM	-6.262 to 20.95
TOP	93.88 to 119.1
LOGEC50	2.844 to 3.090
HILLSLOPE	0.5040 to 5.521
EC50	698.0 to 1230
Goodness of Fit	
Degrees of Freedom	17
R2	0.8991
Absolute Sum of Squares	4239
Sy.x	15.79
Number of points	
Analyzed	21

Figure 2-10: Fetal hemoglobin induced by cucurbitacin I

2x 10^3 k562 cell were treated with different concentration cucurbitacin E for 6 days. Fetal hemoglobin were quantified by ELIZA method. GraphPad Prism® software was used to plot the sigmoidal dose-response curve. EC50 of Hydroxyurea was estimated as 0.9 ng/ml.

2.4.3. RESULTS of quantity of mRNA (real time PCR)

2.4.3.1 Effect of cucurbitacin B, D, E & I on the gene expression of different hemoglobin in k562 cell

Cucurbitacin B, D, E & I were able to induce total hemoglobin production in K562 cells and increase the fetal hemoglobin. We further investigate whether these compounds were also able to induce the expression of different hemoglobin genes. Cell lysate samples were prepared as mention above. We used cucurbitacin B, D & I, to induce k562 cell with the dosage 3.125ng/ml, while Cu E induced k562 cell with the dosage100 ng /ml. The result of globins mRNA level was normalized by GAPDH. Each test was triplicated. Each group result included error bar with the mean and standard deviations. The induced k562 samples were compared with untreated one. Statistical T tests in GraphPad Prism®software was used for comparing the control and test. The relative mRNA levels of different compounds were showed in figure2-11 to figure 2-29.

Cu B, D, & I showed a significant inducing effect in alpha, beta, epsilon, gamma & zeta globins mRNA levels, cucurbitacin E only showed promising result in alpha and beta globin mRNA levels, but not on epsilon, gamma & zeta globin mRNA levels. The T test P values of each test were shown in table 2-3.

Table 2-3: P values of T test of cucurbitacin B, D, E,& I induction of different hemoglobin expression in K562 cells compared with control.

Globin	Cu B P<	Cu D P<	Cu E P<	Cu I P<
Alpha	0.016	0.001	0.037	0.020
Beta	0.026	0.039	0.049	0.041
Epsilon	0.060	0.010	0.181	0.041
Gamma	0.030	0.022	0.188	0.007
Zeta	0.046	0.007	0.117	0.018

Globins mRNA level was normalized by GAPDH. Statistical T tests in GraphPad Prism®software was used for comparing the control and test. The triplicate results were plotted as a graph with mean and standard deviation.

Cucurbitacin B induce alpha globin in k562

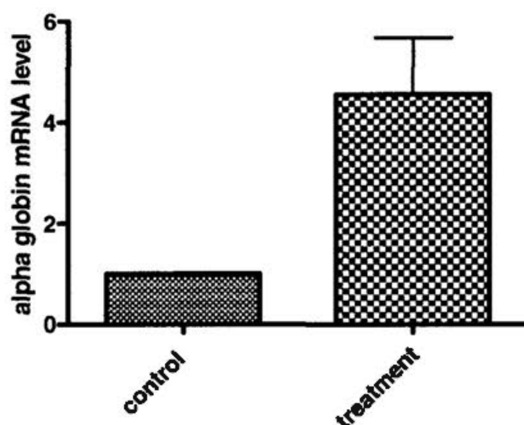


Table Analyzed	cucurbitacin B induce apha globin in K562
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0.0157
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=5.5 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-3.6
95% confidence interval	-6.3 to -0.78
R squared	0.94
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-11: Alpha globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin B for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of alpha globin and GAPDH were measured by real-time PCR. Alpha globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P<0.016).

Cucurbitacin B induce delta globin in k562

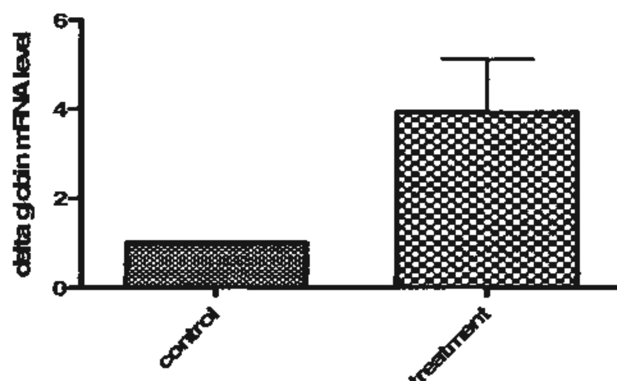


Table Analyzed	cu B induce delta globin
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0.0251
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=4.3 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-2.9
95% confidence interval	-5.9 to 0.0062
R squared	0.90
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-12: Delta globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin B for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of delta globin and GAPDH were measured by real-time PCR. Delta globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P<0.026)

Cucurbitacin B induce epsilon globin in k562

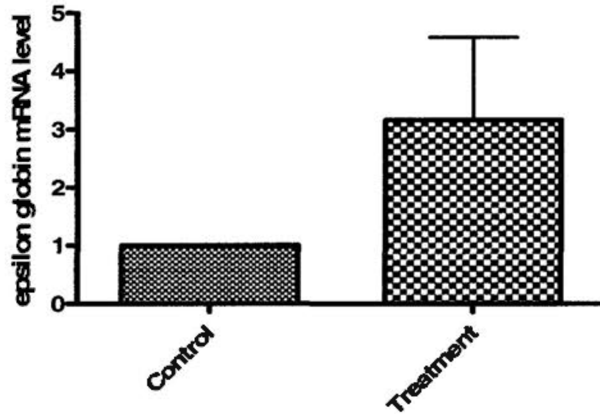


Table Analyzed	cu B induce epsilon globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.0599
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=2.622 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-2.160
95% confidence interval	-5.705 to 1.385
R squared	0.7746
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-12: **Epsilon globin level was normalized by GAPDH**

K562 was treated with 3.125ng/ml cucurbitacin B for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of epsilon globin and GAPDH were measured by real-time PCR. Epsilon globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P<0.06)

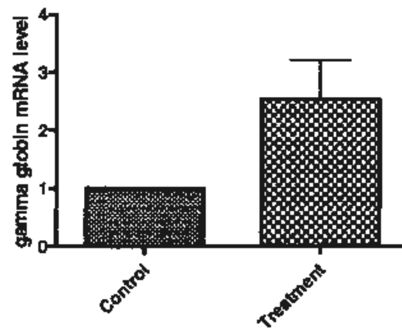


Table Analyzed	cu B induce gamma globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.0298
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=3.9 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-1.5
95% confidence interval	-3.2 to 0.15
R squared	0.88
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points.
P Value (one tailed)	
P value summary	

Figure 2-13: Gamma globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin B for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of gamma globin and GAPDH were measured by real-time PCR. Gamma globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P<0.03)

Cucurbitacin B induce zeta globin in k562

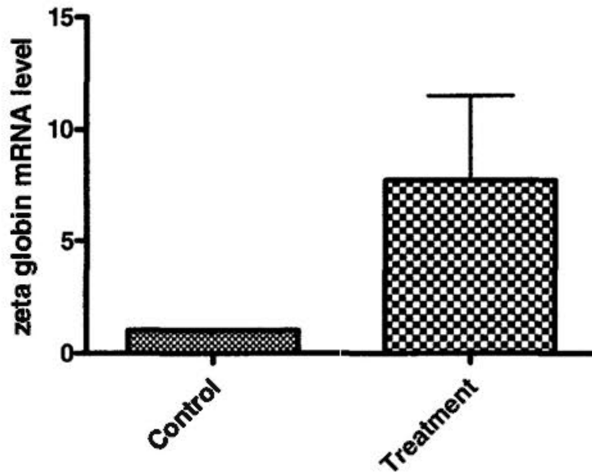


Table Analyzed	cu B induce zeta globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.0454
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=3.088 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-6.730
95% confidence interval	-16.11 to 2.649
R squared	0.8266
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points.
P Value (one tailed)	
P value summary	

Figure 2-14: Zeta globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin B for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of zeta globin and GAPDH were measured by real-time PCR. Zeta globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P>0.046)

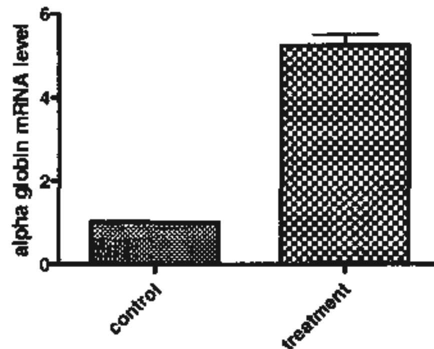


Table Analyzed	cu D induce alpha globin
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0.0007
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=27 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-4.2
95% confidence interval	-4.9 to -3.6
R squared	1.0
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points.
P Value (one tailed)	
P value summary	

Figure 2-15: Alpha globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin D for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of alpha globin and GAPDH were measured by real-time PCR. Alpha globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P>0.001)

Cucurbitacin D induce delta globin in k562

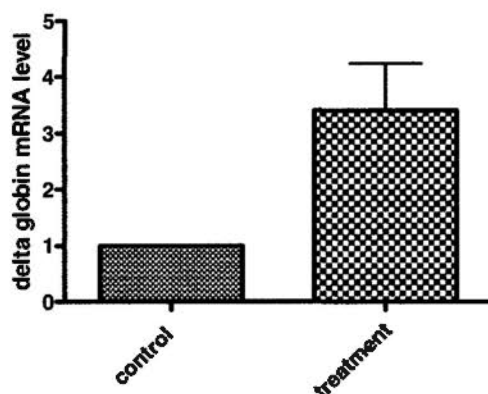


Table Analyzed	cu D induce delta globin
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0.0381
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.0 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-2.4
95% confidence interval	-4.5 to -0.33
R squared	0.93
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points.
P Value (one tailed)	
P value summary	

Figure 2-16: Delta globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin D for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of delta globin and GAPDH were measured by real-time PCR. Delta globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P<0.039)

Cucurbitacin B induce epsilon globin in k562

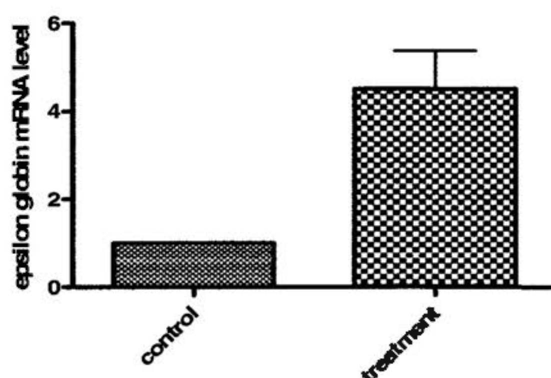


Table Analyzed	cu D induce epsilon globin
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0 0098
P value summary	**
Are means signif different? (P < 0 05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=7.037 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-3.507
95% confidence interval	-5.651 to -1 362
R squared	0.9612
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-17: Epsilon globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin D for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of epsilon globin and GAPDH were measured by real-time PCR. Epsilon globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of epsilon globin (P<0.01)

Cucurbitacin D induce gamma globin in k562

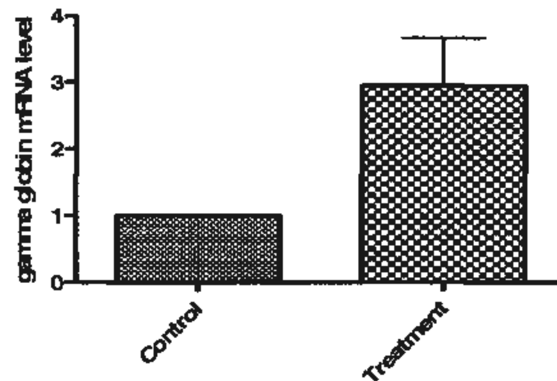


Table Analyzed	cu D induce gamma globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.0210
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=4.7 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-2.0
95% confidence interval	-3.7 to -0.17
R squared	0.92
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points.
P Value (one tailed)	
P value summary	

Figure 2-18: Gamma globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin D for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of gamma globin and GAPDH were measured by real-time PCR. Gamma globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of gamma globin (P<0.022)

Cucurbitacin D induce zeta globin in k562

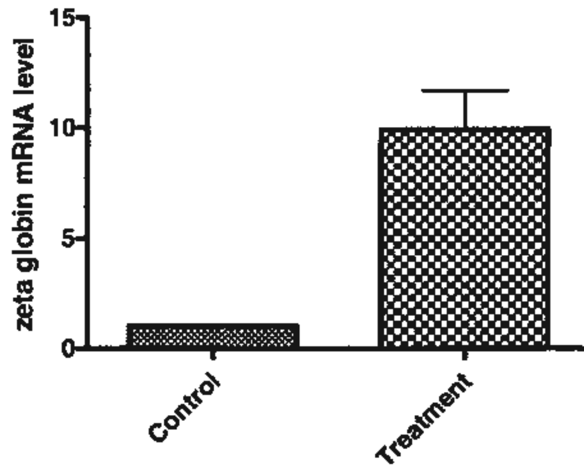


Table Analyzed	cu D induce zeta globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.0065
P value summary	**
Are means signif. different? (P < 0.05	Yes
One- or two-tailed P value?	One-tailed
t, df	t=8.7 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-8.9
95% confidence interval	-13 to -4.5
R squared	0.97
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-19: Zeta globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin D for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of zeta globin and GAPDH were measured by real-time PCR. Zeta globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of zeta globin (P<0.07)

Cucurbitacin E induce alpha globin in k562

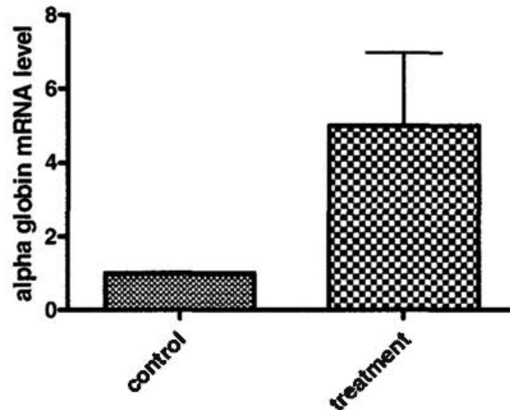


Table Analyzed	cu E induce alpha globin
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0.0367
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=3.5 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-4.0
95% confidence interval	-8.9 to 0.94
R squared	0.86
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-10: Alpha globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin E for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of alpha globin and GAPDH were measured by real-time PCR. Alpha globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P<0.037)

Cucurbitacin E induce delta globin in k562

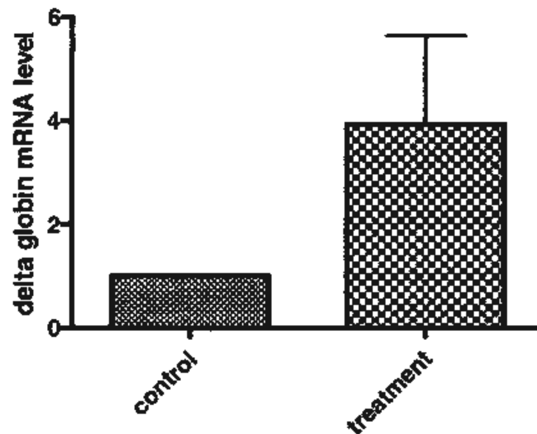


Table Analyzed	cu E induce delta globin
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0.0485
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=3.0 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-2.9
95% confidence interval	-7.2 to 1.3
R squared	0.82
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-21: Delta globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin E for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of delta globin and GAPDH were measured by real-time PCR. Delta globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of delta globin (P<0.049)

Cucurbitacin E induce epsilon globin in k562

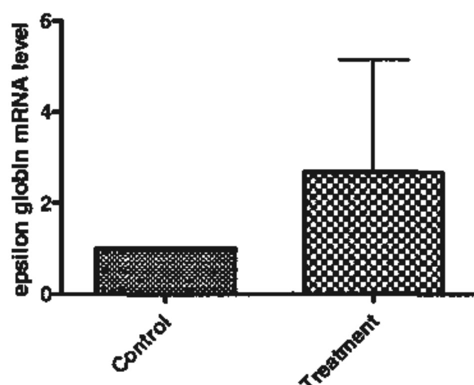


Table Analyzed	cu E induce epsilon globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0 1806
P value summary	ns
Are means signif different? (P < 0 05)	No
One- or two-tailed P value?	One-tailed
t, df	t=1 2 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-1 7
95% confidence interval	-7 8 to 4 5
R squared	0 41
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-22: Epsilon globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin E for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of epsilon globin and GAPDH were measured by real-time PCR. Epsilon globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of epsilon globin (P<0.187)

Cucurbitacin E induce gamma globin in k562

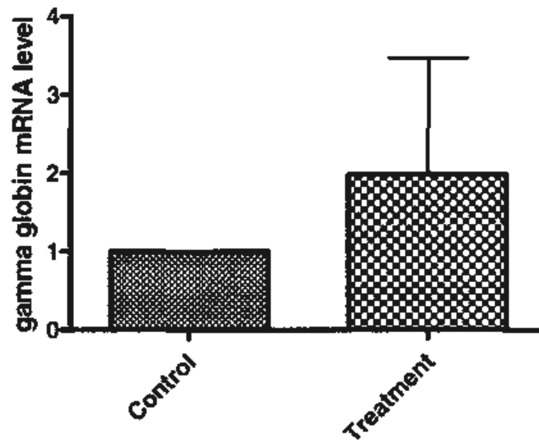


Table Analyzed	cu E induce gamma globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.1873
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=1.1 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-0.98
95% confidence interval	-4.7 to 2.7
R squared	0.39
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points.
P Value (one tailed)	
P value summary	

Figure 2-23: Gamma globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin E for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of gamma globin and GAPDH were measured by real-time PCR. Gamma globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of gamma globin (P<0.188)

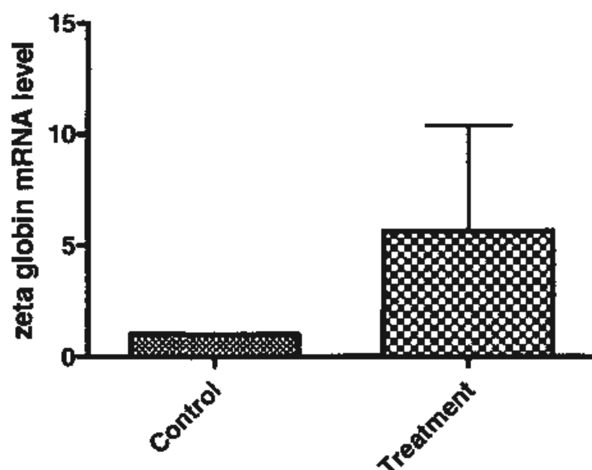


Table Analyzed	cu E induce zeta globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.1162
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
t, df	t=1.7 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-4.6
95% confidence interval	-16 to 7.2
R squared	0.59
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-24: Zeta globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin E for 48 hours. mRNA was collected and synthsised the cDNA. Expression level zeta globin and GAPDH were measured by real-time PCR. Zeta globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of zeta globin (P<0.117)

Cucurbitacin I induce alpha globin in k562

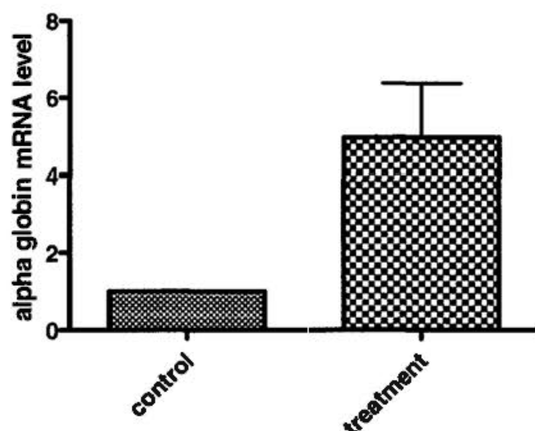


Table Analyzed	cu I induce alpha globin
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0.0199
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=4.9 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-4.0
95% confidence interval	-7.5 to -0.45
R squared	0.92
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-25: Alpha globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin I for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of alpha globin and GAPDH were measured by real-time PCR. Alpha globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of alpha globin (P<0.02)

Cucurbitacin I induce delta globin in k562

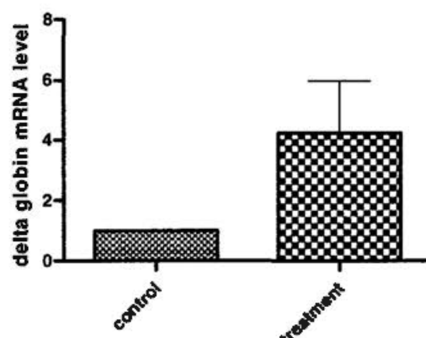


Table Analyzed	cu I induce delta globin
Column A	control
vs	vs
Column B	treatment
Paired t test	
P value	0.0406
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=3.3 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-3.3
95% confidence interval	-7.5 to 1.0
R squared	0.84
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-26: Delta globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin I for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of delta globin and GAPDH were measured by real-time PCR. Delta globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of delta globin (P<0.041)

Cucurbitacin I induce epsilon globin in k562

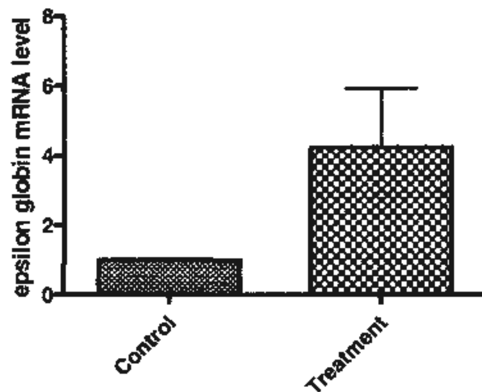


Table Analyzed	cu I induce epsilon globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.0402
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=3.3 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-3.2
95% confidence interval	-7.4 to 0.97
R squared	0.85
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-27: Epsilon globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin I for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of epsilon globin and GAPDH were measured by real-time PCR. Epsilon globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of epsilon globin (P<0.041).

Cucurbitacin I induce gamma globin in k562

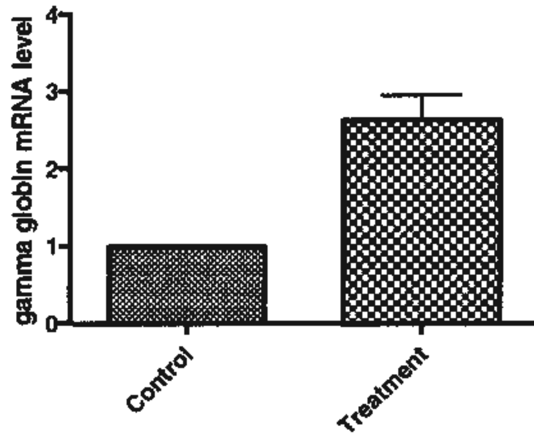


Table Analyzed	cu I induce gamma globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.0066
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=8.607 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-1.633
95% confidence interval	-2.450 to -0.8168
R squared	0.9737
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points
P Value (one tailed)	
P value summary	

Figure 2-28: Gamma globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin I for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of gamma globin and GAPDH were measured by real-time PCR. Gamma globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of gamma globin (P<0.007)

Cucurbitacin I induce zeta globin in k562

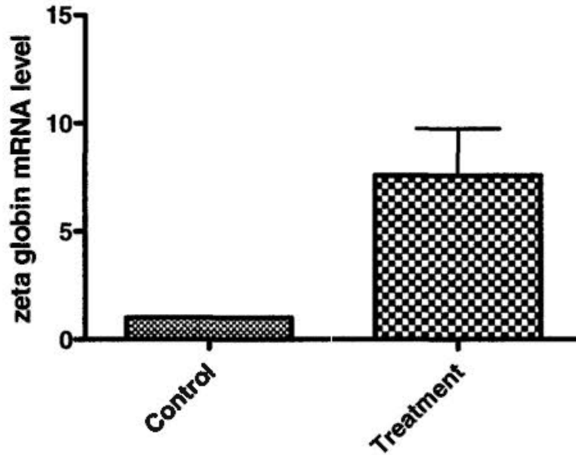


Table Analyzed	cu I induce zeta globin
Column A	Control
vs	vs
Column B	Treatment
Paired t test	
P value	0.0178
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
t, df	t=5.2 df=2
Number of pairs	3
How big is the difference?	
Mean of differences	-6.6
95% confidence interval	-12 to -1.1
R squared	0.93
How effective was the pairing?	
Correlation coefficient (r)	Linear correlation requires at least four points.
P Value (one tailed)	
P value summary	

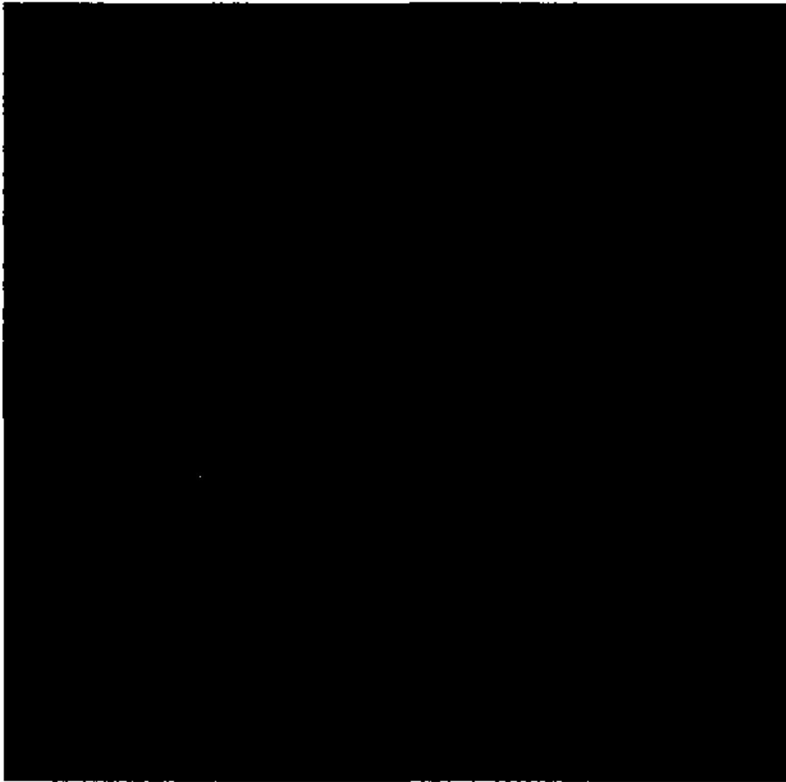
Figure 2-29: Zeta globin level was normalized by GAPDH

K562 was treated with 3.125ng/ml cucurbitacin I for 48 hours. mRNA was collected and synthsised the cDNA. Expression level of zeta globin and GAPDH were measured by real-time PCR. Zeta globin level was normalized by GAPDH. GraphPad Prism® software was used for statistical T test comparing the treated and untreated normalized expression level of zeta globin (P<0.018)

2.4.4. RESULT of Confocal Microscopic Examination

2.4.4.1 Effect of cucurbitacin D & hydroxyurea on inducing fetal hemoglobin in thalassemias major patient's CD 34+ stem cell

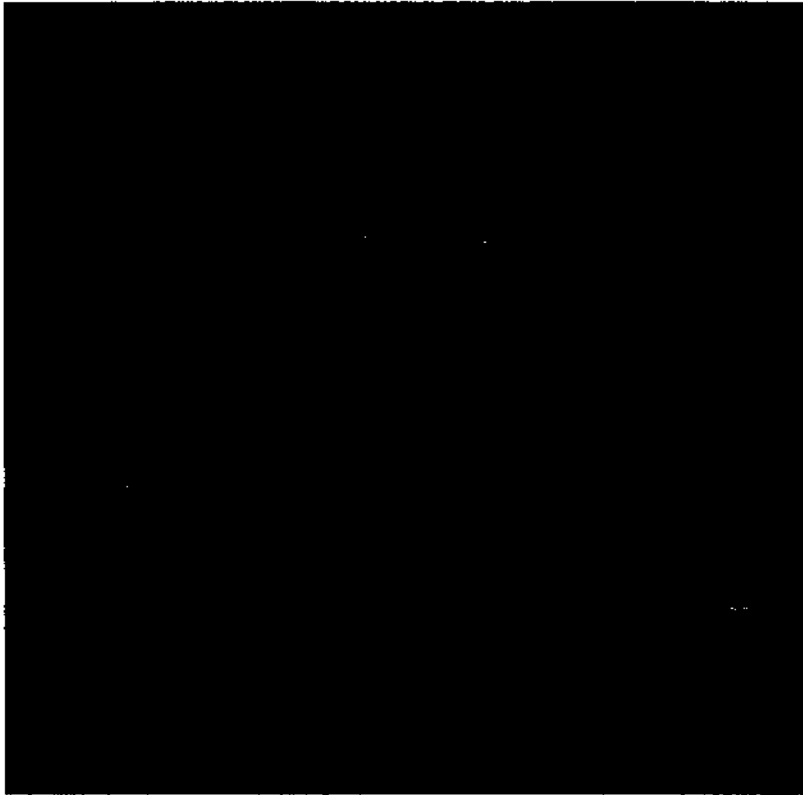
25 ng/ml cucurbitacin D and 25 ug/ml hydroxyurea were used to treat beta-thalassemia major patient progenitor cells ($CD34^+$) independently for 6 days, the cells were stained with anti-human fetal hemoglobin antibody with PE label. The image was observed under the immunofluorescence confocal microscrope. The results were shown as figures 2-30 and 2-31. The significant fetal hemoglobin induction effect was observed in the cucurbaticin D treated patient samples, but with a very weak response in hydroxyurea treated patient samples.



(Patient sample from Prince of Wales Hospital; Dr. KW CHIK)

Figure 2-30: **Fetal hemoglobin induction in patient progenitor cells by cucurbaticin D.**

25 ng/mlLC978 treated beta-thalassemia major patient progenitor cells ($CD34^+$) for 6 days, the cells were stained with anti-human fetal hemoglobin antibody with PE .label. Fetal hemoglobin expressing cells were stained red.



(Patient sample from Prince of Wales Hospital; Dr. KW CHIK)

Figure 2-31: **Fetal hemoglobin induction in patient progenitor cells by hydroxyurea.** 25 μg/ml

Hydroxyurea treated beta-thalassemia major patient progenitor cells ($CD34^+$) for 6 days, the cells were stained with anti-human fetal hemoglobin antibody with PE .label. Fetal hemoglobin expressing cells were stained red.

2.5 Discussion

Previously our laboratory demonstrated that cucurbitacin D can induce gamma globin gene expression and fetal hemoglobin production. In this study we have further extend to compare the induction potency of different cucurbitacin derivatives and hydroxyurea was used for comparison. The potency was evaluated at three levels by comparing the total hemoglobin production, fetal hemoglobin production and the gene expression level of different hemoglobin genes.

Based on the induction of fetal hemoglobin, cucurbitacin B was the most potent compound with the lowest ED_{50}, however, cucurbitacin D was more potent in producing total hemoglobin (Table 2-4). It is like that cucurbitacin B might induce more gamma globin production than cucurbitacin D, but cucurbitacin D may induced more production of other hemoglobins.

The steepness of a dose response curve can reflect the effectiveness of a drug.

A steep curve correlates with a higher effectiveness, since a small change in the dose could cause a greater response, while a flat curve correlates with a lower effectiveness since a large change in the dose can only cause a small response. From the steepness of the dose response curve, cu B is more potent than cu D in the induction of fetal hemoglobin.

Table 2-4 comparison of ED50 of different cucurbitacins for the induction of fetal hemoglobin and total hemoglobin

	Fetal hemoglobin production			Total hemoglobin production		
	ED_{50}		curve steepness	ED_{50}		curve steepness
Cucurbitacin B	0.36	ng/ml	0.749	0.76	ng/ml	2.941
Cucurbitacin D	1.5	ng/ml	1.750	0.60	ng/ml	2.278
Cucurbitacin E	21.1	ng/ml	0.036	13.96	ng/ml	1.800
Cucurbitacin I	0.9	ng/ml	0.535	1.1	ng/ml	2.157
Hydroxyurea	9.0	ug/ml	0.040	12.24	ug/ml	0.298

Based on the ED_{50} comparison between cucurbitacins and Hydroxyura, cucurbitacins were more potent than hydroxyurea in fetal hemoglobin production and total hemoglobion production. The results were shown as table 2-5.

We have also compared the induction of expression of different globin genes by different cucurbitacins in K562 cells (Table 2-6). From the two set of comparisons, we could conclude that cucurbitacins were significantly inducing total hemoglobin and fetal hemoglobin. The potency of cucurbitacins was much higher than hydroxyurea. And also had the promising effect increased the mRNA level of the globins.

Table 2-5: Comparation the increasing fold of total Hb & Hb-F between Hydroxyurea and cucurbitacins in k562 cell.

	Fetal hemoglobin production	Total hemoglobin production
	ED_{50} Cu/ HU	ED_{50} Cu/ HU
Cucurbitacin B	25000	16105
Cucurbitacin D	6000	20400
Cucurbitacin E	427	877
Cucurbitacin I	10000	11128
Hydroxyurea	1	1

Table 2-6: Comparation the increasing fold of mRNA of globins alpha, beta, epsilon, gamma, zeta between treated and untreated in k562 cell.

mRNA increasing fold after drug	Alpha globin treated/untreated	Beta globin treated/untreated	Epsilon globin treated/untreated	Gamma globin treated/untreated	Zeta globin treated/untreated
Cucurbitacin B	4.6	3.9	3.2	2.5	7.7
Cucurbitacin D	5.2	3.4	4.5	3	9.9
Cucurbitacin E	5.0	3.9	2.7	2.0	5.6
Cucurbitacin I	5	4.3	4.2	2.6	7.6

Finally we also tested whether cuD can induce fetal hemoglobin production in the bone marrow progenitor cells of the thalassemia major patient. Cucurbitain D showed a better induction of fetal hemoglobin production in the bone progenitor cells of the thalassemia major patient than hydroxyurea.

Chapter 3: Discovery of a potent fetal hemoglobin inducer from Chinese herbs

3.1. Introduction

There was a long history for using herb in healing disease in Chinese. Thousands of herbs were used for solving the complicated problem in human physical system. Chinese physicians have been using of herbs to maintain the physical condition in balance status for thousand years. Many Chinese medical regimes have been proved to be able to cure some diseases particular the chronic diseases. Thus it attracted many researchers and companies to explore the treasure of Chinese medicine. 2003 year, cucurbitacin D, in our laboratory, was discovered. It could induce the fetal hemoglobin and anti- cancer. However, the process of extraction was complicated.

It increased the difficulty to purify the compound and raise the cost of the production of cucurbitacin D. Moreover, *Trichosanthis* contained only very little amount of cucurbitacin. All the reasons advance us to unearth another source to improve the the production of cucurbitacin. On the other side, we made progressed in developing the new extraction and purification method.

Herbal extraction was an important step. The bioactive components preservation during the extraction process was the major concern. The various polarities of extraction solvents were selected. Due to the characteristic of the chemicals were different. The solvent selection hold up the category of component could be identified.

On the other hand, the different parts of the plant also made influenced on the process of the extraction. The complexity of tissue in the herbal would increase the difficulty to

purify the compound. The technical problem would increase the cost and decrease the production. The quality and purity of the production affected the characterization of the compound. In this chapter we explored to identify other herbal medicine that may contain more cucurbitacin or contain a more potent compound that can induce fetal hemoglobin production.

3.2. Materials

24 selected Chinese herbs for the inducing hemoglobin assay in k562 cell

No.	名稱	Scientific Name
3	天門冬	*Asparagus cochinchinensis*
8	防杞	*Stephania tetrandra S.Moore*
9	白朮	*Atractylodes macrocephala Koidz*
22	紅棗	*Ziziphus jujuba Mill.*
33	知母	*Rhizoma Anemarrhenae*
40	阿膠	*Colla Asini (Gelatinum Asini)*
43	茜草	*Rubia cordifolia L*
44	北耆	*Astragalus*
61	田七	*Panax Notoginseng*
62	貫仲	*Dryopteris crassirhizoma Nakal*
70	熟地	*Rehmannia glutinosa*
83	石葦	*Pyrrosia lingua*
84	當歸	*Angelica sinensis*
87	黃柏	*Phellodendron chinense Schneid*
99	絲瓜子	*Luffa cylindria*
100	胡蘆瓜子	*Lagenaria siceraria*
101	胡蘆	*Lagenaria siceraria*
118	蒼朮	*Aractylodes lancea*
124	南棗	*Ziziphus jujuba Mill*
125	黑棗	*Zizyphi Sativae Fructus*

126	蛇形絲瓜子	*Luffa cylindrica*
127	短棒瓠瓜子	*Cotyledon of Lagenaria sicieraria var.makinoi*
128	瓜蔞	*Fructus Trichosanthis*
129	蔞皮	*Pericarpium Trichosanthis*
130	青瓜子A	*Cucumi sativus A*
131	青瓜子B	*Cucumi sativus B*

Chemicals and Reagents	Company & Lot No.
Acetonitrile (ACN, HPLC grade)	BDH 152856K
Methanol (MeOH, HPLC grade)	BDH 15250
Ethanol (EtOH, Industrialized)	CSR 95
Ethanol (HPLC grade)	BDH 15338
Trifluoroacetic Acid (TFA)	BDH 15311

Equipments	Company & Lot No.
Bench-top microcentrifuge	Eppendorf 5415C
Bench-top centrifuge	Beckman
Freezing Dryer	Labconco
High Performance Liquid Chromatography System	Waters alliance 2695
Incubator	SHELDON 1535
Rotary Evaporator	Buchi R144 A
Solid Phase Extraction (SPE) System	Waters

3.3. Method

Herbs preparation

25 herbs were selected for the experiment.

Two different polarity solvents were used to prepare the extraction, one was milli-Q water, and the other was absolute ethanol. The extractions were prepared for treating k562 cell. They were verified their efficiency of inducing hemoglobin in k562 bioassay. All Chinese herbs for experimental use were dry form. When the herbs were collected, they were coarsely chopped into smaller pieces. Then measured their weight and made the extraction.

3.3.1. Extractions

Watery extraction—extraction A

1. 5 grams of herbs were immersed into milli- Q water and boiled them for 1.5 hrs, always checked the water level during the boiling process. Ensure the water always laid over the herbs.
2. After boiling, threw away all the solid material. Span down the liquid compartment at 3000 r.p.m. Boiled the herbs continuously, until the volume droped to about 10ml.
3. Spin down the precipitation, and filter the extraction by Millipore 0.45μm ester cellulose.
4. At last, lyophilized the extraction become power.
5. Melts the power in 2ml milli Q water firstly, however, some of herbal extractions were found not melts in good condition, Then add more water and stored them in 65℃ incubator for two days and boiled it again to 5ml.

Ethanol extraction —extraction B

1. 5 gm herbs put into 95% industry grade ethanol for four days, incubated the mixture in 65 ℃. During the incubation period, sonication was used for extraction.
2. After incubation, the solid material was removed away, spin down the precipitation. Rotary evaporation removed the ethanol, the extraction became very viscose.
3. Added back 95% ethanol to extraction until the final volume of extractions were about 5ml. Made sure that in the rotary evaporate process, the extraction could not separate into two phase or formed precipitation.

3.3.2. K562 cell assay

1. The method was the same as the chapter II mention. Take 100 ul of 1g/ml extraction as the initial dosage.
2. Make serial two fold dilution until the 1/2048 times of the original dosage. 96 wells plate would be used for the experiment.
3. Hydroxyurea was used as a positive control and compared with the 23herbs.
4. Statistical T tests analyzed the variance between hydroxyurea and one of the selected herb. If the variences were not significant, ($P > 0.05$) they were considered as a positive result.
5. The null hypothesis assumed that hydroxyurea and 23 herbs both could induced the hemoglobin in k562 cell. If the result was against the null hypothesis, it meant the herbs could not produce the hemoglobin in k562 cell.

3.3.3. Reversed -phased chromatography (solid phase extraction)

Reversed –phase chromatography was one of separation method. The crude extract contain abundant of ingredients. It exhibited the different solubility in polar or non polar solvent. The fluid flowed through the column named mobile phase. The material of adsorbent inside the column called stationary phase. When the molecules passed through the column, the different compound would adsorb to the stationary phase in certain affinity. The different compounds were eluted from the adsorbent material in a specify condition, such as pH, polarity and molecular size etc. Due to the different migration rates of the compound, so we could collect the isolated compound in elution under a specify condition. The process was involved optimizing the binding and eluting condition.

C18 column would be used for isolating the interested compound, C18 was a hydrophobic material coated on silica particles. It contained 18 carbon chain attached to the silica surface and exhibited the attraction of non-polar molecule.

Concentrated the elution by votary evaporation and followed with lyophilization. The eluted samples were used for k562 cell assay and assess the efficacy of the compound

Sample preparation for solid phase extraction

5ml *Luffa cylindria(絲瓜子)* ethanol extraction was diluted to 100ml with ~50% ethanol, it was used for SPE purification. Repeated SPE step until all 100ml dilution had finished.

Fractionation

1. C 18 solid phase extraction (SPE) column was used for purify the compound. First equilibrate the new column with absolute ethanol (HPLC grade), then milli-Q water followed.
2. Conditional the column first with 20 ml 40% methanol, 15ml herb extraction followed, and then added 15 ml 40% methanol, the elution buffer collected as **fraction X**
3. The column further washed with 15ml 60 % methanol, the elution buffer collected as **fraction Y**
4. 100 % methanol displaced 60 % methanol binding buffer, the elution was discarded. The column finally washed by milli-Q water, the elution was discarded.
5. Concentrated the fraction X & Y with vaccum evaporation to about 1ml, then dissolved them 40 % ethanol and adjusted the final volumn to 2.5ml, and then filtered the sample with 0.22 um filter membrane.
6. The filtered fraction X & Y were the drugs for k562 cell assay. Due to fraction X & Y showed the significant induction hemoglobin effect, mixing them together, then the mixture was further purified by HPLC method.

3.3.4. High-pressure liquid chromatography (HPLC)

A high pressure pump forced the solvent through the tightly packed HPLC column; the electronic UV detectors monitored the appearance of the elution. The improvement of the column with uniform, spherical particles, the small size and special design increased the surface area for adsorption. The coated silica microspheres within the column were trended toward to adsorb the compound in a certain condition.

1. After repeated several times of solid phase extraction, 90 ml fraction X & Y were collected separately.
2. They were concentrated by vacuum evaporation mixed them together; the volume was about 5ml.
3. 10ul of 5ml concentrated fraction X & Y was injected into HPLC machine. After injection, samples were collected each 5 minutes.
4. About 20 ml of each fraction was collected and placed in 65℃ incubator. The samples were dried up and the volumes were down to 1ml.
5. The samples were placed at -70°C and lyophilized. 100 ul of dried up samples were used for k562 cell assay. The working method and the results were mention as below.

HPLC method information

Workstation:	Waters® Millennium^{32} chromatography workstation
machine:	Waters® alliance 2690/5 separation module
Detector:	PDA 996 UV / Visible detector
PDA acquisition:	210-400 nm, 2.4nm spectral resolution, maximum plot
Stationary phase:	Nova-Pak® C_{18}, 7.8×300 mm
Mobile Phase:	Milli-Q water and Methanol
Solvent A :	Methanol
Solvent B :	Milli-Q water
Flow Rate:	3.0 ml/min
Gradient table:	
0.00 min	100%degas milli-Q water and 0.0%methanol
10.00 min	100%degas milli-Q water and 0.0%methanol
10.01 min	100% methanol and 0.0 % degas milli-Q water
20.00min	10 ul sample injectionWater-40.0%
20.01 min	45.0%methanol and 55% degas milli-Q water
55.00 min	45.0%methanol and 55% degas milli-Q water
55.01 min	100.0%methanoland 0.0% degas milli-Q water
65.00 min	0.0%methanoland100.0% degas milli-Q water

3.4. Results

3.4.1. Hemoglobin induction activities of 25 herbs in k562 cell

Water or ethanol extracts of 24 herbs were prepared and tested on the ability of inducing hemoglobin production in K562 cells. Total hemoglobin was detected by TMB staining. Hydroxyurea was used as a comparison of the potency. 10 herbs showed positive result, $P < 0.05$. The summarized result was shown in table 3-1 and table3-2. The P values showed in table3-3 to table 3-19.

Dose-response curves were drafted by GraphPad Prism®4.0 software .The data were normalized to 100%. The ED_{50} results were shown as table 3-2, the dose-response curves were shown in figure 3-1 to figure 3-18.

Ethanol extract of *Luffa cylindria* showed a very high potency of inducing hemoglobin production in k562. The ED_{50} of *Luffa cylindria* extract was 9.90e-005 mg/ml while *Fructus Trichosanthis* was 0.31 mg/ml. The ED_{50} of crude extraction of *Luffa cylindria*絲瓜子was 31935 times higher than the crude extraction of Fructus *Trichosanthis*瓜蔞

Table 3-1 Hemoglobin induction of 25 herbs in k562 cell

No.	名稱	Scientific Name	Results
3	天門冬	*Asparagus cochinchinensis*	Negative
8	防杞	*Stephania tetrandra S.Moore*	Positive
9	白朮	*Atractylodes macrocephala Koidz*	Positive
22	紅棗	*Ziziphus jujuba Mill.*	Negative
33	知母	*Rhizoma Anemarrhenae*	Positive
40	阿膠	*Colla Asini (Gelatinum Asini)*	Negative
43	茜草	*Rubia cordifolia L*	Negative
44	北耆	*Astragalus*	Negative
61	田七	*Panax Notoginseng*	Negative
62	貫仲	*Dryopteris crassirhizoma Nakal*	Negative
70	熟地	*Rehmannia glutinosa*	Negative
83	石葦	*Pyrrosia lingua*	Negative
84	當歸	*Angelica sinensis*	Negative
87	黃柏	*Phellodendron chinense Schneid*	Negative
99	絲瓜子	*Luffa cylindria*	Positive
100	胡蘆瓜子	*Lagenaria siceraria*	Negative
101	胡蘆	*Lagenaria siceraria*	Positive
118	蒼朮	*Aractylodes lancea*	Positive
124	南棗	*Ziziphus jujuba Mill*	Negative
125	黑棗	*Zizyphi Sativae Fructus*	Positive
126	蛇形絲瓜子	*Luffa cylindrica*	Negative
127	短棒瓠瓜子	*Cotyledon of Lagenaria sicieraria var.makinoi*	Positive

128	瓜蔞	*Fructus Trichosanthis*	Positive
129	蔞皮	*Pericarpium Trichosanthis*	Positive
130	青瓜子A	*Cucumi sativus A*	Negative
131	青瓜子B	*Cucumi sativus B*	Negative

24 herbs were selected to induce hemoglobin in k562. The extraction of the herbs was titrated in different concentration and treated 2 x10^3 k562 cells for 6 days. The experiment performed in 96 well plate. The cells were lysed and stained by TMB. The intensity of color was compared with untreated control cell lysate. If the color intensity was significantly higher than control, the result would be indicated as positive.

Table 3-2: Hemoglobin induction of 10 herbs in k562

Comparison to HU	t-tests, P value	ED_{50} (mg/ml, dry weight to volume of extract)
8A 防杞	0.0713	~33.9
8B 防杞	0.4417	0.31
9A 白朮	0.1584	----
9B白朮	0.4274	2.926e-007
33A 知母	0.4676	44.09
33B 知母	0.1626	1.2
43B 茜草	0.4676	1.08
99A 絲瓜子	0.1350	0.06
99B 絲瓜子	0.1059	9.90e-005
101A 胡蘆	0.2318	0.12
101B 胡蘆	0.1001	0.99
118A 蒼朮	0.4325	1.085e-011
125A黑棗	0.4734	3329
127A短棒瓠瓜子	0.0926	27.10
127B短棒瓠瓜子	0.4353	1.484e-006
128A 瓜蔞	0.5	0.84
128B 瓜蔞	0.18	0.31
129A 蔞皮	0.3119	1.44
129B 蔞皮	0.1801	0.51

Extraction of 10 herbs induced hemoglobin in k562. Tests compared the significant of inducing effect between the herbs and hydroxyurea. The dose-response curves drafted by GraphPad Prism4.0 software. ED_{50}s were calculated from the curves.

3.4.2. Hemoglobin induction ability of partially purified Luffa cylindria extract

C 18 solid phase extraction (SPE) column was used for purify the hemoglobin activity of the Luffa cylindria. 絲瓜子After added the crude extract to the column, the column was rinsed with 40% & 60 % methanol. The elution of 40% & 60 % methanol were collected and concentrated. The concentrated elutes were used to treat k562 cell.
T tests was used to analyze variances of hemoglobin production between *Trichosanthes rosthornii Harms*瓜蔞crude extract (positive control) and elutes in 40 % & 60 % methanol. The P values were 0.1301 & 0.2352. There was no significant different between positive control and elutes. They both could induce hemoglobin in k562 cell assay.
The comparison between 60% methanol elute & the original Luffa cylindria crude extract, P> 0.4, they both could induce hemoglobin in k562 cell assay.
The comparison between 40% methanol elute & the untreated cell lysate, P< 0.01, there was significant difference in inducing hemoglobin effect in k562 cell. All the t tests results from reverse- phase chromatography were shown as table 3-20 to 3-23.

3.4.3. HPLC elution for hemoglobin induction in k562 cell

After solid phase extraction, 40 and 60 % methanol elution fractions were pooled together, concentrated and separated by HPLC as described in the methods. In HPLC chromatography the samples were collected at each 5 min and used to induce k562 cell for hemoglobin production.

The fraction collected at 15min and 25 min showed the induction effect in the cell assay, while the other fractions did not show any induction activities. The dose-response curve was draft by statistic program *Prism® 4.0* and ED_{50} was calculated. The HPLC result was shown in figure 3-21. The arrows indicated the location of the peaks. Dose-response curves of fractions collected at 15min and 25 min were shown as figure 3-19 & 3-20.

ED_{50} of fraction collected at 15 min fraction was 1.95 mg/ml

ED_{50} of fraction collected at 25 min fraction was 1.254 mg/ml

Comparison efficacy in hemoglobin production between 8A防杞extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 8A/Hu
Column A	8A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.0713
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	45.5 , 45.5
Mann-Whitney U	9.50

Table 3-1: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb8A 防杞in 6 days k562 bioassay. The P>0.07 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 8B防杞extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 8B/Hu
Column A	8B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.4417
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	57.5 , 33.5
Mann-Whitney U	18.5

Table 3-2: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 8B 防杞in 6 days k562 bioassay. The P> 0.44 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 9A 白朮 extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 9A
Column A	9A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.1584
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	75.5 , 29.5
Mann-Whitney U	14.5

Table 3-3: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 9A 白朮 in 6 days k562 bioassay. The P> 0.15 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 9B 白朮 extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 9B/Hu
Column A	9B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.4274
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	34.5 , 31.5
Mann-Whitney U	13.5

Table 3-4: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 9B 白朮 in 6 days k562 bioassay. The P> 0.42 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 33A知母extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 33A/Hu
Column A	33A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.4676
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	44.5 , 33.5
Mann-Whitney U	16.5

Table 3-5: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb33A 知母 in 6 days k562 bioassay. The P>0.46 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 33B知母extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 33B/Hu
Column A	33B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.1626
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	24.5 , 20.5
Mann-Whitney U	5.50

Table 3-6: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 33B 知母 in 6 days k562 bioassay. The P> 0.16 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 43B茜草extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 43B/Hu
Column A	43B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.4676
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	44.5 , 33.5
Mann-Whitney U	16.5

Table 3-7: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb43B 茜草in 6 days k562 bioassay. The P> 0.46 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 99A絲瓜子extraction and hydroxyurea in k562 cell.

Table Analyzed	99A/HU
Column A	99A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.1272
Exact or approximate P value?	Exact
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	90 , 30
Mann-Whitney U	15

Table 3-8: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 99A 絲瓜子 in 6 days k562 bioassay. The P> 0.13 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 99B絲瓜子extraction and hydroxyurea in k562 cell.

Table Analyzed	99B/HU
Column A	99B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.0631
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	333 , 45
Mann-Whitney U	30

Table 3-9: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 99B 絲瓜子 in 6 days k562 bioassay. The P>0.11 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between101A胡蘆extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 101A/Hu
Column A	101A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.2318
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	61.5 , 29.5
Mann-Whitney U	14.5

Table 3-10: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 101A 胡蘆in 6 days k562 bioassay. The P> 0.23 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 101B胡蘆extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 101B/Hu
Column A	101B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.1001
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	43.5 , 22.5
Mann-Whitney U	7.50

Table 3-11: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 101B 胡蘆in 6 days k562 bioassay. The P>0.1 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 118A蒼朮extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 118A/Hu
Column A	118A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.4325
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	91.5 , 44.5
Mann-Whitney U	25.5

Table 3-12: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 118A 蒼朮in 6 days k562 bioassay. The P>0.43 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 125A黑棗extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 125A/Hu
Column A	125A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.4734
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	67.5 , 37.5
Mann-Whitney U	22.5

Table 3-13: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 125A黑棗n 6 days k562 bioassay. The P> 0.47 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 127A短棒瓠瓜子extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 127A/Hu
Column A	127A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.0962
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	81.5 , 54.5
Mann-Whitney U	15.5

Table 3-14: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 127A短棒瓠瓜子in 6 days k562 bioassay. The P>0.096 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 127B 短棒瓠瓜子extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 127B/Hu
Column A	127B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.4353
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	44 , 34
Mann-Whitney U	16.0

Table 3-15: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 127B短棒瓠瓜子in 6 days k562 bioassay. The P> 0.43 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 128A瓜蔞extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 128A/Hu
Column A	128A
vs	vs
Column B	HU
Mann Whitney test	
P value	0.5000
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	56.5 , 34.5
Mann-Whitney U	19.5

Table 3-16: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb128A 瓜蔞 in 6 days k562 bioassay. The P> 0.49 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 128B瓜蔞 extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 128B/Hu
Column A	128B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.1801
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	41.5 , 24.5
Mann-Whitney U	9.50

Table 3-17: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 128B 瓜蔞 in 6 days k562 bioassay. The P> 0.18 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between101A胡[illegible]extraction and hydroxyurea in k562 cell

Table Analyzed	Normalize of 129A/Hu
Column A	129A
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.3119
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	75.5 , 44.5
Mann-Whitney U	20.5

Table 3-18: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb 129A 蘡皮 in 6 days k562 bioassay. The P> 0.31 showed that the variance was not significant. The null hypothesis was accepted.

Comparison efficacy in hemoglobin production between 129B 蘡皮 extraction and hydroxyurea in k562 cell.

Table Analyzed	Normalize of 129B/Hu
Column A	129B
vs	vs
Column B	Hu
Mann Whitney test	
P value	0.1801
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	41.5 , 24.5
Mann-Whitney U	9.50

Table 3-19: Statistical T tests from GraphPad Prism 4.0 analyzed the variance between hydroxyurea and the herb129B 蘡皮 in 6 days k562 bioassay. The P> 0.18 showed that the variance was not significant. The null hypothesis was accepted.

Hemoglobin induction by crude extract 8A 防杞 in k562 cell Line.

	8A
log(agonist) vs response -- Variable slope	Ambiguous
Best-fit values	
BOTTOM	20 67
TOP	~ 5120
LOGEC50	~ 0 5312
HILLSLOPE	~ 2 822
EC50	~ 3 397
Span	~ 5099
Std Error	
BOTTOM	3 060
TOP	~ 6 540e+006
LOGEC50	~ 206 1
HILLSLOPE	~ 25 19
Span	~ 6 540e+006
95% Confidence Intervals	
BOTTOM	14 24 to 27 10
TOP	(Very wide)
LOGEC50	(Very wide)
HILLSLOPE	(Very wide)
EC50	(Very wide)
Span	(Very wide)
Goodness of Fit	
Degrees of Freedom	18
R2	0 8335
Absolute Sum of Squares	2260
Sy x	11 21
Number of points	
Analyzed	22

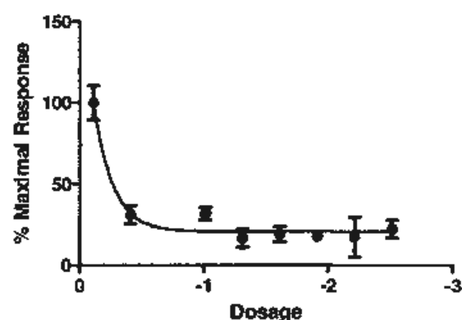


Figure 3-1: The serial two fold dilution of crude extract of 8A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated ~33.9mg/ml.

Hemoglobin induction by crude extract 8B 防杞 in k562 cell line

	8B
log(agonist) vs response -- Variable slope	
Best fit values	
BOTTOM	26 61
TOP	100 6
LOGEC50	-1 509
HILLSLOPE	2 289
EC50	0 03096
Span	73 98
Std Error	
BOTTOM	4 156
TOP	3 833
LOGEC50	0 05582
HILLSLOPE	0 6109
Span	6 244
95% Confidence Intervals	
BOTTOM	17 94 to 35 28
TOP	92 59 to 108 6
LOGEC50	-1 628 to -1 393
HILLSLOPE	1 014 to 3 563
EC50	0 02368 to 0 04046
Span	60 96 to 87 01
Goodness of Fit	
Degrees of Freedom	20
R2	0 9348
Absolute Sum of Squares	1574
Sy x	8 870
Number of points	
Analyzed	24

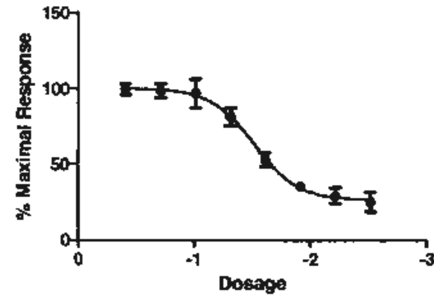


Figure 3-2: The serial two fold dilution of crude extract of 8B treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 0.31mg/ml.

Hemoglobin induction by crude extract 9B 白朮 in k562 cell Line.

	9B
log(agonist) vs response – Variable slope	Ambiguous
Best-fit values	
BOTTOM	6 517
TOP	~ 12653
LOGEC50	~ 7 534
HILLSLOPE	~ -0 4270
EC50	~ 2 926e-008
Span	~ 12646
Std Error	
BOTTOM	161 2
TOP	~ 1 122e+007
LOGEC50	~ 942 6
HILLSLOPE	~ 3 062
Span	~ 1 122e+007
95% Confidence Intervals	
BOTTOM	-339 2 to 352 3
TOP	(Very wide)
LOGEC50	(Very wide)
HILLSLOPE	(Very wide)
EC50	(Very wide)
Span	(Very wide)
Goodness of Fit	
Degrees of Freedom	14
R2	0 7129
Absolute Sum of Squares	4152
Sy x	17 22
Number of points	
Analyzed	18

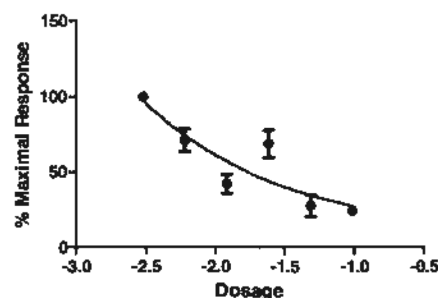


Figure 3-3: The serial two fold dilution of crude extract of 9B treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0.

The data was normalized. ED $_{50}$ was calculated 2.926 e-007mg/ml.

Hemoglobin induction by crude extract 33A 知母 in k562 cell Line.

	33A
log(agonist) vs. response - Variable slope	Ambiguous
Best-fit values	
BOTTOM	36 79
TOP	~ 3400
LOGEC50	~ 0 6443
HILLSLOPE	1.273
EC50	~ 4 409
Span	~ 3363
Std Error	
BOTTOM	19 43
TOP	~ 981694
LOGEC50	~ 106 2
HILLSLOPE	4 671
Span	~ 981706
95% Confidence intervals	
BOTTOM	5 549 to 79 12
TOP	(Very wide)
LOGEC50	(Very wide)
HILLSLOPE	-8 906 to 11 45
EC50	(Very wide)
Span	(Very wide)
Goodness of Fit	
Degrees of Freedom	12
R2	0 4136
Absolute Sum of Squares	8799
Sy.x	27 08
Number of points	
Analyzed	16

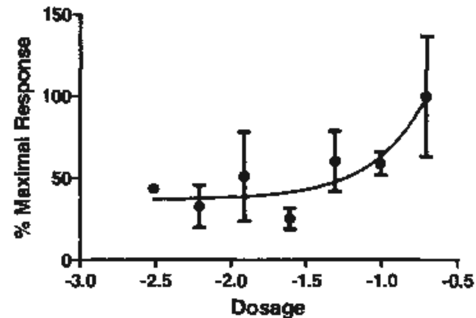


Figure 3-4: The serial two fold dilution of crude extract of 33A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated ~44.09mg/ml.

Hemoglobin induction by crude extract 33B 知母 in k562 cell Line.

	33B
log(agonist) vs response – Variable slope	
Best-fit values	
BOTTOM	35 22
TOP	108 1
LOGEC50	-0 9215
HILLSLOPE	4 243
EC50	0 1198
Span	72 91
Std Error	
BOTTOM	8 612
TOP	158 7
LOGEC50	0 7957
HILLSLOPE	23 27
Span	182 5
95% Confidence Intervals	
BOTTOM	15 36 to 55 08
TOP	-257.9 to 474 2
LOGEC50	-2 756 to 0 9134
HILLSLOPE	-49 41 to 57 90
EC50	0 001752 to 8 192
Span	-301 9 to 447 7
Goodness of Fit	
Degrees of Freedom	8
R2	0 5279
Absolute Sum of Squares	3712
Sy x	21 54
Number of points	
Analyzed	12

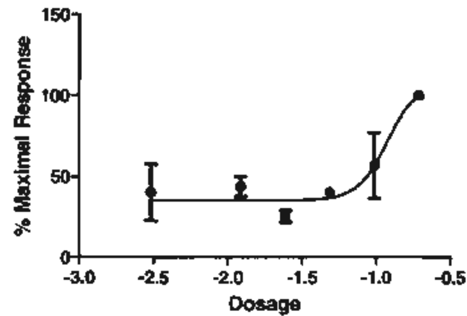


Figure 3-5: The serial two fold dilution of crude extract of 33B treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED_{50} was calculated 1.20 mg/ml.

Hemoglobin induction by crude extract 43B 茜草 in k562 cell Line.

	43B
log(agonist) vs. response -- Variable slope	
Best-fit values	
BOTTOM	25 30
TOP	135 6
LOGEC50	-0 9688
HILLSLOPE	1 120
EC50	0 1075
Span	110 3
Std Error	
BOTTOM	14 34
TOP	139 8
LOGEC50	0 9711
HILLSLOPE	1 261
Span	149 9
95% Confidence Intervals	
BOTTOM	-5 687 to 56 27
TOP	-166 4 to 437 6
LOGEC50	-3 066 to 1 129
HILLSLOPE	-1 603 to 3 844
EC50	0 0008581 to 13 45
Span	-213 4 to 434 1
Goodness of Fit	
Degrees of Freedom	13
R2	0 7088
Absolute Sum of Squares	3222
Sy x	15 74
Number of points	
Analyzed	17

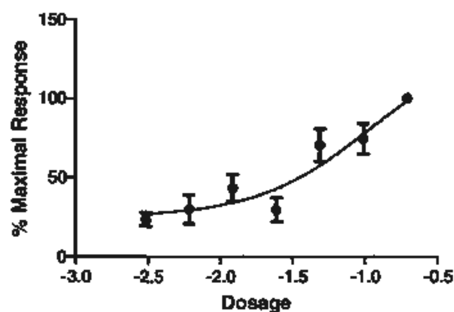


Figure 3-6: The serial two fold dilution of crude extract of 43B treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED_{50} was calculated 1.08 mg/ml.

Hemoglobin induction by crude extract 99A 絲瓜子 in k562 cell Line.

	99A
log(agonist) vs. response -- Variable slope	
Best-fit values	
BOTTOM	6 484
TOP	98 39
LOGEC50	2 200
HILLSLOPE	1 922
EC50	0 006302
Span	89 91
Std Error	
BOTTOM	3 915
TOP	6 817
LOGEC50	0 06639
HILLSLOPE	0 5260
Span	8 895
95% Confidence intervals	
BOTTOM	0 3838 to 18 58
TOP	84 28 to 112 5
LOGEC50	-2 342 to 2 059
HILLSLOPE	0 8341 to 3 011
EC50	0 004550 to 0 008730
Span	71 92 to 107 9
Goodness of Fit	
Degrees of Freedom	23
R2	0 9237
Absolute Sum of Squares	2777
Sy x	10 99
Number of points	
Analyzed	27

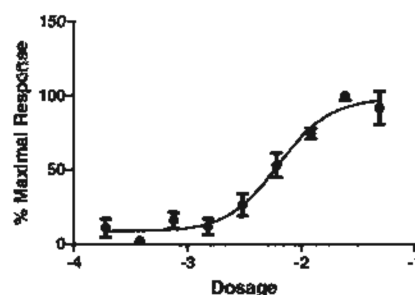


Figure 3-7: The serial two fold dilution of crude extract of 99A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 0.06 mg/ml.

Hemoglobin induction by crude extract 99B 絲瓜子 in k562 cell Line.

	99B
log(agonist) vs. response -- Variable slope	
Best-fit values	
BOTTOM	5 381
TOP	78 31
LOGEC50	5 004
HILLSLOPE	0 7087
EC50	9 898e-006
Span	72 93
Std. Error	
BOTTOM	3 349
TOP	4 416
LOGEC50	0 1557
HILLSLOPE	0 1692
Span	6 281
95% Confidence Intervals	
BOTTOM	1 315 to 12 08
TOP	69 47 to 87 14
LOGEC50	5 316 to -4 693
HILLSLOPE	0 3703 to 1 047
EC50	4 833e-006 to 2 027e 005
Span	60 37 to 85 48
Goodness of Fit	
Degrees of Freedom	62
R2	0 6522
Absolute Sum of Squares	9481
Sy.x	12 35
Number of points	
Analyzed	66

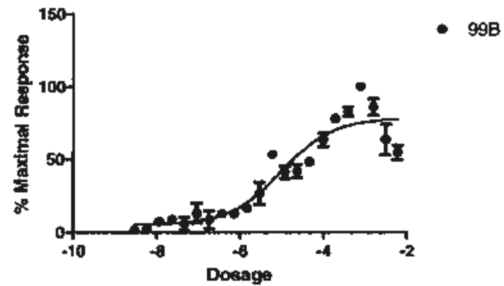


Figure 3-8: The serial two fold dilution of crude extract of 99B treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized.

ED $_{50}$ was calculated 9.90e⁻005mg/ml.

Hemoglobin induction by crude extract 101A 胡蘆 in k562 cell Line.

	101A
log(agonist) vs. response – Variable slope	
Best-fit values	
BOTTOM	19 96
TOP	90 09
LOGEC50	-1 939
HILLSLOPE	2 514
EC50	0 01150
Span	70 13
Std. Error	
BOTTOM	9 350
TOP	3 636
LOGEC50	0 08448
HILLSLOPE	1 075
Span	10 81
95% Confidence Intervals	
BOTTOM	0 4538 to 39 46
TOP	82 50 to 97 67
LOGEC50	-2 116 to -1 763
HILLSLOPE	0 2716 to 4 756
EC50	0 007664 to 0 01726
Span	47 59 to 92 67
Goodness of Fit	
Degrees of Freedom	20
R2	0 6639
Absolute Sum of Squares	2606
Sy.x	11 42
Number of points	
Analyzed	24

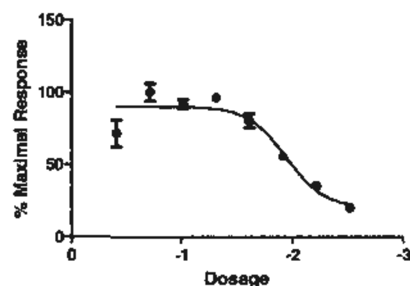


Figure 3-9: The serial two fold dilution of crude extract of 101A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 0.12 mg/ml.

Hemoglobin induction by crude extract 101B 胡蘆 in k562 cell Line.

	101B
log(agonist) vs response Variable slope	Ambiguous
Best fit values	
BOTTOM	1 815
TOP	96 97
LOGEC50	~ 1 005
HILLSLOPE	~ -15 97
EC50	~ 0 09888
Span	95 15
Std Error	
BOTTOM	3 440
TOP	2 264
LOGEC50	~ 1 239
HILLSLOPE	~ 3391
Span	4 536
95% Confidence intervals	
BOTTOM	5 517 to 9 148
TOP	92 14 to 101 8
LOGEC50	(Very wide)
HILLSLOPE	(Very wide)
EC50	(Very wide)
Span	86 48 to 104 8
Goodness of Fit	
Degrees of Freedom	15
R2	0 9610
Absolute Sum of Squares	659 7
Sy x	6 632
Number of points	
Analyzed	19

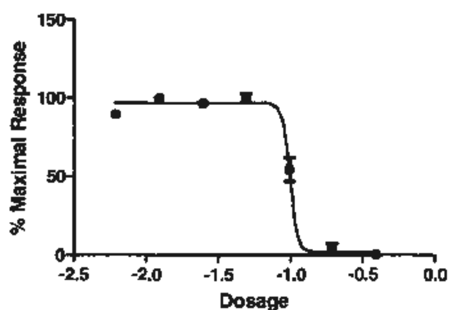


Figure 3-10: The serial two fold dilution of crude extract of 101B treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED_{50} was calculated 0.99 mg/ml.

Hemoglobin induction by crude extract 118A 蒼朮 in k562 cell Line.

	118A
log(agonist) vs response -- Vanable slope	Ambiguous
Best-fit values	
BOTTOM	~ -4100
TOP	~ 176 4
LOGEC50	~ -11 96
HILLSLOPE	~ 0 1492
EC50	~ 1 085e-012
Span	~ 4277
Std. Error	
BOTTOM	~ 2 256e+006
TOP	~ 1752
LOGEC50	~ 1845
HILLSLOPE	~ 4 446
Span	~ 2 257e+006
95% Confidence Intervals	
BOTTOM	(Very wide)
TOP	(Very wide)
LOGEC50	(Very wide)
HILLSLOPE	(Very wide)
EC50	(Very wide)
Span	(Very wide)
Goodness of Fit	
Degrees of Freedom	15
R2	0 5963
Absolute Sum of Squares	8353
Sy x	23 60
Number of points	
Analyzed	19

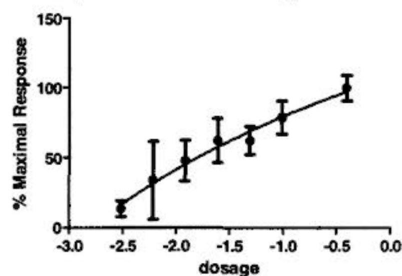


Figure 3-11: The serial two fold dilution of crude extract of 118A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 1.085e-011mg/ml.

Hemoglobin induction by crude extract 125A 黑棗 in k562 cell Line.

	125A
log(agonist) vs response -- Variable slope	Ambiguous
Best-fit values	
BOTTOM	18 56
TOP	~ 10856
LOGEC50	~ 2 522
HILLSLOPE	~ 0 6059
EC50	~ 332 9
Span	~ 10848
Std Error	
BOTTOM	104 8
TOP	~ 1 232e+007
LOGEC50	~ 845 5
HILLSLOPE	~ 4 536
Span	~ 1 232e+007
95% Confidence Intervals	
BOTTOM	-212 1 to 249 3
TOP	(Very wide)
LOGEC50	(Very wide)
HILLSLOPE	(Very wide)
EC50	(Very wide)
Span	(Very wide)
Goodness of Fit	
Degrees of Freedom	11
R2	0 5159
Absolute Sum of Squares	6783
Sy x	24 83
Number of points	
Analyzed	15

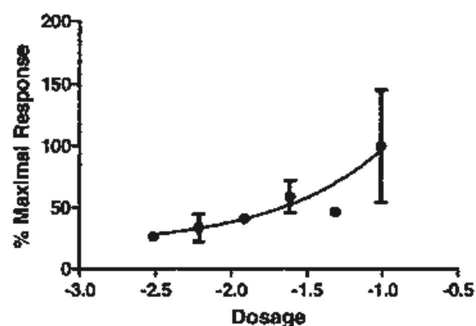


Figure 3-12: The serial two fold dilution of crude extract of 125A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 3329.0 mg/ml.

Hemoglobin induction by crude extract 127A 短捧瓠瓜子 in k562 cell Line.

	127A
log(agonist) vs response -- Variable slope	
Best fit values	
BOTTOM	7 168
TOP	88 63
LOGEC50	0 4330
HILLSLOPE	2 516
EC50	2 710
Span	81 47
Std Error	
BOTTOM	2 480
TOP	4 242
LOGEC50	0 04709
HILLSLOPE	0 6027
Span	5 188
95% Confidence Intervals	
BOTTOM	2 089 to 12 25
TOP	79 95 to 97 32
LOGEC50	0 3366 to 0 5295
HILLSLOPE	1 282 to 3 750
EC50	2 171 to 3 384
Span	70 84 to 92 09
Goodness of Fit	
Degrees of Freedom	28
R2	0 9373
Absolute Sum of Squares	2457
Sy x	9 367
Number of points	
Analyzed	32

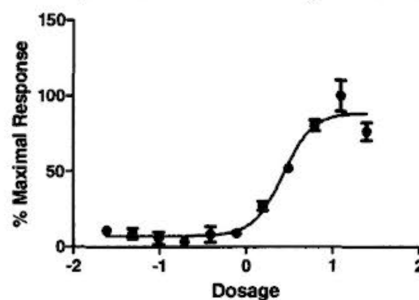


Figure 3-13: The serial two fold dilution of crude extract of 127A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 27.10 mg/ml.

Hemoglobin induction by crude extract 127B 短捧瓠瓜子 in k562 cell Line.

	127B
log(agonist) vs response – Variable slope	Ambiguous
Best-fit values	
BOTTOM	11 69
TOP	~ 10126
LOGEC50	~ -6 828
HILLSLOPE	~ -0 4816
EC50	~ 1 484e-007
Span	~ 10115
Std Error	
BOTTOM	131 9
TOP	~ 9 328e+006
LOGEC50	~ 869 9
HILLSLOPE	~ 3 681
Span	~ 9 328e+006
95% Confidence intervals	
BOTTOM	-273 3 to 296 7
TOP	(Very wide)
LOGEC50	(Very wide)
HILLSLOPE	(Very wide)
EC50	(Very wide)
Span	(Very wide)
Goodness of Fit	
Degrees of Freedom	13
R2	0 4669
Absolute Sum of Squares	12185
Sy x	30 62
Number of points	
Analyzed	17

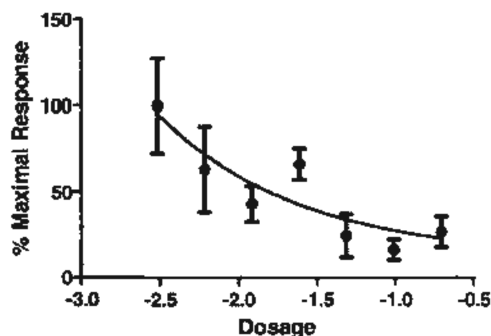


Figure 3-14: The serial two fold dilution of crude extract of 127 Btreated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized.

ED $_{50}$ was calculated 1.484e-006 mg/ml.

Hemoglobin induction by crude extract 128A 瓜蔞 in k562 cell Line.

	128A
log(agonist) vs response – Vanable slope	
Best-fit values	
BOTTOM	1 064
TOP	84 11
LOGEC50	1 078
HILLSLOPE	1 917
EC50	0 08365
Span	83 04
Std. Error	
BOTTOM	20 99
TOP	6 257
LOGEC50	0 1683
HILLSLOPE	1 075
Span	24 13
95% Confidence Intervals	
BOTTOM	-43 95 to 46 08
TOP	70 69 to 97 53
LOGEC50	-1 439 to -0 7166
HILLSLOPE	-0 3883 to 4 223
EC50	0 03643 to 0 1921
Span	31 27 to 134 8
Goodness of Fit	
Degrees of Freedom	14
R2	0 8316
Absolute Sum of Squares	2660
Sy x	13 78
Number of points	
Analyzed	18

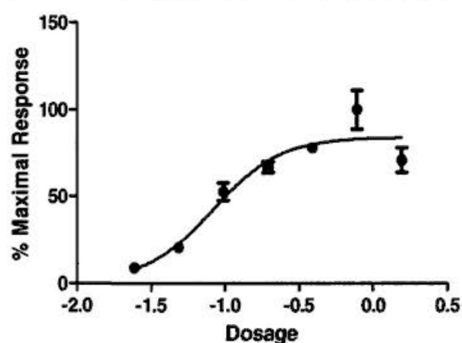


Figure 3-15: The serial two fold dilution of crude extract of 128A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 0.84 mg/ml.

Hemoglobin induction by crude extract 128B 瓜蔞 in k562 cell Line.

	128B
log(agonist) vs response -- Variable slope	
Best-fit values	
BOTTOM	45 97
TOP	97 27
LOGEC50	-1 513
HILLSLOPE	2 382
EC50	0 03070
Span	51 30
Std Error	
BOTTOM	12 63
TOP	7 816
LOGEC50	0 1486
HILLSLOPE	1 813
Span	17 15
95% Confidence intervals	
BOTTOM	18 45 to 73 50
TOP	80 24 to 114 3
LOGEC50	-1 837 to -1 189
HILLSLOPE	-1 569 to 6 333
EC50	0 01457 to 0 06472
Span	13 93 to 88 67
Goodness of Fit	
Degrees of Freedom	12
R2	0 7397
Absolute Sum of Squares	1958
Sy.x	12 77
Number of points	
Analyzed	16

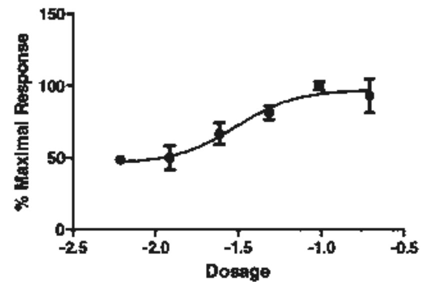


Figure 3-16: The serial two fold dilution of crude extract of 128B treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 0.31mg/ml.

Hemoglobin induction by crude extract 129A 蔓皮 in k562 cell Line.

	129A
log(agonist) vs response - Variable slope	
Best-fit values	
BOTTOM	11 21
TOP	91 78
LOGEC50	-0 8417
HILLSLOPE	2 657
EC50	0 1440
Span	80 57
Std Error	
BOTTOM	4 516
TOP	4 338
LOGEC50	0 06209
HILLSLOPE	0 8549
Span	6 615
95% Confidence Intervals	
BOTTOM	1 905 to 20 51
TOP	82 84 to 100 7
LOGEC50	-0 9696 to -0 7138
HILLSLOPE	0 8962 to 4 418
EC50	0 1073 to 0 1933
Span	66 94 to 94 20
Goodness of Fit	
Degrees of Freedom	25
R2	0 8981
Absolute Sum of Squares	4141
Sy x	12 87
Number of points	
Analyzed	29

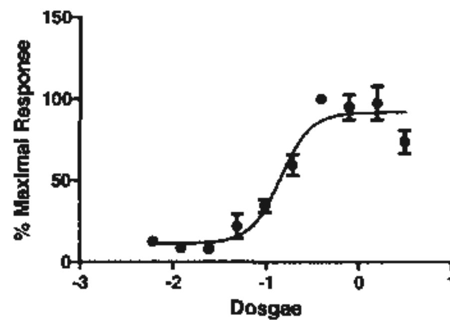


Figure 3-17: The serial two fold dilution of crude extract of 129A treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 1.44 mg/ml.

Hemoglobin induction by crude extract 129B 蔞皮 in k562 cell Line.

	129B
log(agonist) vs. response -- Variable slope	
Best-fit values	
BOTTOM	34.96
TOP	82.88
LOGEC50	-1.289
HILLSLOPE	7.431
EC50	0.05136
Span	47.91
Std. Error	
BOTTOM	13.45
TOP	6.426
LOGEC50	0.1339
HILLSLOPE	42.33
Span	16.17
95% Confidence Intervals	
BOTTOM	4.990 to 64.93
TOP	68.56 to 97.19
LOGEC50	-1.588 to -0.9910
HILLSLOPE	-88.89 to 101.7
EC50	0.02584 to 0.1021
Span	11.90 to 83.93
Goodness of Fit	
Degrees of Freedom	10
R2	0.6098
Absolute Sum of Squares	2871
Sy.x	16.94
Number of points	
Analyzed	14

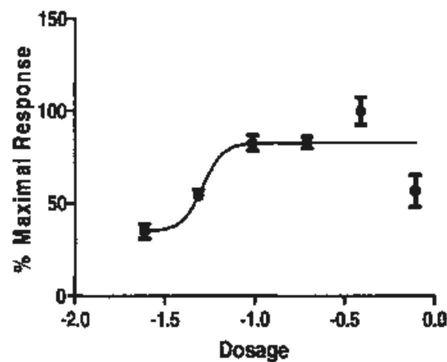


Figure 3-18: The serial two fold dilution of crude extract of 129B treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 0.51mg/ml.

Hemoglobin induction by crude extract cucurbitacin D in k562 cell Line.

	978
log(agonist) vs response – Variable slope	
Best-fit values	
BOTTOM	6 229
TOP	92 06
LOGEC50	-1 036
HILLSLOPE	1 757
EC50	0 09205
Span	85 82
Std Error	
BOTTOM	1 683
TOP	3 358
LOGEC50	0 05227
HILLSLOPE	0 3210
Span	3 806
95% Confidence Intervals	
BOTTOM	2 851 to 9 608
TOP	85 31 to 98 79
LOGEC50	-1 141 to 0 9310
HILLSLOPE	1 112 to 2 401
EC50	0 07229 to 0 1172
Span	77 98 to 93 66
Goodness of Fit	
Degrees of Freedom	53
R2	0 9370
Absolute Sum of Squares	4636
Sy x	9 355
Number of points	
Analyzed	57

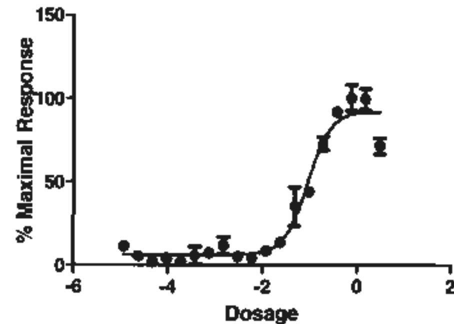


Figure 3-19: The serial two fold dilution of crude extract of cucurbitacinD treated k562 cell for 6 days. The experiment was performed in 96 well plate. The trend of hemoglobin induction was analyzed by non-liner regression method and drafted the dose-response curve by GraphPad Prism 4.0. The data was normalized. ED $_{50}$ was calculated 0.92 mg/ml.

Hemoglobin induction of 40% methanol elution from reversed –phased chromatography in k562 cell

Table Analyzed	Transform of solid phase extraction
Column A	128A
vs	vs
Column B	40%---1
Mann Whitney test	
P value	0.2352
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	163 , 137
Mann-Whitney U	59.0

Table 3-20:t tests analyzed of hemoglobin production in k562 cell between 128A crude extract (positive control) and 40 % methanol elution of 99B絲瓜子solid phase extraction. P > 0.23. It was no significant different between positive control and 40 % methanol elution. They both could induce hemoglobin in k562 cell assay.

Hemoglobin induction of 60% methanol elution from reversed –phased chromatography in k562 cell

Table Analyzed	Transform of solid phase extraction
Column A	128A
vs	vs
Column D	60%--1
Mann Whitney test	
P value	0.1301
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,D	170 , 130
Mann-Whitney U	52.0

Table 3-21:t tests analyzed of hemoglobin production in k562 cell between 128A瓜蔞 crude extract (positive control) and 60 % methanol elution of 絲瓜子99B solid phase extraction.P > 0.13. It was no significant different between positive control and 40 % methanol elution. They both could induce hemoglobin in k562 cell assay.

Hemoglobin induction of 40% methanol elution from reversed –phased chromatography in k562 cell

Table Analyzed	Transform of solid phase extraction
Column B	40%---1
vs	vs
Column F	cell alone
Mann Whitney test	
P value	0.0014
Exact or approximate P value?	Exact
P value summary	**
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	One-tailed
Sum of ranks in column B,F	143 , 67
Mann-Whitney U	12.0

Table 3-22: t tests analyzed of hemoglobin production in k562 cell between untreated cell lysate(negative control) and 40 % methanol elution of 99B絲瓜子solid phase extraction.P <0.002. It was significant different between negative control and 40 % methanol elution. Only the elution could induce hemoglobin in k562 cell assay.

Hemoglobin induction of 60% methanol elution from reversed –phased chromatography in k562 cell

Table Analyzed	Transform of 25B--2 solid phase
Column A	60%--1
vs	vs
Column B	pre-loading
Mann Whitney test	
P value	0.4091
Exact or approximate P value?	Exact
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	37 , 41
Mann-Whitney U	16.0

Table 3-23: t tests analyzed of hemoglobin production in k562 cell between 99B絲瓜子crude extract (preloading) and 60 % methanol elution of 99B絲瓜子solid phase extraction. P > 0.4. It was no significant different between preloading and 60 % methanol elution. They both could induce hemoglobin in k562 cell assay.

Elution of HPLC 15 min. for hemoglobin induction in k562 cell

log(agonist) vs response – Variable slope	
Best-fit values	
BOTTOM	-4 110
TOP	92 08
LOGEC50	0 2900
HILLSLOPE	1 957
EC50	1 950
Span	96 19
Std Error	
BOTTOM	16 51
TOP	6 903
LOGEC50	0 1245
HILLSLOPE	0 8644
Span	20 05
95% Confidence Intervals	
BOTTOM	-42 19 to 33 97
TOP	76 16 to 108 0
LOGEC50	0 003054 to 0 5770
HILLSLOPE	-0 03673 to 3 950
EC50	1 007 to 3 776
Span	49 96 to 142 4
Goodness of Fit	
Degrees of Freedom	8
R2	0 9455
Absolute Sum of Squares	928 9
Sy x	10 78
Number of points	
Analyzed	12

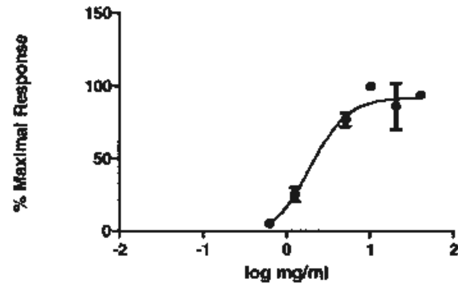


Figure 3-19: Dose-response curve of HPLC 15 min elution was drafted by statistic program *Prism®* 4.0 software and ED_{50} was calculated. The obvious sigmoid curve was presented.ED_{50}= 1.95 mg/ml.

Hemoglobin inducing activity of elution fraction at 25 min

	25 min elution
log(agonist) vs response -- Variable slope	Ambiguous
Best-fit values	
BOTTOM	35.29
TOP	78.73
LOGEC50	~ 0.09820
HILLSLOPE	~ 40.79
EC50	~ 1.254
Span	43.44
Std. Error	
BOTTOM	75.74
TOP	13.50
LOGEC50	~ 3.699e+008
HILLSLOPE	~ 2.774e+012
Span	84.55
95% Confidence Intervals	
BOTTOM	-159.4 to 230.0
TOP	44.01 to 113.5
LOGEC50	(Very wide)
HILLSLOPE	(Very wide)
EC50	(Very wide)
Span	-173.9 to 260.8
Goodness of Fit	
Degrees of Freedom	5
R2	0.3883
Absolute Sum of Squares	2735
Sy.x	23.39
Number of points	
Analyzed	9

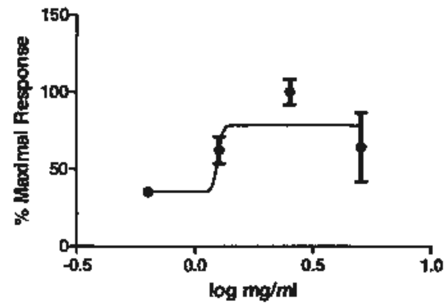


Figure 3-20 Dose-response curve of HPLC 25 mins elution was drafted by statistic program *Prism®* 4.0 software and ED_{50} was calculated. The obvious sigmoid curve was presented. ED_{50}= 1.254 mg/ml.

the diagram of HPLC separation of extraction of Luffa cylindria絲瓜子

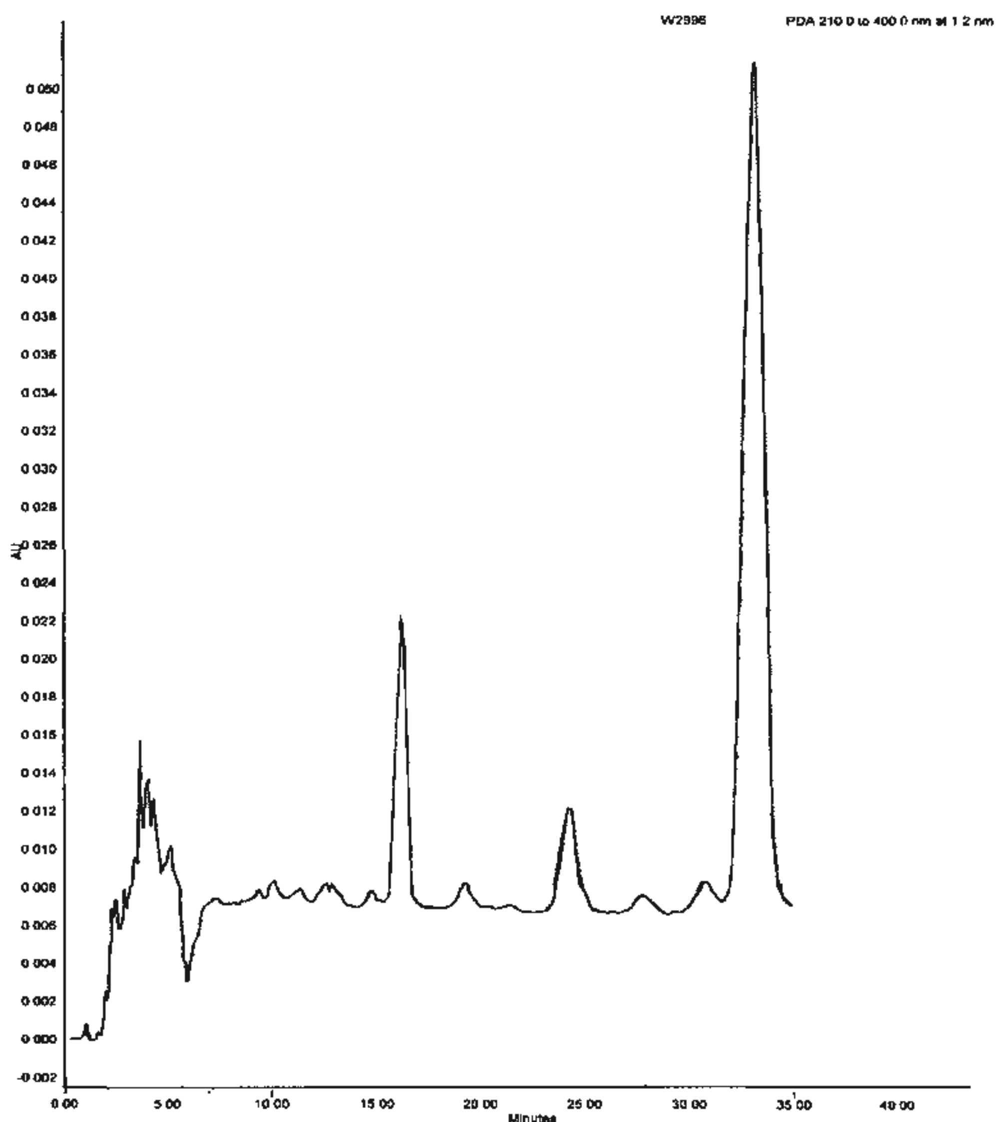


Figure 3-21: The compounds in extraction of Luffa cylindria絲瓜子were isolated by HPLC after the solid- phase purification. The arrows indicated that the peaks of lead compounds could induce hemoglobin in k562 cell assay. Fractions were collected at 15mins and 25 mins. However, diagram showed that there were a lot of impurities. Indeed, further HPLC purification was required.

3.5. Discussion

25 herbs were selected for testing the induction potency of hemoglobin. It was divided into two group of extraction, water extraction A and ethanol extraction B. The extracts were used to treated k562 cell for 6 days. The results were compared with hydroxyurea. 9B (ethanol extracts of Atractylodes macrocephala Koidz) 白朮, 99B (ethanol extract of Luffa cylindria) 絲瓜子, 118A (water extract of Aractylodes lancea蒼朮and 127B (ethanol extract of Cotyledon of Lagenaria sicieraria var.makinoi) 短棒瓠瓜子extracts showed the lowest ED_{50} among all the other extracts. However, the ED_{50}s were not represented high efficacy all the time. There were another two parameters required to consider the efficacy of the drug action. One was R2, the other was the steepness of the hill slope. R2 measured goodness of fit of the data. The total performance of the data was assessed by their consistence, the trend of the slope under the measuring criteria. R2= 1 meant perfect set of data. The lowest R2 reflected the lowest quality of the data was. Another consideration was the steepness of the slope in a sigmoid curve. The small change of the dosage would lead the great therapeutic response. The more steepness of the slope indicated the higher efficacy of the compound. The dose-response curves of白朮9B, 蒼朮118A and 短棒瓠瓜子127B were very flat. The hill slopes were 24.0, 9.9 and 18.4, respectively. The hill slope of絲瓜子99B was 16526.0. Only the extraction of 99B showed very low ED_{50} 9.90e^{-}005, high steepness of the slope 16526.0 and R2 was 0.9.

The extraction of 9A showed the inducing effect in k562, but the data could not draft the dose-response curve, because the data did not show the trend of the sigmoid. The result of 9A might be another pathway to activate the hemoglobin or by random error. All the positive results were as shown in table 3-23.

The results of herbs for inducing hemoglobin in k562

Comparison to HU	t-tests, P=	ED_{50}= mg/ml	R2	Hill slope
8A 防杞	0.0713	33.9	0.8	1.8
8B 防杞	0.4417	0.31	0.9	24.7
9A 白朮	0.1584	-----	-----	-----
9B白朮	0.4274	2.926e-007	0.7	24.0
33A 知母	0.4676	44.09	0.4	1.8
33B 知母	0.1626	1.2	0.5	36.2
43B 茜草	0.4676	1.08	0.7	4.3
99A 絲瓜子	0.1350	0.06	0.9	87.54
99B 絲瓜子	0.1059	9.90e-005	0.9	16526.0
101A 胡蘆	0.2318	0.12	0.9	53.7
101B 胡蘆	0.1001	0.99	1.0	121.9
118A 蒼朮	0.4325	1.085e-011	0.6	9.9
125A黑棗	0.4734	3329	0.5	3.5
127A短棒瓠瓜子	0.0926	27.10	0.9	0.4
127B短棒瓠瓜子	0.4353	1.484e-006	0.4	18.4
128A 瓜蔞	0.5	0.84	0.8	9.6
128B 瓜蔞	0.18	0.31	0.7	22.7
129A 蔞皮	0.3119	1.44	0.9	6.3
129B 蔞皮	0.1801	0.51	0.6	123.6

Table 3-23: The extractions of 25 herbs induced k562 cell, the dose-responses compared with Hydroxyurea, P value as shown. The ED50s, R2s and hill slopes were calculated

Since only 99B (ethanol extract of Luffa cylindria) 絲瓜子showed a very potent hemoglobin induction activity, this extract was subjected to further purification by reverse-phase (solid phase) chromatography and HPLC using C18 column. In the solid phase separation, the induction activities could be eluted by 40% and 60% methanol (figure 3-19 to 3-21). These elutents were pooled and subjected to further HPLC separation. Induction activities were found in the 15min and 25 min fractions. These two fractions have similar ED50 value and likely to be equally potent for the induction of hemoglobin production. However, the content of 25min fraction is more than the 15 min fraction.

We are not sure whether these two fraction may also contain cucurbitacin at a higher concentration or contain other compounds that is very potent for inducting hemoglobin production. It is required further purification and activity analysis. However, because our project ran out of resources for further research, we did not continue to further purify the active ingredients.

Chapter 4: Evaluation of compound toxicity and efficacy Using Cell and animal models

4.1. Introduction

In the development of a new discovery drug, the pharmacologic and toxicological data had to submit to the Food and Drug Administration (FDA) in USA. It needs to demonstrate the drug is safe and effective. Cucurbitacin D was proven its efficacy in stimulating fetal hemoglobin production in sickle cell mouse model. However, the toxicity of cucurbitacin needs to be evaluted. Studies reported that cucurbitacin bound on glucocorticord receptors and developed the cytotoxic effect. It showed the linear relationship between the binding affinities and cytotoxic activities of cucurbitacin. (Witkowski and Konopa 1981) The stronger binding affinity, the more serious is the cytotoxic effect. The preclinical studies to evaluate the toxicity of the compound included normal human cell assay and C elegan assay. From the cellular toxicity data, which would predict the toxic events happen in the cell.

The toxicity of the different cucurbitacins (B, D, E, I) were evaluated, while Sodium Lauryl Sulfate was used as a positive control, and cell alone as negative control. The toxicity effect of compound was expressed as IC_{50}. Correlation coefficient measured the association of two groups of variables (cucurbitacin and positive control)

4.1.1. The Principle of two cytotoxicity assay

Alamar blue cytotoxicity cell assay

Alamar blue is non-toxic agent which absorb by cell. The cell permeable compound was blue in colour and non-fluorescent. Once it entered the cell, the viable cell would convert the ingredient of alamar blue, resazurin to resorufin which was bright pink fluorescent molecular. The bright pink colour was proportional to the number of livings cell. The dead cell could not reduce resazurin to resorufin and it remained the blue colour. From the colour produced by the cell could predict the viable cell number. The toxicity of the compound to the cell could be determined.

Neutral Red Up-take Cytotoxicity Assay

Neutral red uptake cytotoxicity test was base on the non-toxic; weak cationic dye was incorporated into the living cell. It accumulated in lysosomes. If the living cell was damaged by the compound, the binding neutral red ability was weakening. The toxicity of chemical would change of the cell membrane integrity and cellular metabolism. If the level of damage was serious, the injury would become irreversible. The neutral cannot enter the cell or accumulate in lysosomes. The proportion of pink colour indicated the viable cell number, and the toxicity of the compound to the cell could be verified.

4.1.2. Toxicity study using *Caenorhabditis elegans* (C elegans)

The toxicity of chemical would change of the cell membrane integrity and cellular metabolism. The toxicity study of cucurbitacin D was performed in the cell cytoyoxicity assay. In this chapter, another toxicity study was applied to a simple multi-cellular organism. *Caenorhabditis elegans* (C elegans) was a type of nematode, it lived in wet soil. Because of the short life span and easy to keep it as a culture, C elegans was used as a model to access the toxicity of a drug or xenobiotic chemical. (Ura *et al.*, 2002) The endpoint bioassay depended on the lethality of the worm under the exposure of the compound. IC_{50} would be determined by the dose-response curve. The lethal and safety dosage would be given by the result.

4.1.3. Preclinical study using sickle cell mouse model

This chapter adopted the sickle cell mouse model obtained from Jason laboratory and prof. Kan's laboratory. The transgenic mice carried the human beta-globin gene which contained the base pair mutation in codon 6. It caused chain polymerization when the beta-globin was deoxygened. The mouse also expressed the similar symptom as human, anemia, multi-organ dysfunction. The mouse process the truncated mouse alpha and beta hemoglobin genes, and then reconstructed with human alpha and beta sickle hemoglobin genes.

Homozygous transgenic mice genotype

(mouse alpha -/-, mouse beta -/-; human transgene Tg/-)

Heterozygous transgenic mouse genotype

(Mouse alpha -/-, mouse beta +/-; human transgene Tg/-).

The mouse models were used for cucurbitacin D pharmacokinetic study. It verified whether the drug can increase the total fetal hemoglobin level.

4.2. Materials

cell line	Lot No. and company
CRL1656 **Mustela putoris furo (ferret), brain**	ATCC: CRL- 1656 (Adherent)
CRL 2120 **Homo sapiens (skin fibroblast)**	ATCC: CRL- 2120 (Adherent)
CRL 2115 **Homo sapiens (skin fibroblast)**	ATCC: CRL- 2115 (Adherent)
cc 2507 **NHEK Neo-keratinocyte-neonatal pooled**	ATCC: cc- 2507 (Adherent)

Equipment

Falcon 50ml	Falcon
Laminar flow clean bench (biological hazard)	
96-well flat bottom tissure culture microtiter plate	Corning/COSTAR tissue culture treated
Multi-channel , single channel pipette	eppendorf
Multi-channel reservoir	Corning
Tissue culture flasks (75-80 cm^2, 25 cm^2)	Nunc
shaker	
Hemocytometer	Sigma Z35 962-9
Petri dish	NUNC 150255
Waterbath	Fisher Scientific (Model 102S)
Whatman® chromatography paper 3MM Chr, W × L 20 cm × 20 cm	Sigma 270849
X-ray film 8 × 10 inch (20 × 25 cm) film	Sigma Z380156

Whatman®Westran®PVDF membranes	Sigma Z671010
Coulter R A C.T diff ™ analyzer	Beckman Coulter
Plastic Clad Micro Hematocrit Tube Heparinized	Becton Dickinson Vacutainer Systems
96 well NUNC Maxsorp plate	Nunc

Solution

Chemical medium and serum	Lot No. and company
Basal medium (Eagle) in Earle's balanced salt solution	Sigma M2075
NHEK Neonatal Pooled, cryopreserved in Keratinocyte Growth Medium (KGM)	Cambrex Bio Science Walkersville, Inc CC-2507
Keratinocyte Growth Medium	Clonetics CC-3104
Minimum Essential Medium Eagle	Sigma M2279
GIBCO™ Lamb Serum	Invitrogen 16070096
Neutral red Dye (power form)	Sigma N4638
Alamar blue	AbD Serotec BUF012B
sodium lauryl sulfate	Sigma L4390-25g
Cucurbitacin (B,D, E, I) 5 mg	Chromadex 03910-805,212,401

Ethanol (ETOH) HPLC grade	Sigma 459828-2L
Trypsin	
LB agar (bacterial culture)	Sigma S9938
Bacto-yeast extract	BD 288620
Bacto-tryptone	BD 25922
Sodium chloride (NaCl)	Sigma 24886
Potassium chloride (KCl)	Sigma P9541
Sodium Acetate (NaOAc)	Sigma S2889
Bacto-peptone	BD 211677
Calcium Chloride (CaCl)	Sigma C1016
Potassium Phosphate (KPO_4)	Sigma P2222
Magnesium Sulfate ($MgSO_4$)	Sigma M2643
Cholesterol	Sigma 362794
Hydrochloric Acid (HCl)	Sigma H1758
Cadmium chloride (CdCl)	Sigma 439800
c elegan and E.coli OP50 bacteria	K chow's lab
Tris-HCl	Sigma 93363
Ethylenediaminetetraacetic acid tetrasodium salt hydrate(EDTA)	Sigma E5391

Sodium hydroxide (NaOH)	Sigma s8045
SSC buffer	powder blend (Sigma) s0902
Sodium phosphate dibasic (Na2HPO4)	Sigma s7907
Ethidium bromide	Sigma 160539
TAE buffer (10X)	Sigma 93296
Dextran sulfate	Sigma D8787
Sheared Salmon Sperm	Sigma 31149
Bovine serum albumin (BSA)	Sigma A7888
Bgl II restriction enzyme	New England Biolabs RO144S
New methylene blue	Sigma 556416
SeaKem LE Agarose	Cambrex 12001-870
Human Fetal Hemoglobin ELISA Quantitation Kit.	Bethy Laboratories.Inc
ELISA Starter Accessory Kit	E101 Bethy Laboratories.Inc
Genomic DNA Purification Kit	Gentra System
GoTaq PCR kit	Promega

Chemical preparation

Keratinocyte Growth Medium (KGM)

Final 500ml medium add 0.5ug/ml hydrocortisone, 5ug/ml insulin and 0.0001ng/ml human recombinant grow factor.1% PSF and 10% fetal calf serum. Store in 4°C.

Basal medium (Eagle) in Earle's balanced salt solution

Final 500 ml medium, add 70 ml lamb sera and 5 ml PSF. Store in 4°C.

Minimum Essential Medium Eagle

Final 500 ml medium, add 50ml fetal calf sera and 5ml PSF Store in 4°C.

Sodium Lauryl Sulfate (SLS)

600mg SLS melts into 10 ml sterilize milli-Q water. Store in 4°C.

Cucurbitacin (B,D, E, I) 5 mg

5mg cucurbitacin melts in 2.5 ml 100% HPLC grade ethanol, it works as a stock, store in-70 °C. Take 8 ul stock melts into 992 ul sterile milli-Q water, it works as a working solution. Store in-20 °C.

Neutral red stock

0.165 mg neutral red power melts in 50ml sterile milli-Q water.(store in dark at room temperature up to two months

Neutral red working solution

Freshly prepare 1 ml stock and add into 99 ml complete medium, then filter the olution and pre-warm in 37°C. Before adding into the cell.

Ethanol/Acetic acid solution (NR Desorb)

Add 5 ml flacial acetic acid into 245ml sterile milli-Q water, then mix together with 250ml absolute ethanol.

LB agar for OP50 culture

7.5 g agar, 2.5 g NaCl, 2.5g bacto-yeast extract, 5g bacto-tryptone, add into 500 ml distilled water, adjusted pH 7.5, the sterilized by autoclave. Cool down and dispense into petri dishes under sterile condition.

L broth for OP50

2.5g NaCl, 2.5g bacto-yeast extract, 5g bacto-tryptone, add into 500 ml distilled water, adjusted pH 7.0, the sterilized by autoclave.

5mg/ml cholesterol in ethanol

0.25g cholesterol add into 50 ml absolute ethanol. Sterile by filtration through 0.22um syringe filter under sterile condition.

NGM agar plate

8.5 g LB agar, 1.5 g NaCl, 1.25g bacto-peptone, add into 487.5 ml distilled water, sterilize by autoclave. Cool down the mixture in 55 °C waterbath, add 0.5ml 1M

$CaCl_2$ solution

0.5ml 1M $MgSO_4$ solution, 12.5 ml 1M KPO_4 solution, 0.5 ml 5mg/ml cholesterol in ethanol. Mix well and dispense the mixture into petri dishes under the sterile condition.

0.8% agarose

3.2 g agarose, 20ml 10x TAE buffer, fill up to 200 ml distilled water. Heat up in microwave mechine until all the agarose melts in the liquid.

1M Tris-HCl

121.1 g Tris-HCl melts into 800 ml distill water, adjust the pH to 7.5

0.5M EDTA

Dissolve 37.22 g EDTA 150 ml distilled water, adjusted the pH to 8.0 with NaOH. Fill up to 200 ml distilled water.

5M NaCl

146.1 g NaCl melts in 450 ml distilled water by stirring, add the water to final volume 500ml.

TE buffer

10 mM Tris and 0.5 mM EDTA mixed, adjusted the pH at 7.5

0.25N HCl

21.5 ml con HCl add into 1000 ml distill water

Alkaline solution

87.66 g NaCl ,20.00 g NaOH add into 1000 ml distill water

Hybridization buffer

3.5g SDS, 2.76g NaP , 12.5 ug sheared salmon sperm DNA, up to 50 ml distill water. Freshly prepared.

Staining solution

Dissolve 1 g of New methylene blue in 100 ml iso-osmotic phosphate buffer (pH 7.4)

4.3. Methods

4.3.1. Procedure of Alamar Blue Cytotoxity Cell Assay

1. Take out the cell from liquid nitrogen, as soon as possible put into 37°C. Then seed the thawed cell into 10ml pre-warmed complete medium. Spin down the cell at 300g. Remove the supernatant and collect the cell pellet.
2. Transfer it into 25 cm^2 flasks. Incubate the cell at 37°C., 90% humidity, and 5 % CO^2/air until the cell attach to the bottom of the flask. Change the medium a period of time. Until about 70% area was occupied by the cell.
3. Add 1ml trypsin to remove the adherent cell. Then counting the cell until the number of cell was sufficient to the biological assay.
4. The experiment performed in 96 well plate. Each well was seed 1.2 x 10^3 cells. Until the cells adhere to the bottom of the well, add the drug to each well.
5. The initial drug concentration is 1.6 ug /ml. The serial two fold dilution fraction was carried in each row of 96 well plate. The plate must be included positive control, negative control and reagent only.
6. After adding the drug, the plate was incubated in CO2 incubator for more 24 hrs. Then add 20 ul alamar blue in each well and incubate at 37°C continuously for 10 hrs.
7. Read the plate under the absorbance at 570nm. The total experiment interval time could not over one doubling time of cell proliferation.

4.3.2. Procedure of Neutral Red Up-take Cytotoxity Cell Assay

1. Take out the cell from liquid nitrogen, as soon as possible put into 37°C. Then seed the thawed cell into 10ml pre-warmed complete medium. Spin down the cell at 300g. Remove the supernatant and collect the cell pellet.
2. Transfer it into 25 cm^2 flasks. Incubate the cell at 37°C., 90% humidity, and 5 % CO^2/air until the cell attach to the bottom of the flask. Change the medium a period of time. Until about 70% area was occupied by the cell.
3. Add 1ml trypsin to remove the adherent cell. Then counting the cell until the number of cell was sufficient to the biological assay.
4. The experiment performed in 96 well plate. Each well was seed 1.2 x 10^3 cells. Until the cells adhere to the bottom of the well, add the drug to each well.
5. The initial drug concentration is 1.6ug /ml. The serial two fold dilution fraction was carried in each row of 96 well plate. The plate must be included positive control, negative contol and reagent only.
6. After adding the drug, the plate was incubated in CO2 incubator for more 24 hrs. Remove all the medium and drug from each well, add back 200ul neutral red (NR) into each well, then incubate the plate at 37°C for 3 hrs.
7. Aspirated out the NR medium, rinsed the cell with pre-warmed phosphate buffer saline (PBS) carefully.
8. Removed the PBS, added 100 ul NR desorb in each well. Shake the plate on shaker for 20 mins. It extracted the NR from the cell and forms a homogenous solution. The process must be protected from light.
9. The plate read at 540 nm.

10. The statistical measurement through non-liner regresstion drafted the dose-response curve. It calculates the IC_{50} by GrapPad $PRISM_R$ 4.0.

Comparison between the drug and the control was through Correlation coefficient method. It gave the association of two groups of variances. The value was between +1 to -1. A value of +1 indicated the perfect association. -1 indicate perfect inverse correlate. And 0 indicated no correlation. The drug perfectly correlated with positive control, that the toxic of the drug is significant under the dosage. If the drug perfectly correlates with negative control, it indicated the drug is non-toxic under the dosage. The strength of correlation set at 0.5. If the value was under 0.5, the weak correlations sign. If the value was over 0.5, the strong correlations sign.

4.3.3. Procedure of toxicity test on C.elegans

1. E.coli OP50 was the food source of C. elegans. We prepared the food source for the C elegans. Take the single colony of E coli OP50 from a starter plate. Inoculate the colony into 100ml L broth and incubate at 37 °C 18 hrs with shaking 120 rpm. The liquid culture was seed on NGM plate. All the procedure must be performed at aseptic environment.

2. The C elegans were washed out from a start plate with K medium. Aspirate the mixture into a 50 ml falcon and span down the worm at 300 g for 3 mins. Remove the supernatant.

3. Wash the worm twice with distilled water, then K medium once. Span down the worm again and aspirate the supernatant out.

4. The worm spread on the NGM plate which had been cultured OP50. The thick layer bacteria culture ensured abundance food for the C. elegans.

5. Harvested the worm on 4th or 5th day. Estimate the number of the worm under the microscope.
6. Synchronized L1 larval of C. elegans in K medium which was suppied with E.Coli OP50, the concentration was 2500 worms/ ml. 200 ul of the mixture was dispensed in 48 well plate.
7. Dissolved the Cadmium chloride (CdCl) in the K medium, make the final concentration was 20 mM.

8. Independently, Hydroxyurea was melted the in K medium. The concentration is 57.7 ug /ml.
9. *Trichosanthes rosthornii Harms.* 瓜蔞 (crude extract) was separately dissolved in K medium, the concentration was 4406.64 mg/ml.
10. The three set of drug dilutions was made in a serial two fold fraction. 200 ul of different concentration drugs were dispensed into each well of the plate. The worms were exposed in the drugs for 24 hours at 20 ℃. The percentages of death worms were counted

4.3.4. Procedure of toxicity of mouse

The mouse from UCSF Dr Kan yuet wai's laboratory

1. 11 homozygous transgenic mice and 2 heterozygous transgenic mice were obtained from Professor Kan' laboratory.
2. Four mice as a group. Two test group and one control group, each mouse was given 1.5 ug cucurbitacin D (cu D) per gram of mouse. For example, mouse 272 weight 22g.22x 1.5=33. 33ug cu D was given to the mouse 272. The other group was given 1.0 ug for each gram of mouse. Control group was given medicine solvent (10% EtOH in 90 ul PBS).
3. The drug administrate as IP injection. It was maintained for 28 days the skin lesion were examined.
4. Southern Blot detected the mouse genotype.

4.3.4.1. Southern Blot

1. 5-10 ug genomic DNA was extracted from the mouse's tissue.
2. Ethanol precipitation was performed and washed DNA with 70 % EtOH, then resuspended it in 30 ul TE buffer, stored in 4℃
3. It was digested with restriction enzyme Bgl II in 25 ul reaction, incubation at appropriate temperature for 4 hrs.
4. The digestion mixture was as below:

	Volume (ul)
DNA	10
10X buffer	2.5
BSA	2.5
Bgl II	1
H_2O	9

5. DNA eletrophoresis in 0.8 % agarose gel, then the gel was transfered into 0.25N HCl for 15 mins with shaking, and then rinsed with distilled water.

6. Soaked the gel in alkaline solution again for 15 mins with shaking. Cut the suitable size of membrane, soak in water, then transferred to alkaline solution.
7. Assemble the transfer unit in the following order. The bottom is paper towels, following is 3mm paper, then the membrane, the top is the gel. Only the two end of 3mm paper submerged into alkaline solution. Make sure to squeeze out of any bubble between the membrane and gel. The process was maintained for three hours.
8. Neutralize the membrane with 2X SSC buffer, soak the membrane again in 1 N NaP buffer for 20 mins assist with shaking. Rinsed with 2XSSC and blotted dry.
9. Warm the hybridization buffer to 65℃. Put the membrane into the hybridization buffer in 65℃ for 15mins,
10. add the probe into hybridization buffer. Then shaking them in 65 ℃ for overnight. Rinse the membrane in 2XSSC for 20 mins. at room temperature.
11. Washed the membrane in 2XSSC & 1% SDS at 65 ℃ for 1 hr. Blotted dry the membrane.
12. Wrap the membrane with plastic sheet. Place it and the X-ray film in a cassette in dark. Signal for a typical genomic blot will be generate after exposure.

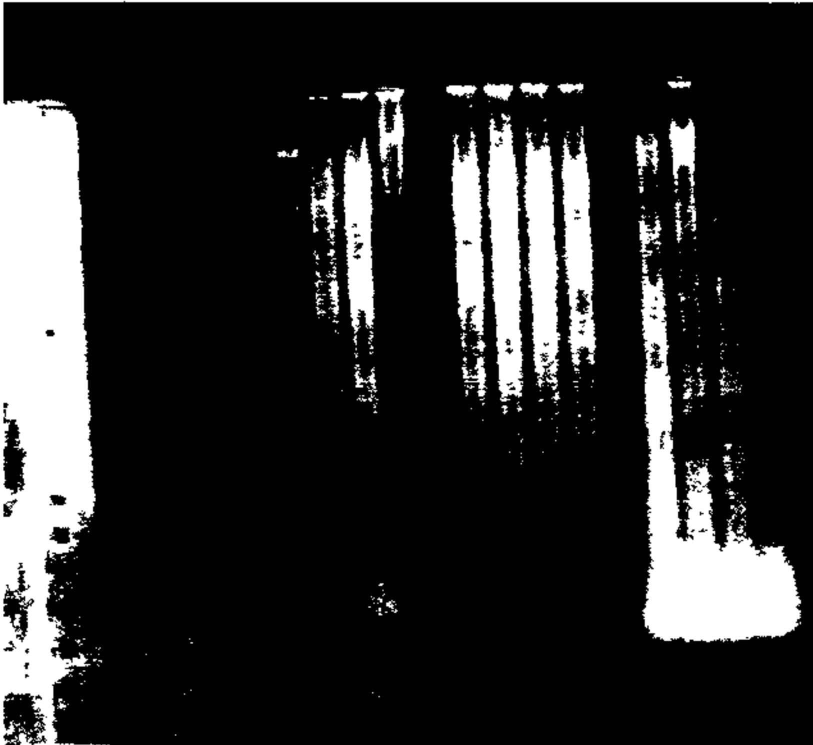
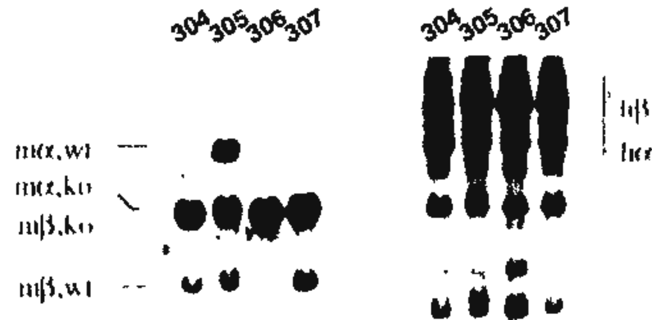


Figure 4-1: DNA extract from the mouse tissue before the restriction enzyme digestion. It shows that it is non-degrade.

4.3.4.2 Transgenic mouse genotyping by Southern blot

$m\alpha$	$m\beta$	$h\alpha$	$h\beta^{S}$
-/-	-/-	+/0	+/+



The photo was provided by Dr. Kan's laboratory

Figure4-2:

The southern blot data showed the transgenic mouse genotype, the genotype shown as figure 4-2.

Homozygous transgenic mouse genotype 306

(mouse alpha -/-, mouse beta -/-; human transgene : human alpha +/ Human Beta sickle+)

Heterozygous transgenic mouse genotype 305

(Mouse alpha +/-, mouse beta +/-; human transgene : human alpha +/ Human Beta sickle+).

Heterozygous transgenic mouse genotype 304

(Mouse alpha -/-, mouse beta +/-; human transgene : human alpha +/ Human Beta sickle+).

4.3.4.3 Genotype of Sickle Cell mice used in the experiment

Mouse name	weight	Dosage 1 1.5 ug/ g	Genotype mouse α/α, β/β
Group 1			
272	24	36 ug in 100ul	(-/-, -/-)
280	22	33 ug in 100ul	(-/-, -/-)
281	22	33 ug in 100ul	(-/-, -/-)
289	22	33 ug in 100ul	(-/-, -/-)
		Dosage 2 1 ug/ g	
Group 2			
249	23	23 ug in100 ul	(-/-, -/-)
263	22	22 ug in 100ul	(-/-, -/-)
265	22	22 ug in 100ul	(-/-, -/-)
273	23	23 ug in 100ul	(-/-, -/-)
Group control 3		solvent	
236	23	100 ul of 10% EtOH-PBS	(-/-, -/-)
237	22	100 ul of 10% EtOH-PBS	(-/-, -/-)
270	27	100 ul of 10% EtOH-PBS	(-/-, -/-)
306	29	100 ul of 10% EtOH-PBS	(-/-, -/-)
Group control 4		No injection	
267	30	---	(-/-, +/-)
46	---	---	(-/-, +/-)
271	29	---	(-/-, +/-)

Table 4-1 The experimental mouse list: It included the mouse name, weight, the dosage the mouse taken and their genotype.

4.3.5. Sickle cell mouse from Jason lab. demonstrated fetal hemoglobin level was increased by cucurbitacin D

4.3.5.1 General procedure

1 Six transgenic mice bought from Jason laboratory.

2 Mouse model No : C57 JE3342

3 Strain name : STOCK Hba^{tm1Paz} Hbb^{tm1Tow} Tg(HBA-HBBs)41Paz/J

3.1. The mouse alpha and .beta gene were deleted and replaced by neomycin cassette. Therefore, the mouse could not express the alpha and beta hemoglobin. It was rescued by engineered human transgene. It included hemoglobin A1, gamma A, gamma G, delta, and beta sickle.

3.2. Homozygous transgenic mouse genotype

mouse alpha -/-, mouse beta -/-;

human alpha +/ Human Beta sickle+

3.3. Heterozygous transgenic mouse genotype

Mouse alpha -/-, mouse beta +/-;

human alpha +/ Human Beta sickle+

4 The set of experiment use twenty mice. Twenty mice were divided into three groups.

4.1 The first group five mice were given 0.5 ug cucurbitacin D per gram of mouse weight.

4.2 The second group five mice were given 0.1 ug cucurbitacin D per gram. After taking the medicine, 50 ulof 0.6 g/ml sucrose was given to them continuously.

4.3 Control group has ten mice. They just were given the surcrose.

5. Oral feeding was the drug administration route. The feeding was maintained for 30 days.

6 Each fifth day collected the blood sample from the mouse. The microtube of blood samples were span down.

7 Fix amount of RBC were lysied in lysing buffer, the samples were further test for ELISA.

8 The buffy coats were lysied and extracted DNA for genotyping.

9 After lysing the RBC, the WBCs were lysied in lysing buffer. The protein was precipitated in 4℃. Span down the precipitation and kept the supernatant for DNA precipitation.

10 1.5 part volume of Isopropanol was added into the tube and mixed with up-side –down, Span the tube with 14K rpm for 15mins .

11 Then washed the DNA pellet with 70 % EtOH. Dry the pellet in room temperature. Suspended the DNA pellet in 30 ul TE buffer. Kept in 4℃.

12 The procedure of ELISA test was as mention in chapter 2.

4.3.5.2 Determination the genotype of sickle cell mouse

1. The genotypes of 20 mice were verified by PCR method. The Primer pairs were provided by Jason Laboratory Company.

PCR profile: 94℃ 1min

54℃/58℃ 1min

72℃ 1min

Repeated 35 cycles.

2. The detail primers list was provide by Jason laboratory LTD.

Table 4-2

Mouse JE3342		Primer list
Mouse alpha globin	1137	5'-AGT GGG CAG CTT CTA ACT ATG C-3'
	1138	5'-GTC CCA GCG CAT ACC TTG-3'
	1139	5'-ATA GAT GGG TAG CCA TTT AGA TTC C-3'
	1140	5'-CCG GGT TAT AAT TAC CTC AGG TC-3'
Mouse Beta globin	1141	5'-TTA GGT GGT CTT AAA ACT TTT GTG G-3'
	1142	5'-ACT GGC ACA GAG CAT TGT TAT G-3'
	1143	5'-AGA TGT TTT TTT CAC ATT CTT GAG C-3'
	1144	5'-AAT GCC TGC TCT TTA CTG AAG G-3'
Human transgene	1145	5'-GTA TGG GAG AGG CTC CAA CTC-3'
	1146	5'-GTA TGG GAG AGG CTC CAA CTC-3'

3. Genotyping of Sickle Cell Mouse from Jason's lab

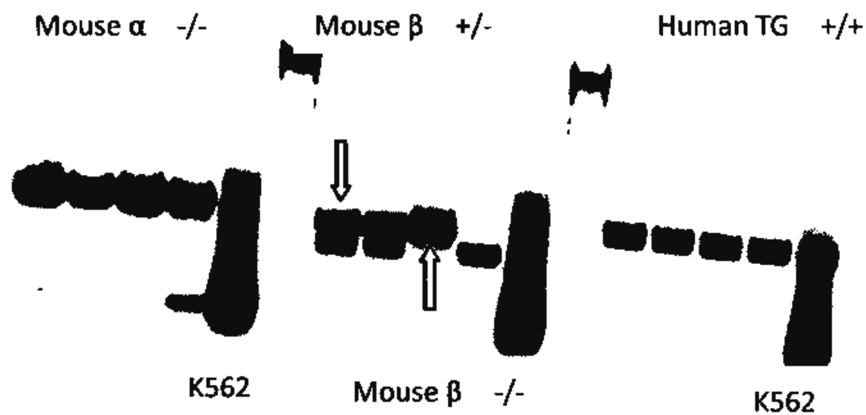


Figure 4-3: DNA extracted from Jason Lab. Sickle cell mouse, genotype revealed in gel picture (ethidium bromide staining)

PCR method determine the mouse genotype

Primers were provided by Jason Laboratory LTD. DNA samples were extracted by the mice blood, and then PCR amplified the mouse wild-type hemoglobin gene, truncated mouse hemoglobin gene and the human transgene. The different genotypes were shown in the photo.

4.3.5.3 Genotypes of Sickle cell mice (Jason Laboratory) used for the experiment

Mouse genotype table (to be continued)

Mouse	Mouse alpha	Mouse beta	Human transgene	Date of birth
Gp 1	Group 0.5ug/g	N=5		
51	–/–	–/+	Positive	16-2-07
85	–/–	–/+	Positive	4/07/07
93	–/–	–/+	Positive	21/4/07
97	–/–	–/+	Positive	21/4/07
99	–/–	–/+	Positive	13/4/07
Gp 2	Group 0.1ug/g	N=5		
62	–/–	–/+	Positive	16/3/07
77	–/–	–/+	Positive	4/07
75	–/–	–/+	Positive	3/4/07
95	–/–	–/+	Positive	21/4/07
110	–/–	–/+	Positive	3/5/07

Mouse	Mouse alpha	Mouse beta	Human transgene	Date of birth
Gp 3	Control group	N=10		
56	– / –	– / +	Positive	16/3/07
79	– / –	– / +	Positive	4/07/07
84	– / –	– / +	Positive	4/07
86	– / –	– / +	Positive	4/07
90	– / –	– / +	Positive	21/4/07
92	– / –	– / +	Positive	21/4/07
96	– / –	– / +	Positive	21/4/07
100	– / –	– / +	Positive	13/4/07
107	– / –	– / +	Positive	3/5/07
112	– / –	+ / +	Positive	24/5/07

Table 4-3: Three group of experimental mice, information included their name, hemoglobin genotype, the date of birth, and their treatment.

4.4. Results

4.4.1. Toxicity of cucurbitacin B, D, E and I on human normal cell line

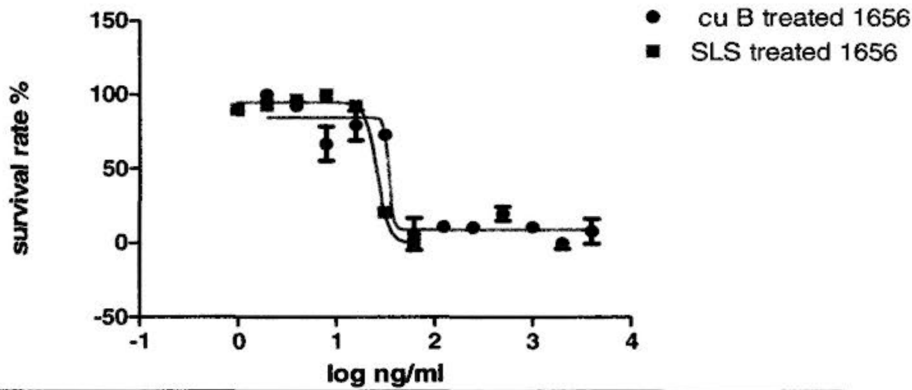
Four cell lines 1656, 2115, 2507 and 2120 were used to access the toxicity of cucurbitacins. IC_{50} and correlation coefficient (C.C.) were two parameters to evaluate the compound toxicity. IC_{50s} were calculated from dose-response curves. % of C.C. was the association of tendency between the positive control curve and toxicity curve of the compound upon a specific cell line. Toxicity dose-response curves were shown in figure 4-4to figure 4-35. The lowest toxicity to the four cell lines was cucurbitacin E. The most toxic one was cucurbitacin B. Comparison of IC_{50} and % of C.C. of cucurbitacin B, D, E, I was shown in table 4-1.

Toxicity of cucurbitacin B, D, E & I

Cell line	cucurbitacin	IC_{50} ng/ml Alamar blue	C.C. % correlation coefficient	IC_{50} ng/ml Neutral Red	C.C. % correlation coefficient
1656	B	34.74	0.86	120.3	0.96
	D	146.7	0.92	181.5	0.99
	E	5628	0.6	1084.0	0.97
	I	12464	0.54	139.3	0.95
2115	B	2221	0.64	123.0	0.99
	D	19711	0.89	157.4	0.96
	E	2134	0.73	984.3	0.95
	I	63.73	-0.59	127.0	0.95
2507	B	40.63	0.75	33.1	0.96
	D	198.4	0.76	124.4	0.99
	E	948.9	0.63	474..1	0.95
	I	100.78	0.79	44.0	0.94
2120	B	78.55	0.92	60.3	0.98
	D	125.0	0.92	157.9	0.99
	E	0	0.63	741.4	0.96
	I	1650	0.88	80.7	0.7

Table 4-5: The IC_{50} was calculated from dose-response curve. The non-liner regression method drafted the curve in GrapPad PRISM$_R$ 4.0. The % of C.C. was calculated in excel program. The toxic effects of different cucurbitacins were evaluated with 1656, 2115, 2507 and 2120 cell lines.

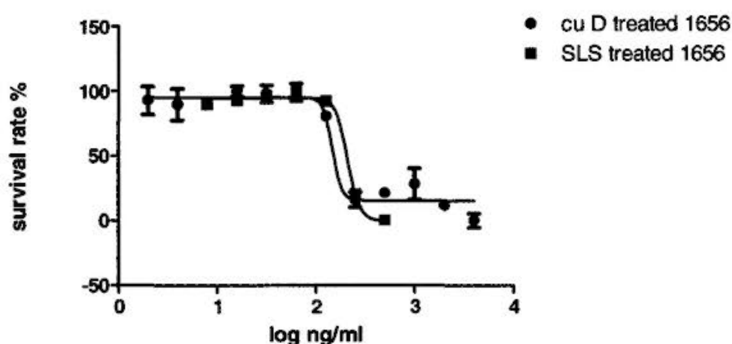
cu B & SLS matching cytotoxicity curve (Alamar Blue)



	cu B treated 1656	SLS treated 1656
log(inhibitor) vs. response -- Variable slope	Ambiguous	
Best-fit values		
BOTTOM	8.879	-0.1813
TOP	84.68	94.80
LOGIC50	~ 1.535	1.416
HILLSLOPE	~ -18.50	-7.143
IC50	~ 34.25	26.09
Span	75.80	94.98
Std. Error		
BOTTOM	3.383	4.483
TOP	3.783	2.200
LOGIC50	~ 23.06	0.03692
HILLSLOPE	~ 10704	3.083
Span	5.094	5.068
95% Confidence Intervals		
BOTTOM	1.961 to 15.80	-14.44 to 14.08
TOP	76.94 to 92.41	87.80 to 101.8
LOGIC50	(Very wide)	1.299 to 1.534
HILLSLOPE	(Very wide)	-16.95 to 2.667
IC50	(Very wide)	19.91 to 34.19
Span	65.38 to 86.22	78.85 to 111.1
Goodness of Fit		
Degrees of Freedom	29	3
R2	0.8993	0.9944
Absolute Sum of Squares	4979	57.66
Sy.x	13.10	4.384
Number of points		
Analyzed	33	7

Figure 4-4: The cytotoxicity assay of Cucurbitacin B and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin B and Sodium Lauryl Sulfate. IC 50 of cu B was 34.25 ng/ml. The correlation coefficient between two toxicity curves was 0.86. It indicated the toxicity result of cu B in 1656 cell assay was significant.

cu D & SLS matching cytotoxicity curve (Alamar Blue)

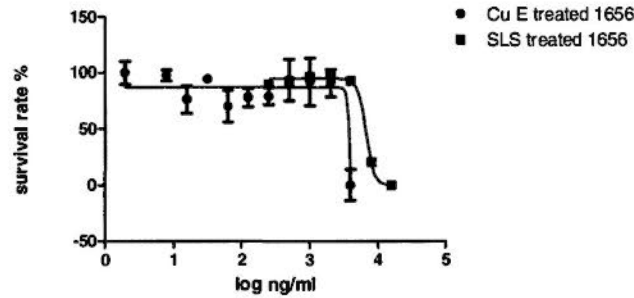


	cu D treated 1656	SLS treated 1656
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	15.41	-0.1813
TOP	95.09	94.80
LOGIC50	2.166	2.320
HILLSLOPE	-9.455	-7.143
IC50	146.7	208.7
Span	79.68	94.98
Std. Error		
BOTTOM	3.770	4.483
TOP	3.079	2.200
LOGIC50	0.1777	0.03692
HILLSLOPE	23.86	3.083
Span	4.891	5.068
95% Confidence Intervals		
BOTTOM	7.733 to 23.10	-14.44 to 14.08
TOP	88.82 to 101.4	87.80 to 101.8
LOGIC50	1.804 to 2.528	2.202 to 2.437
HILLSLOPE	-58.07 to 39.16	-16.95 to 2.667
IC50	63.71 to 337.7	159.2 to 273.5
Span	69.71 to 89.64	78.85 to 111.1
Goodness of Fit		
Degrees of Freedom	32	3
R2	0.9069	0.9944
Absolute Sum of Squares	5452	57.66
Sy.x	13.05	4.384
Number of points		
Analyzed	36	7

Figure 4-5: The cytotoxicity assay of Cucurbitacin D and Sodium Lauryl Sulfate

Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin D and Sodium Lauryl Sulfate. IC 50 of cu D was 146.7 ng/ml. The correlation coefficient between two toxicity curves was 0.92. It indicated the toxicity result of cu D in 1656 cell assay was significant.

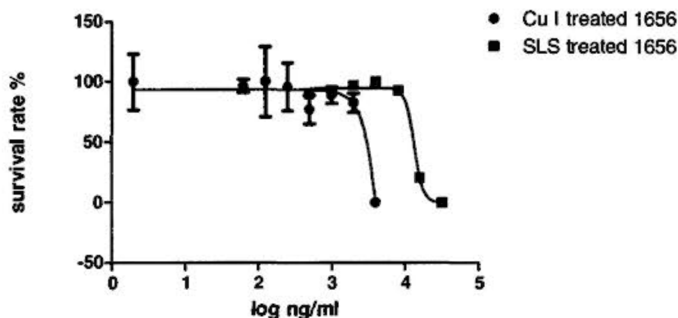
cu E & SLS matching cytotoxicity curve (Alamar Blue)



	cu E treated 1656	SLS treated 1656
log(inhibitor) vs. response -- Variable slope	Ambiguous	
Best-fit values		
BOTTOM	~ -159600	-0.1813
TOP	86.85	94.80
LOGIC50	~ 3.802	3.825
HILLSLOPE	~ -16.34	-7.143
IC50	~ 6336	6679
Span	~ 159719	94.98
Std. Error		
BOTTOM	~ 1.605e+014	4.483
TOP	4.869	2.200
LOGIC50	~ 2.672e+007	0.03692
HILLSLOPE	~ 688434	3.083
Span	~ 1.605e+014	5.068
95% Confidence Intervals		
BOTTOM	(Very wide)	-14.44 to 14.08
TOP	76.82 to 96.88	87.80 to 101.8
LOGIC50	(Very wide)	3.707 to 3.942
HILLSLOPE	(Very wide)	-16.95 to 2.667
IC50	(Very wide)	5096 to 8753
Span	(Very wide)	78.85 to 111.1
Goodness of Fit		
Degrees of Freedom	25	3
R2	0.6312	0.9944
Absolute Sum of Squares	11852	57.66
Sy.x	21.77	4.384
Number of points		
Analyzed	29	7

Figure 4-6: The cytotoxicity assay of Cucurbitacin E and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin E and Sodium Lauryl Sulfate. IC 50 of cu E was 6336 ng/ml. The correlation coefficient between two toxicity curves was 0.6. It indicated the toxicity result of cu E in 1656 cell assay was significant.

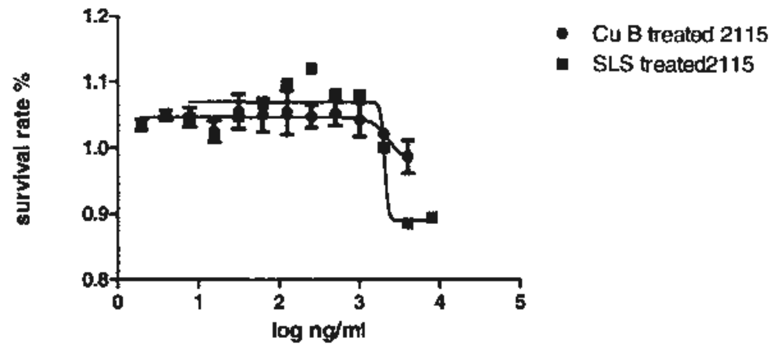
cu I & SLS matching cytotoxicity curve (Alamar Blue)



	cu I treated 1656	SLS treated 1656
log(inhibitor) vs. response -- Variable slope	Ambiguous	
Best-fit values		
BOTTOM	93 55	-0.1813
TOP	~ -4803	94.80
LOGIC50	~ 4.194	4 126
HILLSLOPE	~ 2 890	-7.143
IC50	~ 15637	13357
Span	~ -4897	94.98
Std. Error		
BOTTOM	7 183	4.483
TOP	~ 3.220e+006	2 200
LOGIC50	~ 104 0	0.03692
HILLSLOPE	~ 15.96	3 083
Span	~ 3.220e+006	5.068
95% Confidence Intervals		
BOTTOM	78.57 to 108.5	-14 44 to 14.08
TOP	(Very wide)	87 80 to 101.8
LOGIC50	(Very wide)	4 008 to 4.243
HILLSLOPE	(Very wide)	-16.95 to 2.667
IC50	(Very wide)	10192 to 17506
Span	(Very wide)	78 85 to 111 1
Goodness of Fit		
Degrees of Freedom	20	3
R2	0.6239	0.9944
Absolute Sum of Squares	13433	57.66
Sy.x	25 92	4 384
Number of points		
Analyzed	24	7

Figure 4-7: The cytotoxicity assay of Cucurbitacin I and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin I and Sodium Lauryl Sulfate. IC 50 of cu I was 15637 ng/ml. The correlation coefficient between two toxicity curves was 0.54. It indicated the toxicity result of cu I in 1656 cell assay was significant.

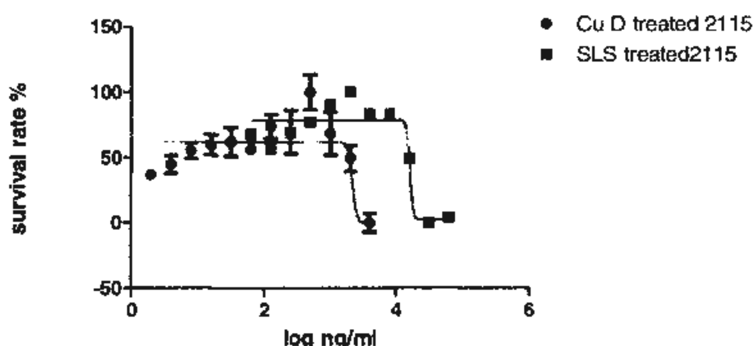
cu B & SLS matching cytotoxicity curve (Alamar Blue)



	cu B treated 2115	SLS treated2115
log(inhibitor) vs. response -- Variable slope		Ambiguous
Best-fit values		
BOTTOM	0.9822	0.8900
TOP	1.046	1.070
LOGIC50	3.347	~ 3.311
HILLSLOPE	-4.402	~ -19.13
IC50	2221	~ 2048
Span	0.06367	0.1796
Std. Error		
BOTTOM	0.03869	0.03092
TOP	0.003834	0.01177
LOGIC50	0.2201	~ 64.95
HILLSLOPE	10.17	~ 119702
Span	0.03993	0.03433
95% Confidence Intervals		
BOTTOM	0.9034 to 1.061	0.8169 to 0.9631
TOP	1.038 to 1.054	1.042 to 1.097
LOGIC50	2.898 to 3.795	(Very wide)
HILLSLOPE	-25.14 to 16.33	(Very wide)
IC50	790.6 to 6241	(Very wide)
Span	-0.01769 to 0.1450	0.09844 to 0.2608
Goodness of Fit		
Degrees of Freedom	32	7
R2	0.4586	0.8751
Absolute Sum of Squares	0.01241	0.007516
Sy.x	0.01969	0.03277
Number of points		
Analyzed	36	11

Figure 4-8: The cytotoxicity assay of Cucurbitacin B and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin B and Sodium Lauryl Sulfate. IC 50 of cu B was 2221 ng/ml. The correlation coefficient between two toxicity curves was 0.64. It indicated the toxicity result of cu B in 2115 cell assay was significant.

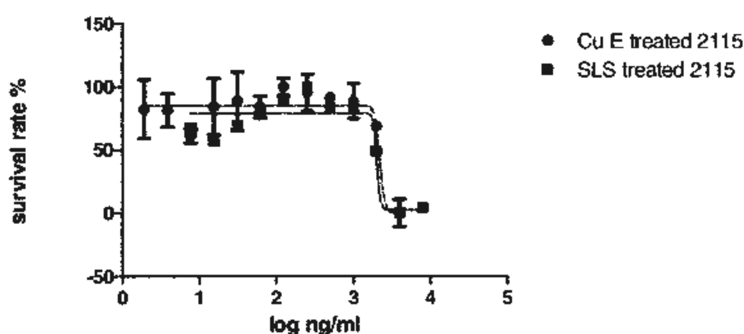
cu D & SLS matching cytotoxicity curve (Alamar Blue)



	cu D treated 2115	SLS treated2115
log(inhibitor) vs. response -- Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	-0.009928	2.127
TOP	61.48	78.56
LOGIC50	~ 3.343	~ 4.215
HILLSLOPE	~ -14.66	~ -19.13
IC50	~ 2201	~ 16387
Span	61.49	76.44
Std. Error		
BOTTOM	212.6	13.16
TOP	4.190	5.006
LOGIC50	~ 90.18	~ 64.95
HILLSLOPE	~ 31764	~ 119706
Span	214.0	14.61
95% Confidence Intervals		
BOTTOM	-434.1 to 434.0	-28.99 to 33.24
TOP	52.93 to 70.04	66.72 to 90.40
LOGIC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
IC50	(Very wide)	(Very wide)
Span	-375.4 to 498.4	41.89 to 111.0
Goodness of Fit		
Degrees of Freedom	30	7
R2	0.4401	0.8751
Absolute Sum of Squares	13167	1361
Sy.x	20.95	13.94
Number of points		
Analyzed	34	11

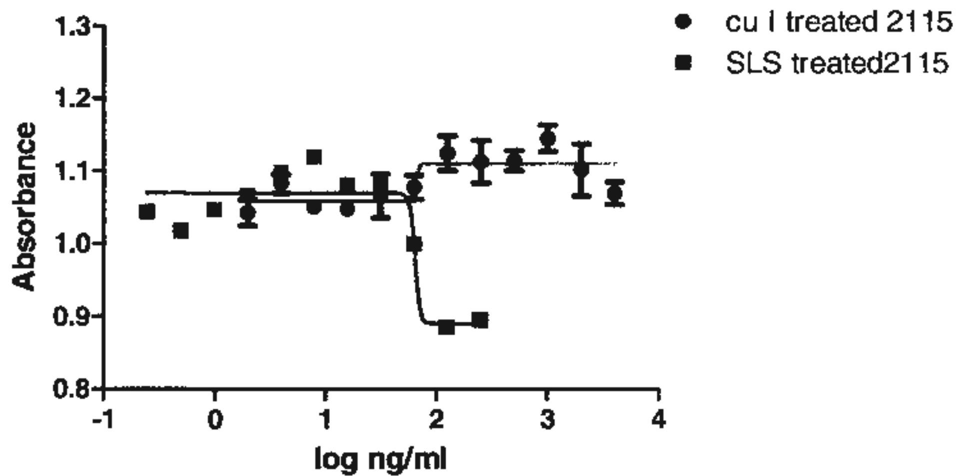
Figure 4-9: The cytotoxicity assay of Cucurbitacin D and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin D and Sodium Lauryl Sulfate. IC 50 of cu D was 2201 ng/ml. The correlation coefficient between two toxicity curves was 0.86. It indicated the toxicity result of cu D in 2115 cell assay was significant.

cu E & SLS matching cytotoxicity curve (Alamar Blue)



	cu E treated 2115	SLS treated2115
log(inhibitor) vs. response -- Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	84.97	2.127
TOP	-0.02060	78.56
LOGIC50	~ 3.345	~ 3.311
HILLSLOPE	~ 14.10	~ -19.13
IC50	~ 2214	~ 2048
Span	-85.00	76.44
Std. Error		
BOTTOM	5.001	13.16
TOP	264.9	5.006
LOGIC50	~ 58.85	~ 64.95
HILLSLOPE	~ 18731	~ 119707
Span	266.6	14.61
95% Confidence Intervals		
BOTTOM	74.73 to 95.22	-28.99 to 33.24
TOP	-542.5 to 542.4	66.72 to 90.40
LOGIC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
IC50	(Very wide)	(Very wide)
Span	-630.9 to 460.9	41.89 to 111.0
Goodness of Fit		
Degrees of Freedom	28	7
R2	0.5385	0.8751
Absolute Sum of Squares	16806	1361
Sy.x	24.50	13.94
Number of points		
Analyzed	32	11

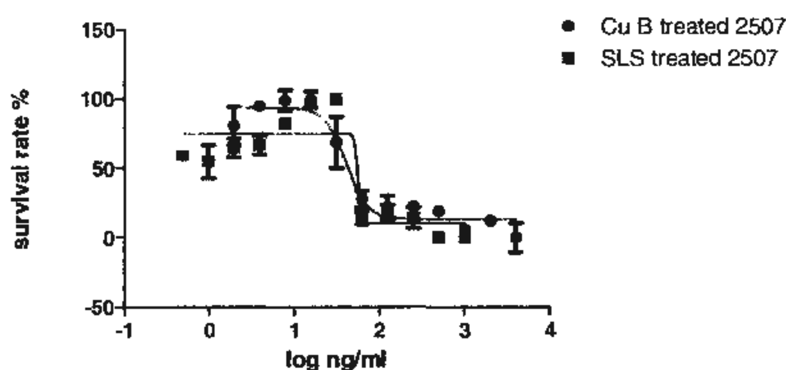
Figure 4-10: The cytotoxicity assay of Cucurbitacin E and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin E and Sodium Lauryl Sulfate. IC 50 of cu E was 2214 ng/ml. The correlation coefficient between two toxicity curves was 0.73. It indicated the toxicity result of cu E in 2115 cell assay was significant.



	cu I treated 2115	SLS treated2115
log(agonist) vs. response -- Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	1.058	0.8900
TOP	1.110	1.070
LOGEC50	~ 1.804	~ 1.806
HILLSLOPE	~ 26.13	~ -19.13
EC50	~ 63.73	~ 64.01
Span	0.05236	0.1796
Std. Error		
BOTTOM	0.01128	0.03092
TOP	0.01008	0.01177
LOGEC50	~ 9979	~ 64.95
HILLSLOPE	~ 3.087e+007	~ 119702
Span	0.01556	0.03433
95% Confidence Intervals		
BOTTOM	1.035 to 1.081	0.8169 to 0.9631
TOP	1.090 to 1.131	1.042 to 1.097
LOGEC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
EC50	(Very wide)	(Very wide)
Span	0.02016 to 0.08455	0.09844 to 0.2608
Goodness of Fit		
Degrees of Freedom	23	7
R2	0.3641	0.8751
Absolute Sum of Squares	0.02848	0.007516
Sy.x	0.03519	0.03277
Number of points		
Analyzed	27	11

Figure 4-11: The cytotoxicity assay of Cucurbitacin I and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin I and Sodium Lauryl Sulfate. IC 50 of cu I was 63.75ng/ml. The correlation coefficient between cu I and L.S.L. was -0.59. They were inverse correlated.

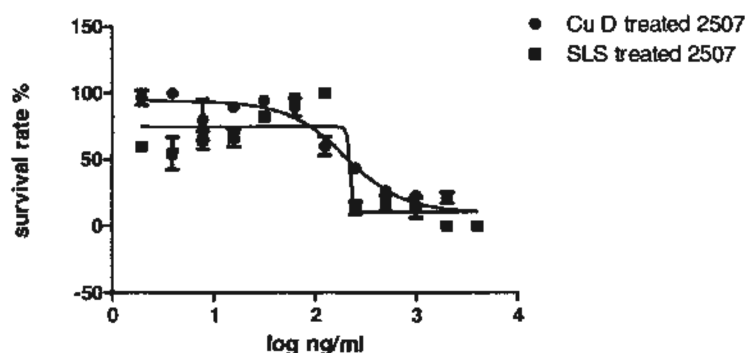
cu B & SLS matching cytotoxicity curve (Alamar Blue)



	cu B treated 2507	SLS treated2507
log(inhibitor) vs. response -- Variable slope		Ambiguous
Best-fit values		
BOTTOM	13.38	10.68
TOP	93.88	74.91
LOGIC50	1.609	~ 1.747
HILLSLOPE	-3.522	~ -25.43
IC50	40.63	~ 55.85
Span	80.50	64.24
Std. Error		
BOTTOM	3.681	6.790
TOP	4.681	4.801
LOGIC50	0.05948	~ 1419
HILLSLOPE	1.308	~ 737896
Span	6.091	8.319
95% Confidence Intervals		
BOTTOM	5.876 to 20.88	-3.589 to 24.94
TOP	84.34 to 103.4	64.83 to 85.00
LOGIC50	1.488 to 1.730	(Very wide)
HILLSLOPE	-6.187 to -0.8571	(Very wide)
IC50	30.73 to 53.71	(Very wide)
Span	68.09 to 92.92	46.76 to 81.71
Goodness of Fit		
Degrees of Freedom	32	18
R2	0.8701	0.8043
Absolute Sum of Squares	7222	4980
Sy.x	15.02	16.63
Number of points		
Analyzed	36	22

Figure 4-12: The cytotoxicity assay of Cucurbitacin B and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin B and Sodium Lauryl Sulfate. IC 50 of cu B was 40.63 ng/ml. The correlation coefficient between two toxicity curves was 0.75. It indicated the toxicity result of cu B in 2507 cell assay was significant.

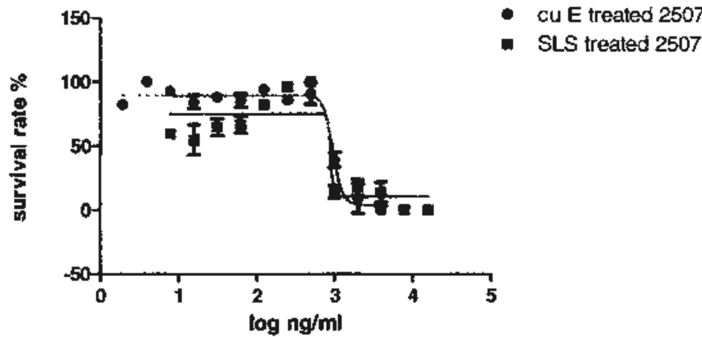
cu D & SLS matching cytotoxicity curve (Alamar Blue)



	cu D treated 2507	SLS treated2507
log(inhibitor) vs. response -- Variable slope		Ambiguous
Best-fit values		
BOTTOM	10.49	10.68
TOP	94.39	74.91
LOGIC50	2.298	~ 2.349
HILLSLOPE	-1.472	~ -25.43
IC50	198.4	~ 223.4
Span	83.90	64.24
Std. Error		
BOTTOM	5.661	6.790
TOP	3.618	4.801
LOGIC50	0.08478	~ 1419
HILLSLOPE	0.3832	~ 737896
Span	7.426	8.319
95% Confidence Intervals		
BOTTOM	-1.049 to 22.02	-3.589 to 24.94
TOP	87.02 to 101.8	64.83 to 85.00
LOGIC50	2.125 to 2.470	(Very wide)
HILLSLOPE	-2.253 to -0.6908	(Very wide)
IC50	133.3 to 295.4	(Very wide)
Span	68.77 to 99.04	46.76 to 81.71
Goodness of Fit		
Degrees of Freedom	32	18
R2	0.9048	0.8043
Absolute Sum of Squares	4257	4980
Sy.x	11.53	16.63
Number of points		
Analyzed	36	22

Figure 4-13: The cytotoxicity assay of Cucurbitacin D and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin D and Sodium Lauryl Sulfate. IC 50 of cu D was 198.4 ng/ml. The correlation coefficient between two toxicity curves was 0.76. It indicated the toxicity result of cu D in 2507 cell assay was significant.

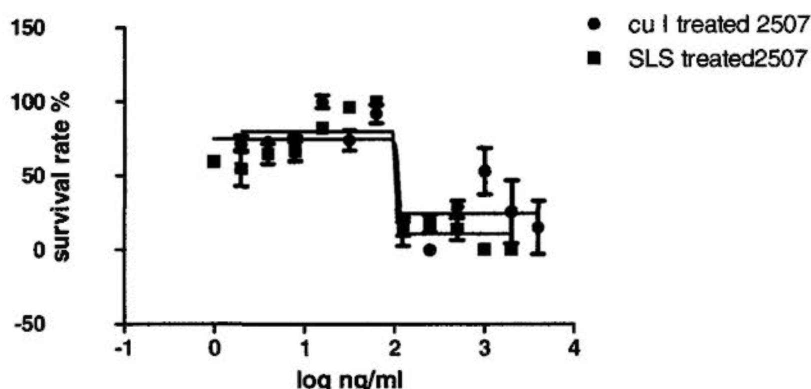
cu E & SLS matching cytotoxicity curve (Alamar Blue)



	cu E treated 2507	SLS treated2507
log(inhibitor) vs. response -- Variable slope		Ambiguous
Best-fit values		
BOTTOM	4.332	10.68
TOP	89.40	74.91
LOGIC50	2.977	~ 2.951
HILLSLOPE	-7.242	~ -25.43
IC50	948.9	~ 893.5
Span	85.06	64.24
Std. Error		
BOTTOM	4.295	6.790
TOP	2.005	4.801
LOGIC50	0.03510	~ 1419
HILLSLOPE	10.22	~ 737897
Span	4.916	8.319
95% Confidence Intervals		
BOTTOM	-4.420 to 13.08	-3.589 to 24.94
TOP	85.31 to 93.48	64.83 to 85.00
LOGIC50	2.906 to 3.049	(Very wide)
HILLSLOPE	-28.07 to 13.59	(Very wide)
IC50	804.8 to 1119	(Very wide)
Span	75.05 to 95.08	46.76 to 81.71
Goodness of Fit		
Degrees of Freedom	32	18
R2	0.9255	0.8043
Absolute Sum of Squares	3114	4980
Sy.x	9.864	16.63
Number of points		
Analyzed	36	22

Figure 4-14: The cytotoxicity assay of Cucurbitacin E and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin E and Sodium Lauryl Sulfate. IC 50 of cu E was 948.9 ng/ml. The correlation coefficient between two toxicity curves was 0.63. It indicated the toxicity result of cu E in 2507cell assay was significant.

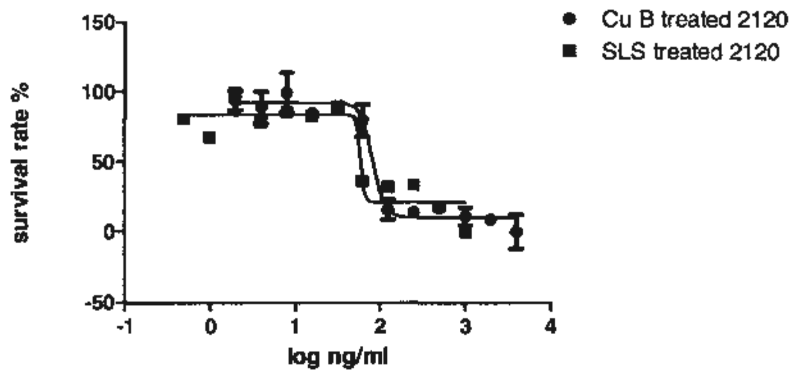
cu I & SLS matching cytotoxicity curve (Alamar Blue)



	cu I treated 2507	SLS treated2507
log(inhibitor) vs. response -- Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	24.47	10.68
TOP	79.72	74.91
LOGIC50	~ 2.002	~ 2.048
HILLSLOPE	~ -181.2	~ -25.43
IC50	~ 100.4	~ 111.7
Span	55.25	64.24
Std. Error		
BOTTOM	5.209	6.790
TOP	5.209	4.801
LOGIC50	~	~ 1419
HILLSLOPE	~	~ 737897
Span	7.366	8.319
95% Confidence Intervals		
BOTTOM	13.86 to 35.09	-3.589 to 24.94
TOP	69.11 to 90.34	64.83 to 85.00
LOGIC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
IC50	(Very wide)	(Very wide)
Span	40.24 to 70.26	46.76 to 81.71
Goodness of Fit		
Degrees of Freedom	32	18
R2	0.6533	0.8043
Absolute Sum of Squares	15627	4980
Sy.x	22.10	16.63
Number of points		
Analyzed	36	22

Figure 4-15: The cytotoxicity assay of Cucurbitacin I and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin I and Sodium Lauryl Sulfate. IC 50 of cu I was 100.4 ng/ml. The correlation coefficient between two toxicity curves was 0.79. It indicated the toxicity result of cu I in 2507 cell assay was significant.

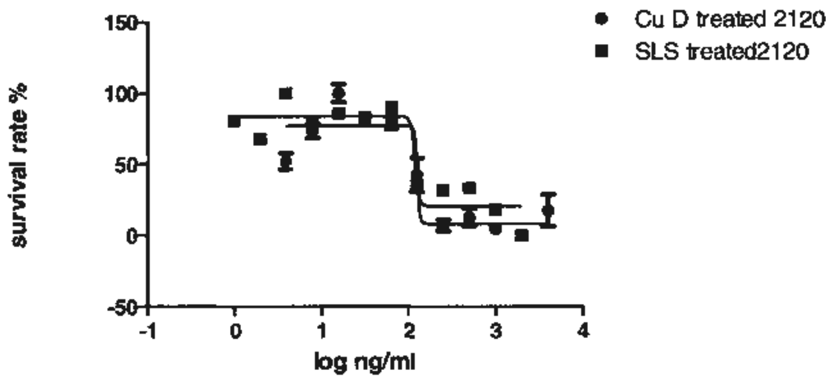
cu B & SLS matching cytotoxicity curve (Alamar Blue)



	cu B treated 2120	SLS treated2120
log(inhibitor) vs. response -- Variable slope		Ambiguous
Best-fit values		
BOTTOM	10.01	20.97
TOP	92.33	83.80
LOGIC50	1.916	~ 1.770
HILLSLOPE	-6.149	~ -19.02
IC50	82.32	~ 58.88
Span	82.33	62.83
Std. Error		
BOTTOM	3.507	6.431
TOP	3.774	5.239
LOGIC50	0.05585	~ 73.99
HILLSLOPE	2.486	~ 54346
Span	5.174	8.379
95% Confidence Intervals		
BOTTOM	2.847 to 17.17	6.141 to 35.80
TOP	84.63 to 100.0	71.72 to 95.88
LOGIC50	1.801 to 2.030	(Very wide)
HILLSLOPE	-11.22 to -1.073	(Very wide)
IC50	63.31 to 107.0	(Very wide)
Span	71.76 to 92.89	43.51 to 82.15
Goodness of Fit		
Degrees of Freedom	30	8
R2	0.9068	0.8894
Absolute Sum of Squares	5467	1319
Sy.x	13.50	12.84
Number of points		
Analyzed	34	12

Figure 4-16: The cytotoxicity assay of Cucurbitacin B and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin B and Sodium Lauryl Sulfate. IC 50 of cu B was 83.32 ng/ml. The correlation coefficient between two toxicity curves was 0.92. It indicated the toxicity result of cu B in 2120 cell assay was significant.

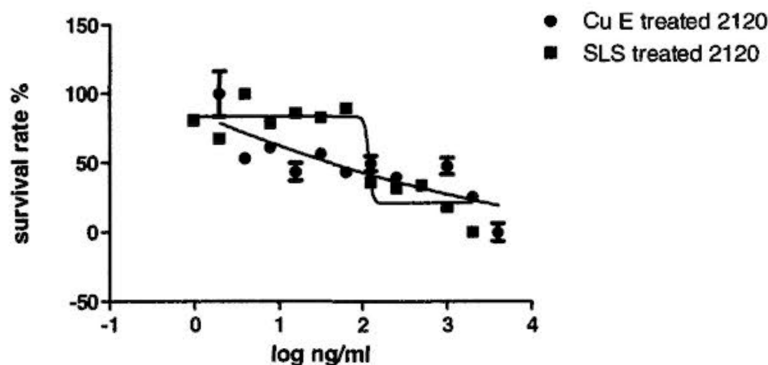
cu D & SLS matching cytotoxicity curve (Alamar Blue)



	cu D treated 2120	SLS treated2120
log(inhibitor) vs. response -- Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	8.471	20.97
TOP	77.15	83.80
LOGIC50	~ 2.097	~ 2.071
HILLSLOPE	~ -31.47	~ -19.02
IC50	~ 125.0	~ 117.8
Span	68.68	62.83
Std. Error		
BOTTOM	4.198	6.431
TOP	4.191	5.239
LOGIC50	~ 1441	~ 73.99
HILLSLOPE	~ 4.359e+008	~ 54346
Span	6.253	8.379
95% Confidence Intervals		
BOTTOM	-0.1153 to 17.06	6.141 to 35.80
TOP	68.58 to 85.72	71.72 to 95.88
LOGIC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
IC50	(Very wide)	(Very wide)
Span	55.89 to 81.47	43.51 to 82.15
Goodness of Fit		
Degrees of Freedom	29	8
R2	0.8387	0.8894
Absolute Sum of Squares	6804	1319
Sy.x	15.32	12.84
Number of points		
Analyzed	33	12

Figure 4-17: The cytotoxicity assay of Cucurbitacin D and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin D and Sodium Lauryl Sulfate. IC 50 of cu D was 125 ng/ml. The correlation coefficient between two toxicity curves was 0.92. It indicated the toxicity result of cu D in 2120 cell assay was significant.

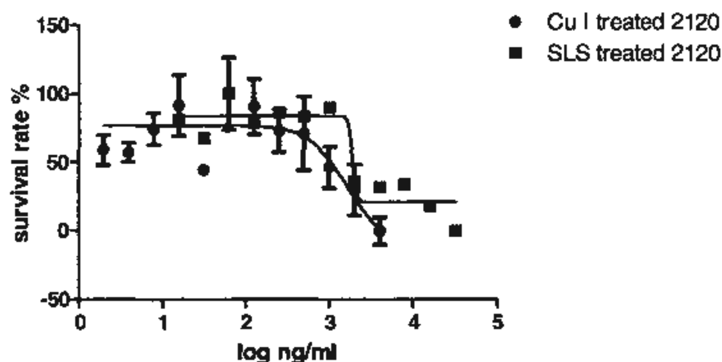
cu E & SLS matching cytotoxicity curve (Alamar Blue)



	cu E treated 2120	SLS treated2120
log(inhibitor) vs. response -- Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	~ -37.34	20.97
TOP	~ 15578	83.80
LOGIC50	~ -22.08	~ 2.071
HILLSLOPE	~ -0.09494	~ -19.02
IC50	~ 0.0	~ 117.8
Span	~ 15615	62.83
Std. Error		
BOTTOM	~ 1057	6.431
TOP	~ 3.667e+007	5.239
LOGIC50	~ 11352	~ 73.99
HILLSLOPE	~ 2.428	~ 54346
Span	~ 3.667e+007	8.379
95% Confidence Intervals		
BOTTOM	(Very wide)	6.141 to 35.80
TOP	(Very wide)	71.72 to 95.88
LOGIC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
IC50	(Very wide)	(Very wide)
Span	(Very wide)	43.51 to 82.15
Goodness of Fit		
Degrees of Freedom	29	8
R2	0.5900	0.8894
Absolute Sum of Squares	8315	1319
Sy.x	16.93	12.84
Number of points		
Analyzed	33	12

Figure 4-18: The cytotoxicity assay of Cucurbitacin E and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin E and Sodium Lauryl Sulfate. IC 50 of cu E was 0 ng/ml. The correlation coefficient between two toxicity curves was 0.63. It indicated the toxicity result of cu E in 2120 cell assay was significant.

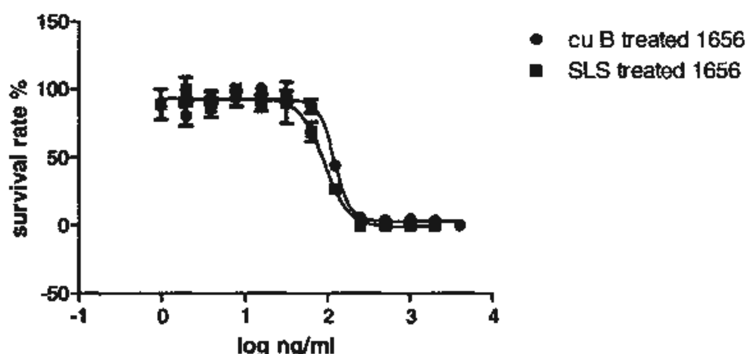
cu I & SLS matching cytotoxicity curve (Alamar Blue)



	cu I treated 2120	SLS treated2120
log(inhibitor) vs. response -- Variable slope		Ambiguous
Best-fit values		
BOTTOM	-12.61	20.97
TOP	76.85	83.80
LOGIC50	3.217	~ 3.275
HILLSLOPE	-1.902	~ -19.02
IC50	1650	~ 1884
Span	89.47	62.83
Std. Error		
BOTTOM	63.86	6.431
TOP	6.963	5.239
LOGIC50	0.4615	~ 73.99
HILLSLOPE	2.213	~ 54346
Span	65.95	8.379
95% Confidence Intervals		
BOTTOM	-143.4 to 118.2	6.141 to 35.80
TOP	62.59 to 91.12	71.72 to 95.88
LOGIC50	2.272 to 4.163	(Very wide)
HILLSLOPE	-6.434 to 2.631	(Very wide)
IC50	187.2 to 14539	(Very wide)
Span	-45.60 to 224.5	43.51 to 82.15
Goodness of Fit		
Degrees of Freedom	28	8
R2	0.4273	0.8894
Absolute Sum of Squares	25085	1319
Sy.x	29.93	12.84
Number of points		
Analyzed	32	12

Figure 4-19: The cytotoxicity assay of Cucurbitacin I and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin I and Sodium Lauryl Sulfate. IC 50 of cu I was 1650 ng/ml. The correlation coefficient between two toxicity curves was 0.88. It indicated the toxicity result of cu I in 2120 cell assay was significant.

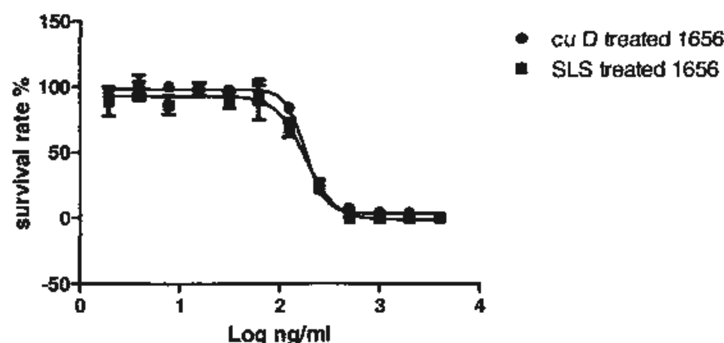
cu B & SLS matching cytotoxicity curve (Neutral Red)



	cu B treated 1656	SLS treated 1656
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	3.031	-0.9878
TOP	92.43	92.96
LOGIC50	2.080	1.957
HILLSLOPE	-4.573	-2.991
IC50	120.3	90.57
Span	89.40	93.94
Std. Error		
BOTTOM	1.980	3.454
TOP	1.836	2.701
LOGIC50	0.01895	0.03970
HILLSLOPE	1.316	0.6605
Span	2.813	4.523
95% Confidence Intervals		
BOTTOM	-1.004 to 7.066	-8.193 to 6.217
TOP	88.69 to 96.17	87.32 to 98.59
LOGIC50	2.042 to 2.119	1.874 to 2.040
HILLSLOPE	-7.255 to -1.891	-4.369 to -1.613
IC50	110.0 to 131.4	74.85 to 109.6
Span	83.67 to 95.13	84.51 to 103.4
Goodness of Fit		
Degrees of Freedom	32	20
R2	0.9749	0.9655
Absolute Sum of Squares	1638	1531
Sy.x	7.155	8.751
Number of points		
Analyzed	36	24

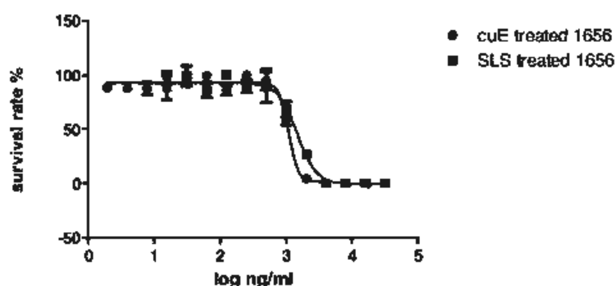
Figure 4-20: The cytotoxicity assay of Cucurbitacin B and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin B and Sodium Lauryl Sulfate. IC 50 of cu B was 120.3 ng/ml. The correlation coefficient between two toxicity curves was 0.96. It indicated the toxicity result of cu B in 1656 cell assay was significant.

cu D & SLS matching cytotoxicity curve (Neutral Red)



	cu D treated 1656	SLS treated 1656
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	3.612	-0.9878
TOP	97.97	92.96
LOGIC50	2.259	2.258
HILLSLOPE	-4.509	-2.991
IC50	181.5	181.1
Span	94.36	93.94
Std. Error		
BOTTOM	1.572	3.454
TOP	1.269	2.701
LOGIC50	0.01707	0.03970
HILLSLOPE	0.4898	0.6605
Span	2.040	4.523
95% Confidence Intervals		
BOTTOM	0.4088 to 6.816	-8.193 to 6.217
TOP	95.38 to 100.6	87.32 to 98.59
LOGIC50	2.224 to 2.294	2.175 to 2.341
HILLSLOPE	-5.507 to -3.511	-4.369 to -1.613
IC50	167.5 to 196.6	149.7 to 219.2
Span	90.20 to 98.51	84.51 to 103.4
Goodness of Fit		
Degrees of Freedom	32	20
R2	0.9873	0.9655
Absolute Sum of Squares	893.8	1531
Sy.x	5.285	8.751
Number of points		
Analyzed	36	24

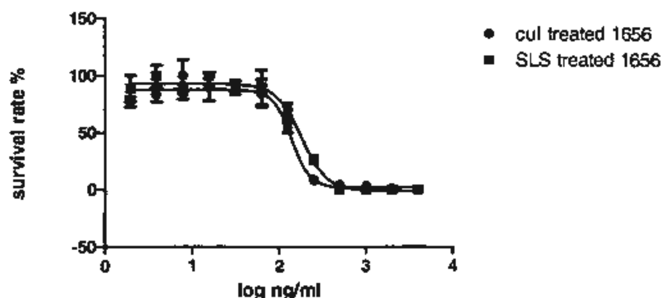
Figure 4-21: The cytotoxicity assay of Cucurbitacin D and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin D and Sodium Lauryl Sulfate. IC 50 of cu D was 181.5 ng/ml. The correlation coefficient between two toxicity curves was 0.99. It indicated the toxicity result of cu D in 1656 cell assay was significant.



	cuE treated 1656	SLS treated 1656
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	1.031	-0.9878
TOP	92.73	92.96
LOGIC50	3.035	3.161
HILLSLOPE	-6.154	-2.991
IC50	1084	1449
Span	91.70	93.94
Std. Error		
BOTTOM	4.368	3.454
TOP	1.516	2.701
LOGIC50	0.02993	0.03970
HILLSLOPE	4.031	0.6605
Span	4.768	4.523
95% Confidence Intervals		
BOTTOM	-7.870 to 9.933	-8.193 to 6.217
TOP	89.65 to 95.82	87.32 to 98.59
LOGIC50	2.974 to 3.096	3.078 to 3.244
HILLSLOPE	-14.37 to 2.060	-4.369 to -1.613
IC50	942.3 to 1248	1198 to 1754
Span	81.99 to 101.4	84.51 to 103.4
Goodness of Fit		
Degrees of Freedom	32	20
R2	0.9555	0.9655
Absolute Sum of Squares	1918	1531
Sy.x	7.742	8.751
Number of points		
Analyzed	36	24

Figure 4-22: The cytotoxicity assay of Cucurbitacin E and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin E and Sodium Lauryl Sulfate. IC 50 of cu E was 1084 ng/ml. The correlation coefficient between two toxicity curves was 0.97. It indicated the toxicity result of cu E in 1656 cell assay was significant.

cu I & SLS matching cytotoxicity curve (Neutral Red)

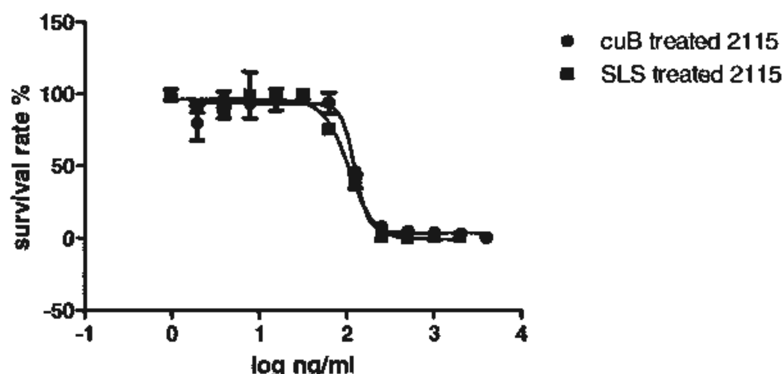


	cuI treated 1656	SLS treated 1656
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	1.908	-0.9878
TOP	87.58	92.96
LOGIC50	2.144	2.258
HILLSLOPE	-4.188	-2.991
IC50	139.3	181.1
Span	85.67	93.94
Std. Error		
BOTTOM	3.417	3.454
TOP	2.873	2.701
LOGIC50	0.03735	0.03970
HILLSLOPE	1.597	0.6605
Span	4.609	4.523
95% Confidence Intervals		
BOTTOM	-5.055 to 8.871	-8.193 to 6.217
TOP	81.72 to 93.43	87.32 to 98.59
LOGIC50	2.068 to 2.220	2.175 to 2.341
HILLSLOPE	-7.442 to -0.9327	-4.369 to -1.613
IC50	116.9 to 166.0	149.7 to 219.2
Span	76.28 to 95.06	84.51 to 103.4
Goodness of Fit		
Degrees of Freedom	32	20
R2	0.9301	0.9655
Absolute Sum of Squares	4322	1531
Sy.x	11.62	8.751
Number of points		
Analyzed	36	24

Figure 4-23: The cytotoxicity assay of Cucurbitacin I and Sodium Lauryl Sulfate

Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin I and Sodium Lauryl Sulfate. IC 50 of cu I was 139.3 ng/ml. The correlation coefficient between two toxicity curves was 0.95. It indicated the toxicity result of cu I in 1656 cell assay was significant.

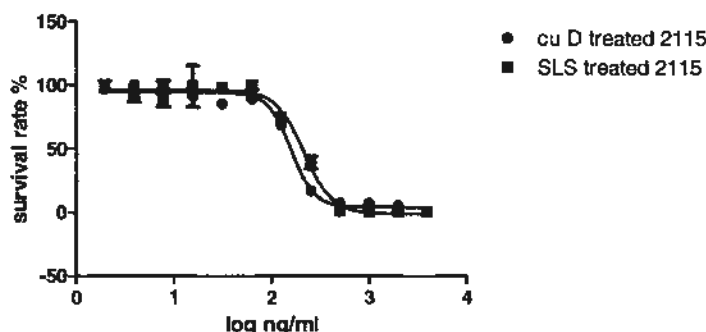
cu B & SLS matching cytotoxicity curve (Neutral Red)



	cuB treated 2115	SLS treated 2115
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	3.461	-1.129
TOP	93.35	96.12
LOGIC50	2.090	2.026
HILLSLOPE	-5.740	-3.047
IC50	123.0	106.3
Span	89.89	97.25
Std. Error		
BOTTOM	2.529	3.066
TOP	2.323	2.290
LOGIC50	0.01936	0.03213
HILLSLOPE	3.678	0.6017
Span	3.590	3.967
95% Confidence Intervals		
BOTTOM	-1.692 to 8.615	-7.525 to 5.267
TOP	88.62 to 98.09	91.35 to 100.9
LOGIC50	2.050 to 2.129	1.959 to 2.093
HILLSLOPE	-13.23 to 1.754	-4.302 to -1.792
IC50	112.3 to 134.7	91.06 to 124.0
Span	82.58 to 97.21	88.98 to 105.5
Goodness of Fit		
Degrees of Freedom	32	20
R2	0.9600	0.9754
Absolute Sum of Squares	2721	1140
Sy.x	9.221	7.551
Number of points		
Analyzed	36	24

Figure 4-24: The cytotoxicity assay of Cucurbitacin B and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin B and Sodium Lauryl Sulfate. IC 50 of cu B was 123 ng/ml. The correlation coefficient between two toxicity curves was 0.99. It indicated the toxicity response.

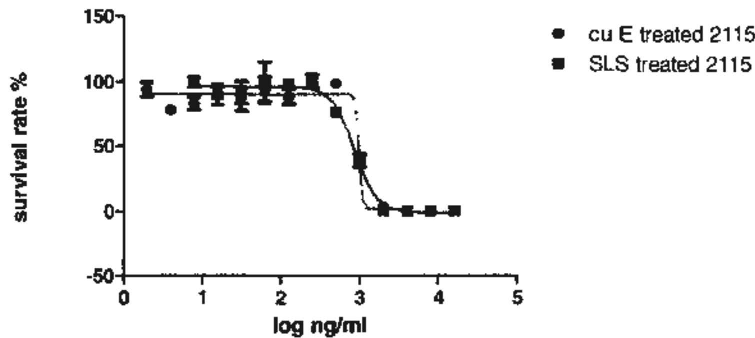
cu D & SLS matching cytotoxicity curve (Neutral Red)



	cu D treated 2115	SLS treated 2115
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	4.153	-1.129
TOP	94.22	96.12
LOGIC50	2.197	2.327
HILLSLOPE	-3.724	-3.047
IC50	157.4	212.5
Span	90.06	97.25
Std. Error		
BOTTOM	1.663	3.066
TOP	1.355	2.290
LOGIC50	0.01899	0.03213
HILLSLOPE	0.4747	0.6017
Span	2.193	3.967
95% Confidence Intervals		
BOTTOM	0.7635 to 7.542	-7.525 to 5.267
TOP	91.45 to 96.98	91.35 to 100.9
LOGIC50	2.158 to 2.236	2.260 to 2.394
HILLSLOPE	-4.691 to -2.756	-4.302 to -1.792
IC50	144.0 to 172.1	182.1 to 248.0
Span	85.59 to 94.53	88.98 to 105.5
Goodness of Fit		
Degrees of Freedom	32	20
R2	0.9847	0.9754
Absolute Sum of Squares	966.9	1140
Sy.x	5.497	7.551
Number of points		
Analyzed	36	24

Figure 4-25: The cytotoxicity assay of Cucurbitacin D and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin D and Sodium Lauryl Sulfate. IC 50 of cu D was 157.4 ng/ml. The correlation coefficient between two toxicity curves was 0.96. It indicated the toxicity result of cu D in 2115 cell assay was significant.

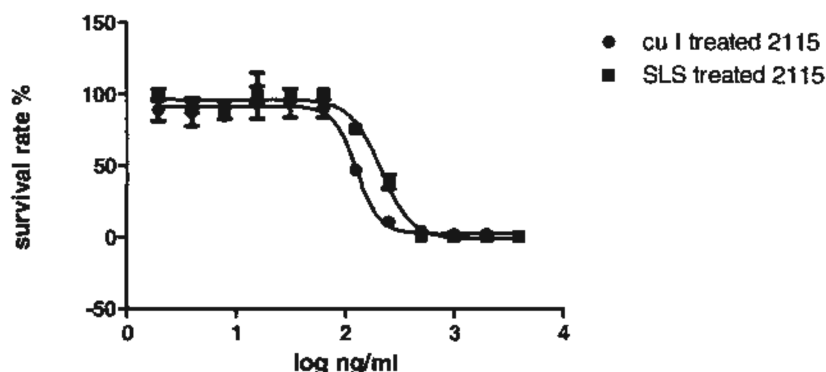
cu E & SLS matching cytotoxicity curve (Neutral Red)



	cu E treated 2115	SLS treated 2115
log(inhibitor) vs. response -- Variable slope	Ambiguous	
Best-fit values		
BOTTOM	1.611	-1.129
TOP	90.29	96.12
LOGIC50	~ 2.991	2.929
HILLSLOPE	~ -19.94	-3.047
IC50	~ 978.5	850.0
Span	88.68	97.25
Std. Error		
BOTTOM	4.632	3.066
TOP	2.207	2.290
LOGIC50	~ 31.98	0.03213
HILLSLOPE	~ 67412	0.6017
Span	5.311	3.967
95% Confidence Intervals		
BOTTOM	-7.828 to 11.05	-7.525 to 5.267
TOP	85.79 to 94.79	91.35 to 100.9
LOGIC50	(Very wide)	2.862 to 2.996
HILLSLOPE	(Very wide)	-4.302 to -1.792
IC50	(Very wide)	728.5 to 991.8
Span	77.86 to 99.50	88.98 to 105.5
Goodness of Fit		
Degrees of Freedom	32	20
R2	0.9184	0.9754
Absolute Sum of Squares	3779	1140
Sy.x	10.87	7.551
Number of points		
Analyzed	36	24

Figure 4-26: The cytotoxicity assay of Cucurbitacin E and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin E and Sodium Lauryl Sulfate. IC 50 of cu E was 978.5 ng/ml. The correlation coefficient between two toxicity curves was 0.95. It indicated the toxicity result of cu E in 2115 cell assay was significant.

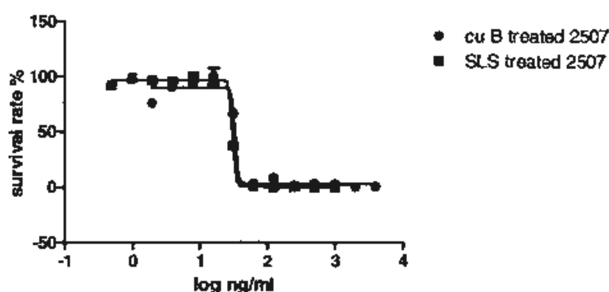
cu I & SLS matching cytotoxicity curve (Neutral Red)



	cu I treated 2115	SLS treated 2115
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	2.517	-1.129
TOP	91.61	96.12
LOGIC50	2.104	2.327
HILLSLOPE	-4.065	-3.047
IC50	127.0	212.5
Span	89.09	97.25
Std. Error		
BOTTOM	2.592	3.066
TOP	2.301	2.290
LOGIC50	0.02627	0.03213
HILLSLOPE	1.242	0.6017
Span	3.613	3.967
95% Confidence Intervals		
BOTTOM	-2.766 to 7.800	-7.525 to 5.267
TOP	86.92 to 96.29	91.35 to 100.9
LOGIC50	2.050 to 2.157	2.260 to 2.394
HILLSLOPE	-6.597 to -1.534	-4.302 to -1.792
IC50	112.3 to 143.6	182.1 to 248.0
Span	81.73 to 96.45	88.98 to 105.5
Goodness of Fit		
Degrees of Freedom	32	20
R2	0.9597	0.9754
Absolute Sum of Squares	2611	1140
Sy.x	9.033	7.551
Number of points		
Analyzed	36	24

Figure 4-27: The cytotoxicity assay of Cucurbitacin I and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin I and Sodium Lauryl Sulfate. IC 50 of cu I was 127 ng/ml. The correlation coefficient between two toxicity curves was 0.95. It indicated the toxicity result of cu I in 2115 cell assay was significant.

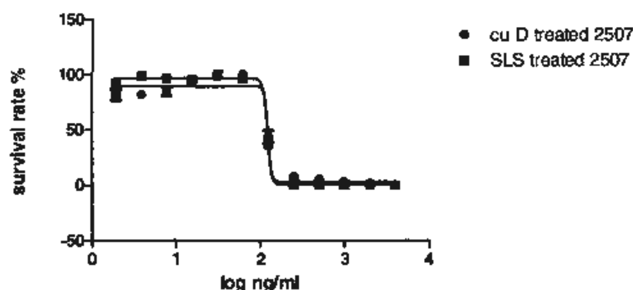
cu B & SLS matching cytotoxicity curve (Neutral Red)



	cu B treated 2507	SLS treated 2507
log(inhibitor) vs. response – Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	2.921	0.1935
TOP	90.00	96.51
LOGIC50	~ 1.517	~ 1.484
HILLSLOPE	~ -19.35	~ -18.36
IC50	~ 32.89	~ 30.45
Span	87.08	96.31
Std. Error		
BOTTOM	1.683	0.9523
TOP	2.069	0.9279
LOGIC50	~ 20.91	~ 4.130
HILLSLOPE	~ 18261	~ 6733
Span	2.703	1.374
95% Confidence Intervals		
BOTTOM	-0.5075 to 6.350	-2.002 to 2.390
TOP	85.78 to 94.22	94.37 to 98.65
LOGIC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
IC50	(Very wide)	(Very wide)
Span	81.57 to 92.59	93.14 to 99.48
Goodness of Fit		
Degrees of Freedom	32	6
R2	0.9738	0.9986
Absolute Sum of Squares	1634	35.18
Sy.x	7.147	2.097
Number of points		
Analyzed	36	12

Figure 4-28: The cytotoxicity assay of Cucurbitacin B and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin B and Sodium Lauryl Sulfate. IC 50 of cu B was 32.89 ng/ml. The correlation coefficient between two toxicity curves was 0.96. It indicated the toxicity result of cu B in 2507 cell assay was significant.

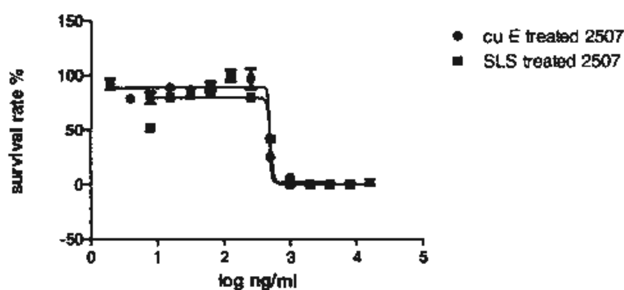
cu D & SLS matching cytotoxicity curve (Neutral Red)



	cu D treated 2507	SLS treated 2507
log(inhibitor) vs. response -- Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	3.475	0.1935
TOP	89.66	96.51
LOGIC50	~ 2.094	~ 2.086
HILLSLOPE	~ -23.21	~ -18.36
IC50	~ 124.3	~ 121.8
Span	86.18	96.31
Std. Error		
BOTTOM	1.992	0.9523
TOP	1.849	0.9279
LOGIC50	~ 56.59	~ 4.130
HILLSLOPE	~ 531923	~ 6733
Span	2.847	1.374
95% Confidence Intervals		
BOTTOM	-0.5847 to 7.535	-2.002 to 2.390
TOP	85.89 to 93.42	94.37 to 98.65
LOGIC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
IC50	(Very wide)	(Very wide)
Span	80.38 to 91.98	93.14 to 99.48
Goodness of Fit		
Degrees of Freedom	32	8
R2	0.9721	0.9986
Absolute Sum of Squares	1747	35.18
Sy.x	7.389	2.097
Number of points		
Analyzed	36	12

Figure 4-29: The cytotoxicity assay of Cucurbitacin D and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin D and Sodium Lauryl Sulfate. IC 50 of cu D was 124.3 ng/ml. The correlation coefficient between two toxicity curves was 0.99. It indicated the toxicity result of cu D in 2507 cell assay was significant.

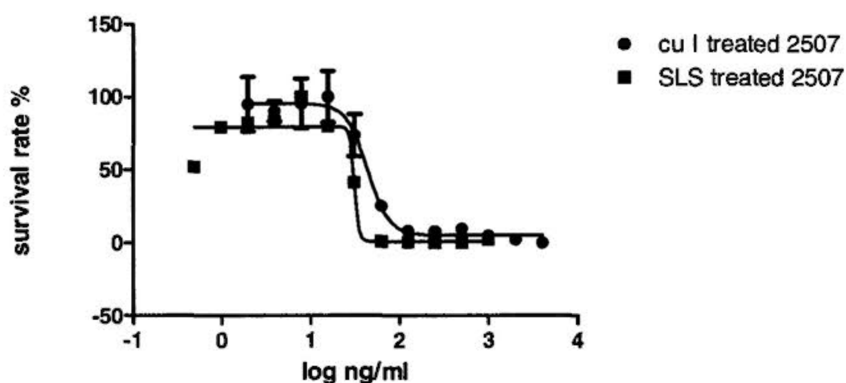
cu E & SLS matching cytotoxicity curve (Neutral Red)



	cu E treated 2507	SLS treated 2507
log(inhibitor) vs. response -- Variable slope	Ambiguous	Ambiguous
Best-fit values		
BOTTOM	2.320	0.7691
TOP	89.32	79.55
LOGIC50	~ 2.687	~ 2.701
HILLSLOPE	~ -37.11	~ -17.09
IC50	~ 486.2	~ 502.1
Span	87.00	78.78
Std. Error		
BOTTOM	3.041	5.810
TOP	1.983	5.182
LOGIC50	~ 1.596e+006	~ 2.525
HILLSLOPE	~ 4.893e+009	~ 24267
Span	3.676	8.161
95% Confidence Intervals		
BOTTOM	-3.878 to 8.517	-12.63 to 14.17
TOP	85.27 to 93.36	67.60 to 91.50
LOGIC50	(Very wide)	(Very wide)
HILLSLOPE	(Very wide)	(Very wide)
IC50	(Very wide)	(Very wide)
Span	79.51 to 94.49	59.96 to 97.60
Goodness of Fit		
Degrees of Freedom	32	8
R2	0.9533	0.9346
Absolute Sum of Squares	2648	1184
Sy.x	9.096	12.17
Number of points		
Analyzed	36	12

Figure 4-30: The cytotoxicity assay of Cucurbitacin E and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin E and Sodium Lauryl Sulfate. IC 50 of cu E was 486.2 ng/ml. The correlation coefficient between two toxicity curves was 0.95. It indicated the toxicity result of cu E in 2507 cell assay was significant.

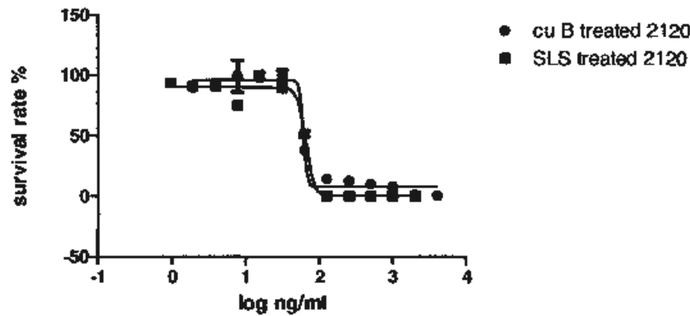
cu I & SLS matching cytotoxicity curve (Neutral Red)



	cu I treated 2507	SLS treated 2507
log(inhibitor) vs. response -- Variable slope		Ambiguous
Best-fit values		
BOTTOM	5.079	0.7691
TOP	95.48	79.55
LOGIC50	1.643	~ 1.497
HILLSLOPE	-3.623	~ -17.09
IC50	43.98	~ 31.38
Span	90.40	78.78
Std. Error		
BOTTOM	3.830	5.810
TOP	4.793	5.182
LOGIC50	0.05506	~ 2.526
HILLSLOPE	1.205	~ 24272
Span	6.253	8.161
95% Confidence Intervals		
BOTTOM	-2.726 to 12.88	-12.63 to 14.17
TOP	85.72 to 105.3	67.60 to 91.50
LOGIC50	1.531 to 1.755	(Very wide)
HILLSLOPE	-6.078 to -1.167	(Very wide)
IC50	33.96 to 56.94	(Very wide)
Span	77.66 to 103.1	59.96 to 97.60
Goodness of Fit		
Degrees of Freedom	32	8
R2	0.8880	0.9346
Absolute Sum of Squares	7803	1184
Sy.x	15.62	12.17
Number of points		
Analyzed	36	12

Figure 4-31: The cytotoxicity assay of Cucurbitacin I and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin I and Sodium Lauryl Sulfate. IC 50 of cu I was 43.98 ng/ml. The correlation coefficient between two toxicity curves was 0.94. It indicated the toxicity result of cu I in 2507 cell assay was significant.

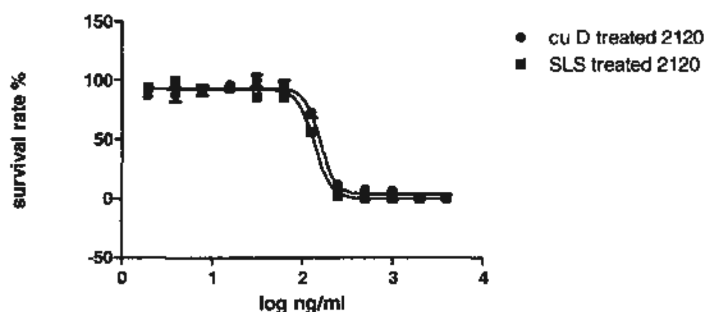
cu B & SLS matching cytotoxicity curve (Neutral Red)



	cu B treated 2120	SLS treated 2120
log(inhibitor) vs. response -- Variable slope	Ambiguous	
Best-fit values		
BOTTOM	7.510	0.02660
TOP	95.91	89.85
LOGIC50	~ 1.780	1.813
HILLSLOPE	~ -17.74	-8.011
IC50	~ 60.31	64.95
Span	88.40	89.83
Std. Error		
BOTTOM	1.927	3.223
TOP	2.325	2.758
LOGIC50	~ 8.340	0.04499
HILLSLOPE	~ 9540	19.83
Span	3.098	4.414
95% Confidence Intervals		
BOTTOM	3.582 to 11.44	-7.406 to 7.459
TOP	91.17 to 100.6	83.49 to 96.21
LOGIC50	(Very wide)	1.709 to 1.916
HILLSLOPE	(Very wide)	-53.74 to 37.72
IC50	(Very wide)	51.15 to 82.47
Span	82.09 to 94.71	79.65 to 100.0
Goodness of Fit		
Degrees of Freedom	32	8
R2	0.9682	0.9844
Absolute Sum of Squares	2108	347.2
Sy.x	8.116	6.588
Number of points		
Analyzed	36	12

Figure 4-32: The cytotoxicity assay of Cucurbitacin B and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin B and Sodium Lauryl Sulfate. IC 50 of cu B was 60.31 ng/ml. The correlation coefficient between two toxicity curves was 0.98. It indicated the toxicity result of cu B in 2120 cell assay was significant.

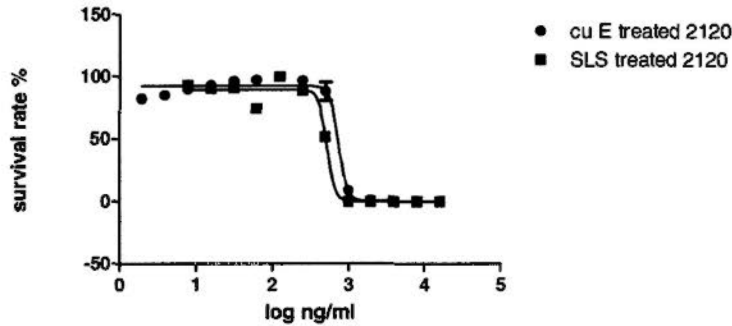
cu D & SLS matching cytotoxicity curve (Neutral Red)



	cu D treated 2120	SLS treated 2120
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	3.670	-0.2199
TOP	93.01	92.80
LOGIC50	2.198	2.134
HILLSLOPE	-5.217	-4.626
IC50	157.9	136.0
Span	89.34	93.02
Std. Error		
BOTTOM	1.862	1.986
TOP	1.519	1.662
LOGIC50	0.02213	0.01887
HILLSLOPE	0.9147	1.159
Span	2.426	2.893
95% Confidence Intervals		
BOTTOM	-0.1255 to 7.465	-4.799 to 4.360
TOP	89.91 to 96.10	88.93 to 96.68
LOGIC50	2.153 to 2.243	2.090 to 2.177
HILLSLOPE	-7.081 to -3.353	-7.297 to -1.954
IC50	142.3 to 175.2	123.1 to 150.4
Span	84.39 to 94.28	86.81 to 99.23
Goodness of Fit		
Degrees of Freedom	32	8
R2	0.9802	0.9946
Absolute Sum of Squares	1294	124.8
Sy.x	6.359	3.949
Number of points		
Analyzed	36	12

Figure 4-33: The cytotoxicity assay of Cucurbitacin D and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin D and Sodium Lauryl Sulfate. IC 50 of cu D was 157.9 ng/ml. The correlation coefficient between two toxicity curves was 0.99. It indicated the toxicity result of cu D in 2120 cell assay was significant.

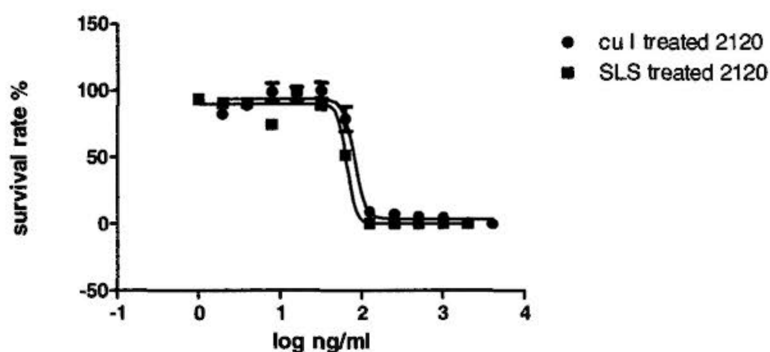
cu E & SLS matching cytotoxicity curve (Neutral Red)



	cu E treated 2120	SLS treated 2120
log(inhibitor) vs. response -- Variable slope		
Best-fit values		
BOTTOM	0.5892	0.02660
TOP	92.76	89.85
LOGIC50	2.870	2.716
HILLSLOPE	-7.683	-8.011
IC50	741.4	519.6
Span	92.17	89.83
Std. Error		
BOTTOM	2.718	3.223
TOP	1.353	2.758
LOGIC50	0.03125	0.04499
HILLSLOPE	1.658	19.83
Span	3.040	4.414
95% Confidence Intervals		
BOTTOM	-4.949 to 6.127	-7.406 to 7.459
TOP	90.01 to 95.52	83.49 to 96.21
LOGIC50	2.806 to 2.934	2.612 to 2.819
HILLSLOPE	-11.06 to -4.304	-53.74 to 37.72
IC50	640.3 to 858.5	409.2 to 659.8
Span	85.98 to 98.37	79.65 to 100.0
Goodness of Fit		
Degrees of Freedom	32	8
R2	0.9745	0.9844
Absolute Sum of Squares	1403	347.2
Sy.x	6.621	6.588
Number of points		
Analyzed	36	12

Figure 4-34: The cytotoxicity assay of Cucurbitacin E and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin E and Sodium Lauryl Sulfate. IC 50 of cu E was 741. ng/ml. The correlation coefficient between two toxicity curves was 0.96. It indicated the toxicity result of cu E in 2120 cell assay was significant.

cu I & SLS matching cytotoxicity curve (Neutral Red)



	cu I treated 2120	SLS treated 2120
log(inhibitor) vs response -- Variable slope		
Best-fit values		
BOTTOM	3 618	0 02660
TOP	93 66	89 85
LOGIC50	1 907	1 813
HILLSLOPE	-6 386	-8 011
IC50	80 71	64 95
Span	90 04	89 83
Std Error		
BOTTOM	2 100	3 223
TOP	2 102	2 758
LOGIC50	0 03184	0 04499
HILLSLOPE	1 595	19 83
Span	2 983	4 414
95% Confidence Intervals		
BOTTOM	-0 6602 to 7 897	-7 406 to 7 459
TOP	89 37 to 97 94	83 49 to 96 21
LOGIC50	1 842 to 1 972	1 709 to 1 916
HILLSLOPE	-9 637 to -3 135	-53 74 to 37 72
IC50	69 51 to 93 71	51 15 to 82 47
Span	83 96 to 96 12	79 65 to 100 0
Goodness of Fit		
Degrees of Freedom	32	8
R2	0 9702	0 9844
Absolute Sum of Squares	2095	347 2
Sy x	8 091	6 588
Number of points		
Analyzed	36	12

Figure 4-35: The cytotoxicity assay of Cucurbitacin I and Sodium Lauryl Sulfate Non-liner regression method drafted the dose-response cytotoxicity curves of Cucurbitacin I and Sodium Lauryl Sulfate. IC 50 of cu I was 80.71 ng/ml. The correlation coefficient between two toxicity curves was 0.7. It indicated the toxicity result of cu I in 2120cell assay was significant.

4.4 Results

4.4.2. Toxic effect of cucurbitacin D, *Trichosanthes rosthornii Harms.* 瓜蔞 (crude extract) and hydroxyurea on C. elegans.

Dose-response curve was sketched by non-linear regression method. Statistical analyzed the dose-response curve of the C elegans assay which exposed to hydroxyurea, *Trichosanthes rosthornii Harms.* 瓜蔞 (crude extract)

and Cadmium chloride (CdCl). The IC_{50s} of hydroxyurea, *Trichosanthes rosthornii Harms.* 瓜蔞 (crude extract) and Cadmium chloride (CdCl) were calculated from the dose-response curves. The data was shown as figure 4-33, 4-34, and 4-35.

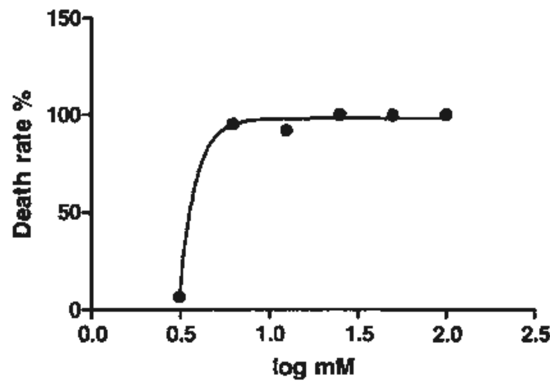
IC_{50} of hydroxyurea = 0.00639mg/ml,

IC_{50} of *Trichosanthes rosthornii Harms.* 瓜蔞 (crude extract) = 470.5 mg/ml.

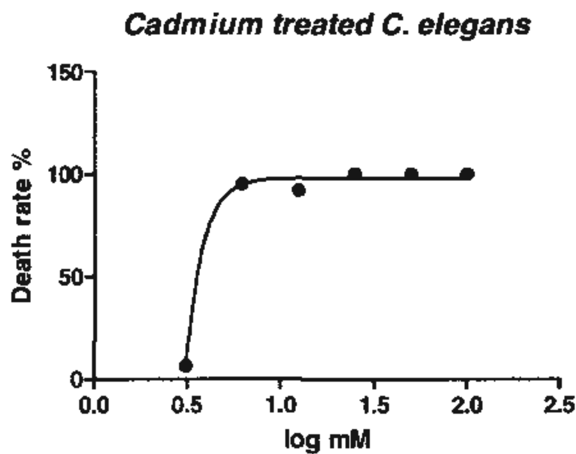
IC_{50} of Cadmium chloride (CdCl) = 0.016mg/ml

Statistical T tests analyzed the death rate of C. elegans exposured between the k medium and 0.5 mg/ml cucurbitacin D treated the C. elegans by GrapPad $PRISM_R$ 4.0. The $P > 0.3$. T tests result was shown in table 4-2. It did not show any significant difference between the K medium and 0.5 mg/ml cucurbitacin D treated C elegans.

Cadmium treated C. elegans



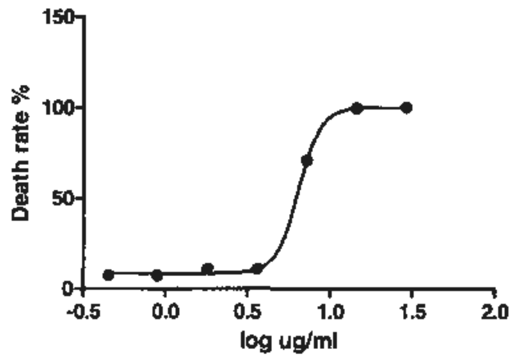
	Cadmium treated C elegans
log(inhibitor) vs response -- Variable slope	Ambiguous
Best-fit values	
BOTTOM	93 69
TOP	100 0
LOGIC50	~ -0 05163
HILLSLOPE	~ 109 7
IC50	~ 0 8879
Span	6 307
Std Error	
BOTTOM	0 5661
TOP	4 025
LOGIC50	~ 3 082e+018
HILLSLOPE	~ 6 549e+021
Span	4 064
95% Confidence Intervals	
BOTTOM	92 45 to 94 94
TOP	91 14 to 108 9
LOGIC50	(Very wide)
HILLSLOPE	(Very wide)
IC50	(Very wide)
Span	-2 639 to 15 25
Goodness of Fit	
Degrees of Freedom	11
R2	0 8713
Absolute Sum of Squares	21 15
Sy x	1 387
Number of points	
Analyzed	15



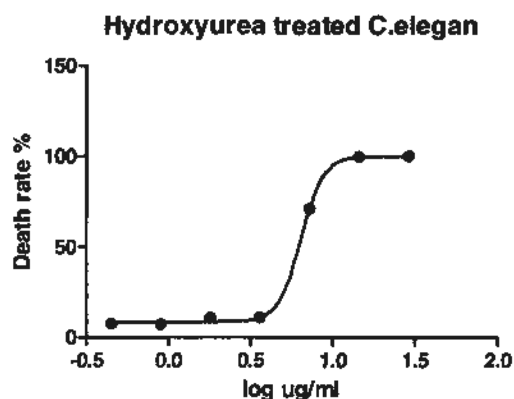
	Cadmium treated C. elegans
log(inhibitor) vs. response -- Variable slope	Ambiguous
Best-fit values	
BOTTOM	93.69
TOP	100.0
LOGIC50	~ -0.05163
HILLSLOPE	~ 109.7
IC50	~ 0.8879
Span	6.307
Std. Error	
BOTTOM	0.5661
TOP	4.025
LOGIC50	~ 3.082e+018
HILLSLOPE	~ 6.549e+021
Span	4.064
95% Confidence Intervals	
BOTTOM	92.45 to 94.94
TOP	91.14 to 108.9
LOGIC50	(Very wide)
HILLSLOPE	(Very wide)
IC50	(Very wide)
Span	-2.639 to 15.25
Goodness of Fit	
Degrees of Freedom	11
R2	0.8713
Absolute Sum of Squares	21.15
Sy.x	1.387
Number of points	
Analyzed	15

Figure 4-36: Survival curve of C.elegans under the exposure of Cadmium Chloride.

The lethality of C. elegans in different concentration CdCl was acted as a positive control for toxicity test. The non-linear regression drafted the survival curve of c.elegans. IC_{50} is 0.89 mM. It demonstrated that the C. elegans could be a toxicity model.



	HU treated C elegan % of death
log(inhibitor) vs. response -- Variable slope	
Best-fit values	
BOTTOM	8.570
TOP	100.1
LOGIC50	0.8055
HILLSLOPE	6.366
IC50	6.390
Span	91.50
Std. Error	
BOTTOM	0.7272
TOP	0.9170
LOGIC50	0.008500
HILLSLOPE	0.9028
Span	1.217
95% Confidence Intervals	
BOTTOM	7.036 to 10.10
TOP	98.14 to 102.0
LOGIC50	0.7876 to 0.8235
HILLSLOPE	4.461 to 8.271
IC50	6.132 to 6.660
Span	88.94 to 94.07
Goodness of Fit	
Degrees of Freedom	17
R2	0.9977
Absolute Sum of Squares	80.14
Sy.x	2.171
Number of points	
Analyzed	21



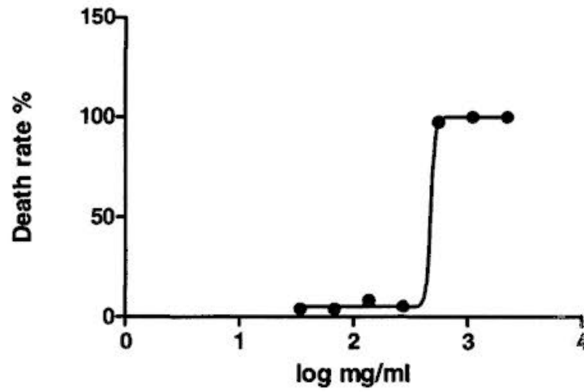
	HU treated C elegan % of death
log(inhibitor) vs. response -- Variable slope	
Best-fit values	
BOTTOM	8.570
TOP	100.1
LOGIC50	0.8055
HILLSLOPE	6.366
IC50	6.390
Span	91.50
Std. Error	
BOTTOM	0.7272
TOP	0.9170
LOGIC50	0.008500
HILLSLOPE	0.9028
Span	1.217
95% Confidence Intervals	
BOTTOM	7.036 to 10.10
TOP	98.14 to 102.0
LOGIC50	0.7876 to 0.8235
HILLSLOPE	4.461 to 8.271
IC50	6.132 to 6.660
Span	88.94 to 94.07
Goodness of Fit	
Degrees of Freedom	17
R2	0.9977
Absolute Sum of Squares	80.14
Sy.x	2.171
Number of points	
Analyzed	21

Figure 4-37: Survival curve of C.elegans under the exposure of Hydroxyrea (HU).

The endpoint assay depended on the lethality of C. elegans in certain concentration of HU .The non-liner regression drafted the survival curve of c.elegans.

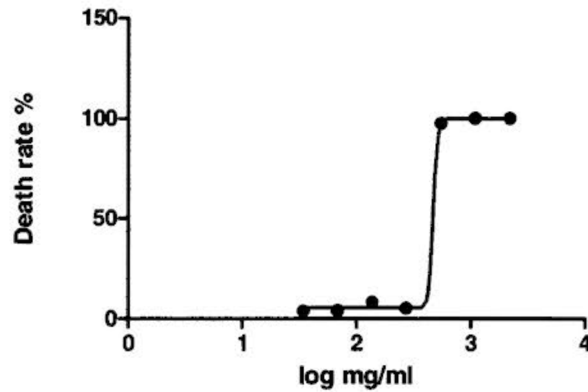
IC_{50} was 6.39 ug/ml. It demonstrated that the C. elegans could be a toxicity model.

Trichosanthes rosthornii Harms. 瓜蔞 (crude extract) treated C.elegans



	Trichosanthes crud Vs C elegan
log(inhibitor) vs response -- Variable slope	Ambiguous
Best-fit values	
BOTTOM	5 388
TOP	100 0
LOGIC50	~ 2 673
HILLSLOPE	~ 23 09
IC50	~ 470 5
Span	94 61
Std Error	
BOTTOM	0 5635
TOP	0 6901
LOGIC50	~ 11 96
HILLSLOPE	~ 4032
Span	0 8910
95% Confidence Intervals	
BOTTOM	4 199 to 6 577
TOP	98 54 to 101 5
LOGIC50	(Very wide)
HILLSLOPE	(Very wide)
IC50	(Very wide)
Span	92 73 to 96 49
Goodness of Fit	
Degrees of Freedom	17
R2	0 9989
Absolute Sum of Squares	48 58
Sy x	1 690
Number of points	
Analyzed	21

Trichosanthes rosthornii Harms.* 瓜蔞 *(crude extract) treated C.elegans



	Trichosanthes crud Vs C elegan
log(inhibitor) vs response -- Variable slope	Ambiguous
Best-fit values	
BOTTOM	5 388
TOP	100 0
LOGIC50	~ 2 673
HILLSLOPE	~ 23 09
IC50	~ 470 5
Span	94 61
Std Error	
BOTTOM	0 5635
TOP	0 6901
LOGIC50	~ 11 96
HILLSLOPE	~ 4032
Span	0 8910
95% Confidence Intervals	
BOTTOM	4 199 to 6 577
TOP	98 54 to 101 5
LOGIC50	(Very wide)
HILLSLOPE	(Very wide)
IC50	(Very wide)
Span	92 73 to 96 49
Goodness of Fit	
Degrees of Freedom	17
R2	0 9989
Absolute Sum of Squares	48 58
Sy x	1 690
Number of points	
Analyzed	21

Figure 4-38: Survival curve of C.elegans under the exposure of *Trichosanthes rosthornii Harms.* 瓜蔞 (crude extract)

The lethality of C. elegans in different concentration extraction was demonstrated as a toxicity test. The non-liner regression drafted the survival curve of c.elegans.

IC_{50} was 470.5 mg /ml.

Table Analyzed	mortality of C elegan
Column A	K medium
vs	vs
Column B	0.5mg/ml LC978
Mann Whitney test	
P value	0.3429
Exact or approximate P value?	Exact
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	One-tailed
Sum of ranks in column A,B	20 , 16
Mann-Whitney U	6.0

Table 4-6

	% of death			Mean
0.5mg/ml cu D	6.25	5.72	5.43	5.80
Control	6.03	5.04	7.24	6.10

Table 4-7

Table 4-2: Statistical T tests compared the variance between K medium only

K medium (negative control)and 0.5mg/ml LC978 treated C elegans. The mean % death rate of C elegans in 0.5 mg/ml cu D or in K medium were no significant diffferent. P > 0.3, therefore, the dosage within 0.5mg/ml cu D did not show toxicity effect toward C. elegans. The result was shown as table 4-6 to 4-7.

(The Group data was provided by Lau Ka Yee, Liu Shuk Ming, Yuen Ka Leung)

4.4 Results

4.4.3. Cucurbitacin D toxicity ---Transgenic Mouse Skin lesion

Cucurbitacin D caused Skin lesion

After 28 days, the skin lesions were discovered on the injection site. The skin lesion was observed as shown in figure 4-39 and 4-40.

It has been reported that cucurbitacin could lead to the skin allergy with unknown mechanism (Raikhlin-Eisenkraft. & Bentur, 2000) The same result was observed in our experiments. The phenomenon did not find on the control group. Skin lesions were observed in the mice treated with 1.5 ug/g of cucurbitacin D IP injection, but not in the group with lower dosage.

Figure 4-39 lesion of homozygous sickle cell mice

Figure 4-40 Skin lesion of homozygous sickle cell mice

The mice was IP injected the cucurbitacin for 28 days. There was an imflammation on the injection site.

4.4 Results

4.4.4. Fetal hemoglobin induction in sickle cell mice (Jason lab)

Since homozygous (i.e. mouse beta -/-) mice were very difficult to breed and hard to collect enough mice for the experiment, therefore we had to use the heterozygous (i.e. mouse beta -/+) mice for the experiment. Mice were orally fed with CuD at 0.5 or 0.1 ug/g orally, daily for 30 days. Blood samples were collected before and after 10, 15, 20, 25 and 30 days of treatment for fetal hemoglobin assay as described in methods. 0.5 ug/g oral treatment of the mice could significantly increase the fetal hemoglobin level after treatment, but not the 0.1 ug/g dosage (figure 4-41 to 4-43). The result also indicated that the response was the highest after 10 days of treatment and then gradually decrease, however the overall level of fetal hemoglobin after the whole treatment was significantly increased (figure 4-41).

The summaries of the groups results were shown as table 4-8 to 4-9.

Fetal hemglobin of Sickle cell mice (Jason Laboratory) before & after treatment

	Day 0	Day1 0	Day 15	Day 20	Day 25	Day 30
0.5 ug/g followed sucrose	**69.2±0.5**	**67.5±0.7** **p>0.2**	**92.9±1.2** **P<0.004**	**78.6±0.9** **P>0.12**	**93.7±5.2** **p<0.016**	**54.8±2.5** **P<0.028**
0.1 ug/g followed sucrose	**76.8±2.2**	**64.3±2.4** **p>0.07**	**89.7±3.7** **P<0.048**	**80.4±1.5** **p>0.3**	**90.9±4.6** **P<0.07**	**66.9±0.9** **P<0.016**
Surcose	**94.1±0.8**	**95.7±5.4** **p>0.2**	**122.4±5.2** **P<0.0033**	**119.5±2.7** **P<0.038**	**121.5±8.4** **p>0.022**	**74.6±0.3** **P<0.0006**

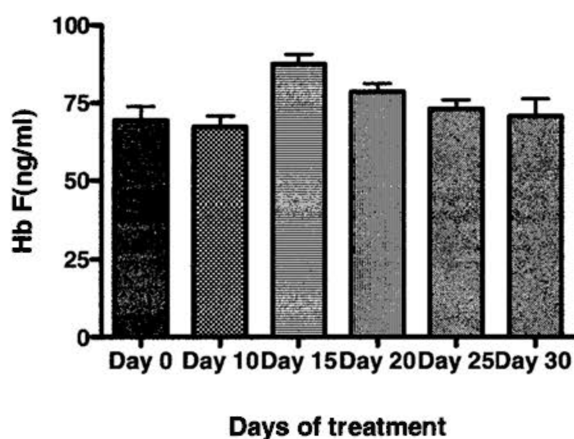
Table 4-8: Mice were orally fed with CuD at 0.5 or 0.1 ug/g orally, daily for 30 days. Blood samples were collected before and after 10, 15, 20, 25 and 30 days of treatment for fetal hemoglobin assay as described in methods. The means value of fetal hemoglobin were calculated. The results from day 10 to day 30 compared with day 0, the Ps were calculated by t test of GraghPad prism 4.0.

Fetal hemoglobin change in the sickle cell mouse after orally fed with cucurbitacin D

Dosage	Fetal hemoglobin induction after treatment, P=
0.5 ug/g in sucrose solution	0.0106
0.1 ug/g in sucrose solution	0.2569
Surcose	0.1265

Table 4-9: One way ANOVA statistical analyzed the change of fetal hemoglobin after drug.

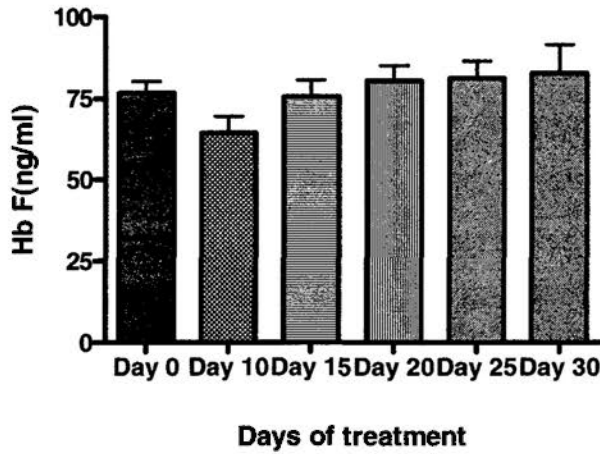
cucurbitacin D (0.5ug/g) treated transgenic mice fetal hemoglobin level



Parameter	Value	Data Set-B	Data Set-C
Jason mice-0.5ug/g-Hb F			
One-way analysis of variance			
P value	0.0106		
P value summary	*		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	6		
F	3.846		
R squared	0.4448		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	3.060		
P value	0.6907		
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	1422	5	284.5
Residual (within columns)	1775	24	73.96
Total	3197	29	

Figure 4-41: One way ANOVA statistical analysis the fetal hemoglobin level 0.5 ug cucurbitacin D / gram of mouse weight were given to the transgenic mouse (Jason laboratory) for 30 days. Compare the fetal hemoglobin level after drug. The significant different between the control and case data sets were examined. P < 0.011.

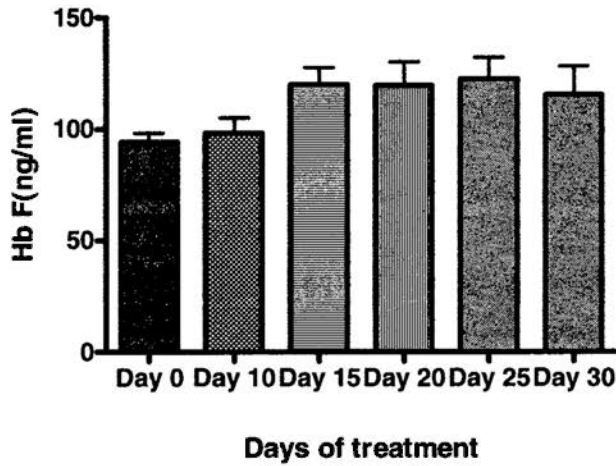
cucurbitacin D (0.1ug/g) treated transgenic mice fetal hemoglobin level



Parameter	Value	Data Set-B	Data Set-C
Jason mice-0.1ug/g-Hb F			
One-way analysis of variance			
P value	0.2569		
P value summary	ns		
Are means signif. different? (P < 0.05)	No		
Number of groups	6		
F	1.408		
R squared	0.2268		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	3.520		
P value	0.6204		
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	1116	5	223.3
Residual (within columns)	3806	24	158.6
Total	4922	29	

Figure 4-42: One way ANOVA statistical analysis the fetal hemoglobin level 0.1 ug cucurbitacin D / gram of mouse weight were given to the transgenic mouse (Jason laboratory) for 30 days. Compare the fetal hemoglobin level after drug. However, there was no significant different between the data sets. P > 0.25.

Untreated transgenic mice fetal hemoglobin level



Parameter	Value	Data Set-B	Data Set-C
Jason mice control			
One-way analysis of variance			
P value	0.1265		
P value summary	ns		
Are means signif. different? (P < 0.05)	No		
Number of groups	6		
F	1.810		
R squared	0.1435		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	11.73		
P value	0.0387		
P value summary	*		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	7524	5	1505
Residual (within columns)	44900	54	831.5
Total	52430	59	

Figure 4-43: One way ANOVA statistical analysis the fetal hemoglobin level 50ul of 0.6g/ml surcose were given to the transgenic mouse (Jason laboratory) for 30 days. Compare the fetal hemoglobin level after surcose. However, there was no significant different between the data sets. P > 0.12.

4.5. Discussion

4.5.1 Toxicity effect—cell model

The Alamar blue data indicated the toxicity of cucurbitacin of B, D, E and I on four normal cell lines, 1656, 2115, 2507 and 2120. Dose and response curves were drafted by non-linear regression method and IC_{50} was calculated. The larger number of IC_{50}, the lower toxicity was, and vise versa. By the result of alamar blue assay, Cucurbitacin B and D were more toxic. Cucurbitacin E and I were less toxic. However, the data was not consistent. We repeated the toxicity test using Neutral red toxicity assay and the result was consistent. The toxicity order from the highest was Cu B, I, D and E. The percentages of correlation coefficiency were around 0.7 to 0.99. 1.0 = 100% similarity between control and test curves tendency. If the range of % correlation coefficiency was 0.7 to 0.99, it meant the toxicity of cucurbitacins was significant. The results were reasonable, because cucurbitacin was commonly used as insecticide for long time.

We adopted four different cell lines to test the cucurbitacin D toxicity, 1656, 2115, 2507 and 2120. Cucurbitacin B and I showed a stronger toxicity in 2507 & 2120 cell line. Different cell line may have different sensitivity to cucurbitacins.

4.5.2 Toxicity effect—C elegans model

Hydroxyurea is a drug for the hemoglobinopathy patient, however many adverse effects were reported. The myelotoxicity and carcinogenesis effects were reported in the long term user. Nearly 30 % of patient was non-responder. It was yearn for a more safe and effective new drug in the market. The IC_{50} of hydroxyurea = 0.00639mg/ml and IC_{50} of *Trichosanthes rosthornii Harms.* 瓜蔞 (crude extract) = 470.5 mg/ml. By

compare with their IC_{50}, there was 73630 fold differences between Hydroxyurea and *Trichosanthes rosthornii Harms.* 瓜蔞 (crude extract)The effective dosage of cucurbitacin D was 12.5 ng/ml. From our data, 0.5 mg/ml cucurbitacin D did not show any toxicity to the *C. elegans.* Although it was requested to find out the toxic dosage to the *C elegans,* it supposed the toxic dosage was larger than 0.5 mg/ml of cucurbitacin D Only compared the ED50 of cucurbitacin D and low toxicity level, 0.5mg/ml of cucurbitacin D, there was > or = 40 fold difference between the effective dosage and toxic dosage in the test. The safety dosage limitation was large. It was a major criterion for development a new drug.

Summarize the *C. elegans* toxicity data, it was demonstrated that the cucurbitacin D was safer than hydroxyurea. And the effectiveness of CuD on sickle cell mouse model, CuD may be a good candidate to develop as a drug to treat beta hemoglobinopathies.

4.5.3 Sickle cell mouse model

In the sickle cell mouse model experiment done in Prof. Kan's laboratory, the result indicated that there was no significant increase in total hemoglobin level after 28 days of i.p. injection with CuD. The RBC counts also fluctuated through out the experiment. This may due to the treatment of the mice with CuD in 100% ethanol solvent and the control of using the same volume (100ul) of 100% ethanol i.p. injection daily for 28 days. The data was not shown. The high percentage of reticulocyte background appeared in the sickle cell mice model. It was around 40% to 70%. The normal one in human was about 10^{-5}%. There was no reduction of the reticulocyte number after treatment.

We therefore modified the treatment protocol by oral feeding instead of injection.

Since Cu D is bitter and mice do not like to ingest, therefore we mix Cu D with sucrose solution to feed the mice,, and the mice like to ingest the solution. We found that 0.5ug/g dosage, daily for 30 days of treatment could increase the fetal hemoglobin level. However, the response was the highest after 10 days of treatment and then it was gradually decrease. It seems there is a feed back mechanism to reduce the response of CuD in the sickle cell mouse model.

Table 4-10 The therapeutic index of HU an d cucurbitacin B, D, E, I were shown

	HU	Cu B	Cu D	Cu E	Cu I
IC_{50}	6.39	120.3	181.5	1084	139.3
ED_{50}	12.24	0.76	0.6	13.96	1.1
therapeutic index	0.5	158	302	77	126

The comparasion the IC_{50}/ED_{50} was made, the best therapeutic index of cucurbitacin D was302, while the poor one was hydroxyurea 0.5.

Chapter 5: Cucurbitacins and Glucocorticoid Hormone

5.1. Introduction

Drug response was defined as ligand and receptor response. Many receptors located on the target cell surface or its cytosol. The ligand bound on the receptor triggers series events inside the cell. The conformational change of receptor started the signal transduction. Finally, the morphology of the cell was changed. Some studies reported that cucurbitacin could act on glucocorticoid receptor and modified prostaglandin and adrenocorticosteroid translation. (Duncan *et al*., 1996) Another study reported cucurbitacin bound on glucocorticord receptors and developed the cytotoxic effect. It showed the linear relationship between the binding affinities and cytotoxic activities of cucurbitacin. (Witkowski and Konopa 1981)
This chapter used four steroid hormones to verify whether cucurbitacins acted on the glucocorticord receptor and the effect on hemoglobin production. Dexamethasone, Hydrocortisol, Progesterone and Mifepristone (Mife.) are lipid soluble steroid hormones. Mifepristone was antagonist to the receptor. Cortisol and progesterone were the agonists. The genes turn on by Progesterone, but turn off by Mifepristone. Mifepristone was used as an abortion medicine. It bound to the steroid receptor. It causes embryo to detach from endometrium because uterus is no longer to secrete chorionic gonardotropin (HCG) and leads to endometrium break down. It induced the abortion.

The most abundance of glucocorticoid is cortisol, also named hydrocortisol. It is produced by adrenal cortex. It associates with both gluconeogenesis and lipolysis. It depresses the

immune response, especially, the cell mediate immune response. Cortisol plays a role of stress and can elicit many metabolic events. It increases protein and triacylglycerol break down and decreases the glucose uptake. Finally, cortisol increases the plasma concentrations of amino acid, glucose and free fatty acid.

Dexamethasone is also a glucocorticoid, but more potency than hydrocortisol, 20-30 time of hydrocortisol. It has the same function of hydrocortisol, suppressing the immune response. Sometimes it is used for suppressing the side effect of anti-cancer treatment. Cucurbitacins may induce hemoglobin in k562 cell model through binding to the glucocorticoid receptor. The objective of this chapter was to understand the possible interaction of cucurbitacin and glucocorticoid receptor in the induction of hemoglobin production.

5.2. Materials

Chemical	Company & Lot No.
Dexamethasone 1mg	Sigma D8893
Progesterone	Sigma P6149
Hydrocortisone	Sigma H0888
Mifepristone	Sigma M8046
Cucurbitacin B	ChromaDex
Cucurbitacin D	ChromaDex 03914-401
Cucurbitacin E	ChromaDex 03910-805
Cucurbitacin I	ChromaDex 03915-212

5.3. Methods

1. The optimal dosages of cucurbitacins (cu) were determined in k562 cell assay before.
   cucurbitacin B = 3.125 ng/ml
   cucurbitacin D =12.5 ng/ml
   cucurbitacin E = 100 ng/ml
   cucurbitacin I = 3.125 ng/ml

2. Fixed the Cu B,D, E& I concentration in a optimal dosage, dissolve the optimal dosage of cucurbitacins in complete medium separately.

3. Make the serial two-fold dilution of Progesterone, Hydrocortisone, Dexamethasone and Mifepristone independently with complete medium. The initial concentration of them was 5.5 nmol. They were performed in 96 well plate. The titration final volume was 50 ul.

4. Added 50 ul of cucurbitacin B or D or E or I in each well.

5. There were 16 mixtures.
   Optimal dosage of cu B was added into titration of Dexamethasone.
   Optimal dosage of cu B was added into titration of Hydrocortisone.
   Optimal dosage of cu B was added into titration of Progesterone.
   Optimal dosage of cu B was added into titration of Mifepristone

   Optimal dosage of cu D was added into titration of Dexamethasone.
   Optimal dosage of cu D was added into titration of Hydrocortisone.
   Optimal dosage of cu D was added into titration of Progesterone.
   Optimal dosage of cu D was added into titration of Mifepristone

   Optimal dosage of cu E was added into titration of Dexamethasone.
   Optimal dosage of cu E was added into titration of Hydrocortisone.
   Optimal dosage of cu E was added into titration of Progesterone.
   Optimal dosage of cu E was added into titration of Mifepristone

   Optimal dosage of cu I was added into titration of Dexamethasone.
   Optimal dosage of cu I was added into titration of Hydrocortisone.
   Optimal dosage of cu I was added into titration of Progesterone.
   Optimal dosage of cu I was added into titration of Mifepristone

6. Mixed the cucurbitacins with the titration of Progesterone, Hydrocortisone ,Dexamethasone and Mifepristone.

7. Add 100 ul of k562 into the mixture; the cell concentration was 2 x 10^4

8. Each set of experiment was repeated three times.

9. The plates were incubated in 37°C, 5% CO_2 humidify incubator for 6 days.

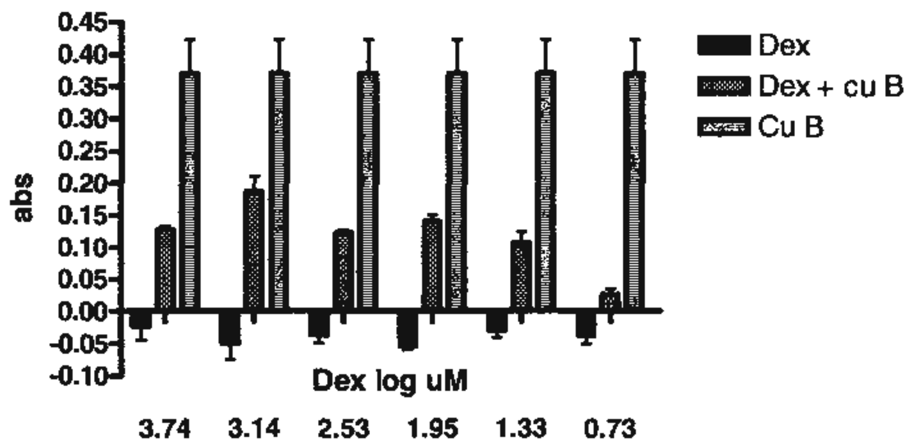
10. K562 was stained by TM and the cell lysates were read under spectral absorbance 600 nm.

5.4. Results

In the experiment the optimal doses of cucurbitacins were chosen to induce K562 cells in production of hemoglobin. Different concentration of four steroid hormones were added to the assay to observe its effect on cucurbaticin induce hemoglobin production in K562 cells. One way ANOVA was used to analyze the effect of four steroid hormones on the cucurbitacins induced hemoglobin production in k562 cell.

Dexamethasone, hydrocortisol and progesterone could suppress the hemoglobin induction in K562 cells by the optimal dose of cucurbitacin B, D, E and I (all $p<0.0001$) (figure 5-1 to 5-3, 5-7 to 5-11, 5-13 to 5-15). In contrast, mifepristone in stead of suppression, it could enhance the hemoglobin induction in K562 cells by the optimal dose of cucurbitacin B, D and I (all $p<0.0001$) in a less degree (figure 5-4, 5-8 and 5-16). At a high dose of mifepristone, the enhancing effect was not observed. Mifepristone showed different effect on CuE. The effect was similar to those of dexamethasone, hydrocortisol and progesterone, but suppress the induction of hemoglobin to a less extend and only at high dosage (figure 5-12).

Cu B & dexamethasone competition assay

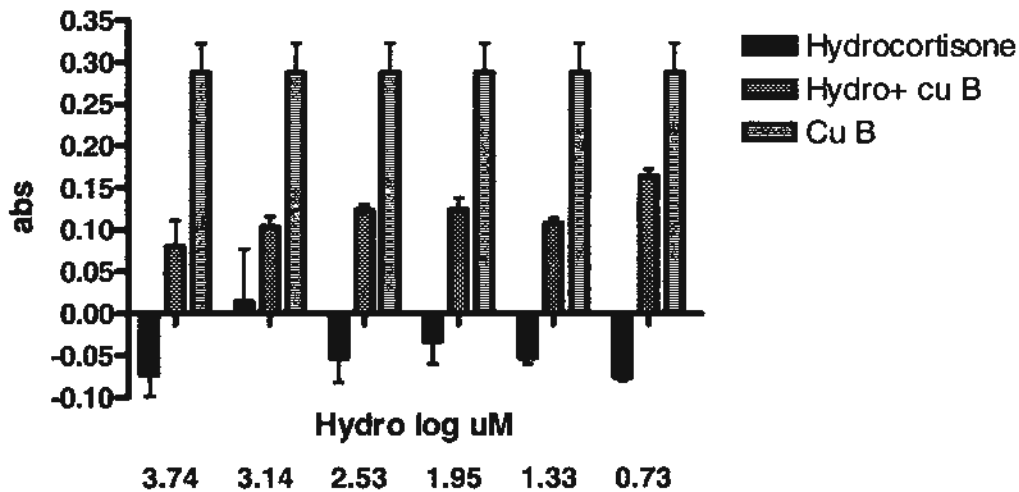


Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Dex+Cu B			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	264.6		
R squared	0.9724		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.5092	2	0.2546
Residual (within columns)	0.01443	15	0.0009622
Total	0.5237	17	

Figure 5-1: Cucurbitacin (cu)B and Dexmethasone (Dex.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu B in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

Cu B & hydrocortisone competition assay



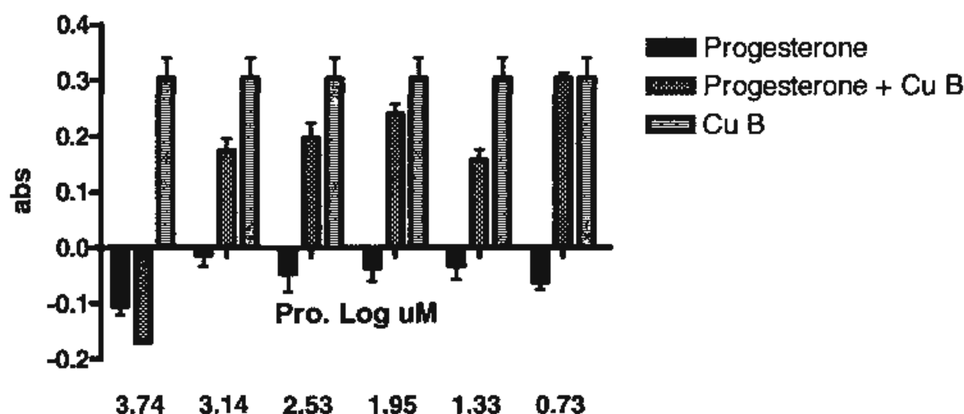
Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu B & hydrocortisone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	266.1		
R squared	0.9726		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.3323	2	0.1661
Residual (within columns)	0.009365	15	0.0006243
Total	0.3416	17	

Figure 5-2: Cucurbitacin (cu) B and Hydrocortisol (Hc.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu B in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant.

P< 0.0001.

Cu B + Progesterone competition assay



Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu B + Progesterone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	19.90		
R squared	0.7263		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	142.2		
P value	P<0.0001		
P value summary	***		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.3767	2	0.1884
Residual (within columns)	0.1420	15	0.009465
Total	0.5187	17	

Figure 5-3: Cucurbitacin (cu) B and Progesterone (Prog.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu B in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

cu B & Mifepristone competiton assay

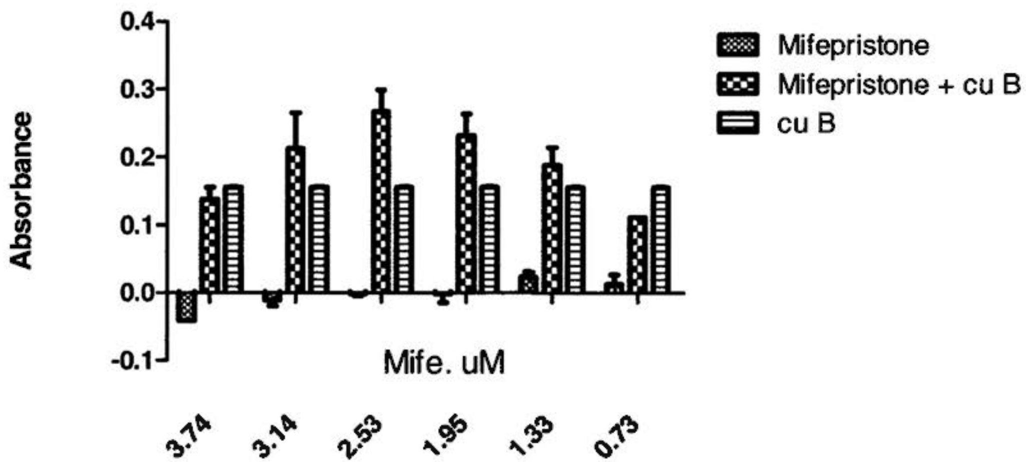
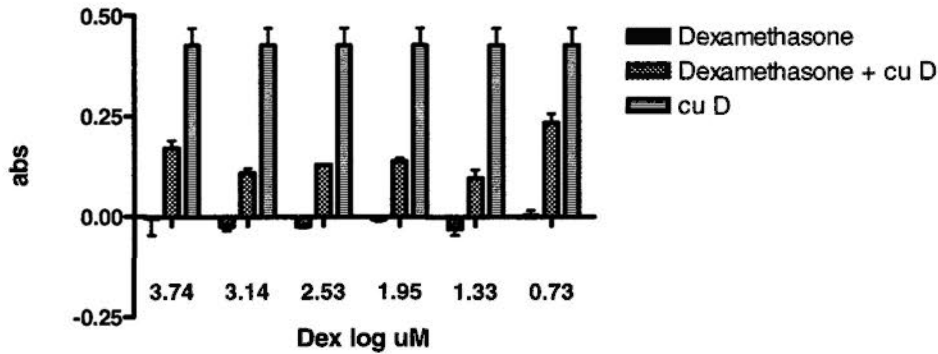


Table Analyzed	cu B + Mifepristone		
Repeated Measures ANOVA			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	51.09		
R squared	0.9109		
Was the pairing significantly effective?			
R squared	0.04666		
F	1.098		
P value	0.4188		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.1287	2	0.06433
Individual (between rows)	0.006914	5	0.001383
Residual (random)	0.01259	10	0.001259
Total	0.1482	17	

Figure 5-4: Cucurbitacin (cu) B and Mifepristone (Mife.) competition assay

The result indicated that the Mife can increased the maxium response of hemoglobin inducion by cu B in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

cu D & dexamethasone competition assay

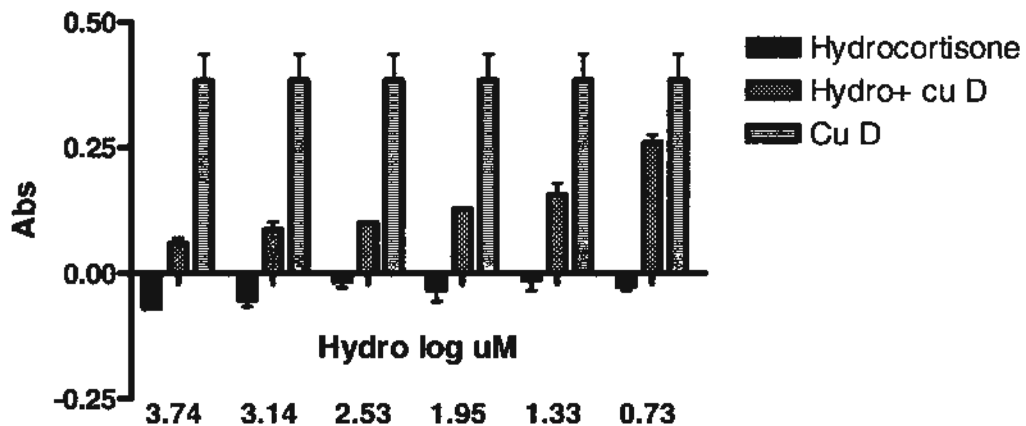


Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
cu D & Dex			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	339.8		
R squared	0.9784		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.5968	2	0.2984
Residual (within columns)	0.01317	15	0.0008783
Total	0.6100	17	

Figure 5-5: Cucurbitacin (cu) D and Dexmethasone (Dex.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu D in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

Cu D + Hydrocortisone competition assay

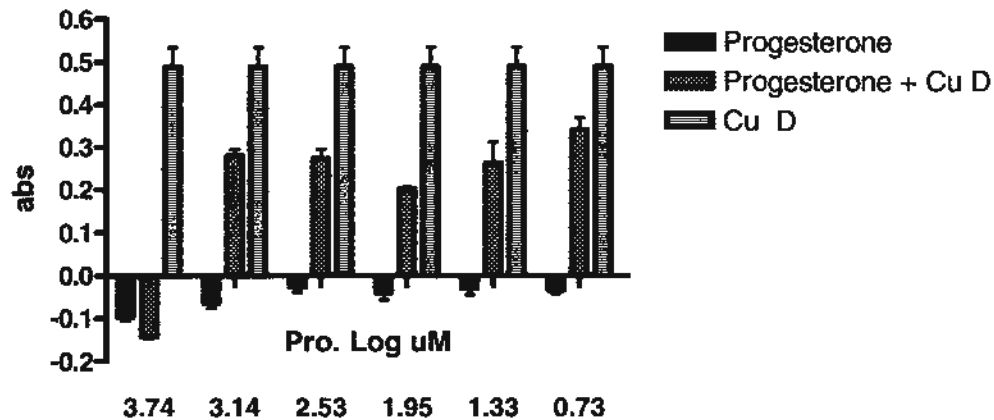


Parameter	Value	Data Set-B	Data Set-C
Cu D + Hydrocortisone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	144.4		
R squared	0.9506		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.5335	2	0.2668
Residual (within columns)	0.02772	15	0.001848
Total	0.5613	17	

Figure 5-6: Cucurbitacin (cu) D and Hydrocortisol (Hc.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu Din k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

Cu D + progesterone competition assay



Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu D + progesterone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	41.62		
R squared	0.8473		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.8707	2	0.4354
Residual (within columns)	0.1569	15	0.01046
Total	1.028	17	

Figure 5-7: Cucurbitacin (cu) D and Progesterone (Prog.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu Din k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

cu D & Mifepristone competition assay

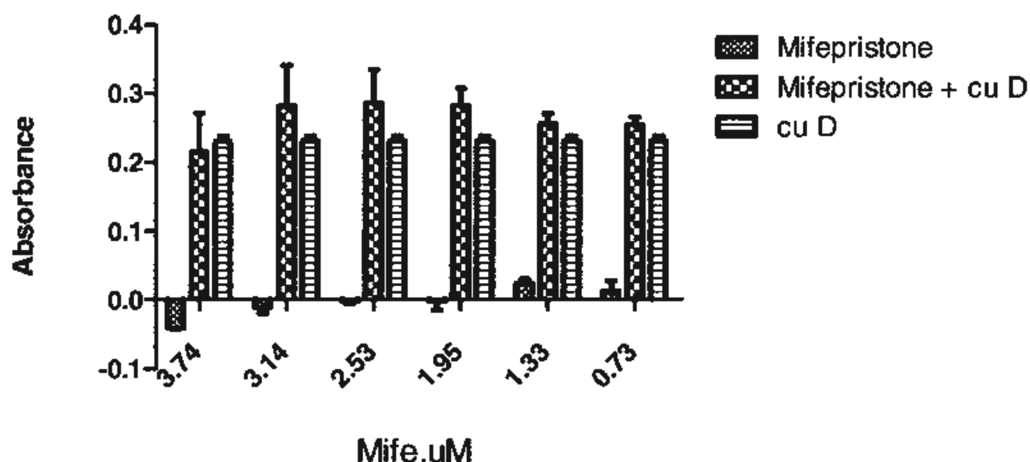
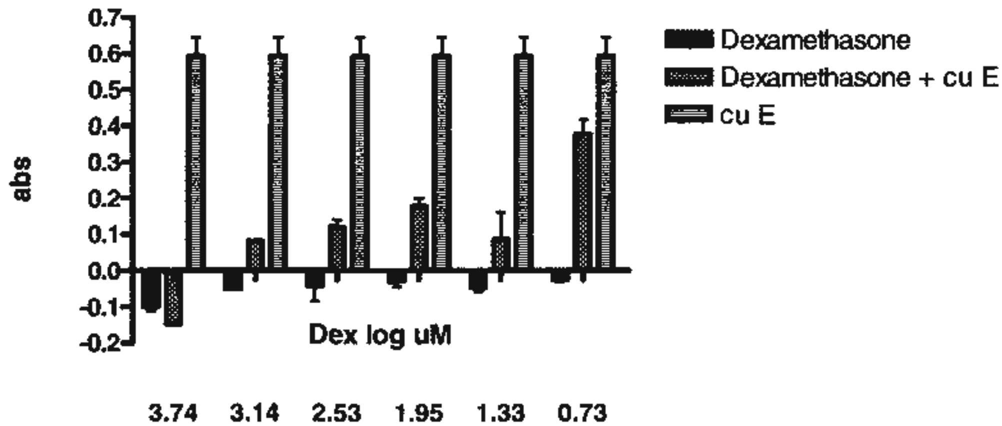


Table Analyzed	cu D + Mifepristone		
Repeated Measures ANOVA			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	396		
R squared	0.988		
Was the pairing significantly effective?			
R squared	0.0115		
F	1.87		
P value	0.1870		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.253	2	0.127
Individual (between rows)	0.00299	5	0.000598
Residual (random)	0.00320	10	0.000320
Total	0.259	17	

Figure 5-8: Cucurbitacin (cu) D and Mifepristone (Mife.) competition assay

The result indicated that the Mife can increased the maxium response of hemoglobin inducion by cu D in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

Cu E + Dexamethasone competition assay



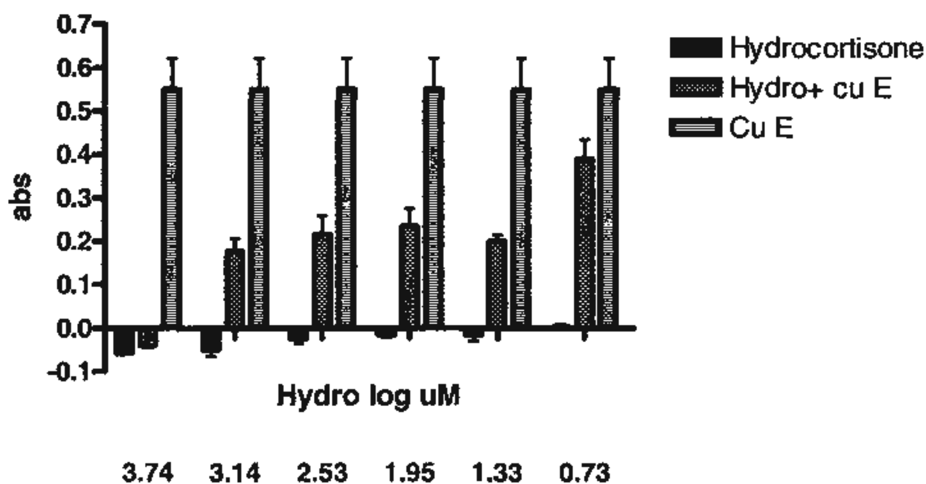
Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu E + Dexamethasone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	68.56		
R squared	0.9014		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	1.337	2	0.6687
Residual (within columns)	0.1463	15	0.009753
Total	1.484	17	

Figure 5-9: Cucurbitacin (cu) E and Dexmethasone (Dex.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu E in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant.

P< 0.0001.

Cu E + Hydrocortisone competition assay



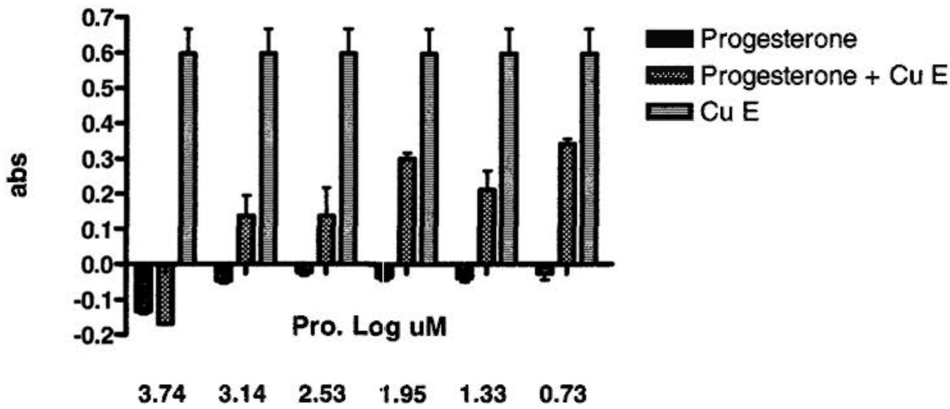
Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu E + Hydrocortisone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	77.56		
R squared	0.9118		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	1.017	2	0.5083
Residual (within columns)	0.09831	15	0.006554
Total	1.115	17	

Figure 5-10: Cucurbitacin (cu) E and Hydrocortisol (Hc.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu E in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant.

P< 0.0001.

Cu E + Progesterone competition assay



Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu E + Progesterone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	56.84		
R squared	0.8834		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	1.311	2	0.6554
Residual (within columns)	0.1730	15	0.01153
Total	1.484	17	

Figure 5-11: Cucurbitacin (cu) E and Progesterone (Prog.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu E in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

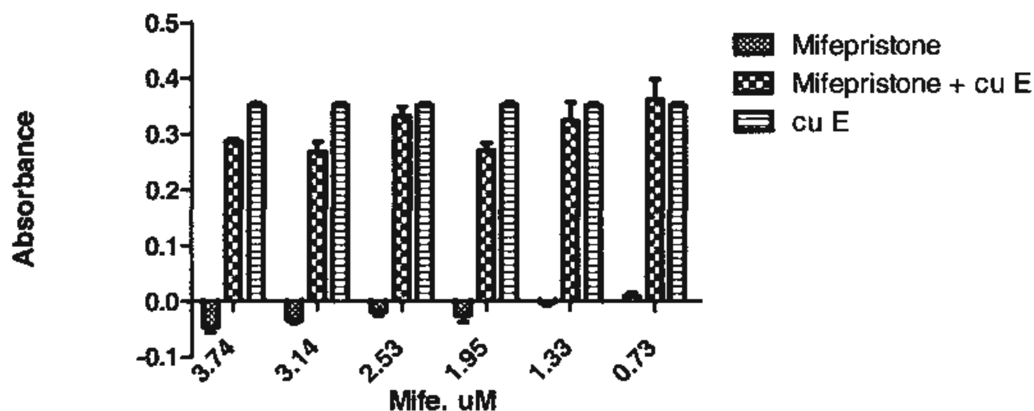
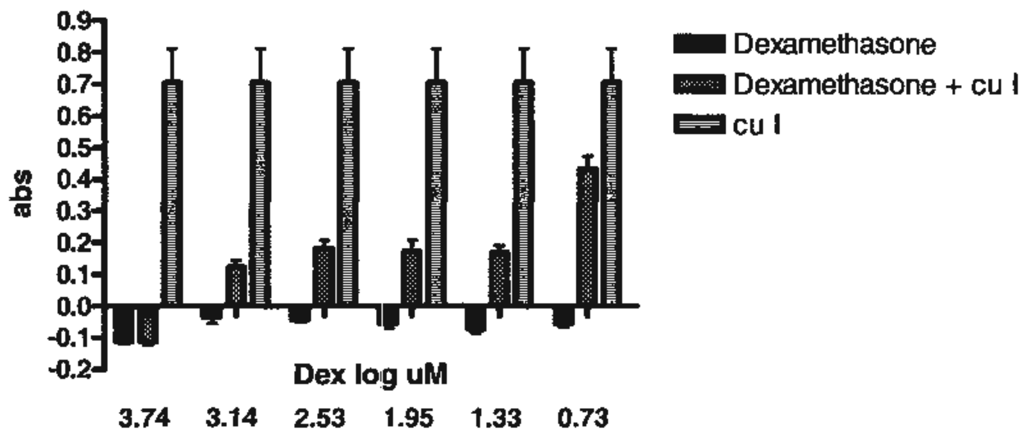


Table Analyzed	cu E + Miferistone		
Repeated Measures ANOVA			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	606		
R squared	0.992		
Was the pairing significantly effective?			
R squared	0.0105		
F	2.60		
P value	0.0930		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.494	2	0.247
Individual (between rows)	0.00530	5	0.00106
Residual (random)	0.00407	10	0.000407
Total	0.503	17	
Post test for linear trend			
Slope	0.186		
R squared	0.824		
P value	P<0.0001		
P value summary	***		
Is linear trend significant (P < 0.05)?	Yes		

Figure 5-12: Cucurbitacin (cu) E and Mifepristone (Mife.) competition assay

The result indicated that the Mife. could inhibit the hemoglobin inducion by cu E in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

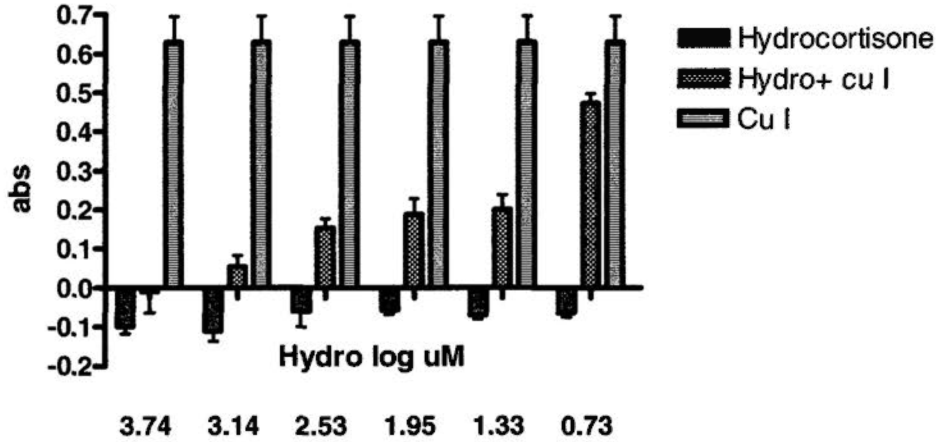


Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu I + Dexamethasone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	91.05		
R squared	0.9239		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	1.864	2	0.9322
Residual (within columns)	0.1536	15	0.01024
Total	2.018	17	

Figure 5-13: Cucurbitacin (cu) I and Dexmethasone (Dex.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu I in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

Cu I + Hydrocortisone competition assay

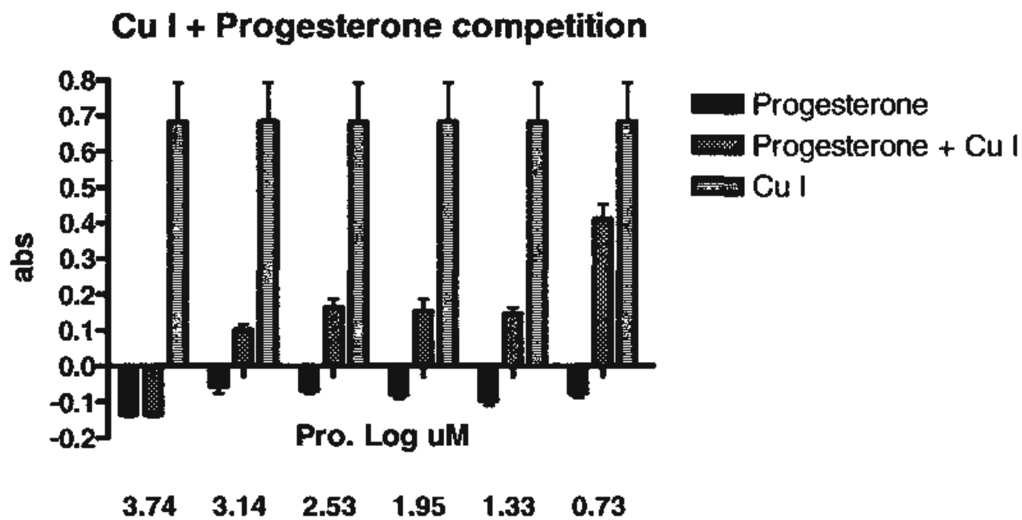


Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu I+ Hydrocortisone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	81.37		
R squared	0.9156		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	139.0		
P value	P<0.0001		
P value summary	***		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	1.539	2	0.7693
Residual (within columns)	0.1418	15	0.009454
Total	1.680	17	

Figure 5-14: Cucurbitacin (cu) I and Hydrocortisol (Hc.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu I in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It means the inhibition effect is significant.

P< 0.0001.



Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Cu I + Progesterone			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	91.37		
R squared	0.9241		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)			
P value			
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	1.872	2	0.9362
Residual (within columns)	0.1537	15	0.01025
Total	2.026	17	

Figure 5-15: Cucurbitacin (cu)I and Progesterone (Prog.) competition assay

The result indicated that the Dex. could inhibit the hemoglobin inducion by cu I in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that the inhibition effect was significant. P< 0.0001.

cu I & Mifepristone competition assay

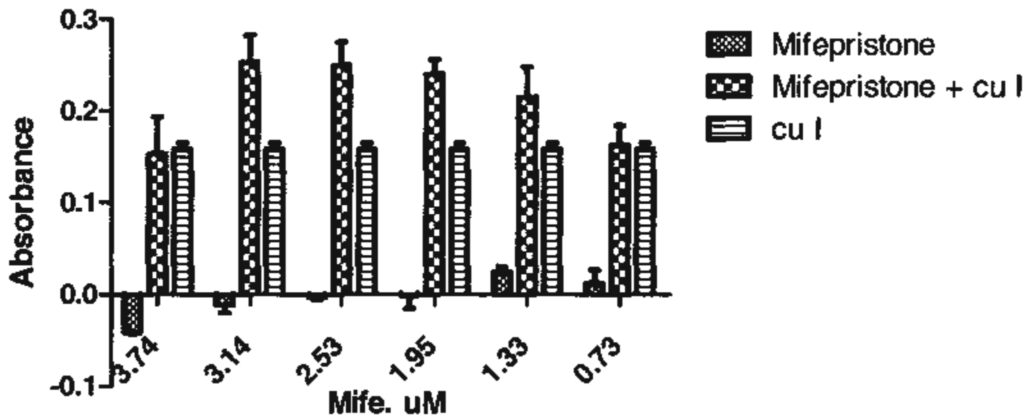


Table Analyzed	cu I + Mifepristone		
Repeated Measures ANOVA			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	104		
R squared	0.954		
Was the pairing significantly effective?			
R squared	0.0300		
F	1.34		
P value	0.3223		
P value summary	ns		
Is there significant matching? (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.151	2	0.0756
Individual (between rows)	0.00490	5	0.000980
Residual (random)	0.00729	10	0.000729
Total	0.163	17	
Post test for linear trend			
Slope	0.0808		
R squared	0.480		
P value	P<0.0001		
P value summary	***		
Is linear trend significant (P < 0.05)?	Yes		

Figure 5-16: Cucurbitacin (cu) I and Mifepristone (Mife.) competition assay

The result indicated that the Mife could increased the maxium response of hemoglobin inducion by cu I in k562 cell assay. One way ANOVA statistcal analyze the result. The varience within group or between groups are significant. It meant that synergisticeffect was significant. P< 0.0001.

5.5. Discussion

Dexamethasone, hydrocortisol and progesterone could inhibit the inducing hemoglobin effect of cucurbitacins. Mifepristone, an antagonist of dexamethasone, hydrocortisol and progesterone, could increase the maxium response which induced by cucurbitacin B, D and I. We speculated that dexamethasone, hydrocortisol and progesterone may compete with cucurbitacins for the same receptor and thus suppress the signal of cucurbitacins, or the signal generates by these hormones show a negative cross-talk with cucurbitacins. The mifepristone, which also binds on the glucocorticoid receptor, in stead of competing cucurbaticins for the same receptor to suppress its effect it, enhanced the hemoglobin induction activities of cucurbitacins. This observation supports the later agurment.

The explanation of this phenomenon was that, it might be interrupted the signal transduction of inducing reaction. If mifepristone or progesterone occupied the receptor which cucurbitacin bound to it, both of them would inhibit the inducing effect. However, one was inhibition of the effect, the other was synergistic effect. The conclusion of the results, it was the signal interference. The further investigation had to carry out.
The optimal dosages of cucurbitacin B, D, E & I were calculated by statistical program. Non-liner regression method sketched the dose-response curve. Mifepristone could increase the maximum response of cucurbitacin B, D, I. However, it did not show the increasing effect when it mixed with cucubitacin E.

Chapter 6: General Discussion

Sickle cell anemia and beta-thalassemia are co-localized with malaria. Sickle cell anemia is highly prevalent in Africa while beta-thalassemia is highly prevalent in south East Asia (Fucharoen *et al.*,2004), where malaria has been epidemic for very long time. Both sickle cell anemia and beta-thalassemia are caused by the mutation of beta-globin gene (Perrine *et al.*,1993). In the past, these beta-hemoglobinopathies were treated by regular blood transfusion (Feng *et al.*, 2006), or bone marrow transplantation (Feng *et al.*, 2006). During embryo development, gamma globin gene is expressed, which is functionally the same as beta-globin to form fetal hemoglobin with alpha globin. After birth, the gamma globin gene is shut down and beta globin gene is turn on to replace gamma globin forming adult hemoglobin with alpha globin. However, some people have mutation in the gamma globin gene promoter will continuously express the gamma globin gene through out the life and is phenotypically normal (Forget, 2006). Based on this observation scientists proposed to treat the beta-hemoglobinopathies by turning on the gamma globin gene, which is normal in beta-hemoglobinopathy patients to cure these diseases. This type of gene therapy is called "gene switch" therapy (Testa, 2006). Hydroxyurea, an anti-cancer drug, was accidentally found to be able to ameliorate the sickle cell anemia(Bakanay *et al.*,2005). However, only 30% of patients response to this drug and this drug can cause many adverse effects (Martin *et al.*, 2003). Therefore many scientists are screening and testing different compounds to treat beta-hemoglobinopathies, such as butyrate, 5-azacytidine and short chain fatty acid. (Atweh *et al.*, 1999)
(Carr *et al.*, 1987) (Cao *et al.*, 2005)

Comparison of fetal hemoglobin potency of different cucurbitacins

Previously, our team had purified cucurbitacin D from *Trichosanthes rosthornii Harms.*, which can induce fetal hemoglobin production in K562 cells (Xing *et al.*, 2008). Cucurbitacin family has many derivatives such as cucurbitacin B, D, E and I(Chen *et al.*, 2005). In this study we have compared the fetal hemoglobin inducing activities of cucurbitacin B, D, E and I. Cucurbitacin B has the lowest ED50, followed by I, D and E (Table 2-2). However, the curve steepness of the dose response curve of E and I was very flat. Taking the curve steepness into account, the potency of inducing fetal hemoglobin of CuB and CuD are comparable, but CuB has a lower ED50.

Discovery of a potent fetal hemoglobin inducer from Chinese herbs

Since the herb, Trichosanthis, only contains little cucurbitacin D, therefore extraction and purification of cucurbitacin D from this herb is quite difficult and expensive. Other members of this family or genus also contain cucurbitacins, and other herbal medicine may contain other hemoglobin inducing activities. Therefore we had screened a number of other herbal medicine and some other members belong to the same family or genus of Trichosanthis for the hemoglobin inducing activities. Extracts of *Atractylodes macrocephala Koidz, Luffa cylindria* and *Cotyledon of Lagenaria sicieraria var.makinoi* contains hemoglobin inducing activities (table 3-23). Among these three herbs, Luffa cylindria contains the most potent hemoglobin inducing activities. The extract of Luffa cylindria was further purified by chromatography using C18 column. The hemoglobin inducing activities could be eluted by 40% and 60% methanol. These hemoglobin inducing activities were further purified by HPLC and two fractions collected at 15 min and 25 min corresponding to two peaks (figure 3-21) of the HPLC elution profile contain the hemoglobin inducing activities.

ED_{50} of fraction collected at 15 min fraction was 1.95 mg/ml

ED_{50} of fraction collected at 25 min fraction was 1.254 mg/ml

These two fractions may contain very potent hemoglobin inducers. However, due to the limitation of our resources we were not able to further purify and identify the active ingredients.

Toxicity study of cucurbitacins

Cucurbitacin B and cucurbitacin D are potential candidates that can be developed as drug to treat beta-hemoglobinopathies. Before further development of these compounds as drugs, toxicity analysis is needed. We have applied four human normal cell lines to study their cytotoxicity and C. elegans as a model for in vivo toxicity analysis. At the time of toxicity analysis, we did not have large quantity of the compounds and they are very expensive to purify or purchase from chemical companies. Therefore in stead of using mouse for the in vivo toxicity analysis, we applied the C. elegans in vivo toxicity assay model for the study (William.,1988). Our results indicated that cucurbitacins showed a certain degree of toxicity to human normal cells. The IC50s of cucurbitacin B, D, E and I and hydroxyurea are 120.3ng/ml, 181.5 ng/ml, 1084 ng/ml, 139.3 ng/ml and 12.24 ug/ml, respectively (figure 4-20 to 4-35, table4-5). It is not surprise that cucurbitacins are cytotoxic since they have been used as insecticides for a long time (Torkey *et al.*, 2009). However, the therapeutic index of CuB and CuD are 158 and 302, respectively (table 4-10), which are much better than hydroxyurea (table4-10).

In the C. elegans in vivo toxicity assay, we had used up to 0.5 mg/ml of CuD in the assay and the survival of C. elegans was comparable to the culture medium control

and showed no significant difference (table4-6). Our data at least indicates that CuB and CuD are good potential drug candidates for the treatment of beta-hemoglobinopathies with good therapeutic index.

Efficacy of cucurbitacin D for treating sickle cell anemia

We had evaluated the efficacy of cucurbitacin D on inducing fetal hemoglobin in a sickle cell anemia mouse model. We had first tried to administrate CuD through i.p. injection. However, high dose (1 ug/g) daily for 30 days could lead to skin lesion. Therefore we changed our protocol to oral administration. Cucurbitacins are bitter and mice do not like to ingest the drug. We then mixed CuD with 2% sucrose solution for oral feeding. The result indicated that oral feeding with 0.5ug/g daily, but not 0.1ug/g daily could enhance the production of fetal hemoglobin (figure 4-41 to 4-43). The increase was obvious at day 10 and then gradually slightly decreased. This may due to a feed back mechanism of the body or tolerant of the drug after long term administration such as the absorption resistance of artemisininn (Wong JW & Yuen KH, 2001). Improved oral bioavailability of artemisinin through inclusion complexation with β- and γ-cyclodextrins. Modification of the compound or the deliver method is needed to improve the efficacy of CuD.

Interaction of cucurbitacins and glucocorticoid Hormone

It has been reported that cucurbitacins can bind on glucocorticoid hormone receptor (Witkowski *et al.*, 1981). Glucocorticoids like dexamethasone, hydrocortisol and progesterone are used as drugs to treat Addison's Disease, Cushing's Syndrome and Congenital Adrenal Hyperplasia (Rivkees., 2010). Therefore it is important to

investigate whether cucurbitacins will interact with these glucocorticoids. We had tested the effect of different dose of dexamethasone, hydrocortisol and progesterone on the effect of hemoglobin induction of CuB, CuD, CuE and CuI at their optimal concentrations. The results indicated that dexamethasone, hydrocortisol and progesterone could suppress the hemoglobin induction activities of CuB, CuD, CuE and CuI (figure 5-1 to 5-3, 5-7 to 5-11, 5-13 to 5-15). It could be due to the competition of the same receptor. To test this possibility, we also tested the effect of mifepristone, which is an antagonist of dexamethasone, hydrocortisol and progesterone on the hemoglobin inducing activities of CuB, CuD, CuE and CuI. Mifepristone, in stead of suppressing, enhanced the hemoglobin inducing effect of CuB, CuD and CuI, but not CuE (figure 5-4, 5-8 and 5-16). It is likely that dexamethasone, hydrocortisol and progesterone induce cell signals that cross-talk with the CuB, CuD, CuE and CuI cell signals and lead to a suppression of their activities.

Summary

In addition to CuD, other cucurbitacins may also have hemoglobin inducing activities and may have a higher potency. So far we have found CuB can also induce hemoglobin production with a higher potency. We have further search for more potent inducer from the Chinese herbs and discovery that *Luffa cylindria* contains very potent hemoglobin inducing activities. Two fractions were separated by HPLC demonstrate high hemoglobin inducing activities. These fractions may contain one or more active ingredients, which need to be further, purify and identify their chemical structures. Cucurbitacins are cytoxic but not as toxic as hydroyurea and

CuD did not show toxic effect to C. elegans even at a very high concentration. Based on the ED50 and IC50 values, CuB and CuD both have good therapeutic index than hydroxyurea. CuD can induce fetal hemoglobin production in sickle cell anemia mice, although a feed back effect is observed in long term administration. Further modification of the compound or delivery method may improve the efficacy. The last but not the least important finding is that cucurbitacins can interact with glucocorticoids, in which glucocorticoids can suppress the hemoglobin inducing effect of cucurbitacins. So cucurbitacins as a hemoglobin inducer should be not used together with glucocorticoids.

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