EFFECTS OF CHRYSIN ON THE mRNA EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARα) AND *CYP* GENES, CELL CYCLE AND CELL MIGRATION IN COLORECTAL CANCER HCT-116 CELLS

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by

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LIST OF SYMBOLS

%	Percent sign
ºC	Degree celcius
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
mg	Milligram
ml	Millilitre
mM	Millimolar
g	Gram
L	Litre

LIST OF ABBREVIATIONS

3-MA	3-methyladenine
5-FU	5-fluorouracil
AHR	Aryl hydrocarbon receptor
AKT	Protein kinase B
ALT	Alanine aminotransferases
AST	Aspartate aminotransferases
AMPK	AMP-activated protein kinase
ATCC	American Type Culture Collection
BAX	Bcl-2-associated X protein
BUN	Blood urea nitrogen
CARLA	Coactivator-dependent receptor ligand assay
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CYP	Cytochrome P450
DBD	DNA-binding domain
ddH_2O	Deionized distilled water
DMEM	Dulbecco's modified Eagle's medium
DMH	Dimethylhydrazine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediamine-tetra acetic acid
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FLAP	5-lipoxygenase
HCC	Hepatocellular carcinoma
HK-2	Hexokinase-2
hTERT	Human telomerase reverse transcriptase
LBD	Ligand-binding domain
LDH	Lactate dehydrogenase
L-NAME	N^{ω}-nitro-l-arginine methyl ester
mTOR	Mammalian target of rapamycin

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetyl-cysteine
NcoR	Nuclear receptor corepressor
NF	Nuclear factor
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-small cell lung cancer
PAH	Polycyclic aromatic hydrocarbon
PBR	PPAR binding region
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin/Streptomycin
PGE2	Prostaglandin E2
PHH	Primary human hepatocyte
PI	Propidium iodide
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator responsive element
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SALL4	Sal-like protein 4
siRNA	Small interfering ribonucleic acid
SMART	Silencing mediator for retinoid and thyroid hormone receptor
SRC	Steroid receptor coactivator
TBE	Tris-Borate-Ethylenediamine-tetra acetic acid
TNF	Tumor necrosis factor
TOP1	Topoisomerase 1
UV	Ultraviolet
VDAC-1	Voltage-dependent anion-selective channel-1

KESAN CHRYSIN PADA EKSPRESI mRNA PEROXISOME PROLIFERATOR RESEPTOR YANG DIAKTIFKAN ALPHA (PPARα) DAN GEN-GEN *CYP*, KITARAN SEL DAN MIGRASI SEL PADA SEL-SEL KANSER KOLOREKTAL HCT-116

ABSTRAK

Peroxisome proliferator reseptor-alpha (PPARα) yang diaktifkan telah dilaporkan mengawal transkripsi gen sitokrom P450 dalam hati dan usus manusia, yang mana ia telah menyumbang 70-80% daripada enzim yang terlibat dalam metabolisme dadah. Chrysin, sebaliknya, adalah sejenis flavonoid fitokimia semulajadi yang mungkin mempunyai kesan sederhana untuk digunakan sebagai agen antikanser. Kajian ini bertujuan untuk mengkaji kesan metabolisme chrysin dengan menyiasat tahap ungkapan mRNA PPARα dan mekanisme selular yang berkaitan dengan sel HCT-116 yang dirawat. Ungkapan mRNA PPARα dalam sel HCT-116 yang dirawat dengan chrysin atau kombinasi chrysin dan MK886 mula-mula ditentukan dengan menggunakan PCR konvensional dan dianalisis secara kuantitatif oleh Bioanalyzer. Kemudian, tahap ungkapan mRNA yang diukur dinormalisasikan dengan GAPDH (gen pengemasan). Sitometri aliran digunakan untuk mengesan fasa kitaran sel HCT-116 yang dirawat dengan chrysin atau kombinasi chrysin dan MK886. Aktiviti penghijrahan sel HCT-116 selepas rawatan dengan chrysin atau kombinasi chrysin dan MK886 juga disiasat oleh ujian penyembuhan luka. Akhirnya, ungkapan mRNA gen *CYP* yang berkaitan dengan metabolisme dalam chrysin atau kombinasi chrysin dan MK886 yang dirawat sel HCT-116 ditentukan oleh PCR. Ungkapan mRNA PPARα secara signifikan diinduksi dalam sel HCT-116 setelah rawatan dengan

chrysin selama 36 jam, menunjukkan bahawa HCT-116 yang dirawat chrysin mampu mengawal transkripsi cytochrome P450. Ungkapan mRNA PPARα terhalang apabila sel-sel dirawat dengan kombinasi chrysin dan MK886 (PPARα inhibitor) telah membuktikan bahawa penggabungan MK886 menurunkan tahap ungkapan PPARα, maka memungkinkan kita mempelajari fungsi PPARα. Populasi sel fasa G_0/G_1 dikesan meningkat dengan ketara dalam sel yang dirawat oleh chrysin, yang mana ia disertai dengan penurunan peratusan populasi sel fasa S pada 12 jam rawatan. Walau bagaimanapun, rawatan sel HCT-116 dengan chrysin atau kombinasi chrysin dan MK886 tidak menunjukkan keadaan bertentangan dalam populasi sel fasa G_0/G_1 dan S, menunjukkan bahawa ungkapan PPARα tidak boleh dikaitkan dengan kitaran sel dalam sel yang dirawat. Kadar penghijrahan pada sel HCT-116 yang dirawat dengan chrysin dikurangkan dengan ketara pada 24 dan 36 jam rawatan. Walau bagaimanapun, aktiviti itu dihidupkan kembali apabila ungkapan PPARα dihalang, menunjukkan bahawa aktiviti penghijrahan sel yang dirawat chrysin mungkin dikaitkan dengan ungkapan PPARα, *CYP2S1* dan *CYP1B1* dalam sel-sel, yang mana inhibitor PPARα dapat mengubahsuai ungkapan gen *CYP* ini. Kesimpulannya, rawatan sel HCT-116 dengan chrysin mendorong ungkapan PPARα, yang dapat dilemahkan dengan mengawasi kombinasi chrysin dengan MK886 dalam sel-sel. Ungkapan PPARα dalam sel HCT-116 mungkin berkorelasi dengan penghijrahan sel dan ekspresi *CYP2S1* dan *CYP1B1*. Fenomena ini menunjukkan bahawa PPARα, *CYP2S1* dan *CYP1B1* mungkin memainkan peranan penting dalam metabolisme chrysin dan juga mengawalselia aktiviti penghijrahan sel-sel kanser kolorektal.

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ABSTRACT

Peroxisome proliferator-activated receptor-alpha (PPARα) is reported to regulate the transcription of cytochrome P450 genes in human liver and intestine, which accounts for 70-80% of the enzymes involved in drug metabolism. Chrysin, on the other hand, is a natural phytochemical flavonoid that may possess a mild effect to be used as an anticancer agent. This study aimed to examine the effect of chrysin by investigating the mRNA expression level of PPARα and its related cellular mechanisms in treated HCT-116 cells, where camptothecin was used as a positive control The mRNA expressions of PPARα in HCT-116 cells treated with chrysin or a combination of chrysin and MK886 (PPARα inhibitor) were first determined using conventional PCR and then quantitatively analysed by Bioanalyzer. Each quantified level of mRNA expression was then normalised to GAPDH (housekeeping gene). Flow cytometry was used to detect the cell cycle phases of HCT-116 cells treated with chrysin or combination of chrysin and MK886. The migration activity of HCT-116 cells post-treatment with chrysin or combination of chrysin and MK886 were also investigated by cell migration assay. Finally, the mRNA expression of *CYP* genes in chrysin or combination of chrysin and MK886 treated HCT-116 cells were determined by PCR. The mRNA expression of PPARα was significantly induced in HCT-116 cells following treatment with chrysin for 36 hours, indicating that chrysin-treated HCT-

116 was able to regulate the transcription of cytochrome P450. The mRNA expression of PPARα was inhibited when the cells were treated with a combination of chrysin and MK886 proving that the incorporation of MK886 lowers the expression level of PPARα, thus enabling us to study the function of PPARα. The cell population of the G_0/G_1 phase was detected to be significantly increased in chrysin-treated cells, which was accompanied by a decrease in the percentage of S phase cell population at 12 hours of treatment. However, treatments of HCT-116 cells with chrysin or combination of chrysin and MK886 did not show the opposite situation in the G_0/G_1 and S phase cell populations, indicating that the expression of PPARα may not be associated with the cell cycle in the treated cells. The migration rate in chrysin treated HCT-116 cells was reduced significantly at 24 and 36 hours of treatments. However, the activity was revived when the expression of PPARα was inhibited, indicating that the migration activity of chrysin-treated cells is likely correlated with the expressions of PPARα, *CYP2S1* and *CYP1B1* in the cells, whereby the PPARα inhibitor could modify the expression of these *CYP* genes. In conclusion, treatment of HCT-116 cells with chrysin induced the expression of PPARα, which could be attenuated by administrating a combination of chrysin with MK886 to the cells. The expression of PPARα in HCT-116 is likely correlated with cell migration and the expressions of *CYP2S1* and *CYP1B1*. This phenomenon indicates that PPARα, *CYP2S1* and *CYP1B1* may play an essential role in regulating the migration activity of colorectal cancer cells.

CHAPTER 1

INTRODUCTION

1.0 General introduction

The peroxisome proliferator-activated receptors alpha (PPARα) is a transcription factor which belongs to the nuclear hormone receptors superfamily. The activation of PPARα is mainly through ligand binding (Contreras et al., 2013). PPARα plays an important role in the regulation of metabolism, growth and inflammation control (Chinetti et al., 2000). In addition to metabolic and inflammatory response control, evidence shows that activation of PPARα by specific ligand is able to inhibit the growth of tumor cells (Strakova et al., 2005; Grabacka et al., 2006; Grau et al., 2006). Upon binding of specific ligands, PPARα is activated and heterodimerize with retinoid X receptors (RXRs), forming a complex. The translocation of the complex to the nucleus then takes place and subsequently bind to peroxisome proliferator response elements (PPREs), which regulates the transcription of the target gene (Kostadinova et al., 2005; Bernardo & Minghetti, 2006). PPARα has been demonstrated to be able to regulate the transcription of cytochromes P450 (*CYP*s) genes such as *CYP3A4* and *CYP2C8* (Thomas et al., 2013; Thomas et al., 2015). *CYP*s are major enzymes that play an important role in drug metabolism. The enzymes are primarily found in liver, lung, intestines and kidneys, which involve in the metabolism of xenobiotics and endogenous substances (Danielson, 2002; Nebert & Russell, 2002).

Chemotherapy is one of the approaches used in cancer treatments. However, the risk of death increased from current chemotherapeutic agents are due to high toxicity, which leads to serious drug adverse effects (Ohe, 2002). Researchers have

found a large number of different drugs that possess significant anticancer properties. Natural products or compounds have been utilised as one of the most important sources of anticancer agents, which is estimated to become the major alternative for reducing cancer death in the 21st century (Mukherjee et al., 2001). Chrysin, also known as 5,7-dihydroxyflavone, is a naturally occurring common flavonoid belonging to the apigenin family. Accumulation of high-level content of flavonoids have been identified in many kinds of plants, propolis and honey (Siess et al., 1996; Williams et al., 1997). Multiple studies have shown that chrysin possess antiinflammation, antioxidant and anticancer properties (Shao et al., 2012; Rehman et al., 2014; Xu et al., 2017; AL-Ani et al., 2018; Sithisarn et al., 2019). Perhaps, chrysin may possess a mild effect to be used as an anticancer agent (Khoo et al., 2010).

Realising the importance of studying chrysin, in this study, the drug metabolising effect of chrysin in HCT-116 was investigated. HCT-116 is a colorectal cancer cell line that was reported to demonstrate the highest susceptibility to chrysin-induced cell death (Ronnekleiv-Kelly et al., 2016). As such, the investigation was carried out. Firstly, polymerase chain reaction (PCR) was done to evaluate the mRNA expression of PPARα post-treated with chrysin and combination of chrysin and MK886 (PPARα inhibitor) in HCT-116 cells. The cell cycle analysis was carried out by using flow cytometry to correlate the mRNA expression of PPARα with the phases of arrested HCT-116 cells in the cell cycle post-treatment with chrysin and combination of chrysin and MK886. Next, cell migration assay was performed to determine the migration activity of the HCT-116 cells after the cells were treated with chrysin and combination of chrysin and MK886. Finally, the mRNA expression of

*CYP*s was measured by using PCR in HCT-116 cells treated with chrysin and combination of chrysin and MK886.

1.1 Problem Statement

Chemotherapy remains as one of the most common strategy that is employed in cancer therapy. However, the risk that is associated with this treatment remains high due to the toxicity of the chemotherapy drugs used. Previous studies have shown that poor drug metabolism which leads to drug accumulation creates greater potential for adverse drug effects (Ohe, 2002). Because of that, PPARα became a popular target as it was reported to regulate the transcription of cytochrome P450 genes in human liver and intestine, which is involved in drug metabolism (Thomas et al., 2013; Thomas et al., 2015). Chrysin, a natural phytochemical flavonoid that have been demonstrated to show broad anticancer effects in various types of cancer, including colon, lung and liver cancers (Shao et al., 2012; Bahadori et al., 2016; Xu et al., 2017). Chrysin was selected to be used in this study as naturally occuring phytochemicals were reported capable of modulating the expression of PPARα (Rigano et al., 2017). Therefore, it is beneficial to investigate the effect of chrysin on the regulation of PPARα mRNA expression, as well as the mechanisms involved to further improve the therapeutic value of chrysin.

1.2 Objectives

This study aimed to investigate the drug metabolising effect of chrysin by investigating the mRNA expression level of PPARα and its related cellular mechanisms in HCT-116. The specific objectives of this study are:

1. To evaluate the effect of chrysin on the mRNA expression of PPARα in HCT-116 cells.
2. To determine the mRNA expression of PPARα with phases of the cell cycle induced by chrysin alone or combination of chrysin and MK886 in HCT-116 cells.
3. To investigate the migration activity of HCT-116 cells post-treatment with chrysin alone or combination of chrysin and MK886.
4. To determine the mRNA expression of metabolism-related *CYP* genes in chrysin-treated or combination of chrysin and MK886 treated HCT-116 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 PPAR

PPARs were reported to be involved in proliferation of peroxisome and regulates metabolic functions in the cells, such as metabolism and homeostasis of lipid (Yoon, 2009; Liu et al., 2018; Villapol, 2018). In 1969, De Duve reported peroxisome proliferation was shown in rats treated with a hypolipidemic agent, clofibrate. Several compounds which are able to induce proliferation of peroxisomes were subsequently discovered later on therefore the compounds were named as peroxisome proliferators and the first receptor for the peroxisome proliferator was called PPAR (Reddy, 2004).

PPAR comprised of three different subtypes: PPARα, PPARβ/δ and PPARγ . All PPAR isoforms function mainly as transcription factors to regulate numerous biological processes (Feige et al., 2006). These three PPARs constitute the 1C group of the nuclear hormone receptor superfamily that consists of 48 nuclear receptor members. PPARα (NR 1C1), PPARβ/δ (NR 1C2), and PPARγ (NR 1C3), contain four major functional domains (Figure 2.1), similar as other nuclear receptors. The domains are made up of the N-terminal ligand-independent transactivation domain (AF-1) in A/B domain, the DNA binding domain (DBD) in C domain, the co-factor docking domain (D domain), and the C-terminal E/F domain including ligand binding domain (LBD) and the ligand-dependent transactivation domain (AF-2). The high degrees of sequence conservation of these subtypes across numerous species have been characterized. The C domain of the three PPAR subtypes are 80% identical, while the E/F domain exhibit a lower degree of identity, approximately 65% (Tachibana et al.,

2008). The PPAR subtypes are differentially regulated in the body and displayed different ligand selectivity and biologic actions. Due to the relatively high discrepancy among the PPAR subtype-specific E/F domains, differential activation of subtypes by endogenous and exogenous compounds may account for the specific biological activity (Desvergne & Wahli, 1999; Willson et al., 2000).

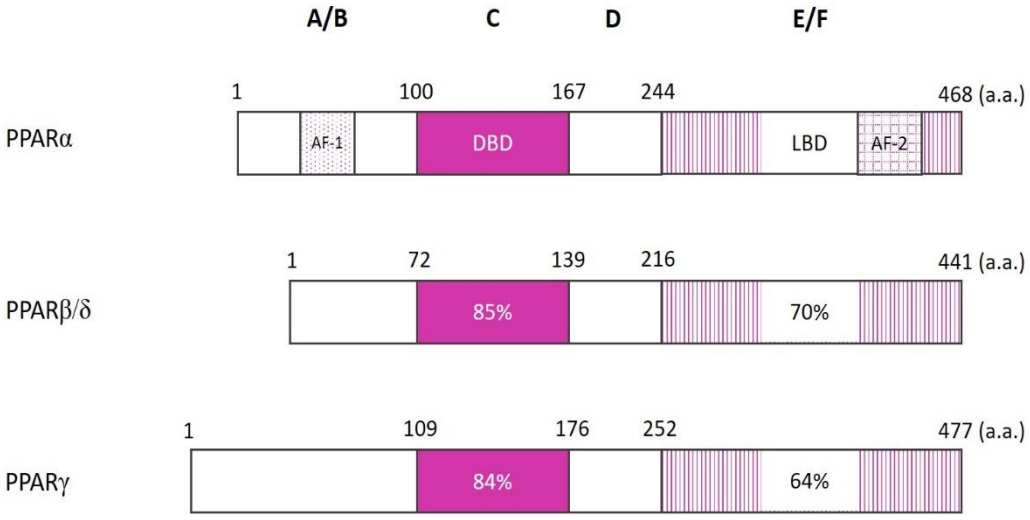


Figure 2.1: Schematic representation of human PPAR. Structure and functional domain of human PPARs. A/B, C, D, and E/F indicate AF-1; DBD; the hinge region; LBD and AF-2 respectively. The number inside each domain corresponds to the amino acid sequence identity of human PPARβ/δ and PPARγ relative to PPARα. This figure was adapted and modified from Tachibana et al. (2008).

2.1.1 PPARα

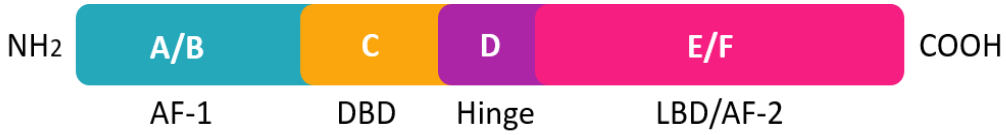
PPARα showed an elevated expression in the liver and was originally identified as the molecular target of xenobiotics by the induction of peroxisome proliferation in rodents (Issemann & Green, 1990). High expression of PPARα are commonly found in tissues with high occurance of fatty acid oxidation, for example liver, kidney, heart, skeletal muscle, and brown adipose. Besides that, PPARα is also expressed in other types of cells and tissues, which includes intestine, vascular endothelium, smooth muscle and immune cells (Lefebvre et al., 2006). The gene plays a part in controlling the gene networks expression in homeostasis of energy and lipid, differentiation of adipocyte and inflammation (Lalloyer & Staels, 2010; Wahli & Michalik, 2012).

2.1.2 Molecular characteristics of PPARα

Similar to other members of the nuclear receptor family, PPARα contains four main domains shown in Figure 2.2(a). The phosphorylation of the A/B domain is able to change the transcriptional activity and ligand binding to the receptor (Diradourian et al., 2005). The C domain contain PPARα DNA binding domain (DBD) and in charge for the interaction with DNA, allowing PPARα/RXRα complex to bind to specific PPREs that is made up of two AGGTCA-like sequences, which is interspaced by a single nucleotide (IJpenberg et al., 1997; Wan et al., 2000). D domain or the hinge region acts as a docking site for cofactors and connects the DBD to the ligand binding domain (LBD). The E/F domain harbours the ligand-binding pocket that is important for RXR dimerization and the ligand-dependent transactivation domain in physical interactions with coregulatory proteins (Xu et al., 2002). The synergy of the two activation domains, AF-1 (amino terminus) and AF-2 (carboxyl terminus) play a vital role in increasing the transcription and expression of target gene.

PPARα heterodimerizes with RXRα and binds to PPRE, which composed of two AGGTCA-like sequences directionally aligned with a single nucleotide spacer in promoter regionm of target DNAs. Under the unliganded state, PPARα/RXRα heterodimers are bound to corepressor complex encompassing histone deacetylase activity, for example nuclear receptor corepressor (NcoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMART). The histone deacetylases strengthens the association of histones to DNA, therefore hindering the gene expression. When PPARα ligand is present, the PPARα changes its conformation, leading to the release of corepressor complex and the recruitment of coactivators such as steroid receptor coactivator (SRC) and often have intrinsic histone acetyltransferase that weakens the association of histones to DNA, promoting the activation of gene transcription (Figure 2.2b) (Viswakarma et al., 2010; Mottis et al., 2013).

(a)



(b)

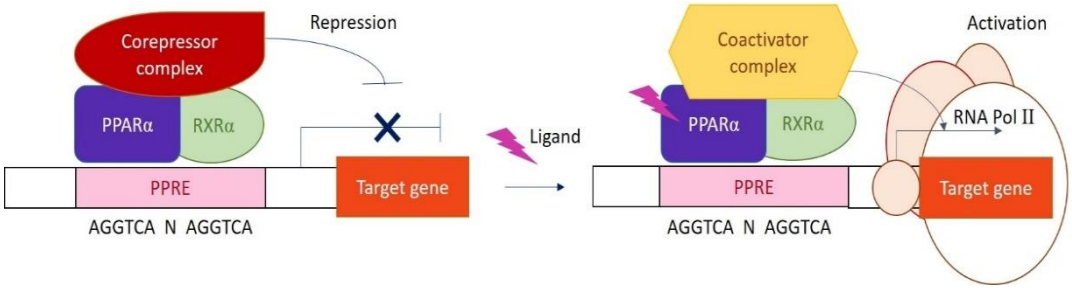


Figure 2.2: (a) Schematic representation of the principal domains of PPARα (b) PPARα mediated gene regulation. PPARα/RXRα heterodimers bind to a PPRE located in the promoter of target genes through the C domain. Unliganded PPARα associates with the corepressor complex. In the presence of ligand, the ligand-bound E/F domain associates with the coactivator complex. Ligand, chrysin; RXRα, retinoid x receptor α; PPRE, PPARα response element; RNA Pol II, RNA polymerase II. This figure was adapted and modified from Tachibana et al. (2008).

2.1.3 The inhibition of PPARα by MK886

MK886 was initially recognised as the inhibitor of 5-lipoxygenase (FLAP), a fatty-acid binding protein (Datta et al., 1999). A study by Kehrer et al. (2001) demonstrated that MK886 possess the ability to inhibit the expression of PPARα, PPARβ/δ and PPARγ by using reporter assay systems. Transient transfection reporter assay was carried out in CV-1 cells and keratinocyte 308 cells; and stable transfection system in CV-1 cells. Both transient and stable transfection system indicated that the activation of PPARα by WY-14,643 (PPARα ligand) was reduced by 80%, which was the most pronounced compared to another two PPAR subtypes. According to coactivator-dependent receptor ligand assay (CARLA), conformational changes were not observed when PPARα was bound to MK886. On the other hand, MK886 recruited non-competitive inhibition towards PPARα.

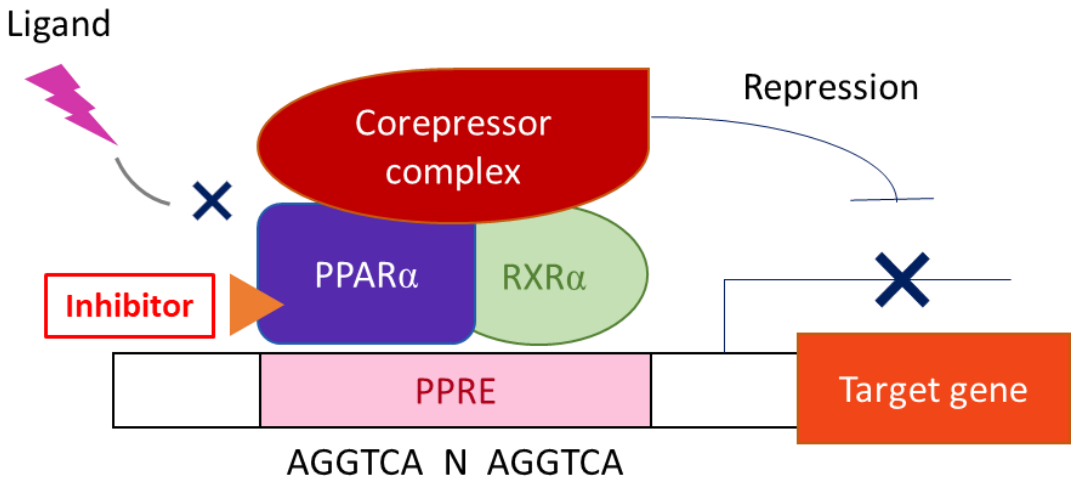


Figure 2.3: MK886 binds to PPARα in a non-competitive manner. The inhibitor binds to nearby ligand binding site, blocking the conformational change that is necessary for the formation of the active complex. Ligand: chrysin; Inhibitor: MK886; RXRα: retinoid x receptor α; PPRE: PPARα response element (Kehrer et al., 2001).

2.1.4 PPARα in cancers

PPARα was found to play a role in the breast cancer-specific survival period. Loss of PPARα expression in breast carcinomas was associated with basal-like tumours, which have the worst prognosis, results in shorter breast cancer-specific survival period (Baker et al., 2013). Similarly, increased PPARα expression in patients with hepatocellular carcinoma were reported to have longer survival period (Xiao et al., 2018).

The agonist of PPARα, fenofibrate possess the ability to suppress the cell proliferation of MDA-MB-231 by inducing apoptosis and cell cycle arrest *via* increasing the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), subsequently activate caspase-3 (Li et al., 2014). Although the antiproliferative activity of fenofibrate was independent of PPARα activity, clofibrate induces apoptosis in human hepatocarcinoma HepG2 (Maggiora et al., 2010) and inhibition of tumor progression by WY-14643 (Pozzi et al., 2007) were both dependent on PPARα.

Furthermore, by treating human non-small cell lung cancer (NSCLC) with PPARα agonists, e.g. bezafibrate and WY-14643, metastatic growth of NSCLC cells and tumor angiogenesis were found to be reduced by downregulation of *CYP2C44* (Skrypnyk et al., 2014). Additionally, the induction of p53 mediated by PPARα enhanced the antiproliferative effect of N-Acetyl-Cysteine (NAC) in NSCLC (Hann et al., 2013).

2.1.5 PPARα regulates gene expression of Cytochrome P450

*CYP*s are a major enzyme family, which are able to catalyze the oxidative biotransformation of several endogenous substances and exogenous substances, as well as foreign compounds (You, 2004; Fukami et al., 2008; Zanger & Schwab, 2013; Bishop-Bailey et al., 2014; Chen et al., 2014; Koe et al., 2014). The enzymes are classified based on the similarity in gene sequences. The gene family number is then indicated followed by the letters *CYP* (eg., *CYP1*, *CYP2*, *CYP3*), subsequently a subfamily letter. A number is then placed after the subfamily letter to indicate the specific gene eg., *CYP1A1*, *CYP2C9*, *CYP3A4* (McDonnell & Dang, 2013; Basheer & Kerem, 2015). A study by Luthra et al. (2011) has shown that the reduction of cytochrome P450 during the exposure of carbon monoxide resulting in a 450 nm Soret peak, hence the name CYP450. *CYP*s are integral proteins that usually resides in the membrane of the endoplasmic reticulum, cell surface and mitochondria (Neve & Ingelman-Sundberg, 2010). *CYP*s are primarily found in human liver, kidney and small intestine (Thelen & Dressman, 2009; Renaud et al., 2011). *CYP* enzymes are a phase I metabolic enzymes, which act as monooxygenases. Through the addition of polar functional groups, the enzymes are capable of catalysing the metabolic activity of xenobiotics and endogenous substances *via* oxidation and reduction, This act also promotes the hydrophilicity of molecules and enhances the clearance from the body, hence reducing toxicity (Brown et al., 2008; Gillet & Gottesman, 2010).

Thomas and colleagues revealed that PPARα directly regulates the transcription of *CYP3A4* by using chromatin immunoprecipitation (ChIP) assay, revealing that the promoter region of *CYP3A4* was occupied by PPARα in primary

PHH. It was observed that the transcription activation of *CYP3A4* was due to a significant enrichment of PPARα binding in PBRs (Thomas et al., 2013).

In addition to that, a report also showed that PPARα was capable of regulating the transcription of *CYP2C8*, which is highly expressed in human liver. *CYP2C8* was not only able to metabolize clinically used drugs, but also endogenous substances, such as retinoic acid and arachidonic acid (Thomas et al., 2015). The functional impact of PPARα on *CYP2C8* was examined by using WY-14,643 as induction and siRNA-mediated knockdown of PPARα for reduction of the gene. According to Thomas et al. (2015), the mRNA expression and protein level of *CYP2C8* were significantly up-regulated when HepaRG cells were treated with ligand WY-14,643. On the other hand, the mRNA expression and protein level were significantly reduced when HepaRG cells were transfected with siRNA, which targeted PPARα compared to the non-silenced cells. Furthermore, ChIP assay revealed the presence of PBRs in the upstream of transcriptional start site in HepaRG human hepatocyte cells, confirming the expression of *CYP2C8* was directly regulated by PPARα (Thomas et al., 2015). Although *CYP*s usually facilitate detoxification reactions, some isoforms activate the substrates to carcinogenic products (Chun & Kim, 2016). For example, polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants, in which the carcinogenic potential has been elucidated in *in vitro* and *in vivo* studies (Bauer et al., 2018; Wang & Xue, 2015). *CYP1A1* and *CYP1B1* were reported to be able to catalyse the activation of PAHs into highly reactive metabolites, which can bind to cellular proteins and DNA, subsequently initiate the transformation of cells (Shimada & Fujii-Kuriyama, 2004). Besides, the mRNA levels of *CYP1A1* and *CYP1B1* were also found to be overexpressed in colon and bladder tumor compared to normal tissues. Hence,

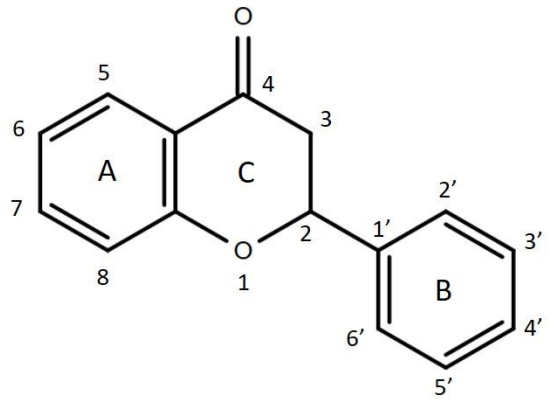
the role of *CYP*s was studied in cancer progression (Androutsopoulos et al., 2013; Patel & Gooderham, 2015). *CYP1A1* and *CYP1B1* can be transcriptionally activated *via* cytosolic aryl hydrocarbon receptor (AHR) when there is the presence of substrate, such as inhalation chemicals and environmental pollutants, (Yang et al., 2008; Androutsopoulos et al., 2009). Prior studies had shown that *CYP1A1* and *CYP1B1* played vital roles in breast cancer progression, e.g. *CYP1A1* and *CYP1B1* knockdown significantly inhibited the cell proliferation and increased apoptosis in MDA-MB-231, MCF-7 and MCF-10A cells (Rodriguez & Potter, 2013; Kwon et al., 2016).

2.2 Chrysin

Chrysin, also known as 5,7-dihydroxyflavone, is a naturally occurring common flavonoid belonging to the flavone class. Flavonoid is a natural antioxidant, which can possess free radical scavenging ability. The flavonoid is widely present in foods and beverages originated from plants, such as fruits, vegetables, tea, cocoa and others (Panche et al., 2016). It is an important class of plant secondary metabolites comprised of a large group of 15-carbon skeleton polyphenolic compounds. The flavonoid consists of two benzene rings linked *via* a heterocyclic pyrane ring. A number of studies have shown that the phenolic structure of flavonoids are accountable for various important biochemical and pharmacological activities in chemoprevention and therapeutic (Kasala et al., 2015). The flavonoids can be divided into a variety of classes, such as flavones, flavonols, flavanones, isoflavonoid and anthocyanidins (Kale et al., 2008). Chrysin belongs to the flavone class, which is one of the most important subgroups of flavonoid. Chrysin and flavone share a common structure with two hydroxy groups at the 5th and 7th position of the A ring. A phenyl B ring is

attached to the 2nd position of the C ring (Khoo et al., 2010). Chrysin has been reported to be present in many kinds of plants, such as *Pelargonium crispum*, *Passiflora caerulea*, *Passiflora incarnata* and *Oroxylum indicum* (Pereira et al., 2012; Pasini et al., 2013; Stepanic et al., 2015).

(a)



(b)

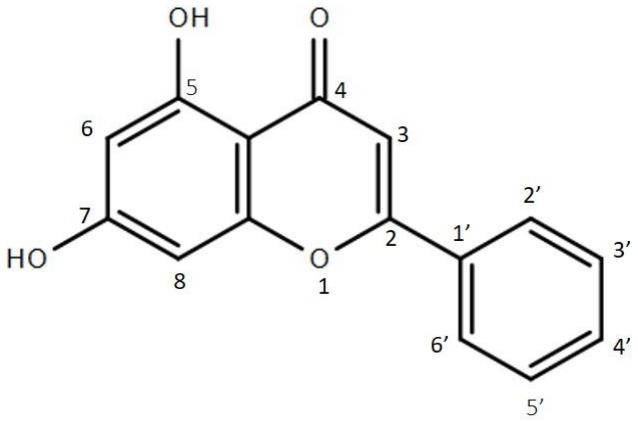


Figure 2.4: The chemical structure of flavone and chrysin. (a) The structure of flavone (b) Chrysin is in the flavone subgroup flavonoids. Two hydroxy groups are present at positions 5 and 7 (Mani & Natesan, 2018).

2.2.1 Pharmacological activities of chrysin

A number of *in vitro* and *in vivo* studies have been carried out and demonstrated the therapeutic effects of chrysin. Generally, chrysin possesses many biological activities and pharmacological effects, including anticancer, antioxidant, antibacterial activities and others (Mani & Natesan, 2018).

Suppression of the development and progression of cancer cells by chrysin were shown in a number of studies by selectively mediating the cell signalling pathway (Ji et al., 2015). Bahadori et al. (2016) revealed that chrysin induced cell death in CT26 colon cancer cells through the intrinsic apoptotic pathway *in vitro*, whereas *in vivo* assay showed that the volume of tumor in chrysin-treated mice was reduced compared to control by downregulating the mRNA expression of sal-like protein 4 (sall4) and upregulation of the Bcl-2-associated X protein (Bax). Another study elucidated that AMP-activated protein kinase (AMPK) was induced post-treatment by chrysin, resulting in growth inhibition and apoptosis in A549 lung cancer cells, subsequently inhibiting the activation of protein kinase B (Akt; Shao et al., 2012). Hexokinase-2 (HK-2) was reported to be overexpressed in hepatocellular carcinoma cell (HCC) compared to normal hepatocytes in a previous report (Xu et al., 2017). The high level of HK-2 in HCC cells demonstrated high aerobic glycolysis, which shown by increased in glucose uptake and production of lactate (Dai et al., 2015). The anticancer effect of chrysin against HCC was exerted by inhibiting the attachment of HK-2 and voltage-dependent anion-selective channel 1 (VDAC-1) on mitochondria. Bax gained access to VDAC-1, forming VDAC-Bax complex, which was able to pass other pro-apoptotic proteins, resulting in apoptosis induction. Downregulation of HK-2 by

chrysin was also reported to decrease the glucose uptake and the production of lactate, leading to the suppression of glycolysis in HCC (Xu et al., 2017).

Chrysin was reported as one of the major flavonoids containing in *Oroxylum indicum* plant, which is a medicinal plant found in tropical countries, such as Malaysia, Sri Lanka, India and China (Harminder et al., 2011). The whole food ethanolic extract of *O. indicum* displayed antibacterial effects against *Staphylococcus intermedius*, *Streptococcus suis*, *Pseudomonas aeruginosa*, and *β-Escherichia coli* to treat diseases, e.g. meningococcal meningitis and soft tissue bacterial meningitis (Sithisarn et al., 2019). In addition, ethanolic extract of propolis samples, which contain flavonoids and polyphenols have shown to to play key role in the inhibition of bacterial cell wall synthesis, by comparing with the antibiotics namely vancomycin and oxacillin against *Streptococcus pyogenes*, Methicillin-resistant *Staphylococcus aureus* and Vancomycin-resistant enterococci (AL-Ani et al., 2018).

A study has elucidated that chrysin had an antihypertensive effect as it was capable of counteracting oxidative stress, which mediated by free radical. The reaction was caused by lipid peroxidation, results in the damage of lipid membrane. This scenario was reversed when N^{ω}-nitro-l-arginine methyl ester (L-NAME) induced hypertensive rats were treated with chrysin, by reducing the product of lipid peroxidation. As a consequence, the rise in antioxidants level was observed, which leads to vessel wall relaxation, and blood pressure was controlled. Chrysin was believed to possess the antioxidation activity due to hydroxyl groups in the 5^{th} and 7^{th} position (Veerappan & Senthilkumar, 2015). Moreover, chrysin possesses the hepatoprotective efficiency by lowering the hepatic marker enzyme activity, and the

product of lipid peroxidation, that in turn elevated the activities of free-radical scavenging enzymes in d-galactosamine-intoxicated rats (Pushpavalli et al., 2010).

Apart from these pharmacological activities of chrysin, it has also been demonstrated to protect numerous tissues against injuries caused by drugs. The hepatotoxicity induced by cisplatin (an antitumor drug) was improved with the supplementation of chrysin in rats. Chrysin reduced the tissue damage in the liver by reducing the inflammatory response and oxidative stress caused by cisplatin (Rehman et al., 2014). Another *in vivo* study showed that chrysin was able to reduce the side effects caused by doxorubicin, such as nephrotoxicity. Chrysin reduced the serum toxicity markers, e.g. serum creatinine, blood urea nitrogen (BUN), alanine aminotransferases (ALT), aspartate aminotransferases (AST) and lactate dehydrogenase (LDH). In addition to that, the antioxidant content in renal tissues was found to be increased post-treatment of chrysin (Rashid et al., 2013). The studies are summarised in **Table 2.1**.

Table 2.1: The effects of chrysin on colorectal cancer cells.

Cell model	Remark	References
CT26	Chrysin demonstrated a cytotoxic effect on CT26 cells through the inhibition of cell proliferation. The cytotoxicity of chrysin was associated with the induction of the intrinsic apoptotic pathway in CT26 cells. Chrysin (4, 8, 10 $mg.kg^{-1}$) reduces the volume of tumor dose-dependently in the animal model study by downregulating sal-sall4 and up-regulation of the Bax.	Bahadori et al., 2016
Caco-2	The combination of resveratrol and chrysin exert an antiproliferative effect in Caco-2 cells. Real-time RT-PCR analysis revealed that mRNA expression of UGT1A1 was elevated 22-folds, when Caco-2 cells were treated with the combination of 20 µM resveratrol and 32 µM chrysin.	Iwuchukwu et al., 2011
Caco-2 & SW480	EC50 values for the toxic potency in SW480 were estimated at chrysin (165 µM) > acacetin (160 µM) > kampferol (100 µM) > luteolin (90 µM) > quercetin (85 µM); a stronger toxic effect was found for the combination of chrysin at 80 µM with apigenin at 20 µM. DNA flow cytometric analysis indicated that treatment of the cells with chrysin at 0-80 µM for 48 h resulted in a cell-cycle arrest at the G_2/M phase in a dose-dependent manner. When chrysin at doses between 5-30 µM was combined with apigenin at 20 µM, there was an increase of 22% in the proportion of G_2/M cells.	Wang et al., 2004
Colo205	Chrysin-Organogermanium (IV) complex (Chry-Ge) inhibited the proliferation and migration of Colo205 cells. The induction of apoptosis was reported by disrupting the mitochondrial membrane potential and recruitment of caspase 3/9 activation in Colo205 cells treated with Chry-Ge. Simultaneously, the changing of morphology, cytoskeleton and nucleus of Colo205 was observed post-treated with Chry-Ge. Moreover, Chry-Ge	Yang et al., 2015

	were demonstrated to be able to destruct the vessel formations, implying that Chry-Ge possesses antiangiogenesis effect.	
DLD-1, HCT-116 & SW837	Chrysin-induced up-regulation of tumor necrosis factor (TNF) α and β gene expression, which consequent activation of the TNF-mediated transcriptional pathway in chrysin-induced apoptosis, was dependent on the aryl hydrocarbon receptor (AHR).	Ronnekleiv-Kelly et al., 2016
HT-29, SW48, SW480, SW620 & HCT-116	Chrysin achieved similar effects on the attenuation of cell viability with a combination of 5-Fluorouracil (5-FU) and oxaliplatin in HT-29, SW48, SW480, SW620 and HCT-116 cells. Chrysin induced the production of reactive oxygen species (ROS) which subsequently lead to autophagy by upregulation of LC3-II and inhibition of protein kinase B (Akt)/mammalian target of rapamycin (mTOR).	Lin et al., 2018
SW480	The nano-encapsulation of curcumin and chrysin have shown to induce cytotoxicity in SW480 cells compared to free forms of the compounds. The anticancer effect of nanocapsulated curcumin and chrysin was promoted by significantly reduced the gene expression of human telomerase reverse transcriptase (hTERT) in SW480 cells *via* the improvement of bioavailability and the solubility.	Bagheri et al., 2018
Rat	Chrysin plays a role in decreasing the number of pre-neoplastic colorectal lesions in 1,2-dimethylhydrazine (DMH) induced rats. The reduction of dysplasia in intestinal crypts was observed, and it was associated with lower cell proliferation in the groups exposed to chrysin. In addition, the levels of antioxidant minerals, e.g. copper, zinc, selenium and magnesium in intestinal mucosa were shown to be significantly induced in chrysin treated groups.	Sequetto et al., 2013

2.3 Camptothecin

Camptothecin molecule was originally isolated from the stem wood of *Camptheca acuminata* tree native in China back in the mid-1950s (Wall et al., 1966). Hsiang et al. (1985) discovered that camptothecin has the ability to induce DNA damage by interacting with DNA topoisomerase 1 (Top1). Top1 is an enzyme that plays a pivotal role in DNA topology during replication, recombination and transcription. During the process, the phosphotyrosine bond was formed between Top1 and DNA, followed by the cleavage of DNA to allow unwinding and religation (Tse et al., 1980). Camptothecin reversibly bound to the enzyme/DNA complex, preventing the religation step from occurring prior transcription step (Hsiang et al., 1985). Although camptothecin has been shown to possess potent anticancer properties, however, it was prevented from full clinical utilization due to poor solubility and stability (Wall et al., 1966). Moreover, camptothecin has been reported to have high toxicity, resulting in adverse drug effects such as diarrhoea, vomiting, myelosuppression and haemorrhagic cystitis (Srivastava et al., 2005). One of the common causes of adverse drug effects is due to differential regulation of Cytochrome P450. Camptothecin was reported to block the induction of human pregnane X receptor, which attenuates the transcription of *CYP3A4*, which is the most abundant *CYP* that metabolizes the majority of drugs (Chen et al., 2014).