TURKISH REPUBLIC OF NORTHERN CYPRUS

NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

ASSESSING THE POTENTIAL INHIBITORY EFFECTS OF AUXINS ON HUMAN PLACENTAL GLUTATHIONE S-TRANSFERASE P1-1

PAVEL HUSAMADIN

MASTER OF SCIENCE THESIS

MEDICAL BIOCHEMISTRY PROGRAM

NICOSIA

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The Directorate of Institute of Graduate Studies,

This study has been accepted by the thesis committee in Medical Biochemistry program as a Master of Science Thesis.

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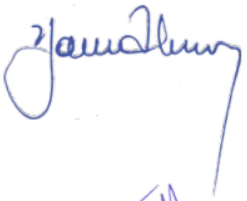
Member:	Professor Yasemin AKSOY Hacettepe University	
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DECLARATION

I hereby declare that the work in this thesis entitled "**Assessing the potential inhibitory effects of Auxins on Human Placental Glutathione S-Transferase P1-1"** is the product of my own research efforts undertaken under the supervision of Professor Ozlem Dalmizrak. No part of this thesis was previously presented for another degree or diploma in any university elsewhere, and all information in this document has been obtained and presented in accordance with academic ethical conduct and rules. All materials and results that are not original to this work have been duly acknowledge, fully cited and referenced.

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ABBREVIATIONS

ABC	ATP binding cassette
ARE	Antioxidant responsive element
ASK1	Apoptosis signal-regulating kinase 1
BSA	Bovine serum albumin
CDNB	1-chloro-2,4-dinitobenzene
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	Deoxyribonucleic acid
15d-PGJ_2	15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2
EDTA	Ethylenediaminetetraacetic acid
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	L-glutathione reduced
G-site	Glutathione binding site
GST	Glutathione S-transferase
4-HNE	4-Hydroxynonenal
HOCl	Hypochlorous acid
$HOO^{\bullet}$	Hydroperoxyl radical
H_2O_2	Hydrogen peroxide
*hp*GSTP1-1	Human placental glutathione transferase P1-1
HRP	Horseradish peroxidase
H-site	Electrophilic substrate binding site
IAA	Indoleacetic acid
IBA	Indolebutyric acid
IC_{50}	Half maximum inhibitory concentration
IKK-B	Inhibitor of κB kinase
IPA	Indolepropionic acid
JNK	c-jun-N terminal kinase

Keap1	Kelch- like ECH-associated protein
K_i	Inhibition constant
K_m	Michaelis-Menten constant
LT	Leukotrienes
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	Mitogen activated protein kinase
MDR	Multidrug resistance
MGST1	Microsomal glutathione S-transferase 1
MRP	Multidrug resistance protein
1-NAA	1-napthaleneacetic acid
NF-KB	Nuclear factor κB
Nrf2	Nuclear factor 2
$O_2^{-\cdot}$	Superoxide anion
$^{\bullet}OH$	Hydroxyl radical
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
P-gp	P glycoprotein
PPARγ	Peroxisome proliferator-activated receptor γ
$RO^{\bullet}$	Alkoxy radical
$ROO^{\bullet}$	Peroxy radical
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TAM	Tryptamine
TRAF2	Tumor necrosis factor receptor associated factor 2
UGT	UDP-glucuronosyltransferases
V_m	Maximum Velocity
WHO	World Health Organization

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Thesis Title: Assessing the Potential Inhibitory Effects of Auxins on Human Placental Glutathione S-Transferase P1-1

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ABSTRACT

Aim: Glutathione S-transferases (GSTs) is a family of phase II enzymes which detoxify xenobiotics in the body through catalyzing their conjugation to glutathione (GSH), which result in the formation of harmless glutathione complexes that are excreted from the body. Implication of GSTs in detoxification process can result in a multifactorial phenomenon termed as multidrug resistance (MDR) in cancer patients. Due to the reason that GST overexpression is related to MDR in cancer, GST superfamily is now extensively targeted in the attempt to develop more competent chemotherapeutic agents for treating cancer. Auxins which are a class of plant hormones that play crucial role in plant growth and development, with indoleacetic acid (IAA) being the most prominent member of these phytohormones tends to have the ability to bind to GST in plants and regulate its function. In this study three classes of auxins, indoleacetic acid (IAA), indolepropionic acid (IPA) and indolebutyric acid (IBA) were examined for their inhibitory effect on *hp*GSTP1-1 as a mean to reduce its activity and combat MDR in patients undergoing chemotherapy.

Materials and Methods: In this study, different auxin concentrations (0.3125 – 10 mM) were tested for the estimation of IC_{50} values. Then inhibitory kinetic experiments were carried out at four chosen inhibitor concentrations (0.25, 0.5, 1 and 2 mM) while keeping one substrate variable and other constant and vice versa. The inhibition types and kinetic parameters were determined from graphs and SPSS version 20.

Results: It appeared that all three classes of auxins inhibit the activity of *hp*GSTP1-1 in a competitive manner in respect to both substrates. IAA had an IC_{50} of 9.7 mM with a V_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 332 ± 17 and 182 ± 5 µmol/min-mg protein, respectively, K_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 2.32 ± 0.17 and 1.14

± 0.06 mM, respectively and a K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 4.00 ± 0.62 and 3.30 ± 0.24 mM, respectively. IPA had an IC_{50} value of 7.2 mM with a V_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 229 ± 8 and 251 ± 11 µmol/min-mg protein, respectively, K_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 1.63 ± 0.1 and 1.38 ± 0.11 mM, respectively and a K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 3.33 ± 0.23 and 3.52 ± 0.24 mM, respectively. IBA has an IC_{50} of 7.0 mM with a V_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 229 ± 7 and 266 ± 13 µmol/min-mg protein, respectively, K_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 1.63 ± 0.08 and 1.53 ± 0.13 mM, respectively and a K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 3.33 ± 0.22 and 2.14 ± 0.16 mM, respectively.

Conclusion: Our *in vitro* study reveals the ability of auxins in inhibiting *hp*GSTP1-1, so that this class of plant hormones could be considered in the development of novel drugs which are less cytotoxic and effective at low concentrations.

Key Words: Human placental glutathione S-transferases, Indoleacetic acid, Indolepropionic acid, Indolebutyric acid, Inhibition kinetics

Tez Başlığı: Oksinlerin İnsan Plasental Glutatyon S-Transferaz P1-1 Enzimini İnhibe Etme Potansiyellerinin Değerlendirilmesi

Öğrencinin Adı/Soyadı: Pavel Awat Husamadin

Danışman: Prof. Dr. Özlem Dalmızrak

Bölüm: Tıbbi Biyokimya Anabilim Dalı

ÖZET

Amaç: Glutatyon S-transferazlar (GST) ksenobiyotiklerin, glutatyon (GSH) ile birleşmesini katalizleyerek zararsız glutatyon kompleksleri şeklinde vücuttan atılmasını sağlayan faz II enzim ailesidir. GST detoksifikasyondaki rolü nedeniyle kanser hastalarında çoklu ilaç direnci olarak adlandırılan çok etmenli bir olayda oldukça önemlidir. Kanserde çoklu ilaç direnci ile GST'nin aşırı ifadelenmesi arasındaki ilişki nedeniyle, GST ailesi yeni kemoterapötik ajanların geliştirilmesinde hedef durumundadır. Bitki hormonu olan oksinler bitki büyümesi ve gelişmesinde görev yapmaktadır. Bu fitohormonlar arasında, indolasetik asit (IAA) bitkilerde GST'ye bağlanma ve fonksiyonunu düzenleme yeteneğinden dolayı öne çıkmaktadır. Çalışmamızda üç oksin grubunun, indolasetik asit (IAA), indolpropiyonik asit (IPA) ve indolbütirik asit (IBA)'nın *hp*GSTP1-1 üzerine olan inhibe edici etkisi araştırılmıştır.

Gereç ve Yöntem: Farklı oksin derişimleri (0.3125 – 10 mM) kullanılarak IC_{50} değerleri hesaplanmıştır. Daha sonra seçilmiş olan dört inhibitör derişimini (0.25, 0.5, 1 and 2 mM) kullanarak ve substratlardan bir tanesini sabit, diğerini değişken tutarak (ve de tam tersini de uygulayarak) inhibisyon kinetiği çalışmaları gerçekleştirilmiştir. İnhibisyon türü ve kinetik parametreler grafiklerden ve aynı zamanda SPSS versiyon 20 kullanılarak bulunmuştur.

Bulgular: Oksinlerin hepsi *hp*GSTP1-1 enzimini kompetitif olarak inhibe etmektedir. IAA'nın IC_{50}'si 9.7 mM, V_m değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla 332 ± 17 ve 182 ± 5 µmol/dk-mg protein, K_m değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla 2.32 ± 0.17 ve 1.14 ± 0.06 mM ve K_i değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla 4.00 ± 0.62 ve 3.30 ± 0.24 mM olarak bulunmuştur. IPA'nın IC_{50}'si 7.2 mM, V_m değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla

229 ± 8 ve 251 ± 11 µmol/dk-mg protein, K_m değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla 1.63 ± 0.1 ve 1.38 ± 0.11 mM and a K_i değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla 3.33 ± 0.23 ve 3.52 ± 0.24 mM olarak hesaplanmıştır. IBA'nın IC_{50}'si 7.0 mM, V_m değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla 229 ± 7 ve 266 ± 13 µmol/dk-mg protein, K_m değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla 1.63 ± 0.08 ve 1.53 ± 0.13 mM ve K_i değeri $[CDNB]_f$-$[GSH]_v$ ve $[GSH]_f$-$[CDNB]_v$ için sırasıyla 3.33 ± 0.22 ve 2.14 ± 0.16 mM olarak bulunmuştur.

Sonuçlar: *In vitro* çalışmamız oksinlerin *hp*GSTP1-1 üzerine inhibe edici etkisini ortaya koymakta ve bu moleküllerin sitotoksisitesi az, çok düşük derişimlerde bile etkili yeni ilaçların geliştirilmesinde yapı taşı olarak kullanabileceğini göstermektedir.

Anahtar Kelimeler: İnsan plasental glutatyon S-transferazı, İndolasetik asit, İndolpropiyonik asit, İndolbütirik asit, İnhibisyon kinetiği

1. INTRODUCTION

Glutathione S-transferases (E.C.2.5.1.18) consist of a large family of eukaryotic and prokaryotic metabolic isoenzymes which are profoundly known to be responsible for phase II detoxification of xenobiotics, through the ability to catalyze the conjugation of xenobiotics to reduced form of glutathione (GSH), which provides a final neutralization of the noxious compounds and further promote their elimination from the body through bile or urine (Sheehan et al., 2001). Nevertheless, there are abundant activities that are associated with glutathione S-transferases (GSTs), including steroid and leukotriene biosynthesis, peroxide degradation, double-bond cis-trans isomerization, dehydroascorbate reduction, Michael addition, and non-catalytic "ligandin" activity (ligand binding and transport). GSTs are comprised of three super families that are classified based on their biological, immunological and structural properties. The cytosolic GSTs (which make up the largest group of the superfamily), the mitochondrial GSTs and the microsomal GSTs which is identified as integral membrane proteins, that are membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEGs). The known classes of cytosolic GSTs are alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, tau, phi and omega. Cytosolic GST isoenzymes classes of alpha, zeta, theta, mu, pi, sigma and omega are found in human beings. Concurrently the mitochondrial GSTs share a deep evolutionary relationship with the cytosolic GSTs, both cytosolic GST and mitochondrial GST form dimers. However, heterodimers of cytosolic GSTs have been identified containing chains belonging to the same class. The MAPEG family comprise of four subgroups (I–IV) and in humans six MAPEG isoenzymes have been identified that belong to subgroups I, II, and IV. Like the cytosolic and mitochondrial GSTs, several MAPEGs such as MGST1 catalyzes the conjugation of GSH to a number of electrophilic compounds. Other members additionally catalyze the reactions in leukotriene and prostaglandin biosynthesis (Oakley, 2011). One of the important properties of GSTs is that their activities are inducible, however not entirely but usually through the metabolized electrophiles. GSTs function therefore is one of the important factors in protecting cells from acute toxic chemical assaults. In addition, GSTs can also

be protective against cancer since for a cancer cell to form it requires the covalent modification of DNA by electrophiles derived from carcinogens or clastogens which are detoxified by GSTs (Whalen and Boyer, 1998). However, it has been stated that in several cases elevated level of GSTs were involved in drug resistant. Evidence suggest that GST isoenzymes are capable of having different roles in the body, apart from their catalytic work to protect cell against xenobiotics, GST isoenzyme can function in the detoxification of the chemotherapeutic agent, initiating the induction of drug resistance through the inactivation of chemotherapeutic compound through GSH conjugation. Overexpression of particularly GSTP1-1 is associated with the resistance to some chemotherapeutic agents in human tumor cells including colon, stomach, pancreas, uterine, cervix, breast, lung cancers, melanoma, and lymphoma. This indicates that GST isoenzymes are capable of forming chemotherapeutic drug resistance in tumors (Townsend and Tew, 2003).

Since chemotherapy provides the most effective treatment method for cancer, resistance to anticancer chemotherapy can be a serious obstacle in treating cancer. Primary and acquired resistance of tumor cells to anticancer drugs can be a serious cause of the limited efficiency of chemotherapy. Potentially tumors can be intrinsically drug resistant or develop resistance during the treatment. This is a phenomenon that is known as multidrug resistance (MDR). The problem with acquired resistance is that tumors not only become resistant to the drugs originally used in treatment but also become cross-resistant to other drugs. Whereas, inhibiting the activity of GST has the potential to be used as a therapeutic strategy to reverse MDR (Chen et al., 2013).

Given that auxins which are a class of plant hormones may possibly be able to inhibit GST activity, it’s of great importance to explore its effects. At a molecular level all auxins possess an aromatic ring and a carboxylic acid group. Indole-3-acetic acid (IAA) is the most prominent member of the auxins, having most of the auxin effect in an intact plant (Simon and Petrasek, 2011). Auxins can be generally found in different concentrations in all parts of plants. The different concentrations in each part of the plant mean important developmental information, that’s why it is sophistically regulated in both metabolism and transport. As a result, this creates a patterns of auxin concentration,

which is known as maxima and minima in the plant body. That in turn guide further development of respective cells, and ultimately of the plant as a whole. This auxin pattern within the plant and its distribution is a dynamic and environmental response. Respectively, it's the most crucial factor for plant growth, its reactions to its environment, and most importantly for developmental process of organs (such as leaves and flowers). Furthermore, these phytohormones are not exclusive to plants, due to humans being continuously exposed to these molecules, it is not foreign to human physiology. It has been stated that it can be endogenously produced by human and human cell cultures. IAA the most prominent member of the auxins, can be originated in the intestine after the ingestion of a vegetable rich diet, or it can be a product of tryptophan metabolism which is considered as a uremic toxin (Cernaro et al., 2015). When IAA is synthesized from tryptophan it can be detectable in urine, blood plasma, and even in the central nervous system. The degradation of high protein dietary peptides from meat and dairy products, can cause an increase in tryptophan concentration which is followed by the production of IAA through either the tryptamine (TAM) pathway (decarboxylation mechanism) or the indole-3-pyruvic acid pathway (transamination metabolism). However, this does not entirely indicate IAA detectability; it has been also stated that IAA can be produced by the liver, hippocampus, kidney, cerebrospinal fluid, and the midbrain. Moreover, patients with neuromuscular diseases, phenylketonuria, diabetes mellitus, hereditary syndrome with symptoms of mental deterioration, intermittent cerebellar ataxia, liver injury and cancer can produce very high amount of IAA endogenously (Kim et al., 2020).

However, based on the natural circulating IAA in human body multiple propitious cancer treatments have come to the fore. As IAA can particularly be oxidized by plant peroxidases present in modified cancer cells, or by photosensitizing dyes used in photodynamic therapy. The oxidation of IAA can cause an increase in cytotoxic radical's species in cancer cells, which in both cases will cause targeted cell death without harming other healthy cells. Given that the lower oxygen level in cancer cells is one of the most important limitation, IAA upturns treatment effectiveness and has appeared to be a promising agent in cancer therapy (Chanclud and Lacombe, 2017).

Furthermore, IAA alone is not considerably cytotoxic, although oxidative decarboxylation by horseradish peroxidase (HRP) can turn it into its active form. Studies recently established that the combination of both IAA and HRP stimulates the apoptosis of G361 human melanoma cells. Biochemically, HRP exists in the ferric form as a heme containing peroxidase enzyme in its native state. It can oxidize a great deal of substrates in the presence of hydrogen peroxide through catalyzing one-electron oxidation reactions via its complex I and II forms, simultaneously *in vitro* IAA can be metabolized to release reactive oxygen species (ROS) by peroxidase. Stating that IAA and its synthetic derivatives could be oxidized by HRP to form cytotoxic species such as indolyl radical cation, which can be of great importance and valuable for discovery of novel cancer therapeutic agent (Lin and Tan, 2011). Accordingly, the three classes of the auxins that are being examined against human placental glutathione S-transferase pi (*hp*GSTP1-1) are indoleacetic acid, indolepropionic acid and indolebutyric acid (IAA, IPA, and IBA), *hp*GSTP1-1 is a member of the pi-class family and are among those isoenzymes that its overexpression was found to be correlated with the resistance to chemotherapeutic agents in human tumor cells. Evaluating data through different kinetic models, obtaining an IC_{50} value and predicting their binding modes can be used to combat chemotherapeutic resistance in tumor cells.

2. GENERAL INFORMATION

2.1. Oxidative Stress and the Antioxidant System

Oxidative stress can be termed as a disrupt in the homeostasis of oxidant and antioxidant systems, favouring oxidants. An imbalance in this homeostatic system can be detrimental and impose injury on membrane lipids, proteins and nucleic acids. In human physiology, oxygen is required as the final acceptor of electrons in mitochondrial electron transport, this process forms harmful metabolites called reactive oxygen species (ROS) which leaks from mitochondria to the cytoplasm and oxidizes DNA, proteins, lipids and carbohydrates, eventually leading to tissue damage. Reactive oxygen species are divided into two categories, the free radicals and non-radicals. Chemical species contain one or more unpaired electron in its outer orbital is called free radical. However, when two free radicals share their unpaired electrons, the non-radical form is produced (Birben et al., 2012).

Although, ROS are crucial for normal biological processes in low or moderate concentrations, as they are generated as a part of normal cellular metabolism and are important mediators in signal transduction pathways and cellular immunity, uncontrollable level of ROS can be very harmful due to their highly reactive nature (Cao et al., 2005). Reactive oxygen species can be of endogenous sources which are produced from molecular oxygen as a result of normal cellular metabolism such as superoxide anion, hydrogen peroxide, hydroxyl radical, hypochlorous acid, peroxyl radical and hydroperoxyl radical (Table 2.1). Some exist through exogenous sources such as cigarette smoke, hyperoxia, ionization radiation, heavy metal ions and ozone exposure. These circumstances lead to the initiation of oxidative stress in which the level of ROS remains high without appropriate mechanisms to combat their reactivity (Birben et al., 2012).

To detoxify ROS there are enzymatic and non-enzymatic antioxidant mechanisms that exist both intracellularly and extracellularly. Certain properties of these antioxidants are scavenging radicals, donating electron or hydrogen, decomposing peroxides, inhibiting enzymes, act as a metal chelating agent or as synergist. Through their free

radical scavenging property, antioxidants can delay or entirely stop cellular damage. These antioxidant molecules are so stable that neutralize free radicals by donating an electron which reduces their ability to cause damage. They can successfully intervene in free radical chain reaction before there is any vital damage. Some of these antioxidants are the products of normal metabolism which include glutathione, ubiquinol, and uric acid and some should be supplied through diet, such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), and β-carotene (Lobo et al., 2010). However, there are also several enzyme systems in the body, among which the most crucial enzymes to scavenge free radicals are superoxide dismutase (SOD), glutathione transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx). Human body is fortified with all of these antioxidants to be protected and counterbalance the effect of oxidants (Yan et al., 2008).

Table 2.1. Major endogenous oxidants (Birben et al., 2012).

Oxidant	Formula	Reaction Equation
Superoxide anion	$O_2^{-\cdot}$	$NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^{-\cdot} + H^+$ $2O_2^{-\cdot} + H^+ \rightarrow O_2 + H_2O_2$
Hydrogen peroxide	H_2O_2	$Hypoxanthine + H_2O + O_2 \rightleftharpoons xanthine + H_2O_2$ $Xanthine + H_2O + O_2 \rightleftharpoons uric\ acid + H_2O_2$
Hydroxyl radical	•OH	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$
Hypochlorous acid	HOCl	$H_2O_2 + Cl^- \rightarrow HOCl + H_2O$
Peroxyl radicals	ROO•	$R\bullet + O_2 \rightarrow ROO\bullet$
Hydroperoxyl radical	$HOO^{\cdot}$	$O_2^- + H_2O \rightleftharpoons HOO^{\cdot} + OH^-$

2.2. Glutathione S-Transferases

Glutathione S-transferase enzyme (EC 2.5.1.18) family comprises 1% of the total cellular proteins and exists in most aerobic eukaryotes and some prokaryotes. GSTs are considered as xenobiotic metabolizing enzymes (Salinas and Wong, 1999), through the ability that these enzymes catalyze nucleophilic attack on the sulfur atom in a glutathione (GSH) on a nonpolar compound with an electrophilic carbon, nitrogen, or sulfur atom. Two main families of these enzymes which are indiscreetly related are distributed in cytosol and mitochondria, which are also considered as soluble enzymes. Microsomal GST which is also known as membrane associated proteins in eicosanoid

and glutathione (MAPEG) is the third family. In their three-dimensional structure cytosolic and mitochondrial GSTs show similarity. However, MAPEG does not represent any structural resemblance but, simultaneously catalyzes the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB). The largest family of GSTs are the cytosolic GSTs which catalyze thiolysis of 4-nitrophenyl acetate; exhibit thiol transferase activity; reduce trinitroglycerin, de-hydroascorbic acid and monomethylarsonic acid and catalyze isomerization of maleylacetoacetate and Δ^5-3-ketosteroids. Simultaneously, alongside MAPEG enzymes catalyze isomerization of a number of unsaturated compounds and are associated with the synthesis of prostaglandins and leukotrienes (Hayes et al., 2005).

GSTs have significant pharmacological and toxicological importance, since they have the ability to target anti-asthmatic and antitumor treatments, metabolizing herbicides, insecticides, cancer chemotherapeutic agents, carcinogens and generally by products of oxidative stress. Concurrently, they have the ability to form bond with several hydrophobic compounds such as bilirubin, heme, polycyclic aromatic hydrocarbons and dexamethasone with high affinity. As a result, specific GST subunits are stimulated by numerous xenobiotics (e.g., phenobarbital, 3-methylcholanthrene, trans-stilbene oxide) which causes their expression in a tissue specific manner. That's why the GST gene family can be a valuable model to study its stimulation and tissue specific regulation of gene expression (Pickett and Lu, 1989).

2.3. Distribution of Glutathione S-Transferases

Glutathione S-transferase isoenzymes are broadly distributed in every tissue concerning aerobic organisms and they have been characterized in a wide range of species, including microbes, trematodes and nematodes, insects, plants, fish and mammals (Buetler and Eaton, 1992) and their expression is upregulated by pro-oxidant exposure (Hayes et al., 2005). It was in 1961 where they were firstly discovered in animals and claimed to be involved with detoxification of xenobiotics and drugs. However, beside detoxification there are additional functions and features attributed to this complex enzyme family (Enayati et al., 2005). From various organs in a variety of

species, numerous molecular forms of GSTs have been classified and its subunits are diversely expressed in a tissue specific manner and its existence differs in various tissues (Table 2.2) (Awasthi et al., 1994). Although mitochondrial and microsomal GST are known, the majority of the enzyme family are cytosolic GST and exist as homodimers or heterodimers (Tsuchida and Sato, 1992).

2.4. Nomenclature

The diversity exists in designating proper names to three major classes of GST isoenzymes by different laboratories. Formerly researchers have given Greek (Kamisaka et al., 1975; Stockman et al., 1985) or English letters (Dao et al., 1984; Tsuchida et al., 1990) to differentiate the numerous GST isoenzymes of human tissues, or using other principles such as pI values (Warholm et al., 1981; Warholm et al., 1983) and electrophoretic mobilities of subunits in SDS (McLellan and Hayes, 1987) or starch gels (Board, 1981; Laisney et al., 1984). This perplex way of identifying different GST isoenzymes have shown to be inevitable, due to the existence of numerous human GST isoenzymes and their expression in different locations (Awasthi et al., 1994).

Currently the likenesses of primary structure and dividing of GSTs into classes which are more associated together is set as a basis of GST nomenclature. Each class is identified by Greek letters: Alpha, mu, pi, etc., which is shortened in Roman capitals: A, M, P, etc. Class members are differentiated through Arabic numerals and the dimer protein structures are designated in accordance with their subunit composition (Mannervik et al., 2005). However, apart from functional and structural likenesses, there are certain features that facilitate division of the enzymes into classes, such that the alpha class has an alkaline isoelectric point, while the pI values for the mu class enzymes are close to neutral. The pi class enzymes tend to have acidic (<7.0) pI values and also are comprised of subunits with a low molecular weight relatively regarding the other isoenzymes (Mannervik et al., 1985). Whilst these characteristics can be beneficial for the GST isoenzymes for differentiation purpose, they cannot be completely correct, due to the fact that some class forms have unusual pI value (*e.g.*, monkey mu enzyme has a pI of 9.5 (Hoesch and Boyer, 1988), that is why all of the features concerning GST

isoenzymes should be considered before identifying them to a certain class (Boyer, 1989).

Table 2.2. Abundance of various GST classes in human tissue from (Awasthi et al., 1994)

Tissues	Alpha	Mu	Pi
Adrenal gland	++++	+	++
Aorta	?	+ (three)	++++
Bladder	+	+	++++
[a]Brain	+ (one)	++ (two)	++++
Breast	–	–	++++
Colon	+ (one)	++ (two)	++++
Cornea	++ (two)	?	++++
Diaphragm	+	++	++++
Duodenum	++	+	++++
Erythrocytes	–	–	++++
Fetal fibroblasts	++++	?	++
[c]Gastric mucosa	+++	+	++++
Heart	+++ (three)	++ (five)	++++
Ileum	+	+	++++
[d]Kidney	++++ (six)	++ (two)	++ (two)
Lens	++++	–	+++
[d]Liver	++++ (at least six)	+++ (two)	+
Lung	++ (three)	+ (two)	++++
Muscle	–	+++ (four)	+++ (two)
Pancreas	+++ (two)	++	+++
Placenta	–	–	++++
Platelets	– –	– –	++++ (two)
Prostate gland	++	+	++++
Retina	++++	–	+
Salivary gland	+	++	++++
Skin	+ (two)	–	++++
Small intestine	+++	+	++++
Spleen	+	++	++++
Stomach	+	+	++++
[f]Testis	++++	++	+
Thyroid gland	– –	– –	++++
Uterus	+ (two)	+ (two)	++++

2.5. Classification of Glutathione S-Transferases

Several attempts have been employed to create a general classification for the isoenzymes of GST. Enzymatic activity, immunological methods, chromatography and lately amino acid sequencing and molecular cloning were all different techniques used to characterize GST isoenzymes (Figure 2.1). However, different techniques undertaken by different laboratories to designate GST isoenzymes have led to more than one classification and nomenclature (Buetler and Eaton, 1992).

GSTs are classified according to the basis of protein sequence and structure. In cytosolic GSTs, among the members of the same class there is more than 40% amino acid sequence identity. Amid classes, there is less than 25% sequence identity of protein. Presently documented classes of cytosolic GSTs are pi (Reinemer et al., 1992), sigma (Ji et al., 1995), alpha (Sinning et al., 1993), beta (Rossjohn et al., 1998a), theta (Wilce et al., 1995; Rossjohn et al., 1998b), omega (Board et al., 2000), zeta (Polekhina et al., 2001), tau (Thom et al., 2002), epsilon (Sawicki et al., 2003) and nu (Schuller et al., 2005). The mitochondrial GSTs have an evolutionary association with the cytosolic GSTs which are designated as class kappa.

Cytosolic GST isozymes in classes alpha, zeta, theta, mu, pi, sigma and omega are discovered in humans. Dimer is formed in cytosolic GST and mitochondrial GST; heterodimers of cytosolic GSTs have been discovered, comprising of chains belonging within the same class. The MAPEG family is comprised of four subgroups (I–IV). Amid each subgroup, there is less than 20% sequence identity. Six MAPEG isozymes have been classified, they fit in the I, II, and IV subgroups which have been discovered in human (Jakobsson et al., 1999). Similar to the cytosolic and mitochondrial GSTs, some MAPEGs, such as MGST1, is involved in catalyzing the conjugation of GSH to a several electrophiles. Furthermore, other members are also involved in catalyzing reactions concerning leukotriene and prostaglandin biosynthesis (Hayes et al., 2005; Oakley, 2011).

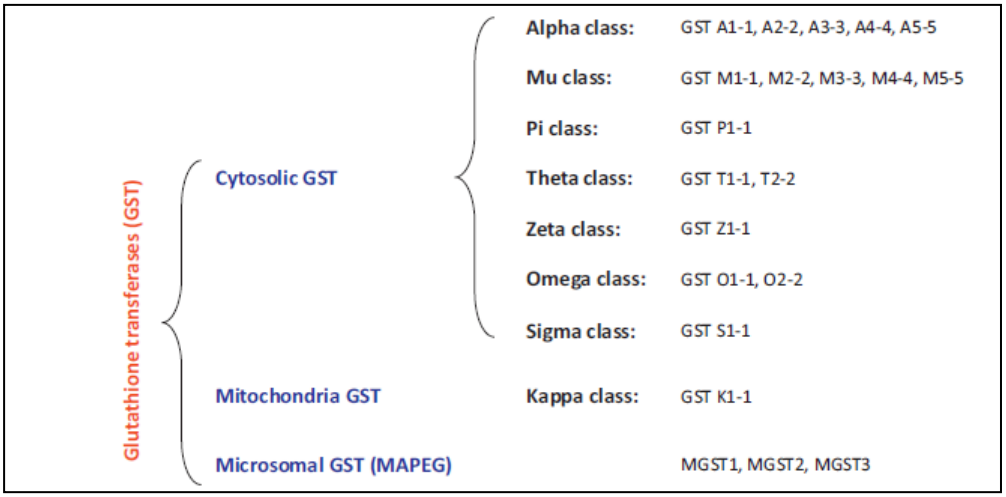

Figure 2.1. Classification of human GSTs established on amino acid sequence from (Wu and Dong, 2012)

2.6. Structure of Glutathione S-Transferases

Three-dimensional structure of human placental GSTP1-1 was identified in 1992 for the first time (Reinemer et al., 1992). Subsequently, the structures of human alpha (GST A1-l) and mu enzymes (GSTM2-2) have been determined (Sinning et al., 1993; Raghunathan et al., 1994). The structure of a blowfly enzyme of the theta class has also been solved as has a sigma class enzyme from squid (Wilce et al., 1995; Ji et al., 1995). The amino acids involved in binding of GSH in theta class enzymes differ from those of the alpha, mu, pi and sigma classes. Based on an analysis of the primary structures of the GSTs, it is likely that the theta class enzymes are evolutionarily older than those of other classes (Pemble and Taylor, 1992).

The overall tertiary structures of all of the soluble GSTs described to date are similar. GSTs are globular dimeric proteins with one catalytic site per subunit and range in molecular weight from about 23,000 to 29,000 Daltons per subunit. Each subunit is formed by about 200 to 240 amino acids. The subunit interactions of most GSTs are extensive along the dimer interface and contacts from opposite subunits are typically involved in the structure of the active site of the opposite subunit. GSTs are typically homodimers; however, heterodimers can form between some closely related members of

the same class and are known to occur among alpha and mu class enzymes (Whalen and Boyer, 1998). The polypeptide chain of each GST subunit forms two domains connected by short linker regions (Figure 2.2). The N-terminal domain is composed of amino acids arranged in a P-sheet and three α-helices and makes most of the contacts for binding of GSH (the G site) and some of the contacts for binding of the hydrophobic electrophile (the H site). The C-terminal domain includes the remaining amino acids in five or six helices and makes most of the contacts for the H site. The amino acid residues that contribute to the G site of alpha, mu, and pi class enzymes are well conserved. The hydroxyl group of a conserved tyrosine residue near the N-terminus forms a hydrogen bond to the sulfur atom of GSH in all of the crystal structures. The tyrosine has been shown by substitution mutagenesis experiments to be involved in the catalytic mechanism which promotes the activation of GSH through formation of a thiolate anion that attacks the electrophilic α substrate (Armstrong, 1997; Orozco et al., 1997).

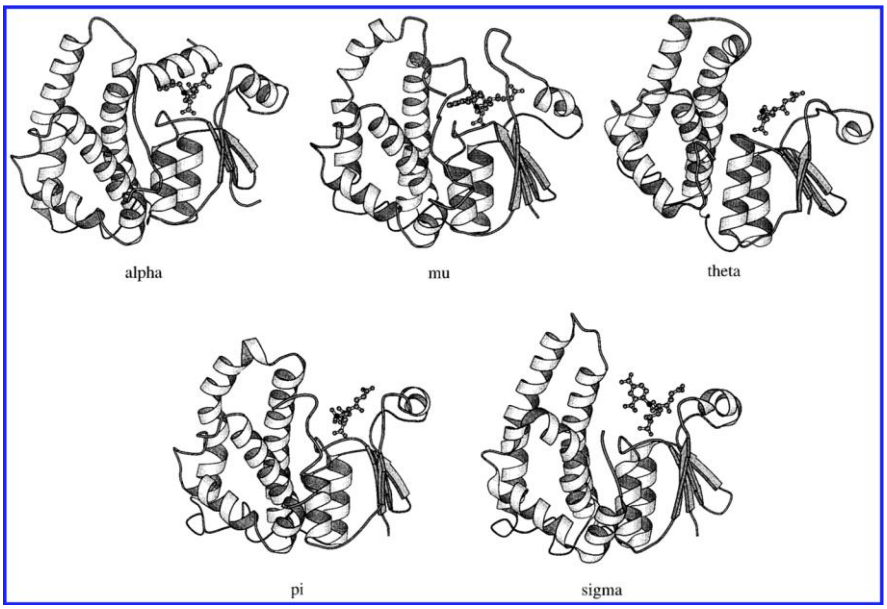

Figure 2.2. A three-dimensional structure of alpha, mu, theta, pi and sigma classes ribbon diagram. These views are from single subunits which are perpendicular toward the two-fold axis of each dimer (Armstrong, 1997).

2.6.1. Active sites of glutathione S-transferases

Each GST subunit has its own active site which is composed of a binding site for GSH and a contiguous substrate binding site for electrophiles, which are referred to as G-site and H-site that are shown in Figure 2.3 (Mannervik and Danielson, 1988). The G site is explicit toward GSH while H site is generally accessible to a broader range of electrophiles as substrates. Tyrosine residue at the N terminus was designated as the catalytic site for pi, mu, alpha and sigma classes (Prade et al., 1997), whilst a serine residue was identified as the catalytic site in theta class (Wilce et al., 1995). GSTs are very specific regarding GSH as thiol substrate. Molecularly recognizing GSH or its analogues binding to the G site includes a complex interface of particular polar connection among tripeptide and other protein moieties of domain I subunit and one (pi and mu classes) or two (alpha class) amino acid of domain II in the adjacent subunit. In GSH many functional groups are isolated and hidden by protein apart from the Gly amide nitrogen (peptide site P7; classes pi and alpha) and its γGlu α-carbonyl oxygen (peptide site P3) in alpha and mu classes. Conserved within a gene class is the amino acid residues involved in the binding of GSH to the G site which also termed as the G site ligands, whilst several G-site ligands are preserved or substituted between classes. The H site structure slightly changes among different gene classes in terms of molecular details. However, the gathering of nonpolar amino acid side chains offers the very hydrophobic protein surface which in the nonexistence of xenobiotic substrate is available to bulk solvent. Fundamental elements that make up the structural architecture of the H site is ascending from domain I and domain II of the identical subunit and its involved in the active site loop which interfaces α1 and β1 (Dirr et al., 1994). Simultaneously, residues from the C terminal domain greatly contributed to the formation of H site (Board and Menon, 2013). An outcome of the variable sequence among gene classes results in the presence of distinctive H site topologies, that would elucidate the different xenobiotic-substrate specificities indicated by the different gene classes. Since recognizing substrates within the H site is facilitated mainly through hydrophobic interaction, binding to this site seems to be less constrained, consequently giving it the flexibility to accept a wide range of structurally distinct hydrophobic

substrates (Dirr et al., 1994). However, activity and structural studies and the exploration of the importance of hydrophobic interaction within the H site explain that the cytosolic GSTs show higher binding affinity as a result of increased hydrophobicity of 4-hydroxyalkenal substrates (Danielson et al., 1987) and of the alkyl group in S-alkylated glutathione competitive inhibitors (Askelof et al., 1975).

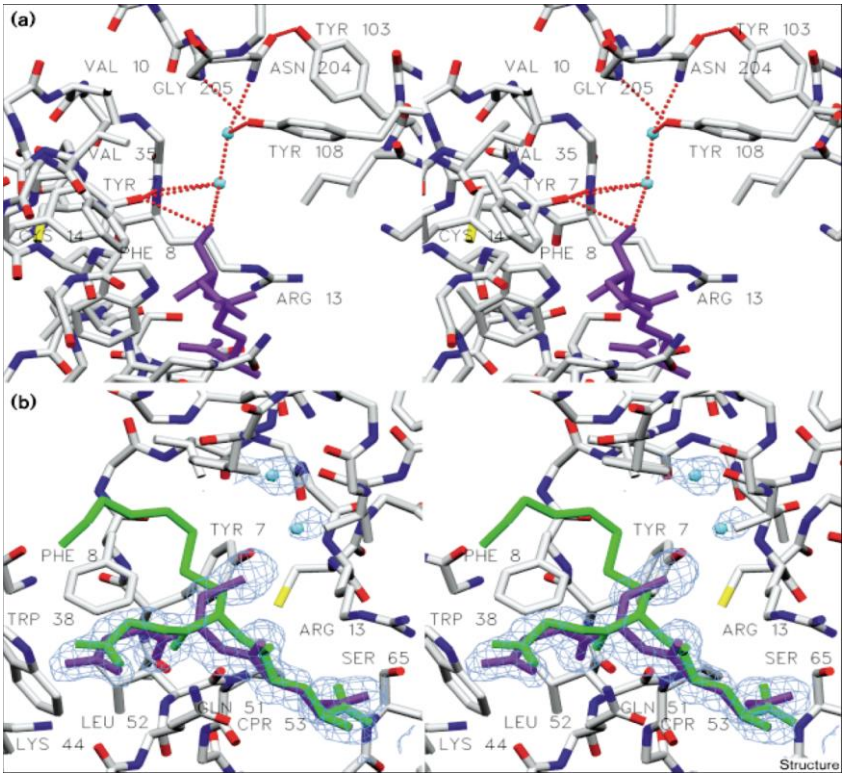

Figure 2.3. Representation of pi class human placental GST in conjugation with glutathione (purple). (a) α2 helix into the H-site view, two water molecules appearing in blue spheres and the hydrogen bonds are the red dotted lines. (b) View of the other monomer toward the whole active site (Prade et al., 1997).

2.7. Reaction Mechanism of Glutathione S-Tranferase

Subunits that originate from the same class of isoenzymes form homodimers or heterodimers of GSTs, where each monomer has its own binding site that function distinctively toward particular substrates (Figure 2.4) (Dourado et al., 2008). The subunit

that binds and activates GSH is termed as the G site, whereas the adjacent subunits that binds xenobiotics are termed the H site (Wu and Dong, 2012).

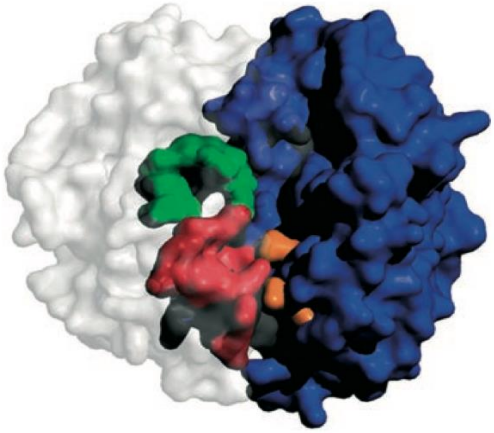

Figure 2.4. View of GSTA1-1 monomers (white and blue). The G site is red and the H site is green which are identified and the residues which is shown in orange belong to the G site (Dourado et al., 2008).

2.7.1. GSH binding site

Since the GSH binding site (G site) is situated in a cleft amid the N-terminal domain of one subunit and the C-terminal domain of the next, its completion occurs after the dimerization process and the binding of GSH to GST enzymes is highly specific (Dourado et al., 2008). The activation of the GSH into anionic thiolate form is the first step, which turns GSH into a strong nucleophile to function toward electrophilic substrate. Once the GSH and G site complex forms, pK_a value of the thiol group decreases from 9.2 to 6.2-6.6 (Caccuri et al., 1999). This transition stimulates the deprotonation of GSH and the composition of the residue that accepts proton from thiol group, thus acting like a base (Dourado et al., 2008). At the termination of the β-sheet with the γ-glutamyl moiety positioning for the protein core is the GSH which is bound in a stretched-out conformation, and it is fixated chiefly by several hydrogen bonds within the β3β4α3 motif. However, the detailed hydrogen bonding is distinctive to each GST subfamily, there are some notable common features. Firstly, the GSH foregoing

antiparallelly the β3 strand loop, whereas a bond of hydrogen pair is formed amid the main GSH residue and central protein. Secondly, a turn is formed through β4 and α3 of the protein which are hydrogen bonded to the GSH through the γ-glutamyl residue by two other residues of a glutamine or glutamate and serine or threonine. Thirdly, in the termination of α1 helix where the GSH sulfur atom is situated at the N-terminal which regularly binds the catalytic residue through hydrogen bonding within the protein. This hydrogen bonding is an important aspect of how stabilized the GSH (GS-) is in GST catalysis (Wu and Dong, 2012). Meanwhile this residue is composed of a tyrosine in the alpha, pi, sigma, and mu classes, a cysteine in the omega class and a serine in zeta and theta classes (Armstrong, 1997; Rossjohn et al., 1998b).

2.7.2. Electrophilic substrate binding site

Electrophilic substrate binding site (H site) is predominantly found in the C-terminal domain and depending on the type of GST isoenzymes, its structure varies. The H site permits a wide variety of electrophilic compounds and GST reaction is explained by Equation 2.1.

$$GSH + R - X \text{ (electrophilic substrate)} \rightarrow GSR + H + X^{-\cdot} \qquad \text{(Equation 2.1)}$$

To form a more soluble GSR compound, the electrophilic substrate reacts with GSH which in turn forms a less toxic compound and facilitates its elimination. The process of releasing the GSR is regulated by the C-terminal region (Nieslanik et al., 1999). The most important issue of understanding the catalysis concerning GST and electrophiles is how the enzyme structure influences the substrate selectivity. Stereoselectivity and regioselectivity of GST isoenzymes in response to numerous different reactions or the structurally variable substrates are best elucidated through how these isoenzymes fulfill the transition state for a certain reaction. To define the xenobiotic binding site, it is important to understand the three-dimensional structure of the product complex. The structure of numerous enzymes in combination with the products have led to the explanation of three distinctive binding mode for the peptide conjugate (GSR) which is

referred to (in) and (out), and (far-out). In both (in) and (out) modes the peptidyl segment of the molecule is engaged in the GSH binding site, although the location of the R group is different. The R group in the (in) mode is engaged in the occupation of domain I cavity residues that provide the loop which attach the primary β strand with α-1 helix and construct the floor of that cavity and the formation of the wall requires the contribution of one side of α-4 helix and the C-terminal tail which is situated in domain II. Essentially this cavity is designated as the catalytic xenobiotic substrate site. However, this xenobiotic substrate site is structurally distinctive, in respect to different GST classes (Armstrong, 1997). In alpha class, α9-helix is involved in the formation of a ceiling in the electrophilic binding site. However, in the sigma class due to having a shorter C-terminal, the active site is more accessible. A feature of the (out) binding mode is that the R group extended out of the active site in between the two dimeric subunits where the solvent channel located which is seen in *S*-(2,4-dinitropheyl) glutathione (Ji et al., 1993; Ji et al., 1995). In (far-out) binding mode the GSH binding site is not occupied by the peptidyl segment nor does the xenobiotic binding site occupied by the R group which is a unique feature to sigma class from squid, whereas the S-(3-iodobenzyl) glutathione is firmly bound to both of the active sites whilst the third molecule binds to where the two subunits meet (Ji et al., 1996). Although domain II is termed as xenobiotic binding domain given that it has very slight involvement with GSH binding, nonetheless provides numerous elements that interact the electrophilic substrate (Armstrong, 1997).

2.8. Role of Glutathione S-Transferases on Endogenous Metabolism

Apart from their enzymatic functions, GSTs are ligandins, and they are capable of binding numerous hydrophobic compounds without showing enzymatic activity. As GSTs are expressed in different concentrations regarding the tissue type and the existence of various classes and substrate specificity regarding those classes have crucial physiological relevance (Prade et al., 1997). GST activities are upregulated in different types of tumors, which is believed to be involved in chemotherapeutic resistance (Coles et al., 1990; Hayes and Pulford, 1995). From an evolutionary standpoint, it is possible

that glutathione emerged as a crucial biomolecule when oxygen level greatly increased in the atmosphere. Accordingly, it is expected that glutathione dependent enzymes, particularly transferases have evolved to counter attack the toxic products of oxygen metabolism in an aerobic organism (Mannervik, 1987). Concurrently, substances that contain aromatic groups or carbon-carbon double bond and polyunsaturated fatty acids are among those biomolecules that are potentially capable of forming reactive oxidation products (Mannervik et al., 1988).

2.8.1. Degradation of aromatic amino acids

The amino acid phenylalanine in mammals, is degraded to fumaric acid and acetoacetate which in turn results in five intermediates termed as tyrosine, maleylacetoacetate, 4-hydroxyphenylpyruvate, homogentisate and fumarylacetoacetate. The maleylacetoacetate isomerase have been designated as cytosolic GST from zeta class, hence catalyzing the last step of phenylalanine and tyrosine catabolism (Fernandez-Canon and Penalva, 1998).

2.8.2. Synthesis of steroid hormones

The synthesis of progesterone and testosterone is initiated from cholesterol metabolite 3β-hydroxy-5-pregnene-20-one. The formation of Δ^5-androstene-3,17-dione, which is an intermediate in the synthesis of testosterone, occurs when the compound's side-chain undergoes oxidation and cleavage of the 3β-hydroxyl group. Simultaneously, the 3β-hydroxyl group oxidation can result in the formation of Δ^5-pregnene-3,20-dione which is an intermediate in the progesterone synthesis. The cytosolic GST converts these 3-keto-Δ^5-steroids into 3-keto-Δ^4-steroid isomers (Johansson and Mannervik, 2001). The generation of 3-keto-Δ^5-steroids through the action of 3β-hydroxysteroid dehydrogenase which also appears to have keto-steroid isomerase action, hence accountable for the steps in isomerization. However, it appears that in steroidogenic tissue alpha class GSTs are potentially 230-fold more efficient in catalysis and therefore

isomerizing 3-keto-steroids when compared to 3β-hydroxysteroid dehydrogenase (Johansson and Mannervik, 2001).

2.8.3. Synthesis of eicosanoids

It is clear now in the biosynthesis of crucial arachidonic acid metabolites glutathione transferases are involved through exhibiting remarkable specificity toward these reactions. Although previously it was suggested that numerous GSTs were involved in the isomerization of PGH_2 to a mixture of PGD_2 and PGE_2, or $PGF_{2\alpha}$, in the reduced form. The cytosolic GST provides a great paradigm for identifying mammalian GSH-dependent prostaglandin D_2 synthase (Jowsey et al., 2001). This is an interesting remark since the enzyme is implicated in PGD_2 formation, which performs different biological functions and simultaneously, cytosolic GST present in human brain appears to have PGE_2 synthase activity (Beuckmann et al., 2000). Additionally, some members of MAPEG family are chiefly involved in the formation of PGE_2 (Jakobsson et al., 1999), while enzyme activated by membrane-bound GSH exhibits $PGF_{2\alpha}$ synthase activity (Nakashima et al., 2003). Other biomolecules that contain cyclopentenone ring such as isoprostanes and prostaglandins in a glutathione-conjugation reaction represents GST substrates (Bogaards et al., 1997). These alterations assist the removal of eicosanoids from cells via multidrug resistance proteins (MRP1 and MRP3) (Paumi et al., 2003).

Another product of arachidonic acid is leukotrienes (LTs) which is another group of eicosanoids. In the synthesis of LTs, MAPEGs are chiefly involved since the activation of 5-lipoxygenase is performed exceptionally through one of their members, and some others are involved in the catalysis of LTC_4 formation (Hayes et al., 2005).

2.8.4. Modulation of signaling pathways

Metabolism of endogenous lipid mediators by GST has biological significance since it effects diverse signaling pathways. While the effect of prostaglandins (PGD_2, PGE_2, and $PGF_{2\alpha}$) are mediated by specific G protein-coupled receptors, the cyclopentenone

prostaglandins apply its effects using a different route. The effect of transferases on the synthesis and removal of eicosanoids, make GST a main regulator. The downstream metabolite the prostaglandin (PGD_2), termed as 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) possess an important property which is capable of serving as a ligand that activates peroxisome proliferator-activated receptor γ (PPARγ) (Figure 2.5). GST overexpression is a factor that reduces the transactivation of 15d-PGJ_2 gene expression which is mediated by PPARγ through GSH-prostanoid bound (Paumi et al., 2004).

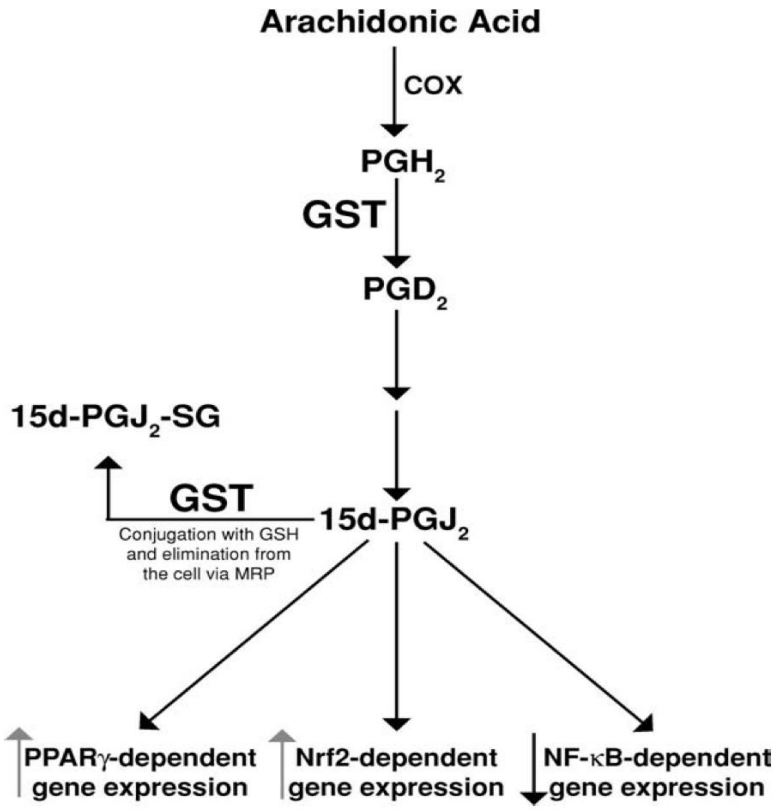

Figure 2.5. Synthesis of 15d-PGJ_2 (Hayes et al., 2005).

15d-PGJ_2 is capable of inducing the nuclear factor 2 (Nrf2)-mediated gene expression by antioxidant responsive element (ARE) (Jowsey et al., 2003; Itoh et al., 2004). This happens since 15d-PGJ_2 is capable of modifying Kelch- like ECH-associated protein 1 (Keap 1) cysteine residues, therefore developing the potential in Keap1 to aim for proteasomal degradation in Nrf2 (Itoh et al., 2004; Wakabayashi et al.,

2004). However, the bond formation between 15d-PGJ_2 and GSH eliminates the ability to alter Keap1. In a comparable mechanism which exhibits the capability of 15d-PGJ_2 to deactivate the β subunit of the inhibitor of κB kinase (IKKβ) and prevent nuclear factor κB (NF-κB)-dependent gene expression (Rossi et al., 2000).

A product of lipid peroxidation, 4-hydroxynonenal (4-HNE) is assumed to function as a signaling molecule intracellularly (Uchida, 2003; Awasthi et al., 2003), consequently when bound to GSH will affect numerous pathways. Similarly to 15d-PGJ_2, this 2-alkenal is an α,β-unsaturated carbonyl that can induce gene expression by ARE (Tjalkens et al., 1999). Commonly with the 15d-PGJ_2 it is possible that Nrf2 stimulation is mediated through ARE-driven gene through 4-HNE (Ishii et al., 2004; Levonen et al., 2004). Through the prevention of IκB phosphorylation, aldehyde will prevent the activation of NF-κB. It has been stated that it is involved in the activation of epithelial growth factor receptor, regulating numerous cell-surface receptors and to activate platelet-derived growth factor-β receptor and also modulating growth factor receptor β1 transformation. 4-HNE induces numerous constituents of signal transduction pathways (*e.g.* p38, JNK, protein kinase C), simultaneously leading to an increase in p53 protein and promote apoptosis. It is believed that the 4-HNE and GSH conjugation will have effect on numerous transduction pathways and regulating function of transcription factors such as Nrf2, NF-κB and c-Jun (Awasthi et al., 2003).

2.9. Glutathione S-Transferase and Multi Drug Resistance

Numerous studies state the implication of phase II detoxification enzyme systems particularly GSTs in the multifactorial phenomenon termed as multi drug resistance (MDR). GST overexpression may be involved in minimizing the efficiency of several different kinds of anticancer drugs. Consequently, GST superfamily is now the extensively targeted by pharmaceutical organizations in the attempt to develop more competent chemotherapeutic agents for treating cancer (Sau et al., 2010).

2.9.1. Mechanism of multi drug resistance

In cancer therapy it is greatly significant to generate a novel drug that is capable of suppressing or escaping multi drug resistance (MDR), either alone or with the help of other drugs. There are multiple factors for the occurrence of chemotherapeutic resistance and numerous mechanisms have been proposed for its existence. One of the mechanisms is when cancer cell procures resistance through the elevated level of enzymes that are involved in the detoxification and evading the cytotoxic effect of anticancer drugs. GST enzymes are significantly involved in the detoxification process and at present, numerous alkylating agents that are used in cancer treatments are identified as GST substrates (Figure 2.6) (Dirven et al., 1996). Accordingly, it is clear that an overly expressed level of GSTs and GSH in cancer is associated with MDR development (Townsend and Tew, 2003).

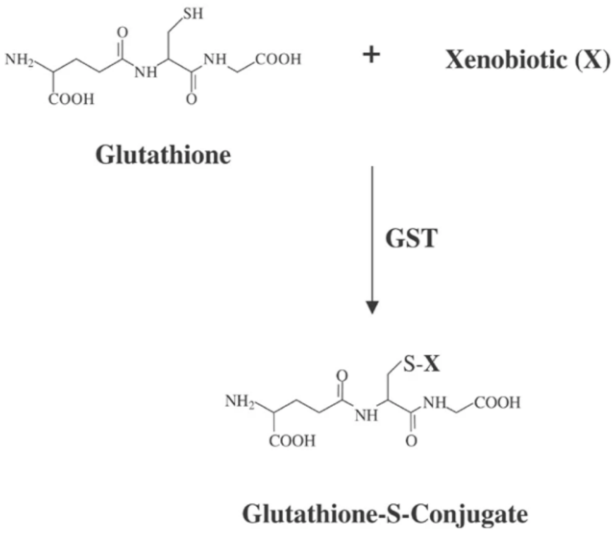

Figure 2.6. An illustration of detoxifying mechanism of GST enzymes and the formation of glutathione-S-conjugate (Townsend and Tew, 2003).

2.9.2. Role of glutathione S-transferase in drug detoxification

Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) activates the transcription that causes GST level to increase. Normally, Nrf2 is sequestered inside Keap1 in the cytoplasm and during cell stimulation, Nrf2 and Keap1 dissociate from each other. Nrf2

travels to the nucleus where it stimulates a set of cytoprotective genes (Hayes et al., 2005; Pool-Zobel et al., 2005). Although, recently it is suggested that due to the high affinity binding of Keap1 and Nrf2 is unfitting. This data suggests that it is not the electrophilic or the oxidative stress that alter their affinity. However, it influences the ubiquitylation between Keap1 mediated Nrf2 which leads to the accumulation of Nrf2 in the nucleus (Eggler et al., 2005; Kobayashi and Yamamoto, 2006). It's existence in the nucleus causes the formation of a heterodimer between Nrf2 and Maf protein which stimulates the transcription of phase II enzymes while collaborating with ARE which is situated in the gene promoter (Hayes et al., 2005).

As GSTs are catalyzing the nucleophilic attack of the sulfur in a GSH up on the electrophiles of various noxious biomolecules, it is also capable of detoxifying drugs via the drug's metabolite rather than the molecules that makes up the drug, consequently decreasing its reactivity and creating a water-soluble compound that is more easily eliminated by the body. Various anticancer drugs are among those drugs that GST catalysis plays a crucial role in their detoxification. GSTP1-1 is elevated to a greater extend compared to the other GST isoenzymes in human cancer (Tew et al., 1996). Its highly elevated level is in relation with the various chemotherapeutic responses and cancer vulnerability to chlorambucil or cisplatin, which are the examples of anticancer drugs (Black et al., 1990; Bai et al., 1996). Inhibiting the expression of pi class of GST via antisense cDNA, appears to increase tumor sensitivity towards melphalan, cisplatin, adriamycin and etoposide (Ban et al., 1996). Elevated levels of mu, alpha, theta and microsomal GSTs may also protect cancerous cells. Although, elevated level of mu class GST is implicated in chlorambucil resistance in ovarian carcinoma cell line (Horton et al., 1999). Alpha class GST overexpression is involved in the resistance toward doxorubicin (Sargent et al., 1999; Sharma et al., 2006) and alkylating agents (Lewis et al., 1988). Furthermore, patients having breast cancer, with theta (GSTT1)-null and GST mu (GSTM1) genotypes present reduced death rate compared those patients with alleles (Ambrosone et al., 2001). Microsomal GST has been stated to guard cells from melphalan, chlorambucil, and cisplatin (Johansson et al., 2007).

2.9.3. Glutathione S-transferase and its noncatalytic function

Apart from its catalytic role in detoxifying anticancer drugs, extensive studies state that GSTs, specifically GSTP1-1 and GSTM1-1, regulate apoptosis via the c-Jun-N-terminal kinase (JNK) signaling pathway (Mcllwain et al., 2006). JNK is induced through several different stimuli resulting in different and ambiguous cellular responses. JNK is involved in promoting cell survival and cell proliferation, on the contrary it may also lead to apoptosis and also needed for the cytotoxic effects of various chemotherapeutic agents (Liu and Lin, 2005).

It is stated that GSTP1-1 overexpression is associated with the inhibition of JNK activity and protection cancerous cell from apoptosis (Adler et al., 1999; Yin et al., 2000), which clarifies the reason that even though GST mediated MDR is seen within anticancer drugs which are not GST substrates but needs the activation of mitogen activated protein kinase (MAPK) pathway to stimulate apoptosis (Townsend and Tew, 2003; Yu et al., 2009). Concurrently, GSTP1-1 is also associated with tumor necrosis factor receptor associated factor 2 (TRAF2) of human cervical carcinoma HeLA cells. Particularly, TRAF2 is needed to activate apoptosis signal-regulating kinase 1(ASK1) which is an apoptosis signal regulating kinase (Nishitoh et al., 1998). Consequently, activating MKK4/7-JNK and MKK3/4/6-p38 pathways, which both are profoundly known to mediate cellular response during environmental stress (Ichijo et al., 1997). These findings indicate that GSTP1-1 is significant in modulating extrinsic and intrinsic signaling pathways and elucidate how the increase in GSTP1-1 is involved in the resistance against apoptosis originating from various stimuli. Additionally, GSTM1-1 interaction with N-terminal segment of ASK1 will inhibit the activity. It appears that thermal shock will dissociate GSTM1-1 and ASK1 complex, consequently triggering ASK1 and phosphorylating p38 and JNK in the process (Dorion et al., 2002).

2.9.4. Glutathione S-transferase and efflux pumps

Regularly in a number of different types of cancer, overexpression of efflux pumps is observed that facilitates the extrusion of a broad range chemotherapeutic agents used to combat cancer. Numerous studies state the fact that synergistically efflux pumps and

phase II enzymes act toward developing MDR (O'Brien et al., 2000). Furthermore, coordination of expressing GSTs, efflux transporter protein and γ-glutamylcysteine synthetase (γ-GCS) which is the enzyme regulating glutathione synthesis, will deliver a competent protective phenotype and it is often seen in drug resistant cells. Elevated level and an increase in the activity of these proteins will result in less drug buildup in the cell and facilitate drug resistance in tumor cell. ATP binding cassette (ABC) transporters MRPs, P-gp and ABCG2 which is also recognized as mitoxantrone-resistance protein are among the common efflux transporters (Meijerman et al., 2008). MRPs are implicated in transporting GSH, byproduct of phase II detoxification enzymes such as glucuronate and the organic anion conjugated sulfate, UDP-glucuronosyltransferases (UGT) and sulfotransferases. MRPs are also involved in exporting GSH and transporting with GSH is needed for MRP extrusion of anticancer drugs (Morrow et al., 2006).

Several examples are given in literature indicating the interaction between human GST isoenzymes and MRPs. The synergy between GSTA1-1 and these pumps have been seen in resistance development against chlorambucil, in which detoxification by GSTA1-1 needs the removal of the glutathione conjugate by one of the MRP transporter (MRP1or MRP2) (Paumi et al., 2001; Smitherman et al., 2004). GSTM1-1 synergistically interacts with MRP1 to shield tumor cell from the noxious effects of vincristine (Depeille et al., 2004). Elevated levels of GSTP1-1 and MRP1 together will develop resistance toward vincristine, chlorambucil, etoposide and ethacrynic acid (O'Brien et al., 2000; Depeille et al., 2005).

2.9.5. Inhibitors of glutathione S-transferase

As a strategy, it is of great significance to find molecules that are able to inhibit GSTs and export pumps in an attempt to overcome resistance against anticancer drugs in cancerous cell. The first ever inhibitor to be used against GST was ethacrynic acid, which was previously utilized as a diuretic. Whilst ethacrynic acid is known to be one of the substrates of several GST isoenzymes, it has remarkable inhibitory effect on GST enzymes. However, there is considerable side effects with its utilization, for that reason it not utilized in clinical practice (Tew et al., 1997).

GSH-peptidomimetics which benefit from the high affinity of GSH for GSTs, are certainly more specific for GSTs (Adang et al., 1990).

2.10. Auxins

The plant hormone auxin is crucial for plant growth since it regulates numerous developmental processes. Although variety of synthetic and natural products exhibit auxin-like function, indoleacetic acid (IAA) is profoundly known as the most crucial auxin in a majority of plants. In animals IAA can be synthesized via the degradation of tryptophan, either through the tryptamine (TAM) pathway or the indole-3-pyruvic acid pathway. Higher plants are capable of storing IAA in the form of IAA complex or IBA. IAA can be obtained through β-oxidation of IBA, which is the second endogenous auxin. IAA can also be obtained from hydrolyzing IAA conjugates, where IAA is linked to peptides, amino acids or sugar. Hence, IAA inactivation requires conjugation or direct oxidation (Woodward and Bartel, 2005). As a key hormone in plants, auxins orchestrate diverse processes, such as tropical responsiveness to light, root and shoot architecture in relation to gravity, vascular development, organ patterning and general growth (Davies, 1995). While auxins are needed for plant growth, it is seemingly vital for human nourishment. In experimental plant research auxin is considered as one of the oldest field of plants experimentation. Early auxin experimentation was performed by Charles Darwin to perceive the effects of hypothetical substances regulating plant shoot elongation to facilitate tropic growth toward light (Darwin, 1880).

2.11. Plant Glutathione S-Transferases and Auxins

It has been stated that in plants GSTs are crucially involved in cellular detoxification of noxious compounds and protection against oxidative stress (Rushmore and Pickett, 1993). GSTs implication have been stated in detoxification of herbicides (Timmerman, 1989), as well as defending tissue against infectious microorganisms (Levine et al., 1994). In *Arabidopsis,* investigation of four plant GST subclasses indicate that gene expression is influenced by dehydration (Kiyosue et al., 1993), ethylene and pathogen (Zhou and Goldshrough 1993). Additionally, their gene product potentially

bind auxin; IAA was among those substances that was capable of inhibiting plant GST5 activity in a competitive inhibition mode when GSH was the variable. GST5 activity was inhibited by 55% with an IC_{50} value of 1.3 mM with 2 mM IAA (Watahiki et al., 1995).

3. MATERIALS AND METHODS

3.1. Chemicals

Glutathione S-Transferase P1-1 from human placenta (*hp*GSTP1-1), 1-Chloro-2,4-dinitrobenzene (CDNB), L-Glutathione reduced (GSH), Sodium phosphate monobasic, Sodium phosphate dibasic dodecahydrate, Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), Sodium azide, Bovine serum albumin (BSA), 3-Indoleacetic acid (IAA), 3-Indolepropionic acid (IPA), Indole-3-butyric acid potassium salt (IBA) were all purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA.

3.2. Methods

3.2.1. Preparation of the solutions

- Buffers: Sodium phosphate (200 mM) buffer was prepared by using both sodium phosphate monobasic and sodium phosphate dibasic. pH was adjusted to 6.5. EDTA was added with a concentration of 2 mM and 0.02% sodium azide was used to prevent bacterial growth. This buffer solution (200 mM sodium phosphate pH 6.5 + 2 mM EDTA) was diluted 1:1 ratio to prepare enzyme preparation buffer (100 mM sodium phosphate pH 6.5 + 1 mM EDTA). Finally, 0.05% BSA was added to obtain enzyme dilution buffer.

- Substrates: GSH and CDNB were dissolved in filtered distilled water and ethanol, respectively to prepare the required concentrations of each.

- Enzyme: The *hp*GSTP1-1 enzyme was prepared by dissolving 2 mg in 1 mL of 100 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA. This stock enzyme was aliquot and kept in -20^{o}C. In all the experiments, the enzyme (2 mg/mL) was diluted by 20 or 35 times with 100 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA and 0.05% BSA before use for IC_{50} determination and inhibitory kinetic studies, respectively.

-Inhibitors: 3-indoleacetic acid and 3-indolepropionic acid were dissolved in ethanol, while indole-3-butyric acid was dissolved in distilled water.

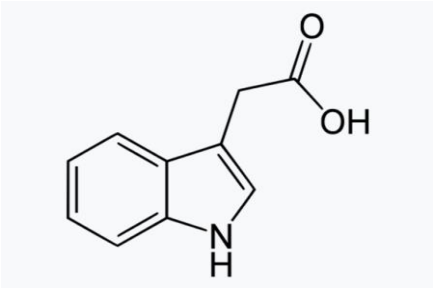

Figure 3.1. Indoleacetic acid

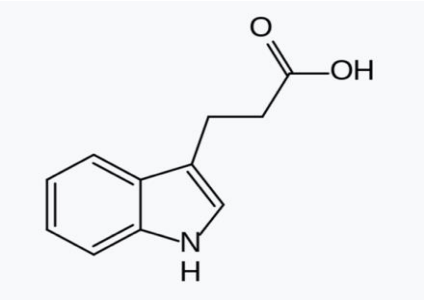

Figure 3.2. Indolepropionic acid

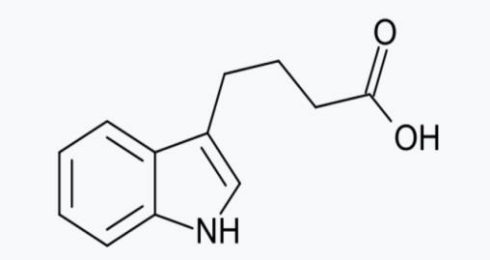

Figure 3.3. Indolebutyric acid

3.2.2. Determination of the *hp*GSTP1-1 enzyme activity

*hp*GSTP1-1 enzyme catalyzes the conjugation of the natural substrate L-glutathione reduced (GSH) to the synthetic substrate (CDNB) through the thiol group that GSH possess. The increased rate of absorption by GS-DNB conjugate is directly proportional with the *hp*GST1-1 activity (Habig and Jakoby, 1981).

$$\text{GSH + CDNB} \xrightarrow{\textit{GST}} \text{GS-DNB Conjugate + HCl}$$

*hp*GSTP1-1 activity was determined by using Perkin Elmer Lambda 25 UV/VIS Spectrophotometer. Increase in absorbance at 340 nm was monitored for 20 seconds to determine the activity of *hp*GSTP1-1 (Habig and Jakoby, 1981). Simultaneously, a non-enzymatic reaction was run, containing all the constituents of the reaction mixture excluding only the *hp*GSTP1-1 enzyme. Values obtained from non-enzymatic reactions were subtracted from the enzymatic reactions. All of the measurements were performed at 37ºC and in triplicates. The average activity (U/mL) values were converted to specific

activity (U/mg protein) and were used to draw Michaelis-Menten, Lineweaver-Burk and other plots (Segel, 1975). One unit of the *hp*GSTP1-1 activity interpret the amount of enzyme that catalyzes the formation of one micromole of product per minute at pH 6.5 and at 37ºC. The formula used for the calculation of the specific activity is shown below:

Specific Activity (U mg^{-1} protein):

$$\frac{\Delta Abs_{340}/min \times Vt}{9.6 \times Vs \times [protein]}$$

Where,

ΔAbs_{340}/min = Absorbance change per minute at 340 nm

Vt = Total volume of the reaction mixture

Vs = Sample volume used to measure enzyme activity

9.6 = Extinction coefficient of GS-DNB conjugate

[protein] = Protein concentration

3.2.3. Effect of 3-indoleacetic acid, 3-indolepropionic acid and indole-3-butyric acid on *hp*GSTP1-1 and IC_{50} determination

The inhibition of *hp*GSTP1-1 was measured by adding increasing concentrations of 3-indoleacetic acid, 3-indolepropionic acid, and indole-3-butyric acid, ranging from (0.3125 – 10 mM). Reaction mixture (800 µL) comprised of 100 mM sodium phosphate pH 6.5 containing 1 mM EDTA, 1 mM CDNB, 1 mM GSH, and the appropriate amount of enzyme (Habig and Jakoby, 1981). In all of the experiments enzyme dilution factor was 1:20 and the enzyme was diluted with 100 mM sodium phosphate buffer pH 6.5, containing 1 mM EDTA and 0.05% BSA. Data obtained in triplicates for each inhibitor concentration. Logarithm percent remaining activity were used to plot against concentrations of inhibitors for the determination of IC_{50} values (Segel, 1975).

3.2.4. Inhibitory kinetic experiments with 3-indoleacetic acid, 3-indolepropionic acid and indole-3-butyric acid

In both absence and presence of 3-indoleacetic acid, 3-indolepropionic acid and indole-3-butyric acid, inhibitory kinetic studies were conducted. Suitable amounts of inhibitors dissolved in their specific solvents, then added to reaction mixture and incubated for temperature equilibration. Final concentration of inhibitors were 0 (control), 0.25, 0.5, 1 and 2 mM. The reaction mixture comprised of 100 mM sodium phosphate buffer pH 6.5 with 1 mM EDTA, 3-indoleacetic acid, 3-indolepropionic acid and indole-3-butyric acid concentrations (as described above), 1 mM $[CDNB]_f$ –$[GSH]_v$, or 1 mM $[GSH]_f$ –$[CDNB]_v$ and the appropriate amount of enzyme The variable concentrations of GSH and CDNB were 0.1, 0.2, 0.4, 0.8 and 1.6 mM in the reaction mixture in each case.). In all of the experiments enzyme dilution factor was 1:35 and the enzyme was diluted with 100 mM sodium phosphate buffer pH 6.5, containing 1 mM EDTA and 0.05% BSA. The increase in absorption due to the formation of the GS-DNB conjugate was followed at 340 nm for 20 seconds. All of the measurements were performed at 37ºC and in triplicates (Habig and Jakoby, 1981). The data obtained with and without inhibitors were used to calculate specific activity (U/mg protein) and draw Michaelis-Menten, Lineweaver-Burk and other plots (Segel, 1975).

3.2.5. Statistical analysis

Michaelis-Menten, Lineweaver-Burk and secondary graphs were plotted; through using intercept points on these graphs inhibition types and kinetic parameters were estimated. Statistical analysis was also performed to confirm the inhibition type and kinetic parameters. For this purpose, non-linear regression module of IBM SPSS Statistics (version 20) was used (SPSS Inc. Chicago, IL, USA).

4. RESULTS

4.1. Inhibitory Kinetic Interaction of *hp*GSTP1-1 with Indoleacetic Acid

The inhibitory effect of IAA on *hp*GSTP1-1 was investigated through the addition of different concentrations of IAA (0.3125, 0.625, 1.25, 2.5, 5 and 10 mM) into the reaction mixture, having 800 µL as the final volume. The reaction mixture was comprised of 100 mM sodium phosphate buffer pH of 6.5 with 1 mM EDTA, 1 mM GSH, 1 mM CDNB, distilled water and appropriate amount of *hp*GSTP1-1 enzyme. Triplicate assays were performed to obtain the data for each IAA concentration. The IC_{50} value of IAA confirmed as 9.7 mM once log % remaining activity versus concentrations of IAA was plotted (Figure 4.1).

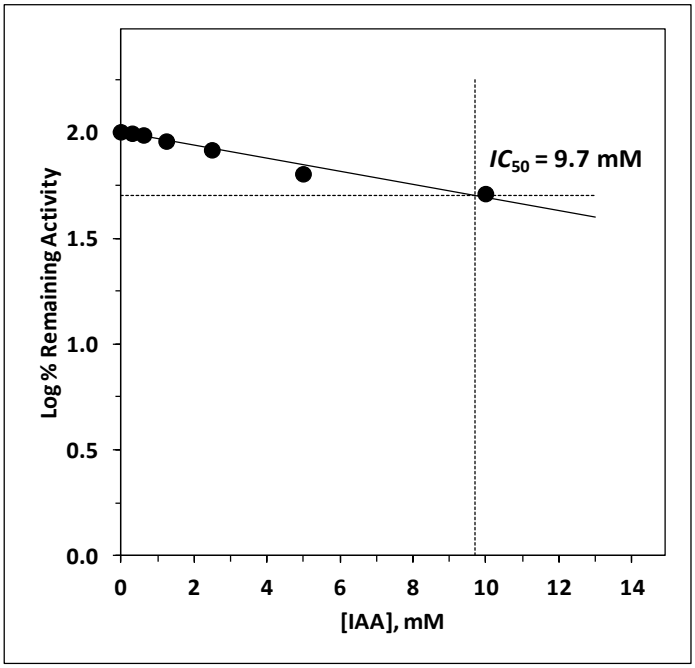

Figure 4.1. Dose dependent inhibition of *hp*GSTP1-1 with IAA.

Four particular IAA concentrations (0.25, 0.5, 1 and 2 mM) were utilized in the inhibitory kinetic studies to elucidate the inhibition type, V_m, K_m and K_i values, in the presence or absence of IAA. In this experiment, the reaction mixture was composed of 100 mM sodium phosphate buffer pH 6.5 with 1 mM EDTA, the four IAA concentrations, 1 mM $[CDNB]_f$-$[GSH]_v$ or $[GSH]_f$-$[CDNB]_v$, distilled water and appropriate amount of enzyme. In each case the reaction mixture had variable concentrations (0.1, 0.2, 0.4, 0.8 and 1.6 mM) of CDNB or GSH. At 340 nm the absorbance was followed for 20 seconds which showed an increase due to GS-DNB conjugate formation. Different kinetic parameters and plots including Michaelis- Menten (Figure 4.2 and 4.5), Lineweaver-Burk (Figure 4.3 and 4.6) and secondary plots (Figure 4.4 and 4.7) were drawn and kinetic parameters were also calculated by using SPSS version 20. The V_m for $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 332 ± 17 and 182 ± 5 µmol/min-mg protein, respectively. K_m for $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 2.32 ± 0.17 and 1.14 ± 0.06 mM, respectively. K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 4.00 ± 0.62 and 3.30 ± 0.24 mM, respectively (Table 4.1). IAA inhibits the activity of *hp*GSTP1-1 via competitive inhibition, in respect to both of the substrates (Table 4.1).

Table 4.1. Kinetic parameters of *hp*GSTP1-1 inhibition by indoleacetic acid

	Indoleacetic Acid	
Parameters	$[GSH]_v$	$[CDNB]_v$
IC_{50}, mM	9.7 mM	
Inhibition type	Competitive	Competitive
V_m, µmol/min-mg protein	332 ± 17	182 ± 5
K_m, mM	2.32 ± 0.17	1.14 ± 0.06
K_i, mM	4.00 ± 0.62	3.30 ± 0.24

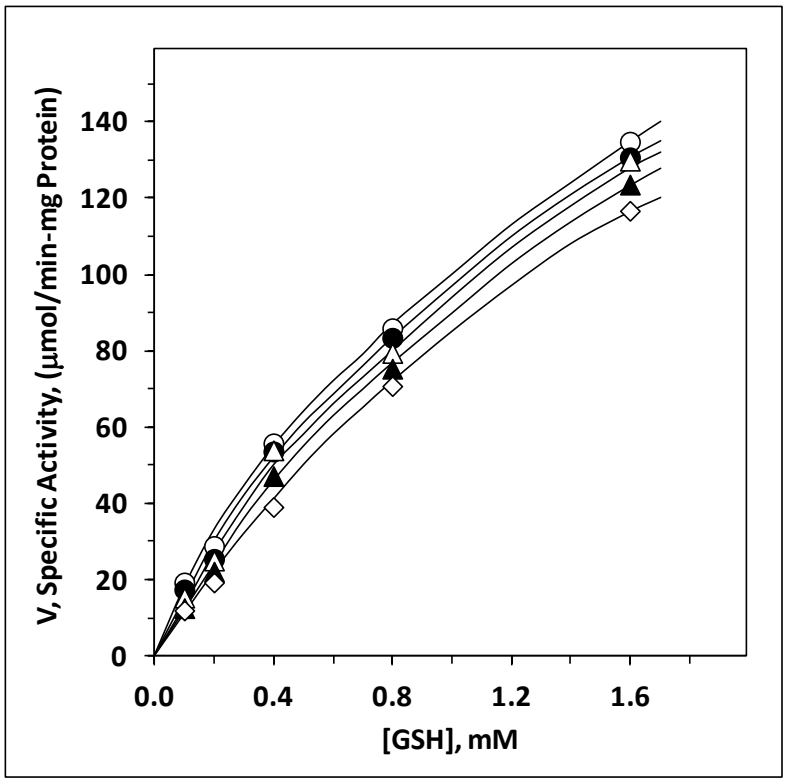

Figure 4.2. A Michaelis-Menten plot of *hp*GSTP1-1 with variable concentrations of IAA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of GSH (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant CDNB concentration (1 mM).

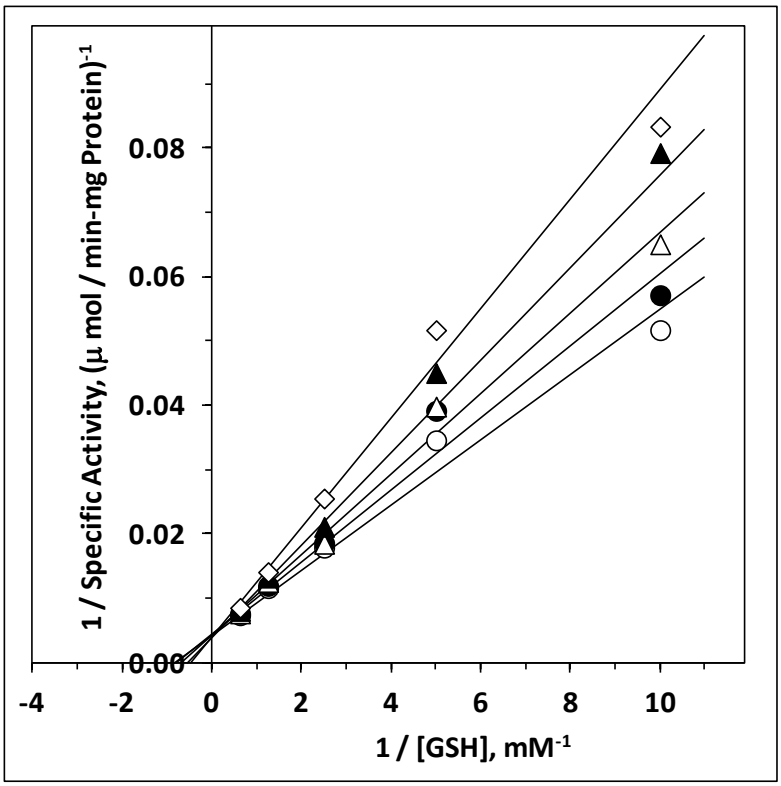

Figure 4.3. A Lineweaver-Burk plot of *hp*GSTP1-1 with variable concentrations of IAA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of GSH (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant CDNB concentration (1 mM).

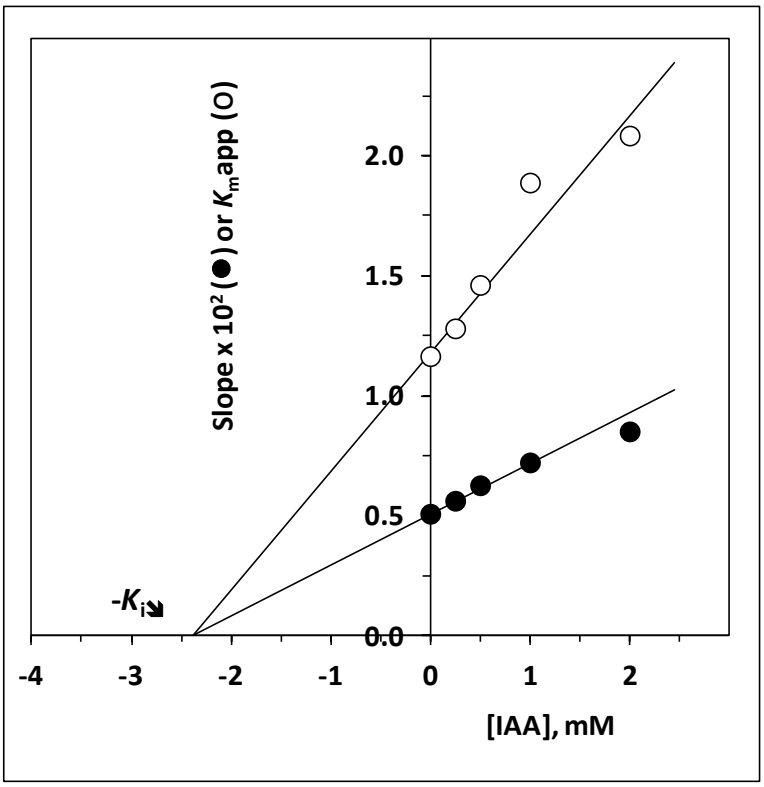

Figure 4.4. Slope and intercept against [IAA] at 1 mM $[CDNB]_f$ and $[GSH]_v$ designating the K_i as 2.38 mM.

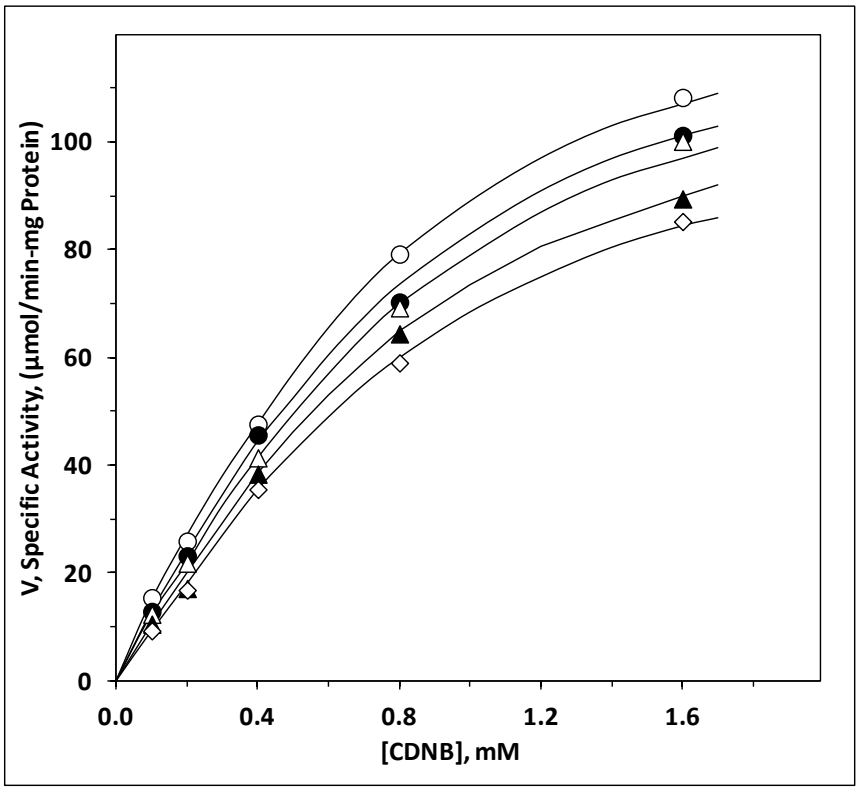

Figure 4.5. A Michaelis-Menten plot of *hp*GSTP1-1 with variable concentrations of IAA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of CDNB (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant GSH concentration (1 mM).

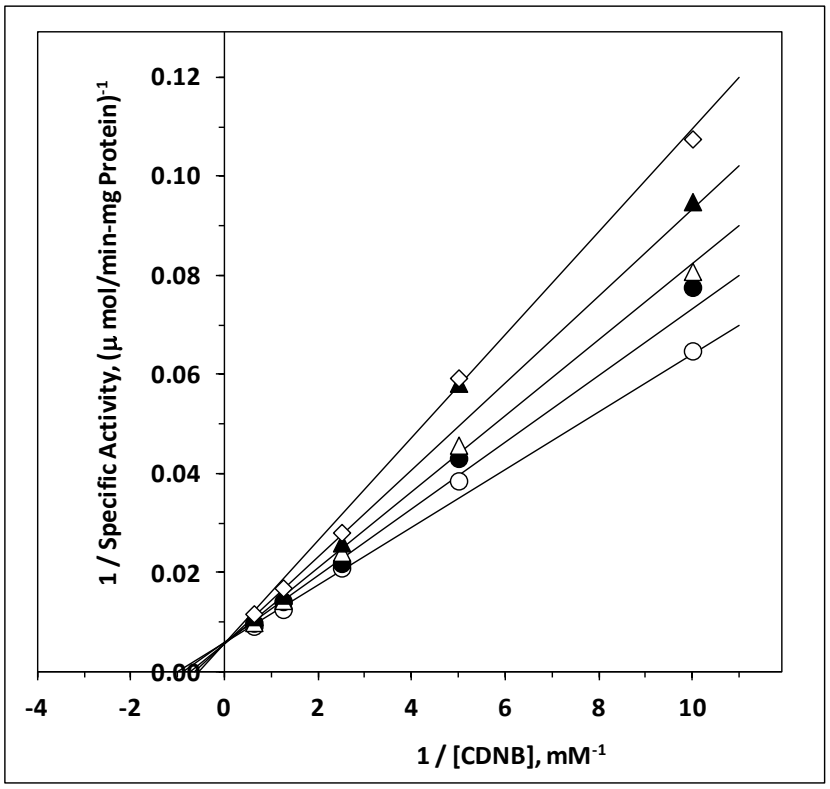

Figure 4.6. A Lineweaver-Burk plot of *hp*GSTP1-1 with variable concentrations of IAA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of CDNB (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant GSH concentration (1 mM).

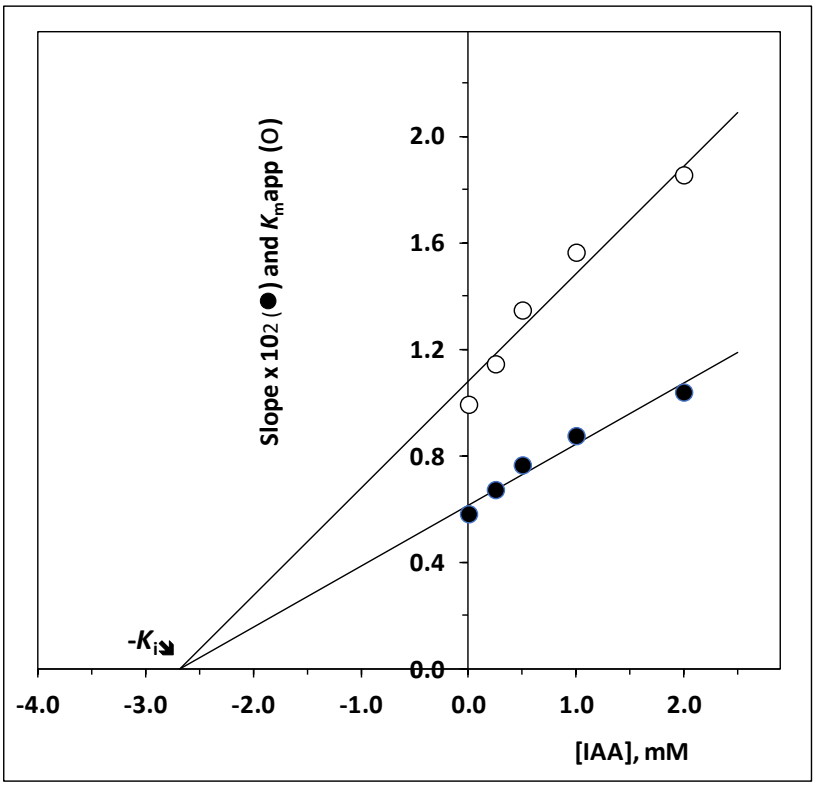

Figure 4.7. Slope and intercept against [IAA] at 1 mM $[GSH]_f$ and $[CDNB]_v$ designating the K_i as 2.68 mM.

4.2. Inhibitory Kinetic Interaction of *hp*GSTP1-1 with Indolepropionic Acid

The inhibitory effect of IPA on *hp*GSTP1-1 was investigated through the addition of different concentrations of IPA (0.3125, 0.625, 1.25, 2.5, 5 and 10 mM) into the reaction mixture, having 800 μL as the final volume. The reaction mixture was comprised of 100 mM sodium phosphate buffer pH 6.5 with 1 mM EDTA, 1 mM GSH, 1 mM CDNB, distilled water and appropriate amount of *hp*GSTP1-1 enzyme. Triplicate assays were performed to obtain the data for each IPA concentration. The IC_{50} value of IPA was confirmed as 7.2 mM (Figure 4.8) once log % remaining activity versus concentrations of IPA was plotted.

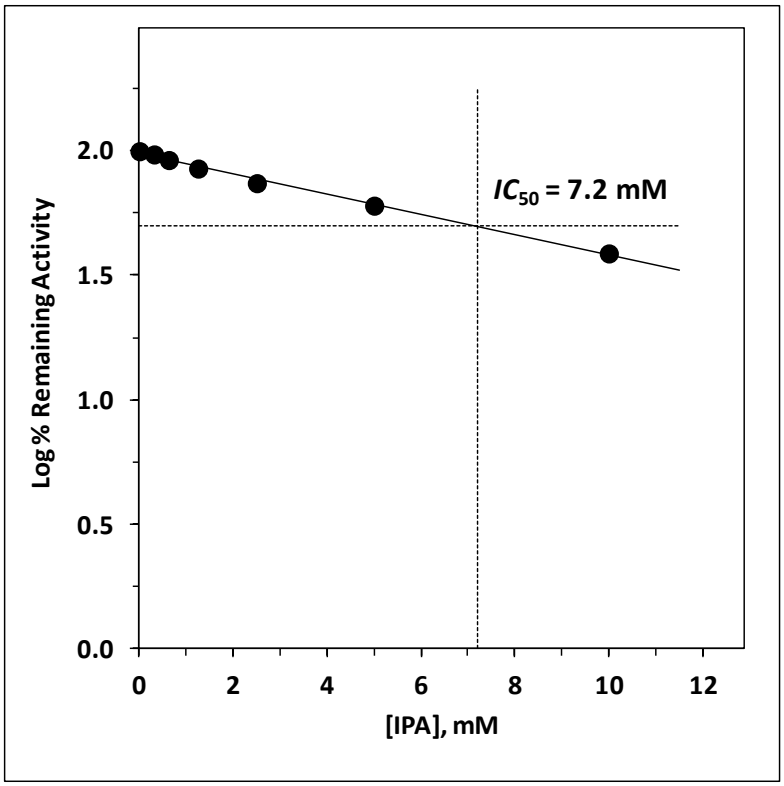

Figure 4.8. Dose dependent inhibition of *hp*GSTP1-1 with IPA

Four particular IPA concentrations (0.25, 0.5, 1 and 2 mM) were utilized in the inhibitory kinetic studies to elucidate the inhibition type, V_m, K_m and K_i values in the presence or absence of IPA. In this experiment, the reaction mixture was composed of 100 mM sodium phosphate buffer pH 6.5 with 1mM EDTA, the four IPA concentrations 1 mM $[CDNB]_f$-$[GSH]_v$ or $[GSH]_f$-$[CDNB]_v$, distilled water and appropriate amount of enzyme. In each case the reaction mixture had variable concentrations (0.1, 0.2, 0.4, 0.8 and 1.6 mM) of CDNB or GSH. At 340 nm the absorbance was followed for 20 seconds which showed an increase due to GS-DNB conjugate formation. Different kinetic parameters and plots including Michaelis-Menten (Figure 4.9 and 4.12), Lineweaver-Burk (Figure 4.10 and 4.13) and secondary plots (Figure 4.11 and 4.14) were drawn and kinetic parameters were also calculated by using SPSS version 20. The V_m for $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 229 ± 8 and 251 ± 11 µmol/min-mg protein, respectively. K_m for $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 1.63 ± 0.1 and 1.38 ± 0.11 mM, respectively. K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 3.33 ± 0.23 and 3.52 ± 0.24 mM, respectively (Table 4.2). IPA inhibits the activity of *hp*GSTP1-1 via competitive inhibition, in respect to both of the substrates (Table 4.2).

Table 4.2. Kinetic parameters of *hp*GSTP1-1 inhibition by indolepropionic acid

	Indolepropionic Acid	
Parameters	$[GSH]_v$	$[CDNB]_v$
IC_{50}, mM	7.2 mM	
Inhibition type	Competitive	Competitive
V_m, µmol/min-mg protein	229 ± 8	251 ± 11
K_m, mM	1.63 ± 0.1	1.38 ± 0.11
K_i, mM	3.33 ± 0.23	3.52 ± 0.24

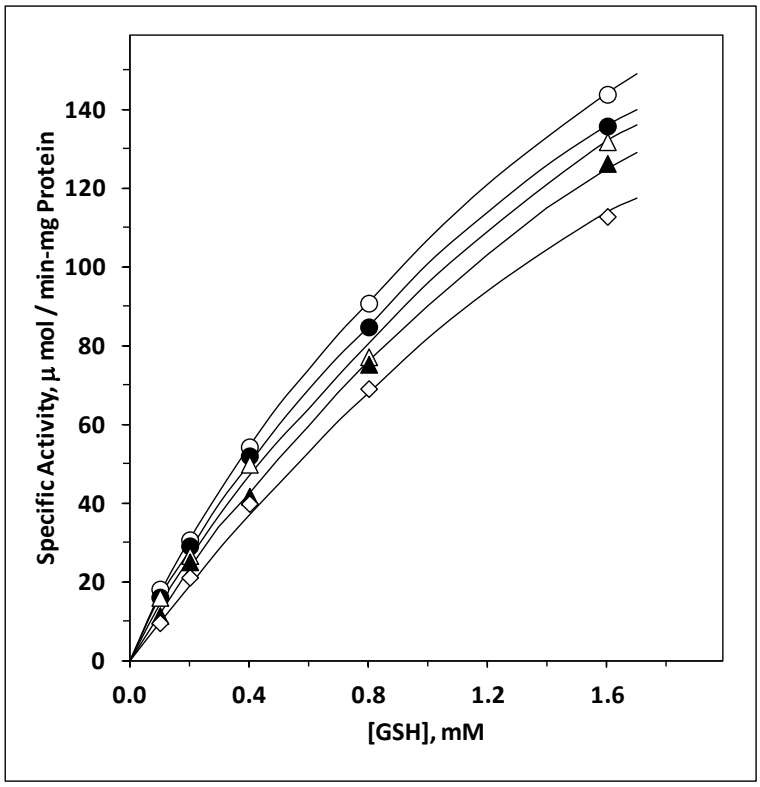

Figure 4.9. A Michaelis-Menten plot of *hp*GSTP1-1 with variable concentrations of IPA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of GSH (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant CDNB concentration (1 mM).

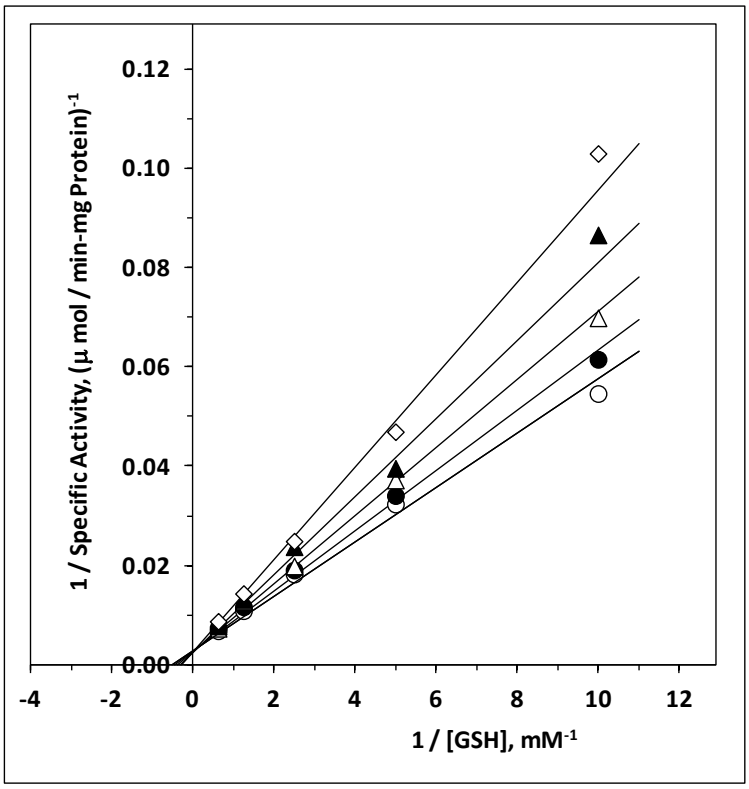

Figure 4.10. A Lineweaver-Burk plot of *hp*GSTP1-1 with variable concentrations of IPA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentration of GSH (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant CDNB concentration (1 mM).

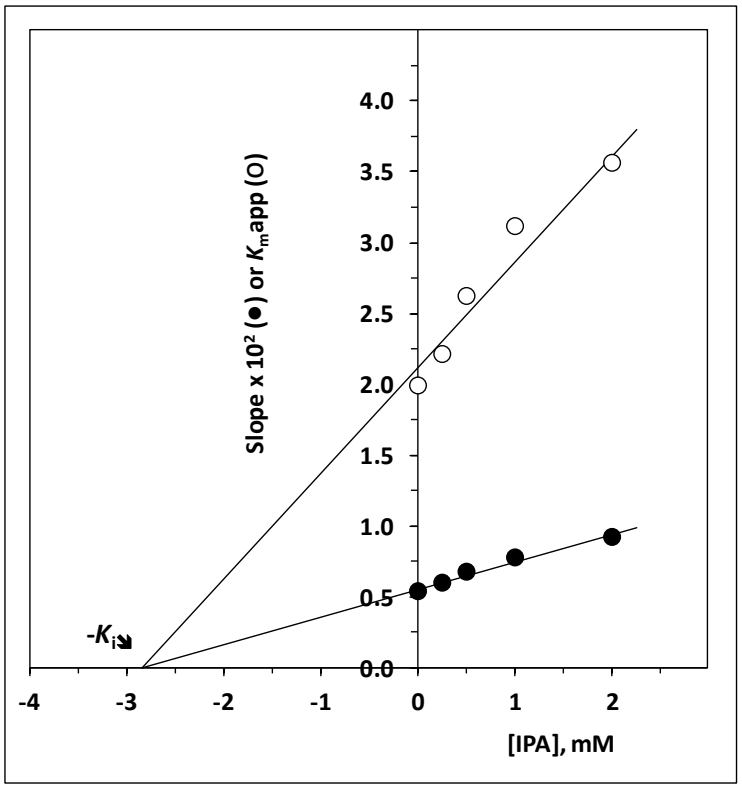

Figure 4.11. Slope and intercept against [IPA] at 1 mM $[CDNB]_f$ and $[GSH]_v$ designating the K_i as 2.84 mM.

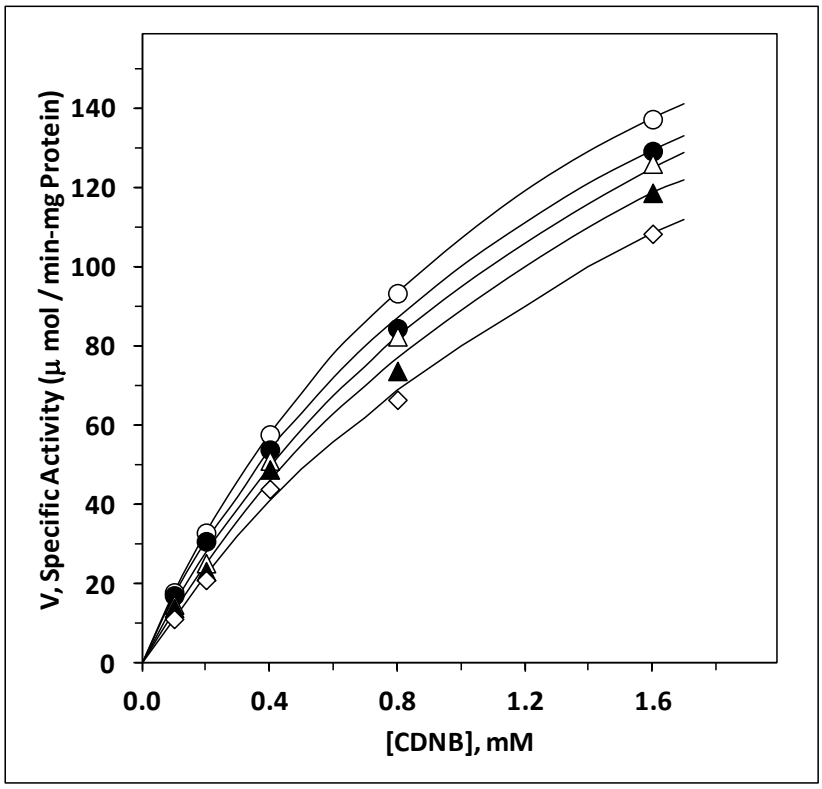

Figure 4.12. A Michaelis-Menten plot of *hp*GSTP1-1 with variable concentrations of IPA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of CDNB (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant GSH concentration (1 mM)

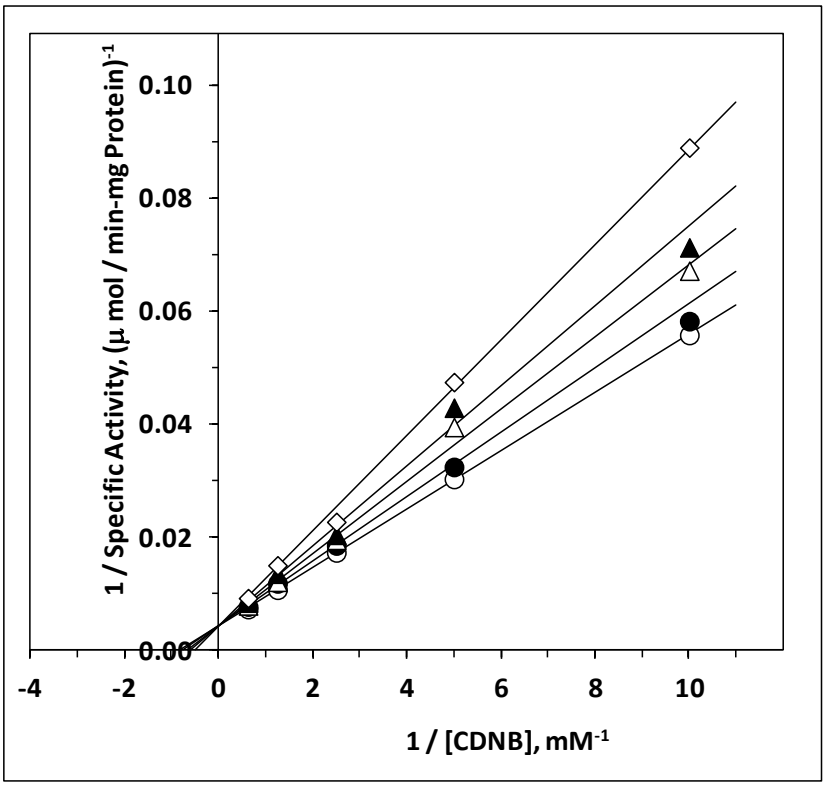

Figure 4.13. A Lineweaver-Burk plot of *hp*GSTP1-1 with variable concentrations of IPA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of CDNB (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant GSH concentration (1 mM).

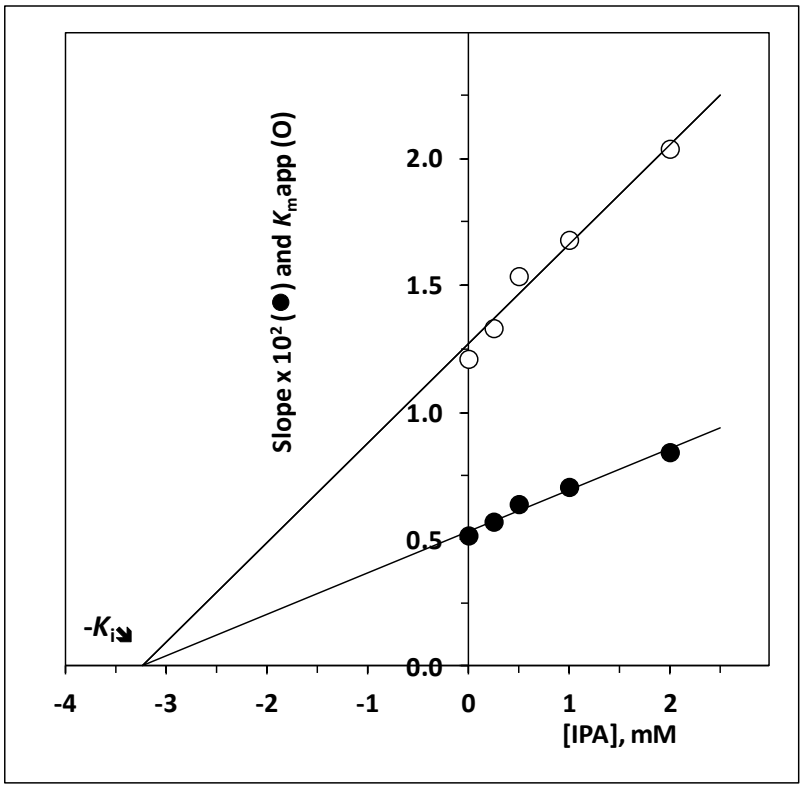

Figure 4.14. Slope and intercept against [IPA] at 1 mM $[GSH]_f$ and $[CDNB]_v$ designating the K_i as 3.23 mM.

4.3. Inhibitory Kinetic Interaction of *hp*GSTP1-1 with Indolebutyric Acid

The inhibitory effect of IBA on *hp*GSTP1-1 was investigated through the addition of different concentrations of IBA (0.3125, 0.625, 1.25, 2.5, 5 and 10 mM) into the reaction mixture, having 800 μL as the final volume. The reaction mixture was comprised of 100 mM sodium phosphate buffer pH 6.5 with 1 mM EDTA, 1 mM GSH, 1 mM CDNB, distilled water and appropriate amount of *hp*GSTP1-1 enzyme. Triplicate assays were performed to obtain the data for each IBA concentration. The IC_{50} value of IBA confirmed as 7.0 mM (Figure 4.15) once log % remaining activity versus concentrations of IBA was plotted.

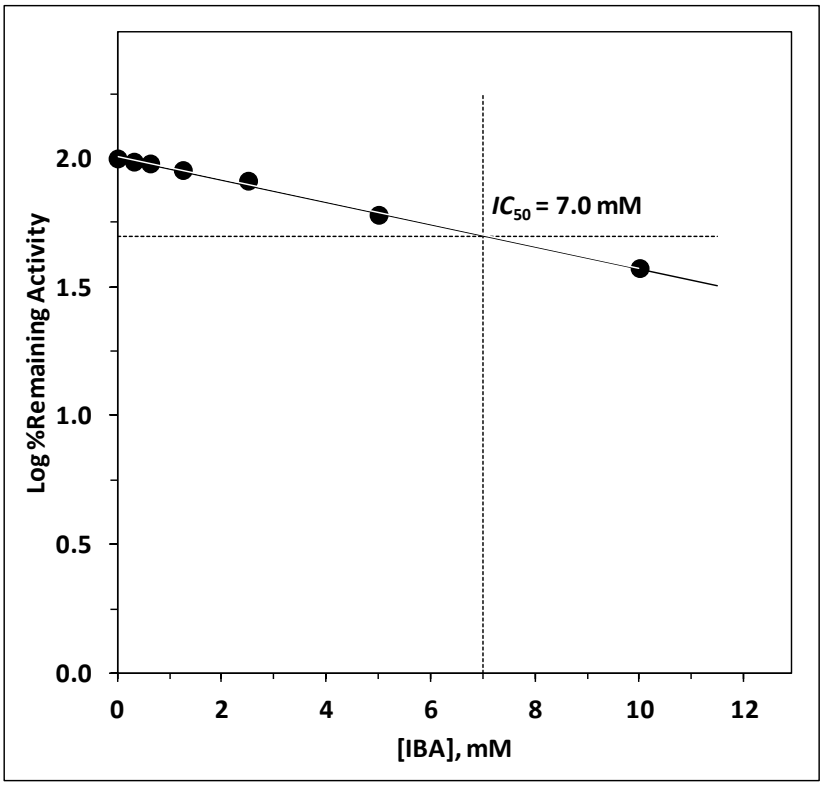

Figure 4.15. Dose dependent inhibition of *hp*GSTP1-1 with IBA

Four particular IBA concentrations (0.25, 0.5, 1 and 2 mM) were utilized in the inhibitory kinetic studies to elucidate the inhibition mode, V_m, K_m, K_i values in the presence or absence of IBA. In this experiment, the reaction mixture was composed of 100 mM sodium phosphate buffer pH 6.5 with 1 mM EDTA, the four IBA concentrations, 1 mM $[CDNB]_f$-$[GSH]_v$ or $[GSH]_f$-$[CDNB]_v$, distilled water and appropriate amount of enzyme. In each case the reaction mixture had variable concentrations (0.1, 0.2, 0.4, 0.8 and 1.6 mM) of CDNB or GSH. At 340 nm the absorbance was followed for 20 seconds which showed an increase due to GS-DNB conjugate formation. Different kinetic parameters and plots including Michaelis-Menten (Figure 4.16 and 4.19), Lineweaver-Burk (Figure 4.17 and 4.20) and secondary plots (Figure 4.18 and 4.21) were drawn and kinetic parameters were also calculated by using SPSS version 20. The V_m for $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 229 ± 7 and 266 ± 13 µmol/min-mg protein, respectively. K_m for $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 1.63 ± 0.08 and 1.53 ± 0.13 mM, respectively. K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 3.33 ± 0.22 and 2.14 ± 0.16 mM, respectively (Table 4.3). IBA inhibits the activity of *hp*GSTP1-1 via competitive inhibition, in respect to both of the substrates (Table 4.3).

Table 4.3. Kinetic parameters of *hp*GSTP1-1 inhibition by indolebutyric acid

	Indolebutyric Acid	
Parameters	$[GSH]_v$	$[CDNB]_v$
IC_{50}, mM	7.0 mM	
Inhibition type	Competitive	Competitive
V_m, µmol/min-mg protein	229 ± 7	266 ± 13
K_m, mM	1.63 ± 0.08	1.53 ± 0.13
K_i, mM	3.33 ± 0.22	2.14 ± 0.16

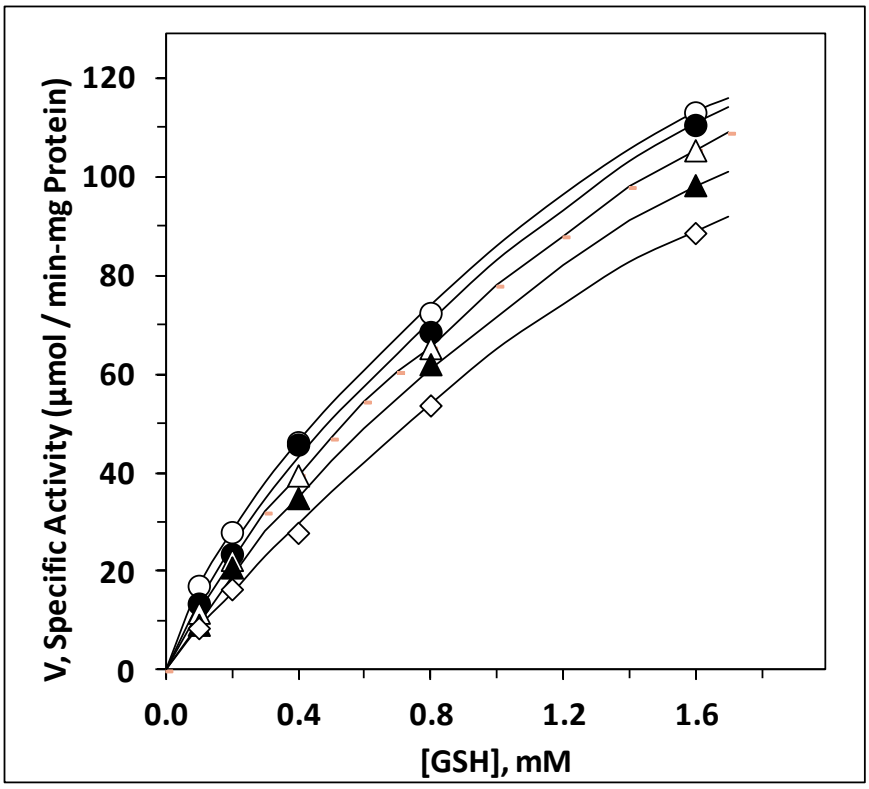

Figure 4.16. A Michaelis-Menten plot of *hp*GSTP1-1 with variable concentrations of IBA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of GSH (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant CDNB concentration (1 mM).

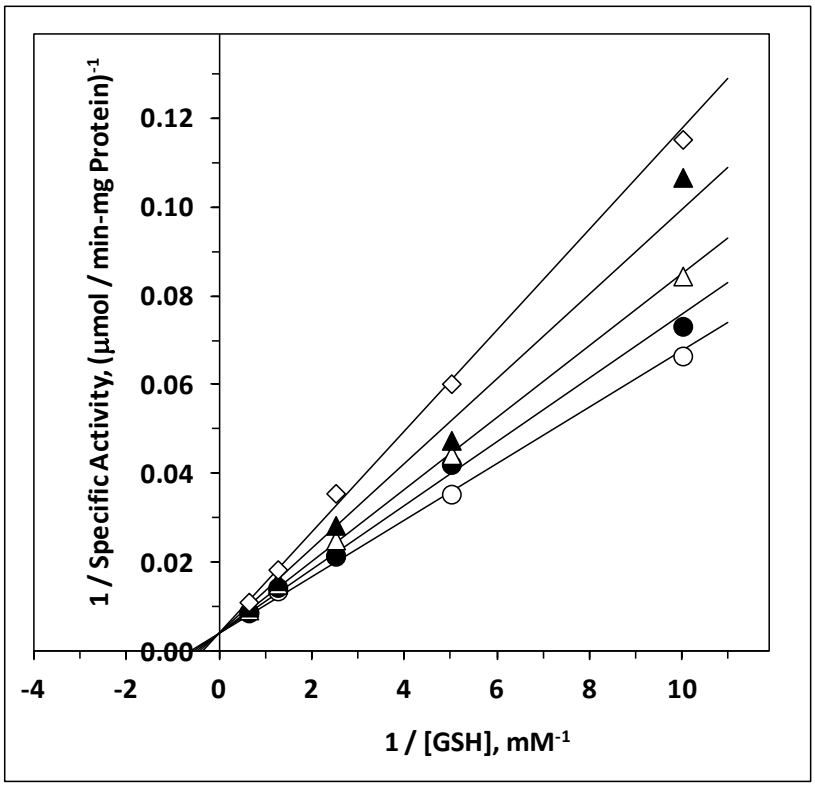

Figure 4.17. A Lineweaver-Burk plot of *hp*GSTP1-1 with variable concentrations of IBA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of GSH (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant CDNB concentration (1 mM).

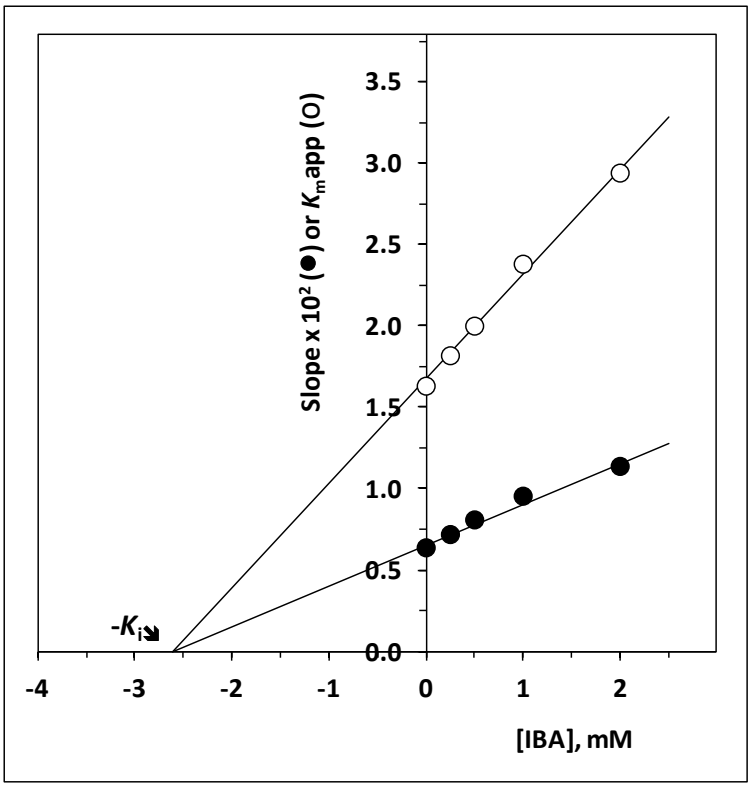

Figure 4.18. Slope and intercept against [IBA] at 1 mM $[CDNB]_f$ and $[GSH]_v$ designating the K_i as 2.61 mM.

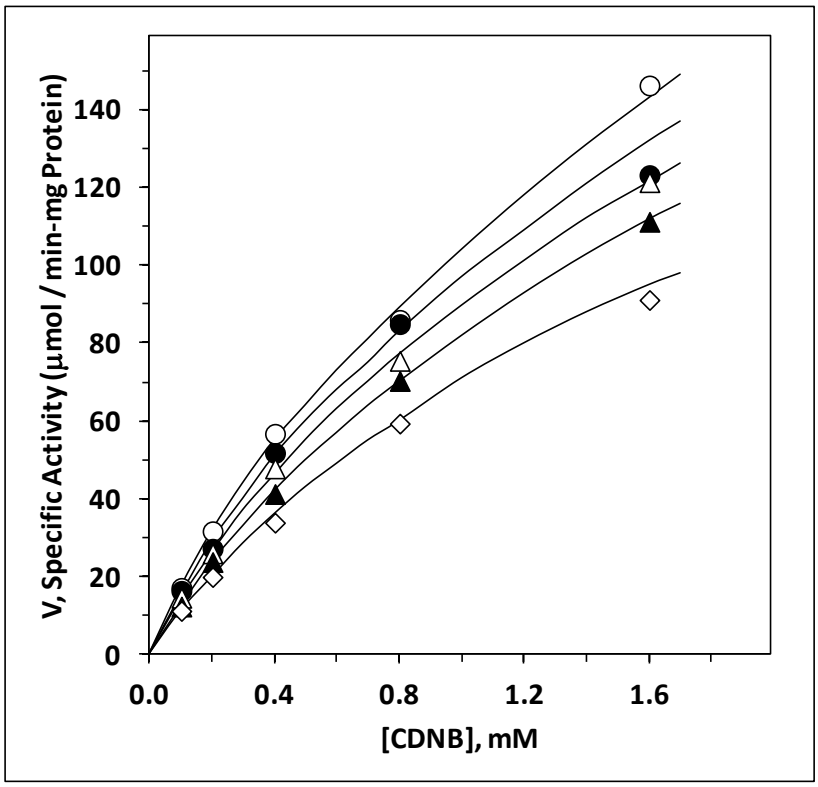

Figure 4.19. A Michaelis-Menten plot of *hp*GSTP1-1 with variable concentrations of IBA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of CDNB (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant GSH concentration (1 mM).

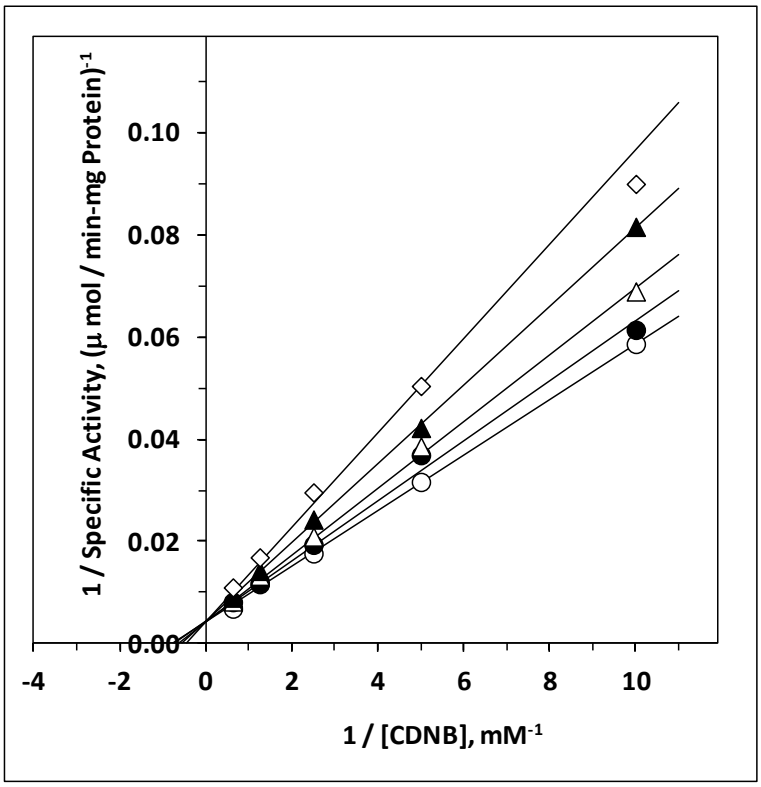

Figure 4.20. A Lineweaver-Burk plot of *hp*GSTP1-1 with variable concentrations of IBA [(○), 0; (●), 0.25; (Δ), 0.5; (▲), 1; (□), 2 mM] at variable concentrations of CDNB (0.1, 0.2, 0.4, 0.8 and 1.6 mM) and constant GSH concentration (1 mM)

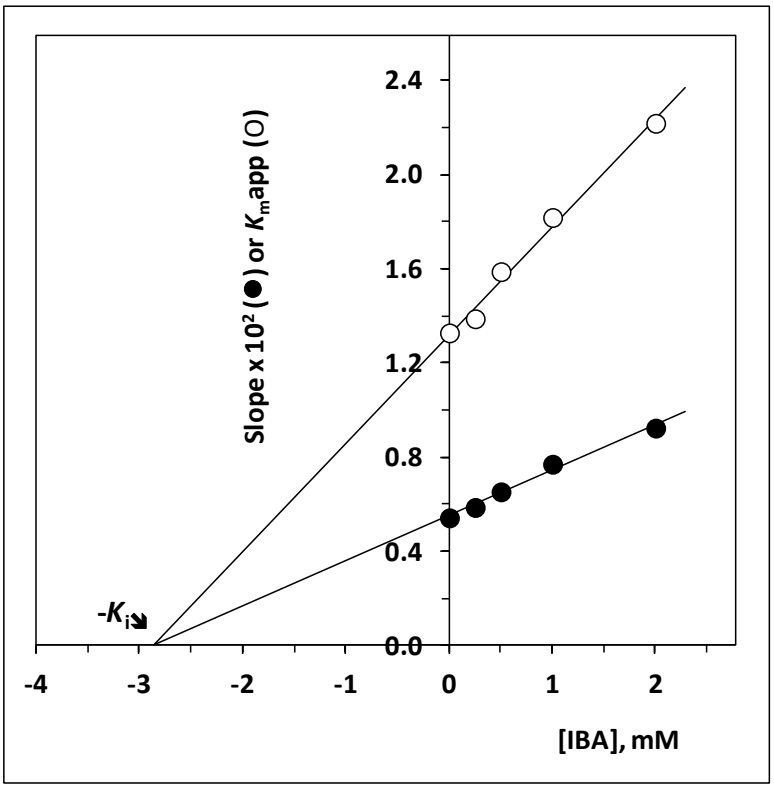

Figure 4.21. Slope and intercept against [IBA] at 1 mM $[GSH]_f$ and $[CDNB]_v$ designating the K_i as 2.86 mM.

5. DISCUSSION

Cancer is believed to be the leading cause of mortality which is among one of those noncommunicable diseases thats currently accountable for most of global deaths. In 2015, World Health Organization (WHO) ranked cancer as first or second in causing death prior to 70 years of age in more than half of the countries around the world and also majorly accountable as a cause of death in the remaining countries (Ferlay et al., 2018). Even with all the innovations in cancer research including treatment, prevention and detection, it is still one of the leading causes of death. Currently, cancer therapy includes removing the cancerous tissues through surgery followed by the use of radiotherapeutic agents or chemotherapeutic agents to remove the remaining cancerous tissues. These treatments will result in critical progress regarding the patient condition and mortality rate. On the contrary, this is not always the case; in several cases there are unfortunate response mechanisms toward this approach. The leading causes for cancer treatment failure are the late detection of the cancer and the resistance to the chemotherapeutic drugs utilized. Among the main drug resistance mechanisms is the increased drug detoxification via detoxifying enzymes such as glutathione S-transferases (GSTs), which this feature can also be exploited by cancerous cell to increase their survival via obtaining resistance against the anti-cancer drugs. Detoxification of drugs occurs through several processes with the use of several enzymes, particularly including GSTs. These superfamily of Phase II enzymes detoxify pesticides, anticancer drugs and genotoxic molecules via catalyzing their conjugation to glutathione (GSH), which in turn will form a less toxic glutathione complexes which facilitates its excretion from the body (Tew and Gate, 2001). Particularly, successful treatment of metastatic cancers includes utilization of toxic chemotherapeutic agents in different types since resistance to one drug can occur very often. That is why for the past 35 years, cancer biologist has been trying to give a clear explanation to multidrug resistance (MDR) mechanisms (Szakács et al., 2006). From 1985, GSTs implication in cancer resistance have been defined (Wang and Tew, 1985). GSTs over-expression and its role in detoxifying anticancer drugs in a broad range of cancer tissues, incite an interest in the finding or synthesizing GST inhibitors or prodrugs that potentially overcome the development of

resistance. Ethacrynic acid was the first inhibitor with promising inhibitory effect *in vitro* however due to its side effects and limited isoenzyme specificity, clinical utilization could not be possible (Tew and Gate, 2001).

Beside GST overexpression which facilitates chemotherapeutic drug detoxification, the synergistic involvement of both GSTs and efflux pumps appears to have been seen in numerous studies (Meijerman et al., 2008; Sau et al., 2010). Concurrently, anticancer drug detoxification through GSTs is significantly related to the discharge rate of the detoxified drugs from the help of the efflux transporters. The formed glutathione conjugates are removed from the cells by the efflux transporters that include P-glycoprotein and MRP1 which is among one of the ATP-binding cassette transporters superfamily (Keppler, 1999; Meijerman et al., 2008). These findings indicate the synergetic development of MDR in cancer patients. Additionally, GSTs are even involved in developing MDR through their non-catalytic function which regulates JNK signaling pathway. The inhibition of this pathway through GSTs protects cancer cells from apoptosis (Allocati et al., 2018).

To overcome this obstacle for years researchers have been finding and synthesizing various types of inhibitors with the potential to inactivate GSTs, among them some plant derived inhibitors are profoundly recognized as potent inhibitors. A study that examined plant derived flavonoids and polyphenols (2-hydroxl chalcone, morin, tannic acid, quercetin and butein) showed that each had a different level of potency in inhibiting rat liver GST. Tannic acid was the most potent with an IC_{50} value of 1.044 μM. Tannic acid inhibited rat liver GST in a competitive way regarding GSH and exhibited noncompetitive inhibition toward CDNB. Coming in second was 2-hydroxyl chalcone with an IC_{50} value of 6.758 μM. Butein, morin and quercetin were able to inhibit rat liver GST with an IC_{50} values of 9.033, 13.710 and 18.732 μM, respectively, coming after the tannic acid and 2-hyrdoxyl chalcone. A pH of 6.0 to 6.5 was optimum for four of the compounds except quercetin, in the meantime a pH of 8.0 appeared to be an optimum pH for quercetin. The study showed that all of the inhibitors shared common features such as not having a sugar moiety or poly hydroxylation substitutions (Zhang and Das, 1994).

Inhibitory effect of quercetin has also been investigated on human GSTP1-1 which appeared to be capable of inhibiting human GSTP1-1 regarding time and concentration. Quercetin is a natural polyphenol and an important constituent of vegetables, fruits, red wine, nuts and tea. In order for quercetin to completely inactivate human GSTP1-1, 100 μM of quercetin was incubated with GSTP1-1 for 1 hour. However, 25 μM quercetin was also capable of completely inactivating GSTP1-1 when it was incubated for 2 hours. Therefore, 2 hours of incubation with 1 and 10 μM quercetin were able to inhibit the activity of GSTP1-1 by 25 and 42%, respectively. Even upon complete inactivation the addition of glutathione appeared to slightly restore GSTP1-1 activity. Furthermore, the addition of tyrosinase with quercetin would significantly increase inactivation rate and the addition of glutathione or ascorbic acid would even weaken quercetin ability to inhibit human GSTP1-1 (van Zanden et al., 2003).

Furthermore, curcumin and ellagic acid which are two natural plant products were able to inhibit the activities of GSTM1-1, M2-2, A1-1, A2-2 and P1-1 in a concentration and time dependent manner, with an IC_{50} values range between 0.04 - 5 μM, curcumin being the more potent inhibitor. Curcumin and ellagic acid appear to have shown more of mixed type inhibition to a lesser extend uncompetitive inhibition mode with respect of the H and G sites (Hayeshi et al., 2007). These studies elucidate the potential inhibitory effect of plant derived compounds on human GSTs, that's why it is significant to study the effect of phytohormones as a mean to reduce the activity of *hp*GSTP1-1. The human pi class glutathione S-transferase is essentially an important class of GSTs, since it can be used as a target for finding or synthesizing an inhibitor that could be utilized to enhance chemotherapeutic efficiency and also as mean to evade MDR in patients with metastatic cancer (van Zanden et al., 2003).

An investigation in 1993 stated that in plants, auxin can bind to GSTs, and it was then when auxin binding to GSTs purpose was explained. The two functions of auxin binding to GSTs that was clarified were how firstly, auxin is capable of binding to GSTs as a substrate which in turn will result in the formation of GS-conjugate, or in the second case it would bind as a non-substrate also termed as ligand. The formation of this conjugate will regulate GST activity (Bilang et al., 1993; Zettl et al., 1994). In the first

case the binding of indoleacetic acid (IAA) to GSTs of plant as a substrate will result in its metabolism and influencing the intracellular levels of IAA. However, when the auxin binds as a ligand it could initiate an alteration of GSH concentrations and regulate cellular redox which potentially influences various cellular processes (Bilang et al., 1993).

To evaluate the physiological importance of auxin binding plant GSTs, inhibitory effects of auxins were investigated on GSTs expressed in *Escherichia coli.* Cloning of cDNA was performed in the fifth gene (*GST5*) of GST in *Arabidopsis.* Even though the altered amino acid sequence on the *GST5* was slightly resemble the original *Arabidopsis* GSTs, the *GST5* protein formed in the *E. coli* expressed competent GST activity. *GST5* K_i values were 0.86 and 1.29 mM for GSH and CDNB, respectively. 2,4-dichlorophenoxyacetic acid (2,4-D), IAA, 1-napthaleneacetic acid (1-NAA) and (2-NAA) were capable of inhibiting plant GST in competitive inhibition mode in respect to GSH. IAA K_i appeared to be 1.56 mM (Watahiki et al., 1995).

All these findings from past researches elucidated the significance of examining the inhibitory effect of auxins on GSTs. The three types of auxins investigated in this study were comprised of indoleacetic acid (IAA), indolepropionic acid (IPA) and indolebutyric acid (IBA). All endogenous natural occurring auxins are compounds with an aromatic ring and a carboxylic acid group, with IAA being the most prominent of the group and significantly effecting an intact plant. However, the six concentrations of IAA that were used ranging from 0.3125 to 10 mM, was the least potent, capable of inhibiting *hp*GSTP1-1 activity with an IC_{50} value of 9.7 mM (Figure 4.1). IAA K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 4.00 ± 0.62 and 3.30 ± 0.24 mM, respectively. IPA was more potent in inhibiting *hp*GSTP1-1 with an IC_{50} value of 7.2 mM (Figure 4.8). IPA K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 3.33 ± 0.23 and 3.52 ± 0.24 mM, respectively. However, the most potent auxin in inhibiting the activity of *hp*GSTP1-1 was IBA with an IC_{50} value of 7.0 mM (Figure 4.15). IBA K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ was 3.33 ± 0.22 and 2.14 ± 0.16 mM, respectively. All three of the auxins examined in this study inactivated *hp*GSTP1-1 in a competitive inhibition mode with respect to both of the substrates.

6. CONCLUSION

In this study, the inhibitory effect of IAA, IPA and IBA was investigated on *hp*GSTP1-1, as a mean of finding a novel therapeutic drug to overcome multi drug resistance mechanism in cancer patients. It appeared that all three classes of auxins (IAA, IPA and IBA) investigated inhibit the activity of *hp*GSTP1-1 in a competitive manner in respect to both substrates. IAA had an IC_{50} of 9.7 mM with a V_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 332 ± 17 and 182 ± 5 µmol/min-mg protein, respectively, K_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 2.32 ± 0.17 and 1.14 ± 0.06 mM, respectively and a K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 4.00 ± 0.62 and 3.30 ± 0.24 mM, respectively. IPA had an IC_{50} value of 7.2 mM with a V_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 229 ± 8 and 251 ± 11 µmol/min-mg protein, respectively, K_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 1.63 ± 0.1 and 1.38 ± 0.11 mM, respectively and a K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 3.33 ± 0.23 and 3.52 ± 0.24 mM, respectively. IBA has an IC_{50} of 7.0 mM with a V_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 229 ± 7 and 266 ± 13 µmol/min-mg protein, respectively, K_m $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 1.63 ± 0.08 and 1.53 ± 0.13 mM, respectively and a K_i values $[CDNB]_f$-$[GSH]_v$ and $[GSH]_f$-$[CDNB]_v$ of 3.33 ± 0.22 and 2.14 ± 0.16 mM, respectively. However, *in vitro* study of these auxins reveals their ability to inhibit *hp*GSTP1-1 activity, there is still certain criteria that should be considered as to develop a novel therapeutic drug which include the cytotoxicity of the drug along with the application of preclinical trials then followed by clinical trials.

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