GENE EXPRESSION CHANGES INDUCED BY THE TUMORIGENIC PYRROLIZIDINE ALKALOID RETRORSINE IN LIVER OF F344 RATS

ENAS ESSAM JEWAID

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by

Enas Essam Jewaid

Supervisors

Dr. Joseph Banoub

&

Dr. Dawn Marshall

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are naturally occurring chemical compounds which are found in various plant species worldwide. Although not all PAs are toxic, retrorsine is another representative retrorncine-type PAs consider as one of the most toxic member of the PAs family which has historically received most of the attention to have a genotoxic, hepatotoxic effects on animals and a serious health problem to human as a veno occlusive disease and liver cancer. The mechanism of hepatotoxicity induced by PAs in which PAs convert to the toxic form in the liver has been extensively studied. It has been shown that a genotoxic mechanism is correlated to the tumorigenicity of retrorsine through the formation of the DHP-derivaed DNA adducts. Cytochrome P450 is playing an essential key role in mechanism of liver genotoxicity. The genotoxicity activity of PAs has made them targets for studies designed to determine genes involved in the metabolic actiavation of retrorsine. Microarray studies are now playing a powerful approach in gene expression and discovery. Hence, to better understanding the mechanism of genotoxic effects of retrorsine treated rats, this microarray analysis together with the real time PCR provide qualification and quantification information of the liver metabolizing enzymes activities, including those of the cytochrome P450 enzymes, and identification of genes involved in liver cancer induced by retrorsine treatment. The present study represents the first in vivo examination of chronic transcriptional response of the liver to retrorsine exposure. The available evidence on the metabolism and target-tissue specificity for retrorsine's tumorigenesis suggests that active metabolites of retrorsine interact with endothelial cells in the liver which cause cell toxicity, followed by compensatory proliferation of DNA-damaged endothelial cells causing mutations in these cells. We have identified 53 genes in the liver of retrorsine-treated rats that were differentially expressed. Our findings suggest that these genes may play an important role in the metabolism of retrorsine. The genes identified in this study are involved in many diverse processes, including apoptosis, angiogenesis, cell growth, cell death, adhesion, and cell movement of endothelial cells, oxidative stress, liver development, catalytic activity, and signal transducer activity. P450 2E1 enzyme is the major metabolizing enzymes responsible for metabolism of retrorsine which was confirmed to be increased in gene expression by Real-Time PCR, these findings suggest that pyrrolizidine alkaloids retrorsine is metabolically activated by P450 2E1 to form chemically reactive dehydrogenated intermediates.

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LIST OF ABBREVIATIONS

260.....Absorption at 260 nm

260/280.....Ratio of absorption at 260 to 280

cDNA.....Complementary DNA

CT.....Confidence threshold

DHP.....6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine

GO.....Gene ontology

mRNA.....Messenger RNA

NCBI.....National Center for biotechnology information

P450.....cytochrome P450

PA.....Pyrrolizidine Alkaloid

PCA.....Principle component analysis

PCR.....Polymerase Chain Reaction

qPCR.....quantitative real time polymerization chain reaction

UV.....Ultraviolet

WHO.....World Health Organisation

GLOSSARY OF TERMS

260/230: ratio of sample absorbance at 260 and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

Assay ID: Identifier assigned by Applied Biosystems to TaqMan® Gene Expression Assays and TaqMan® SNP Genotyping Assays.

Concentration: Concentration of the cRNA product. (ug/ul)

Endogenous control: This is an RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantitation of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.

Exogenous control: This is a characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an in vitro construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase. Whether or not an active reference is used, it is important to use a passive reference containing the dye ROX in order to normalize for non-PCR-related fluctuations in fluorescence signal.

Gene Ontology (GO): is a controlled vocabulary to describe gene and gene product attributes of any organism. The GO project is a collaborative effort to address the need for consistent descriptions of gene products in different databases. The three organising principles of GO are molecular function, biological process and cellular component.

Hepatectomy: Removal of the liver.

Hepatocytomegaly: The production of abnormal hepatocytes (the most common cell type) in the liver. its genome.

ng/ul: sample concentration in ng/ul based on absorbance at 260 nm and the selected analysis constant. See the "Concentration Calculation (Beer's Law)" in the appendix for more details on this calculation.

Reference: A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification. The active reference has its own set of primers and probe.

Standard: A sample of known concentration used to construct a standard curve.

T-test: T-test unpaired is chosen as a test of choice with a kind of experimental grouping shown in Table 1. Upon completion of T-test the results are displayed as three tiled windows.

TaqMan® reagents: PCR reaction components that consist of primers designed to amplify the target and a TaqMan® probe designed to detect amplification of the target.

Transgenic: An animal that carries a foreign gene that has been deliberately inserted into

Veno-occlusive disease: Blockage of the small veins in the liver, resulting in liver damage.

DEDICATION

To the soul of my beloved DAD who was always there for me.
Thank you for great loving & helping through the rough times.
You were everything a Father should be
I love you

God saw you getting tired
and a cure was not meant to be
so He put His arms around you
and whispered, "Come to Me".
With tearful eyes I watched you,
you pass away.
Although I loved you dearly....
I could not make you stay.
A golden heart stopped beating,
hard working hands at rest.
God broke my heart to prove to me
He only takes the best.

CHAPTER I

1. Introduction

1.1 Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) are regarded as naturally occurring chemical compounds which are found in various plant species of *Senecio, Crotalaria, Erechtites* and other related genera worldwide (ANZFA, 2001; Joseilson, *et al.,* 2004; Kaleab, *et al.,* 2004; Smith and Culvenor, 1981). It is well documented that the various PAs are hepatoxic through the function of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) -derived DNA adduct formation (Jeffrey, *et al.,* 1991; Peter, *et al.,* 2002; Peter, *et al.,* 2004; Yu-Ping, *et al.,* 2005). Although not all PAs are toxic, retrorsine is a representative of retroencine-type PAs, considered to be one of the most toxic member of the PAs family. It has historically received much attention and is thought to have a genotoxic, hepatotoxic effect on animals, and to present a serious health problem to humans by causing veno occlusive disease (VOD) and liver cancer (Chen and Huo, 2010; Rasenack, *et al.,* 2003; Zhe and Ji-Rong, 2010). Retrorsine toxicity has historically been a significant problem worldwide. Thus, recent studies have been established to study the tumorigenicity and hepatotoxicity of PAs including Retrorsine (RTS) *in vivo* and *in vitro.*

The mechanism of hepatotoxicity induced by PAs in which PAs convert to the toxic form in the liver has been extensively studied (Yu-Ping, *et al.,* 2005; Mattocks and Butler, 1973). It has been shown that a genotoxic mechanism is correlated to the tumorigenicity of retrorsine through the formation of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)-derived DNA adducts (Yan, *et al.,* 2008). Also, it has been reported that riddelline and retrorsine share the same metabolic activation in rats which can serve as biomarkers for the tumorigenicity (Yu-Ping, *et al.,* 2005). Cytochrome P450 plays an essential key role in the mechanism of liver genotoxicity. The genotoxicity activity of PAs has

made them targets for studies designed to determine genes involved in metabolic activation of retrorsine.

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms that have a pharmacological effect on humans and other animals. Humans have been using alkaloids in the form of plant extracts for poisons, narcotics, stimulants and medicines for at least the last several thousand years. More than 10,000 alkaloids of different structures are now known to have been used as drugs worldwide (Schardl, *et al.,* 2007; Toni, 1995). Currently 25% of *materia medica* have traditionally been of interest and are known to have been used as drugs, either as pure compounds (such as the narcotic analgesic morphine, the analgesic and antitussive codeine, and the chemotherapeutic agents vincristine and vinblastine) or as teas and extracts. Plant constituents have also served as models for modern synthetic drugs and continue to result in new drug discoveries. Nevertheless, certain plant drugs are still extremely important, such as atropine for tropicamide, quinine for chloroquine, and cocaine for procaine and tetracaine. Alkaloids are basic in character and are sometimes toxic to animals when eaten, resulting in significant financial and production losses each year (Holstege, *et al.,* 1995). The biological role of alkaloids in plants is still a matter of speculation, but they are thought to have evolved as a defensive mechanisim against predators (Ober and Hartmann, 1999).

Pyrrolizidine alkaloids are hepatotoxins found in many species of plants throughout the world and are produced by a large variety of organisms, including bacteria, fungi, and animals and are part of the group of natural products (also called secondary metabolites). More than 350 PAs have been identified in over 6,000 plants in the *Broaginaceae* (many genera), *Asteraceae (*tribes *Senecioneae* and *Eupatorieae*), *Orchidaceae* (nine genera) and *Fabaceae* (mainly the genus *Crotalaria*) (Dharrnananda, 2002). More than 95% of the PA containing plants investigated thus far belonged to these four families (Ober and Hartmann, 1999; Stegelmeier, 1999).

Each year alkaloids cause considerable economic loss to the livestock industry and possible

adverse effects to human health though exposure to these compounds as contaminants of grain, milk, herbal teas, honey, and herbal medicines (Dale, 2006; John, *et al.,* 2002; Prakash, *et al.,* 1999; Stegelmeier, 1999). While all livestock are susceptible to PA toxicity, goats and sheep are less susceptible, while the most frequently and greater risk of severe infection is for horses and cattle. Particularly those parts of the world with arid climates and poor rainfall are susceptible to grain contamination and promotion of PA containing plant growth as weeds among cultivated crops. The first recorded instance of PA poisoning was in 1920 when many people in the Western Cape province of South Africa suffered from liver cirrhosis after eating bread made with wheat probably contaminated with *Senecio burchellii.* (Willmot and Robertson, 1920). In 1974 it was reported that the largest outbreak of human intoxication by PAs was in Afghanistan when an estimated 35000 people were affected after grain was contaminated with Heliotropium plant material. Among 7200 cases examined, 1600 were affected and many died three to nine months after the onset of clinical signs (Mohabbat, *et al.,* 1976). A variety of animal products can be contaminated by pyrrolizidine alkaloid and also enter the human food chain, leading to possible long- term toxic effects on humans.

Milk residue is one of the most frequently encountered products of animals that have ingested PA-containing plants (Dickinson, *et al.,* 1976). another source is eggs which can be contaminated with PAs from chickens eating contaminated grain and honey which has been found to contain high PA levels, causing a risk to those who consume large amounts of honey (Deinzer, *et al.,* 1977; Mattocks, *et al.,* 1971). PA toxicity has historically been a significant problem, but with modern herbicides and better grazing management practices this problem has been minimized in some areas.

1.2 Structure of pyrrolizidine alkaloids

The name of pyrrolizidine alkaloids came from their inclusion of a pyrrolizidine nucleus (a pair of linked pyrrole rings) as their back bone. Each pyrrole consists of a five-sided structure with four carbons and one nitrogen forming the ring. Generally, PAs are esters of hydroxylated methyl pyrrolizidines, consisting of a necine base and a necic acid moiety. The necine base can either be 1,2-unsaturated or saturated. The unsaturated necine bases are further classified as two types; retronecine-type (or heliotridinetype, a 7(S)-isomer of 7(R)-retroneeine) and otonecine-type alkaloids (Mattocks, 1986). Pyrrolizidine alkaloid bases can also exist as N-oxides, which are often found together with the basic alkaloids in plants. The structures of retrorsine and retrorsine *N*-oxide are shown in Figure 1-1.

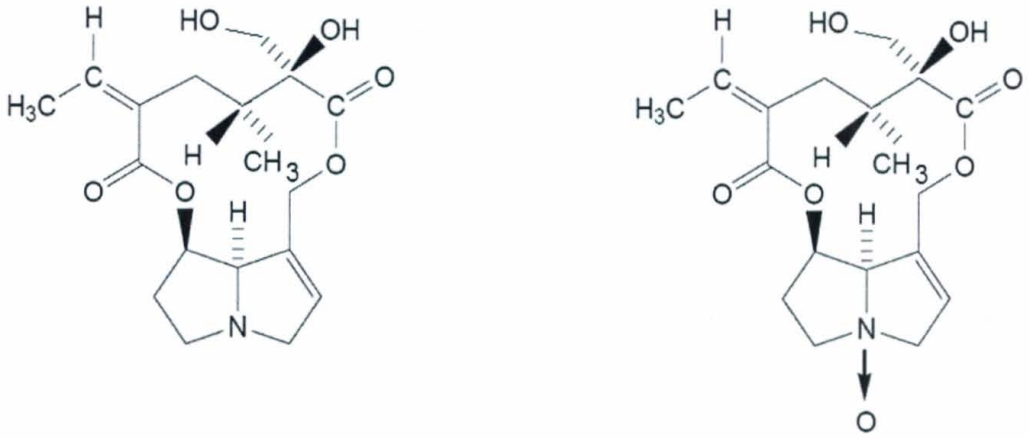


Figure 1-1: Structures of retrorsine (left) and retrorsine *N*-oxide (right)

Necic acids are branched-chained mono- or di-carboxylic acids containing four to six carbon atoms and are typically unsaturated, hydroxylated, or epoxidized. The four most common types of necine bases found in PAs are platynecine, retronecine, heliotridine, and otonecine. Retronecine and heliotrine are enantiomers and have been studied the most because of their abundance and toxicity (Fu, *et al.,* 2004).

Retrorsine consists of two fused five member rings with a nitrogen atom at the bridgehead position and a 1, 2 double bond. This pyrrolizidine ring system has a hydroxymethyl group at the 1-position and a hydroxyl group at the 7-position, through which the esterifying acid is attached.

1.3 Toxicity of pyrrolizidine alkaloids

Structures of naturally toxic PAs have been proposed for more than 350 individual compounds. Determining the potency of these PAs is strongly dependent on knowing their molecular structures features. These toxic PA products share a basic structure derived from esters of four necine bases: platynecine, retronecine, heliotridine and otonecine. The acid moieties of the esters are termed necic acids. The toxicity of the metabolite, once formed is determined by the metabolic route of PAs. The major metabolic routes of unsaturated PAs are catalysed by cytochrome CYP P450 enzymes in the liver. Toxicity also depends on the particular alkaloid and the nature of exposure (Peterson, and Jago, 1984). PAs are fairly stable chemically and require metabolic activation to exert toxicity (WHO, 1988). PAs associated with adverse effects also have a pronounced effect upon activity. Cyclic diesters are the most toxic with non-cyclic diesters being of intermediate toxicity and the monoesters the least toxic. The amino alcohols are not toxic.

There are different factors preventing pyrrolizidine alkaloid hydrolysis which influence toxicity including the presence of carbonyl groups with branching and the rigidity of the acid chain due to cyclic diester rings or unsaturation. Beside the physical and chemical properties of PAs that prevent pyrrolizidine alkaloid hydrolysis, hepatic microsomal enzymes such as cytochrome P450 oxidases play a major role in conversion of these hydrophilic PAs to pyrroles and N-oxides. The enzyme activity of the animal and the type of ester may be one of the important factors influencing the ratio of N-oxide to pyrrole (Wang, *et al.,* 2005). There is evidence that acute hepatotoxicity of some PAs can be contributed to by other metabolites, such as 4-hydroxy-2, 3-unsaturated aldehydes (Segall, *et al.,* 1985).

However, this has still to be confirmed.

PAs need to have certain essential features in addition to the animal enzyme activation before they can be converted to toxic metabolites (Fu, *et al.,* 2004; Guengerich, 1977; Jieyu, *et al.,* 2010; Yu-Ping, *et al.,* 2005).

Structural features of PAs associated with hepatotoxicity in rats (Prakash, *et al.,* 1999) are shown in figure 1-2 and include:

1. An unsaturated 3-pyrrole ring. The other ring is not essential for toxicity and can even be absent.
2. One or two hydroxyl groups or substituted hydroxyl groups attached to the pyrrole ring via one carbon atom.
3. The hydroxyls must be esterified and diesters.
4. A branched chain of the acid moiety must exist.

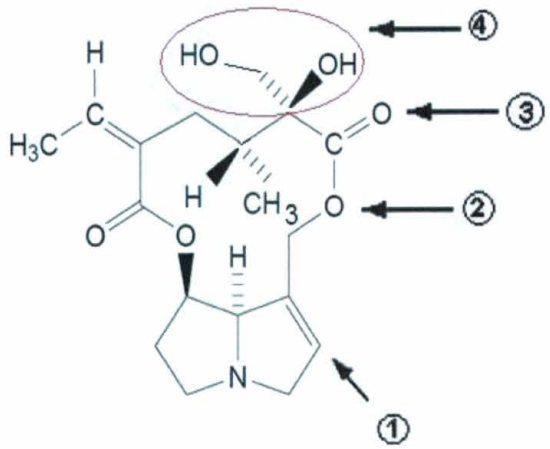


Figure 1-2: Essential structures for hepatotoxicity

1.4 Metabolic activation of pyrrolizidine alkaloids

Activation of cytochrome P450 enzymes and detoxification pathways are considered as the most important factors that determine the amount of didehydropyrrolizidine formation in the target tissues. Other factors affecting the chronic hepatotoxicity of pyrrolizidine alkaloids have been identified (Jago, 1971).

Apart from these factors, structural activity of PAs is required for hepatotoxicity and toxic metabolites (Culvenor, *et al.,* 1976). Numerous reports have identified the formation of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)-derived DNA adduct in the liver of rats treated with different pyrrolizidine alkaloids (Ming, *et al.,* 2003; Yu-Ping, *et al.,* 2005). Dehydropyrrolizidine alkaloids are less stable metabolites that can react with nucleophilic cellular macromolecules in the liver, such as hydroxy-, mercapto- or amino groups of enzymes, globulins, or haemoglobin and purine- or pyrimidine bases of DNA and RNA (Fu, *et al.,* 2002a). This may cause cross-linkages in DNA and RNA, leading to hepatotoxicity or further biotransformations and modifications of the genetic material to generate less toxic or nontoxic metabolites (Thomas, *et al.,* 1998). More stable metabolites can disperse more before exerting an effect. Thus, monocrotaline frequently damages lung tissue, whereas retrorsine, which yields more reactive pyrrolic metabolites, causes only liver damage (Mattocks, 1992).

Detoxification of the pyrrolic metabolites is possible via different mechanisms which can be summarized as follows (Fu, *et al.*. 2002a):

- Alkylation of PA metabolites with constituents such as glutathione which renders more polar products that are easily excreted in urine (Figure 1-3d) page 14.
- Polymerization- it has been shown that metabolites that polymerize easily are also less toxic.
- Hydrolysis- pyrrolic alcohols, rather than DHP, are formed in an aqueous environment.

1.5 Comparative responses

Animal species have different susceptibilities to the toxic effects of PAs (McKnight *et al,* 1991).

1.5.1 The same species responds differently to different PAs

Susceptibility to different plant containing PAs varies across animal species and may not be predictable. However, some plants containing extreme amounts of PAs are very palatable to livestock. Thus, all animals are susceptible. It was found that Guinea pigs are resistant to monocrotoline and susceptible to *Senecio* PAs. Rabbits, pigs, chickens, and other domestic animals however are also resistant to comfrey PA toxicity, while rats appear to be more susceptible (Cheeke, 1979; Grobner, *et al.,* 1985; Yeong, *et al.,* 1993). Additionally, orally administered PAs tend to lead to toxicity, but have less effectiveness compared to the injected PAs. This could be due to overwhelming detoxification pathways when the PAs are delivered to the liver in a bolus. A study showed that rabbits succumb to a single injection of purified *Senecio* alkaloids while they are relatively resistant to chronic feeding of the plant (Schoental and Kelly, 1959).

1.5.2 Species differ in response to the same PAs

Several species feed on PA-containing plants. It has been shown, however, that pigs and chickens are the most susceptible to PA poisoning followed by other poultry, cattle, horses, and goats (Johnson, 1979; King, *et al.,* 1979; Mattocks, 1981), whereas mice and sheep are resistant which could be due the presence of specialized rumen bacteria that can detoxify the alkaloids before they are absorbed. This variation is possibly due to the differences in age and gender which can also influence the susceptibility of animals to the toxic effects of PAs. PA toxicity, however, appears to act differently in different species including humans (Mei and Heflich, 2004); suggesting that the toxin may be a liver metabolite unique for each different species.

1.6 Clinical effects of pyrrolizidine alkaloid toxicity in human

In humans, both acute and chronic toxicity has occurred from ingesting foods contaminated with PAs, particularly herbal products and grains and flours (Bras, *et al.,* 1954; Conradie, *et al.,* 2005; Culvenor, 1983; Huxtable, 1989; Mayer, and Luthy, 1993; Steenkamp, *et al.,* 2000; Tandon, *et al.,* 1978). The liver is the primary target organ in humans, experimental animals, and livestock. Veno-occlusive disease is a characteristic lesion in humans poisoned by PAs (Rollins, 1986; Stewart and Steenkamp, 2001). Other common effects in humans include ascites, splenomegaly, hepatomegaly, centrilobular hepatic necrosis, and cirrhosis. The lungs are the second most common site of PA toxicity, but not all PAs affect the lungs. The primary site of damage is the pulmonary vasculature. Monocrotaline is particularly active in the lung but only at doses that were equal to or greater than doses causing liver toxicity (Ono and Voelkel, 1991).

Pyrrolizidine alkaloid poisoning was discovered in 1954. There are many possible symptoms of PA poisoning and it can take from 2-13 weeks for onset of symptoms after ingestion (Fox, *et al.,* 1978). The severity of symptoms varies depending on the amount of plant consumed and the body size of the person. Children tend to suffer more severe symptoms due to their smaller body size. For most people it is usually severe and even deadly. Many cases of plant poisoning involve plants that are used in medicine (Datta, *et al.,* 1978): traditional Chinese medicine (Kumana, *et al.,* 1985); traditional African medicine (Schoental, 1972); and the folk medicines of Jamaica and Mexico (Fox, *et al.,* 1978; Stillman, *et al.,* 1977).

Veno-occlusive disease was first described in the 1950s in Jamaican children with centrilobular cirrhosis (Rollins, 1986; Stewart and Steenkamp, 2001). Clinically, many patients initially experience the sudden onset of right upper quadrant pain, liver enlargement, and ascites. Further investigation revealed that these patients had a history of ingesting a tea known as bush tea

made from local plants. The bush teas were made from leaves of *Crotalaria* or *Senecio* and contained PAs (Mayer and Luthy, 1993). Other symptoms of PA poisoning may include weakness, abdominal pain and swelling, diarrhea, vomiting, hepatomegaly, and ascites (Ridker, *et al.,* 1985). Veno-occlusive disease has also consistently been associated with ingestion of comfrey teas (Bach, *et al.,* 1989; Selzer and Parker, 1951). 20 cases of veno-occlusive disease in South African children are thought to be caused by exposure to traditional remedies. Hepatic vein occlusion disease has also been found to affect Egyptian children (Panter, *et al.,* 1990) and the presence of PAs was confirmed in the urine of 4 children for whom an on-admission urine specimen was available. Also in South Africa, retrorsine was determined to be present in the traditional herbal remedies administered to two sets of twin infants (a boy and a girl in each set) with veno-occlusive liver disease (Bras, *et al.,* 1954).

The clinical picture of veno-occlusive disease is varied; acute hepatocellular disease have been related to the occurrence of liver disease caused by PAs, but clinical diagnosis at this stage can only be confirmed with biopsy which cannot be easily obtained in this setting. One author hypothesizes that the high incidence of atypical malnutrition cases in Africa (20% of cases) in children could be due to pyrrolizidine alkaloid poisoning (Schoental, 1972).

Veno-occlusive disease was also reported in two infants (2 month old boy and 6 month old girl) in the United States who had consumed herbal tea prepared from *S. longilobus*, a plant known to contain riddelline. The 2-month-old boy developed ascites, splenomegaly, hepatomegaly, and centrilobular hepatic necrosis and died after 6 days in the hospital. The 6-month-old girl initially showed signs of recovery but developed extensive liver fibrosis after 2 months and cirrhosis after 8 months.

At least one case of human embryotoxicity has been reported (Weston, *et al.,* 1987). The mother drank one cup of herbal tea daily throughout her pregnancy. The tea contained 0.6 mg senecionine per kg dry weight and no signs of toxicity were observed; however, the infant was born with fatal veno-

occlusive disease. Toxicity is exacerbated by chronic, small doses, and infants are particularly susceptible. The majority of patients have mild symptoms that resolve without long-term sequelae; however, in severe cases, liver failure from cirrhosis and veno-occlusive disease commonly occurs months to years after exposure (Huxtable 1989); it is estimated that a daily dose of > 1 mg/day for 2 weeks, or > 0.1 mg/day for longer periods could cause liver disease in humans.

1.7 Determination and quantification of toxic pyrrolizidine

Determination of pyrrolizidine alkaloid (PAs) toxicology, tumorigenicity, and hepatotoxicity has attracted the interest of scientists worldwide (Colin, *et al.,* 2010; Johan, *et al.,* 2005) with emphasis on the important aspects of human health. Although the effects of PA toxicity variy considerably between species, pyrrolizidines are seldom suspected at an early stage after poisoning which makes it difficult to identify the suspected toxic plants. Likewise, there have been few attempts to analyse the metabolites of PAs in humans due the lack of toxicity data. Retrorsine, monocrotaline, and senecioic acid are the only readily available commercial products.

Mass spectrometry has been used since early in the 1900s to study the chemical makeup of PAs and their metabolites, the highly toxic nature of many of these PAs has been identified in many different species (Betz, *et al.,* 1994; Colin, *et al.,* 1997; Rashkes, *et al.,* 1978; Winter, *et al.,* 1988; Zuckerman, *et al.,* 2002).

1.8 Biological properties of pyrrolizidine alkaloids

The biosynthetic precursors of most alkaloids are amino acids. In particular, most alkaloids are derived from four different amino acids; lysine, phenylalanine, tyrosine, and tryptophan. (Peter, 2001).

Pyrrolizidine alkaloids are synthesized in plants during amino acid metabolism as N-oxides in the roots of most of the PA producing plants and are translocated to the aerial parts where they are converted into the species-specific alkaloids (Ober and Hartmann, 1999).

1.9 Absorption and metabolic routs of retrorsine activation

1.9.1 Absorption

In plants, pyrrolizidine alkaloids are mostly stored in their non-toxic form. For example, retrorsine is stored mostly in the form of its N-oxide (Mattocks and White, 1971), N-oxides are water soluble and do not dissolve well in the intestine wall, because the inner surface of intestines contains a relatively high concentration of fat. However, PAs do not usually stay in their N-oxide form in the intestines - they are reduced, which means that the oxygen molecule splits off from the nitrogen. The resulting molecule can be absorbed through the intestine wall and will end up in the bloodstream, which passes through the liver where they are metabolized.

1.9.2 Metabolic routes of retrorsine activation

Studies showed that the highest concentrations of pyrrolizidine alkaloids were found in the liver, lungs, kidneys and spleen. In the liver, three major metabolic routes can affect a pyrrolizidine alkaloid molecule as shown in Figure 1-3; each requires some sort of activation, depending on the structure of the acid moiety. Many of these activation reactions consist of biochemical oxidation or hydroxylation. Retrorsine is metabolized to toxic pyrrolic metabolites through the action of the P450 enzymes (Lin, *et al.,* 1998). The principal metabolic pathways involve (i) hydrolysis of the ester functional groups to form the corresponding necine bases and acidic metabolites, which will result in a molecule that is not toxic; (ii) N-oxidation of the necine bases to the corresponding N-oxides, and (iii) formation of the corresponding dehydropyrrolizidine (pyrrolic) derivatives through hydroxylation at the C-3 or C-8 position of the necine base to form 3- or 8-hydroxynecine derivatives followed by dehydration.

1.9.2.1 Hydrolysis

Metabolic reactions occur in the liver. A study showed that esterase hydrolysis (Figure 1-3a) plays a minor role in the formation of Necine base and that a common metabolic pathway may exist between pyrrolic metabolite and INA formation (Pak-Sin, *et al.,* 1993).

The acid moiety structure of PA molecule, with a short and unbraced acid chain esters are more easily hydrolyzed than those where hydrolysis is hindered (Mattocks, 1992).

1.9.2.2 N-Oxidation

N-Oxidation is another metabolic pathway of retronecine-type PAs. The retronecine-type can be catalysed by cytochrome P-450 (CYP) monooxygenases; CYP2E1 is the major subfamily mediating both C- and N-oxidation and oxidative N-demethylation of retronecine-type PAs, and CYP3A of all other three types of PAs (Fu, *et al.,* 2004). N-Oxidation of the necine bases of retronecine-type PAs is also formed metabolically from the alkaloids by oxidation of the nitrogen atom of the molecule (Figure 1-3b), this means that an oxygen atom will bind with the nitrogen generating the corresponding PA N-oxides (Fu, *et al.,* 2002); which are water soluble and will be excreted by the body. It has been shown that the ratio of N-oxide to pyrrolic metabolites varies, depending on the type of ester (Mattocks and Bird, 1983). N-oxides molecules can be metabolically reduced back to the parent PAs in the body and then undergo the aforementioned metabolic activation to form pyrrolic esters, leading to hepatotoxicity and tumorigenicity (Lin, *et al.,* 2000; Xia, *et al.,* 2006).

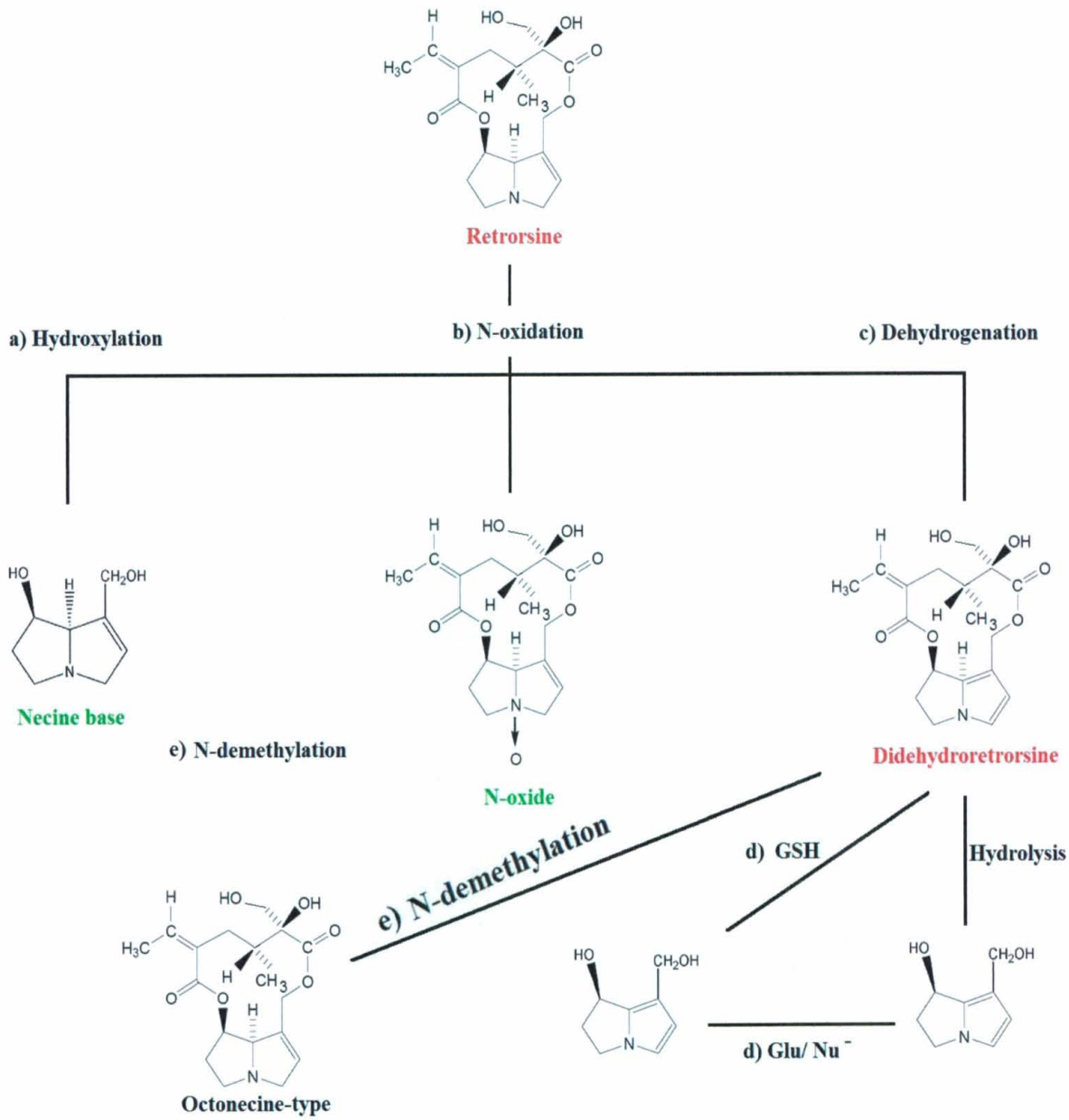


Figure 1-3: Metabolic pathway of 1,2-unsaturated PAs to toxic and non-toxic bases (adapted from Fu, et al, 2002a).

1.9.2.3 Dehydrogenation

As illustrated in Figure 1-3 dehydrogenation starts by converting the retrorsine-heliotridine-type PA by the action of cytochrome P450 enzymes leading to the formation of didehydropyrrolizidine. This metabolite (didehydropyrrolizidine) can further react with glutathione to form four glutathione conjugates and this biotransformation is considered as a detoxification process (Robert, *et al.,* 1975). The intermediate molecules that are formed during the dehydrogenization process are very toxic, because they can damage different kinds of tissue. Because these intermediate molecules are quickly transferred into other molecules, they cannot be found in samples taken from an animal. Only the presence of dehydro-PAs can be shown.

1.9.2.4 Conjugation

Although these dehydro-PAs are more stable than the molecules that are formed in the process in which N-oxides are converted into dehydro-PAs, they can also react with a lot of other molecules such as glutathione (GSH), with which they form DHP-GSH conjugates which are more water-soluble and can be ultimately transfer into urine (Sheweita and Tilmisany, 2003). This is because DHP-GSH contains a double bond, which makes it easier for this molecule to bind and oxidize other molecules, damaging them. Once formed, the pyrrolic ester metabolites can rapidly bind with DNA, leading to DNA cross-linking, DNA-protein cross-linking, and DNA adduct formation (White and Mattocks, 1972; Yang, 2001). The dehydroretronecine (DHR)-derived DNA adducts formed from metabolism of riddelliine, retrorsine, monocrotaline, riddelleiine N-oxide, and retrorsine N-oxide were measured in parallel; the levels of DHP-derived DNA adduct formation were in the order riddelliine> retrorsine>monocrotaline>retrorsine N-oxide or riddelliine N-oxide heliotrine. DHP-derived DNA adducts formation are considered as a potential common biomarkers of pyrrolizidine alkaloid exposure and tumorigenicity (Fu, *et al.*, 2008).

1.9.2.5 N-Demethylation

Some of the pyrrolizidine alkaloids, the octonecine-type PAs, undergo N-demethylation with the eventual formation of a dehydropyrrolizidine alkaloid (Figure 1-3e). It has been found that toxicity decreases when octonecine-type PAs are conjugated with GSH, as they must first be N-demethylated (Lin, *et al.,* 1998).

1.10 Mechanisms of genotoxicity and tumorigenicity

The mutagenicity of PAs has been extensively studied. Although the mechanisms of actions have not been fully identified, DNA adduct formation plays an effective role by which pyrrolizidine alkaloids exert genotoxicity and tumorigenicity consistent with metabolic activation (Fu, *et al.,* 2002; Ming, *et al.,* 2003; Yu-Ping, *et al.,* 2005).

Metabolites and analogues of PAs have been shown to induce tumors in experimental animals in addition to having carcinogenic and genotoxic properties (Fu, *et al,* 2002; Fu, *et al.,* 2007). Several PAs have been shown to share common metabolic activation pathways. Further evidence in support of the genotoxicity and carcinogenic effects are similar to those observed with riddelliine.

1.11 Genetic damage and related effects

DNA adduct formation may plays a role in the genotoxicity of retrorsine and many other PAs. PAs have been tested for genotoxicity in a number of *in vitro* and *in vivo* test systems including prokaryotic and several mammalian systems, where the genetic and related effects of PAs have been reviewed. PAs were found to induce a higher frequency of mutations in endothelial cells than in parenchymal cells in transgenic Big Blue rats (Nan and Tao, 2007). The predominant mutations in the liver cII gene were G·C T·A transversions, which is consistent with PA-induced formation of DNA adducts involving G·C base pairs (Mei and Heflich, 2004).

In rats given PA-N-oxide, the levels of DNA adducts were lower by a factor of 2.6 than in rats

given the same dose of PA. These results indicate that PA-N-oxide, through its conversion to PA, is a potential genotoxic carcinogen. Reference DHR also was reported to be mutagenic in *S. typhimurium*, to induce sister chromatid exchange in human lymphocytes without exogenous metabolic activation, and to induce DNA-DNA and DNA-protein crosslinks (Cheeke, 1976).

1.12 Risk of pyrrolizidines

The risk of chemical contaminants in food should reflect the toxicity of the chemical compound as well as the potential daily intake in a normal diet. Comfrey has been involved in a number of poisonings in people and is considered as the most serious risk of pyrrolizidine alkaloid poisoning in the United States. Cases of human poisoning by comfrey have now been published widely (Dennis, *et al.,* 1993). With all of the concerns about comfrey toxicity, some recommended precautions should be taken in order to benefit from comfrey and it's healing properties without any risk of toxicity. For example, the mature leaves or herb of common comfrey is preferable to be used than any other parts; also, it should be used in combination with other herbs. Furthermore, pregnant or nursing women, or infants under the age of one year, or anyone with a serious liver condition should never consume comfrey or any other related PAs source. This is because the safe level of PA consumption during pregnancy has not been determined and the lack of public health information regarding consumption for small children is cause for concern. Furthermore, the balance between the metabolic activation and detoxification pathways of PA producing non-toxic N-oxides might play a role key in resistance (St-Pierre and De Luca, 1995).

To keep things in perspective, a public Report on Health and Safety was issued in 1978 by the Henry Doubleday Research Association (growers and marketers of comfrey in the United Kingdom) which concluded that until further research clarifies the long-term health hazard from comfrey ingestion, "No human being or animal should eat, drink, or take comfrey in any form" (Henry, 1979).

The death of the great philosopher Socrates which was considered as a major event in Greek history involved alkaloids. After being sentenced to death by poison for corrupting the minds of Athenian youth, Socrates drank a cup of hemlock extract poison containing the potent alkaloid neurotoxin coniine in front of friends (Boer, 1950; Ober, 1977).

1.13 Treatment

Definitive diagnosis requires the histological examination of liver biopsy samples. However, some of the histological changes are characteristic and can be detected. For instance, changes in liver structure produced by the PAs can be used to describe the liver cancer. There are no currently accepted specific medical antidotes, thus the treatment is mainly supportive. Prevention is the best "cure" for PA intoxication. However, one should be clearly aware that diagnosis for cases involving PA poisoning in time in order to prevent lead exposure and poisoning of different PAs and feeding of PAs is discontinued (Stewart and Steenkamp, 200).

A study of rats given monocrotaline has shown success using picroliv, a glycoside from the plant *Picorrhiza kurora,* as a treatment to protect the liver from monocrotaline poisoning (Dwivedi, *et al.,* 1991), however, the treatment was required to be concurrent with the toxin. Currently, Hostege method is being used (Hostege, *et al.,* 1995) to confirm the presence of pyrrolizidines in unknown herbal remedies.

1.14 Retrorsine and human exposure

Retrorsine is a PA that occurs in many medicinal plants (primarily of the genus *Senecio*) that are found in most areas of the western United States and other parts of the world. The available information on human exposure to retrorsine and other PAs is based primarily on case reports of liver toxicity associated with ingestion of herbal products and contaminated grains and flours. The diagnosis of PA toxicity is difficult to establish, and additional cases of poisoning by PAs have probably occurred

(Fu, *et al.,* 2002). *Symphytum officinale, Tussilago farfara, Adenosttyles alliariae, Senecio burchelli, Senecio ilicifolius, Heliotropium lasiocarpum, Heliotropium eichwaldi, Crotalaria Fulva, Crotalaria retusa, Crotalaria nana, and Crotalaria juncea* are plants that have been recorded as causing toxicity in humans (Bach, *et al.,* 1989; Datta, *et al.,* 1978; Ridker, 1989; Stuart and Bras, 1956; Tandon, *et al.,* 1978).

Retrorsine *N*-oxide also is discussed in this section and throughout the document because it can be converted back to retrorsine after ingestion. The quantities of PA *N*-oxides present in plants are highly variable (Fu, *et al.,* 2002) but often can be nearly equal to or even greatly exceed the quantities of parent PAs; in some cases, plants may contain only the *N*-oxide form. Of particular concern is that PA *N*-oxides are much more water soluble than the corresponding PAs (Culvenor, *et al.,* 1981). When plants containing PAs and PA *N*-oxides are used as herbal tea or herbal medicine (e.g., in Chinese herbal medicine), much more PA *N*-oxide than PA will be extracted and ingested. Consequently, it is important to assess the risk to humans posed by drinking herbal teas (including bush teas, comfrey teas, or herb-derived decoctions) that contain PAs and PA *N*-oxides.

1.15 Retrorsine

Retrorsine and retrorsine *N*-oxide have no known commercial uses and are not available from vendors. However, retrorsine-containing plants have occurred in folk medicines and herbal teas in the United States and other parts of the world. The retrorsine-containing plant *Senecio longilobus* has been used in medicinal herbal preparations in the United States (Roger and Coulombe, 2003). Retrorsine has been found to be used in medicinal preparations in other parts of the world especially modern Western and Eastern countries which base medicine on many pharmaceutical preparations and processing Chinese herbs for medicinal preparations. Although retrorsine-containing plants are not used for food in the United States, it has been reported that two plants of the *Senecio* genus (*S. burchellii* and *S.*

inaequidens) have been used as spinach in South Africa (Zuckerman, 2002) and introduced into other parts of the world including Western Europe, Australia, and New Zealand (Bryan, *et al.,* 1999). Accidental exposure has been reported to be caused by ingestion of contaminated flour (Tandon, *et al.,* 1978; Willmot, and Robertson, 1920), milk (Dickinson, *et al.,* 1979) from animals such as goats, which are resistant to the toxin, and honey produced by bees that have fed on pyrrolizidine-containing weeds (Deinzer, *et al.,* 1977; and Ridker, *et al.,* 1989). Russian comfrey, *Symphytum x uplanidum,* have also been found to be used in salads (Yeong, *et al.,* 1993) and herbal teas containing comfrey (McDermott and Ridker, 1990) and the *Crotalaria spp.*, *C. retusa*and *C. fulva* (bush teas). The amount of alkaloid in the leaves and roots of pyrrolizidine-containing plants varies with season, soil type, and climate.

A number of PA-contaning plants are currently used in traditional medicine for the treatment of a wide range of diseases and conditions. Leaves are applied to swellings, cuts, burns, sores, and diseases of the eyes. Infusions (teas) are taken for skin eruptions, chest pains, colic remedy, "madness", nausea, palpitations, to speed childbirth, induce abortion, or for the treatment of constipation and worms. *Heliotropium* spp are used in Indian medicine for intractable fevers, wounds, inflammation, edema, or urticaria. A World Health Organization report on self-medication is available (Estep, *et al.,* 1990).

1.16 Objectives of this study

The first part of the investigation involved the study of the gene expression in rats after exposure to retrorsine; including genes putatively involved in liver abnormalities and carcinogenesis, and genes involved in metabolic activities after retrorsine treatment (through the action of the P450 enzymes), including cytochrome P450 enzymes, using the GeneChip Rat Genome 230 2.0 Array which is a powerful tool for toxicology applications using rat as a model organism.

To gain further insights into the altered regulation of genes the gene expression of the drug

metabolizing genes (ABCB1B, Cyclin G1, CYP2E1, and NQO1) were confirmed using quantitative real-time PCR in the second part of this study. To better understand the biological impact and drug metabolizing enzyme function and expression of retrorsine exposure, genes were associated by Ingenuity Pathway Analysis in which functional annotations of the transcriptional responses were analyzed.

CHAPTER II

2. Materials and Methods

2.1 Samples

A total of six RNA samples, extracted from Male Fischer 344 rat livers were included in this study. Three rats received no treatment (Controls; Control-1, Control-2, Control-3), and three rats were treated with retrorsine (Retrorsine-treated Rats; RTS-1, RTS-2, RTS-3), according to a standard RTS-based protocol for liver repopulation (Laconi *et al.*, 1999).

2.2 Treatment procedure

The retrorsine treatment schedule was based on a standard RTS-based protocol utilized by Laconi *et al.* (1998) at University of Cagliari, Italy. Six-week-old male Fischer 344 rats (approximately 100 g body weight) were randomized into retrorsine treatment (n= 3) and control (n= 3) groups at the outset of the experiment. The treatment schedule for RTS was 2 doses; 30 mg/kg each, 2 weeks apart; Control rats received no RTS treatment. Rats were killed 2 weeks after the second dose. This is the classical treatment for liver repopulation (Laconi, *et al.*, 1999).

2.3 Methods

2.3.1 Isolation of RNA from liver tissues

Total RNA was extracted from retrorsine-exposed and control liver tissues using QIAGEN's RNeasy Total RNA Isolation kit (QIAGEN, Inc., Canada). Purified RNA was stored at –80°C. Quantity and quality of the RNA extracted is discussed below.

2.3.2 Quantification and determination of quality of total RNA

After isolation of RNA, quantification and analysis of quality are necessary to ascertain the approximate quantity of RNA obtained and the suitability of RNA sample for further analysis. This is

important for many applications including RT-PCR and microarray experimentation. The most commonly used methodologies for quantifying the amount of nucleic acid in a preparation are: (i) gel electrophoresis; (ii) spectrophotometric analysis; and (iii) BioAnalyzer analysis.

2.3.2.1 Quantification of RNA

Obtaining high quality, intact RNA is the first and often the most critical step in performing experiments, including RT-PCR and microarray analysis. RNA yield was quantified by spectrophotometric assays (Nanodrop ND-1000; Thermo-Scientific Inc., USA). The absorbance was checked at 260 and 280 nm for determination of sample concentration and purity. The 260/280 ratio was taken for each sample as seen in Table 2-1. Purity was considered good when the ratio was greater than 1.8. This is because nucleic acid is detected at 260 nm, whereas proteins are detected at 230 and 280 nm. A 260/280 ratio of > 1.8 therefore indicates that our extracted is RNA devoid of any of these contaminants.

Table 2-1 summarizes the Quantification of RNA samples. All RNA samples met the assay quality standards to ensure the highest quality RNA is hybridized to the gene expression arrays.

Table 2-1: Purity and Integrity of RNA Isolated from RNA samples.

Sample ID	µg/ul	260/280
Control-1	4.06050	1.81
Control-2	4.14525	1.82
Control-3	2.89040	1.91
Retrorsine treated-1	2.26288	1.94
Retrorsine treated-2	3.87838	1.80
Retrorsine treated-3	4.12576	1.81

2.3.2.2 Visualization of RNA preparations on 1.5% agarose gels

Agarose gel electrophoresis is a convenient method for visualizing the molecular mass of the extracted RNA. Two µl of each RNA sample was loaded into wells of a 1.5% agarose gel, after mixing with 2 µl of 5X loading dye (Promega, USA), which helps keep samples to the bottom of the wells and enables us to follow the progress of electrophoresis run. Also, 2 µl of 100 bp ladders (Qiagen, NEB Inc., Canada) was loaded into gel. 1X Tris borate EDTA (TBE) running buffer was used to electrophorese the gel under a constant voltage (80 V) for 1 hour. 5 µl ethidium bromide (Biorad, NEB Inc., Canada), an intercalating agent was added to the buffer to stain RNA, and the gel was visualized on the UV transluminator provided with the gel documentation system (Biorad, USA).

2.3.3.3 BioAnalyzer

RNA quality was determined using a BioAnalyzer 2100, which was supported by The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada (TCAG).

2.4 Microarray experiment

Six GeneChip Rat Genome 230 Arrays for rat expression analysis were used in this study. GeneChip Rat Genome 230 2.0 Arrays in cartridge format have been used extensively for a wide variety of applications including the discovery of new target genes involved in cardiac ischemia, global analysis of gene expression in skeletal muscular activity, transcriptional profiling of liver disease and analysis of signaling pathways related metabolism and development.

Microarray experiments using the Affymetrics protocol consist of three basic steps which were supported by The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada: sample preparation and labeling, sample hybridization and washing, and microarray image scanning and processing.

2.4.1 Sample preparation and labeling

Sample preparation involves extracting and purifying the mRNAs from rats liver tissues. Due to a number of challenges, this procedure can be quite variable (Amaratunga and Cabrera, 2003); Stekel, 2003). mRNA degrades very quickly. To address this rapid degradation, the mRNA is usually reverse-transcribed into more stable cDNA (for cDNA microarrays) immediately after extraction (Amaratunga and Cabrera, 2003). To allow detection of which cDNAs are bound to the microarray, the sample undergoes a platform-dependent labeling process. For the Affymetrix platform, a biotin-labeled complementary RNA is constructed for hybridizing to the GeneChip. The protocols are very carefully defined by Affymetrix to ensure that every Affymetrix laboratory follows identical steps. Experimental results obtained in different Affymetrix laboratories should therefore be reliably comparable (Stekel, 2003).

2.4.2 cRNA synthesis

All RNA samples were labeled using the Eukaryotic Poly-A RNA Control Kit (Affymetrix) which is designed specifically to provide exogenous positive controls to monitor the entire eukaryotic target labelling process; a set of poly-A RNA controls was supplied in the Kit. Each sample was reverse transcribed, using a T7-oligo (dT) primer, and double-stranded cDNA was synthesized. The double-stranded cDNA, with the incorporated T7 promoter, was then used as a template in the subsequent *in vitro* transcription reaction. The protocol was supported by The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada.

2.4.3 Hybridization and washing

Hybridization is the step in which the RNA probes on the microarrays and the labeled RNA targets form heteroduplexes according to the Watson-Crick base-pairing rule (Stekel, 2003). The essential principle here is that a single-stranded RNA molecule will bind to another single-stranded

RNA molecule with a precisely matching sequence with much higher affinity than that to an imperfectly matching sequence (Amaratunga and Cabrera, 2003). However, hybridization is a complex process and a RNA segment may bind well to a sequence similar but not identical to its complementary target, a phenomenon called cross-hybridization. This is influenced by many conditions, including temperature, humidity, salt concentration, formamide concentration, target solution volume, and hybridization operator (Stekel, 2003). Hybridization may be performed either manually or by a robot. Robotic hybridization provides much better control over the temperatures of the target and slide. The consistent use of a single hybridization station also reduces the variability which arises from multiple hybridizations and various operators (Stekel, 2003). After hybridization, the microarray is removed from the chamber or station and is then washed to eliminate any excess labelled sample so that only the RNA complementary to the probes remains bound on the array. Finally, the microarray is dried using a centrifuge or by blowing clean compressed air (Amaratunga and Cabrera, 2003).

2.4.4 Microarray image scanning

After the completion of hybridization, the surface of the hybridized array is scanned to produce a microarray image. As previously mentioned, samples are labeled with biotin or fluorescent dyes that emit detectable light when stimulated by a laser. The emitted light is captured by the photo-multiplier tube (PMT) in a scanner, and the intensity is recorded. Most scanners contain one or more lasers that are focused onto the array (for two-channel microarrays, the scanner uses two lasers) (Stekel, 2003).

Although the scanner is only intended to detect light emitted by the target RNA strands which are bound to their complementary probes, it will capture incidental light from various other sources. These other sources may include labeled RNA sample which has hybridized non-specifically to the glass slide, residual (unwashed) labeled sample which has adhered to the slide, various chemicals used in processing the slide, and even the slide itself. This incidentally-captured light is called background

(Stekel, 2003). The scanned output of an Affymetrix chip is usually a monochrome image. With two-channel microarrays, the output is a pair of monochrome images. Each image is from one of the lasers in the scanner. The two monochrome images are combined to create the false color images of microarrays Both monochrome and two-color images are usually stored in the tagged image file format (TIFF).

2.4.5 Analysis of microarray data

Microarray analysis was performed on gene expression changes in liver samples (control n=3, and retrorsine-treated n=3). Gene expression alterations caused by exposure to retrorsine were compared to controls using GeneSpring GX.11 Software (Agilent Technologies, Inc., Canada).

2.4.5.1 Normalization of microarray data

Normalization is usually the first step required in microarray data analysis and it attempts to reduce the experimental variability across different array spots while maintaining biological variability (Hegde, *et al.,* 2000). Figure 2-1 shows the distribution of normalized values of the probe sets within each sample which is displayed in the box-Whisker Plot using the RMA algorithm, with the six samples on the X-axis and the Log Normalized Expression values on the Y axis. Entities with intensity values beyond 1.5 times the inter-quartile range are shown in red.

Figure 2-2 shows the $\log_2$ raw intensity data of the six samples (3 controls and 3 retrorsine-treated samples). The results of the scatter plotting of the overall gene expression profiles demonstrated the high quality and reproducibility of microarray data obtained.

The diagonal elements of the correlation matrix will be 1 since they are the correlation of a column with itself. The correlation matrix is symmetric since the correlation of each column on the x-axis of the six samples with the column of each sample on the y-axis is the same as the correlation of each column on the y-axis with each column on the x-axis.

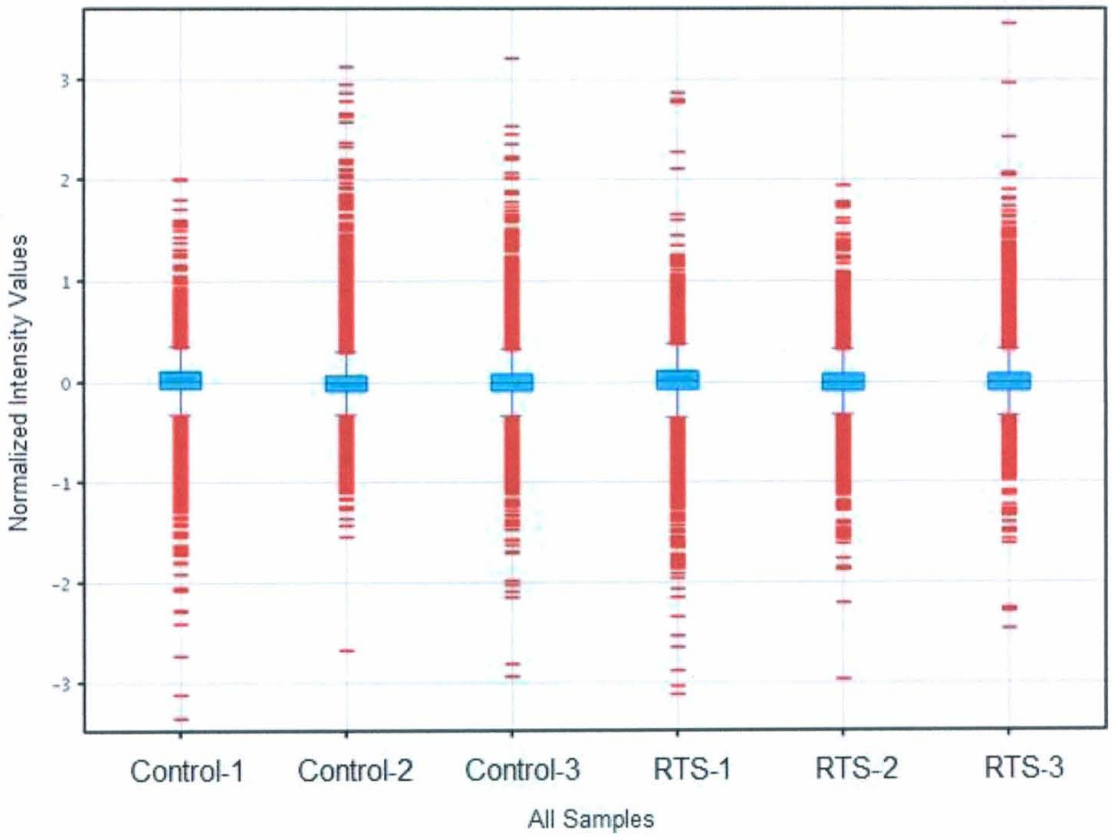


Figure 2-1: Box-Whisker plot, with the samples on the X-axis and the log normalized expression values in the Y-axis.

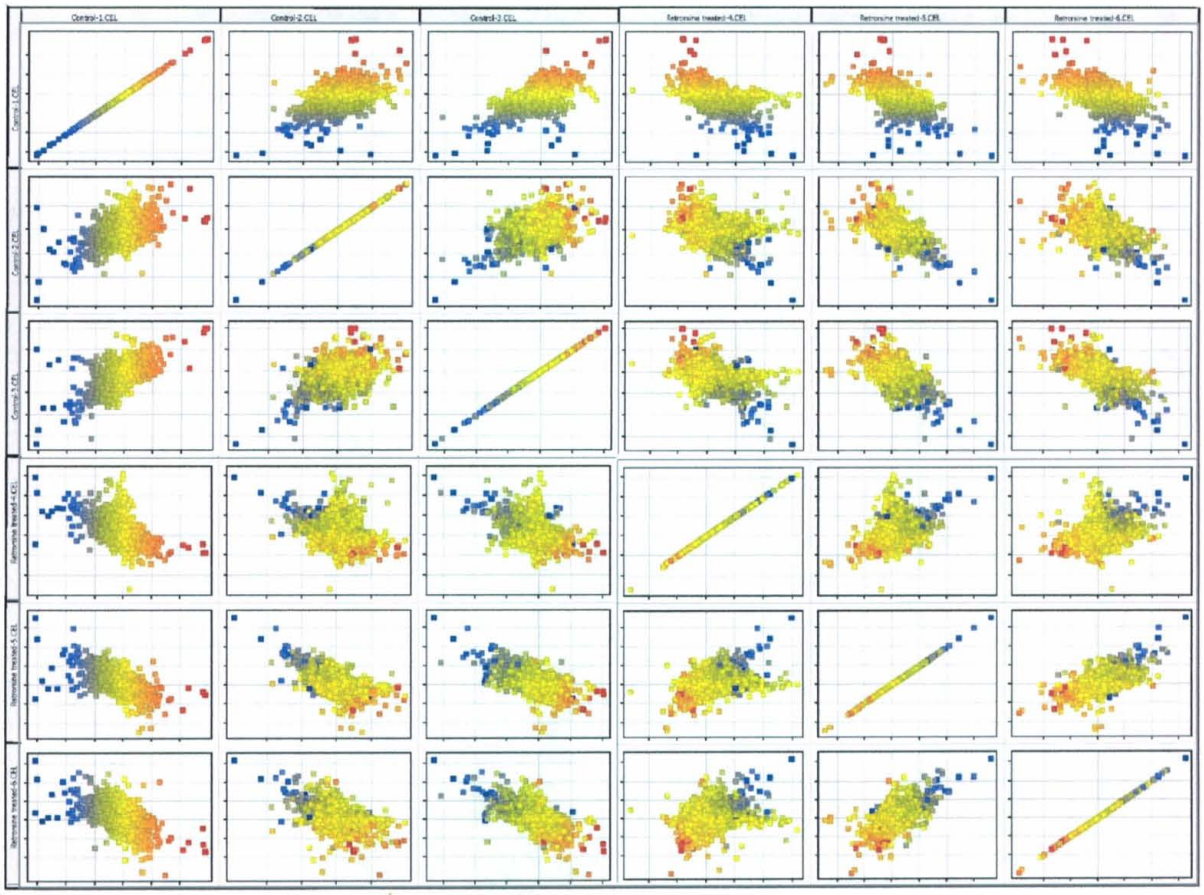


Figure 2-2: The $\log_2$ raw intensity data of the six samples (Control 1, 2, 3, and RST 4, 5, 6) were plotted against each other.

2.5 Quantitative Real-Time PCR analysis (qPCR)

2.5.1 Preparing cDNA

2.5.1.1 First strand cDNA synthesis from total RNA

Complementary DNA (cDNA) was synthesized from 1-2 µl of total RNA. Each sample was reverse transcribed, using the High Capacity_RNA-to-cDNA_Kit (Applied Biosystems, Canada). For each sample, 10 µl 2X RT Buffer was mixed with 1 µL 20X Enzyme Mix, and 7-8 µl nuclease-free water was added to the mix, for a final reaction volume of 20 µl.

2.5.1.2 Thermocycler settings

Reverse transcription was performed using the thermal cycler under the conditions shown in Table 2-2; these conditions are optimized for use with the High Capacity RNA-to-cDNA Kit. Prepared cDNA RT tubes were placed at -15 to- 25°C for long-term storage after amplification.

Table 2-2: Thermal cycler conditions of RNA-to-cDNA Reverse transcription.

	Step 1	Step 2	Step 3
Temperature (°C)	37	95	4
Time	60 min	5 min	∞

2.5.1.3 cDNA quantification

cDNA concentration and quality were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop ND-1000, ThermoScientific etc). Table 2-3 shows the good quality of the cDNA from each sample in which 260/280 ratio are 1.8 or greater and 260/230 are 2.0 or greater.

Table 2-3: Purity and Integrity of cDNA yields from reverse transcription.

Sample ID	µg/µl	260/280	260/230
Control-1	1.75223	1.85	2.26
Control-2	1.60802	1.86	2.26
Control-3	1.51396	1.86	2.26
Retrorsine treated-1	1.55707	1.85	2.28
Retrorsine treated-2	1.56447	1.85	2.23
Retrorsine treated-3	1.62617	1.85	2.25

2.5.2 Gene expression analysis using TaqMan® Assays

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, but in nonprotein coding genes such as rRNA genes or tRNA genes, the product is a structural or housekeeping RNA. In addition, small non-coding RNAs (miRNAs, piRNA) and various classes of long non-coding RNAs are involved in a variety of regulatory functions. Real-time polymerase chain reaction (RT-PCR) is used to confirm the changes of gene expression – increases or decreases – of the genes investigated with microarrays by measuring the abundance of the gene-specific transcript. The investigation monitors the response of a gene to treatment with a retrorsine.

2.5.2.1 TaqMan® Gene Expression Assay chemistry

Real-time PCR reactions were performed using TaqMan® Gene Expression Assays (Applied Biosystems AB) and TaqMan® Gene Expression Assays protocol (Applied Biosystems, USA). TaqMan® Gene Expression Assays have been pre-designed by the Applied Biosystems (Applied Biosystems, USA) with optimized parameters such as %GC content, melting temperature, and amplicon length to ensure that all of the TaqMan® assays have high amplification efficiency. The TaqMan® chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. Expression Assays are based on 5' nuclease chemistry and consist of a FAM™ dye-labeled TaqMan® MGB probe (250 nM, final concentration), and two unlabeled PCR primers (900 nM each, final concentration). All probes contain a FAM reporter dye at the 5' end of the MGB probe and a non-fluorescent quencher at the 3' end. The TaqMan® MGB probes and primers were premixed to concentrations of 18 μM for each primer and 5 μM for the probe. Amplifications were carried out in a final reaction volume of 20 μl. IDs for gene assays and gene symbols are explained in Appendix B. All components were quality-control (QC) tested and formulated into a single

20X mix, and designed to run under universal conditions for two-step RT-PCR by the company.

Detection of real-time PCR product using TaqMan® Assays is based on binding of the TaqMan® probe to a complementary target sequence and release of the fluorescent reporter dye from FRET by DNA polymerase. In addition, no fluorescence signal is produced unless the TaqMan® probe is cleaved by the DNA polymerase as it extends the PCR primer.

2.5.2.2 Performing PCR amplification

2.5.2.2.1 Assay optimization

To ensure efficient and accurate quantification of the target template, RT-PCR assays was optimized and validated. cDNA from control samples (Control-1, Control-2, Control-3) were used as a template for optimization reactions. The cDNA was diluted in a 5-fold dilution range to obtain a dilution series with known amounts of cDNA. Table 2-4 shows the volumes of each PCR reaction mix components that were used to prepare four replicates of each 20-µL PCR reaction mix of each dilution for each assay.

Table 2-4: PCR reaction mix components for Q-RT-PCR with TaqMan Gene Expression Assay.

PCR reaction mix component	Volume per 20-µL reaction (µL)	
	Single reaction	Three replicates‡
20X TaqMan® Gene Expression Assay	1.0	4.0
cDNA template§ + RNase-free water	9.0	36.0
2X TaqMan® Gene Expression Master Mix#	10.0	40.0
Total Volume	20.0	80.0

2.5.2.2.2 Thermal cycling conditions

PCR amplification was performed using the StepOne™ Real-Time thermal cycler under the following conditions shown in (Table 2-5); these conditions are optimized for use with Applied Biosystems StepOne™ Real-Time.

Table 2-5: StepOne™ system thermal cycling conditions.

Run type	Reaction plate	Stage	Temp (°C)	Time
Standard	96-well standard	Hold‡	50	2 min
		Hold	95	10 min
		Cycle (40 cycles)	95	15 sec
			60	1 min

‡ Required for optimal AmpErase® UNG activity; not needed when UNG is not in the reaction.

2.5.2.2.3 qPCR analysis methods

The software for the StepOne™ Systems supports a variety of analysis methods, including: Absolute Quantitation (Standard curve), and Relative Quantitation (Comparative C_T). The absolute quantitation assay is used to quantitate unknown samples by interpolating their quantity from a standard curve to know the exact copy number of the target RNA in the sample in order to monitor the progress of retrorsine treatment.

2.5.2.2.3.1 PCR reaction efficiency

The amplification efficiency of the PCR reaction is one of the major concerns regarding any real-time PCR-based assay (including TaqMan® probe and primers). The slope of the standard curve is used to determine reaction efficiency. Since the PCR reaction is based on exponential amplification, if the efficiency of PCR amplification is 100% the amount of template will double with each cycle, and the standard curve plot of the log of starting template vs. PCR cycles which generate a linear fit with a slope between approximately –3.1 and –3.6 are typically acceptable for most applications requiring accurate quantification (90–110% reaction efficiency).

To address this concern, efficiency values were measured using the C_T slope method. This method involves generating a dilution series of the target template (cDNA) and determining the C_T value for each dilution. A plot of C_T versus log cDNA concentration is constructed. With this method,

the expected slope for a 10-fold dilution series of cDNA is -3.32, when PCR amplification efficiency = 1.0. The StepOne software calculated the slope, amplification efficiency, R^2 value (correlation coefficient), and C_T values for all samples of each assay.

Figure 2-3 illustrates a five-fold dilution series standard curve over the five assays; Endogenous Control (ACTB), ABCB1B, Cyclin G1, CYP2E1, and NQO1 obtained by real-time PCR with a slope range between -3.19 to -3.474 which is well within the acceptable range of -3.1 to -3.6 and an amplification efficiency values (R^2) of 94.023%, 105.793%, 100.754%, 98.028%, and 101.085% respectively, again, within the acceptable parameters >0.985. Amplification efficiency was calculated from the slope of this graph using the equation: Ex = 10(-1/slope) – 1.

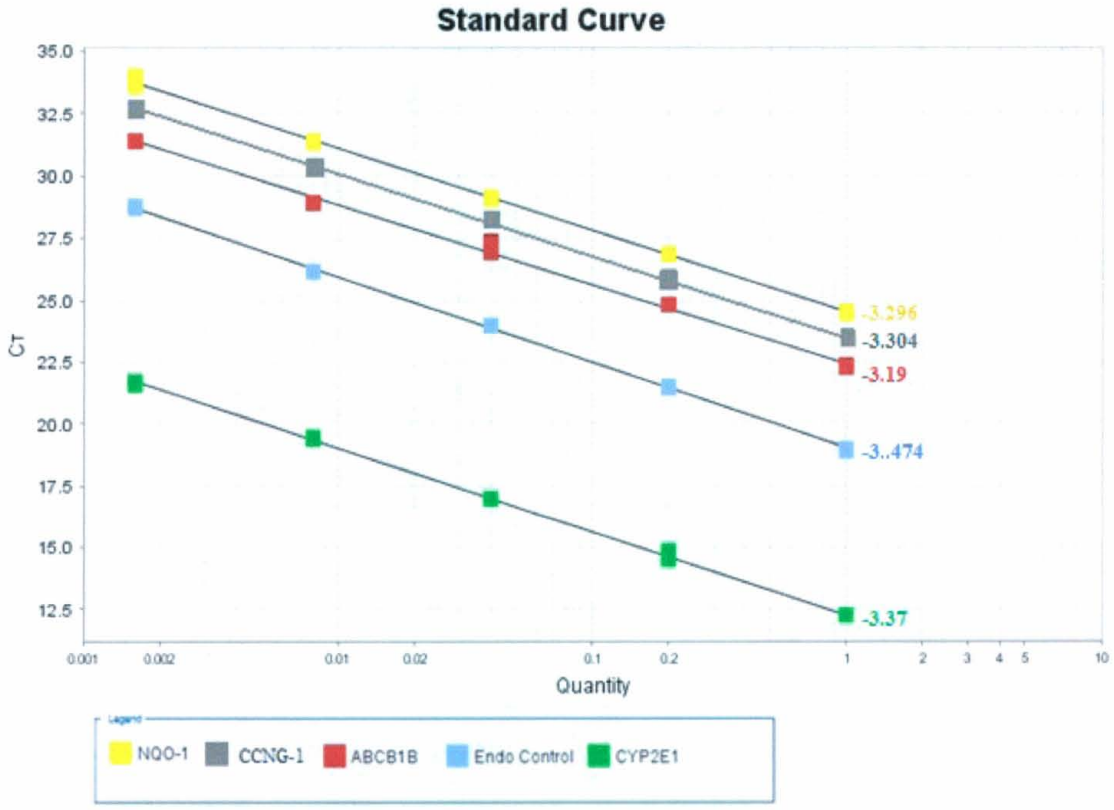


Figure 2-3: Standard curves with five points of each Gene Expression Assay (NQO-1, CCNG-1, ABCB1B, Endogenous Control, and CYP2E1) obtained by real-time PCR (RT-PCR).

A serial dilution of Endogenous Control (ACTB), ABCB1B, Cyclin G1, CYP2E1, and NQO1 cDNA ranging from 1:5, 1:25, 1:125, 1:625, and 1:3125 was analyzed by StepOne Q-RT-PCR. The assays had amplification efficiency values (R^2) of 0.997, 0.999, 0.995, 0.997, and 1 respectively; the R^2 value is a measure of the closeness of fit between the regression line and the individual C_T data points of the standard reactions. A value of 1 indicates a perfect fit between the regression line and the data points. An R^2 value >0.99 is desirable. For the precision analysis, the dilutions were chosen according to the ranges of C_T values (19, 22, 27, 22, and 28; respectively), that were characteristic for the expression levels of the particular reference genes (Endogenous Control (ACTB), ABCB1B, Cyclin G1, CYP2E1, and NQO1) in the samples. The dilution series for untreated samples were done in triplicate to PCR amplify the target sequence (ABCB1B, Cyclin G1, CYP2E1, and NQO1), and Endogenous Control. The C_T value was indicated on the Y axis and the amount of cDNA on the X axis as seen in (Figure 2-3). A C_T value >8 and <35 is desirable. A C_T value <8 indicates that there is too much template in the reaction. A C_T value >35 indicates a low amount of target in the reaction; the results here showed that all C_T values were between 24 and 26.

The threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets the threshold as seen in Figure 2-4. The threshold of each assay was automatically set by the StepOne software to be in the region where the plots are all linear and where they are all as close as possible to being parallel to one another and not too high that it crosses any of the plots where they are starting to plateau and no longer linear.

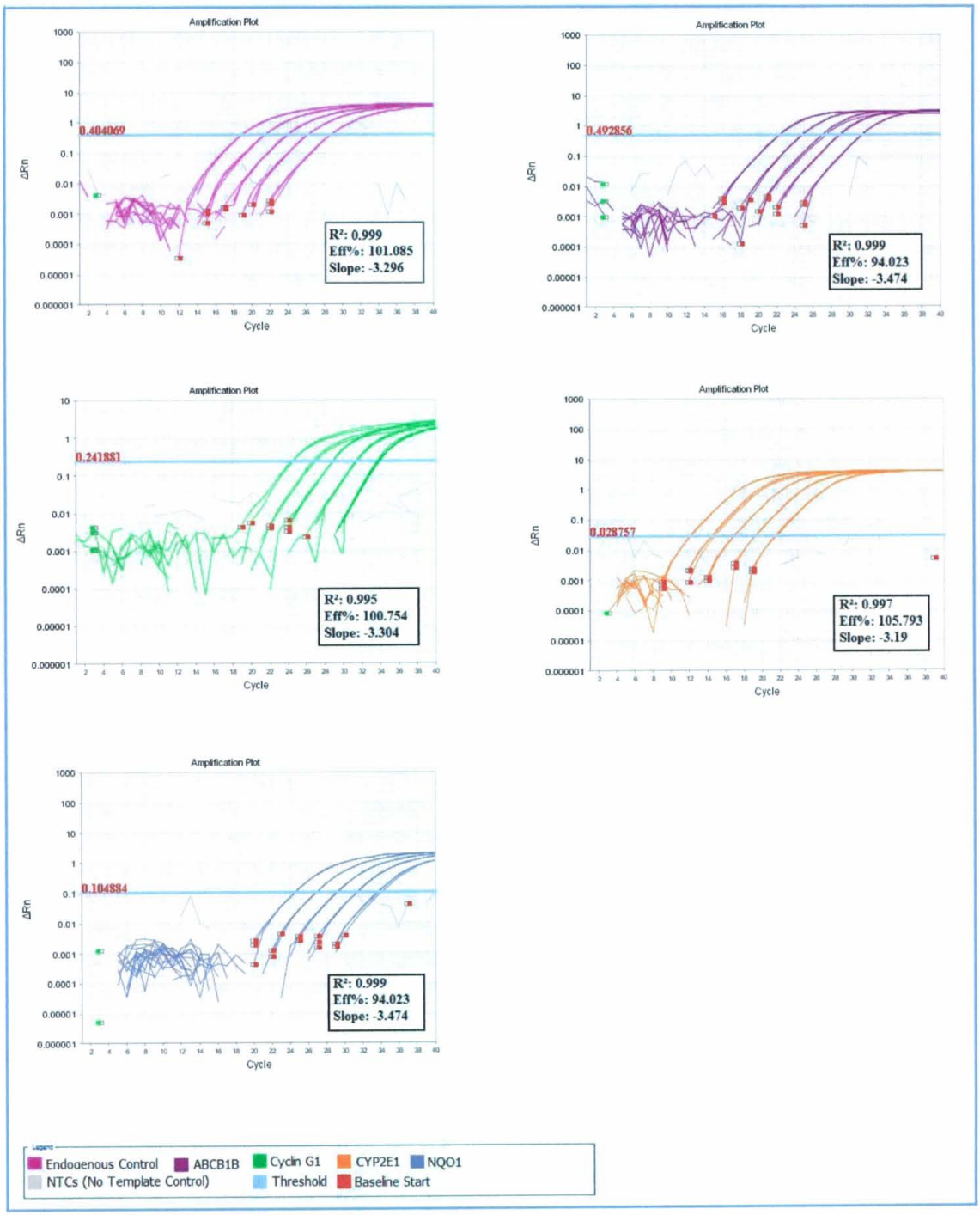


Figure 2-4: Amplification plots viewed with the Y Axis set to a log scale of five-fold dilution series of each assays (Endogenous Control, ABCB1B, Cyclin G1, CYP2E1, NQO1). The optimal setting for the threshold is the point where all the log plots are linear and parallel, as shown in where the threshold is set here for each assay.

2.5.2.2.3.2 Comparative quantification

Another quantification approach is termed the comparative C_T method. This involves comparing the C_T values of the retrorsine treated samples (RST-1, RST-2, RST-3) with the non-treated samples (Control-1, Control-2, Control-3). The C_T values of both the calibrators and the samples of interest are normalized to an appropriate endogenous housekeeping gene (ACTB). The comparative C_T method is also known as the 2–[delta][delta]C_T method, where [delta][delta]C_T = [delta]$C_{T,sample}$ - [delta]$C_{T,reference}$. Here, [delta]$C_{T,sample}$ is the C_T value for any sample normalized to the endogenous housekeeping gene and [delta]$C_{T,reference}$ is the C_T value for the calibrator also normalized to the endogenous housekeeping gene.

Relative quantification was used to determine the changes in cDNA levels of the ABCB1B, Cyclin G1, CYP2E1, NQO1 genes across retrorsine treated/untreated samples and expresses it relative to the levels of Endogenous Control. Calculations were based on the comparison of the distinct cycle determined by cycle threshold values (C_T) at a constant level of fluorescence to determine C_T the expression of a target genes in relation to a specific housekeeping gene (ACTB) actingas an endogenous control. Figure 2-5 shows a typical reading from the StepOne qPCR thermal cycler of differences of ABCB1B, Cyclin G1, CYP2E1, and NQO1 gene expression and Endogenous Control (ACTB) gene expression in liver tissues as a C_T values. The lower a C_T value, the more copies are present in the specific sample.

For each gene (ABCB1B, Cyclin G1, CYP2E1, NQO1 and Endogenous Control), samples of three non-treated (control-1, control-2, control-3) and three retrorsine-treated (retrorsine-treated-1, retrorsine-treated-2, retrorsine-treated-3) were run in quadruplicates. A no-template control (NTCs) was performed which showed no contamination (Figure 3-5). A positive control using a housekeeping gene (ACTB) that is relatively abundant in all samples was also performed to allow for comparisons

between samples. The quantification arises by measuring the amount of amplified product at each stage during the PCR cycle. cDNA from genes with higher copy numbers will appear after fewer PCR cycles as seen in Figure 2-5.

Target mRNAs from retrorsine-treated (n=3) and control (n=3) samples are assayed simultaneously for all genes ABCB1B, Cyclin G1, CYP2E1, NQO1 and Endogenous Control, each serving as a competitor for the other, making it possible to compare the relative abundance of target between samples. From Figure 2-5 all ABCB1B, Cyclin G1, CYP2E1, NQO1 genes for all retrorsine-treated samples showed a higher copy number and a lower C_T values compared with the non-treated samples.

2.5.2.2.3.3 Statistical analysis

Data handling was simplified by automating all calculations in an Excel® worksheet, which enabled the rapid calculation of P values for each gene using GraphPad Prism software (GraphPad Prism).

All data are expressed as mean standard error of measurement (SEM). Unpaired t-tests were used and differences were considered significant for values of $P< 0.001$. Mean fold changes between the two groups (retrorsine-treated and untreated) were calculated by averaging the quadruplicate measurements for each gene. The relative fold difference calculation used the $2^{-\Delta\Delta CT}$ method (Livak, 2001).

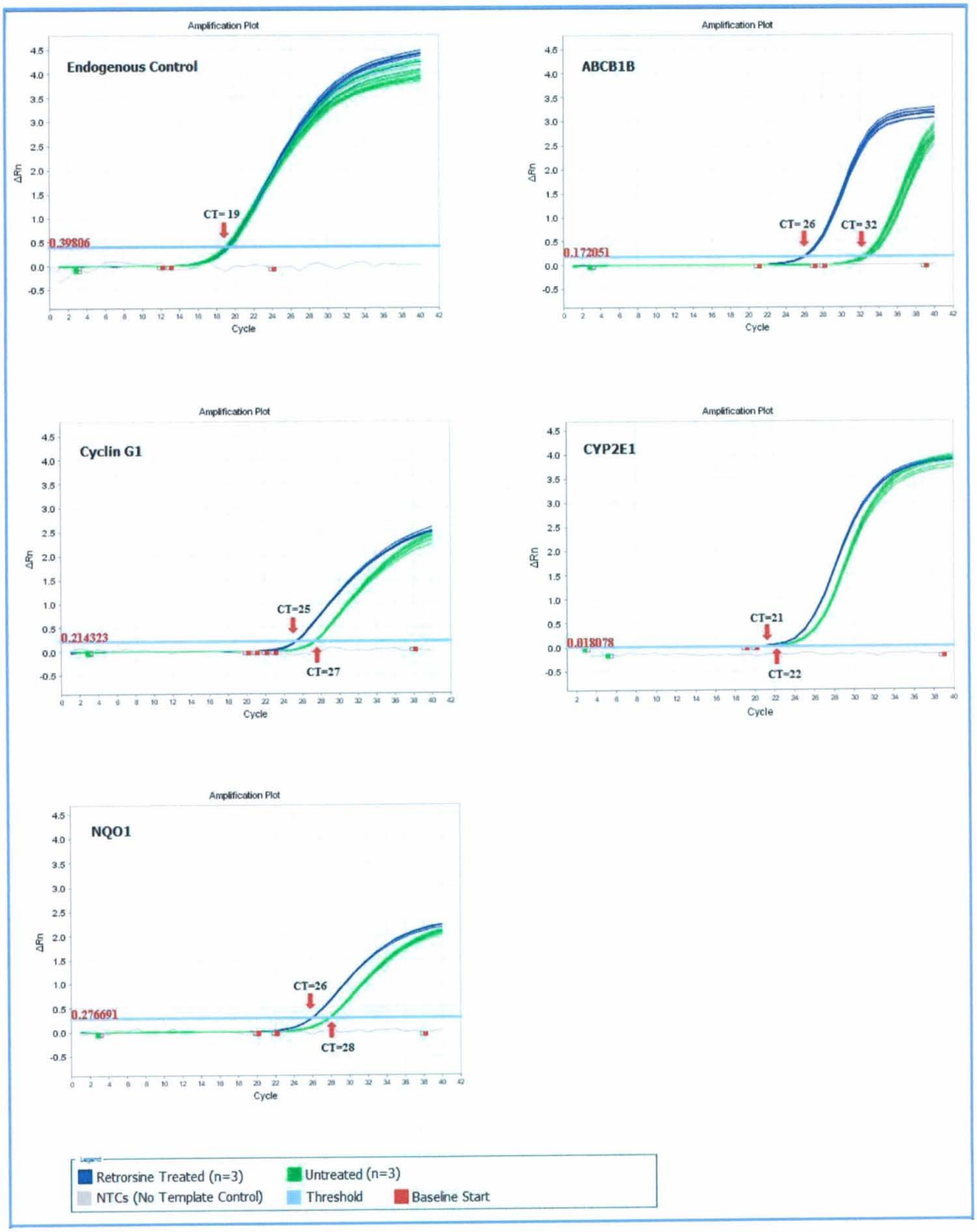


Figure 2-5: Effect of retrorsine treatment on ABCB1B, Cyclin G1, CYP2E1, and NQO1 gene expression and on Endogenous Control (ACTB) gene expression in liver tissues. Expression differences are shown by ΔC_T values.

CHAPTER III

3. Results

3.1 RNA quality

RNA was successfully isolated from the six rat tissue samples. Figure 3-1 shows two distinct ribosomal bands corresponding to 18S and 28S for rats rRNA visible half way down the gel. Non-distinct RNA bands were not seen as a lower molecular weight smear, as a result rRNA degradation was not found. Gel electrophoresis thus provides an indication of the quality of the RNA preparation.

The BioAnalyzer electropherograms showed a high-quality total RNA sample from both controls and retrorsine-treated rats as seen in Figures 3-2 and 3-3; two well-defined peaks with ratios approaching 2:1 corresponding to the 18S and 28S ribosomal RNAs were observed, similar to the denaturing agarose gel as seen in Figure 3-1. The baseline between the internal marker and the 18S rRNA peak is relatively flat and free of small rounded peaks corresponding to smaller RNA molecules that could be degradation products of the rRNA transcripts. The electropherograms were checked for the absence of high-molecular nucleic acids that indicate contamination with DNA. The integrity of total RNA can be easily assessed by evaluation of the 18S and 28S units of ribosomal RNA.

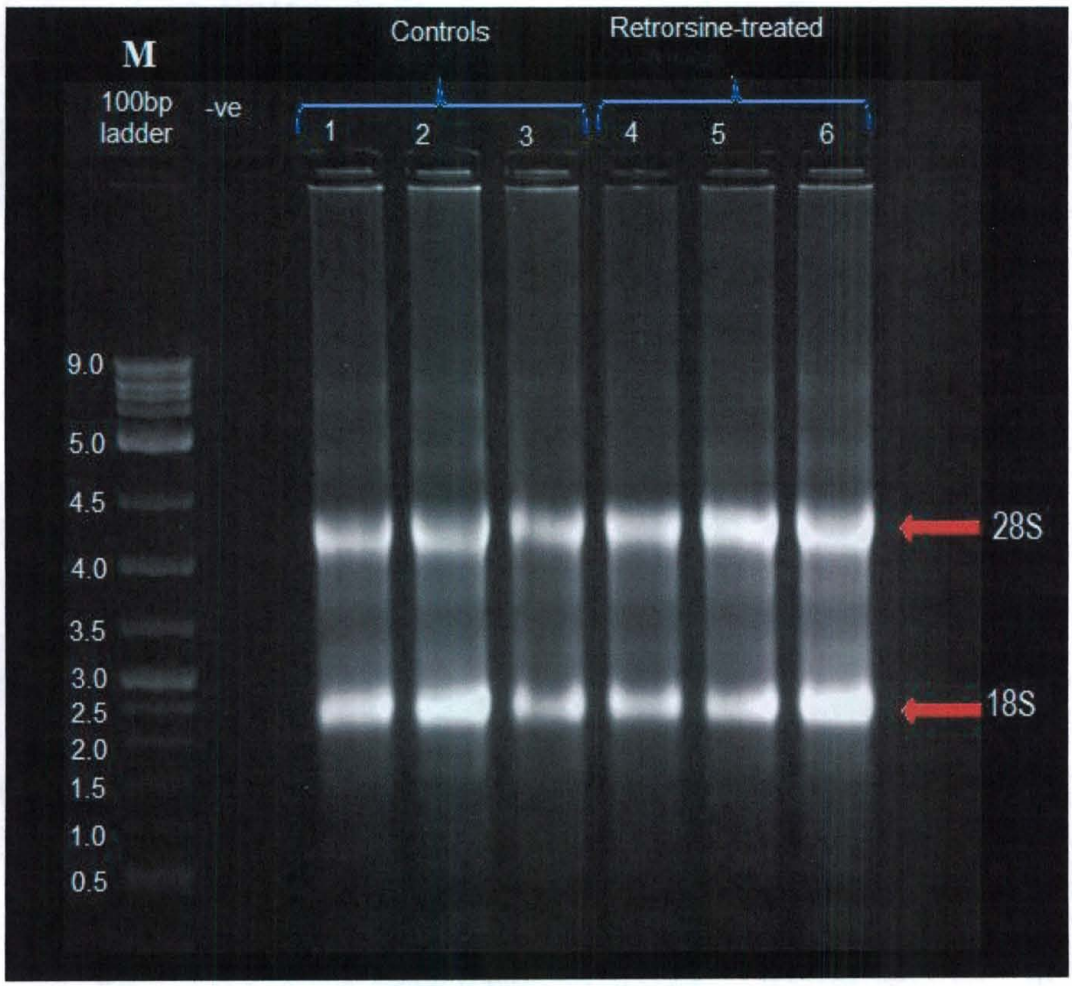
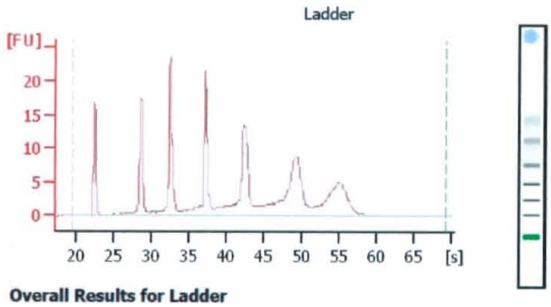


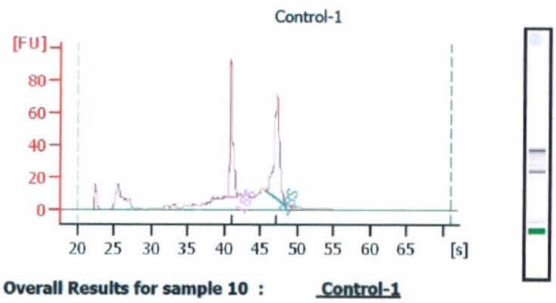
Figure 3-1: Quality of RNA Isolated from Rats liver Tissue. 1.5% Agarose gel of total RNA extracted from rats liver tissue using RNeasy kits. 1.5 µg RNA was loaded per lane, (Lane 1-3) RNA extracted from Controls rats liver tissue, (Lane 4-6) RNA extracted from Retrorsine treated rats liver tissue, M; 100bp marker, -ve; negative control. Bands for (upper arrow) 28S rRNA and (lower arrow) 18S rRNA are shown.

Electropherogram Summary



Overall Results for Ladder

RNA Area:	228.3
RNA Concentration:	150 ng/µl
Result Flagging Color:	
Result Flagging Label:	All Other Samples

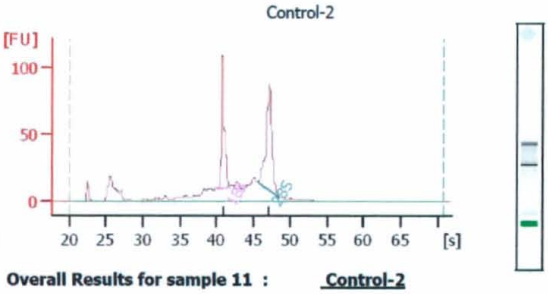


Overall Results for sample 10 : Control-1

RNA Area:	561.0
RNA Concentration:	369 ng/µl
rRNA Ratio [28s / 18s]:	2.0
RNA Integrity Number (RIN):	8.3 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.30

Fragment table for sample 10 : Control-1

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.26	41.86	92.8	16.5
28S	45.77	48.55	120.1	21.4

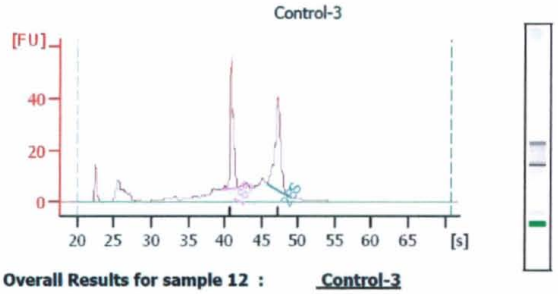


Overall Results for sample 11 : Control-2

RNA Area:	712.6
RNA Concentration:	468 ng/µl
rRNA Ratio [28s / 18s]:	1.9
RNA Integrity Number (RIN):	8.2 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.20

Fragment table for sample 11 : Control-2

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.17	41.76	114.5	16.1
28S	45.62	48.49	144.7	20.3



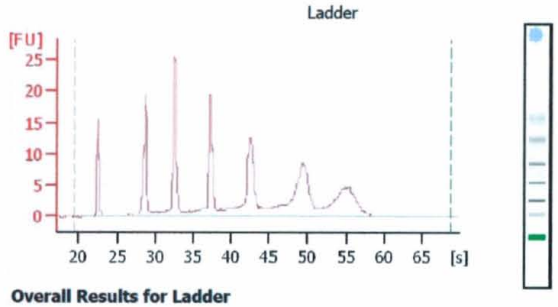
Overall Results for sample 12 : Control-3

RNA Area:	372.8
RNA Concentration:	245 ng/µl
rRNA Ratio [28s / 18s]:	1.9
RNA Integrity Number (RIN):	8.2 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.20

Fragment table for sample 12 : Control-3

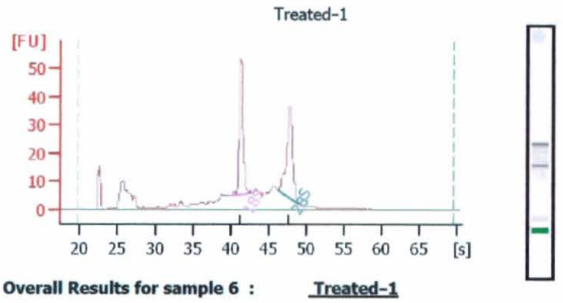
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.66	41.82	61.2	16.4
28S	45.82	48.60	68.0	18.2

Figure 3-2: Electropherogram (from the Agilent 2100 Bioanalyzer) for Rats Control Total RNA. A high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs were observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands.



Overall Results for Ladder

RNA Area:	224.3
RNA Concentration:	150 ng/µl
Result Flagging Color:	
Result Flagging Label:	All Other Samples

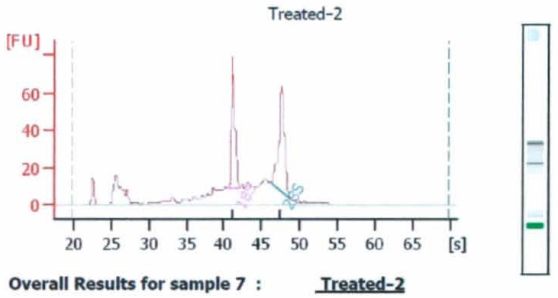


Overall Results for sample 6 : Treated-1

RNA Area:	374.6
RNA Concentration:	250 ng/µl
rRNA Ratio [28s / 18s]:	1.9
RNA Integrity Number (RIN):	8.1 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.10

Fragment table for sample 6 : Treated-1

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.90	42.23	59.6	15.9
28S	46.26	49.09	62.8	16.8

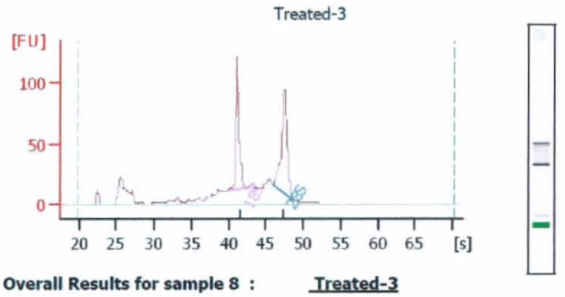


Overall Results for sample 7 : Treated-2

RNA Area:	588.6
RNA Concentration:	394 ng/µl
rRNA Ratio [28s / 18s]:	2.0
RNA Integrity Number (RIN):	7.9 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.90

Fragment table for sample 7 : Treated-2

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.44	42.11	87.1	14.8
28S	46.06	48.85	106.7	18.1



Overall Results for sample 8 : Treated-3

RNA Area:	843.6
RNA Concentration:	564 ng/µl
rRNA Ratio [28s / 18s]:	2.0
RNA Integrity Number (RIN):	7.9 (B.02.07)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.90

Fragment table for sample 8 : Treated-3

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.37	42.61	130.4	15.5
28S	45.97	48.82	157.7	18.7

Figure 3-3: Electropherogram (from the Agilent 2100 Bioanalyzer) for Retrorsine –treated Rats Total RNA. A high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs were observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands.

3.2 Microarray analysis

3.2.1 Principal Component Analysis (PCA)

After data normalization by Quantile normalization which is recommended by the manufacturer, the intensities of the whole rat gene data were analyzed` by Principal Component Analysis (PCA). PCA is a way of identifying the data patterns and highlighting the data's similarity and differences. The main use of PCA is to reduce the dimensionality of a data set while retaining as much information as possible with which it calculates the PCA scores and visually represents them in a 3D scatter plot as seen in Figure 3-4. The scores were used to check data quality. A separation between control and retrorsine-treated groups was observed. Ideally, replicates within Control (untreated) group clustered together (Red) and separately from arrays in RTS-treated groups (Blue), indicating that there was a retrorsine-treatment effect on liver gene expression.

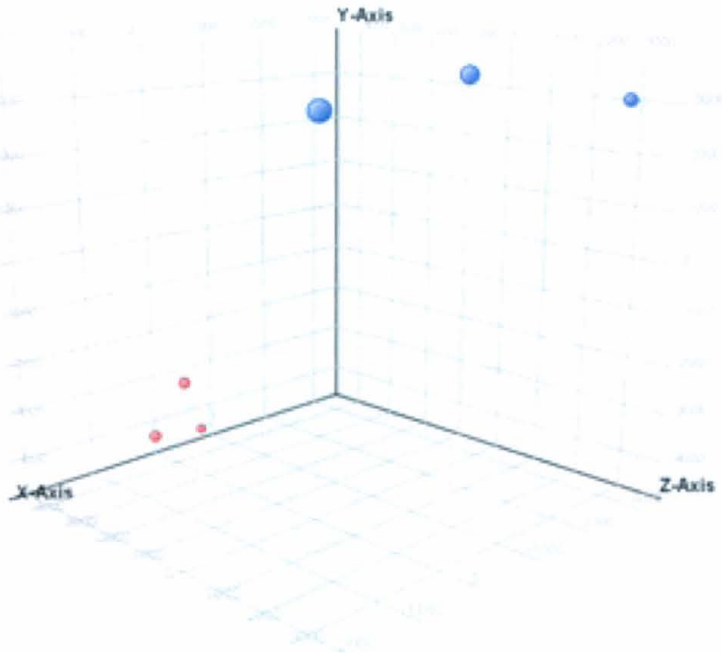


Figure 3-4: Principal Component Analysis (PCA) of gene expression profile for Control and RTS-Treated groups.

The intensity of the entire gene set was used; no specific cut was applied for the analysis. The red and blue dots indicate control and treatment, respectively.

3.2.2 Significance analysis

3.2.2.1 t-test and P values

Unpaired t-tests were chosen as a test of gene expression in RTS-treated versus Control groups. Differentially expressed genes were identified based on the criteria of $P< 0.05$. A total of 206 genes out of 21.532 satisfied the requirement as seen in Table 3-1 and Figure 3-5 (the red and green spots), of which (146) were up-regulated and (60) were down-regulated in response to retrorsine treatment.

Table 3-1: Differential expression analysis report mentioning the test description and Fold Change test (FC). Test has been used for computing P values, type of correction used and P value computation type (*Asymptotic or Permutative*).

	P all	P < 0.05	P < 0.02	P < 0.01	P < 0.0050	P < 0.0010
FC all	21532	206	21	2	2	0
FC > 1.1	9916	203	21	2	2	0
FC > 1.5	1073	105	15	2	2	0
FC > 2.0	275	53	10	1	1	0
FC > 3.0	84	32	9	1	1	0

3.2.2.2 Volcano plot with a fold change

Probe sets that satisfy a fold change cut-off of 2.0 in at least one condition pair are displayed in Table 3-1 and Figure 3-5. Regulation is labeled with respect to expression of controls. As seen in Table 3-1, 53 out of 206 entities with $P < 0.05$ and fold change cut-off of >= 2.0 with [Control] as the control condition were displayed, among of them 47 probes were up regulated after retrorsine treatment, and 6 probes were down regulated after retrorsine treatment.

The Volcano Plot shows the - log10 of P value vs. -log 2 of fold change. 53 of the entities that satisfy the default P value cut off of 0.05 which is equivalent to 1.3010 on the $-\log_{10}$ P value scale and a fold change value of 2.0 are shown in green color, and the rest appear in gray color.

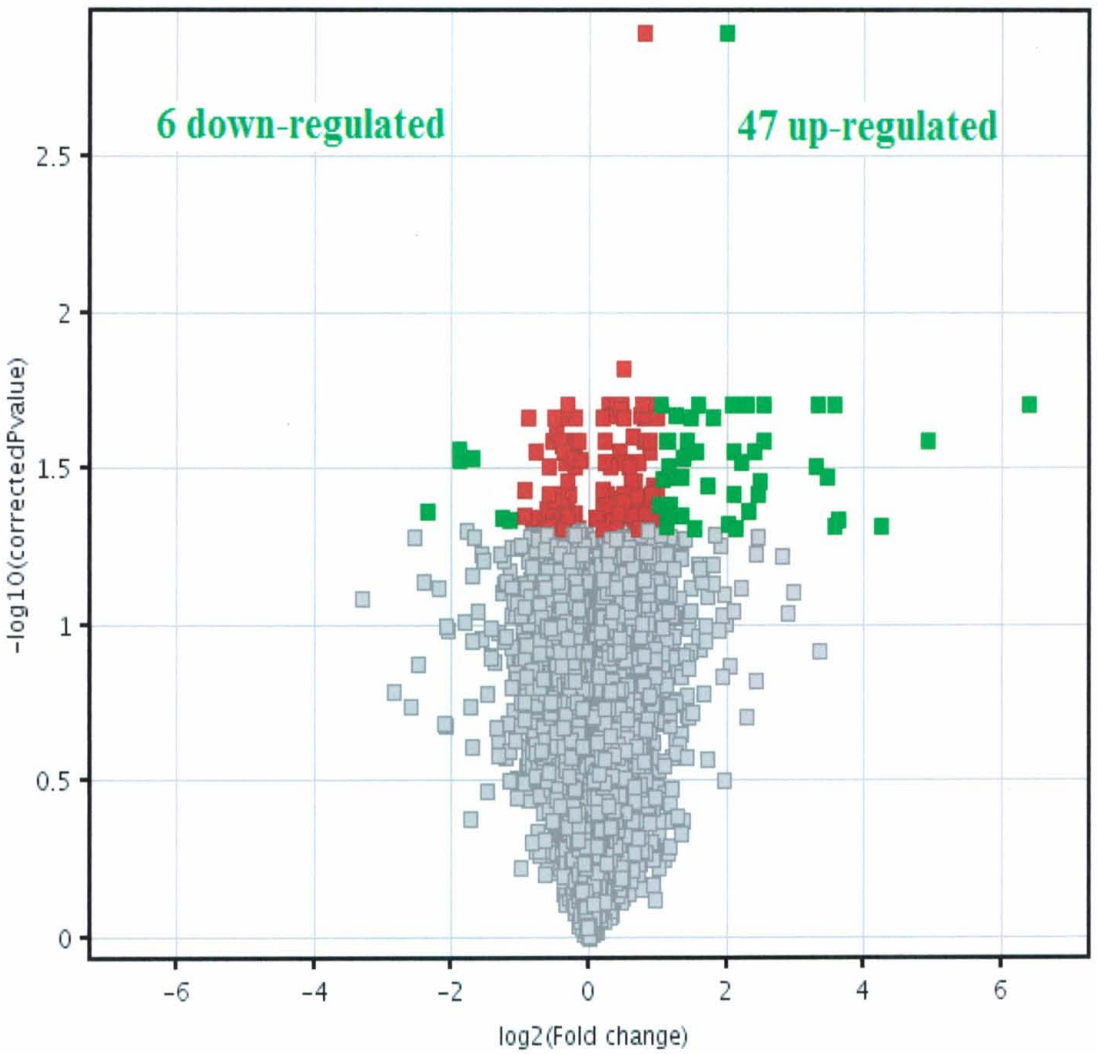


Figure 3-5: Volcano Plot (-log10 P value vs. -log2 fold change). Genes were identified as significantly changed of fold change greater than 2 (up or down) and P value less than 0.05 in comparison to the control group.

3.3 Genes associated with drug metabolism

Since metabolic activation of retrorsine is required to exert its biological effects, I first carried out a detailed analysis of those metabolizing genes that showed expression changes after retrorsine treatment. Table 3-2 shows phase I, II, III drug metabolizing genes whose expression was significantly changed by retrorsine treatment. Up and down-regulation of several cytochrome p540 genes, Ces1,

Table 3-2: The significant changes in gene expression related to phase I, II, III drug metabolizing genes.

Gene symbol	Gene description	Locus ID	Fold Change	P-value
	Phase I Metabolism			
Ces2	carboxylesterase 2	171118	4.25 ↑	0.027
Cyp2e1	cytochrome P450, family 2, subfamily e	25086	1.23 ↑	0.044
Cyp2f4	cytochrome P450, family 2, subfamily f	54246	1.44 ↓	0.048
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	216	12.32 ↑	0.045
ALDH1L1	aldehyde dehydrogenase 1 family, member L1	10840	1.57 ↑	0.034
	Phase II metabolism			
Nqo1	NAD(P)H dehydrogenase, quinone 1	24314	2.48 ↑	0.041
Sult2a11	sulfotransferase family 2A	24902	3.28 ↓	0.029
Sulf2	sulfatase 2	311642	2.19 ↑	0.031
	Phase III metabolism			
Abcb1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	170913	11.91 ↑	0.019
Abcb1b	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	18669	30.39 ↑	0.025
Abcd3	ATP-binding cassette, sub-family D (ALD),member 3	5825	1.35 ↑	0.040
Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	9619	1.39 ↑	0.046
Atp6v1d	ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D	51382	4.00 ↑	0.001
Slc25a36	solute carrier family 25, member	55186	1.39 ↑	0.047
Slc37a4	solute carrier family 37 (glucose-6-phosphate transporter), member 4	29573	1.72 ↓	0.027
Slc6a17	solute carrier family 6 (neurotransmitter -transporter), member 17	613226	1.25 ↓	0.041
Slc5a6	solute carrier family 5 (sodium-dependent vitamin transporter), member 6	8884	1.25 ↑	0.020

*Front color (yellow) indicates common genes between riddiilline and retrorsine treatments. Reference

ATP-binding cassette transporters, and other metabolism-associated genes including NAD(P)H oxidoreductase (Nqo1) and aldehyde dehydrogenes (Aldh1a1) were observed.

3.4 Genes involved in liver abnormalities

To better understand the biological impact and drug metabolizing enzyme function and expression of retrorsine exposure, the genes listed in Table 3-3 were associated by Ingenuity Pathway Analysis in which functional annotation of the transcriptional responses were revealed to be associated with the apoptosis, angiogenesis, cell growth, cell death, adhesion, and cell movement of endothelial cells. Considerable attention was paid to the genes with the highest fold changes after retrorsine treatment.

Table 3-3: Genes involved in carcinogenesis altered by retrorsine treatment in liver.

Gene symbol	gene description	Fold Change
Cell death and apoptosis		
Fas	TNF receptor superfamily, member 6	3.23↑
Phlda3	Pleckstrin homology-like domain, family A, member	5.00↑
Angiogenesis		
Cdh13	Adherin 13, H-cadherin (heart)	4.35↑
Cellular growth		
Ccng1	Cyclin-G1	5.00↑
anatomical structure morphogenesis		
Car2	Carbonic anhydrase II	10.00↑
Dad2	Dopamine receptor D2	2.00↑
Cell movement of endothelial cells and cell adhesion		
Lama5	Laminin, alpha 5	4.50↑
Cdh13	Adherin 13, H-cadherin (heart)	4.35↑
Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	4.30↑
Adam8	Metallopeptidase domain 8	83.7↑
liver development		
Aldh1a1	Aldehyde dehydrogenase 1 family, member A1	12.32↑
Ccnd1	Cyclin D1	4.22↑
Igfbp3	Insulin-like growth factor binding protein 3	2.41↑
Oxidative stress		
Nqo1	NAD(P)H dehydrogenase, quinone 1	2.30↑

3.5 Validation of differential gene expression by quantitative reverse transcription polymerase chain reaction (Q-RT-PCR).

Four differentially expressed genes (ABCB1B, Cyclin G1, CYP2E1, and NQO1) from the final list of 53 genes as seen in Table 3-1 were validated using StepOne Q-RT-PCR. I selected three differently expressed genes (ABCB1B, CYP2E1, and NQO1) related to phase I, II, III drug metabolism genes and Cyclin G1 gene involved in carcinogenesis altered by retrorsine treatment in liver as detected by microarray for verification by Q-RT-PCR.

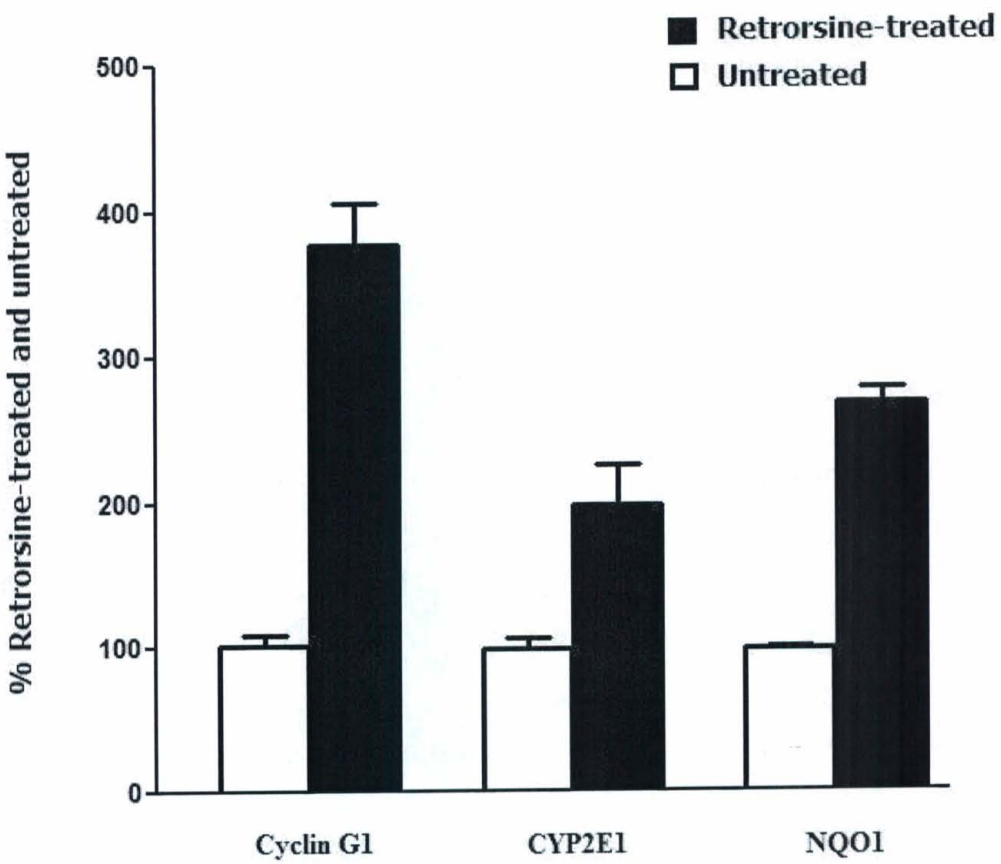


Figure 3-6: Expression of genes related to phase II, III drug metabolizing (CYP2E1, NQO1) and Cyclin G1 gene. Results are expressed as percentage of Retrorsine-treated and untreated rats. Data exprssed as Mean ± SEM with n = 3 per group. P<0.0134, P<0.0001, P< 0.0004 respectively.

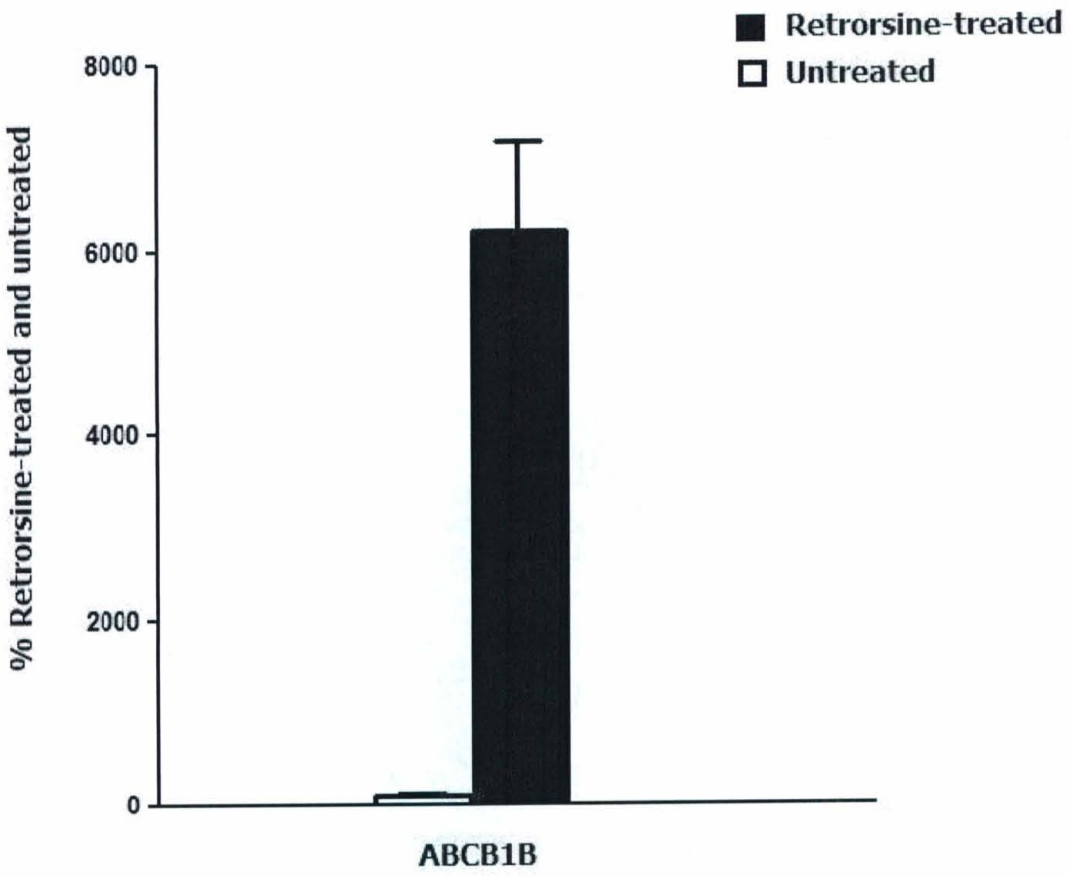


Figure 3-7: Expression of genes related to phase I drug metabolizing (ABCB1B) gene. Results are expressed as percentage of Retrorsine-treated and untreated rats. Data expressed as Mean ± SEM with n = 3 per group. P< 0.0016.

The Q-RT-PCR results were consistent with the microarray data for all the genes verified. There were significant differences in genes expression between retrorsine-treated and untreated rats in all genes (ABCB1B, Cyclin G1, CYP2E1, and NQO1). For example, ABCB1B, CYP2E1, and NQO1 were upregulated in by 30 fold, 4.25 fold, and 2.48 fold by microarray and 30 fold, 4 fold, and 2 fold by Q-RT–PCR. Also, Cyclin G1 was upregulated by 5 fold by microarray and 5 fold by Q-RT–PCR as seen in (Figure 3-6, and Figure 3-7). The expression of ABCB1B, Cyclin G1, CYP2E1, and NQO1 genes were significantly increased in the retrorsine-treated rats (n=3) compared with the

untreated rats (n=3), P value was calculated ($P< 0.0016$, $P< 0.0004$, $P<0.0134$, and $P<0.0001$ respectively.

CHAPTER IV

4. Discussion

Microarray analysis was performed using the Affymetrix GeneChip system to examine gene expression in livers of rats treated with retrorsine, with the goal of identifying genes involved in drug metabolism, injury of endothelial cells, and liver injury and abnormalities, including liver fibrosis and cancer development altered by retrorsine treatment in liver.

Rat RAE 230 2.0 Microarrays were used for six samples, and a total of 31,099 probes covering 26,30 probes verified rat genes were analyzed. The differentially expressed genes between the retrorsine-treated and untreated groups were generated by a two-fold change ranking with a P value cutoff less than 0.05 as the criterion for identifying genes. A total of 53 genes were found to satisfy the requirements; differentially expressed genes in rats exposed to retrorsine included 6 down-regulated genes and 47 up-regulated genes. Among these genes, there were 16 drug metabolizing enzyme associated genes, with 5 Phase I, 3 Phase II, and 8 Phase III. Table 3-5 shows Phase I, Phase II, and Phase III drug metabolism genes whose expression was significantly changed by retrorsine treatment. My findings suggest that these genes may play an important role in the metabolism of retrorsine.

Oxidation of PAs in Phase I appears to occur via cytochrome P450 (CYPs), as my findings show that two out of the five expressed Phase I metabolism associated genes were affected CYPs, genes belonging to the CYP2 superfamily (CYP2E1, and 2F4) were up and down-regulated respectively, two other genes belonging to the Aldh1 superfamily (Aldh1a1, and 1l1) were up-regulated, and one gene belonging to the carboxylesterase superfamily (CES2) was up-regulated.

It is well established that the CYP2 play an important role in catalytic activity, metabolism of xenobiotics, and activation of many toxicological substrates (Irina, and Arthur, 2003). In my results the over-expression of CYP2E1 may explain the promotion of liver cell injury after retrorsine treatment in rats by formation of dehydropyrrolizidines, which are the primary toxic metabolites which may directly

attack DNA and cause DNA damage (Castagnoli, *et al.,* 1997). CYP2E1 was confirmed to be up-regulated with 2 fold change, and (P = 0.0134) by Q-RT-PCR analysis. My finding of increased expression of CYP2E1 is consistent with data from Gordon *et al.* (2000), who showed with RT-PCR that retrorsine caused increased expression of hepatic CYP2E1 in rats. The expression of CYP3A9, CYP3A1, CYP1A2, CYP2B1, CYP4A3, and other CYPs which is believed to be involved in the metabolism of other PAs were unchanged after retrorsine exposure. However, CYP2E1 gene expression is also shown to be increased after riddelliine (another type of PA) treatment (Nan and Tao, 2007). The decrease of CYP2F4 expression after retrorsine exposure suggests that the involvement of these isomers in the bioactivation of retrorsine is possible.

Besides CYP, the CES2 is another major group of Phase I drug-metabolizing enzymes which play a major role in hydrolysis of ester- and amide-bond-containing drugs. This gene was induced about 4-fold after retrorsine treatment which could explain the involvements of the CES2 gene in the detoxification pathway in phase I. CES2 isoenzymes shown to be major intestinal esterases in first pass hydrolysis of ester containing drugs (Masaki, *et al.,* 2007).

I observed a very high fold change in gene expression (both with up-regulated) of aldehyde after retrorsine treatment about 12, and 2-fold for (ALDH1A1, and ALDHA1L1) respectively. These two enzymes are the most important enzymes for aldehyde oxidation in the detoxification pathway in phase I, which could suggest that ALDH1A1 is the major rat liver enzyme involved in retrorsine's phase I metabolic activation. These enzymes are found in many tissues of the body, but are at the highest concentration in the liver.

Among Phase II drug metabolizing genes which are involved in conjugation of the polar functional groups of phase I metabolites, NQO1, and SILF2 were increased 2-fold. In contrast, SULT2AL1 was reduced 3-fold after retrorsine-treatment. Nqo1 was also shown to be increased after reddelline treatment and other PA containing products such as comfrey (Radjendirane, and Joseph,

1998) which might explain that retrorsine can be conjugated by NQO1, and SULF2 enzymes. NQO1 is associated with the mechanisms by which toxicity and carcinogenicity are produced during phase II of major PA products. Altered expression of NQO1 protein has been consistently associated with many tumors and is also associated with Alzheimer disease (AD) (Honda, *et al.,* 2004; Raina, *et al.,* 1999). These results imply that these particular types of NQO1 and SULF2 may therefore be involved in the conjugative detoxification of retrorsine electrophiles and play an essential role in the cellular oxidative defense mechanisms.

In addition, there were four ATP-binding cassette (ABC) transporter genes (ABCB1A, ABCB1B, ABCD3, and ABCG1) that were up-regulated, and four other solute carrier transporter genes, (SLC25A36, SLC5A6) that were up- or down-regulated. These phase III ATP-binding cassette transporters, together with the hepatic microsomal cytochrome P450, as xenobiotic-transporting ATPase activity play a major part in detoxification and a key physiological role in xenobiotic drug metabolism and toxicity, resulting in protection of cells and tissues against xenobiotics. Several studies have demonstrated that ABC transporters directly use the energy of Mg^{2+}/ATP hydrolysis to pump their substrates into the vacuolar lumen (Gang, *et al.,* 2005). My results show ABCB1A, and ABCB1B1 were the major enzymes which ultimately enhance the elimination and clearance of these xenobiotics, suggesting that the overexpression of these genes could be due the DNA-adduct formation. No publications have been found about the relationship between PA treatment and induction of the ATP-binding cassette. To extend these observations, conventional RT-PCR was performed to confirm the expression of ABCB1B gene expression; the up-regulation of gene expression of this gene was confirmed with 60-fold change of and $P < 0.0016$. Future research is encouraged for the role of ATP-binding cassette in PA metabolism and carcinogenesis.

Also, in the present study, gene expression alterations caused by retrorsine exposure were compared with those caused by riddelliine exposure from another study (Nan and Tao, 2007; Guo, *et*

al., 2007). Not surprisingly, there were a strong correlations between the two treatments; in particular, drug metabolizing genes and cancer-related genes in which five common drug metabolizing enzyme associated genes (CYP2E1, ABCB1A, ABCB1B, NQO1,CES2) belonging to Phase I, Phase II, and Phase III, were altered by both retrorsine and riddelliine treatment which suggest that these common genes may share common mechanisms of PA toxicity through the formation of DHP-derived DNA adducts which is reported by Yu-Ping Wang to be responsible for retrorsine-induced liver tumorigenicity as well as the other genotoxicities (Yu-Ping, *et al.,* 2005).

Considerable attention was paid to the high folded genes after retrorsine treatment, which suggest that up- and down-regulated expression of these genes and fold change potentially induce/inhibit metabolism of retrorsine resulting in DNA adduct formation, thus enhancing the toxicity induced by their metabolites. Significantly changed genes were divided into subsets based on functionality, and categories included cell death and apoptosis (FAS and PHLDA3), angiogenesis (CDH13), cellular growth (CCNG1), anatomical structure morphogenesis (LAMA5, CDH13, ALDH1A1, CAR2, CCND1, DAD2), cell movement of endothelial cells (CDH13), cell adhesion (LAMA5, CDH13, LGALS3BP, ADAM8), liver development (ALDH1A1, CCND1, IGFBP3), cell cycle process (CCNG1(M phase), CCND1, PHLDA3), and oxidative stress (NQO1). The over-expression of these genes indicated that these genes were responsible for cell death, whereas the decreased expression indicated that hepatic system development and function were harmed by retrorsine exposure.

Apoptosis, programmed cell death, is known to participate in various biological processes such as development, maintenance of tissue homeostasis and elimination of cancer cells. Reportedly, apoptosis is caused by various inducers such as chemical compounds or proteins. The biochemical pathways of apoptosis are complex and depend on both the cells and the inducers. FAS and PHLDA3 genes play a key role in multiple cellular processes such as cell proliferation, apoptosis, and

transcription. PHLDA3 is putatively involved in apoptosis and FAS expression. Fas is a gene which is now officially known as APT1/ TNFRSF6/ APO-1 and is responsible for activation of pro-apopototic gene products. Also PHLDA3 is required for TP53-dependent apoptosis and AKT repression. The over-expression of PHLDA3 and FAS genes could explain the DNA damage response mediated by P53 gene resulting in induction of apoptosis.

Retrorsine treatment also resulted in 2-fold up-regulation of NQO1 suggesting the induction of oxidative stress. NAD(P)H:quinone oxidoreductase 1 (NQO1) is a flavoenzyme that catalyzes two-electron reductive metabolism and detoxification of quinones and their derivatives leading to protection of cells against oxidative stress. NQO1 has been proposed to stabilize the tumor suppressor gene p53 and has been shown to interact with p53 in a protein-protein interaction (Radjendirane and Joseph, 1998). Thus, the overexpression of NQO1 could explain the role of this enzyme in sensitivity to toxicity and carcinogenesis. NQO1 gene expression is co-ordinately induced with other detoxifying enzyme genes in response to xenobiotions (Jaiswal, 2002).

PA-induced DNA damage prior to DNA synthesis in the liver (endothelial cells and hepatocytes) might produce replication error and mutation which eventually could result in the development of neoplasms in the treated animals. In mammals, the necleotide excision repair process is the most important pathway for elimination of DNA damage caused by exogenous agents, including DNA reactive carcinognous and some endogenously generated oxidative lesions (Pham, 2010). Increased levels of IGF-II were significantly associated with decreased risk of cancer mortality (Pham, 2010; Renehan, 2004) that could explain the increase of the risk in liver cancer with the decrease of the IGF expression level about 2.4-fold change after retrorsine exposure. The eukaryotic cell cycle is governed by cyclin-dependent protein kinases (CDKs) whose activities are regulated by cyclins and CDK inhibitors. CCNG1 has been shown to interact with P16, MDM2, P53 (Zhao *et al.,* 2003) and PPP2R4 (Okamoto *et al.,* 1996) and is induced by DNA damage in a p53 dependent manner (Kimura,

and Nojima, 2002; Chen, 2002; and Sherr, and Weber, 2002), which might explain the overexpression of this protein about 5-fold change in response to DNA damage after the formation of DNA adduct. This suggests that the main role of cyclin G1 is to mediate or regulate the function of p53. In addition, many of the genes exhibited regionalized expression, including those enriched in more distal epididymal regions. In particular, the ADAM family of transmembrane-bound metalloprotease and disintegrin domain-containing proteins were identified, including ADAM8, and ADAM5. Other proteases expressed in the epididymis include the apoptotic FAS, and PHLDA3 were also detected.

Thus, the over-expression of these genes supports a critical role for them in the toxicity of retrorsine.

CHAPTER V

5. CONCLUSIONS

The present study represents the first *in vivo* examination of chronic transcriptional response of the liver to retrorsine exposure. The available evidence on the metabolism and target-tissue specificity for retrorsine's tumorigenesis suggests that active metabolites of retrorsine interact with cells in the liver which cause cell toxicity, followed by compensatory proliferation of DNA-damaged cells causing mutations. I have identified 53 genes in the liver of retrorsine-treated rats that were differentially expressed. My findings suggest that these genes may play an important role in the metabolism of retrorsine. The genes identified in this study are involved in many diverse processes, including apoptosis, angiogenesis, cell growth, cell death, adhesion, and cell movement of endothelial cells, oxidative stress, liver development, catalytic activity, and signal transducer activity. P450 2E1 enzyme is the major metabolizing enzymes responsible for metabolism of retrorsine which was confirmed to be increased in gene expression by Real-Time PCR, these findings suggest that pyrrolizidine alkaloids retrorsine is metabolically activated by P450 2E1 to form chemically reactive dehydrogenated intermediates.

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Appendix A

Appendix A: Rat liver samples and their amount used for cDNA in Microarray Experiment

RNA Name	Used (ng)	IVT Date	Sample ID	cRNA used (ug)	Chip Lot#	Experiment File Name
Control-1	500(4hrs)	7/14/2010	KR-96	15	4098753	RAE2302_071410K_JB1_Control-1
Control-2	500(4hrs)	7/14/2010	KR-97	15	4098753	RAE2302_071410K_JB2_Control-2
Control-3	500(4hrs)	7/14/2010	KR-98	15	4098753	RAE2302_071410K_JB3_Control-3
Retrorsine treated-1	500(4hrs)	7/14/2010	KR-99	15	4098753	RAE2302_071410K_JB4_Retrorsine treated-1
Retrorsine treated-2	500(4hrs)	7/14/2010	KR-100	15	4098753	RAE2302_071410K_JB5_Retrorsine treated-2
Retrorsine treated-3	500(4hrs)	7/14/2010	KR-101	15	4098753	RAE2302_071410K_JB6_Retrorsine treated-3

Appendix B

Appendix B: qPCR genes and gene symbols

Gene Name	Gene Symbol	Amplicon Size	Assay ID
ATP-binding cassette, sub-family B (MDR/TAP), member 1B	ABCB1B	94	Rn01636836_m1
cytochrome P450, family 2, subfamily e, polypeptide 1	CYP2E1	126	Rn01759587_m1
NAD(P)H dehydrogenase, quinone 1	NQO1	109	Rn01432448_g1
Cyclin G1	Cyclin G1	99	Rn00563907_m1
Rat ACTB (actin, beta) Endogenous Control (FAM™ Dye / MGB Probe, Non-Primer Limited)	ACTB	91	NM_031144