AN INVESTIGATION OF THE GENETIC POLYMORPHISMS OF N-ACETYLTRANSFERASE 2 IN HEALTHY MALAYS, CHINESE AND INDIANS IN MALAYSIA AND IN TUBERCULOSIS PATIENTS ON ISONIAZID

by

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List of Abbreviations

TB	- Tuberculosis
WHO	- World Health Organizations
HIV	- Human Immunodeficiency Virus
AIDS	- Acquired Immunodeficiency Syndrome
INH	- Isoniazid
Act-INH	- Acetylisoniazid
NAT2	- Root symbol for N-acetyltransferase 2 protein or mRNA
NAT2	- Root symbol for N-acetyltransferase 2 gene or cDNA
NAT1	- N-acetyltransferase 1 gene
NATP	- N-acetyltransferase pseudogene
SNP	- Single Nucleotide Polymorphism
MDR-TB	- Multi Drug Resistant Tuberculosis
CYP2D6	- Cytochrome P450 2D6 subtype
CYP2C19	- Cytochrome P450 2C19 subtype
CYP3A4	- Cytochrome P450 3A4 subtype
CYP2E1	- Cytochrome P450 2E1 subtype
DNA	- Deoxyribonucleic acid
SLE	- Systemic Lupus Erythematosus
MTB	- Mycobacterium Tuberculosis
PCR	- Polymerase Chain Reaction
PCR-RFLP	- Polymerase Chain Reaction with Restriction Fragment Length Polymorphisms
PCR-ASO	- Polymerase Chain Reaction with Allele-Specific Oligonucleotide Assay
dNTP	- Deoxynucleoside Triphosphate
Mili-Q water	- Distilled and deionized water
1 X	- one time
EDTA	- Ethylenediamine-tetraacetic acid
TE	- Tris-EDTA
TBE	- Tris-Borate
KCl	- Potassium Chloride

OD	- Optical Density
BLAST	- Basic Local Alignment Search Tool
NCBI	- National Centre for Biotechnology Information
dH2O	- distilled water
dDH2O	- double distilled water
V	- volts
U	- units
bp	- base pair
wt	- wild type
mt	- mutant
Tm	- melting temperature
HPLC	- High Performance Liquid Chromatography
SD	- Standard Deviation
iqr	- interquartile range
CI	- Confidence Interval
und	- undetermined
FA	- failed amplification
X2	- Chi square
µl	- microlitres
mM	- milimolar
rpm	- rotations per minute
FDA	- Food and Drug Act
sec	- second
min	- minute

SATU KAJIAN POLIMORFISMA GENETIK N-ACETYLTRANSFERASE 2 DI KALANGAN MELAYU, CINA DAN INDIA YANG SIHAT DI MALAYSIA DAN DI KALANGAN PESAKIT TUBERKULOSIS YANG DIBERIKAN ISONIAZID

ABSTRAK

N-acetyltransferase merupakan enzim yang ditemui dalam hepar, usus kecil, pundi kencing, paru-paru dan kulit. Ia berperanan dalam metabolisme Fasa II untuk bahan asing yang mengandungi amina aromatic atau kumpulan hydrazine. Ia bersifat polimorfik genetik yang disebabkan beberapa SNP. Substrat-substratnya termasuk INH, pro-karsinogen dan karsinogen. Oleh sebab itu, ia berperanan dalam patogenesis sesetengah kanser dan dalam farmakoterapi sesetengah penyakit, contoh yang paling penting adalah tuberkulosis.

Objektif kajian ini adalah untuk menyelidik jenis dan frekuensi polimorfisme NAT2 dikalangan tiga kumpulan etnik penting di Malaysia dan di kalangan pesakit TB untuk membantu meramal pengaruhnya ke atas kadar cepat metabolisme INH.

Sukarelawan sihat Melayu, Cina dan India telah dipilih daripada kalangan penderma darah dan darah mereka diambil untuk tujuan menentukan genotip NAT2. Pesakit yang baru didiagnoskan dengan TB telah juga dipilih. Darah untuk ujian genotip dan fenotip diambil 4 jam selepas pengambilan INH di kalangan pesakit yang dirawat dengan INH. Ujian genotip dilakukan menggunakan kaedah "nested allele-specific multiplex PCR" dan ujian fenotip dilakukan dengan mengukur INH dan Act-INH plasma menggunakan HPLC.

Di kalangan sukarelawan sihat, frekuensi untuk NAT2*4, NAT2*5, NAT2*6, NAT2*7 dan NAT2*12 untuk 212 Melayu, 172 Cina dan 175 India adalah masing-masingnya 43.4%, 10.6%, 25.5%, 16.3% dan 4.3% dikalangan Melayu; 64.0%, 3.2%, 16.3%, 12.2% dan 0.3% dikalangan Cina; dan 22.6%, 30.6%, 30.9%, 6.9% dan 3.4% dikalangan India. Jenis dan frekuensi untuk allel NAT2 dikalangan pesakit TB adalah NAT2*4, NAT2*5, NAT2*6, NAT2*7 dan NAT2*12 pada kadar 47.4%, 14.0%, 21.2%, 12.9% dan 2.3% masing-masingnya. Genotip paling biasa adalah NAT2*4/*4, NAT2*4/*7, NAT2*4/*5 dan NAT2*6/*6 yang mempunyai frekuensi masing-masingnya 25%, 18.9%, 15.2% dan 15.2%. Tiada perbezaan signifikan dari segi frekuensi allel dikalangan pesakit TB dan sukarelawan sehat. Bagi 62 pesakit yang menjalani ujian fenotip, kepekatan INH berkisar daripada 0.31ke 4.17 µg/ml dan bagi Act-INH daripada 0.01 ke 2.48 µg/ml. Terdapat tren untuk nisbah metabolik untuk meningkat dengan genotip yang meramalkan aktiviti lebih lemah. Hubungan di antara genotip dengan MR adalah walau bagaimanapun kurang sempurna.

Kami merumuskan bahawa kami telah berjaya membentuk kaedah-kaedah analisa yang telah diaplikasikan ke atas peserta kajian untuk mengkaji polimorfisme genetik NAT2, kedua-duanya pada tahap molekul dan biokimia. Kedua-duanya kaedah "allele-specific PCR" dan HPLC yang kami bentuk adalah asli, sensitif, spesifik dan praktikal untuk digunakan dalam kajian populasi polimorfisme genetik NAT2 dan untuk diaplikasikan dalam keadaan klinikal. Kajian kami menunjukkan NAT2 adalah polimorfik dikalangan penduduk Malaysia. Polimorfisme ini adalah heterogenus dengan perbezaan etnik yang ketara di antara tiga kumpulan etnik utama. Namun demikian, kami mendapati yang hubungan di antara genotip dan MR adalah tidak sempurna dan kajian

lanjut diperlukan untuk melihat hubungan yang lebih baik demi pemahaman lebih mantap mengenai peranannya dalam farmakoterapi tuberkulosis menggunakan isoniazid.

AN INVESTIGATION OF THE GENETIC POLYMORPHISMS OF N-ACETYLTRANSFERASE 2 IN HEALTHY MALAYS, CHINESE AND INDIANS IN MALAYSIA AND IN TUBERCULOSIS PATIENTS ON ISONIAZID

ABSTRACT

N-acetyltransferases are enzymes found in the liver, the small intestines, urinary bladder, lungs and skin. They mediate Phase II metabolism of xenobiotics containing an aromatic amine or a hydrazine group. They are genetically polymorphic with several important SNP's. Their substrates include INH, pro-carcinogens and carcinogens. They therefore have importance in the pathogenesis of some cancers and in the pharmacotherapy of some diseases, notably tuberculosis.

The objective of our study is to investigate the types and frequencies of NAT2 polymorphisms in the three major ethnic groups in Malaysia and among TB patients to forecast their influence on the rate of metabolism of INH.

Malay, Chinese and Indian healthy volunteers were recruited from blood donation drives and their blood was taken for NAT2 genotyping. Newly diagnosed TB patients were also recruited. Blood for genotyping and phenotyping were collected at 4 hours after INH ingestion in patients who were treated with INH. Genotyping was done using nested allele specific multiplex PCR methods and phenotyping by measuring plasma INH and Act-INH on the HPLC.

Among healthy volunteers, the frequencies for NAT2*4, NAT2*5, NAT2*6, NAT2*7 and NAT2*12 in the 212 Malays, 172 Chinese and 175 Indians were

43.4%, 10.6%, 25.5%, 16.3% and 4.3% respectively among Malays; 64.0%, 3.2%, 16.3%, 12.2% and 0.3% among Chinese; and 22.6%, 30.6%, 30.9%, 6.9% and 3.4% among Indians. The types and frequencies for NAT2 alleles in TB patients were NAT2*4, NAT2*5, NAT2*6, NAT2*7 and NAT2*12 at 47.4%, 14.0%, 21.2%, 12.9% and 2.3% respectively. The most common genotypes were NAT2*4/*4, NAT2*4/*7, NAT2*4/*5 and NAT2*6/*6 with the frequencies of 25%, 18.9%, 15.2% and 15.2% respectively. There was no significant difference in allele frequencies of TB patients and healthy volunteers. For the 62 patients phenotyped, INH concentrations ranged from 0.31 to 4.17 μg/ml and for Act-INH concentration from 0.01 to 2.48 μg/ml. There was a trend for the metabolic ratios to increase with genotypes that predicted poorer activity. Correlation of genotype to MR was however imperfect.

We conclude that we have successfully developed analytical methods that were successfully applied in our subjects to study the genetic polymorphism of NAT2, both at the molecular and biochemical levels. Both our allele-specific PCR and HPLC methods we developed were novel, sensitive, specific and practical for use in population studies of NAT2 polymorphism and for applications in the clinical settings. Our study revealed that NAT2 is polymorphic in our Malaysian population. The polymorphism is heterogenous with clear ethnic differences for the three major ethnic groups studied. We however found that the correlation between genotype and metabolic ratio was imperfect and further studies were needed to better define the relationship for an improved understanding of its role in the pharmacotherapy of tuberculosis with isoniazid.

Chapter 1

Introduction and Review of Literature

1.1 Introduction

N-acetyltransferases are enzymes found in the cytosol of liver and the small intestines. They are also found in smaller amounts in urinary bladder, lungs and skin (Kawakubo and Ohkido, 1998). They are involved in Phase II metabolism. N-acetylation is the major route of biotransformation for xenobiotics containing an aromatic amine ($R\text{-}NH_2$) or a hydrazine group ($R\text{-}NH\text{-}NH_2$), which are converted to aromatic amides ($R\text{-}NH\text{-}COCH_3$) and hydrazides ($R\text{-}NH\text{-}NH\text{-}COCH_3$) respectively. N-acetylation masks the amine with a non-ionizable group so that many N-acetylated metabolites are less water-soluble than the parent compounds. However, N-acetylation of certain xenobiotics such as INH converts its intermediates into more water-soluble derivatives thus facilitates their urinary excretion.

NAT2 is associated with increased susceptibility to develop certain cancers when exposed to certain carcinogen or procarcinogen such as arylamine or heterocyclic amines which can be found in dye substances, well done meat and cigarette smoke. Fast acetylators for instance, are at high risk to develop colonic cancer when exposed to certain procarcinogens (Gil and Lechner, 1998). Conversely, slow acetylators has been suggested to confer increased risk of bladder cancers when exposed to certain carcinogens (Marcus *et al.*, 2000, Rollinson *et al.*, 2001)

Although NAT2 may play a role in environmentally induced diseases like cancers, its importance in pharmacotherapy has probably attracted more attention. In pharmacotherapy, a very important substrate of NAT2 is Isoniazid (INH), a primary drug for tuberculosis (TB) treatment (Petri, 2001). It is also the only drug used in TB prophylaxis (Kinzig-Schippers *et al.*, 2005). It is particularly valued for its efficacy in treating TB, preventing the emergence of drug-resistant organisms and low cost (Jindani *et al.*, 2003). INH has a unique property as compared to other anti-TB drugs where it has the most potent early bactericidal activity especially during the first 2 days of treatment as compared to other anti-TB drugs such as rifampicin and streptomycin (Jindani *et al.*, 2003). It also has the ability to penetrate well into caseous material, pleural, ascetic fluids and meninges (Petri, 2001) as compared to streptomycin, sites frequently affected by the infection.

The importance of INH cannot be overemphasized. Although TB is an ancient disease the incidence of which declined significantly with the introduction of anti-TB medications in 1940's and 1950's, it is re-emerging. The global burden of TB is now growing in many areas of the world, fuelled by HIV/AIDS infections, (Iyawoo, 2004). Immigration from endemic neighboring countries, increase in urban migrations and drug abuse also contribute to the increased incidence of TB. The disease is not only associated with morbidity and mortality. It hits hardest the working-age population, therefore contributing to a loss of economic productivity.

World Health Organizations (WHO) estimated about 9 million new TB cases and 1.7 million TB deaths in the year 2004 alone (WHO, 2006). Some 80% were in Sub-Saharan Africa and Asia, coincident with the HIV pandemic. In 2004, 12.4 % of 11,727 HIV/AIDS patients were positive for TB and 27% of 84,947 TB patients were HIV positive in 41 countries (WHO, 2006). HIV/AIDS causes patients to become more vulnerable to turn *Mycobacterium Tuberculosis* (MTB) infection to active TB and more prone to develop active TB at exposure.

With its increased co-occurrence with HIV/AIDS, it is also expected that the incidence of TB with resistant strains to increase. Globally, the proportion of TB caused by drug resistant strains is increasing (Junien *et al.*, 2000). IN 2004, the incidence of resistant strains was about 1% to INH and 0.1% to multiple drugs (Iyawoo, 2004). The percentages may rise unless appropriate measures are taken to prevent it. Multi-drug resistant TB (MDR-TB) is a virulent, mutated type of TB that is much more difficult to treat. Its spread is often accelerated by HIV infections. Worldwide, the rate of MDR-TB in the year 2000 was estimated at about 3.1% or more than a quarter of a million cases, although very few of these were diagnosed and treated.

Treatment for MDR-TB costs 100 times more and curability cannot be ensured. Frieden *et al.* (1996) reported that In New York City from 1990 to 1993 there were 8,021 TB cases and 13% were multi-drug resistant (MDR). Thirty-five percent of the MDR strains were of the same strain resistant to INH at low concentrations (0.2 mg/dL and 1.0 mg/dL) but susceptible to INH at higher concentrations (more than 5 mg/dL) (Frieden *et al.*, 1996). Drug resistant TB,

particularly disease caused by mycobacteria strains which are resistant to INH and rifampicin (2 most active drugs), is much harder to treat and is often fatal (Frieden *et al.*, 1996). They also require more potent second line drugs which are costly and associated with more side effects.

In Malaysia, the incidence of TB and its death rate are the highest among communicable diseases. About 10% of TB cases notified in Malaysia were however detected among immigrant population who were from high burden neighboring countries (Iyawoo, 2004). The incidence is increasing. In 1985 there were 10,569 TB cases, of which 6,682 were infectious, in 1993 12,075 cases of TB were reported with 6,954 being infectious and in 2000 there were 15,057 TB cases with 8,156 being infectious (MOH, 2001)

INH undergoes metabolism in the liver (Figure 1.1). N-acetyltransferase 2 (NAT2) converts INH to acetylisoniazid (Act-INH). NAT2 enzyme is polymorphic and is coded by the NAT2 gene (Hein, 2002) that has several known Single Nucleotide Polymorphisms (SNP's). To date, 13 SNP's have been described, leading to more than 29 allelic variations in the NAT2 gene. The effects of these variations on the enzyme activity can be grouped into 3 categories: the so-called "fast", "intermediate" and slow acetylator phenotypes. Slow acetylators metabolize INH at a rate much slower than that among fast acetylators. As a consequent, Chen *et al.* (2006) found a 35-fold inter individual differences in INH concentrations when they administered 300mg INH to 46 individuals.

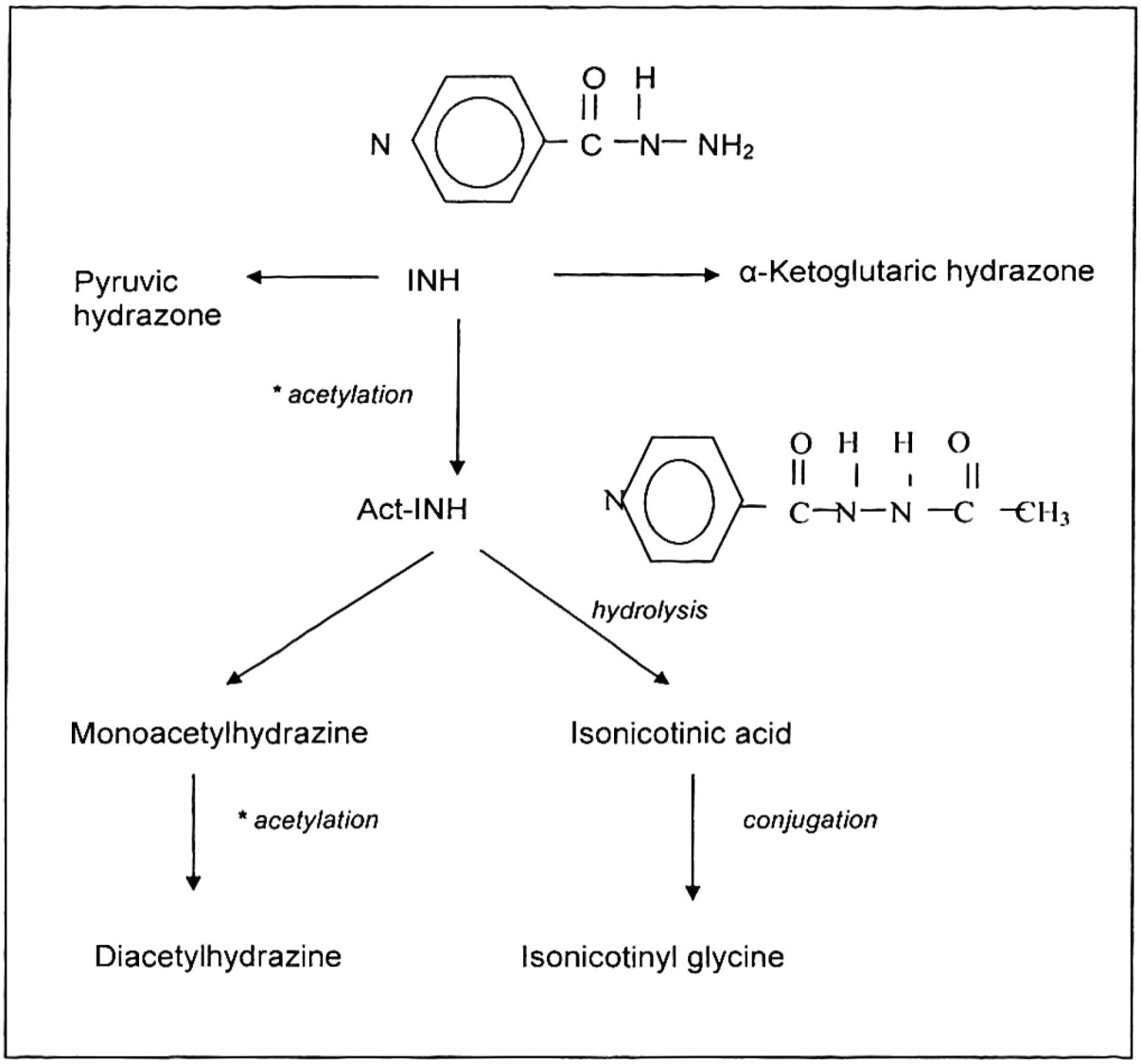

Figure 1.1 INH Metabolism Pathway, adapted from Weber and Hein (1979) * indicate involvement of NAT2 for acetylation

An important feature with NAT2 polymorphism among populations lies in the fact that, on the average more Asians are fast acetylator phenotypes compared to Caucasians. Thus, 90% of Japanese are fast acetylators of INH but 40 to 70% of Caucasians are slow acetylators (Chen *et al.*, 2006). The current practice of giving INH doses based on Caucasian requirement to Asian patients is therefore inherently flawed. Some strains of mycobacteria are resistant to INH at lower concentrations but are susceptible at higher concentration. As suggested by Freiden *et al.* (1996) there is thus a higher possibility for the development of INH-resistant strains among fast acetylator Asian patients, as they are expected to have lower INH concentrations if given doses designed to give adequate concentrations in Caucasian patients.

Another problem with acetylation polymorphisms for INH metabolism is development of side effects in patients who are slow acetylators. Since slow acetylators metabolize INH at slower rate, there are tendency of the drug to accumulate in the body. These accumulations may lead to side effects such as peripheral neuropathy, drug-induced hepatitis, and seizures. The side effects may lead to patients' non-compliance to the drug which may later contribute to development of resistance strains and it also reduces patients' quality of life. The study of pharmacogenetics will hopefully improve the efficacy and safety of the already long existing drug, INH and assist the problem of non-compliance due to side effects. This we hope will also help in preventing morbidity and mortality in TB patients. This therefore suggests a need for further studies to determine the optimum dosage for INH among Asians and to determine its influence on outcomes of INH therapy for TB.

1.2 Literature Review

1.2.1 Drug biotransformation

Once a drug is administered, it is absorbed and distributed to its site of action where it interacts with targets such as receptors and enzymes, undergoes metabolism and is excreted (Figure 1.2). Because of the need for drugs to interact with life components, drugs are usually hydrophobic and this makes it difficult for the body to eliminate them as elimination usually requires water solubility. Metabolism usually converts drugs to metabolites that are more water soluble (Sweeney and Bromilow, 2006) and are thus more easily excreted. Some other drugs however, do not have an inherent pharmacologic activity and requires "activation". Metabolism can also convert these "prodrugs" into therapeutically active compounds. Via the same mechanism, metabolism may even result in formations of toxic metabolites from entities that are pharmacologically active or inactive.

Drug metabolism can generally be classified as Phase I reactions (oxidation, reduction, hydrolysis) and Phase II reactions (acetylation, glucoronidation, sulfation and methylation). Phase II may precede Phase I and occurs without prior oxidation, reduction or hydrolysis if there are polar substrates (Sweeney and Bromilow, 2006). Both, most often, convert relatively lipid soluble drugs into relatively more water-soluble metabolites.

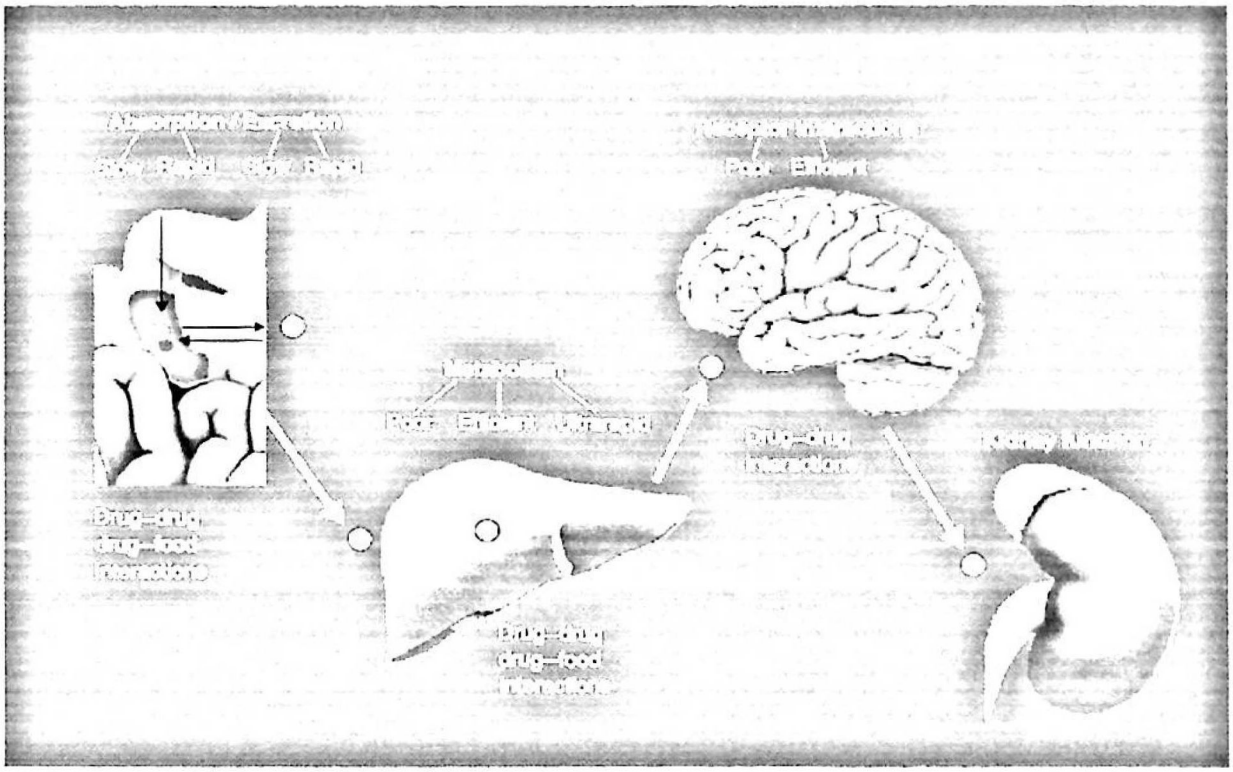

Figure 1.2 The Fate of Drugs in the Human Body

The principal organ of drug metabolism is the liver. However, every tissue has some ability to metabolize drugs. Other tissues that display considerable activity include the gastrointestinal tract, lungs, skin and the kidneys. Following oral administration, most drugs are absorbed intact from the small intestine and are transported, first via the portal system to the liver where they undergo extensive metabolism known as the first pass metabolism. Intestinal metabolism may also contribute to first pass metabolism for certain orally administered drugs which are more extensively metabolized in the intestine than in the liver. Such is the example with nifedipine, a drug that undergoes metabolism by CYP3A4, an enzyme found in large quantities in the gastro-intestinal tracts. The first pass effect may greatly limit the bioavailability of orally administered drugs.

It is not only drugs that undergoes metabolism. Other foreign chemicals or xenobiotics include man-made chemicals and nature's creations. Such are industrial chemicals, pesticides, pollutants, pyrolysis products in cooked food, alkaloids, secondary plant metabolites and toxins produced by molds, plants and animals are also subjected to metabolism. Also included is the conversion of pro carcinogens to carcinogens or non-active compounds to carcinogenic compounds in the body.

The environmental substances humans are exposed to are not only metabolized. A number of them are known to affect the activity of liver enzymes involved in drug metabolism. These substances include certain food, medications, recreational substances such as alcohol, tobacco and pollutants

found in the household and the atmosphere. Other than liver enzymes, several factors are also known to influence the metabolisms of xenobiotics. These include age, sex, hereditary and genetic factors, disease states, dietary and nutritional status, hormonal changes in the body and activity of liver enzymes (Sweeney and Bromilow, 2006).

1.2.2 Pharmacogenetics

In the 1950's, anesthetists who gave patients succinylcholine observed that some patients went into life-threatening respiratory arrests on the operating tables. It was similarly observed that some patients given INH for TB developed peripheral neuropathy. Thus variable response to drug therapy became of concern. Beta-blockers are ineffective in one third of patients. Antidepressants do not work in half the people taking them. Therapeutic doses for warfarin in a given patient can cause severe bleeding in another. Why does one patient suffers an adverse reaction and others don't? Why does a particular drug works well for one patient yet have little or no effect on other patients?

Table 1.1 Factors that Influence Pharmacology

INTRINSIC		EXTRINSIC
Genetic	Physiological and pathological conditions	Environmental
Gender	Age (children-elderly)	Climate
Height Bodyweight		Sunlight Pollution
	Liver Kidney Cardiovascular functions	**Culture** Socioeconomic factors Educational status Language
ADME Receptor sensitivity		
Race		Medical practice Disease definition/Diagnostic Therapeutic approach Drug compliance
Genetic polymorphism of the drug metabolism		
Genetic diseases	Diseases	Regulatory practice/GCP Methodology/Endpoints
Smoking Alcohol Food habits Stress		

The answer lies in the individual variations of human in drug response. Human variability in drug response has been associated with several factors which can be divided into physiological and environmental factor (Table 1.1). Physiological factors include age, gender, ethnicity and body weight and environmental factors include dietary intake, concomitant drug administration and exposure to certain chemicals (Koo and Lee, 2006). Genetic variation is increasingly recognized as an important factor in the variability of drug response. Individuals' functional variants caused by SNPs in genes encoding drug metabolizing enzymes, transporters, ion channels and drug receptors are associated with inter-individual and interethnic variation in drug response, genetic variations in these genes play a role in influencing the efficacy and toxicity of medications (Koo and Lee, 2006). It is now known that much individuality in drug response is inherited. The clinical consequences range from patient discomfort through serious clinical illness to the occasional fatality (Wolf *et al.*, 2000). The genetically determined variability in drug response defines the research area known as pharmacogenetics (Wolf *et al.*, 2000).

Pharmacogenetics has been defined as the study of heredity and response to drugs (Koo and Lee, 2006). The aim of pharmacogenetics is to aid physicians in the prescription of the appropriate dose of the right medicine to a person in an attempt to attain maximum efficacy and minimum toxicity based on genetic tests (Koo and Lee, 2006). In the future, individuals who inherit the enzyme deficiency may benefit from appropriately adjusted doses of the affected drugs based on genetic tests. It is a growing discipline with great potential of improving human health-care, in terms of understanding individual

drug responses, adverse drug reactions associated with genetic so that medicine could be tailored accordingly to prevent side effects and thus reducing cost of therapy and hospitalization. Pharmacogenetics can also provide information about genetic characteristics of a disease which could be used to improve drug design and improve efficacy and safety of existing drugs. In future, pharmacogenetics may help in the determination of risk of disease based on the identification of susceptibility gene early in life and thus measures can be taken to avoid the disease. Pharmacogenetics is important when the drugs prescribed have narrow therapeutic indexes and the metabolism polymorphic. It is also important that when new drugs are developed, pharmacogenetic variations are known early in the developmental stage so as to avoid unnecessary costs of drug misadventures due to genetic traits.

Pharmacogenetics is not a new discipline but its progress has been slow. The concept originated from clinical observations that some patients had very high or very low plasma or urinary drug concentrations followed by realization that the biochemical traits leading to this variation were inherited. The term was coined in 1959 by Vogel to describe the study of genetically determined variations in drug response (Smits *et al.*, 2004, Eichelbaum and Evert, 1996). Over the past 25 years however, pharmacogenetics has progressed rapidly especially in relation to the pharmacogenetics of cytochrome P450 (Smits *et al.*, 2004).

The applications of a pharmacogenetics approach to therapeutics in general clinical practice is still far from being achieved today owing to various

constraints, such as limited accessibility of technology, inadequate knowledge, ambiguity of the role of variants and ethical concerns (Koo and Lee, 2006). So far, pharmacogenetic testing is currently used in only at a limited number of teaching hospitals and specialist academic centers. It is currently most advanced in the Scandinavian countries (Wolf *et al.*, 2000). The most widely accepted application of pharmacogenetic testing is the use of CYP2D6 genotyping to aid individual dose selection for drugs used to treat psychiatric illness. In Malaysia, no pharmacogenetic tests are available routinely currently.

1.2.3 N-acetyltransferases

N-acetyltransferases are enzymes found in the cytosol of liver and the small intestines. They are also found in smaller amounts in urinary bladder, lungs and skin (Kawakubo and Ohkido, 1998). They are involved in Phase II metabolism. N-acetylation is the major route of biotransformation for xenobiotics containing an aromatic amine ($R\text{-}NH_2$) or a hydrazine group ($R\text{-}NH\text{-}NH_2$), which are converted to aromatic amides ($R\text{-}NH\text{-}COCH_3$) and hydrazides ($R\text{-}NH\text{-}NH\text{-}COCH_3$) respectively. N-acetylation masks the amine with a non-ionizable group so that many N-acetylated metabolites are less water-soluble than the parent compounds. However, N-acetylation of certain xenobiotics such as INH converts its intermediates into more water-soluble derivatives thus facilitates their urinary excretion.

N-acetyltransferases are coded by the N-acetyltransferase gene (*NAT*) located on chromosome 8 at locus p21.3 to 23.1 (Hickman *et al.*, 1994). The N-

acetyltransferase gene comprises the *NAT1*, *NAT2* and a pseudo gene *NATP*. *NAT1* and *NAT2* are expressed in an autosomal co-dominant manner (Hein, 2002). Both are polymorphic although *NAT1* initially was believed to be monomorphic (Hein, 2002). They share a high nucleotide sequence homology of 87% with 55 amino acid differences (Hein, 2002). *NAT1* consists of a single exon whereas *NAT2* consists of 2 exons at the coding region and the 3'-region and the other 100 bp are located 8 kb upstream of the translation initiation (Hein, 2002).

NAT2 activities are highest in the liver and gastrointestinal tract and NAT1 is expressed in many extra hepatic tissues as well. As an important feature, NAT1 and NAT2 both catalyze N-acetylation (usually deactivation) and O-acetylation (usually activation) of aromatic and heterocyclic amine carcinogen (Hein, 2002) and may contribute towards risks of developing urinary bladder, colorectal, breast, head and neck and possibly lung cancers. Individual risks associated with *NAT1* and / or *NAT2* genotypes are small but they are increased when considered in conjunction with other susceptibility genes and / or aromatic and heterocyclic amine exposures.

The nomenclature for *NAT* genes for human, eukaryotes and prokaryotes is available at http://www.louisville.edu/medschool/pharmacology/NAT.

1.2.4 N-acetyltransferase *2*

1.2.4.1 NAT2 Phenotype and Genetic Polymorphisms

The polymorphism of the N-acetyltransferase 2 (*NAT2*) gene cluster arises from SNPs which individually or in combinations give rise to the different *NAT2* allelic variants (Dandara *et al.*, 2003). There is more than 15 point-variation on the *NAT2* gene giving rise to at least 28 allelic variants. Generally, when naming the *NAT2* alleles, the most functionally significant nucleotide substitution is considered. The detailed nomenclature for *NAT2* alleles is listed in Appendix 1. Certain variations were more common in certain ethnic groups for example variations at 191 G>A which are assigned to *NAT2*14* were found in African-American only (Bell *et al.*, 1993, Dandara *et al.*, 2003).

The variations in *NAT2* gene result in amino acid substitutions (Fretland *et al.*, 2001, Hein, 2002) that can either cause reduced enzyme activity and expression (Grant *et al.*, 2000, Fretland *et al.*, 2001, Hein, 2002), reduced protein expression (Hein, 2002, Fretland *et al.*, 2001), or reduced protein stability (Fretland *et al.*, 2001, Ferguson *et al.*, 1994). As an example, variations at 191 G>A and 341 T>C were found to have lower N and O acetylation capacity and less stable intrinsically as compared to the reference wild-type allele (Ferguson *et al.*, 1994). Some variations are however silent (Fretland *et al.*, 2001, Grant *et al.*, 1992).

The consequence of SNP's to enzyme activities can be varied. Some may be expressed, others are not. If expressed, their consequence may further depend on the substrates the enzyme acts upon. Thus for NAT2, if the substrate is a carcinogen, slow acetylators may be at increased risks for exposure and may be at higher risks to develop cancers (Figure 1.3). If the substrate is a drug like INH, then slow acetylators may be at increased risks to develop dose-related side effects. On the other hand, if the substrate is a pro-carcinogen or a pro-drug, the opposite will be expected.

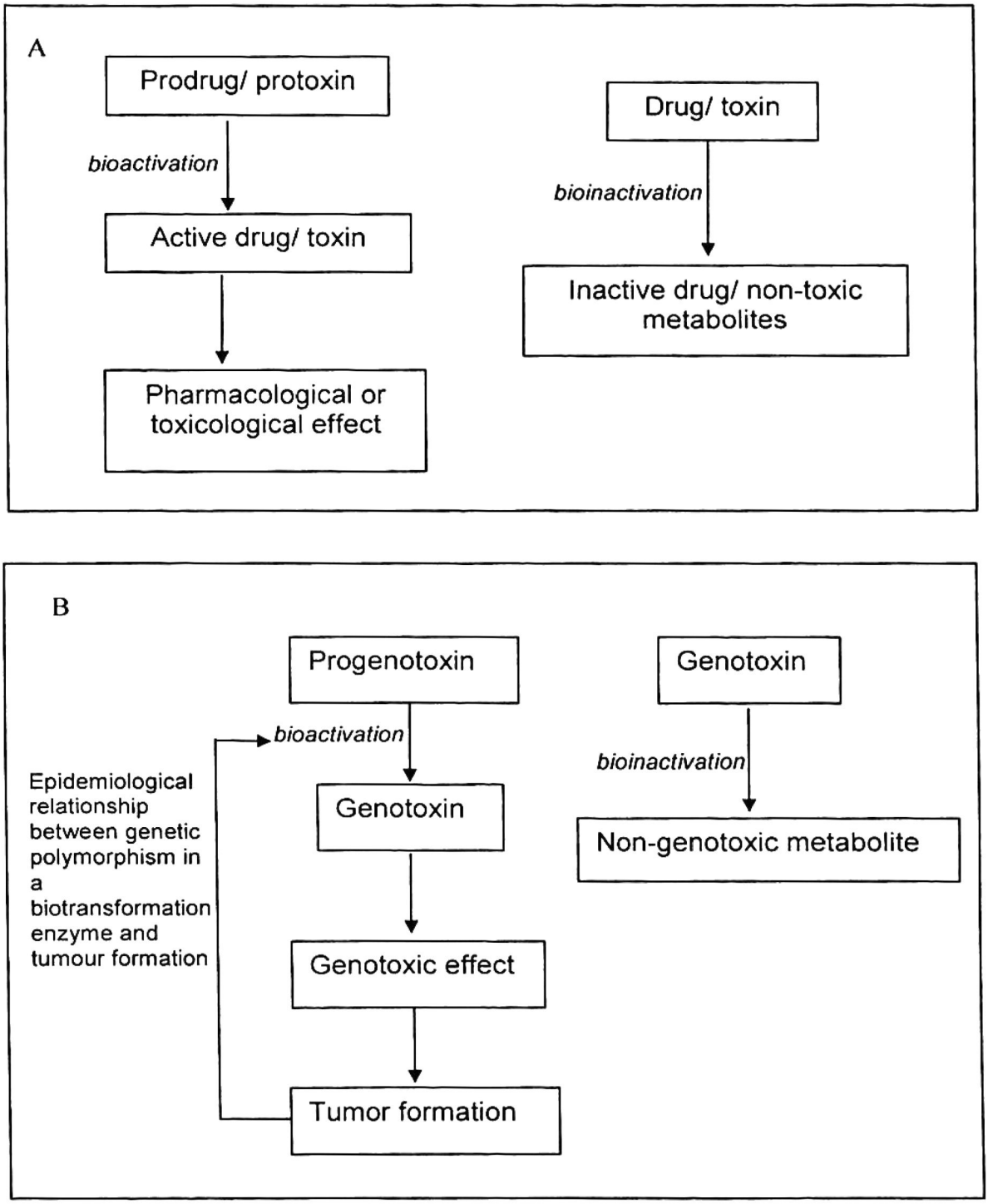

Figure 1.3 Schematic view of the effects of genetic polymorphisms of biotransforming enzymes on metabolism of (pro)drugs and (pro)toxins (A) or (pro)genotoxic compounds (B). Depending on whether the metabolic reaction involved is bioactivating or bioinactivating, genetic polymorphisms might increase or reduce drug efficacy or (geno)toxic effects. Also shown is the often-investigated epidemiological relationship with cancer risk.

The SNPs and their effects on NAT2 activity are listed in Appendix 2. The *NAT2*5* cluster (all possessing 341 T>C) shows the greatest reduction in N-acetylation, O-acetylation and N, O-acetylation followed by the *NAT2*14* cluster (all possessing 191 G>A) and then by the *NAT2*6* cluster (all possessing 590 G>A) (Hein, 2002). The dominant reference allele *NAT2*4* expresses a full enzyme activity. Combinations of the remaining alleles produce enzymes with diminished activity and impaired stability (Meisel *et al.*, 2001).

The expression of the *NAT2* gene has clearly been shown to be involved in the acetylation polymorphism. The stereotypical pattern of metabolism has facilitated the analysis and quantification of an individual's enzymatic status for each drug metabolizing enzyme (DME) using probes, substances which are known to be broken down by the respective enzyme (substrates). The common procedure for NAT2 phenotyping is based on the administration of the probe drug INH and the measurements of the MR of INH to its metabolite either in the plasma or in urine pooled over a certain period of time after drug administration. The ratio is known as metabolic ratio (MR) (Sweeney and Bromilow, 2006). Although many drugs are substrates for more than one enzyme system, some drugs are metabolized primarily by a single enzyme and can therefore be used to measure the activity of the enzyme. NAT2 substrates include INH, dapsone, procainamide, sulfamethazine, hydralazine, aminoglutethemide, nitrazepam, caffeine and phenelzine (Grant *et al.*, 1997, Gaikovitch *et al.*, 2003). However, caffeine are also a substrate for CYP1A2 (Sweeney and Bromilow, 2006).

Although phenotyping should ideally measure enzyme activity that is subsequently explained by relevant variation on the gene of interest, it is not always exact. Concordance between genotype and phenotype is sometimes not seen because, apart from genetic variations, phenotypic expression can be influenced by both intrinsic and extrinsic factors. Zielinska *et al.* (1999) found discordance between acetylator genotype and phenotype for *NAT2* in children and had difficulties phenotyping infants less than 20 weeks. NAT2 is not known to be inducible in humans (O'Neil *et al.*, 1997). Although NAT2 activity is not also influenced by gender or the menstrual cycle phase (Kashuba *et al.*, 1998), it was found to be significantly reduced in early pregnancies (Tsutsumi *et al.*, 2001). NAT2 activity may be altered by progression of HIV and AIDS. O'Neil *et al.* (1997) found that there were discordance between genotype and phenotype of *NAT2* in such patients. A study by Kaufmann *et al.* (1996) however showed concordance between genotypes and phenotypes of *NAT2* in patients with HIV infections and no increase in prevalence of slow acetylation in patients with advanced stages of the disease was observed as would be expected if the HIV status were to impair activity. The discrepancy between the studies may be the result of differences in co-medications of the patients studied. Such a difference may however be important given the frequent co-morbidity of HIV and TB.

1.2.4.2 NAT2 and Other Diseases

As NAT2 is involved in the metabolism of many xenobiotics, and as mentioned above where NAT2 may be involved in the pathophysiology of cancers, NAT2 may be involved in other environmentally induced human

diseases. Thus, NAT2 has also been associated with diseases such rheumatoid arthritis where it was found that the risk for developing rheumatoid arthritis was almost 5-fold greater in slow acetylators compared to fast acetylators (Pawlik *et al.*, 2002). Slow acetylators were also believed to be more prone to develop hydralazine- or procainamide-induced lupus syndrome and haemolytic anaemia due to certain sulfonamides. SLE was observed to occur predominantly in slow acetylator where slow metabolism of one or more unknown dietary or environmental substances over many years was believed to provoke the disease.

1.2.5 Isoniazid (INH)

INH was first synthesized in 1912 by Meyer and Molly but its anti-tuberculous properties were not found until 40 years later when Robitzek *et al.* (1952) tested INH in 92 patients whom he referred to as "mortally ill patients" with extensive pulmonary TB (Evans, 1989). They obtained therapeutic benefits beyond anything they had ever seen with any chemotherapeutic agents ever utilized by them.

INH is a primary drug used for first line treatment of TB, usually in combination with other anti-TB drugs. It is also used alone for prophylaxis of TB. It is available for oral and parenteral administration. The commonly used daily dose for INH is 5 mg/kg to a maximum of 300 mg (Petri, 2001) . It is highly selective for mycobacteria where the minimal tuberculostatic concentration is 0.025 to 0.05 µg/ml compared to concentrations in excess of 500 µg/ml required

to inhibit the growth of other organisms (Petri, 2001). It is bacteriostatic for "resting" bacilli but is bactericidal for rapidly dividing microorganisms and exerts its effect by inhibiting biosynthesis of mycolic acids, an important constituent of mycobacterial cell wall.

When taken either orally or parenterally, INH was rapidly and completely absorbed with peak plasma levels attained at 1 to 2 hours after ingestion and with a half life of 4 hours (Petri, 2001, Weber and Hein, 1979). Absorption occurs mainly in the small intestine. Its absorption is reduced by antacids. It is not protein bound and metabolized in the liver by NAT2 enzyme. It is widely distributed both intra-cellularly and extra-cellularly where significant amounts of the drug were detectable in cerebrospinal fluid, caseous material, pleural (Petri, 2001, Weber and Hein, 1979), ascetic fluids, meninges, saliva and pus (Ellard and Gammon, 1977). This makes INH an important TB drug since it can easily penetrate the caseous materials and other cells to exert its effect in TB infection that may not be reached by other anti-TB drugs. INH also has some other advantages. Its activity is not affected by variations in pH over the range of 5.0 to 8.0, it readily diffuses into macrophages and it is effective against intracellular as well as extra cellular bacilli (Ellard and Gammon, 1977). INH is excreted via the kidneys either as free drugs or as metabolites in an excretion process that was independent of renal function (Weber and Hein, 1979).

In combination therapies with anti-TB drugs during the first 2 days of treatment termed as early bactericidal activity, INH has the greatest killing rate

among all the anti-TB drugs including rifampicin and its activity is not affected by other drugs given concurrently (Jindani *et al.*, 2003).

The metabolism of INH is among the first to be described as being polymorphic. A clearly bimodal distribution of plasma elimination half lives distinguished individuals as phenotypically "rapid" and "slow" inactivator of the drug (Evans *et al.*, 1960) and later, Parkin *et al.* (1997) reported that INH elimination distribution was actually trimodal .

Due to the polymorphic nature of its metabolism, INH 'dose-related' adverse effects vary among individuals. The list of adverse effects is given in Appendix 3. Elevated liver enzymes are frequently reported, however overt clinical hepatitis with symptoms such as gastrointestinal distress, nausea, vomiting and jaundice occurs in less than 5% of patients. Alcohol consumption, advanced age, acetylator status and existing chronic liver disease have been reported to increase risk of anti-TB drug hepatitis (Huang *et al.*, 2003). Its occurrence may also differ in frequencies in different populations, a difference that was thought to be contributed by the genetic polymorphisms of the metabolizing enzyme (Huang *et al.*, 2002).

INH may also cause neurotoxicity, characterized by generalized seizures, coma and metabolic acidosis, that is usually associated with doses in excess of 100mg/kg of body weight. Dvorsek *et al.* (2000) however reported acute INH neurotoxicity during preventive therapy where there was no evidence of INH overdose. Therapeutic doses of INH may also occasionally precipitate

convulsions in patients with known epilepsy or in patients with sub-clinical pyridoxine deficiency, as it occurs in pregnancy, cancer, uremia, alcoholism, chronic liver disease and in advanced age (Martinjak-Dvorsek *et al.*, 2000). Pyridoxine (15 to 50 mg/day) should be administered with INH to minimize adverse drug reactions in malnourished patients and those predisposed to neuropathy such as in elderly, pregnant women, HIV infected individuals, diabetics, alcoholics and uremic patients (Petri, 2001). INH is also known to cause a sensory-dominant peripheral neuropathy. Yamamoto *et al.* (1996) demonstrated that patients who developed INH neuropathy were all slow-acetylator genotype.

NAT2 activity is not known to be inducible or inhibited. INH on the other hand may inhibit the activity of CYP2C19, CYP2D6, CYP3A4 and CYP2E1 (Desta *et al.*, 2001). Some studies suggested that monoacetylhydrazine which is a metabolite of INH may antagonize the anti-TB activity of INH but other metabolites such as Act-INH, isonicotinic acid and diacetylhydrazine were not inhibitory (Weber and Hein, 1979). INH acetylation may be reduced when there is concomitant treatment with other drugs metabolized by NAT2. Such occurred with procainamide where INH half-life was slightly but clinically insignificantly prolonged (Weber and Hein, 1979). Insulin causes interesting alterations in INH pharmacokinetics (Weber and Hein, 1979). It was found to enhance the intestinal uptake of INH and increased concentrations of INH in the lungs and the liver. Maximum concentrations of the drug observed in the kidneys and the brain was however reduced. There were also reports on alterations of INH half-

lives with salicylic acids and chlorpromazine where salicylic acid shortened INH half-life and chlorpromazine prolonged the half-life (Petri, 2001).

1.2.6 Tuberculosis (TB)

TB is a major public health problem worldwide. With the increased incidence of HIV/AIDS and immigration, TB incidence is on the rise. Socioeconomic change and decline of the health-care system also contribute to increase in TB incidence (Frieden *et al.*, 2003). TB is the world's second commonest cause of death from infectious disease after HIV/AIDS. TB has probably killed 100 million people over the past 100 years although cure was available for the second half of the 20th century (Frieden *et al.*, 2003).

TB infection is caused by *Mycobacterium tuberculosis* (MTB). It is spread by airborne droplets. The MTB contained can remain airborne for min to hours after expectoration by people with pulmonary or laryngeal TB. This occurs during coughing, sneezing, singing or talking. The infectious droplet nuclei are inhaled and lodged in the alveoli in distal airways. These are then taken up by alveolar macrophages and results in either successful containment of the infection or progression to active disease (primary progressive TB). The risk of development of active disease varies according to time since infection, age and host immunity. The definitive diagnosis of TB is based on culture for MTB, but rapid diagnosis of infectious PTB by simple sputum smear for acid fast bacilli remains an important tool (Frieden *et al.*, 2003).