CHAPTER II
LITERATURE REVIEW

Pharmacogenetics

Pharmacogenetics, a field that provides the experimental framework for dissecting the effects of genetic variation on human drug response. Its potential affects practice of medicine and opens up for the discovery of better and safer therapies. Firstly, pharmacogenetics was usually limited to investigations of a single gene in small groups of individuals, now such investigation may involve testing of multiple gene loci spanning a large fraction of human genome in many patients and healthy persons. With the maturation of Human Genome Project, the field has experienced a period of rapid growth and redefinition, and it is not uncommon to see pharmacogenetics referred to as pharmacogenomics.

The origins of pharmacogenetics

Pharmacogenetics emerged as an experimental science in 1950s when researchers employed new tools for analyzing person-to-person differences in patterns of drug response. First studied genetic polymorphism was the disposition of succinylcholine, isoniazid, and antimalarial drugs such as primaquine,to explain the predisposition of certain individuals to alcaptonuria and certain in born errors of metabolism. Garrod (1910) proposed his concept of chemical individuality, he was the first to realize and suggest that enzymes were implicated in the detoxification of exogenous chemicals.

Evidence of variations in human response to exogenous chemicals was scarce until Marshall and coworkers reported in 1918 that blacks were much more resistant than wnites to blistering of the skin from exposure to mustard gas. By 1929, additional reports about racial differences in response to cocaine, euphthalmine, ephedrine, and

atropine had appeared. During this same period, hereditary deficits in two modalities of sensory perception, 'odor blindness' and 'taste blindness' , were identified. A genetic study of taste blindness revealed the high specificity, sensitivity, and heritability of the trait as features by characteristic of human responsiveness to exogenous chemicals. Additional studies performed in several African, Asian, Middle Eastern, and European populations showed the frequency of the non-taster phenotype in Europeans is approximately 35-40%, but is appreciably less in Africans, Chinese, Japanese, South-American (Brazil), and Lapps. The latter studies were the first to document the association of race or ethnicity to human response to chemicals (Weber., 2001).

Genetic Polymorphism and Single nucleotide polymorphism (SNPs)

Genetic Polymorphism

A difference in DNA sequence among individuals, groups, or population includes SNPs, sequence repeats, insertions, deletions and recombination. Genetic polymorphism may be the result of chance processes, or may have been induced by external agents (such as viruses or radiation). If a difference in DNA sequence among individuals has been shown to be associated with disease, it will usually be called a genetic mutation. Changes in DNA sequence which have been confirmed to be caused by external agents are also generally called "mutations" rather than "polymorphism".

Single Nucleotide Polymorphism (SNPs)

A Single Nucleotide Polymorphism is a source variance in a genome. A SNP (Snip) is a single base mutation in DNA. SNPs are the most simple form and most common source of genetic polymorphism in the human genome (90% of all human DNA polymorphisms).

There are two types of nucleotide base substitutions resulting in SNPs:

- A transition substitution occurs between purines (A, G) or between pyrimidines

(C, T). This type of substitution constitutes two thirds of all SNPs.

- A transversion substitution occurs between a purine and a pyrimidine.

Phenotype and Genotype

- Phenotype

  The observable properties of an individual as they have developed under the combined influences of the individual's genotype and the effects of environmental factors.

- Genotype

  An exact description of the genetic constitution of an individual, with respect to a single trait or a larger set of traits. The genetic constitution of an organism as revealed by genetic or molecular analysis, i.e. the complete set of genes, both dominant and recessive, possessed by a particular cell or organism.

Pharmacogenetics and cancer chemotherapy

Inter-subject variability in therapeutic drug responses and drug toxicities is a major problem in clinical practice. Such variability is largely due to genetic factors leading to altered drug metabolism and/or receptor expression. Polymorphisms in drug-metabolising enzymes, which appear to be more extensive than those of receptors, result in altered pharmacokinetics of therapeutic agents. Since the discovery of debrisoquine hydroxylase deficiency in 1970s, pharmacogenetic polymorphisms of several drug metabolizing enzyme systems have been identified and characterized. Genetic variations in receptor expression systems, or pharmacodynamic polymorphisms, have been recently identified as major determinants of drug response. The relationship between an individual's capacity to metabolize environmental carcinogens and other xenobiotics and susceptibility to cancer has been extensive studied. The applicability of pharmacogenetics in cancer chemotherapy is critical due to the following reasons:

- anticancer agents generally have a narrow margin of safety;
- many of these agents are prodrugs and are biotransformed to active counterparts by enzyme systems that exhibit genetic polymorphisms;
- the active forms are usually also associated with toxicity;
- certain anticancer agents are detoxified by polymorphic enzyme systems;
- most cancer chemotherapeutic drugs exhibit significant inter-patient variability in pharmacokinetics and toxicity.

Thiopurine methyltransferase pharmacogenetics

Mutations in the thiopurine methyltransferase (TPMT) gene have profound effects on 6-mercaptopurine (6-MP) tolerance and dose intensity in maintenance treatment of acute lymphoblastic leukemia (ALL) in children (figure 1). A recent trial estimated that 71% of patients with bone marrow intolerance to 6-MP were phenotypically-TPMT deficient. When 14 of these patients were typed for open reading frame (ORF) mutations associated with TPMT deficiency (TPMT*2, TPMT*3A, TPMT*3C), 9 of them were found to be positive, with 100% concordance in the homozygous-mutant patients. However, less concordance was found in patients with intermediate TPMT activity. 6-MP dose adjustment strategy in TPMT-deficient patients was applied, and appropriate dose reductions (91% of the reduction in homozygous mutants) allowed patients to tolerate full doses of all other chemotherapy treatments. In 8 wild-type patients who did not tolerate standard 6-MP dosage, median 6-MP dose reduction was 8%, suggesting that toxicity was not caused by genetic differences in 6-MP disposition. Although 6-MP dose intensity was reduced in homozygous mutants compared with either heterozygous or wild-type patients, no differences in survival were observed among the genotypes. After 6-MP dose individualisation in TPMT deficient patients, survival outcomes are similar between deficient and wild-type patients (Innocenti and Ratain., 2002).

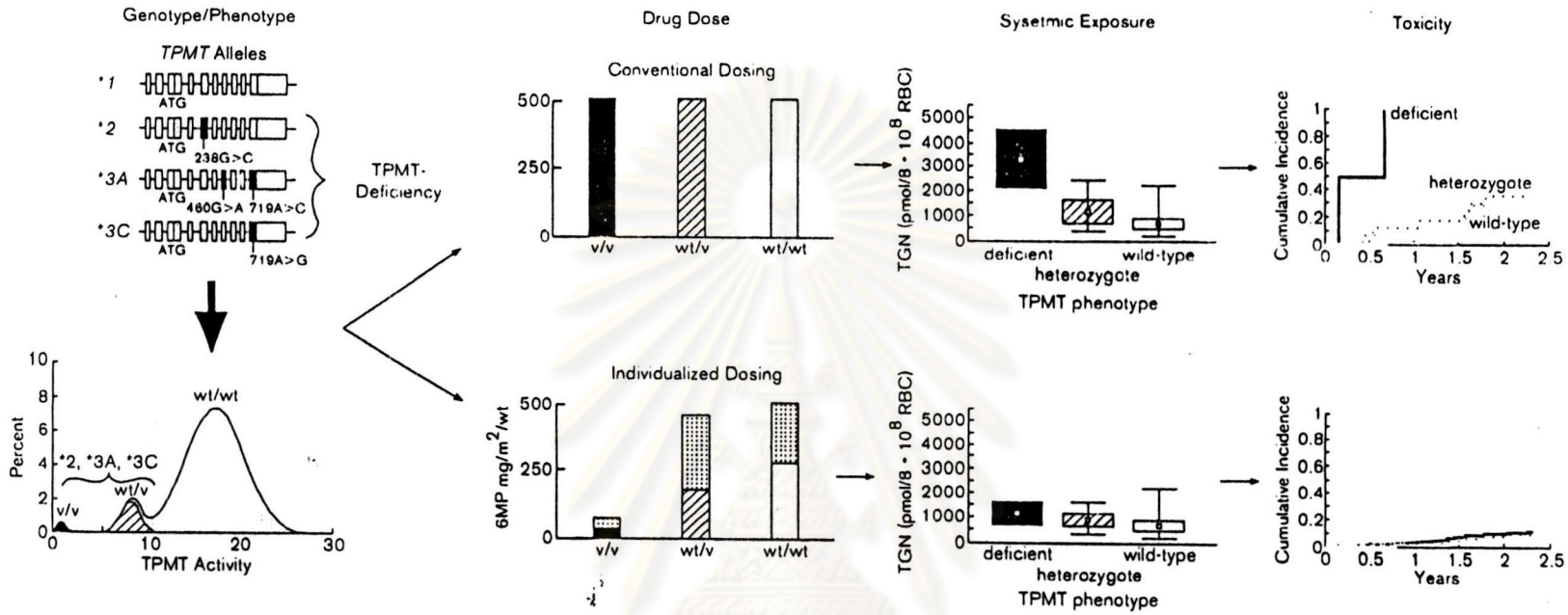


Figure.1 Genetic polymorphism of thiopurine methyltransferase and its role in determining toxicity to thiopurine medications (azathioprine, mercaptopurine, thioguanine). The left panels depict the predominant TPMT mutant alleles causing autosomal codominant inheritance of TPMT activity in humans. As depicted in the subsequent top three panels, when uniform (conventional) dosages of thiopurine medications are administered to all patients, TPMT-deficient patients accumulate markedly higher (10-fold) cellular concentrations of the active thioguanine nucleotides (TGN) and heterozygous patients accumulate approximately two-fold higher TGN concentrations, translating into a significantly higher frequency of toxicity (far right panels). As depicted in the bottom three panels, when genotype-specific dosages of thiopurines are administered, comparable cellular TGN concentrations are achieved, and all three TPMT phenotype can be treated without acute toxicity (in the two panels under the heading "Drug Dose", the solid or striped portion of each bar depicts the mean mercaptopurine (6-MP) doses that were tolerated in patients who presented with haematopoietic toxicity; whereas the stippled portion depicts mean tolerable dosage of all patients in each genotype group, not just patients presenting with toxicity. V, variant; wt, wild-type. From Evans 2002

Thiopurine methyltransferase (TPMT)

Thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) is a cytoplasmic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds, such as the anticancer agents mercaptopurine and thioguanine and the immunosuppressant azathiopurine. TPMT was shown to be present in erythrocytes, kidney and liver. TPMT mRNA was detected in most human tissues e.g. heart, blood cells, placenta, pancreas and intestine. Human TPMT has a molecular mass of 28 kDa and comprises 245 amino acid. The active gene for TPMT was located on chromosome 6p22.3 and approximately 34 kb in length and consisted of ten exons and nine introns (Aarbakke et al., 1997).

- Genetic polymorphism of TPMT

TPMT activity, in human tissue, is controlled by a common genetic polymorphism. The frequency distribution of TPMT activity in a population of white subjects is trimodal, 88.6% of the subjects displayed high activity, 11.1% had intermediate activity, while the lysated of 0.3% did not contain detectable TPMT activity (figure.2) (Yan et al., 2000).

A simple model for such a distribution is that a single genomic locus governs TPMT activity and that there are two alleles for the locus. One allele corresponds to the high activity form ($TPMT^{H}$) and the other ($TPMT^{L}$) to the low activity form. The locus is diploid and both copies are expressed. Individuals that are homozygous for $TPMT^{H}$ display high activity, heterozygous have intermediate activity and $TPMT^{L}$ homozygotes have an activity that is below the detection level of the assay. The validity of this model was suggested by the distribution data that fit with Hardy-Weinberg statistics for inheritance by Mendelian laws. The frequency of $TPMT^{H}$ is 0.94 (0.06 for $TPMT^{L}$) in the group of white subjects studies. To date, eight TPMT alleles have been identified, including three alleles (*TPMT*2, TPMT*3A* and *TPMT*3C*) which account for 80-95% of intermediate or low enzyme activity cases (figure.3) (Keuzenkamp-Jansen et al., 1996).

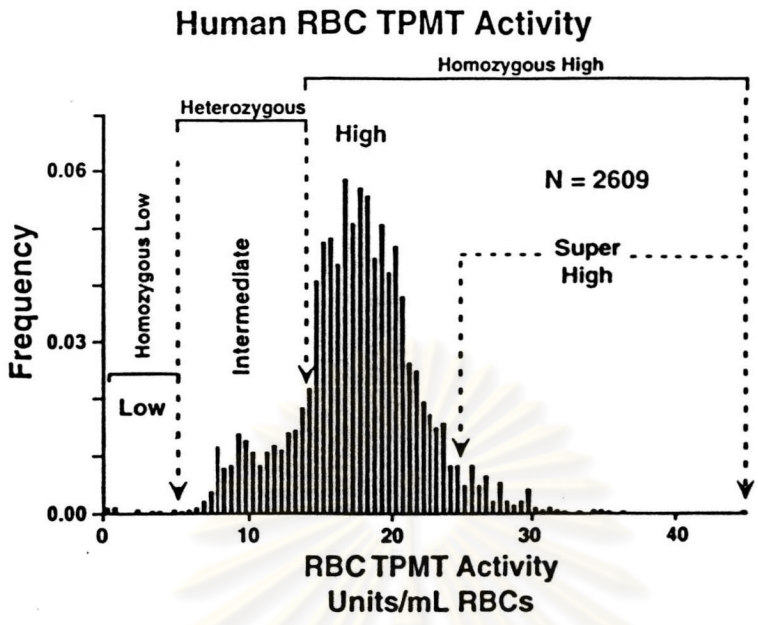


Figure. 2 Human red blood cell (RBC) TPMT activity in 2609 clinical laboratory samples. Mayo Clinic clinical laboratory "cutoff" values are shown for low, intermediate, and high activity phenotypic groups presumed to be homozygous for alleles for low activity, heterozygous for both low and high activity alleles, or homozygous for alleles for the trait of high activity, respectively. An arbitrary defined "super high" subgroup with RBC TPMT activity>25 units/mL RBCs is also shown. From Yan et al., 2000.

The mutant allele TPMT*2 is defined by a single nucleotide transversion (G238C) in the open reading frame, leading to an amino acid substitution at codon 80 (Ala>Pro). When assessed in a yeast heterologous expression system, this mutation lead to a >100-fold reduction in TPMT activity relative to wild-type cDNA, despite a comparable level of messenger RNA expression. The second and more prevalent mutant allele, TPMT*3A, contain two nucleotides transition mutations (G460A and A719G) in the open reading frame, leading to amino acid substitutions at codon 154 (Ala>Thr) and codon 240 (Tyr>Cys). When heterologously expressed in yeast or COS-1 cells, TPMT*3A had >200-fold lower TPMT activity and immunodetectable protein compared to wild-type cDNA. Heterologous expression in yeast established an enhanced rate of proteolysis of mutant TPMT proteins encoded by TPMT*2 and TPMT*3A alleles, with

mutant TPMT proteins encoded by TPMT*2 and TPMT*3A alleles, with degradation half lives of approximately 15 min for both mutant proteins compared with 18 h for the wild-type protein. When cDNAs that contained only the G460A (TPMT*3B) or only A719G (TPMT*3C) were prepared and heterologously expressed, TPMT*3B was associated with approximately a fourfold reduction in protein level and a nine fold decrease in activity, whereas TPMT*3C yielded similar protein levels and about 30% lower catalytic activity compared with the wild-type cDNA. The mutant alleles TPMT*4-8 have also been identified during clinical genotype-phenotype analysis . TPMT*4 has a G>A transition at the intron 9-exon 10 junction which disrupts the final nucleotide of the intron at the 3' acceptor splice site sequence. TPMT*5 was identified as a T146C transition in a heterozygous individual of undefined ethnicity who had intermediate TPMT activity. This mutation results in a Leu>Ser amino acid substitution at codon 49. TPMT*6 was identified in a Korean subject with intermediate activity. This A539T transversion in exon 8 result in a Tyr>Phe substitution at codon 180. TPMT*7 was identified in a single European subject with intermediate activity. This allele contains a T681G transversion in exon 10 which results in a His>Glu amino acid substitution at codon 227. Lastly, TPMT*8 contains a single nucleotide transition (G644A) leading to an amino acid change at codon 215 (Arg>His). This allele has been identified in one African-American individual with intermediate activity (McLeod and Chokkalingam., 2002).

- Measurement of TPMT activity

TPMT activity is typically measured in erythrocytes, as the level of TPMT activity in human liver, kidney, and normal lymphocytes has been shown to correlate with that in erythrocyte. In the previous study, kidney-RBC TPMT activity showed a significant correlation ($r=0.665$, $p<0.001$, figure 4) (Woodson. et al., 1982). The other study presented a significant association between TPMT activity in ALL blasts and erythrocytes ($r=0.75$; $p<0.0001$, figure 5) (McLeod. et al., 1995). The last study, liver-RBC TPMT activity was a significant correlation ($r=0.597$, $p<0.001$, figure 6) (Ferroni. et al., 1996).

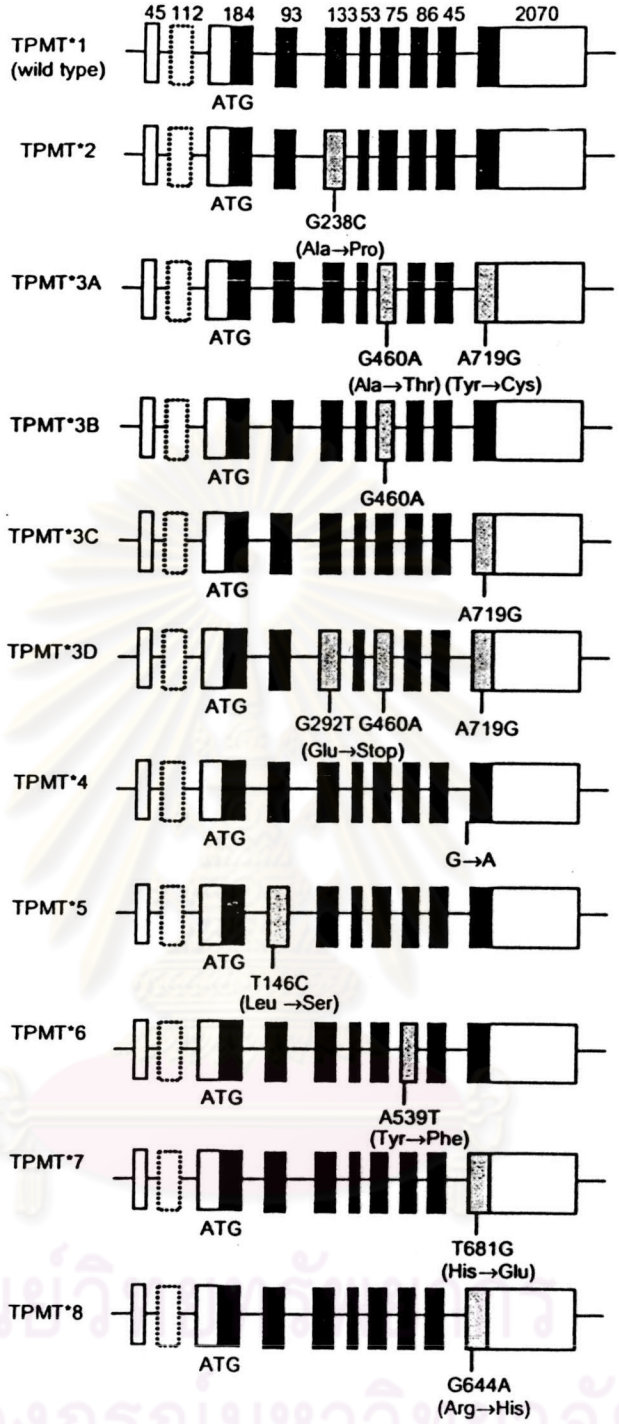


Figure.3 Allele variants at the human TPMT locus. Gray boxes are mutations that result in amino acid changes. TPMT*4 is a 5' splice site mutation for exon 10 that does not alter an amino acid. White boxes are untranslated regions. Black boxes are exons in the ORF. The dashed box is exon 2, which was detected in 1/16 human liver cDNAs during initial evaluation. ORF: Open reading frame; TPMT: thiopurine methyltransferase. From Keuzenkamp-Jansen et al., 1996.

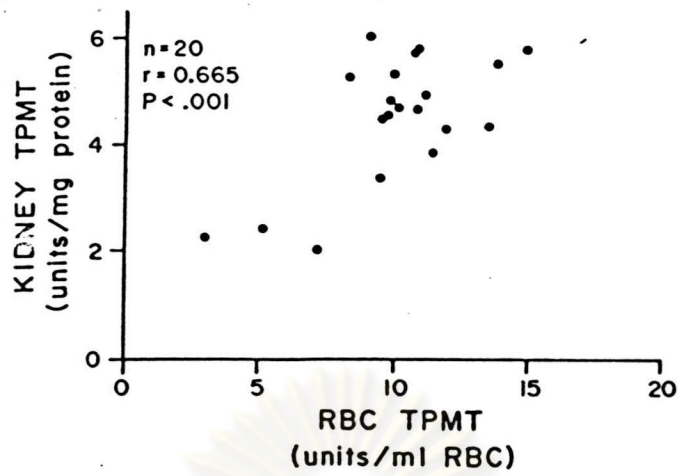


Figure.4 Correlation of RBC with kidney TPMT activity in sample from 20 patients who underwent clinically indicated nephrectomies. From Woodson. et al., 1982.

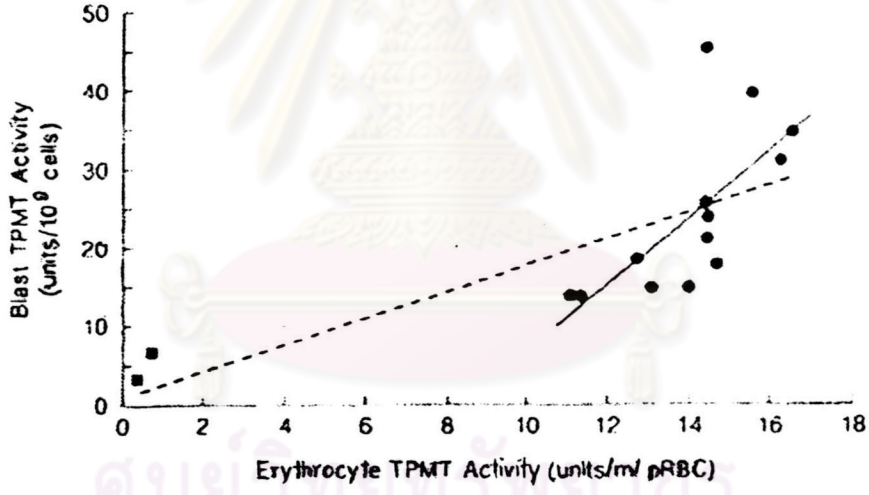


Figure.5 Correlation between erythrocyte and leukemic blast cell TPMT activity in 15 children with ALL. Solid line is best-fit of linear regression of 13 patients whose erythrocytes and ALL blasts were measured before the start of chemotherapy (closed circles; r^2=0.48; p<0.009). These values were also significantly associated when assessed by Spearman's correlation coefficient (r=0.75; p<0.0001). Dashed line depicts the best-fit linear regression including two patients (closed squares) with inherited erythrocyte TPMT deficiency (r^2=0.51; p<0.005; N=15). From McLeod. et al., 1995.

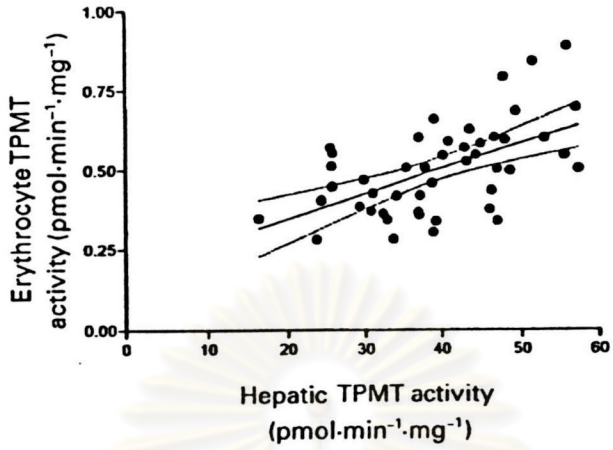


Figure.6 Correlation of hepatic and erythrocyte activities (pmol/min/mg) of TPMT in 47 patients. The broken line indicates the 95% confidence limits of the correlation. The correlation coefficient was 0.597 ($p<0.001$) and the goodness of fit (r^2) was 0.357. From Ferroni. et al., 1996.

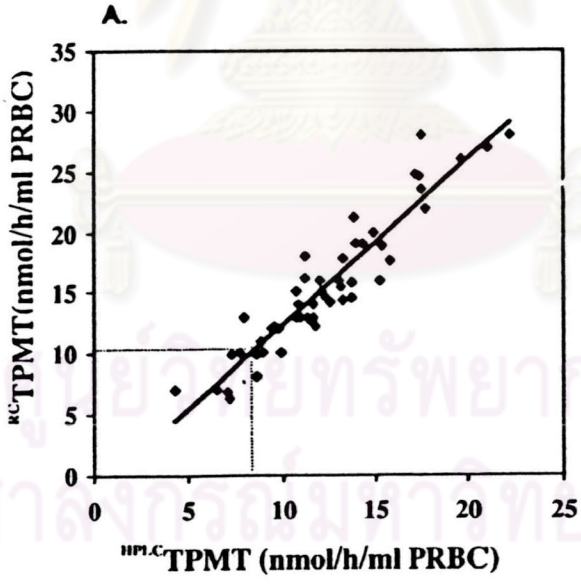


Figure.7 Linear regression of thiopurine mehyltransferase (TPMT) values measured by the cheomatographic (TPMTHPLC) and radiometric (TPMTRC) assays. TPMT activity is expressed as the amount of 6-methylmercaptopurine (nmol) formed per hour and per ml of red blood cell cytosol. The regression line is expressed as $y = 1.33x+0.71$ ($r^2=0.82$; $p<0.0001$). From Ferroni. et al., 1996.

At present, there are two methods to measure TPMT activity: the radiochemical method, which uses [^{3}H]-S-adenosyl-L-methionine (^{3}H-SAM) as methyl donor, and the non-radioactive procedure, which uses SAM and 6-MP as substrates, and the product formed, 6-methylmercaptopurine (6-MMP), is separated and identified by high-performance liquid chromatography (HPLC) equipment. The first method is problematic in many laboratories because the handling of radioactive material is prohibited (Menor. et al., 2001). The second method has been published, later modified. This method used a liquid-liquid extraction, which reduces duration and cost of the analysis. The correlation of TPMT activity using HPLC and radiometric assay described in previous study showed a significant correlation ($r^2 = 0.82$, $p<0.0001$, Figure.7) (Anglicheau. et al., 2002).

- Relationship between TPMT activity and 6-thioguanine nucleotides (6-TGNs)

6- Mercaptopurine are prodrugs that undergo metabolic activation to form 6-thioguanine nucleotides (6-TGNs). Levels of 6-TGNs measured in the RBC were correlated with both thiopurine therapeutic efficacy and toxicity such as myelosuppression. A major factor of individual variation in 6-TGN concentrations was the genetically determined level of TPMT activity (figure 8). Specifically, the higher the level of TPMT activity, the lower the 6-TGN levels in RBC, and the lower the level of TPMT activity results in the higher the RBC 6-TGN levels. There was the first report in 1989 that patients with genetically very low or absent TPMT activity who were treated with "standard" doses of thiopurine drugs were at risk for the development of life-threatening thiopurine toxicity. Furthermore, patients with very high TPMT activity might display decreased therapeutic efficacy when treated with standard doses of thiopurine drugs. Consequently, in some referral centers the measurement of TPMT enzyme activity has become a standard clinical test (Weinshilboum., 2001).

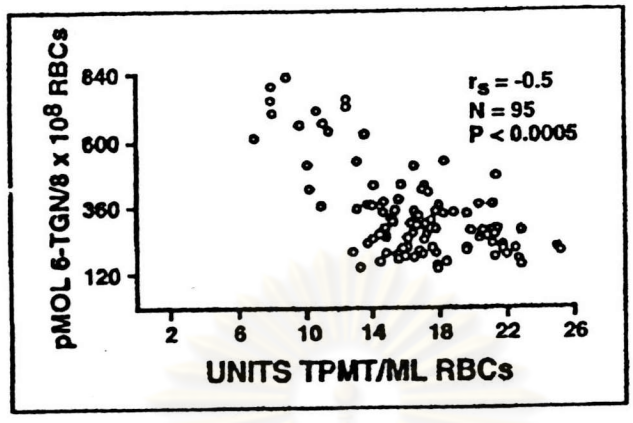


Figure.8 Relationship between RBC TPMT activity and RBC 6-TGN concentrations in 95 children with acute lymphoblastic leukemia (ALL who were treated on protocol UK ALL VIII). From Weinshilboum., 2001.

- Interethnic variations of TPMT alleles

Based on the population genotype-phenotype studies performed to date, assays for the molecular diagnosis of TPMT deficiency have focused on the following alleles TPMT*2, TPMT*3A and TPMT*3C. By using allele-specific polymerase chain reaction (PCR) or polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), three signature mutations in these alleles, detected and identified up to 80-95% of all mutant alleles. Population studies in Caucasian, East and West African, African-American, Chinese, Japanese, Thai and Southwest Asian populations have demonstrated the utility of this approach. However, the frequency and pattern of mutant TPMT alleles is different among various ethnic populations (Table 1). For example, Southwest Asians (Indian, Pakistani) have a lower frequency of mutant TPMT alleles and all mutant alleles identified to date are TPMT*3A. This is in contrast with East and West African populations where the frequency of mutant alleles is similar to Caucasians but all mutant alleles in the African populations are TPMT*3C. Among African-Americans, TPMT*3C is the most prevalent but TPMT*2 and TPMT*3A are also found, reflecting the integration of Caucasian and African-American genes in

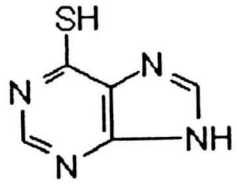
US populations. More data on inter-ethnic variations in TPMT polymorphisms continue to emerge and accordingly genotyping strategies of the future are likely to take into account for such variations (McLeod. et al., 2002).

Table.1 Ethnic variation in TPMT alleles.

Ethnic group	n	wt/wt %	wt/mu %	mut/mut %	TPMT*2 %	TPMT*3A %	TPMT*3C %
British Causasian	199	89.9	9.6	0.5	0.5	4.5	0.3
French Causasian	191	85.9	13.6	0.5	0.5	5.7	0.8
AmericanCausasian	*	92.5	7.4	0.14	0.2	3.2	0.2
African-American	*	90.7	9.2	0.2	0.4	0.8	2.4
Kenyan	101	89.1	10.9	0	0	0	5.4
Ghanaian	217	85.3	14.4	0.5	0	0	7.6
Chinese	192	95.3	4.7	0	0	0	2.3
Japanese	553	97.3	2.4	0.4	0	0	1.5
Thai	75	89	11	0	0	0	5.3
Southwest Asian	99	98	2	0	0	1	0

*: Calculated; mut: Mutant; TPMT: Thiopurine methyltransferase; wt: wild type. From McLeod. *et al.* 2002

6-Mercaptopurine (Puri-Nethol®)



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6-mercaptopurine (6-MP) is indicated for the treatment of acute leukemia. It may be utilized in remission induction and is particularly indicated for maintenance therapy in: acute lymphoblastic leukemia and acute myelogenous leukemia. 6-MP may be used in the treatment of chronic granulocytic leukemia. 6-MP and other thiopurines, azathioprine and 6-thioguanine (6-TG), are all inactive prodrugs, requiring metabolism to thioguanine nucleotides (TGN) in order to exert cytotoxicity (Figure.9). The principal cytotoxic mechanism of these agents is generally considered to be mediated by incorporation of TGN into DNA and RNA. Both 6-MP and 6-TG, TGNs are formed by a multi-step pathway which is initiated by hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Alternatively, these agents can undergo S-methylation catalyzed by thiopurine methyltransferase (TPMT) to methylmercaptopurine (MMP) or oxidation to thiouric acid via xanthine oxidase (XO). Metabolism via either TPMT or XO reduces formation of the active TGNs. TPMT can also S-methylate 6-thioinosine 5' monophosphate (TIMP), yielding the S-methylated derivative (methylTIMP). MethylTIMP is a potent inhibitor of de novo purine synthesis and represents an alternative mechanism for cytotoxicity (McLeod. et al., 2000).

Dosage in adults and children:

For adults and children the usual starting dose is 2.5 mg/kg bodyweight per day, or 50-75 mg/m^2 body surface area per day, but the dose and duration of administration depend on the nature and dosage of other cytotoxic agents given in conjunction with 6-MP. The dosage should be carefully adjusted to suit the individual patient. 6-MP has

been used in various combination therapy schedules for acute leukemia and the literature should be consulted for details.

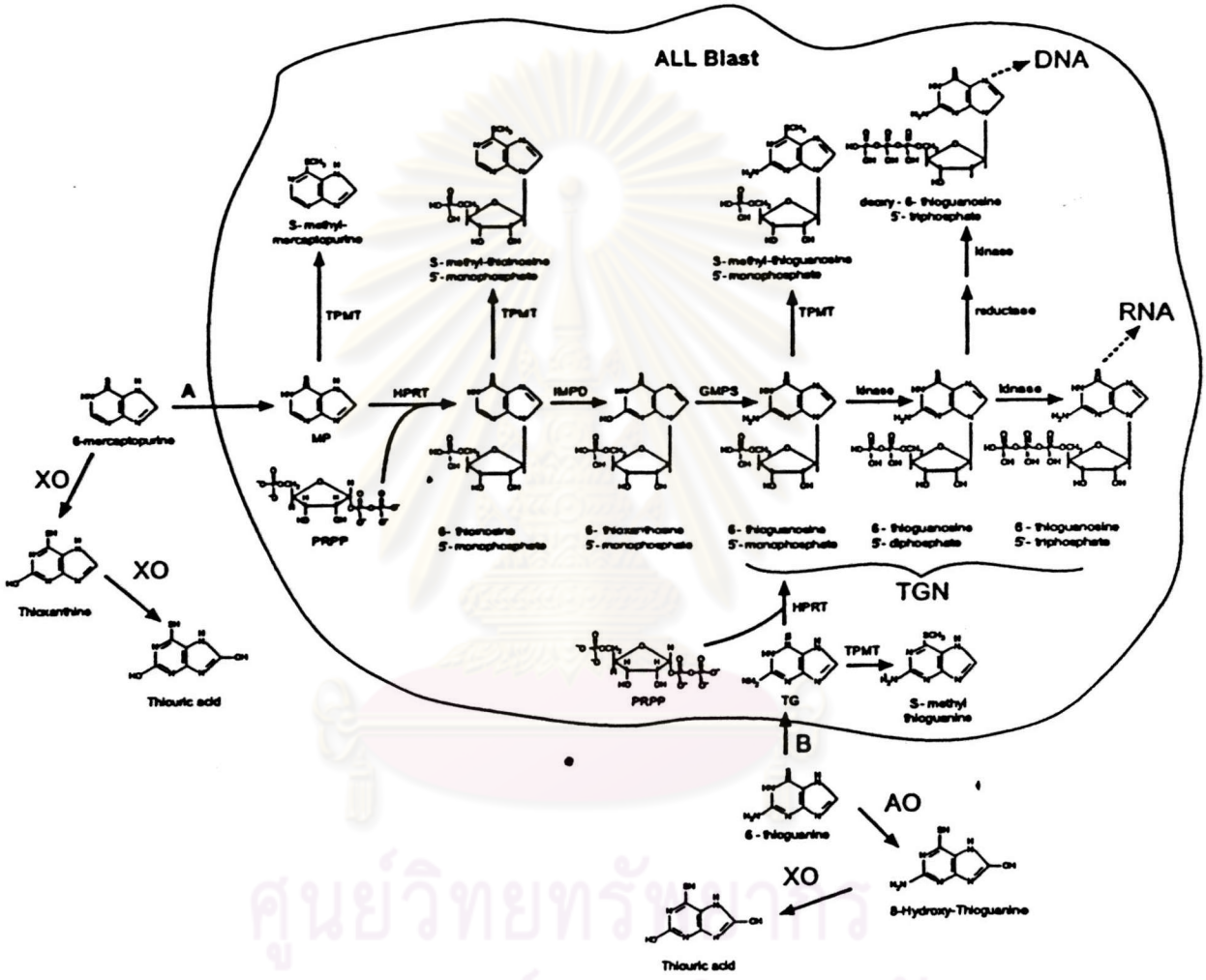

Figure.9 Metabolism of 6-mercaptopurine (A), and 6-thioguanine (B) in human leukemic cells. From McLeod. et al., 2000.

Monitoring:

- Treatment with 6-MP causes bone marrow suppression leading to leucopenia and thrombocytopenia, and less frequently to anemia. Full blood counts must be taken daily during remission induction and careful monitoring of hematological parameters should be conducted during maintenance therapy.

- The leukocyte and platelet counts continue to fall after treatment is stopped, so at the first sign of an abnormally large fall in the counts, treatment should be interrupted immediately.

- Bone marrow suppression is reversible if 6-MP is withdrawn early enough.

- During remission induction in acute myelogenous leukemia the patient may frequently have to survive a period of relative bone marrow aplasia and it is important that adequate supportive facilities are available.

- There are individuals with an inherited deficiency of the enzyme thiopurine methyltransferase (TPMT) who may be unusually sensitive to the myelosuppresive effect of 6-mercaptopurine and prone to develop a rapid bone marrow depression following the initiation of treatment with 6-MP. This problem could be exacerbated by coadministration with drugs that inhibit TPMT, such as olsalazine, mesalazine or sulphasalazine.

- 6-MP is hepatotoxic and liver function tests should be monitored weekly during treatment. More frequent monitoring may be advisable in those with pre-existing liver disease or receiving other potentially hepatotoxic therapy. The patient should be instructed to discontinue 6-MP immediately if jaundice becomes apparent.

- During remission induction when rapid cell lysis is occurring, uric acid levels in blood and urine should be monitored as hyperuricaemia and/or hyperuricosuria may develop, with the risk of uric acid nephropathy.

- Cross resistance usually exists between 6-mercaptopurine and 6-thioguanine.

- The dosage of 6-mercaptopurine may need to be reduced when this agent is combined with other drugs whose primary or secondary toxicity is myelosuppression.

Interaction with other medicinal products ad other forms of interaction

- When allopurinol and 6-MP are administered concomitantly it is essential that only a quarter of the usual dose of 6-MP is given since allopurinol decreases the rate of catabolism of 6-MP.

- Inhibition of the anticoagulant effect of warfarin, when given with 6-MP, has been reported.

- As there is *in vitro* evidence that aminosalicylate derivatives (eg. olsalazine, mesalazine or sulphasalazine) inhibit the TPMT enzyme, they should be administered with caution to patients receiving concurrent 6-MP.

Pharmacokinetic

- The bioavailability of oral 6-mercaptopurine shows considerable inter-individual variability, which probably results from its first-pass metabolism (when administered orally at a dosage of 75 mg/m^2 to 7 pediatric patients, the bioavailability averaged 16% of the administered dose, with a range of 5 to 37%).

- The elimination half-life of 6-mercaptopurine is 90 ± 30 minutes, but the active metabolites have a longer half-life (approximately 5 hours) than the parent drug. The apparent body clearance is 4832 ± 2562 ml/min/m^2. There is low entry of 6-mercaptopurine into the cerebrospinal fluid.

- The main method of elimination for 6-mercaptopurine is by metabolic alteration. The kidneys eliminate approximately 7% of 6-mercaptopurine unaltered within 12 hours of the drug being administered. Xanthine oxidase is the main catabolic enzyme of 6-mercaptopurine. It converts the drug into the inactive metabolite, 6-thiouric acid which is excreted in the urine.