

***AZATHIOPRINE***

## Introduction

Azathioprine is the nitroimidazole derivative of 6 mercaptopurine and is the most widely used cytotoxic immunosuppressant in clinical medicine. In combination with corticosteroids, azathioprine is the primary agent employed to suppress transplantation rejection reactions. More recently, it has been used, with increasing frequency, in the treatment of autoimmune diseases. (*Winkelstein 1979*)

Azathioprine and 6 mercaptopurine (6-MP) are biochemically classified as thiopurines (fig 1). The original compound in this series, 6-MP, a sulfate derivative of hypoxanthine, was initially developed as an antileukemic agent. (*Elion et al 1952*) Shortly thereafter, it was recognized that it possessed immunosuppressive activities. (*Schwartz et al 1958*) Azathioprine was synthesized in 1961; it was reasoned that addition of an imidazole side group to 6 MP would delay metabolism and result in a compound with a longer duration of action. (*Elion et al 1961*) However, experimental studies did not bear out this concept. In mice, approximately 70% of an intravenous dose of azathioprine is converted to 6 MP within five minutes. (*Elion et al 1967*) This process is non-enzymatic, but can be accelerated by sulfhydryl-containing compounds such as glutathione. Small amounts of azathioprine may be degraded by alternate pathways. It has been postulated that compounds generated in these alternate reactions may have intrinsic immunosuppressive properties which are distinct from those related to 6 MP (*Elion et al 1972*)

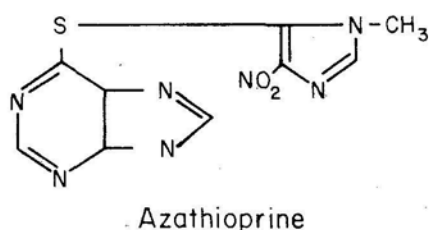
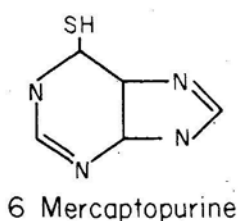
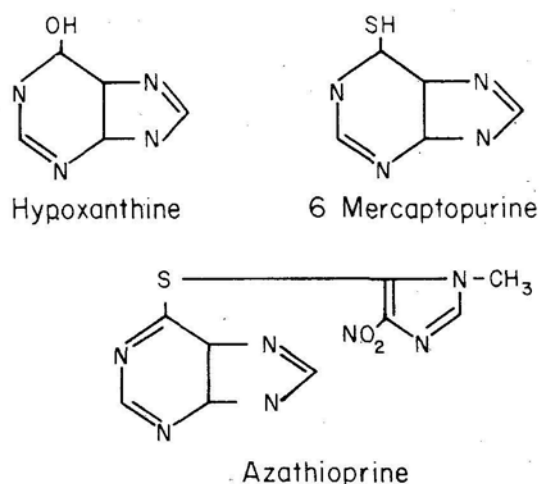


Fig 1 Structure of Hypoxanthine, 6 Mercaptopurine and Azathioprine

Azathioprine has an effect both on immunological response and on tumour growth. Its major role has been as an agent for suppressing the immune response, and although the exact mechanism whereby it achieves its effect is unknown, it has an action with three major components: (*Sir Colin Dollery*)

1. Impairment of the cellular component of the immune response, the B-cell series being the cells most affected, with reduction of both IgM and IgG synthesis.
2. Inhibition of the cellular component of the inflammatory response.
3. Depression of cell proliferation with consequent diminution of non-specific immunity.

Following absorption of azathioprine, it is rapidly converted to 6-MP. Extensive first-pass metabolism occurs in the intestinal mucosa and liver, where xanthine oxidase and thiopurine methyltransferase (TPMT) convert 6-MP to the inactive metabolites 6-thiouric acid and 6-methyl-mercaptopurine (6-MMP), respectively. The subsequent metabolic steps occur intracellularly, where hypoxanthine guanine phosphoribosyl transferase converts 6-MP to 6-thioinosine-5'-monophosphate (TIMP). The latter molecule is converted to the active, but potentially myelotoxic metabolites, the 6-thioguanine nucleotides (6-TGN). The therapeutic and immunosuppressive effects of 6-MP and AZA are thought to be primarily due to the intracellular formation of the 6-TGN after their incorporation into the DNA and RNA of cells. (*Seidman et al 2002*)

Azathioprine has been established as a drug which protects against rejection of human organ transplants. It is usually given initially at 5mg/kg daily, and then reduced to maintenance levels of 1-2 mg/kg daily. (*Sir Colin Dollery*)

Azathioprine finds use in the treatment of rheumatoid arthritis in patients with severe, active, and erosive disease not responsive to conventional management including rest, nonsteroidal anti-inflammatory drugs, and/or disease-modifying antirheumatic drugs (DMARDs). Azathioprine is one of several DMARDs that can be used when DMARD therapy is appropriate. NSAID or salicylate therapy may be continued when treatment with azathioprine is initiated. Azathioprine therapy may allow reduction of corticosteroid requirements. (*AHFS*)

Azathioprine and 6-mercaptopurine have been used in the treatment of inflammatory bowel disease, i.e. ulcerative colitis and Crohn's disease, for more than 30 years. However, widespread use of azathioprine or 6-mercaptopurine in inflammatory bowel disease is of more recent origin, the primary reason being a long-standing debate on the efficacy of these agents in inflammatory bowel disease. Both drugs are slow acting, which is why clinical efficacy cannot be expected until several weeks or even months of treatment have elapsed. Consequently, azathioprine and 6-mercaptopurine have no place as monotherapy in the treatment of acute relapsing inflammatory bowel disease. Today, azathioprine and 6-mercaptopurine are the most commonly used immunomodulatory drugs in the treatment of inflammatory bowel disease. (*Nielsen et al 2001*)

The principal toxic effect of azathioprine is bone marrow depression manifested by leukopenia, macrocytic anemia, pancytopenia, and thrombocytopenia which are dose related. When receiving usual dosages of azathioprine, patients with intermediate levels of thiopurine methyl transferase (TPMT) activity (about 10-11% of the population) may be at increased risk of developing myelotoxicity, while those with low or absent levels of the enzyme (0.3% of the population) are at increased risk of life-threatening myelotoxicity. Reduced dosage is recommended in patients with intermediate TPMT activity, while alternative therapy may be considered in those with low or absent levels of TPMT. The other major toxic effect of azathioprine is hepatotoxicity manifested by increased serum alkaline phosphatase, bilirubin, and/or aminotransferase concentrations may occur in patients receiving azathioprine, principally in allograft recipients. (*AHFS*)

## **Module 2.4**

### **Nonclinical Overview**

#### **NONCLINICAL OVERVIEW**

##### **2.4.1 Overview of the Nonclinical Testing Strategy**

Since the data in this module is provided from published sources, the above section is not applicable.

##### **2.4.2 Pharmacology**

###### **Immunosuppressive Action**

The exact mechanism of immunosuppressive activity of azathioprine has not been determined. Azathioprine which is an antagonist to purine metabolism, may inhibit RNA and DNA synthesis. The drug may also be incorporated into nucleic acids resulting in chromosome breaks, malfunctioning of the nucleic acids, or synthesis of fraudulent proteins. The drug may also inhibit coenzyme formation and functioning, thereby interfering with cellular metabolism. Mitosis may be inhibited by the drug. (*AHFS*) Many of azathioprine's actions are attributed to 6-mercaptopurine (6 MP) to which it is converted in the body. (*Martindale*)

Studies indicate that, in vitro, azathioprine is specifically able to bind murine T lymphocytes; this can be shown by their ability to inhibit their capacity to rosette with sheep erythrocytes. Azathioprine is also a potent inhibitor of mixed lymphocyte culture responses and can readily suppress the in vitro generation of cytotoxic T cells. These observations suggest that drugs exert preferential toxicities for murine T cells. B lymphocytes for mice appear to vary in their susceptibility for thiopurines. By contrast, the activity of human B cells can be readily suppressed with this drug whereas T helper function is comparatively resistant. In addition to immunosuppressive properties, thiopurines are capable of exerting anti-inflammatory activities, primarily by inhibiting the replication of hematopoietic precursors. (*Winkelstein 1979*)

The short-term, in vitro responses of canine peripheral blood lymphocytes to mitogenic stimulation and serum immunoglobulin concentrations were evaluated following treatment with currently recommended doses of cyclophosphamide and azathioprine. Cyclophosphamide had no significant effect on either the serum immunoglobulin concentrations or the blastogenic response of lymphocytes to mitogenic stimulation. Serum immunoglobulin concentrations remained unchanged following azathioprine treatment. The blastogenic response was significantly suppressed following one week of azathioprine therapy and returned to baseline values one week following cessation of treatment. The study concluded that the short-term use of azathioprine, but not cyclophosphamide, in clinically used dosages, suppresses selective aspects of the canine immune system, and the T cells appear to be more susceptible than B cells to the immunosuppressive effect of this drug. (*Ogilvie et al 1988*)

The in vitro antibody response to most T-independent antigens is inhibited by azathioprine only at concentrations of 1-10 µg/ml. In contrast, B cell response to T-dependent antigens and to the T-independent antigen is sensitive to low azathioprine concentrations ( $10^{-2}$  µg/ml). These data suggest the existence of two different B cell activation processes : a. The activation by B cell mitogens or T-independent antigens with a mitogenic moiety which are azathioprine-resistant; b. The activation by T-dependent antigens or T-independent antigens without a mitogenic moiety which are azathioprine-sensitive. (*Galanaud et al 1975*)

A Study (Murthy et al 1991) suggested that long-term treatment with azathioprine may prevent extravasation and cause reduction in neutrophil trafficking which may be beneficial for maintaining remission in IBD. In this study, rats were treated with I.P. injection of azathioprine (1 mg/kg) for 6 weeks. At the end of 2 and 6 weeks rats were injected I.V. immune complex and on the following day the proximal colon was perfused with 2.5% formaldehyde (local irritant 3 ml/hour for 5 mins). Extravasation was measured by Evans' blue technique and neutrophil concentration in the tissue was determined by measuring myeloperoxidase (MPO). Azathioprine did not inhibit extravasation and MPO after 2 weeks of therapy. However, after 6 weeks, azathioprine reduced extravasation to  $20 \pm 2$  µg/gm compared to untreated animals ( $51 \pm 6$  µg /gm tissue) and MPO levels to  $0.3 \pm 13$  compared to untreated rats ( $0.8 \pm 0.32$  mU/gm). There was a good correlation between extravasation and MPO levels.

### **Anti-inflammatory Action**

It has been shown that azathioprine and 6-mercaptopurine (10-500 µg/ml) inhibit in a dose-dependent manner the production of PGE<sub>2</sub>, PGF<sub>2</sub> alpha, 6-keto-PGF<sub>1</sub> alpha and TXB<sub>2</sub> by unseparated spleen cells as well as that of 6-keto-PGF<sub>1</sub> alpha by adherent peritoneal macrophages. This inhibitory effect appears rapidly in vitro (within 15 min of incubation) and is observed in the presence of exogenous arachidonic acid ( $5 \times 10^{-6}$  M). The persistence of this effect in the presence of arachidonic acid, together with the fact that the production of four cyclooxygenase derivatives of acid arachidonic metabolism are inhibited, suggests that these drugs are acting at the cyclooxygenase level. This finding may explain in part the anti-inflammatory effects of azathioprine and 6-mercaptopurine. (*Homo-Delarche et al 1988*)

Significant suppression of in vitro PGE<sub>2</sub> production by rabbit retinas was reported reports with azathioprine at the concentrations of 0.1 and 0.05 µg/ml. However, at concentrations below (0.01 µg/ml) or above (1.10 µg/ml) azathioprine had no inhibitory effect, suggesting a dose-dependent inhibitory effect of azathioprine on prostaglandin E<sub>2</sub> production. (*Dottan et al 1987*)

### 2.4.3 Pharmacokinetics

The plasma concentrations and tissue distribution of thiopurines were studied in mice after oral administration of 50 mg/kg azathioprine using HPLC analysis. Peak concentrations of azathioprine and three other thiopurine metabolites in plasma were observed as early as 10 min after drug application, thus indicating fast absorption and extensive metabolism of azathioprine, and were followed by a rapid decline. The extraction of thiopurines from organs (intestinal mucosa, liver, kidney, testes, spleen, and bone marrow) and from red blood cells (RBCs) was preceded by an acid hydrolysis procedure resulting in the release of thiopurine bases from their corresponding ribonucleotides. 6-MP, 6-thioxanthene (6-TX), 6-thioguanine (6-TG), thiouric acid (TUA) and 8-hydroxy-6-MP (8-OH-6-MP) were extracted from the organs, whereas only 6-MP and 8-OH-6-MP were found in the processed RBCs. Initially, high concentrations of TUA, the endpoint of metabolic azathioprine degradation, were detected in the intestinal mucosa and in the liver. This provides evidence for a first-pass metabolism of azathioprine in these two organs. The initial concentrations of 6-MP extracted from the organs were about 10-fold those found in plasma. This indicates rapid cellular uptake of 6-MP and an accumulation of 6-MP derivatives that can be explained by formation of the 6-MP ribonucleotide thioinosine monophosphate (TIMP). With the exception of plasma and RBCs, 6-TG, which may originate from intracellular 6-thioguanosine nucleotides (TGNs), was extracted from all organs examined in the study. The highest concentrations of 6-TG derivatives were found in the spleen and bone marrow. This correlates with the clinical and experimental observation that azathioprine cytotoxicity mainly affects bone-marrow stem cells and lymphocytes and supports the hypothesis that the incorporation of TGN into DNA is the cytotoxic mechanism of azathioprine and 6-MP. (*Kurowski et al 1991*)

The metabolism of azathioprine in liver-injured rats was studied *in vivo* by measuring the clearance rate (K) of the drug from the blood, and its excretion in the urine. The K values in probenecid, diethyl maleate and carbon tetrachloride (CCl<sub>4</sub>)-treated rats were much smaller than those in control rats. (*Hobara et al 1981*) Probenecid, a known inhibitor of glutathione S-transferase (*Hobara et al 1981, Kaplowitz et al 1978*), inhibited the urine excretion of azathioprine, and diethyl maleate produced a prompt depletion of hepatic reduced glutathione. Reduced levels of both glutathione and glutathione S-transferase activity were observed in rats treated with CCl<sub>4</sub>. Pretreatment with glutathione resulted in no significant change of the K value. These findings, together with the reported kinetic data of the transferase, indicate that the conversion of azathioprine to 6-mercaptopurine *in vivo* may be catalyzed largely enzymatically by glutathione S-transferase in the liver. (*Hobara et al 1981*)

The bioavailabilities and pharmacokinetics of azathioprine (AZA) and 6-MP were studied in the rhesus monkey. Four male rhesus monkeys were used. Four doses of drug were given to each monkey: iv AZA, iv 6-MP, po AZA, and po 6-MP. Monkey I received 10.8 µmol of AZA or 6-MP per kg on all four occasions. Monkeys II, III, and IV received 10.8 µmol/kg for iv AZA, iv 6-MP and po 6-MP, and 32.5 µmol/kg for AZA given orally. This is because initial determinations of 6-MP plasma levels following 10.8 µmol/kg po doses of AZA in three monkeys yielded values that were too low for accurate estimates of bioavailability. Monkeys II and IV also received 10.8 µmol/kg iv doses of 8-hydroxymercaptopurine (8-OHMP). (*Ding et al 1979*)

Following iv 6-MP administration, 6-MP levels were described by a two-compartment body model; mean terminal half-life; plasma clearance (CL<sub>p</sub>), and volume of distribution (V<sub>dss</sub>) were 41.6 ± 12.1 min, 48.4 ± 15.4 ml/min/kg, and 1.76 ± 0.64 liters/kg, respectively. (Table 1) (*Ding et al 1979*)

8-OHMP had a CL<sub>p</sub> twice that for 6-MP, while its V<sub>dss</sub> was similar to that for 6-MP. After an iv dose, AZA is converted to 6-MP to the extent of 15%. The conversion of AZA to 6-MP and 8-OHMP was independent of the route of administration. (*Ding et al 1979*)

Table 1 6-Mercaptopurine kinetics in 4 monkeys following 10.8 μmol/kg iv dosing

Monkey	Weight	t <sub>1/2α</sub>	t <sub>1/2β</sub>	CL <sub>p</sub>	V <sub>d<sub>ss</sub></sub>	R <sup>2*</sup>
	kg	min	min	ml/min/kg	lit/kg	
I	6.8	6.1	47.0	34.6	1.31	0.972
II	4.6	5.0	43.7	35.6	1.28	0.988
III	4.4	11.2	51.7	59.8	2.66	0.996
IV	5.2	2.7	24.2	63.4	1.77	0.997

\* From NONLIN least-square regression analysis.

## 2.4.4 Toxicology

### Single Dose Toxicity

LD<sub>50</sub> Rat oral - 535 mg/kg (*Lewis 1996*)

LD<sub>50</sub> Rat intraperitoneal - 300 mg/kg (*Lewis 1996*)

LD<sub>50</sub> Rat intraduodenal - 630 mg/kg (*Lewis 1996*)

LD<sub>50</sub> Mouse oral - 1389 mg/kg (*Lewis 1996*)

LD<sub>50</sub> Mouse intraperitoneal - 273 mg/kg (*Lewis 1996*)

LD<sub>50</sub> Mouse subcutaneous - 350 mg/kg (*Lewis 1996*)

LD<sub>50</sub> Mouse intraduodenal - 2437 mg/kg (*Lewis 1996*)

The frequency of micronuclei was assessed in polychromatic erythrocytes of bone marrow of rats following single dose of azathioprine. At the maximum tolerated dose in the single-dose study (40 mg/kg) the incidence obtained at 48 h post-treatment was 15.7/1000. (*Henderson et al 1993*)

### Repeated dose toxicity

#### Bone Marrow Depression

Toxicity studies in animals have shown that the haemopoietic system is most affected, with depression mainly of granulopoiesis and relative sparing of megakaryocytes and, hence, platelet formation. (*Sir Colin Dollery*)

Of 60 newborn, random-bred Swiss mice of both sexes, 22 survived day 30 after administration of 40 mg/kg body weight azathioprine (dissolved in 0.1N NaOH and diluted in physiological saline) once a day on days 1-4 after birth. The experiment was terminated after 180-200 days. Of 135 controls treated with identical amounts of physiological saline, 124 survived day 30. Leukemia developed in 4/20 treated mice (20%), and in only 1 of 119 controls. This difference was reported to be statistically significant ( $p < 0.001$ ). Azathioprine was administered under identical conditions to an additional group of 17 newborn mice as 4 single doses of 10 mg/kg on days 1-4 after birth. In the 14 animals that survived day 30, no leukemia occurred; 2 (14%) animals developed lung adenomas, and this type of tumor was reported in 10/119 (8%) controls. (*IARC*)

The main toxic effect of azathioprine (after 45 mg/kg body weight per day in rats or 4 mg/kg body weight per day in dogs) is bone-marrow depression. Lymphocyte depletion in lymphoid tissues has also been observed in mice following chronic administration. Infections have been a frequent complication, especially in animals receiving large doses for a prolonged period, the germ-free mice tolerated larger doses than mice in a conventional environment. Such infections can develop with a large variety of organisms, including those with a facultative intracellular parasitism. (*IARC*)

The frequency of micronuclei was assessed in polychromatic erythrocytes of bone marrow and in polychromatic and normochromatic erythrocytes in peripheral blood of rats following exposure to azathioprine for 28 days. The incidence of micronuclei in bone-marrow polychromatic erythrocytes at the maximum tolerated dose (10 mg/kg)

following exposure for 28 days was 29.5/1000. The incidence of micronucleated polychromatic erythrocytes in the peripheral blood at this dose was 4.4/1000. (*Henderson et al 1993*)

Twelve rabbits weighing between 1.8 and 3.5 kg were divided into two groups (6 rabbits for each group). The rabbits in the test group received daily administration of azathioprine (10 mg/kg) orally for two weeks. The rabbits in the control group received daily administration of azathioprine vehicle (0.01MNaOH in 0.9% NaCl) for two weeks. Blood samples were obtained from the marginal ear vein. On day zero and prior to any treatment, blood counts and hemoglobin estimation were performed. Daily administration of azathioprine (10 mg/kg) for two weeks did not cause any statistically significant change in erythrocyte count, leukocyte count or hemoglobin level. However, there was a significant increase (34.1%) in platelet count in all tested rabbits during the two-week treatment. Platelet count returned back to the basal level on day 45. Moreover, erythrocyte count was significantly depressed (60.2%) on day 45 although azathioprine treatment was discontinued on day 14. All rabbits died between days 47–54 due to severe fatal macrocytic anemia. The mechanism of azathioprine-induced macrocytosis is unclear as there was no deficiency of vitamin B12 and/or folate. Azathioprine-induced macrocytosis may be due to the inhibition of DNA synthesis in bone marrow precursor cells leaving both RNA and protein synthesis intact. (*Al-Safi et al 2002*)

In dogs, 10 mg/kg for 10 days caused death from agranulocytosis. (*Elion et al 1961*) Allied to the effect on haemopoiesis is the effect on the lymphatic system, with atrophy of the lymphoid tissue being seen in rhesus monkeys on a dose of 1 mg/kg daily. (*Sir Colin Dollery*)

Profound neutropenia (less than 600 cells/ $\mu$ l) was observed in 5 cats treated with an azathioprine suspension (2.2 mg/kg of body weight on alternate days). Treatment was discontinued if the WBC count was less than 3,000 cells/ $\mu$ l. Four weeks after discontinuation of azathioprine, 1 treated cat again was given azathioprine at a lower dosage (1.1 mg of azathioprine/kg on alternate days) and neutropenia recurred within 2 weeks. During treatment, 3 cats developed thrombocytosis, and 2 developed thrombocytopenia. In 4 of 5 cats, neutropenia and thrombocytopenia resolved when azathioprine was discontinued. Bone marrow cytologic examination during treatment revealed reduction of the neutrophil line, with relative increase in monocytes. Core bone marrow biopsy at the completion of the study revealed hypocellular marrow with marked decrease in the myeloid series in cats given azathioprine. (*Beale et al 1992*)

### Liver Damage

Sprague-Dawley rats given azathioprine in the diet for 3 to 4 weeks developed severe liver damage. Elevations of serum alkaline phosphatase and gamma-glutamyl transpeptidase activities were associated with increased hepatic glucose 6-phosphate dehydrogenase levels and decreased liver glucose 6-phosphatase activities, i.e., conditions which were commonly observed in various hepatotoxin-induced liver injuries. Light and electron microscopic observations revealed centrilobular necrosis with large scars and the proliferation of the mitochondria and rough endoplasmic reticulum. (*Watanabe et al 1979*)

Azathioprine, like 6-mercaptopurine, may damage the liver. A reversible hepatotoxicity has been seen in dogs. (*Aronsen et al 1969*) Dogs are very susceptible to this reaction, with hepatotoxicity being produced at a dose of only 5 mg/kg daily. (*Sir Colin Dollery*)

## **Reproduction Toxicity**

### ***Effect on Fertility***

Azathioprine has been reported to cause temporary depression in spermatogenesis and reduction in sperm viability and sperm count in mice at doses 10 times the usual human dose; a reduced percentage of fertile matings occurred when animals received 5 mg/kg. (AHFS)

### ***Teratogenic Effects***

Teratogenicity has been seen in a number of animal species, with a varying degree of susceptibility. In rabbits, 5-15 mg/kg daily on days 6-14 of pregnancy produced skeletal abnormalities; in mice and rats, doses of 1-2 mg/kg daily on days 3-12 were lethal to the embryos. (Elion *et al* 1975)

Azathioprine was shown to induce embryoletality in Wistar rats and Swiss albino mice when given at doses of 1-20 mg/kg body weight on days 3-12 of pregnancy. No gross malformations were noted in the surviving fetuses. Intragastric doses of 5-15 mg/kg body weight azathioprine to rabbits on days 6-14 of gestation induced a large variety of skeletal malformations. Under these experimental conditions in rabbits, the rate of resorptions was not increased when compared with controls. (IARC)

A single sc dose of 50 mg/kg body weight azathioprine given on day 10 of gestation was teratogenic in NMRI mice, inducing cleft palates in 90%, malformations of the vertebrae in 70%, malformations of the upper extremities in about 75% and malformations of the lower extremities in 50% of the fetuses. When the same dose was administered orally at the same gestational stage, 80% of fetuses showed cleft palates and about 20% showed abnormalities of the lower extremities; embryomortality was approximately 20% in both treated groups, in comparison to 6% in the control series. (IARC)

### **Humans**

Despite reports of chromosomal aberrations (*The Registration Committee of the European Dialysis and Transplant Association 1980*) or fetal growth retardation (*Pirson et al 1985*) in the offspring of mothers who received azathioprine during pregnancy, there seems to be little evidence that azathioprine is teratogenic in humans. (*Hou 1985; Whittle et al 1986; Alstead et al 1990*) Given the nature of the severe chronic conditions for which azathioprine is generally used, discontinuing therapy in patients who become pregnant may not be necessary or desirable, but it seems prudent to avoid its use where possible during pregnancy. Leucopenia has been reported in neonates whose mothers received azathioprine during pregnancy. (*Davison et al 1985*)

## **Mutagenic Potential**

Azathioprine is mutagenic in animals and in humans. Chromosomal abnormalities have been documented in humans receiving azathioprine, but the abnormalities were reversed following discontinuance of the drug. (*AHFS*)

Azathioprine in a dose of 50 µg/ml produced cytogenetic damage in human lymphocytes in vitro, and cytogenetic changes were found in lymphocytes of rabbits given 5-20 mg/kg daily of azathioprine. (*Obe et al 1971*) Azathioprine was also mutagenic in the Ames test.

## **Oncogenic/Carcinogenic Potential**

Studies were conducted on 100 male and 100 female CDI mice receiving 0, 0.3 and 10 mg/kg daily of azathioprine for 18 months. Mice on high dose were not given the drug for weeks 21-38. There was a dose-related excess of lymphosarcoma in both males and females. A similar study in rats produced carcinomas in treated animals, but the incidence was not dose-related. (*Sir Colin Dollery*)

Azathioprine was administered intramuscularly into the thigh muscles of male inbred NZB mice before onset of autoimmune hemolytic anemia, which develops spontaneously in mice of this strain. Azathioprine was dissolved in 0.1N NaOH and then diluted with physiological saline to a final concentration of 10 mg/ml azathioprine; single doses corresponded to 100 mg/kg body weight. The 16 mice, aged 2 months, were matched into two similar groups on the basis of body weight and hematocrit readings; 8 mice were injected 3 times a week for 4 weeks, followed by 2 injections during the 5th week and then one injection a week, for a total treatment period of 6 months; 8 controls were given the solvent only. Of the treated mice, 6/8 (75%) had malignant thymic lymphomas; 4 of these died during treatment, and in the other 2 animals the tumors were diagnosed after termination of the experiment. No lymphoma was seen in the 6 control mice examined histologically at the end of the study. (*IARC*)

Two groups, each of 25 male and 25 female outbred Swiss-Webster-derived mice, 6 weeks old, were given ip injections of either 7.5 or 15 (females, 30) mg/kg body weight azathioprine as an NaOH-stabilized solution in physiological saline 3 times a week for 6 months. Animals that survived over 100 days were observed for up to 12 further months, at which time they were killed. Controls consisted of 254 untreated mice. The survival times of the treated animals were reported as percentages of that of the controls (no precise definition of the mode of calculation was given): the survival times of treated male mice were reported to be 51-77% that of untreated controls, and the corresponding figures for females were 23-75%. Animals that died before day 100 on test were excluded from evaluation. The incidence of 9/21 (43%) tumor-bearing males and of 25/40 (63%) tumor-bearing females was 1.5-2 times higher than that in controls (26%), in which there was only a small difference between males (28/101) and females (38/153). (*IARC*)

A group of 50 male (6 week old) and 50 female (8 week old) Fischer 344 stain rats were fed ad libitum a diet containing 150 mg/kg azathioprine for 52 weeks. Ten males and 10 females fed a normal diet served as controls. Male rats tolerated the treatment; females rapidly showed weight loss, diarrhea and tachypnea after 5-6 weeks on the diet, and more than 40% died within the first 12 weeks of the study. All surviving animals were killed after 52 weeks. Squamous-cell ear-duct carcinomas developed in 3/25 female and 3/17 male treated rats and in none of 20 controls animals. There was no difference in the incidence of other tumor types. (*IARC*)

Treatment of mice with even low doses of azathioprine was found to increase cell cycle time of promonocytes by over 5 hr and led to a 70% reduction of monocyte production. (*Foye 1995*)

## Humans

Classification of carcinogenicity: 1) evidence in humans: sufficient; 2) evidence in animals: limited. Overall summary evaluation of carcinogenic risk to humans is Group 1: The agent is carcinogenic to humans. (*IARC*)

Immunosuppression, including that with azathioprine, may be associated with an increased risk of certain neoplasms such as lymphomas and skin cancers in transplant recipients (*Kinlen et al 1979*) and in patients with rheumatoid arthritis. (*Silman et al 1988; Asten et al 1999*) Rheumatic diseases may themselves be associated with an increased risk of malignancy that is independent of treatment, but one study (*Asten et al 1999*) concluded that there is a further risk related to the duration of exposure to immunosuppressive drugs, including azathioprine. Conversely, a study in 755 patients given azathioprine for inflammatory bowel disease and followed for up to 29 years failed to show any increased risk of neoplasia. (*Connell et al 1994*)

Skin cancer may be a particular risk in immunosuppressed patients with a history of high sun exposure. A synergistic clastogenic effect has been noted with azathioprine and long-wave ultraviolet light. (*Boyle et al 1984*)

## **Local Tolerance Studies**

Local tolerance data is not available.

## 2.4.5 Impurities

The impurities listed in the DMF file of azathioprine are:

6-Mercaptopurine – Limit NMT 0.2%

Hypoxanthine – Limit NMT 0.2%

5-Chloro-1-methyl nitroimidazole – Limit NMT 0.2%

### Toxicity profile of the impurities

#### 6-Mercaptopurine

Inadequate evidence of carcinogenicity in humans. Inadequate evidence of carcinogenicity in animals. Overall evaluation: Group 3: The agent is not classifiable as to its carcinogenicity to humans. (*IARC*)

Mercaptopurine causes chromosomal aberrations in animals and humans and induces dominant-lethal mutations in male mice. (*AHFS*)

Long-term 6-mercaptopurine treatment in male mice did not impair sperm production and sperm morphology. However, a significantly high rate of embryonic resorption indicated occult sperm damage. (*Ligumsky et al 2005*)

Toxicological data on hypoxanthine and 5-Chloro-1-methyl nitroimidazole not available.

## **2.4.6 Environmental Risk Assessment**

Since azathioprine is an antimetabolite, consideration should be given to handling and disposal according to guidelines issued for hazardous drugs, although there is no general agreement that all of the procedures recommended in such guidelines are necessary or appropriate.

## 2.4.7 Integrated Overview and Conclusions

### Pharmacology

Azathioprine is the nitroimidazole derivative of 6 mercaptopurine and is the most widely used cytotoxic immunosuppressant in clinical medicine. In combination with corticosteroids, azathioprine is the primary agent employed to suppress transplantation rejection reactions. More recently, it has been used, with increasing frequency, in the treatment of autoimmune diseases. Many of azathioprine's actions are attributed to 6-mercaptopurine to which it is converted in the body. Azathioprine and 6 mercaptopurine (6-MP) are belong to the chemical class thiopurines

The exact mechanism of immunosuppressive activity of azathioprine has not been determined. Azathioprine which is an antagonist to purine metabolism, may inhibit RNA and DNA synthesis. The drug may also be incorporated into nucleic acids resulting in chromosome breaks, malfunctioning of the nucleic acids, or synthesis of fraudulent proteins.

In vitro the thiopurines exert preferential toxicities for murine T cells. B lymphocytes for mice appear to vary in their susceptibility for thiopurines. By contrast, the activity of human B cells can be readily suppressed with this drug whereas T helper function is comparatively resistant. Short-term use of azathioprine, in clinically used dosages, suppresses selective aspects of the canine immune system, and the T cells appear to be more susceptible than B cells to the immunosuppressive effect of this drug.

A Study has suggested that long-term treatment with azathioprine may prevent extravasation and cause reduction in neutrophil trafficking which may be beneficial for maintaining remission in IBD.

In addition to immunosuppressive properties, thiopurines are capable of exerting anti-inflammatory activities, by inhibiting cyclooxygenase production.

### Pharmacokinetics

Azathioprine is absorbed fast and extensively metabolized when administered orally in mice. Azathioprine undergoes first pass metabolism in mice indicated by the presence of thiouric acid, the end product of azathioprine metabolism in intestinal mucosa and liver. The initial concentrations of 6-MP extracted from the organs were about 10-fold those found in plasma indicating rapid cellular uptake of 6-MP. The conversion of azathioprine to 6-mercaptopurine in vivo may be catalyzed largely enzymatically by glutathione S-transferase in the liver.

Following iv 6-MP administration in rhesus monkeys, 6-MP levels were described by a two-compartment body model; mean terminal half-life; plasma clearance (CL<sub>p</sub>), and volume of distribution (V<sub>dss</sub>) were  $41.6 \pm 12.1$  min,  $48.4 \pm 15.4$  ml/min/kg, and  $1.76 \pm 0.64$  liters/kg, respectively.

## **Toxicology**

Toxicity studies in animals have shown that the haemopoietic system is most affected, with depression mainly of granulopoiesis and relative sparing of megakaryocytes and, hence, platelet formation. Allied to the effect on haemopoiesis is the effect on the lymphatic system, with atrophy of the lymphoid tissue being seen in rhesus monkeys on a dose of 1 mg/kg daily.

Azathioprine, like 6-mercaptopurine, may damage the liver. Sprague-Dawley rats given azathioprine in the diet for 3 to 4 weeks developed severe liver damage. Elevations of serum alkaline phosphatase and gamma-glutamyl transpeptidase activities were associated with increased hepatic glucose 6-phosphate dehydrogenase levels and decreased liver glucose 6-phosphatase activities. A reversible hepatotoxicity has been seen in dogs.

Azathioprine has been reported to cause temporary depression in spermatogenesis and reduction in sperm viability and sperm count in mice at doses 10 times the usual human dose; a reduced percentage of fertile matings occurred when animals received 5 mg/kg.

Teratogenicity has been seen in a number of animal species, with a varying degree of susceptibility. In rabbits, 5-15 mg/kg daily on days 6-14 of pregnancy produced skeletal abnormalities; in mice and rats, doses of 1-2 mg/kg daily on days 3-12 were lethal to the embryos. There seems to be no evidence of teratogenicity in humans.

Azathioprine is mutagenic in animals and in humans. Chromosomal abnormalities have been documented in humans receiving azathioprine, but the abnormalities were reversed following discontinuance of the drug.

According to International Agency for Research on Cancer azathioprine is carcinogenic to humans.

## **Conclusions**

In conclusion, both azathioprine and 6-MP exhibit immunosuppressive and anti-inflammatory potential in animal studies. Azathioprine is rapidly converted into 6-MP after oral dose, this may be catalyzed largely enzymatically by glutathione S-transferase in the liver. The drug undergoes first pass metabolism. The cellular uptake of 6-MP is high.

Haemopoietic system and liver are the main targets of azathioprine toxicology. Azathioprine is teratogenic in animals but not in humans; mutagenic in both animals and humans and carcinogenic in humans.

## 2.4.8 List of Literature Citations

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