

## Biochemical, Cytotoxic and Genotoxic Effects of a Novel Non-Mercaptopurine Immunosuppressant

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### Abstract:

Administration of MNITMT at a dose of 10 mg/kg for 35 days did not induce any significant change in various biochemical markers including serum alanine transferase, fasting blood glucose, total serum cholesterol and serum amylase. Moreover, MNITMT induced significant decrease in serum creatinine, serum albumin and total serum bilirubin and a significant increase of blood urea. The increased level of blood urea may be attributed to reduced protein biosynthesis and increased tissue wasting due to a possible inhibitory effect of the derivative on protein biosynthesis. MNITMT did not show any cytotoxicity when tested by the shrimp bioassay. However, MNITMT was a weak base-pair substitution mutagen in strain TA 100 of *Salmonella typhimurium* but induced no mutagenicity in TA 1535 strain. This compound did not have any frameshift mutagenic action in TA 98, TA 1537 or TA 1538 of *Salmonella typhimurium* strains when Ames test was used. The safety of this novel derivative as indicated by these preliminary studies as well as by the previous investigations encourages performing more detailed investigations to further confirm its efficacy and safety.

**Key words:** Azathioprine, cytotoxicity, genotoxicity, mutagenicity, immunosuppressant.

**Abbreviation:** MNITMT (3-[1-methyl-4-nitro-1H-imidazol-5-yl]thio]-4-methyl-1, 2, 4-triazole)

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## Introduction

Immunosuppressive agents are used clinically to prevent rejection of organ transplants (Attalla et al., 2005) and to treat autoimmune disorders (Marder and McCune, 2004). Albeit recent progress has been achieved in developing highly potent and effective immunosuppressants, bone marrow toxicity remains the main obstacle (Danesi and Del Tacca, 2004). Azathioprine, the pro-drug of 6-mercaptopurine has been in clinical use since 1961 but its bone marrow toxicity and other toxic effects limit its use (Kerstens et al., 1995). These myelotoxic effects are attributed to the intracellular metabolites 6-mercaptopurine moiety (Pollak et al., 1980).

In 1996 a novel non-mercaptopurine derivative was synthesized (Crawford et al., 1996). This derivative is known as 3-[1-methyl-4-nitro-1H-imidazol-5-yl] thio]-4-methyl-1, 2, 4-triazole (MNITMT). The novel derivative was a more potent immunosuppressant than azathioprine in preventing skin allograft rejection and was devoid of any bone marrow toxicity (Crawford et al., 1996). A further investigation confirmed that MNITMT was effective in inhibiting antibody response in rabbits without inducing any significant changes in the blood picture (Al-Safi et al., 2003).

Since 2003 and to the best of our knowledge no further studies were reported in the literature on this novel and non-toxic immunosuppressive agent. In this project we have re-synthesized MNITMT and investigated its safety profile in rats by monitoring some biochemical parameters as well as its cytotoxicity and mutagenicity.

## Materials and methods

### *Synthesis of MNITMT*

The suggested analog under investigation (Fig. 1) is 3-[1-methyl-4-nitro-1H-imidazol-5-yl] thio]-4-methyl-1, 2, 4-triazole. It was synthesized as previously described (Clark, 1975; Wallach, 1882; Wallach, 1877). The target compound was purified by the appropriate methods and characterized by its melting point and a number of spectroscopic techniques such as IR, NMR, and mass spectrometry. The data obtained were consistent with the reported values in the literature (Crawford et al., 1996).

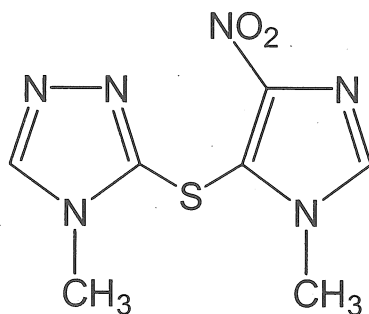


Fig. 1. Chemical structure of MNITMT

### *Animals*

A total of 10 Swiss rats weighing 200 – 300 g (5 males and 5 females) were obtained from the Biological Research Center at Jordan University of Science and Technology. Blood samples were obtained by external heart puncture on day zero before starting drug administration.

Animals were allowed to fast for three hours before the administration of the drug. MNITMT was dissolved in distilled water and administered orally. At the end of the experiment (day 35), the animals were sacrificed after an over night fast. Blood samples were withdrawn by external heart puncture. Blood samples were allowed to clot at room temperature before centrifugation at 3000 rpm for 20 min. Samples of serum were stored at 4° C until analysis.

### *Biochemical Tests*

All biochemical tests except alanine aminotransferase (ALT), were determined enzymatically using Randox kits (Randox laboratory Ltd UK). Plasma enzymatic activity of was determined using commercial kits (Human Gesellschaft für Biochemica und diagnostica mbH, Germany).

### *Brine Shrimp Bioassay*

MNITMT was prepared in three concentrations, 1, 10 and 100µg/ml by dissolving 10 mg of the compound in 2 ml dimethylsulfoxide. Brine shrimp eggs (Living World, Metaframe Inc., Elmwood Park, NJ. USA) were hatched in artificial sea water prepared from a commercial

salt mixture (Instant Oceans, Aquarium System, Inc., Mentor, OH, USA). Appropriate quantities of MNITMT solution were transferred to vials and dried. A disposable pipette was used to transfer 10 shrimps to each sample vial, and artificial sea water was added to make the volume up to 5ml. The vials were maintained under illumination (Meyer et al., 1982). Colchicin was used as a positive control. The nauplii were counted visually after 24 h. The probit analysis method was used to determine the LC<sub>50</sub> and 95% confidence intervals (Finnery, 1972).

### *Mutagenicity assay*

Different standard bacterial strains have been used according the Ames test recommendations to perform the mutagenicity test. *Salmonella typhimurium* strains TA 100, TA 1535, TA 1537, TA 1538, TA 102, and TA 98 were used. All mutant bacterial strains were purchased from Institute Pasteur of Paris, France.

Stock solution was prepared by dissolving 10 mg of MNITMT in 1 ml dimethylsulfoxide. Serial dilutions ranging from 4 mg/ml to 0.01 mg/ml were made. The plate incorporation test was utilized (Maron and Ames, 1983). The top agar was distributed into capped culture tubes, which were held at 45°C in a water bath. To each tube, 0.1 ml of a fresh overnight culture of the tested stain was added, followed by the addition of 0.1 ml of the tested compound. Sodium azide was used as a positive control for TA100 and TA 1535 strains, 4-nitro-o-phenylenediamine for TA 98 and TA 1538 strains, 9-aminoacridine for TA 1537, and Mitomycin for TA 102 strain (Kristien and Errol, 2000).

The test components were mixed by vortexing the tube for 3 seconds at low speed and directly poured onto a minimal glucose agar plates. After 45 minutes, the plates were inverted and placed in a dark incubator at 37°C. The revertant colonies on the treated as well as on the negative control plates were counted. A similar method was described previously for the detection of carcinogens as mutagens (McCann and Ames, 1976).

### Statistical analysis

Data were expressed as mean  $\pm$  SEM (standard error of mean). Student t-test was used for statistical analysis and P values  $< 0.05$  were considered statistically significant.

### Results

Oral exposure of rats to a high dose (10 mg/kg) of MNITMT for 35 days induced significant decrease in serum creatinine, serum albumin and total serum bilirubin levels (Table 1). Blood urea was increased significantly while serum ALT, fasting blood glucose, total serum cholesterol and serum amylase were not altered significantly (Table 1).

The brine shrimp bioassay which was used to determine cytotoxicity showed that MNITMT was inactive and the median lethal concentration ( $LC_{50}$ ) was more than 1000  $\mu\text{g/mL}$  (Table 2).

Mutagenic activities were determined using *Salmonella typhimurium* strains TA 1535 and TA 100 to detect a base-pair substitution, while strains TA 1537, TA 1538 and TA 98 were used to detect frameshift mutation. Moreover, strain TA 102 was used as a nonsense mutation detector. Table 3 shows that MNITMT is a weak base-pair substitution mutagen in strain TA 100 of *Salmonella typhimurium*, at which the number of revertants is equal three times the number of colonies of the negative control. Furthermore, no mutagenic activity was detected in TA 1535.

Table1. Effect of MNITMT (10 mg/kg) on some biochemical parameters.

Test	n	Mean $\pm$ SEM		P value
		Day 0	Day 35	
Serum albumin (g/dL)	10	3.48 $\pm$ 0.16	2.45 $\pm$ 0.19	$< 0.0002$
Blood urea (mg/dL)	10	72.80 $\pm$ 4.40	137.38 $\pm$ 23.62	$< 0.01$
Serum creatinine (mg/dL)	9	2.41 $\pm$ 0.46	1.18 $\pm$ 0.07	$< 0.02$
Total serum bilirubin (mg/dL)	10	0.52 $\pm$ 0.09	0.32 $\pm$ 0.038	$< 0.05$
Serum ALT (U/L)	10	13.77 $\pm$ 1.19	15 $\pm$ 2.14	NS
Fasting blood glucose (mg/dL)	9	99.81 $\pm$ 8.39	118.11 $\pm$ 11.96	NS
Total serum cholesterol (mg/dL)	9	44.49 $\pm$ 4.91	44.01 $\pm$ 3.4	NS
Serum amylase	7	760.13 $\pm$ 33.31	256.40 $\pm$ 96.91	NS

Table 2. Cytotoxic effect of MNITMT.

Chemical	Number of shrimps killed at ( $\mu\text{g/mL}$ )			
	100	10	1	LC <sub>50</sub> *
Negative control	0	0	0	-
Positive control	29	29	7	2.1671
MNITMT	5	5	1	>1000

\* LC<sub>50</sub>: lethal concentration killing 50% of tested shrimps.

Table 3. Mutagenic activity of the chemical compound

Bacterial strain	MNITMT N □	Positive control N □	Negative control N
TA 100 (31 $\mu\text{g/plate}$ )	247*	280	80
TA 1535	23	253	10
TA 1537	4	287	5
TA 1538	13	174	11
TA 102	6	205	9
TA 98	18	275	13

N □ number of colonies per plate after exposure to the chemical compounds, N number of colonies in the negative control plates.

\* An average of three plates after subtracting the background revertants.

## Discussion

Albeit MNITMT has been synthesized and tested in 1996, only two publications are available on this derivative. Accordingly the biochemical, cytotoxic and mutagenic effects of MNITMT will be compared to the parent drug, azathioprine.

The traditional immunosuppressive agent, azathioprine, had been reported to induce pancreatitis which is manifested by elevation of serum amylase (Alexander and Dowling, 2005; Weersma et al., 2004). Moreover, azathioprine is classified as a class I drug that induces pancreatitis (Trivedi and Pitchumoni, 2005). However, the novel derivative, MNITMT seems to be better in this regard as it did not induce any elevation in serum amylase although it was used at a high dose.

The decreased level of serum albumin (29.6%) may be attributed to an inhibitory effect of MNITMT on protein biosynthesis. However, it is unlikely that this effect will be significant clinically if the derivative is used at a therapeutic immunosuppressive dose.

The increased level of blood urea does not indicate the presence of renal toxicity since serum creatinine did not increase. Blood urea elevation may be attributed to endogenous protein breakdown due to a possible inhibitory effect of MNITMT on hepatic protein biosynthesis and increased tissue wasting. However, this effect may not be encountered if the derivative is used at a therapeutic immunosuppressive dose.

MNITMT appears non-hepatotoxic since ALT (index of cell damage), fasting blood glucose and total serum cholesterol were not elevated although a high dose was used. On the other hand azathioprine had been reported to cause elevation of liver enzymes in some patients (Hohlfeld et al., 1988) and to be hepatotoxic (Amin and Hamza, 2005).

Albeit MNITMT was used in a high concentration in the brine shrimp bioassay, it did not induce cytotoxicity (Table 2). However, azathioprine was cytotoxic and depleted NK cells in renal transplant patients but not in patients with rheumatoid arthritis (Alamartine et al., 1990; Dupont, 1984; Czeuz, 1990).

The results in table 3 indicate that MNITMT is a weak base-pair substitution mutagen in strain TA 100 of *Salmonella typhimurium*. This mutagenic effect has not been observed at concentrations less than 31 µg/plate. However, MNITMT did not have any mutagenic action in *Salmonella typhimurium* TA 1535. In addition, no frameshift mutation was detected in TA 98, TA 1538 and TA 1537 strains of *Salmonella typhimurium* after exposure to MNITMT. In a previous study azathioprine was a potent mutagenic in *Salmonella typhimurium* TA 100 and TA 1535 (Pollak et al., 1980). It is notable, that MNITMT mutagenic activity is sufficiently reduced compared to azathioprine and other similar compounds (Clark, 1975; Kristien and Errol, 2000; Fracasso et al., 1993; Hrelia et al., 1988; Smith et al., 1999; Voogd, 1989). This is an important finding since mutagenic compounds are potentially carcinogenic. In addition to the better safety profile of MNITMT, its efficacy was better than azathioprine in preventing skin allograft rejection (Crawford et al., 1996; Nagafuchi and Miyazaki 1991) In conclusion these preliminary results indicate that MNITMT has a better safety profile than azathioprine.

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