Polish Journal of Pharmacology
Pol. J. Pharmacol., 2003, 55, 239–243
ISSN 1230-6002

COMPARISON OF THE EFFECTS OF AZATHIOPRINE AND ITS NOVEL NON-MERCAPTOPURINE ANALOG ON ANTIBODY RESPONSE IN RABBITS

Saafan Al-Safi[1, #], Bassam Tashtoush[2], Mohamad Hassan[3]

[1]Department of Clinical Pharmacy, [2]Department of Pharmaceutical Technology, [3]Department of Medicinal Chemistry & Pharmacognosy, Faculty of Pharmacy, Jordan University of Science & Technology, P.O. Box 3030, Irbid 22110, Jordan

Comparison of the effects of azathioprine and its novel non-mercaptopurine analog on antibody response in rabbits. S. AL-SAFI, B. TASHTOUSH, M. HASSAN. Pol. J. Pharmacol., 2003, 55, 239–243.

Azathioprine (AZA) was originally developed as a pro-drug of the cytotoxic agent 6-mercaptopurine (6-MP). It was assumed that the methylnitroimidazole (MNI) group attached to 6-MP served only as thiol protecting moiety and was pharmacologically inactive. However, in this study we confirm that the novel compound, 3-[(1-methyl-4-nitro-1H-imidazol-5-yl)thio]-4-methyl-1,2,4-triazole (MNITMT) lacking the 6-MP moiety and retaining the MNI group is a better immunosuppressive agent than AZA. Thus, administration of MNITMT (2 mg/kg/day) to rabbits for two weeks caused a statistically significant and consistent inhibition of the antibody response. The onset of immunosuppression was on day 14. However, administration of AZA (2 mg/kg/day) to rabbits for two weeks inhibited the antibody response significantly on day 60 post-treatment. The solvent used to dissolve the above-mentioned drugs had no effect on the antibody response.

Neither AZA nor MNITMT had any effect on the blood picture of the treated rabbits indicating no bone marrow toxicity.

Key words: *azathioprine, methylnitroimidazole, 6-mercaptopurine, antibody titer*

correspondence; e-mail: alsafi52@yahoo.com

Abbreviations: AZA – *azathioprine,* MNITMT – *3-[(1-methyl-4-nitro-1H-imidazol-5-yl)thio]-4-methyl-1,2,4-triazole,* MNI – *methylnitroimidazole,* 6-MP – *6-mercaptopurine,* IU – *international unit*

INTRODUCTION

Azathioprine (AZA) is an immunosuppressive agent used mainly to prevent rejection of organ transplants [27] and to treat various autoimmune disorders [2, 19, 23, 35]. It was originally synthesized as a pro-drug of the cytotoxic agent 6-mercaptopurine (6-MP) [17]. However, various *in vitro* studies showed that there were clear differences between both drugs [3, 4, 7]. Moreover, AZA inhibited the mixed lymphocyte reaction when the lymphocytes were obtained from patients with Lesch-Nyhan syndrome (lack the enzyme HPRTase) that were unable to form active nucleotide metabolites, responsible for cytotoxicity [38]. Taken together, these observations indicate that AZA has an effect clearly different from that which might be expected from the action of 6-MP alone.

Based on the above facts, a group of investigators [13] succeeded in developing a novel derivative of AZA lacking the 6-MP moiety 3-[(1-methyl-4-nitro-1H-imidazol-5-yl)thio]-4-methyl-1,2,4-triazole (MNITMT) (Fig. 1). MNITMT was a more potent immunosuppressive agent than AZA, and was devoid of any bone marrow toxicity [13].

To the best of our knowledge, since 1996 no further studies have been reported in the literature on this novel and non-toxic immunosuppressive agent. In this investigation, we have synthesized MNITMT and studied its immunosuppressive effects on antibody response in rabbits. In addition, we compared its action with AZA.

MATERIALS and METHODS

Azathioprine

AZA pure powder was purchased from Sigma Chemical Co. Limited through its agent in Amman (United Tetra Group). It was dissolved in equimolar concentration of 0.01 M sodium hydroxide solution and then diluted with 0.9% sodium chloride solution.

MNITMT

The suggested analog under investigation (Fig. 1) is MNITMT. It was synthesized as previously described [39, 40] according to Scheme 1.

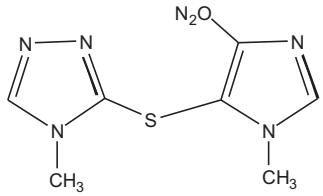
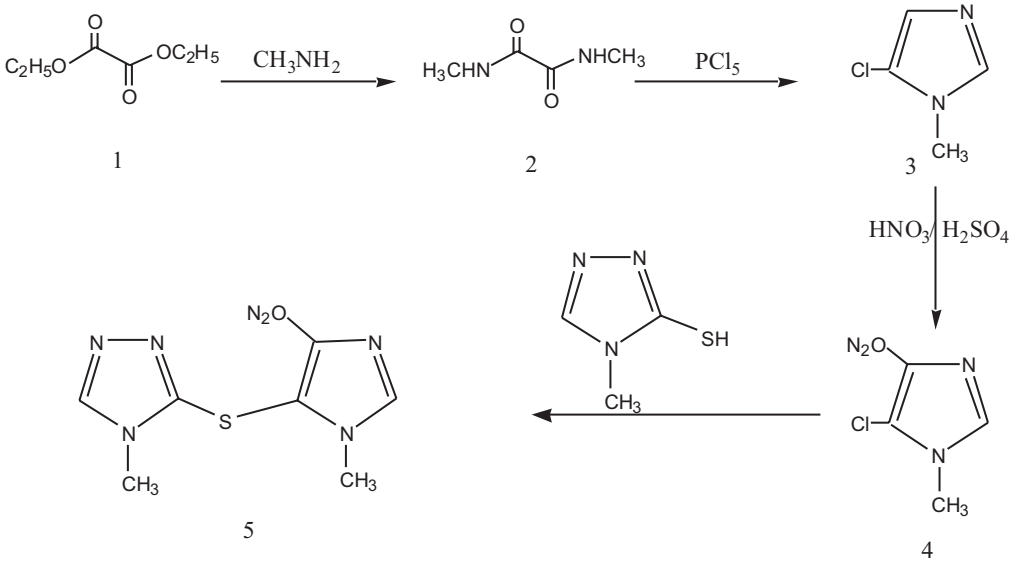


Fig. 1. Chemical structure of MNITMT



Scheme 1. Synthesis of MNITMT

Diethyl oxalate (compound 1) was converted to dimethyl oxamide (compound 2) with aqueous solution of methylamine, which upon treatment with phosphorous pentachloride gave 5-chloro-1-methylimidazole (compound 3). Compound 3 was nitrated using conventional methods to give 5-chloro-4-nitro-1-methylimidazole (compound 4). The target compound (compound 5) was obtained by reaction of the compound 4 with 3-mercapto-4-methyl-4H-1,2,4-triazole in aqueous solution of sodium hydroxide.

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 obtained data were consistent with the values reported in the literature [13].

Rabbits

Eighteen rabbits weighing 1.7–3.2 kg (average: 2.3 kg) were obtained from the Biological Research Center at Jordan University of Science & Technology. These rabbits were divided into three groups (6 rabbits for each group). Blood samples were obtained from the marginal ear vein. All animals were immunized with human IgG (Kabiglobulin^R, Pharmacia) suspended as water-in-oil emulsion according to the standard immunization methods [21, 29]. Each rabbit was injected subcutaneously with a total of 2 mg (1 mg in each thigh) of human IgG. The immunization procedure was repeated 3 times with 10-day intervals. The titer of the antibody response was estimated using the Rheumatoid Factor Kit (Labkit, Spain). The sensitivity of the kit is 8 IU/ml. The approximate titer of the serum expressed in international units (IU), was calculated by multiplying the highest dilution giving a positive agglutination by the kit's sensitivity (8 IU/ml).

On day zero and prior to any drug or solvent treatment, antibody titers, blood cell counts and hemoglobin levels were determined. The first group of rabbits (n = 6) received daily administration (for 14 days) of the solvent (0.01 M NaOH in 0.9% NaCl) used to dissolve both drugs. The second group of rabbits (n = 6) received daily administration of MNITMT at a dose of 2 mg/kg/day for 14 days. The third group of rabbits (n = 6) received daily administration of AZA at a dose of 2 mg/kg/day for 14 days.

Hematological tests

Blood cell counts including erythrocytes, leucocytes and platelets were performed microscopically and hemoglobin was estimated by UV spectroscopy [11].

Statistical analysis

The results were analyzed by a computerized program utilizing the Student's paired *t*-test and were considered statistically significant when $p < 0.05$.

RESULTS

Table 1 shows that daily administration of MNITMT (2 mg/kg) for two weeks inhibited the antibody response significantly by 46.4, 64.3, 82.2, 92.4 and 98.1% on days 14, 25, 36, 48 and 60, respectively. However, AZA (2 mg/kg/day for 14 days) caused a significant inhibition (97.4%) of the antibody response on day 60. Prior to day 60, the decrease in antibody titer in the AZA group was statistically nonsignificant. The vehicle used to dissolve both MNITMT and AZA did not cause any significant change in the antibody titer in the treated rabbits. The solvent, MNITMT and AZA were administered orally to the rabbits.

Table 1. Comparison of the effects of MNITMT and AZA at a dose of 2 mg/kg/day on antibody response

Day	Antibody titer (IU) ± SEM (n = 6 for each group)		
	Solvent	AZA	MNITMT
0*	1024 ± 324	768 ± 280	1195 ± 280
2	1450 ± 278 (NS)	981 ± 251 (NS)	1451 ± 278 (NS)
4	1024 ± 228 (NS)	640 ± 128 (NS)	1451 ± 278 (NS)
10	1109 ± 206 (NS)	810 ± 265 (NS)	1451 ± 278 (NS)
14**	1195 ± 286 (NS)	555 ± 103 (NS)	640 ± 128 ($p < 0.05$)
25	1365 ± 314 (NS)	341 ± 54 (NS)	427 ± 54 ($p < 0.05$)
36	1109 ± 206 (NS)	234 ± 61 (NS)	213 ± 27 ($p < 0.05$)
48	938 ± 244 (NS)	139 ± 32 (NS)	91 ± 17 ($p < 0.02$)
60	938 ± 244 (NS)	20 ± 4 ($p < 0.05$)	23 ± 9 ($p < 0.01$)

* prior to the treatment, ** end of the treatment

There were no significant changes in blood cell counts (erythrocytes, leukocytes, platelets) and in hemoglobin levels of all animals during and after solvent or drug treatment.

DISCUSSION

AZA is an immunosuppressive agent, used to prevent rejection of organ transplants in human recipients particularly kidney allografts [27]. Since AZA was originally synthesized as a pro-drug of the well-known cytotoxic agent 6-MP, it has been assumed that *in vivo* release of this agent is responsible for AZA immunosuppressive activity [16]. Initial clinical observation suggested that AZA-induced immunosuppression differed subtly from that which might have been expected from 6-MP actions [5, 32]. Moreover, further *in vitro* work showed a number of differences between 6-MP and AZA effects on human lymphocytes [3, 4, 6]. The perceived logic behind these observations has always been that the effects obtained with AZA are due to its greater metabolic stability, giving a more controlled release of 6-MP [15].

Interest in investigating the above phenomenon was stimulated by the fact that clinical use of AZA is limited by potentially serious toxicity, particularly to the bone marrow [12, 22, 30, 32]. In 1996, Crawford et al. [13] published their interesting results on a number of rationally designed analogs of AZA. The most effective and safest analog was MNITMT. It was more effective in inhibiting the human mixed lymphocyte reaction and in preventing skin allograft in mice than AZA [13]. Moreover, MNTIMT was devoid of any bone marrow toxicity (13) although it was administered to mice at high doses (100–400 mg/kg/day for 14 days). In our investigations, MNTIMT caused more consistent, significant and quicker inhibition of the antibody response than AZA. Neither MNTIMT nor AZA produced any type of blood dyscrasias indicating no bone marrow toxicity.

The effect of MNTIMT on antibody production had not been investigated previously. Thus, our results are the first to be reported in this respect. However, the effect of AZA on antibody response *in vivo* and *in vitro* had been shown to be controversial. Some investigators [14, 20, 25, 31, 33, 36, 37] demonstrated an inhibitory effect, while other investigators [1, 8–10, 18, 24, 26, 28, 34] showed inconsistent effects or no change in the antibody titer. These controversial results may be attributed to the differences in the conditions used in the above investigations.

We conclude that the antibody response in rabbits was more consistently suppressed at an earlier stage by MNTIMT than by AZA. Neither AZA nor MNITMT induced any bone marrow toxicity during and after the treatment period. More detailed investigations are required to gain in-depth information on this promising novel non-toxic immunosuppressive agent that may have various clinical applications particularly in organ transplantation and for the treatment of autoimmune disorders.

Acknowledgment. We would like to thank the Deanship of Scientific Research at Jordan University of Science & Technology for their financial support to accomplish this project through grant no. 33/99.

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Received: November 12, 2002; in revised form: March 3, 2003.