Department of Pharmaceutical Technology[1], Department of Clinical Pharmacy[2], College of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan

Azathioprine transport through rat skin and its immunosuppressive effect

B. M. Tashtoush[1], S. A. Al-Safi[2], K. J. Al-Fanek

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Bassam M. Tashtoush, Ph. D., Dept. of Pharmaceutical Technology, Faculty of Pharmacy, Jordan University of Science and Technology, P. O. Box 3030, Irbid 22110, Jordan
bmtash@just.edu.jo

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The objective of this research was to study the *in vitro* and *in vivo* percutaneous absorption of azathioprine with and without the effect of penetration enhancers. *In vitro* permeation of azathioprine was studied using a Franz diffusion cell and rat skin. Both azathioprine and 6-mercaptopurine were detected in the receiver solution with a reversed phase HPLC system. The steady state flux of azathioprine, permeability coefficient, and lag time were reported. Penetration enhancers such as dimethylsulfoxide (DMSO), dimethylformamide (DMF), and urea were added to the donor compartment to increase the skin permeation of azathioprine. The flux of azathioprine was increased by 20.7%, and 22.4% using dimethylsulfoxide, and dimethylformamide respectively. The *in vivo* permeation was determined by measurement of antibody titers by the slide latex agglutination test. The *in vivo* permeation study showed that the titers of antibody induced in the rats were not affected by topical application of azathioprine solution. The results show that azathioprine has low flux to exert a systemic effect with and without penetration enhancers. However these results may support the use of topical azathioprine for the treatment of some dermatological disorders with minimum side effects.

1. Introduction

Azathioprine is one of the most widely used immuno-suppressive drugs in organ transplantation (Shoker 1996; Hoffmann et al. 2000), and in the management of several disorders such as rheumatoid arthritis (Case 2001), ulcerative colitis (Ludwig et al. 2000), and also inflammatory bowel disease, especially Crohn's disease (Louis et al. 1999; Hoffmann et al. 2000; Biancone et al 1999) and dermatological disorders (Meggitt et al. 2001; Dutz et al. 1998; Snow et al. 1995) lupus nephritis (Austin et al. 2000; Uppal 1999; Balow et al. 2000; Niaudet 2000), glomerulonephritis (Locattelli et al. 1999), nephrotic syndrome (Cochat et al. 1996), sarcoidosis (Baughman et al. 1997; Selroos 1994) and idiopathic pulmonary fibrosis (Egan 1998; Nicod 1998). Topical azathioprine has also been used in the treatment of immune-mediated chronic oral inflammatory conditions (Epstein et al. 2001).

Azathioprine is rapidly metabolized by non-enzymatic nucleophilic attack of free sulfhydryl or amino groups (Scoik et al. 1985; De Miranda et al. 1970) on the sulfide bond between the purine and the imidazole rings of azathioprine in the liver to the major active metabolite 6-mercaptopurine and an imidazole moiety (Elion et al. 1972). To a less extent azathioprine may split between the purine ring and the sulfur to yield the metabolite 1-methyl-4-nitro-5-thioimidazole by an unknown mechanism (Elion 1972). 6-Mercaptopurine can be metabolized by three known pathways; two pathways metabolize 6-MP to inactive metabolites: 6-thiouric acid via xanthine oxidase and 6-methylmercaptopurine via thiopurine methyltransferase, and the third pathway can convert 6-mercaptopurine to its active form, 6-thioinosinic acid, by hypoxanthine phosphoribosyl transferase and then to a 6-thioguanine nucleotide by other enzymes (Lennar 1992; Foss et al. 1978; Kaplowitz 1977).

Most of the pharmacological effects of azathioprine are due to its cleavage to 6-mercaptopurine. Azathioprine suppresses both immunological response and tumor growth. Its major role has been as an agent for suppressing the immune response (Calne 1982).

Oral administration of azathioprine may cause serious side effects, some of which are assumed to be dose dependent such as bone marrow depression, which may be manifested as leukopenia or thrombocytopenia, or less often anemia (Lawson et al. 1984), hepatotoxicity and thrombocytosis, and also as carcinogenicity (Sherloch 1986). Another side effect, which is related to the direct contact of azathioprine with the gastrointestinal tract, is gastrointestinal toxicity manifested as nausea, vomiting, peptic ulceration, intestinal hemorrhage (Sherloch 1986), and severe life-threatening diarrhea (Marbet et al. 2001).

It is proposed that topical application of azathioprine may minimize gastro-intestinal tract side effects, by both decreasing the administered dose and avoiding the direct contact of the drug with the gastro-intestinal tract. Moreover, minimal systemic absorption may decrease its immunosuppressive and toxic effects making it an excellent choice for some refractory skin disorders.

The objective of this research was to study the *in vitro* and *in vivo* percutaneous absorption of azathioprine with and without the effect of penetration enhancers in order to

Table 1: Fluxes of azathioprine, 6-mercaptopurine and total (AZA & 6-MP) at different donor concentrations

Donor conc. μg/ml	Flux (μg/cm²)		
	6-Mercaptopurine	Azathioprine	Total (AZA + 6-MP)
500	0.005 ± 0.001	0.128 ± 0.001	0.133 ± 0.001
1000	0.107 ± 0.002	0.200 ± 0.01	0.307 ± 0.015
1500	0.149 ± 0.003	0.269± 0.024	0.418 ± 0.024
2000	0.197 ± 0.003	0.310 ± 0.032	0.507 ± 0.021
2500	0.245 ± 0.018	0.342 ± 0.03	0.587 ± 0.032

n = 3 (Mean ± SD)

minimize the unwanted immunosuppressive and toxic side effects associated with the oral route.

2. Investigations, results and discussion

The steady state flux of azathioprine and total (azathioprine + 6-mercaptopurine) was calculated at different donor concentrations of azathioprine from the linear portion of the permeation profile. The fluxes of azathioprine, 6-mercaptopurine and total are shown in Table 1 as a function of donor concentration. The permeation profile at 2000 μg/ml azathioprine for azathioprine and 6-mercaptopurine is shown in Fig. 1 with the lag time for azathioprine being 2.5 h and 4.5 h for 6-mercaptopurine. The total permeability coefficient was calculated as the ratio of the total flux to donor concentration. The permeability coefficient was found to be 0.000268 ± 0.000036 cm/hr. *In vitro* permeation studies showed low flux of azathioprine through rat skin possibly due to lipophilicity and very low water solubility. The results support the hypothesis that a balance between hydrophilicity and lipophilicity is required for percutaneous absorption.

Dimethyl sulfoxide has been used as a penetration enhancer to enhance skin permeation of azapropazone (Nouh et al. 1989) and bepridil (Klamerus et al. 1992) with little or no effect on the penetration of piroxicam (Xu et al. 1991) and haloperidol (Vaddi et al 2001). Urea and its derivatives have been used to enhance the permeation of hydrocortisone (Godwin et al. 1998), salicylic acid (Han et al. 1991) and flurbiprofen (Chi et al. 1995). The enhancing effect of DMSO, and DMF on the skin permeation of azathioprine through rat skin was determined at a concentration of 10%. Urea was used as an enhancer at a concentration of 1%. The cumulative amount of azathioprine permeated through excised rat skin from the azathioprine vehicle as a function of time is shown in Fig. 2. The amount of azathioprine permeated as a function of time and the lag time with the use of 10% DMSO, 10% DMF, and 1% urea as permeation enhancers are shown in Table 2. As a result DMSO was found to increase azathioprine permeation by 91.3% whereas DMF increased it by 107.2%. On the other hand urea was found to decrease the skin permeation of azathioprine. This can be explained by the fact that urea causes two changes in the barrier function of the skin through an increase in the hydration of the stratum corneum which may lead to precipitation of azathioprine because of its low water solubility, and inducement of keratolysis of skin after prolonged contact (Lashmar et al. 1989). It has been found that urea was not a penetration enhancer for hydrophobic drugs such as 5-flourouracil (William et al. 1989).

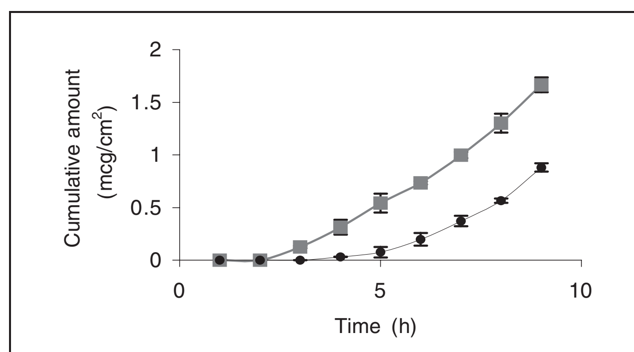

Fig. 1: Permeation profile through rat skin from vehicle containing 2000 mcg/ml azathioprine. Key, azathioprine (■); 6-mercaptopurine (●). n = 3 (Mean ± SD)

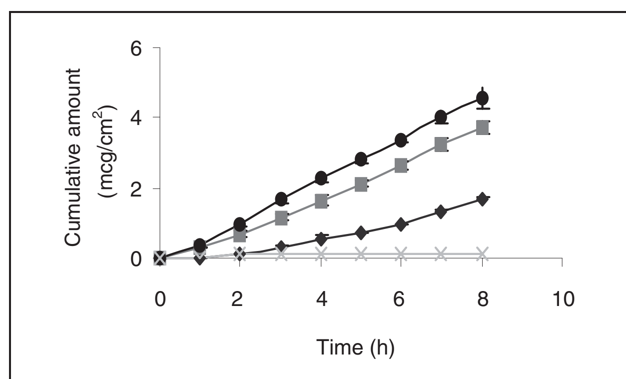

Fig. 2: Effect of permeation enhancer in vehicle containing 2000 μg/ml azathioprine on drug permeation through rat skin. Key: azathioprine (◆); dimethylformamide (10%) (●); dimethylsulfoxide (10%) (■); urea (1%) (×). n = 3 (mean ± SD)

The *in vivo* percutaneous absorption of azathioprine is represented by the titers of antibodies against human IgG as measured by a slide latex agglutination test (Table 3). The results showed that topical application of azathioprine for forty days had no effect on antibody titers as compared to the control. This suggests that there is no systemic immunosuppressive effect of the drug. These results support the *in vitro* permeation studies.

As a conclusion the results showed that the flux of azathioprine was increased using dimethylsulfoxide, and using dimethylformamide. The *in vivo* permeation study showed that the titers of antibody, which were induced in the rats, were not affected by topical application of azathioprine solution.

The results of the *in vitro* and *in vivo* permeation studies showed that azathioprine has a low flux to exert a systemic effect both with and without penetration enhancers. However these results may support the use of topical azathioprine for some dermatological disorders with no or minimum side effects.

Table 2: Effect of penetration enhancers on the permeation parameters of 2000 mcg/ml azathioprine through excised rat skin after transdermal application

Components	Flux (μg/cm² · h) (Mean ± SD)	Lag time (h)
Azathioprine	0.281 ± 0.033	2.5
Azathioprine + dimethylsulfoxide (10%)	0.537 ± 0.036	1.6
Azathioprine + dimethylformamide (10%)	0.583 ± 0.066	1.4
Azathioprine + Urea (1%)	0.004 ± 0.001	...

Table 3: Effect of topical application of azathioprine (2 mg/kg/day) or its vehicle on antibody titers

		Mean ± SEM	
Day	n	AZA	AZA vehicle
0*	8	38 ± 13	36 ± 14 (NS)
8	8	34 ± 7.5 (NS)**	32 ± 8 (NS)
16	8	32 ± 8 (NS)	32 ± 8 (NS)
23	8	30 ± 10.5 (NS)	32 ± 8 (NS)
29	8	28 ± 6 (NS)	30 ± 10.5 (NS)
49	8	28 ± 6 (NS)	28 ± 6 (NS)

* Prior to topical application
** Statistically non-significant ($p > 0.05$)

3. Experimental

3.1. Materials

Azathioprine and 6-mercaptopurine were purchased from Sigma Chemical Company, commercial HPLC grade acetonitrile was purchased from Scharlau Chemie S.A. Phosphoric acid was purchased from Janssen. Acros, Belgium, provided triethylamine, urea, propylene glycol, dimethylsulfoxide (DMSO) and dimethylformamide (DMF). Sodium hydroxide was purchased from Frutarom, UK. Arachis oil was purchased from SNOI Inc, USA. Human normal immunoglobulin was purchased from Globuman, Switzerland. Imuran IV injection was purchased from Wellcome, England. RF latex was purchased from K labkit, Spain. All the reagents were used as received and deionized distilled water was used to prepare stock solutions.

3.2. Methods

3.2.1. In vitro permeation of azathioprine

In vitro permeation was studied using white rat skin and a glass Franz diffusion cell. The skin was excised from a shaved rat under ethyl ether anesthesia. The muscles and subcutaneous fatty tissues were then removed. After that the skin was washed with normal saline and immediately mounted between two diffusion half-cells.

The two compartments were filled with phosphate buffered saline (PBS 0.1M, pH 7.4), 1 h of equilibration was allowed and then the receiver solution was replaced with fresh PBS and the donor solution with drug solution. The drug solution of azathioprine (4 mg/ml) was prepared in an equimolar concentration of 0.01 N NaOH. Dilutions were made with PBS to prepare other concentrations of azathioprine. Samples of 0.4 ml were taken from the receiver solution at the following time intervals: 0, 1, 2, 3, 4, 5, 6, 7, 8 h, and replaced each time with the same volume of drug-free PBS solution to maintain a constant volume through out the permeation experiment. The samples were stored at 4 °C until they were analyzed.

Azathioprine and 6-mercaptopurine were analyzed on a Lichrocart 250-4 mm, HPLC cartridge, Alluspher 100, RP-select B (5 μm). The mobile phase consisted of acetonitrile : water : phosphoric acid : triethyl amine (0.05 : 0.948 : 0.001 : 0.001). The pH was adjusted with phosphoric acid to 2.8 ± 0.1. The flow rate of the mobile phase was 1.5 ml/min and the detection wavelength was 325 nm. The retention time was found to be 4.0 ± 0.1 min for azathioprine and 1.9 ± 0.1 min for 6-mercaptopurine.

The effects of penetration enhancers such as 10% DMSO, 10% DMF and 1% urea on the skin penetration of azathioprine were studied. The permeation data were plotted as the cumulative amount of the drug collected in the receptor compartment as a function of time. The flux value for a given run was calculated from Fick's First Law of diffusion:

$$J_s = dm/dt \cdot = P\Delta C \quad (1)$$

Where:
J_s: steady state flux in ($\mu g/cm^2 \cdot h$)
P: effective permeability coefficient in (cm/s)
S: diffusion area, which is equal to 3.465 cm^2
ΔC: concentration gradient across the membrane in (μg/ml).

3.2.2. In vivo permeation studies

The immunizing agent was prepared according to the following procedure (Hay et al. 2002): Human immunoglobulin (Ig, 0.1 ml) was taken from a vial containing 320 mg/2 ml, and volume was made up to 8 ml with normal saline giving a conc. of 2 mg/ml. Eight ml of dilute immunoglobulin solution was mixed gradually (drop by drop) to 8 ml arachis oil giving a conc. of 1 mg/ml. Stability of the emulsion was checked by adding a drop from the emulsion to a water surface in a beaker. If the drop does not spread this means that the emulsion is stable. Every rat was given 2 mg of Ig/1 kg of rat weight.

Rats were injected with 0.2 ml of human normal immunoglobulin (IgG) in a rachis oil vehicle (prepared as water in oil emulsion) subcutaneously in each thigh (primary immunization), then after one week, secondary immunization, and then tertiary immunization two weeks later. Blood samples were taken from the rats after 10 days and the serum was separated by centrifugation. The antibody titers were determined by the slide latex agglutination test.

100 μl of drug solution (2 mg/ml) were applied topically on two shaved dorsal sites daily for 40 days. At day zero (prior to azathioprine application) and at the following time points: 0, 8, 16, 23, 29, and 40 days, blood samples (500 μl) were collected from the rats tails using an insulin syringe. Serum was separated by centrifugation and frozen until determination of anti-Ig titer with a slide latex kit.

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