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Comparison of suppressive potency between azathioprine and 6-mercaptopurine against mitogen-induced blastogenesis of human peripheral blood mononuclear cells in-vitro

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Abstract

Azathioprine (AZ) is a prodrug of 6-mercaptopurine (6-MP), but little is known about the relative suppressive efficacy of these agents against blastogenesis of human peripheral blood mononuclear cells (PBMCs) in-vitro. We compared the pharmacological efficacy of AZ and 6-MP against T cell mitogen-induced blastogenesis of PBMCs in-vitro. PBMCs were obtained from seven healthy subjects. The potency of AZ and 6-MP to suppress PBMC-blastogenesis in-vitro was compared using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay procedures. Production of 6-MP from AZ in PBMC culture was examined by high-performance liquid chromatography. Both AZ and 6-MP dose-dependently suppressed PBMC blastogenesis. Mean ± s.d. IC50 (concentration of drug that gave 50% inhibition) values for AZ and 6-MP were 230.4 ± 231.3 and 149.5 ± 124.9 nM, respectively. Thus, the potencies of AZ and 6-MP to suppress PBMC blastogenesis were not significantly different. A significant correlation was observed between the IC50 values of AZ and 6-MP ($P < 0.01$, $n = 6$). After incubation of PBMCs with 100 μM AZ for 4 days, 35.3–92.5 μM 6-MP was liberated into the culture medium. The ratio of 6-MP liberation from AZ was influenced by the presence of PBMCs, but not by the medium or fetal bovine serum. The results suggest that the suppressive potency of the prodrug AZ and its converted form 6-MP against blastogenesis of human PBMCs in-vitro is similar, although PBMCs appeared to convert AZ to 6-MP. AZ is suggested to be effective after conversion to 6-MP to express immunosuppressive efficacy in-vitro.

Introduction

Azathioprine (AZ) has been used in combination with glucocorticoid and either ciclosporin or tacrolimus for the prevention of allograft rejection in organ transplantation. AZ is a prodrug of 6-mercaptopurine (6-MP), containing an imidazole group attached to the sulfur atom at the 6-position of the purine ring. There are some other immunosuppressive agents of the prodrug such as mycophenolate mofetil and prednisolone sodium succinate. The bioavailability of mycophenolic acid was improved by esterification to mycophenolate mofetil (Lee et al 1990). Prednisolone sodium succinate has been developed to improve the water solubility of prednisolone.

In our previous study, we compared the immunosuppressive pharmacological efficacy of prednisolone and prednisolone sodium succinate in-vitro using peripheral blood mononuclear cells (PBMCs) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay procedures (Sugiyama et al 2001). The prednisolone sodium succinate potency was demonstrated to be markedly lower than that of prednisolone (Sugiyama et al 2001). If AZ is effective only after conversion to 6-MP in the body, the conversion ratio and its lag time are critical for the clinical efficacy of AZ. On the other hand, in in-vitro studies, AZ is considered to be a more effective immunosuppressant than 6-MP (Hardman et al 1995). Thus, in general, the immunosuppressive potency of AZ is considered to be greater than that of 6-MP (Winkelstein 1979; Hardman et al 1995). However, a comparative study on the suppressive potency of these agents against cellular immunity has not been carried out. Many studies have

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shown that the in-vitro effect of immunosuppressive drugs against blastogenesis of PBMCs correlated with the clinical efficacy of the drugs (Hirano et al 1994, 1997). If so, it might be useful to examine differences in the lymphocyte-suppressing potencies of these drugs and individual PBMC susceptibility to these drugs in-vitro. In the present study, we compared the pharmacological efficacy of AZ and 6-MP against T cell mitogen-induced blastogenesis of human PBMCs in-vitro.

Materials and Methods

Materials

Most of the chemicals including 6-MP were obtained from Wako Chemical Co. (Osaka, Japan), except for the following. AZ was obtained from Nacalai Tesque, Inc (Kyoto, Japan). MTT was from Sigma Chemical Co. (St Louis, MO, USA). Ficoll-Paque was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). RPMI 1640 medium, fetal bovine serum (FBS) and Hank's balanced salt solution (HBSS) were obtained from Gibco Laboratories (Rockville, MD, USA). Concanavalin A was from Seikagaku Kogyo Co. (Tokyo, Japan). All other reagents were of the highest grade available.

Subjects

The present study was carried out in accordance with the Helsinki Declaration of 1975. After informed consent was obtained, heparinized venous blood (20 mL) was taken from seven healthy volunteers (two males and five females) aged 24–33 years. The subjects had no history of taking immunosuppressive agents, including glucocorticoids. To measure the response of PBMCs to drugs in-vitro, a drug sensitivity test (Hirano et al 1994, 1997) was carried out in each subject as described below.

Isolation of PBMCs

Isolation and culture of PBMCs were carried out according to the method described previously (Hirano et al 1994, 1997). In brief, heparinized blood was loaded onto Ficoll-Paque and centrifuged at 900 *g* for 20 min at room temperature. The buffy coat was taken and then rinsed three times with HBSS. PBMCs including lymphocytes were suspended in RPMI 1640 medium containing 10% FBS.

PBMC culture and evaluation of drug potency

A total of 186 μL of the cell suspension prepared as described above was placed into each well of microplates with 96 flat-bottomed wells. Then, 10 μL of saline containing concanavalin A was added to each well to a final concentration of 5.0 μg mL^{-1}. Subsequently, 4 μL of sodium hydroxide solution containing AZ or 6-MP was added to give a final drug concentration of 0.01, 0.1, 1, 10, 100, 1000, 10 000 or 100 000 nM. The same volume of sodium hydroxide solution was added to control wells. The total volume in one well was therefore 200 μL, and a final cell density in each well was adjusted to be 1×10^6 cells mL^{-1}. The plates were incubated for 4 days in 5% CO_2 at 37 °C.

MTT assay

After 4 days of culture, MTT dissolved in saline was added to each well and then reincubated under 5% CO_2 at 37 °C for 4–5 h (Hirano et al 1995). The plates were centrifuged at 375 *g* for 5 min to precipitate cells and formazan produced by growing cells. Aliquots of the supernatant were removed from each well and dimethylsulfoxide was added and the plate was shaken on a microshaker for 10 min to dissolve the formazan crystals. The wells were read using a microplate reader at 550 nm. Dose–response curves were plotted, and the concentration of drug that gave 50% inhibition of cell growth (IC50) was calculated.

Chromatographic measurement of AZ and 6-MP

After incubation with 100 μM AZ for 4 days, the 6-MP concentration in the PBMC culture supernatant was measured by reversed-phase high-performance liquid chromatography (HPLC). The supernatant was centrifuged at 375 *g* for 5 min. The HPLC system (Shimadzu, Kyoto, Japan) comprised a UV-Vis detector SPD-10AV VP, a liquid chromatograph LC-10AD VP, a degasser DGU-14A, an auto injector SIL-10AD VP, and a system controller SCL-10A VP. The detection wavelength was 320 nm. We used a Nova-Pak C18 60 4 μm HPLC column (3.9 × 300 mm) (Waters, Milford, MA, USA). The mobile phase was a mixture of 0.01 M CH_3COONa and CH_3CN (9:1 v/v). The flow rate of the mobile phase was 0.5 mL min^{-1}.

Statistics

The differences between the mean IC50 values of AZ and 6-MP (as shown in Tables 1 and 2) were analysed with the Mann-Whitney *U*-test. The data in Tables 1 and 2 are paired in design. Differences in the mean amount of AZ liberated into the medium under different experimental conditions were compared with Bonnferroni tests. The correlation of two values was examined with Pearson's correlation coefficient test. The data for the correlation between the AZ IC50/6-MP IC50 ratio and the amount of 6-mercaptopurine converted from azathioprine were analysed with linear model as well as non-linear model. In each case, *P* values less than 0.05 were considered to be significant.

Results

Comparison of the immunosuppressive effects of AZ and 6-MP in-vitro

The effects of AZ and 6-MP on in-vitro blastogenesis of PBMCs from healthy subjects were compared. Typical

Table 1 Comparison of immunosuppressive potency between azathioprine and 6-mercaptopurine against blastogenesis of peripheral blood mononuclear cells (PBMCs) from six healthy subjects.

PBMC donor no.	IC50 (nM)		Azathioprine/6-mercaptopurine ratio	6-Mercaptopurine detected in culture medium[a] (μM)
	Azathioprine	6-Mercaptopurine		
1	80.2	109.8	0.73	92.5
2	19.1	21.1	0.91	84.6
3	137.0	25.8	5.31	35.3
4	354.3	248.1	1.43	77.1
5	100.0	119.8	0.83	90.5
6	691.7	372.2	1.86	76.5
Mean ± s.d.	230.4 ± 231.3	149.5 ± 124.9	1.85 ± 1.60	76.1 ± 19.2

[a] After incubation of 100 μM azathioprine with PBMCs for 4 days, the 6-mercaptopurine concentration in the medium was measured using HPLC.

Table 2 Day-to-day variation in IC50 values for azathioprine and 6-mercaptopurine in peripheral blood mononuclear cells (PBMCs) measured on six different days in one healthy subject.

Blood sampling	IC50 (nM)		Azathioprine/6-mercaptopurine ratio	6-Mercaptopurine detected in culture medium[a] (μM)
	Azathioprine	6-Mercaptopurine		
Day 1	127.1	91.5	1.39	60.9
Day 2	98.2	100.0	0.98	67.6
Day 3	253.2	273.6	0.93	75.7
Day 4	166.7	192.7	0.87	56.2
Day 5	58.6	67.4	0.87	74.7
Day 6	143.5	107.1	1.34	65.8
Mean ± s.d.	141.2 ± 60.6	138.7 ± 71.8	1.06 ± 0.22	66.8 ± 6.96

[a] After incubation of 100 μM azathioprine with PBMCs for 4 days, the 6-mercaptopurine concentration in the medium was measured using HPLC.

dose–response curves of AZ and 6-MP on mitogen-induced blastogenesis of PBMCs obtained from one healthy subject are presented in Figure 1. Both of the agents suppressed blastogenesis dose dependently, and the potencies of AZ and 6-MP were almost equal. The IC50 values for AZ and 6-MP were 98.2 nM and 100.0 nM, respectively. To confirm the similarity in the individual suppressive potency against blastogenesis of PBMCs between AZ and 6-MP, we compared the in-vitro effects of these agents using PBMCs obtained from six healthy subjects (Table 1). The range of AZ IC50 values showed large deviations from 19.1 to 691.7 nM between subjects. The maximum difference in the IC50 values of AZ in these cases was 36.2-fold. The mean ± s.d. IC50 of AZ was 230.4 ± 231.3 nM. Similar to AZ, the range of 6-MP IC50 values showed large deviations from 21.1 to 372.2 nM. The mean ± s.d. IC50 of 6-MP was 149.5 ± 124.9 nM. The maximum difference in IC50 values between subjects was 17.6-fold. There was a significant correlation between AZ

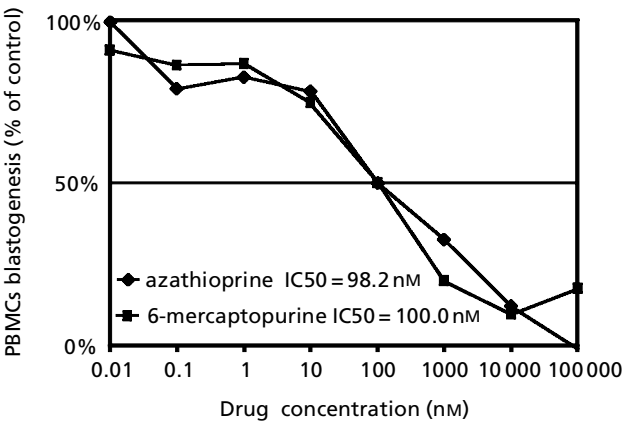

Figure 1 Comparison of dose–response curves between azathioprine and 6-mercaptopurine on mitogen-induced blastogenesis of human peripheral blood mononuclear cells (PBMCs) from one healthy subject. Each point represents the mean of duplicate culture wells.

IC50 values and 6-MP IC50 values in these subjects ($r = 0.943$, $P < 0.01$). The ratio of the AZ IC50 to 6-MP IC50 ranged from 0.73 to 5.31, and the mean ratio was 1.85. The IC50 values of AZ and 6-MP were not largely different, except of donor 3, as shown in Table 1. There was no statistically significant difference between the medians (ranges) of IC50 values of these drugs, as analysed with the Mann-Whitney *U*-test. Neither AZ nor 6-MP IC50 values correlated with PBMC stimulation indices by concanavalin A in these six healthy subjects.

Table 2 shows the day-to-day variation of PBMC sensitivities to the drugs measured on six different days in one healthy subject. The range of AZ IC50 values deviated from 58.6 to 253.2 nM (4.32-fold difference) with a mean ± s.d. of 141.2 ± 60.6 nM, while the range of 6-MP IC50 values was from 67.4 to 273.6 nM, with a mean ± s.d. of 138.7 ± 71.8 nM (4.06-fold difference). There was again a significant correlation between AZ and 6-MP IC50 values ($r = 0.943$, $P < 0.01$). In this case, the ratio of these values (AZ/6-MP) ranged from 0.87 to 1.39. Variation in the ratio was relatively small compared with that presented in Table 1. Thus, the day-to-day variations were smaller compared with those of inter-individual (subject-to-subject) deviations (Table 1).

Conversion of AZ to 6-MP in PBMC cultures

Figure 2 shows the change in concentrations of 6-MP converted from AZ in our PBMC culture from Day 1 to Day 4. AZ (100 μM) was cultured in the presence of PBMCs, and the amounts of both AZ and 6-MP were measured. 6-MP concentrations in cultures of PBMCs obtained from one healthy subject increased gradually from 20.6 (Day 1) to 66.7 μM (Day 4). Thus, approximately 20% of AZ was converted to 6-MP within 1 day under our culture conditions, and the amount of 6-MP increased gradually until 4 days of culture. The amounts of 6-MP converted from AZ were measured in cultures of PBMCs obtained from six healthy subjects (Table 1). The amount of 6-MP released from 100 μM AZ in these cultures for 4 days was from 35.3 to 92.5 μM. Thus, the amount of 6-MP released during the 4 days of culture was markedly different between PBMC donors.

Figure 3 shows that addition of PBMCs, but not FBS, to the culture medium markedly converted AZ to 6-MP, indicating that PBMCs, but not the medium or FBS, have the ability to convert AZ to 6-MP.

There was a significant negative correlation between the AZ IC50/6-MP IC50 ratio and the amount of 6-MP converted from AZ in culture medium (Figure 4) ($r = 0.991$, $P < 0.001$ as analysed with a linear model, and $r = 0.996$, $P < 0.0001$ as analysed with a non-linear model).

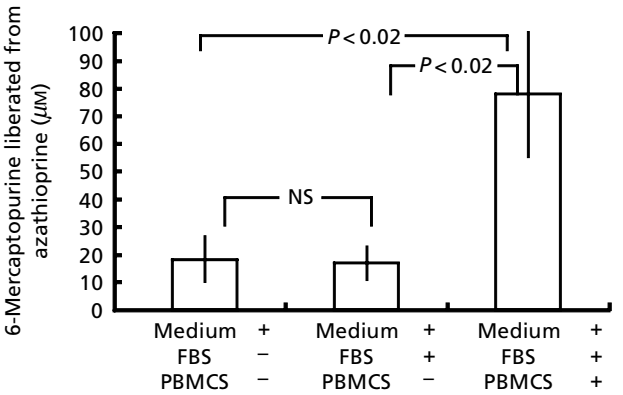

Figure 3 Comparison of the amounts of azathioprine in culture medium in the presence or absence of human peripheral blood mononuclear cells (PBMCs) and fetal bovine serum (FBS). Values are presented as means ± s.d. of six experiments.

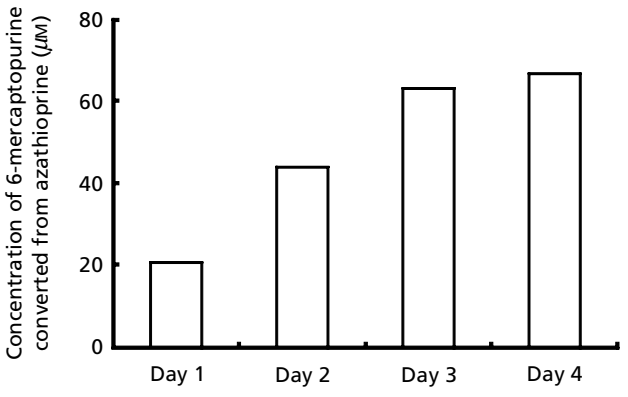

Figure 2 Concentrations of 6-mercaptopurine converted from azathioprine in human peripheral blood mononuclear cell (PBMC) cultures after incubation with 100 μM azathioprine for 1–4 days. Representative data by use of PBMCs from one healthy subject are shown.

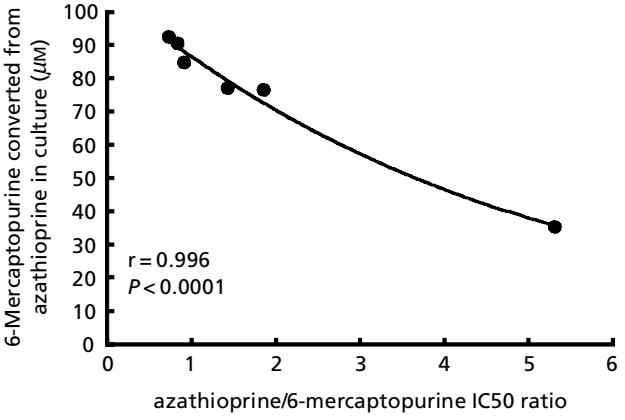

Figure 4 Negative correlation between the azathioprine/6-mercaptopurine IC50 ratio and the amount of 6-mercaptopurine converted from azathioprine in human peripheral blood mononuclear cell (PBMC) culture medium determined using the PBMCs from six healthy subjects.

Discussion

AZ is a prodrug of 6-MP, and the immunosuppressive potency of AZ. In general, AZ is reported to be more effective as an immunosuppressant than 6-MP (Winkelstein 1979; Hardman et al 1995). However, using our PBMC culture system, we demonstrated in the present study that the suppressive potency of AZ against mitogen-induced blastogenesis of human PBMCs in-vitro after an incubation period of 4 days was almost equal to or slightly weaker than that of 6-MP.

Conversion of AZ to 6-MP in-vivo is mainly mediated enzymatically by the glutathione-*S*-transferases in the liver (Kaplowitz & Kuhlenkamp 1978; Gunnarsdottir & Elfarra 1999).

In a recent retrospective study, Regueiro & Mardini (2002) determined whether the initial AZ dosing based on thiopurine methyltransferase genotype or phenotype alters the likelihood of developing acute leukopenia. In addition, Nishida et al (2002) has described TP6 methyltransferase activity in Japanese subjects. We found here that the medium and FBS have no effect on the conversion of AZ to 6-MP in-vitro, whereas conversion of AZ to 6-MP was markedly promoted by the addition of PBMCs, thus suggesting that PBMCs exhibit metabolizing activity for the conversion of AZ to 6-MP. AZ is also reported to be transformed in whole fresh human blood in-vitro to 6-MP (Chrzanowska et al 1985). In addition, the relative glutathione-*S*-transferase activity was found to be higher in lymphocytes than in erythrocytes (al-Turk et al 1987). Thus, it may be possible that glutathione-*S*-transferase of lymphocytes mediated the conversion of the prodrug AZ to 6-MP in the present study. However, although conversion of AZ to 6-MP appeared to occur in PBMCs, the suppressive effects of these compounds against blastogenesis of PBMCs in culture were not significantly different. Prednisolone sodium succinate, a prodrug of prednisolone, was suggested to have little or no pharmacological efficacy itself. Indeed, in a similar in-vitro assay system to that used in the present study, we revealed that the immunosuppressive potency of prednisolone sodium succinate was several fold lower than that of prednisolone in-vitro (Sugiyama et al 2001). In this sense, the relative PBMC suppressive effect of the prodrug AZ and its converted form 6-MP were somewhat different from that of prednisolone sodium succinate and its active form prednisolone.

As expected, AZ IC50 values and 6-MP IC50 values were significantly correlated. However, neither AZ nor 6-MP IC50 values correlated with the amount of 6-MP produced from AZ in PBMC culture. This may be because of intrinsic differences in PBMC sensitivity to 6-MP between the subjects. These observations also suggest that each agent possesses equal immunosuppressive efficacy, working in a similar mechanism to circumvent PBMC blastogenesis in-vitro, and AZ appears to be effective after conversion to 6-MP by PBMCs. Interestingly, our further analysis (Figure 4) suggested that PBMCs having high ability to convert AZ to 6-MP were more sensitive to the suppressive effect of AZ compared with that of 6-MP. In contrast, the PBMCs exhibiting low potency to convert AZ appeared to be relatively insensitive to the suppressive effect of AZ. This observation may be reasonable for the PBMCs to escape from the influence of AZ or 6-MP in-vitro. Further studies, for example measuring glutathione-*S*-transferase activity in PBMCs, may be required to confirm these observations.

Conclusion

We have demonstrated in the present study that the suppressive efficacies of the prodrug AZ and its converted form 6-MP against blastogenesis of human PBMCs in-vitro were almost equal, although the PBMCs appeared to possess marked ability to convert AZ to 6-MP. The results suggest that AZ is effective after conversion to 6-MP to suppress blastogenesis of human PBMCs in-vitro.

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