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Effect of mercaptan nucleophiles on the degradation of azathioprine in aqueous solution

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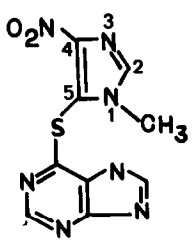
Summary

Azathioprine, a potentially useful antileukemic and immunosuppressive agent, is rapidly cleaved in the body to 6-mercaptopurine. The metabolic breakdown appears to be mediated by glutathione, L-cysteine and other amino acids predominantly in the erythrocytes of the blood and in the hepatocytes of the liver. Studies have been initiated in an effort to understand the mechanism of azathioprine degradation in the presence of a series of mercaptans as well as non-sulfhydryl amino acids in aqueous solution. The reactions were performed with an initial 2×10^{-5} M concentration of azathioprine in solutions containing varying concentrations of nucleophiles ($1-4 \times 10^{-4}$ M) in pH 7.40 phosphate buffer (0.05 M). The final ionic strength of the solutions was adjusted to 0.15 with sodium chloride and the temperature was kept constant at 25 ± 0.5°C. The rates of decomposition of azathioprine were followed by a high pressure liquid chromatographic method. The reactions appeared to follow pseudo-first-order kinetics and were very prone to nucleophilic attack by glutathione, L-cysteine and other mercaptans. Thiolate anion (RS^-) was found to be the predominant attacking species and approximately 24 times more potent nucleophile than the corresponding thiol species (RSH). Non-sulfhydryl amino acids like glycine and phenylalanine seemed to have no effect on the reaction rate.

Introduction

The clinical effectiveness of 6-mercaptopurine and thioguanine in the treatment of human leukemia led to the syntheses and investigation of a large number of related purines as potential chemotherapeutic agents in neoplastic diseases (Goodman et al., 1955; Elion et al., 1956, 1959, 1960). Azathioprine, 6-[(1-methyl-4-nitro-5-imidazolyl)thio]purine (1) was designed to present



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the thiopurines in a masked but therapeutically active form (Clarke et al. 1985; Elion et al., 1960).

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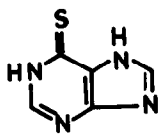
The S-substitution might decrease the inactivation of the compound by S-methylation, non-enzymatic oxidation or conversion to thiourate by xanthine oxidase. Tests on adenocarcinoma 755 revealed that azathioprine had a chemotherapeutic index superior to that of the parent 6-mercaptopurine (2)



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when administered by oral route (Elion et al., 1960).

In the course of further studies, it was found that azathioprine had a superior chemotherapeutic index to that of 6-mercaptopurine as an immunosuppressive agent. Currently it is used as an adjunct in the prevention of rejection of renal transplants and in the treatment of conditions that are thought to have an autoimmune basis such as severe active rheumatoid arthritis.

Azathioprine is rapidly converted to 6-mercaptopurine in the body (Elion, 1967). Metabolism studies have revealed that the breakdown occurs mainly in the red blood cells and in the liver hepatocytes which are rich in glutathione (DeMiranda et al., 1973). The reaction of azathioprine with liver homogenates was demonstrated to be non-enzymatic in nature since 6-mercaptopurine production was not reduced by heating the homogenates at 100°C for 15 min, and formation of 6-mercaptopurine occurred with trichloroacetic acid precipitable liver protein as well as with the supernatant fraction therefrom. Mild performic acid treatment led to inactivation, implicating the sulfhydryl group as the responsible factor (Bresnick, 1959).

Apart from sulfhydryl compounds, proteins (DeMiranda et al., 1973) and possibly peptides and amino acids (Elion et al., 1970; DeMiranda et al., 1970) are also believed to be capable of splitting azathioprine but to a lesser degree. Since the breakdown of azathioprine to 6-mercaptopurine had important biological implications and the reactions are non-enzymatic in nature, a detailed investigation of the effect of amino acids and a series of sulfhydryl compounds on the degradation has been undertaken.

Materials and Methods

Materials

Azathioprine, 6-mercaptopurine and 6-methyl thiopurine were supplied by Burroughs Wellcome, Research Triangle Park, NC and were used as received. Glutathione (reduced), L-cysteine, 3-mercaptopropionic acid, thiomalic acid, thioglycolic acid, L-phenylalanine, and glycine were obtained in free acid form from Sigma Chemicals, St. Louis, MO. Ethyl 2-mercaptoacetate and methylthioglycolate were obtained from Aldrich Chemicals, Milwaukee, WI. Buffer substances and all other chemicals were of reagent grade. For the preparation of HPLC mobile phase, spectral grade acetonitrile (Burdick and Jackson Laboratories, Muskegon, MI) was used. Distilled, deionized water was used for the preparation of buffer solutions as well as mobile phases.

Apparatus

All the pH measurements were made at the temperature of the study using a Corning Model 125 pH-meter equipped with a combination electrode (Corning Science Products, Medfield, MA).

The reactions were followed by a high-pressure liquid chromatographic method using ultraviolet detection. The instrument consisted of a Waters Model M45 solvent delivery system, a Rheodyne 20 μl fixed volume loop injector, a Waters Model 481 variable wavelength detector, a fisher Recordall 5000 strip chart recorder and an Alltech C_{18} econosphere octadecyl silane column.

The mobile phase was made up of a combination of 0.01 M acetate buffer and acetonitrile (90 : 10) and the apparent pH was adjusted to 5.0. The flow rate was kept constant at 2.0 ml/min which generated a column pressure of 1 500 p.s.i. Ultraviolet detection was performed at 254 nm.

Kinetic studies

The kinetic studies were performed in 0.05 M phosphate buffer ($\mu = 0.15$) at 25°C. The pH of the buffer solutions was adjusted to 7.40 by hydro-

chloric acid. 1×10^{-2} M solutions of all the amino acids and sulfhydryl compounds were prepared fresh in distilled deionized water. Triplicate samples, each containing 4.9 ml of phosphate buffer solution with a known concentration of the amino acid or the sulfhydryl compound were placed in a Beckman Thermocirculator at 25 ± 0.1°C. Four different concentrations 1, 2, 3 and 4×10^{-4} M of each compound were used to calculate the rate constant for that compound.

Reactions were initiated by adding 0.1 ml of 1×10^{-3} M stock solution of azathioprine in dimethyl formamide to 4.9 ml of the buffer solution which gave an initial concentration of 5×10^{-5} M. Samples (0.1 ml) were withdrawn every 15 min and added to 0.1 ml of 6-methylthiopurine, an external standard. The final concentration of the external standard was 1×10^{-5} M. After thorough mixing, a 30 μl sample was injected onto the chromatograph. A typical chromatogram has been illustrated in Fig. 1. A baseline separation has been achieved with capacity factors of 5.3 and 7.4 for the external standard and the drug, respectively, which generates a value of 1.4 for the separation factor. A standard curve constructed by plotting peak height ratios against azathioprine concentration was linear with a correlation coefficient of 0.998 over the concentration range of 1×10^{-7} to 1×10^{-5} M.

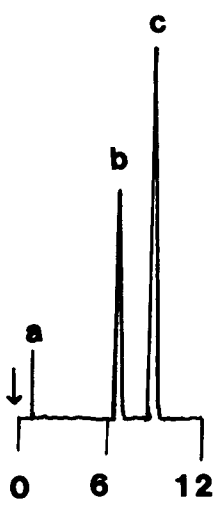


Fig. 1. A typical chromatogram illustrating separation of 6-methyl thiopurine and azathioprine. a, solvent front; b, 6-methyl thiopurine; and c, azathioprine.

Results and Discussion

The breakdown of azathioprine to 6-mercaptopurine in aqueous solution was studied in the presence of a series of sulfhydryl compounds at pH 7.40, 25°C and at an ionic strength of 0.15. The observed rates followed pseudo-first-order behavior and showed direct dependency on the concentration of the sulfhydryl compound. The chemical structures and pK_a values of the compounds studied have been shown in Table 1. The observed rate constants obtained from the plots of the logarithm of azathioprine remaining as a function of time, have also been plotted as a function of the sulfhydryl (nucleophile) concentration. Such a plot has been illustrated in Fig. 2. The excellent linearity of the plots clearly suggests that the breakdown of azathioprine to 6-mercaptopurine is directly dependent on the concentration of the mercaptan in solution. The plot in Fig. 2 tends to suggest that in the absence of mercaptans, the rate constant for the breakdown of azathioprine to 6-mercaptopurine at pH 7.40, 0.05 M phosphate buffer, 25°C, $\mu = 0.15$, is very small, k_{obs} about 6.7×10^{-7} min^{-1}. The rates are approximately 10^3 and 10^4 times faster in presence of $1\text{–}4 \times 10^{-4}$ M mercaptans. The nucleophilic reaction rate constants, k_{NUC} could be obtained from the plots of k_{obs} versus nucleophile concentration according to Eqn. 1.

$$k_{obs} = k_{pH} + k_{NUC}[\text{Nucleophile}] \quad (1)$$

where k_{pH} represents the rate constant independent of nucleophile concentration and the slopes designate the apparent second order rate constants for the nucleophilic attack. It is apparent from the plots in Fig. 2 that the lower the pK_a of the thiol, the higher is the nucleophilic rate constant.

These k_{NUC} values were obtained from the slopes of the plots shown in Fig. 2 and can be plotted against the fraction of the thiolate con-

TABLE 1

POTENTIAL SULFHYDRYL NUCLEOPHILES AND pK_a OF THEIR –SH GROUPS IN WATER AT 25°C

Nucleophile	Chemical structure	pKa of –SH group in water at 25°C	Reference
Glutathione	$H_2N\,CH(CH_2)_2\,CONH\,CHCONHCH_2COOH$ (COOH; CH_2SH)	8.66	Merck Index, 8th edn. (1968)
L-Cysteine	$HOOC-CH(NH_2)-CH_2SH$	8.33	Merck Index, 8th edn. (1968)
Thioglycolic acid	$HOOC-CH_2SH$	10.68	Merck Index, 8th edn. (1968)
3-Mercaptopropionic acid	$HOOC-CH_2-CH_2SH$	10.52	Danehy and Noel (1960)
Thiomalic acid	$HOOC-CH_2-CHSH$ (COOH)	10.83	Danehy and Noel (1960)
Methylthioglycolate	$CH_3-O-COCH_2SH$	7.70	Danehy and Noel (1960)
Ethyl 2-mercaptoacetate	$C_2H_5O-COCH_2SH$	7.88	Kreevoy et al. (1960)

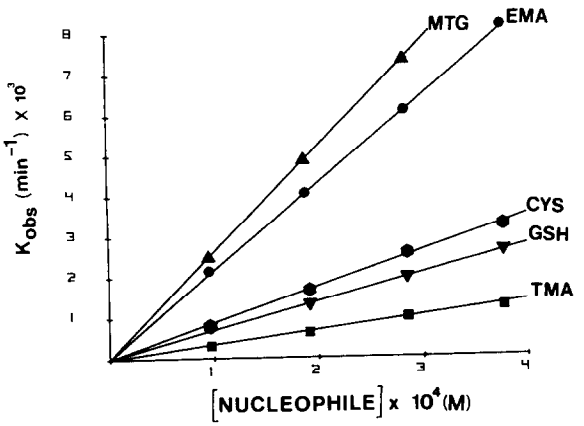


Fig. 2. Effects of varying concentrations of the sulfhydryl nucleophiles on the degradation of azathioprine at pH 7.40, 0.05 M phosphate buffer, 25°C and 0.15 M ionic strength. The points represent the mean values of 3 determinations and the standard deviations are less than the size of the symbols. Symbols: ▲, methylthioglycolate (MTG); ●, ethyl 2-mercaptoacetate (EMA); ●, L-cysteine (CYS); ▼, glutathione (GSH); and ■, thiomalic acid (TMA).

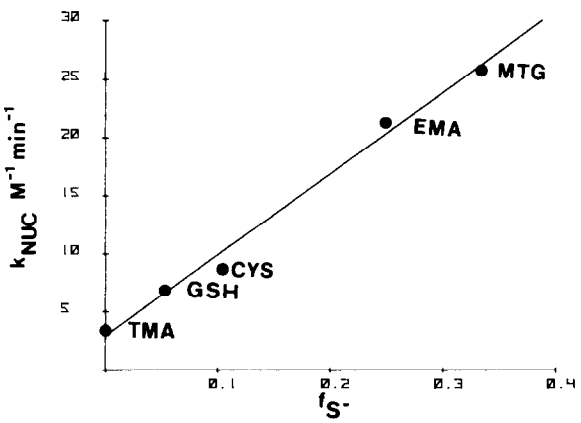


Fig. 3. The dependence of nucleophilic rate constant (k_{NUC}) on the fraction of thiolate anion (f_{S^-}) in solution, for the degradation of azathioprine at pH 7.40, 0.05 M phosphate buffer, 25°C and 0.15 M ionic strength.

TABLE 2

NUCLEOPHILIC RATE CONSTANTS OBTAINED AT pH 7.40, 0.05 M PHOSPHATE BUFFER ($\mu = 0.15$) AT 25 ± 0.5°C AND THE CORRESPONDING FRACTIONS OF THIOLATE ANION (f_{S^-}) IN SOLUTION

Nucleophile	pK_a of –SH group in water at 25°C	f_{S^-} at pH 7.40	k_{NUC} ($M^{-1} \cdot min^{-1}$)
Methyl thioglycolate	7.70	0.338	25.81
Ethyl 2-mercaptoacetate	7.88	0.249	21.30
L-Cysteine	8.33	0.105	8.73
Glutathione	8.66	0.052	6.87
3-Mercaptopropionic acid	10.52	< 0.005	3.48
Thioglycolic acid	10.68	< 0.005	3.11
Thiomalic acid	10.83	< 0.005	3.41

centration in the solution. The numerical values have been tabulated in Table 2 and such a plot has been illustrated in Fig. 3. Excellent linearity could be evidenced by the correlation coefficient of 0.9963 and by the visual inspection of the plot. Eqn. 2 can adequately describe such a plot.

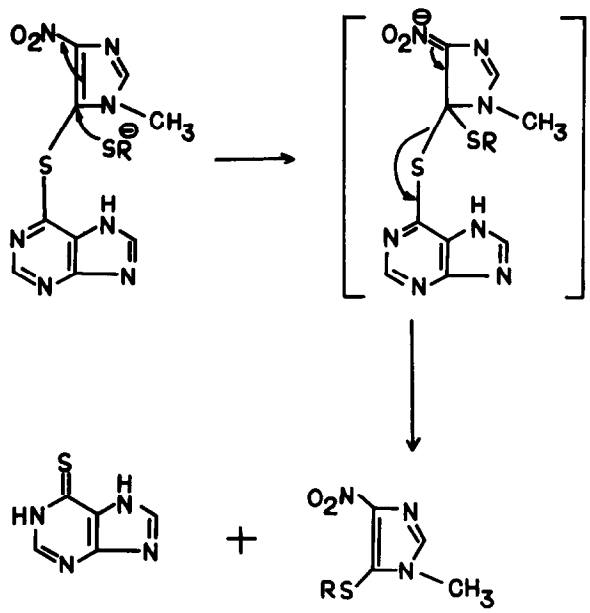
$$k_{NUC} = k_{SH} + (k_{S^-} - k_{SH}) f_{S^-} \quad (2)$$

where f_{S^-} represents the fraction of thiolate anion in the solution and k_{SH} and k_{S^-} represent the apparent second-order rate constants associated with the undissociated thiol and the thiolate anion, respectively. The values for k_{SH} and k_{S^-} obtained from this plot are 2.89 $M^{-1} \cdot min^{-1}$ and 69.22 $M^{-1} \cdot min^{-1}$, respectively. Therefore, it can be concluded that thiolate anion is approximately 24 times more potent nucleophile than undissociated thiol species. Amino acids, peptides or proteins with a larger fraction of thiolate anion concentration at physiological pH will be able to function as very strong nucleophiles.

The carboxylate anion does not seem to participate in the reaction. The two non-carboxylate sulfhydryl compounds, methylthioglycolate and ethyl 2-mercaptoacetate are sufficiently stable at pH 7.4 and do not hydrolyze to any significant extent within the 3 h experimental time. The nucleophilic reaction rate constants (k_{NUC}) for these two nucleophiles are consistent with the rate constants obtained from other carboxylate sulfhydryl nucleophiles when the thiolate anion concentrations were taken into account. This observation tends to suggest that the carboxylate moiety probably does not participate in any intra- or inter-molecular assistance or hindrance to the reaction.

Non-sulfur containing amino acids, i.e. glycine and phenylalanine, were unable to cause any breakdown of azathioprine. Because of the zwitterionic nature of these amino acids at physiological pH, the amino groups are not expected to be potent nucleophiles. Therefore, N-terminal amino acid of peptides and proteins may not be capable of acting as very strong nucleophilic groups.

The possible mechanism of breakdown of azathioprine to 6-mercaptopurine by sulfhydryl compounds has been depicted in Scheme 1. The



Scheme 1

nucleophilic attack at the C-5 carbon of the nitroimidazole moiety by the thiolate anion can initiate the reaction. The attack can be facilitated by the pulling of the π electrons (between C-4 and C-5 bond) towards the electron withdrawing nitro group. This can be followed by the electronic rearrangement with the formation of double bond between C-4 and C-5 with simultaneous breakdown of C–(5)–S bond leading to 6-mercaptopurine. The proposed mechanism suggests that α,β unsaturation or the presence of a carbonyl group (thioester) at the α position to the carbon–sulfur linkage may be required for the breakdown of the compounds by the sulfhydryl nucleophiles. The presence of an electron withdrawing group at C-4 position facilitates the reaction. Therefore, it might be expected that an electron-donating group at the same position might hinder the same reaction. Future design of prodrugs or analogues of thiopurines should take into account the differences in the reactivity of the synthesized compounds towards glutathione, cysteine or other sulfhydryl containing amino acid in the liver or red blood cells. Although glutathione, cysteine or other sulfhydryl-containing amino acids, peptides or proteins are distributed in a

wide variety of tissues, red blood cells and liver are the most accessible body compartments capable of rapidly uptaking and establishing rapid pseudo-equilibrium with the administered drug in systemic circulation.

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