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STUDIES ON THE MECHANISM OF ACTION OF MICONAZOLE—II. INTERACTION OF MICONAZOLE WITH MAMMALIAN ERYTHROCYTES

K. H. SREEDHARA SWAMY, M. SIRSI and G. RAMANANDA RAO*

Microbiology and Cell Biology Laboratory, Indian Institute of Science, Bangalore-560012, India

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Abstract—Interaction of miconazole, an antifungal agent, with mammalian erythrocytes has been studied. Miconazole brings about hemolysis of sheep erythrocytes and 50 per cent hemolysis is observed at a drug concentration of $1{\cdot}58 \times 10^{-4}$ M. The drug-induced hemolysis is dependent on drug:cell ratio. Erythrocytes from different species do not show any significant variation in their sensitivity to miconazole. The uptake of miconazole by erythrocytes is very rapid and major portion of the drug taken up is associated with the cell membrane. Miconazole binds mostly to membrane lipoprotein fractions containing a lipid:protein ratio of 1·0. Miconazole-induced hemolysis is inhibited by serum and the serum components responsible for the inhibition have been identified as albumin and IgG. Bovine serum albumin is found quite effective in protecting erythrocytes against drug-induced hemolysis. The hemolytic activity of miconazole has been compared with polyene antibiotics, digitonin and 2-phenethylalcohol. From these results it is concluded that miconazole interacts directly with the red cell membrane and alters its permeability.

Miconazole nitrate [1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy) phenethyl)imidazole nitrate] has a broad-spectrum of activity against most pathogenic fungi and Gram-positive bacteria [1–3]. Its effective therapeutic use as a topical applicant in treating skin and nail infections and in vaginal candidiasis has been well documented [4–8].

Biochemical and electron microscopic studies on the mechanism of action of miconazole have been reported [9–11]. The drug caused a significant increase in membrane permeability in cells of *Candida albicans* as evidenced by a rapid loss of intracellular materials [9]. At low concentrations, miconazole selectively inhibited the uptake of purines and glutamine by the cells of *C. albicans* [10]. Electron microscopic examination of *C. albicans* cells exposed to miconazole revealed that the earliest drug-induced alterations are seen at the plasma membrane before any other cytoplasmic organelle seems to be involved [11]. These findings clearly reveal that miconazole impairs membrane function by inducing selective permeability changes in the cell membranes of sensitive cells.

In an attempt to determine whether the action of miconazole on membrane permeability is restricted to yeast cells or has a similar action on animal cells, its effect on the permeability of sheep erythrocytes is investigated. The present paper reports a study of various aspects of interaction of miconazole with red blood cells.

MATERIALS AND METHODS

Materials. Crystalline bovine serum albumin, cholesterol, digitonin and sodium dodecyl sulphate were purchased from Sigma Chemical Co., St. Louis, U.S.A. Amphotericin B and nystatin were kindly donated by E. R. Squibb and Sons, Inc., Princeton, U.S.A. Miconazole nitrate was a gift sample from Ethnor Ltd., Bombay, India. [^{3}H]Miconazole (sp. act. 292·3 mCi/m-mole) was a kind donation of Janssen Pharmaceutica, Belgium.

Erythrocyte preparation. Fresh blood from healthy human adults, wistar rats, guinea pigs, rabbits and sheep (for non-nucleated erythrocytes) or from chickens (for nucleated erythrocytes) were obtained using Alsever's solution as an anticoagulant. Immediately after collection, the blood was centrifuged at 1500 *g* in a Sorvall centrifuge model RC-2B for 10 min at 4° and the plasma and the buffy coat were removed. The packed erythrocytes were washed four times with 0·15 M NaCl (isotonic saline) followed by centrifugation. The cells after the last wash were suspended in 0·15 M NaCl to give an erythrocyte suspension containing approximately $1{\cdot}5 \times 10^9$ cells/ml (by hemocytometer count). Unless otherwise specified, all experiments were done with this cell suspension. Aliquots of the cell suspension were pre-incubated for 15 min at 37° before the start of the reaction.

Measurement of hemolysis. Hemolysis was determined by the addition of 0·1 ml of washed erythrocytes to 4·9 ml of 0·15 M NaCl (preincubated at 37° for 15 min) containing miconazole (in 50% ethanol) at various concentrations. Each sample had a final volume of 5 ml and erythrocyte concentration of 3×10^7 cells/ml. Control tubes containing appropriate quantity of ethanol were included in each experiment and the final concentration of ethanol in all the samples was 1 per cent. After the required time of incubation at 37°, the tubes were centrifuged and the hemoglobin released was determined by measuring the absorbance of the supernatant at 540 nm in a Beckman model-DU spectrophotometer. The data were corrected for the release of hemoglobin

* To whom correspondence should be addressed.

observed in controls. To determine the total amounts of intracellular hemoglobin, 0·1 ml of cell suspension was lysed in 4·9 ml of distilled water and centrifuged. The supernatant had an absorbancy of 0·800 at 540 nm. Loss of hemoglobin was expressed as a per centage of total cellular content of hemoglobin in the untreated cells.

Uptake of [^{3}H]miconazole. [^{3}H]Miconazole (sp. act. 292·3 mCi/m-mole) was diluted with unlabeled miconazole to achieve the desired molar concentration of the drug. This diluted solution (4×10^{-4} M) was used to measure the uptake of miconazole at different concentrations in 0·15 M NaCl 0·01 M sodium phosphate buffer, pH 7·4. Erythrocytes ($1·6 \times 10^8$ cells) were incubated at 37° with different concentrations of [^{3}H]miconazole dissolved in 50% ethanol. The final volume of each sample was 4 ml and contained 4×10^7 cells/ml. After 15 min of incubation, 2-ml aliquots of cell suspension were removed and centrifuged. Aliquots (100 μl) of supernatant fraction and total cell suspension were spotted on Whatman 3 MM filter squares, dried under infrared lamp, and counted in Beckman LS-100 liquid scintillation counter using 10 ml scintillation fluid containing 0·4% PPO and 0·005% POPOP in toluene. The difference between the counts in the supernatant and total cell suspension was taken as the amount of drug taken up by the cells.

The amount of miconazole associated with the cytoplasm and bound to the cell membrane was determined in the following way. Cell suspensionn incubated with [^{3}H]miconazole (1×10^{-5} M) was washed twice with 0·15 M NaCl and were then hemolysed in 5 mM sodium phosphate buffer, pH 7·4. An aliquot (100 μl) of the hemolysate was removed for counting and the remainder was centrifuged at 20,000 *g* for 20 min to separate the red cell membrane from the cytoplasm. Radioactivity in 100 μl of the supernatant fraction (cytoplasm) was determined as described above. The difference between the amount of drug taken by the cells and the amount in the cytoplasm is taken as the amount of drug bound to membranes.

Preparation of erythrocyte membranes. Sheep erythrocyte membranes were prepared by the procedure of Dodge *et al.* [12]. The cells were washed thrice with 0·15 M NaCl by centrifugation at 1500 *g* for 10 min at 4°. Membranes were prepared by the osmotic lysis of washed erythrocytes by adding 10 volumes of cold 6·5 mM sodium phosphate buffer, pH 7·4 to 1 volume of packed cells and mixed with a magnetic stirrer for 15 min. The hemolysate was centrifuged at 30,000 *g* at 4° for 40 min in a Sorvall centrifuge model RC-2B. Following centrifugation, the post hemolytic residue was washed four to six times with the same buffer and a final wash with isotonic saline. The milky membrane preparation thus obtained from the last centrifugation, was suspended in isotonic saline to a protein concentration of 13 mg/ml.

Solubilization of erythrocyte membranes and gel filtration. Solubilization of erythrocyte membranes and subsequent gel filtration was done according to the method of Zimmer *et al.* [13]. Membrane preparation containing 9 mg protein was incubated at 37° with 0·55 ml of 1% sodium dodecyl sulphate for 30 min. The concentration of sodium dodecyl sulphate was about 0·6 mg/mg of membrane protein. 0·015 μmoles of [^{3}H]miconazole contained in 0·15 M NaCl was then added and the final volume was made up to 2·5 ml with 0·15 M NaCl. Incubation was continued for a further 30 min and the solubilized membranes were gel filtered.

Sephadex G-100 column (48 × 1·2 cm) was equilibrated with 0·5 M NaCl 0·01 M Tris HCl, pH 8·4. The dissolved membranes were layered onto the column and gel filtration was carried out at 28°. Fractions of about 1·5 ml were collected. The fractions comprising within the peak at 280 nm were analysed for protein, lipid and radioactivity. Protein, phospholipid and cholesterol were determined in 0·1 0·2-ml samples. Radioactivity in 0·1-ml sample was determined as described above.

Incubation of serum with [^{3}H]miconazole and gel filtration. One ml sheep serum was incubated with [^{3}H]miconazole (0·025 μmoles) at 37° for 30 min and gel-filtered at 28° on Sephadex G-200 column (53 × 2 cm) equilibrated with 0·1 M NaCl 0·05 M sodium phosphate buffer, pH 7·4. Fractions of 3 ml were collected and absorbancy at 280 nm and radioactivity determined.

Analytical procedures. Protein was determined by the procedure of Lowry *et al.* [14] with crystalline bovine serum albumin as standard. Membrane lipids were isolated by the method of Bligh and Dyer [15]. Lipid phosphorous was determined by a modification of the method of Bartlett [16], as reported by Marinetti [17] and phospholipid was estimated by multiplying the lipid phosphorus content by 25. Cholesterol was determined by the method of Glick *et al.* [18].

RESULTS

Miconazole-induced hemolysis. The time course of miconazole-induced hemolysis of sheep erythrocytes is shown in Fig. 1a. At a miconazole concentration of 1×10^{-4} M, the hemolysis by miconazole is very rapid and the rate is linear up to 20 min. Complete hemolysis resulted in 40 min. In control tubes containing ethanol at a final concentration of 1% (concentration equivalent to those in experimental tubes) hemolysis is less than 0·5 per cent.

The effect of miconazole concentration on the initial rate of hemolysis is shown in Fig. 1b. The cells are incubated with various concentrations of drug for 5 min at 37°. The extent of hemolysis is markedly influenced by the drug concentration. Up to a drug concentration of $1·2 \times 10^{-4}$ M the rate of hemolysis is very slow and thereafter increases rapidly. The drug concentration required for 50 per cent hemolysis is $1·58 \times 10^{-4}$ M and complete hemolysis occurs at a concentration of $2·2 \times 10^{-4}$ M.

The loss of hemoglobin from the erythrocytes is dependent not only on the concentration of miconazole in the incubation medium, but also on the number of erythrocytes present in the suspension. When miconazole concentration is held constant, and the number of cells per unit volume of suspending medium is increased, the hemolysis is progressively decreased with increasing cell concentration (Fig. 2). The hemolysis is only 60 and 20 per cent at erythro-

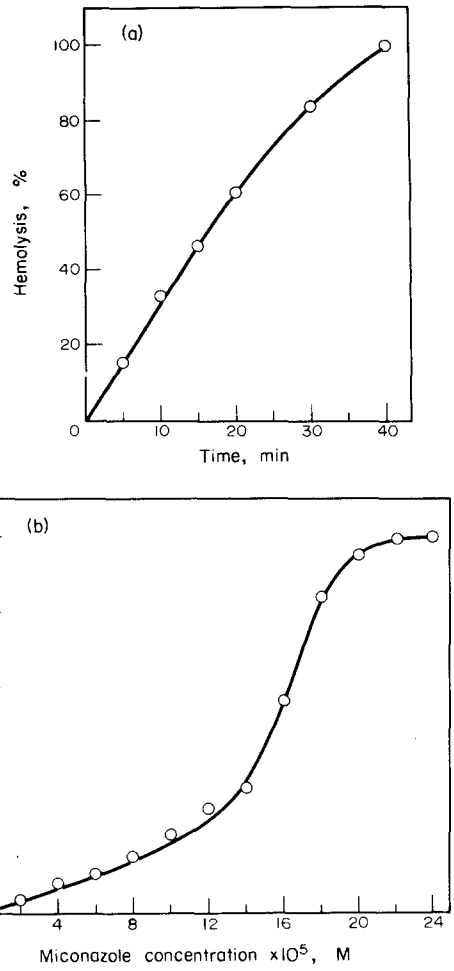

Fig. 1. Miconazole-induced hemolysis of sheep erythrocytes. (a) Time course of hemolysis of sheep erythrocytes by miconazole. Miconazole concentration, 1×10^{-4} M. (b) Effect of miconazole concentration on the initial rate of hemolysis of sheep erythrocytes. Incubation time, 5 min at 37°.

cyte concentration of 3×10^7 and 6×10^7 cells/ml, respectively.

Uptake and distribution of miconazole. When erythrocytes are incubated with [^{3}H]miconazole at non-hemolytic concentrations for 15 min at 37°, the uptake of drug by erythrocytes is very rapid and is linear over the concentrations tested (Fig. 3). Measurements of its uptake by red cells at different concentrations show that more than 60 per cent of the drug added to the red cell suspension is associated with the red cells. The distribution of radioactivity in cellular fractions of erythrocytes incubated with [^{3}H]miconazole was studied. The major portion of the radioactivity taken up by the cells (83%) is found associated with the cell membranes.

Binding of [^{3}H]miconazole to erythrocyte membrane fraction. When sheep erythrocyte membranes are solubilized with sodium dodecyl sulphate and gel filtered on Sephadex G-100, the partial separation of membrane lipoproteins is achieved. As the gel filtration proceeds, the lipid:protein ratio changes from above 1 to about 0·2. This separation of membrane

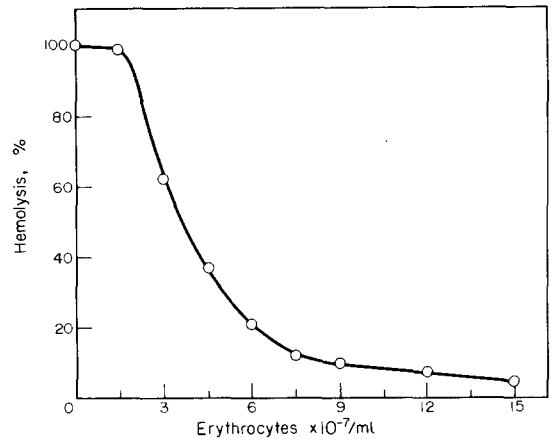

Fig. 2. Effect of sheep erythrocyte concentration on hemolysis by miconazole. Miconazole concentration, 1×10^{-4} M.

lipoproteins indicate that the lipoproteins with the higher lipid content are eluted first. To characterize the miconazole binding site on membranes the solubilized membranes are incubated with [^{3}H]miconazole and gel-filtered. The pattern of elution of lipoproteins and radioactivity are shown in Fig. 4. The radioactivity peak coincided with the membrane lipoprotein peak having a lipid:protein ratio of 1·0. About 90 per cent of the total radioactivity present during the incubation of membranes with the drug got eluted with the liproprotein fractions indicating a strong binding of miconazole to membrane components.

Inhibition of hemolysis by serum. The effect of homologous serum on the miconazole-induced hemolysis of sheep erythrocytes is shown in Fig. 5. The erythrocytes are preincubated with serum and the reaction is started by the addition of miconazole to a final concentration of 1×10^{-4} M and incubated for 20 min at 37°. The serum has a protective action and inhibits the miconazole-induced hemolysis even at low concentrations. At 0·2% serum level, the hemolysis is inhibited by 60 per cent and at 1% the inhibition is more than 90 per cent.

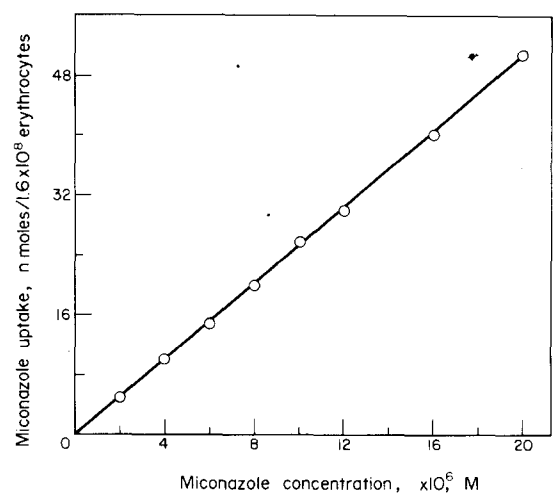

Fig. 3. Uptake of [^{3}H]miconazole by sheep erythrocytes. Incubation time, 15 min at 37°.

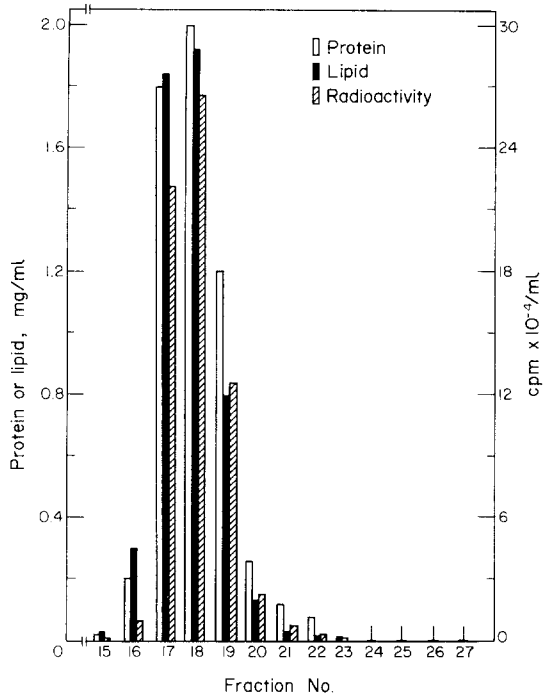

Fig. 4. Binding of [^{3}H]miconazole to erythrocyte membrane lipoprotein fractions. Erythrocyte membranes, solubilized with sodium dodecyl sulphate, were incubated with [^{3}H]miconazole and gel-filtered on Sephadex G-100 column. After gel filtration, individual fractions were analysed for radioactivity, protein and lipid content.

To characterize the serum component(s) responsible for the inhibition of miconazole-induced hemolysis, serum is incubated with [^{3}H]miconazole and gel-filtered on Sephadex G-200 (Fig. 6). The radioactivity of the drug eluted in fractions containing IgG and albumin. About 40 per cent of the total radioactivity is found in IgG fraction and 60 per cent in the albumin. These results indicate that miconazole binds to serum proteins and the inhibitory effect of serum on hemolysis is a consequence of miconazole binding to serum proteins.

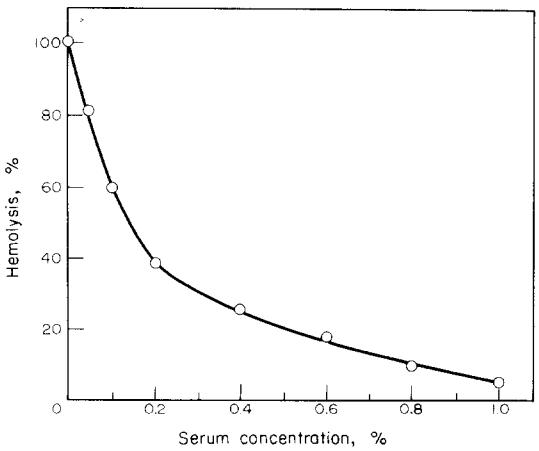

Fig. 5. Inhibition of miconazole-induced hemolysis of sheep erythrocytes by homologous serum. Miconazole concentration, 1×10^{-4} M.

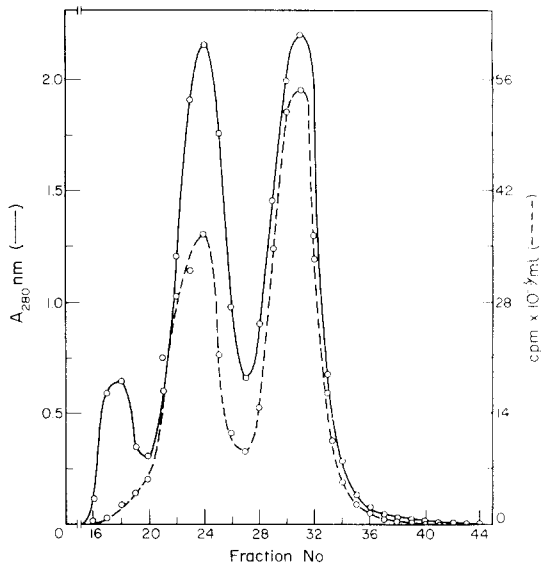

Fig. 6. Binding of [^{3}H]miconazole to serum proteins. One ml sheep serum was incubated with [^{3}H]miconazole and gel-filtered on Sephadex G-200 column. After gel filtration, each fraction was analysed for absorbancy at 280 nm and radioactivity.

Since miconazole binds to albumin it is of interest to see the hemolytic activity of miconazole in the presence of albumin. Erythrocytes are preincubated with various amounts of bovine serum albumin for 30 min at 37° before the addition of miconazole. As shown in Fig. 7 the hemolytic activity of miconazole is inhibited by albumin and at an albumin concentration of 0·4 mg/ml, the hemolysis is inhibited by 68 per cent. The hemolytic activity of miconazole is partially retained at 2 mg/ml albumin concentration.

Relative hemolytic effect of miconazole compared with other membrane-active drugs. For comparison, the effect of other membrane-active agents like polyene antibiotics, digitonin and 2-phenethylalcohol on sheep erythrocytes is studied. The cells are incubated with the different concentrations of each drug for 5 min at 37° and from the graphic plot of the

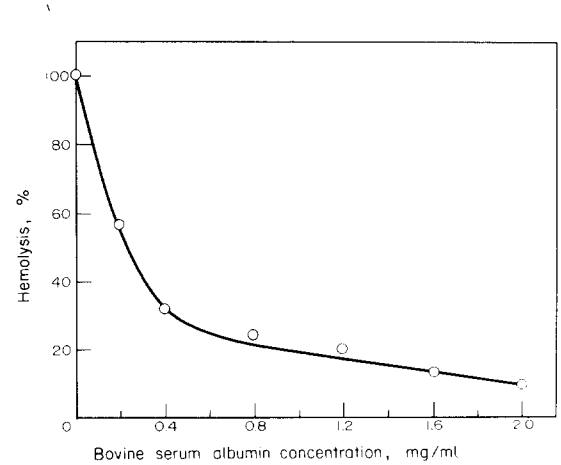

Fig. 7. Effect of bovine serum albumin concentration on hemolysis of sheep erythrocytes by miconazole. Miconazole concentration, 1×10^{-4} M.

Table 1. Comparative hemolytic effect of miconazole with other membrane active agents

Drugs tested	Concentration of drug required for 50% hemolysis
Amphotericin B	$9{\cdot}2 \times 10^{-6}$ M
Nystatin	$8{\cdot}4 \times 10^{-5}$ M
Digitonin	$2{\cdot}6 \times 10^{-6}$ M
2-Phenethylalcohol	$5{\cdot}7 \times 10^{-2}$ M
Miconazole	$1{\cdot}58 \times 10^{-4}$ M

To tubes containing different concentrations of drugs indicated in the table, washed sheep erythrocytes were added to a final concentration of 3×10^7 cells/ml, incubated for 5 min at 37° and immediately centrifuged. The hemoglobin released was measured as described under Materials and Methods. Amphotericin B and nystatin were dissolved in dimethylformamide, digitonin in absolute alcohol and dilutions of 2-phenthylalcohol in 50% ethanol. The control tubes contained the equivalent amount of solvents in which the drug was dissolved.

data, the concentration of each drug needed to bring about 50% hemolysis is calculated. As shown in Table 1, digitonin is a more potent hemolytic agent while 2-phenethylalcohol is least effective. Digitonin, amphotericin B and nystatin cause 50% hemolysis at 60, 17 and 1·8-fold lesser concentration than miconazole, respectively. On the other hand, 2-phenethylalcohol requires about 360-fold higher concentration than miconazole to bring about 50% hemolysis.

Hemolytic effect of miconazole on erythrocytes from different species. Erythrocytes from different species are examined for their susceptibility to miconazole (Table 2). The washed erythrocytes from different species are incubated with different concentrations of miconazole for 5 min at 37° and from a graphic plot of the data, the hemolytic value corresponding to 50% hemolysis is calculated. Among the non-nucleated erythrocytes, the variation in their susceptibility to miconazole-induced hemolysis is not very significant. Erythrocytes from hamster require comparatively lower concentration ($0{\cdot}9 \times 10^{-4}$ M) than erythrocytes from humans which require a 3-fold higher concencentration ($2{\cdot}76 \times 10^{-4}$ M). Erythrocytes from guinea pig, rabbits and sheep are susceptible to the same extent and require about 1·7-fold lower concentration than human erythrocytes. The nucleated erythrocytes from chicken are also equally sensitive to the action of miconazole and 50% hemolysis occurs at a drug concentration of $1{\cdot}48 \times 10^{-4}$ M.

Table 2. Hemolytic effect of miconazole on erythrocytes from different species

Erythrocyte source	Concentration of miconazole required for 50% hemolysis ($\times 10^{-4}$ M)
Rat	1·18
Rabbit	1·58
Guinea pig	1·54
Hamster	0·90
Sheep	1·58
Human	2·76
Chicken	1·48

The washed erythrocytes (3×10^7 cells/ml) from different species were incubated with different concentrations of miconazole for 5 min at 37° and centrifuged immediately. Hemoglobin released was measured as described under Materials and Methods. From the graphic plot of the data, the concentration of drug required for 50% hemolysis was calculated.

DISCUSSION

The drug-induced hemolysis of red cells is currently thought to occur by either of two basic mechanisms [19–22]. The first involves direct interaction of the drug with the red cell membrane which results in changes in membrane structure, increased permeability, osmotic swelling and hemolysis. In the second mechanism, the drug first penetrates into the cell interior where it interferes with cellular metabolism ultimately resulting in membrane damage and hemolysis. The drug-induced hemolysis in the second mechanism may be due to enzyme deficiencies, unstable hemoglobins or immune mechanisms [20, 21].

The present findings reveal that miconazole has a profound effect on erythrocyte membrane structure and brings about rapid hemolysis. This raises the possibility that hemolysis is the result of a direct interaction between miconazole and the plasma membrane of the erythrocytes. A prerequisite to direct alteration of membrane permeability is that miconazole must interact with the cell membrane. Data presented in this paper show that [^{3}H]miconazole at low, non-hemolytic concentrations binds largely to the red cell membrane and thus satisfying a necessary condition.

Identification of the binding site on the erythrocyte membrane surface would help for a better understanding of the mechanism of action of membrane-active drugs. In our effort to characterize the miconazole-binding site on the erythrocyte membrane, it has been shown that miconazole binds to membrane lipoproteins strongly and that miconazole binding is maximum in lipoprotein fraction containing lipid: protein ratio of 1·0. The concentration of sodium dodecylsulphate (0·6 mg SDS/mg membrane protein) employed to solubilize the membrane in the present experiment does not cause any loss of biological activity of the membrane [13, 23], and no disruption of lipoprotein structure of the membrane is evident (Fig. 4). The present experiments, however, do not indicate, the differential binding of miconazole to either lipid or protein part on the membrane.

The protective effect of serum against miconazole-induced hemolysis apparently results from the binding of miconazole to serum proteins. Serum albumin is found quite effective in protecting erythrocytes against miconazole-induced hemolysis. The binding of miconazole to serum proteins would result in a reduction of effective drug concentration to bring about hemolysis of erythrocytes. Several drugs are known to bind to albumin in the serum, but the binding of miconazole to IgG, presented in this paper, raises two possibilities. It may be either due to the binding of miconazole to IgG itself or to the elution of albumin dimers formed in the serum along with IgG. In the latter case the drug primarily binds to albumin, but due to the formation of albumin dimers the radioactivity appears in the IgG peak.

Polyene antibiotics, which have been extensively studied for their membrane damaging properties,

have been compared with miconazole for their hemolytic activity. Among the polyenes, amphotericin B showed higher hemolytic effect than miconazole and nystatin showed almost equal effect. Digitonin, a plant saponin, exerts higher hemolytic activity than miconazole. Polyenes and saponins induce membrane damage by their strong binding to cholesterol in the membrane [24–32]. 2-Phenethylalcohol, which is known to impair the cell membrane function in bacteria [33, 34], yeasts [35], fungi [36], tumor cells [37] and mammalian erythrocytes [38], showed lower hemolytic activity than miconazole.

There is little variation in the hemolytic effect of miconazole on erythrocytes from different species and it is independent of the presence or absence of a nucleus. The basis for the reported differences in the hemolytic tendency among species is not known but variation in the protein and lipid composition of the red cell membrane may account for such differences [39–46].

On the basis of the results presented in this paper we conclude that miconazole interacts directly with the red cell membrane and brings about permeability alterations. Though miconazole binds to membrane lipoproteins strongly, the nature of this interaction is not known. The chemical nature of the drug indicates a possible mode of interaction. Since the drug is hydrophobic in nature, the interaction may be of a hydrophobic type and this would lead to an impairment of membrane function. Further studies are needed to elucidate this aspect.

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