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Changes in erythrocyte membrane permeability induced by verapamil, chlorpromazine, and their combinations with amphotericin B

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Abstract: Hemolysis induced by 2 amphipathic agents, verapamil and chlorpromazine, was investigated in various incubation conditions. Changes in absorbance of erythrocyte suspension were monitored by absorption spectrophotometry at a wavelength of 590 nm. The hemolysis induced by verapamil or chlorpromazine is of the permeability type. The resistance of erythrocytes to verapamil is much higher than their resistance to chlorpromazine. No evident difference is found between human and pig erythrocytes in their resistance to verapamil. Only a small decrease in the rate of hemolysis induced by verapamil is observed in isotonic $CaCl_2$, $MgCl_2$ or K_2SO_4 solutions, compared to 160 mM KCl (the standard incubation medium). The changes in hemolytic activity of chlorpromazine in the presence of the divalent cations and anions are less evident. No decrease in hemolytic activity of chlorpromazine and verapamil is observed in the sucrose medium. The hemolytic activity of both the agents increases when they act in combination with polyene antibiotic amphotericin B. The results indicate a strong synergy between amphotericin B and verapamil or chlorpromazine. By contrast, a combined effect of verapamil and chlorpromazine on erythrocytes leads to a decrease in their hemolytic activity. This indicates antagonism between verapamil and chlorpromazine.

Keywords: verapamil, chlorpromazine, amphotericin B, synergy, erythrocyte membrane hemolysis

INTRODUCTION

Verapamil is a calcium channel blocker, commonly used in cardiovascular diseases (McTavish & Sorkin 1989). It may act as a chemosensitizer of p-glycoprotein or multidrug resistance protein 1 - MRP1 (Speelmans et al. 1995; Salerno et al. 2004, Meier et al. 2006; Perrotton et al. 2007), which are membrane proteins responsible for multidrug resistance (MDR). Verapamil may lead to the reversal of MDR by direct binding to p-glycoprotein (Meier et al. 2006) or by changing the membrane fluidity and permeability (Drori et al. 1995; Pajeva et al. 1996).

Chlorpromazine is a tricyclic phenothiazine derivative, widely used in the treatment of neurological disorders like schizophrenia (Lieberman et al. 2003). It may act

as a chemosensitizer of p-glycoprotein (Aanismaa & Seelig 2007) and it enhances the cytotoxic effect of some anticancer drugs (Wadkins & Houghton 1993; Bebawy et al. 2001).

Both the amphipathic agents, verapamil and chlorpromazine, show a high affinity to membrane anionic phospholipids located within the inner layer of the cell membrane (Anteneodo et al. 1995; Speelmans et al. 1995; Chen et al. 2003; Wiśniewska & Wolnicka-Glubisz 2004; Pickholz et al. 2007). Studies on human erythrocytes have shown that both the agents induce the formation of stomatocytes, i. e. invaginated erythrocytes (Deuticke 1968; Schreier et al. 1992; Chen et al. 1997; Suwalsky et al. 2008, 2010). According to the bilayer couple hypothesis (Sheetz & Singer 1974), stomatocytosis can be explained by favorable interaction of both the agents with the inner layer of the erythrocyte membrane.

The interaction of verapamil and chlorpromazine with membranes is modified by changes in phospholipid distribution or cholesterol content (Mason et al. 1992; Castaing et al. 2003a; Wiśniewska &Wolnicka-Glubisz 2004; Wolnicka-Glubisz et al. 2009). Membrane potential profile (Pohl et al. 1998) and ionic strength (Castaing et al. 2003b) strongly influence the membrane activity of verapamil. Differences in membrane activity of chlorpromazine have been found for its neutral and protonated state (Wajnberg et al. 1988; Bennouna et al. 1997; Ahyayauch et al. 2003; Wiśniewska & Wolnicka-Glubisz 2004).

The effect of verapamil and chlorpromazine on the membrane strongly depends on their concentrations (Zachowski & Durand 1988; Hagerstrand et al. 2006; Michalak et al. 2007; Watts & Handy 2007; Suwalsky et al. 2008). At high concentrations, verapamil and chlorpromazine induce hemolysis (Lieber et al. 1984; Morimoto et al. 1995; Watts & Handy 2007; Suwalsky et al. 2008). There are some studies on the influence of physicochemical conditions of incubation medium on the hemolytic activity of verapamil and chlorpromazine (Lieber et al. 1984; Morimoto et al. 1995; Ahyayauch et al. 2003; Watts & Handy 2007). However, the mechanism of the increase in membrane permeability induced by the amphipaths is still not clear.

The results of our previous studies on mammalian erythrocytes treated with amphipathic polyene antibiotic amphotericin B have revealed a high dependence of the polyene hemolytic activity on the species and specimen features of the erythrocyte membrane (Knopik-Skrocka & Bielawski 2002, 2005; Knopik-Skrocka et al. 2003, 2006; Knopik-Skrocka & Buldańczyk 2004). The rate of hemolysis induced by amphotericin B is highly decreased in media with different-sized markers (Knopik-Skrocka & Bielawski 2002; Knopik-Skrocka et al. 2003, 2007; Knopik-Skrocka & Buldańczyk 2004). The hemolytic activity of amphotericin B is an effect of formation of antibiotic-sterol channels (Brajtburg et al. 1980; Cybulska et al. 1995). Our earlier results suggested that these channels differ in diameter and selectivity.

In the present paper, the hemolytic activity of verapamil and chlorpromazine was investigated in incubation media with various ions or sucrose and the results are compared with those obtained for amphotericin B. With regard to a high affinity of amphotericin B to membrane cholesterol (Charbonneau et al. 2001) and its competition for membrane target with other polyene antibiotics (Brajtburg et al. 1980), changes in the hemolytic activity of verapamil and chlorpromazine as a result of their

combined action with amphotericin B have been investigated. The hemolytic effect of combined action of verapamil with chlorpromazine has been also examined.

MATERIALS AND METHODS

Preparation of erythrocyte suspension

The 1-day concentrate of human erythrocytes in a polyvinyl bag, with reduced plasma content, was obtained from a blood bank. The erythrocyte concentrate was stored at 4°C with the addition of CPD (citric acid monohydrate 206 mg, sodium citrate dihydrate 1.66 g, sodium dihydrogenophosphate monohydrate 140 mg, glucose monohydrate 1.61 g, water 63 mL) and ADSOL formula (sodium chlorate 900 mg, glucose monohydrate 2.2 g, adenine 2.7 mg, mannitol 750 mg, water 100 mL). After 24 h, the concentrate was used to prepare the erythrocyte suspension. Pig blood was obtained from a slaughterhouse freshly after venipuncture. As described earlier (Knopik-Skrocka et al. 2003), pig blood was stored for up to 24 h at 4°C in the presence of citrate and glucose. The procedure of erythrocyte suspension preparation was the same as described earlier (Knopik-Skrocka & Bielawski 2005; Knopik-Skrocka et al. 2007). The final concentration of erythrocytes suspended in the incubation medium was 0.25 µl of total erythrocyte volume/mL (2.8×10^6 erythrocytes/mL).

Preparation of stock solutions

Verapamil hydrochloride and chlorpromazine hydrochloride were obtained from Sigma-Aldrich. The stock solution of verapamil in distilled water was 20 mM and that of chlorpromazine was 7.5 mM. Amphotericin B was from Fluka, and its 0.54 mM stock solution was prepared in dimethylformamide (Fluka). The dimethylformamide in the incubation medium had no effect on the erythrocytes. All the stock solutions were prepared freshly before experiments.

Incubation conditions

The standard incubation medium of erythrocytes was 160 mM KCl. This solution, as well as the other media tested (107 mM $CaCl_2$, $MgCl_2$, K_2SO_4, and 300 mM sucrose), were taken as isoosmotic to the cell interior. The concentration of the stock solution of the salts or sucrose was twice that of isotonicity. The final concentration was obtained by mixing an appropriate volume of the stock solution of the salt, the hemolytic agent and distilled water.

The experiments were performed at 37°C. As described earlier (Knopik-Skrocka & Bielawski 2005; Knopik-Skrocka et al. 2007), erythrocytes were incubated without any buffer added. The pH of the incubation medium was 6.8. This value was obtained by addition of 160 mM KOH or HCl.

Measurements of hemolysis

In standard experiments, the required amounts of verapamil or chlorpromazine stock solutions were preincubated in isoosmotic KCl for 10 min at 37°C in test tubes (inner diameter of 14 mm). Total volume of the medium in the tubes was 10 mL. Next, the erythrocyte suspension was added and mixed with the incubation medium.

The absorbance was measured at selected time intervals. The suspension was mixed frequently to avoid erythrocyte sedimentation. In all the experiments, the erythrocyte suspension absorbance was measured in the control tube, without the hemolytic agent added.

The absorbance of the erythrocyte suspension was measured as described previously (Knopik-Skrocka & Bielawski 2005) with the use of spectrophotometer Epoll 2000 Eco (EMCO, Warsaw) at λ=590 nm. At this wavelength, the absorbance of lysed erythrocytes is very low and independent of pH. Most of the absorbance is due to light scattering, which decreases with increasing in volume and lysis of erythrocytes. Participation of light absorption in the absorbance is low.

Estimation of erythrocyte resistance to verapamil and chlorpromazine

The resistance of erythrocytes to verapamil or chlorpromazine was evaluated as the agent concentration that induces 50% erythrocyte hemolysis during 30 min of incubation (C_{50}). The C_{50} value was interpolated from the agent concentration-hemolysis rate curve (Knopik-Skrocka & Bielawski 2005).

Statistical significance between the groups of results compared was determined using Student's *t* test. $P < 0.05$ was taken as the threshold of significance.

RESULTS

The effect of verapamil on the absorbance of the human erythrocyte suspension in standard incubation conditions is presented in Fig. 1. After addition of erythrocytes at zero time of incubation, there is a slow drop in absorbance. It is followed by a faster drop in absorbance, reaching finally the lowest value (minimal absorbance). During the initial slower drop, swelling of erythrocytes occurs. When the critical volume of the cells is exceeded, hemolysis occurs, monitored as the second, faster drop in absorbance. At 4 mM verapamil, the rate of absorbance drop is rather low (Fig. 1). The first phase, corresponding to erythrocyte swelling, lasts 60 min and the absorbance value decreases from about 0.570 to 0.420. Next, the rate of absorbance drop increases due to cell lysis, leading to complete hemolysis at the absorbance of 0.030. Complete hemolysis takes place at about 100 min.

The increase in verapamil concentration is associated with an increase in the rate of human erythrocyte swelling and lysis (Fig. 1). All the cells are lysed at both high and low verapamil concentrations. Similar results were obtained for pig erythrocytes treated with verapamil (curves not shown).

The kinetics of hemolysis induced by chlorpromazine (Fig. 2) does not differ from the kinetics of hemolysis caused by verapamil. The process expressed by this type of time-absorbance curves (Figs. 1–2) can be classified as the permeability type of hemolysis (Bielawski 1990). This type of hemolysis allows the calculation of the rate of hemolysis as the reciprocal of the time (min^{-1}) at which 50% of erythrocytes are lysed. If correction is made for swelling, it corresponds to the absorbance decrease equal to 55% of the maximal absorbance decrease. The dependence of the rate of human erythrocyte hemolysis on verapamil concentration in standard incubation conditions is presented in Fig. 3. The slope of the curve increases to a concentration

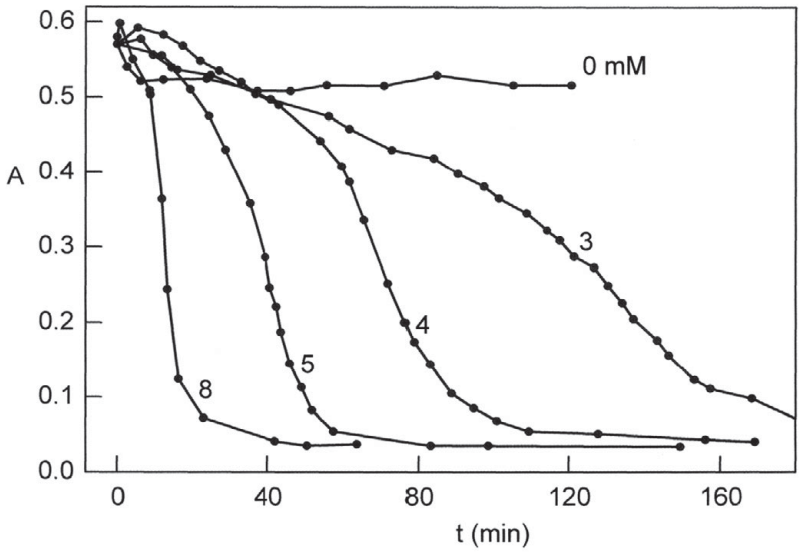

Fig. 1. Influence of verapamil concentration on the dependence of human erythrocyte suspension absorbance (A) on incubation time (t). Incubation medium: 160 mM KCl, 37°C. Verapamil concentrations are given in mM in the figure

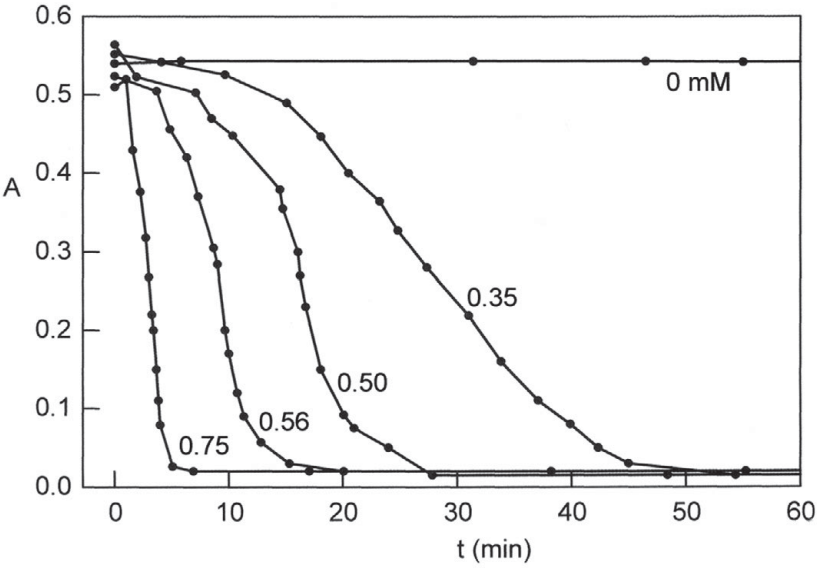

Fig. 2. Influence of chlorpromazine concentration on the dependence of human erythrocyte suspension absorbance (A) on incubation time (t). Incubation medium: 160 mM KCl, 37°C. Chlorpromazine concentrations are given in mM in the figure

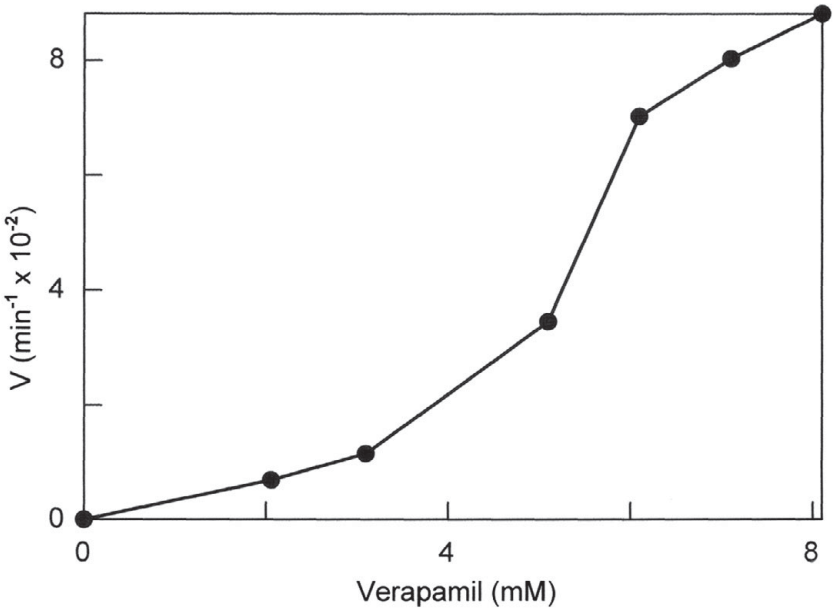

Fig. 3. Influence of verapamil concentration on the rate of human erythrocyte hemolysis (V). Incubation medium: 160 mM KCl, 37°C

of about 6 mM verapamil. At higher concentrations, the slope of the curve gradually decreases. This decrease may be an indication of saturation of the membranes with verapamil. Interpolation of the concentration-rate curves, such as the one in Fig. 3, enables evaluation of the resistance of the erythrocytes to verapamil and chlorpromazine (C_{50}). The resistance was calculated as the concentration that induces lysis of 50 % erythrocytes during 30 min (Table 1). The hemolytic activity of verapamil to human and pig erythrocytes does not differ significantly. It is much lower than the hemolytic activity of chlorpromazine.

There are relatively small differences in the hemolytic activity of verapamil in the media of various chemical compositions. With regard to high erythrocyte resistance to verapamil, we estimate the influence of the medium chemical composition on verapamil hemolytic activity in the experiments with constant verapamil concentration and various concentrations of the solutions tested. The isotonicity of the media was kept constant by mixing appropriate volumes of their isoosmotic solutions with 160 mM KCl. The constant 5 mM verapamil was chosen as close to the C_{50} value. The kinetics of hemolysis induced by this concentration of verapamil in increasing concentrations of $CaCl_2$ in the incubation medium is presented in Fig. 4. An increase in the time of hemolysis is observed when Ca^{2+}, concentration is increased. At 0 mM Ca^{2+} the complete hemolysis is finished after about 50 min. When the concentration of Ca^{2+} equals to 11 mM, the time of hemolysis increases to about 100 min. At higher

Table 1. Resistance (C_{50}) of human and pig erythrocytes to verapamil, chlorpromazine and amphotericin B in 160 mM KCl

Erythrocytes	Verapamil C_{50} (mM)	Chlorpromazine C_{50} (mM)	Amphotericin B C_{50} (mM)
Human	4.785 ±0.664 (10)	0.277 ±0.062 (8)	0.00198 ±0.000318 (10)[a]
Pig	4.417 ±0.461 (7)	-	0.00090 ±0.0010 (11)[b]

C_{50} values are means ±SD calculated from (*N*) measurements.
[a]Results from Knopik-Skrocka & Bielawski (2005).
[b]Results from Knopik-Skrocka et al. (2003).
For human erythrocytes, pair-wise differences between the 3 agents are significant at $P < 0.001$. Differences between human and pig erythrocytes are not significant for verapamil but significant at $P < 0.01$ for amphotericin B.

concentrations of Ca^{2+}, there is a slower drop in absorbance, and the hemolysis is incomplete in 200 min.

No evident increase in absorbance of the erythrocyte suspension is observed before the hemolysis induced by verapamil. Similar results to those in $CaCl_2$ were obtained in the other media tested. Fig. 5 presents the dependence of the rate of hemolysis induced by 5mM verapamil on $CaCl_2$, $MgCl_2$ and K_2SO_4 concentration in the incubation medium. The decrease in the rate of hemolysis is beginning with the low concentrations of Ca^{2+}, Mg^{2+} and SO_4^{2-}. The main difference between the action of these ions consists in the final decrease in the rate of hemolysis. The ratios of the rate of hemolysis induced by verapamil in the solutions tested to that in KCl are presented in Table 2. Divalent cations are slightly more effective than SO_4^{2-} in decreasing of verapamil activity.

Table 2. Effect of replacing KCl in the incubation medium with isoosmotic solutions of several salts and sucrose on the rate of human erythrocyte hemolysis induced by 5.0 mM verapamil

Incubation medium	V/V_{KCl}*
107 mM $CaCl_2$	0.27
107 mM $MgCl_2$	0.23
107 mM K_2SO_4	0.65
300 mM sucrose	1.32

*Ratio of the rate of hemolysis induced by verapamil in the given medium to that in KCl. The values are means of 2 measurements

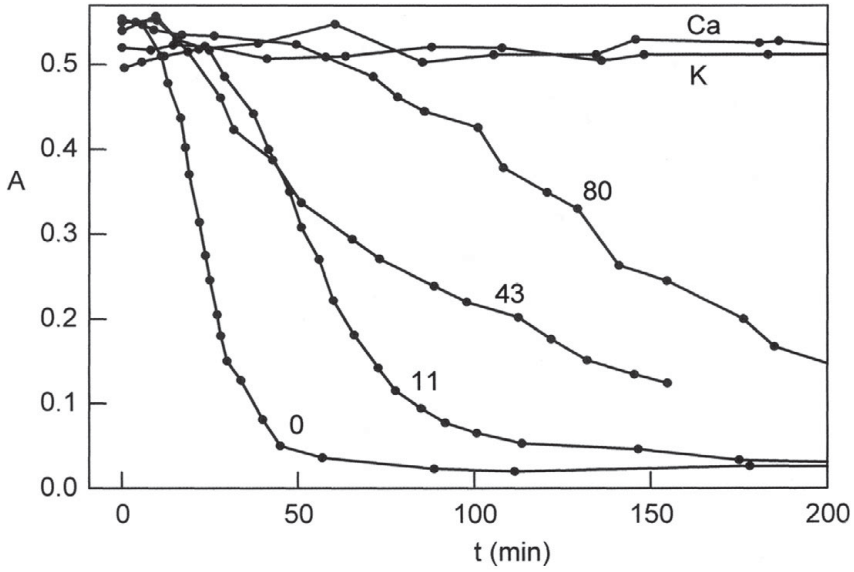

Fig. 4. Effect of $CaCl_2$ concentration in the incubation medium on the dependence of human erythrocyte suspension absorbance (*A*) on incubation time (*t*). Verapamil concentration 5 mM, 37°C. Concentrations of $CaCl_2$ are given in mM in the figure. K = 160 mM KCl without verapamil. Ca = 107 mM $CaCl_2$ without verapamil

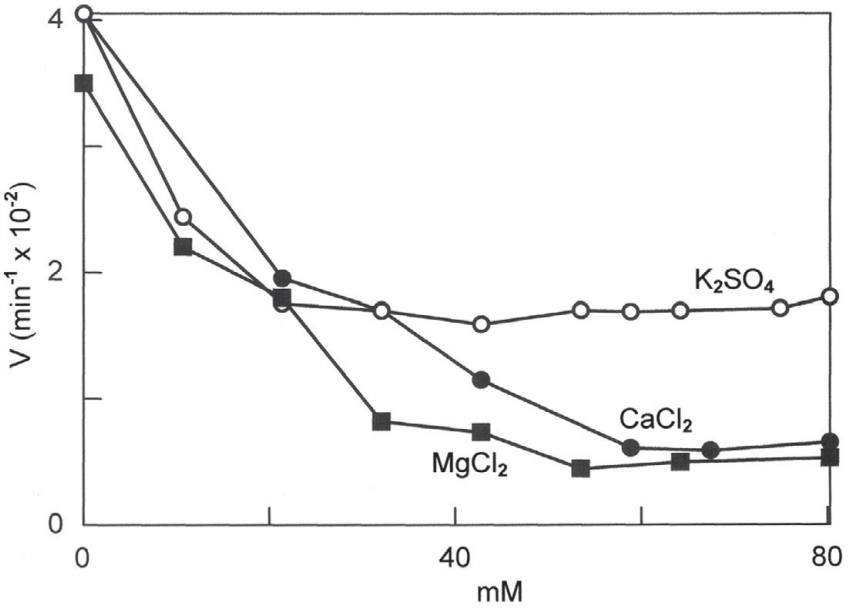

Fig. 5. Effect of $CaCl_2$, $MgCl_2$ and K_2SO_4 concentration in the incubation medium on the rate of hemolysis (*V*) induced by 5 mM verapamil, 37°C

The changes in hemolytic activity of chlorpromazine caused by modifications of the chemical composition of the incubation medium are even less evident than those obtained for verapamil. Like in the standard incubation medium, the C_{50} was calculated in all other media tested from interpolation of concentration-rate curves. The final results are presented as the ratios of C_{50} in 107 mM $CaCl_2$, $MgCl_2$, K_2SO_4 and in 300 mM sucrose to the C_{50} in KCl (Table 3). Only a very small increase in the resistance of the erythrocytes to chlorpromazine is observed in the presence of 107 mM $CaCl_2$, $MgCl_2$ and K_2SO_4. In 300 mM sucrose, the resistance of erythrocytes is lower than in the standard medium.

Table 3. Effect of replacing KCl in the incubation medium with isoosmotic solutions of several salts and sucrose on the resistance of human erythrocytes to chlorpromazine

Incubation medium	C_{50}/C_{50KCl}*
107 mM $CaCl_2$	1.28
107 mM $MgCl_2$	1.46
107 mM K_2SO_4	1.18
300 mM sucrose	0.61

*Ratio of the resistance (C_{50}) in the given medium to that in KCl. The values are means of 2 measurements.

The influence of combined action of verapamil with polyene antibiotic amphotericin B in 160 mM KCl is presented as a competition plot (Fig.6). The reference concentrations of the hemolytic agent verapamil (a_0) and of amphotericin B (b_0) were chosen in such a way that the rate of hemolysis at a_0 and b_0 were close to those at their C_{50} values. Next, the verapamil solution at concentration a_0 was mixed with amphotericin B solution at concentration b_0 in various proportions (p) and the rate of hemolysis in these solutions was measured. In Fig. 6, the coordinate x presents the values of p in the range from 0 to 1. At $p = 0$, verapamil is alone, while at $p = 1$, amphotericin B is alone. At the other p values, the concentrations a and b are varied together in such a way that $a = (1-p)a_0$ and $b = pb_0$. On the coordinate y, the rate of hemolysis (V) is presented. The rate of hemolysis increases gradually with p value increase to $p = 0.5$. In this proportion, the rate of hemolysis is about 10 times higher than the rate of hemolysis at $p = 0$ (verapamil alone) and $p = 1$ (amphotericin B alone). At p values higher than $p = 0.5$, the rate of hemolysis is diminished. The results indicate strong synergy between verapamil and amphotericin B.

Similar results were obtained with chlorpromazine and amphotericin B (Fig. 7). The highest synergistic effect between chlorpromazine and the polyene is observed at $p = 0.5$. The rate of hemolysis is about 14 times higher then, the rate of hemolysis induced by these agents applied singly. An opposite effect on the rate of hemolysis was found for a combination of verapamil and chlorpromazine (Fig. 8). The lowest rate was obtained at $p = 0.5$. The rate of hemolysis at $p = 0$ and $p = 1$ was about 2.5 times higher than at $p = 0.5$.

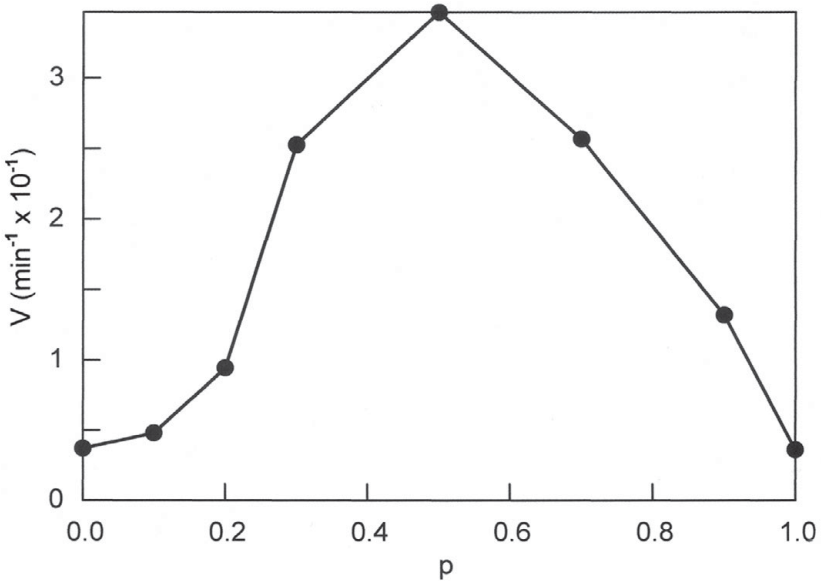

Fig. 6. Influence of combined action of verapamil with amphotericin B on the dependence of the rate of human erythrocyte hemolysis (V) on p (proportion of amphotericin B in the mixture). The reference concentration a_0 (verapamil) was 4.5 mM. The reference concentration b_0 (amphotericin B) was 2.0 μM. The concentrations were chosen so that the effect of verapamil and the polyene, applied singly, was equal to C_{50}. Incubation medium: 160 mM KCl, 37°C

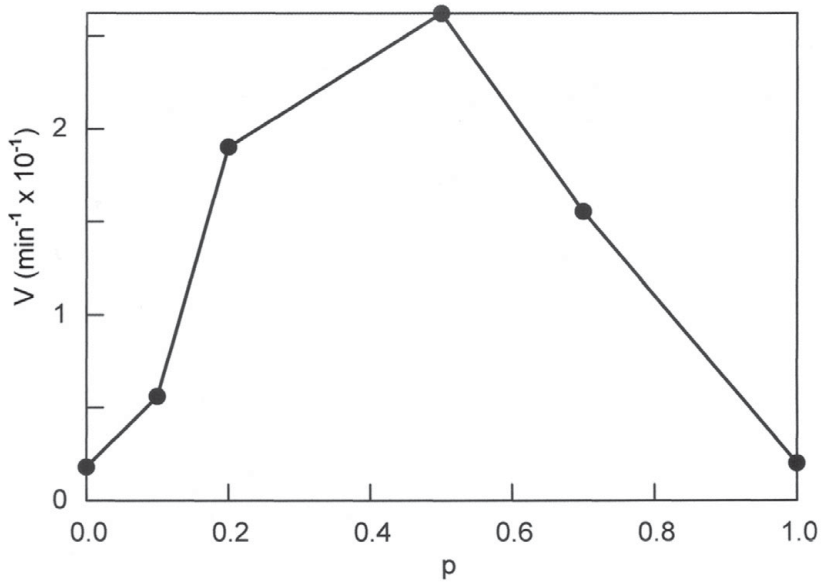

Fig. 7. Influence of combined action of chlorpromazine with amphotericin B on the dependence of the rate of human erythrocyte hemolysis (V) on p (proportion of amphotericin B in the mixture). The reference concentration a_0 (chlorpromazine) was 0.20 mM. The reference concentration b_0 (amphotericin B) was 2.9 μM. Other explanations as in Fig. 6

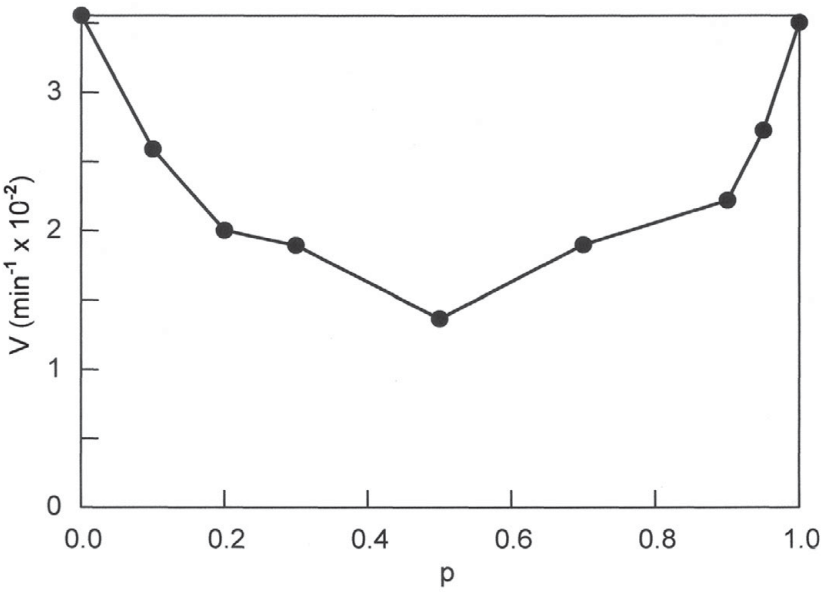

Fig. 8. Influence of combined action of verapamil with chlorpromazine on the dependence of the rate of human erythrocyte hemolysis (V) on p (proportion of chlorpromazine in the mixture). The reference concentration a_0 (verapamil) was 6.0 mM. The reference concentration b_0 (chlorpromazine) was 0.30 mM. Other explanations as in Fig. 6

DISCUSSION

In the present work, we investigated the hemolysis caused by the 2 amphipathic agents (verapamil and chlorpromazine) in various incubation conditions. Hemolytic concentrations of verapamil and chlorpromazine, used in the present paper, are similar to those reported earlier (Khasnobis et al. 1982; Lieber et al. 1984; Hendrich et al. 2002; Watts & Handy 2007; Suwalsky et al. 2008, 2010). They are higher than the therapeutic and toxic concentrations of each of the agents alone. Therapeutic plasma concentrations of chlorpromazine range from 0.1 to 1 μM (Kolakowska et al. 1976), or up to 5 μM (Dorson et al. 1988). Toxic effects of the drug may appear by its plasma concentration equivalent to approximately 3 μM (Rivera-Calimlim et al. 1973). The range of therapeutic concentrations of verapamil at its typical dose during cardiac care is about 4 μM. However, overdosing would result in circulating verapamil concentration of around 325 μM (Watts & Handy 2007). Its toxicity appears above approximately 2 μM verapamil in plasma (Musshoff et al. 2004). Such low concentrations of verapamil are not able to induce hemolysis. It was found that 5 μM verapamil only altered the shape of erythrocytes inducing stomatocytosis, while hemolysis is caused by 5 mM verapamil (Suwalsky et al. 2010). The last result is in agreement with our mean verapamil C_{50} value.

The resistance of erythrocytes to verapamil is evidently higher than their resistance to chlorpromazine. The difference is most probably a consequence of higher chlorpromazine lipophilicity (Pyka et al. 2006). Both the agents are much less effective towards erythrocytes than the polyene antibiotic amphotericin B, studied earlier (Knopik-Skrocka & Bielawski 2005; Knopik-Skrocka et al. 2006). The polyene is hemolytically active only in the self-associated form (Bolard et al. 1991). Present results compared to critical micellar concentrations of chlorpromazine and verapamil (Aanismaa & Seelig 2007) indicate that monomer forms of these agents are able to induce hemolysis.

No evident difference was found in the resistance of human and pig erythrocytes to verapamil. Similar results were earlier described for chlorpromazine-induced hemolysis in erythrocytes of various species of mammals (Biery et al. 1978). In contrast, our previous studies with amphotericin B have revealed a high dependence of the polyene hemolytic activity on species-specific properties of the mammalian erythrocyte membrane (Knopik-Skrocka et al. 2003; Knopik-Skrocka & Buldańczyk 2004). Hence, it can be assumed that the hemolytic activity of verapamil and chlorpromazine, compared to amphotericin B, is much less sensitive to the species-specific differences in the molecular organization or composition of the mammalian erythrocyte membranes.

The kinetics of hemolysis induced by verapamil is very similar to that caused by chlorpromazine and can be classified as the permeability type of hemolysis (Bielawski 1990). The process corresponds to that caused by amphotericin B (Knopik-Skrocka & Bielawski 2005). However, the rate of hemolysis induced by the polyene is greatly decreased in the presence of divalent cations, anions and disaccharides. The hemolysis caused by the polyenes is preceded by a high increase in erythrocyte suspension absorbance (Knopik-Skrocka et al. 2003, 2007; Knopik-Skrocka & Buldańczyk 2004). This increase is an effect of the faster efflux of K^+, compared to the slower influx of divalent ions or disaccharides, and erythrocyte shrinkage. It has been interpreted that amphotericin B forms with membrane cholesterol highly selective channels (Knopik-Skrocka & Bielawski 2002; Knopik-Skrocka et al. 2003, 2007).

By measuring chlorpromazine-induced uptake of mono- and disaccharides, Lieber et al. (1984) concluded that chlorpromazine induces colloid osmotic hemolysis resulting not from the direct creation of large holes but from the opening of small holes, which lead to a selective increase in membrane permeability. According to the results of Katsu et al. (2007), chlorpromazine, similar to the polyene antibiotic filipin, forms in liposomes pores permeable simultaneously to K^+ and calcein. Their radius is higher than that of pores formed by amphotericin B (Katsu et al. 2007). From our previous kinetics studies, we concluded that filipin-induced hemolysis is of the damage type, which can be explained by formation of large perforations, permeable to substances of low molecular weight as well as macromolecules, including hemoglobin (Knopik-Skrocka & Bielawski 2002; Knopik-Skrocka et al. 2003).

The present results show a low sensitivity of the hemolytic activity of verapamil and chlorpromazine to changes in the chemical composition of the incubation medium. Among the media examined, $CaCl_2$ and $MgCl_2$ are distinguished by the highest

decrease in hemolytic activity of verapamil. Only a small increase in the resistance of erythrocytes to chlorpromazine is observed in the presence of divalent ions. In the sucrose medium, instead of the decrease, an increase in the hemolytic activity of chlorpromazine and verapamil is observed. We suggest that hemolysis induced by chlorpromazine as well as verapamil is an effect of the formation of pores with low selectivity. However, their diameter is not large enough to cause hemoglobin escape, typical for filipin action. The erythrocyte membranes treated with verapamil or chlorpromazine are more permeable to sucrose than to divalent cations. Divalent cations are known agents that interact with erythrocyte membranes and inhibit the activity of many pore-forming substances by screening of the membrane charges (Bashford et al. 1988). With regard to the protonated state of verapamil (Pohl et al. 1998), as well as chlorpromazine (Ahyayauch et al. 2003; Wiśniewska & Wolnicka-Glubisz 2004), at pH under and near the physiological value, the activity of both the amphipaths in the media of divalent cations is not modified by salt formation.

The permeabilizing action of verapamil is increased in the presence of other MDR modulators (Castaing et al. 2007). Combinations of chemical agents may exhibit a greater effect than that expected from the action of single agents (Berenbaum 1989). A useful method for assessing synergy or antagonism is the competition plot proposed by Castaing et al. (2007). This method was earlier described as a simple test of enzyme-catalyzed reactions (Chevillard et al. 1993; Cardenas 2001). In the present study, the competition plot was also used to investigate the changes in hemolytic activity of verapamil and chlorpromazine, acting in combination with amphotericin B. We found that the hemolytic activity of verapamil and chlorpromazine is markedly enhanced in the presence of the polyene. The results indicate strong synergy between verapamil and amphotericin B, as well as between chlorpromazine and the polyene.

Amphotericin B is able to inhibit the hemolytic action of the polyene antibiotic filipin (Brajtburg et al. 1980). The antagonism between these polyenes is interpreted as the effect of competition for cholesterol as a membrane target (Brajtburg et al. 1980). The increase in membrane cholesterol content decreases the binding of verapamil to the membrane lipid bilayer (Mason et al. 1992). Cholesterol alters the lipid bilayer hydrocarbon core structure in a manner that makes verapamil partitioning into the membrane less energetically favorable. Castaing et al. (2003a) show that cholesterol modifies the permeabilizing activity of verapamil and other MDR modulators. This effect is dependent on cholesterol proportion in the membrane. The interaction of chlorpromazine with lipid membranes is also reduced by the presence of cholesterol (Luxnat & Galla 1986; Takegami et al. 1999; Wiśniewska & Wolnicka-Glubisz 2004; Wolnicka-Glubisz et al. 2009). This effect was explained by the rigidifying effect of cholesterol and a decrease in amphipath partition coefficient.

One possible explanation of the potentiation of hemolytic activity of verapamil and chlorpromazine in combination with amphotericin B, may be associated with changes in the molecular organization of membrane cholesterol and increased accessibility of anionic phospholipids to verapamil or chlorpromazine. The increase in hemolytic activity of the polyene acting with verapamil or chlorpromazine can be explained by facilitation of the amphotericin-B–cholesterol interaction.

The results obtained for the combination of verapamil with chlorpromazine indicate antagonism between these agents. A competition for the anionic phospholipids as membrane targets may be a reasonable explanation of this effect.

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