Effects of ginseng-spikenard heart-nourishing capsule on inactivation of C-type Kv1.4 potassium channel

Zhi-Quan Wang[1]*, Xue-Jun Jiang[2], Guang-Yu Zhang[1], Ming Chen[1], Chuan-Feng Tong[1] and Dong Zhang[1]

[1]Department of Cardiology, Zhongnan Hospital, Wuhan University, Wuhan, Hubei Province, China
[2]Department of Cardiology, Renmin Hospital, Wuhan University, Wuhan, Hubei Province, China

Abstract: This research is to explore the effects of traditional Chinese medicine Ginseng-spikenard heart-nourishing capsule on the inactivation of c-type Kv1.4 channels (Kv1.4ΔN) in Xenopus laevis oocytes with two-electrode voltage-clamp technique. Defolliculated oocytes (stage V-VI) were injected with transcribed cRNAs of ferret Kv1.4ΔN channels. During recording, oocytes were continuously perfused with ND96 solution (control group) and solution prepared from Ginseng-spikenard heart-nourishing capsule (experimental group). Results found that, at the command potential of +50 mV, the current of experimental group was reduced to 48.33±4.0% of that in control group. The inactivation time constants in control and experimental groups were 2962.56±175.35 ms and 304.13±36.22ms, respectively (P<0.05, n= 7). The recovery time of fKv1.4ΔN channel after inactivation in control group and experimental groups was 987±68.39 ms and 1734.15±98.45 ms, respectively (P<0.05, n=5). Ginseng-spikenard heart-nourishing capsule can inhibit the Kv1.4ΔN channel, which may be one of the mechanisms of underlying antiarrhythmia.

Keywords: Ginseng-spikenard heart-nourishing capsule, potassium channel, inactivation; mechanism.

INTRODUCTION

Shensongyangxin Capsule, an activating collateral drug, is compound preparation made from 12 kinds of traditional Chinese medicine, including ginseng, radix ophiopogonis, spikenard, *Taxillus chinensis, Fructus corni, Rhizoma coptidis, Semen zizyphi* spinosae, fossil fragments, the root of red-rooted salvia, radix paeoniae rubra, *Schisandra chinensis* and ground beeltle, among which spikenard, *Taxillus chinensis* and *Fructus corni* have anti-arrhythmic effect. Animal model has discovered that Shensongyangxin Capsule obviously inhibits drug-induced arrhythmia and cures ischemia-reperfusion-induced arrhythmia (Wu, 2004). At the same time, randomized double-blind clinical trial has demonstrated Shensongyangxin Capsule has a significant efficacy on cardiac arrhythmia in coronary heart disease (Wu *et al*., 2007; Li *et al*., 2007). To further explore its mechanism underlying antiarrhythmia, recently it has reported that Shensongyangxin Capsule markedly inhibits cardiac transient outward potassium current (Ito) in ion channel level. As for Ito research, potassium channel Kv1.4 is always studied and discussed because it is the main component of the repolarization current Ito in repolarization process of action potential (Chen *et al*., 2013; Fan *et al*., 2013). Our research utilized oocytes and N-terminal truncated K14 (Kv1.4ΔN) and employed two-electrode voltage-clamp technique to investigate the effects of Shensongyangxin Capsule on inactivation and reactivation of c-type Kv1.4 channel. Our study facilitated the understanding of the effects of Shensongyangxin Capsule on electrical activities in myocardial tissue, as well as the pharmacology and toxicology effects, which may provide more sufficient and reliable experimental bases for clinical application.

Corresponding author: e-mail: zhiquanwangcn@126.com

MATERIALS AND METHODS

Materials

Kv1.4ΔN channel was obtained from ferret Kv1.4 channel in which 2-146 bases were truncated from N-terminal (fKv1.4ΔN, provided by Laboratory of cardiology in People's Hospital of Wuhan University). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University.

Digestion and transcription of fKv1.4ΔN plasmid

The plasmid DNA was linearized by restriction endonuclease *Xho* I (New England Biolabs Inc., MA, USA). Then *in vitro* transcription was performed using T3 kit (Ambition Company, NY, USA) (Rasmusson *et al*., 1995; Madeja *et al*., 1991; Fan *et al*., 2012). The cRNA products from transcription were diluted to 1g/l and kept under -70ºC for reservation.

Xenopus oocytes isolation and micro injection (Li et al., 2003; Iwashita et al., 2013)

Tricaine with the concentration of 1.5g/l was used to narcotize the frogs (female Xenopus, 2 years old, purchased from the Experimental Animal Center of

Genetics and Developmental Research Institute, Chinese Academy of Sciences, Beijing, China) for 15-20min. When the frog does not respond to mechanical stimuli any more, it is placed on ice for surgery. A 0.5-1.0cm long longitudinal incision is made into the skin at lower abdomen, from which about $1cm^3$ oocyte tissue was isolated and put into OR_2 solution (82.5mM NaCl, 2mM KCl, 1mM $MgCl_2$ and 5mM HEPES, the pH was adjusted to 7.4 using NaOH). solution with 1.5g/l collagenase I (Sigma-Aldrich Inc., MO, USA) to perform shaking digestion for two hours after being washed in OR_2 solution (82.5mM NaCl, 2mM KCl, 1mM $MgCl_2$ and 5 mM HEPES, the pH was adjusted to 7.4 using NaOH). After digestion, the oocytes were washed in OR_2 solution repeatedly and the ones from grading IV-V were selected for further injection. Nanoliter 2000 micro injector (WPI Inc., MA, USA) was applied to inject 27-34 nl cRNA into each oocyte. The oocytes were incubated in Barth's solution (88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 0.82 mM $MgSO_4$, 1.5mM $CaCl_2$ and 5mM Hepes, pH=7.4) with 100 u/ml penicillin in 96-well plate at 18ºC.

Current recording using two-electrode clamping technique

The oocytes could be used for two-electrode clamping experiment after 24-72 hours incubation. The instruments adopted mechanical 3D micromanipulator (M3301L and M3301R), using Digidata 1322A A/D converter (Axon Inc., CT, USA), CA-1B dual-electrode clamp amplifier (Dagan Corp., MN, USA) and PP-83 electrode puller (Narishige Inc., Tokyo, Japan). The electrode filling solution was 3M KCl, and the resistance of electrode V_1 (clamping electrode) was 0.5-1 MΩ, V_i (recording electorde) was 1-2 MΩ. The content of control solution ND96 was: 96mM NaCl, 2mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$ and 10mM HEPES and the pH was adjusted to 7.4 using NaOH. The perfusion speed of extra cellular solution was 3-5 ml/min and the washing out time of medicine perfusion was 10 min. The data was collected and recorded by PCLAMP 9.0 software (Axon Inc., CT, USA). Shensongyangxin solution preparation: Shensongyangxin powder from capsule was dissolved into ND96 solution as following mass concentrations, 0.1, 0.5, 1.5 and 10g/l and the pH of each solution was adjusted to 7.4. After centrifuge, the supernatant of each solution was isolated for further experiment. The perfusion was performed using RC-11 perfusion groove (Warner Inc., NY, USA), using ND96 solution and Shensongyangxin solution in control group and experimental group, respectively.

STATISTICAL ANALYSIS

Experimental data was analyzed by Clamp fit 9.0 software. The inactivation time constant was fitted by exponential formula (τ=Aexp (-v/x) +τ_0) using fitting program within Clampfit9.0. The statistical analysis was performed using statistical software SPSS 11.5. The data was presented as x ±s. The comparison between the data in two groups was performed by t-test where the mean values of two sampling groups were analyzed; $P<0.05$ indicated statistical difference. Standard current was adopted in all the statistics of current values mentioned in this research.

RESULTS

Shensongyangxin concentration-dependent curve of fKv1.4ΔN

The effects of Shensongyangxin at different perfusion concentrations on fKv1.4ΔN channel were shown in fig. 1. Holding potential was -80mV, from which depolarization was performed till +50mV within 5 s. The concentrations of Shensongyangxin, 0.1, 0.5, 1, 5, 10g/l (n=5), were plotted on x-axis and channel blocking (%) was plotted on y-axis, which was present as percentage of current peak after drug application (experimental group) and current peak before drug application (control group). The graph displayed that the inhibition of fKv1.4ΔN channel by current gradually enhanced and the channel blocking strengthened along with the increasing reperfusion concentration of Shensongyangxin.

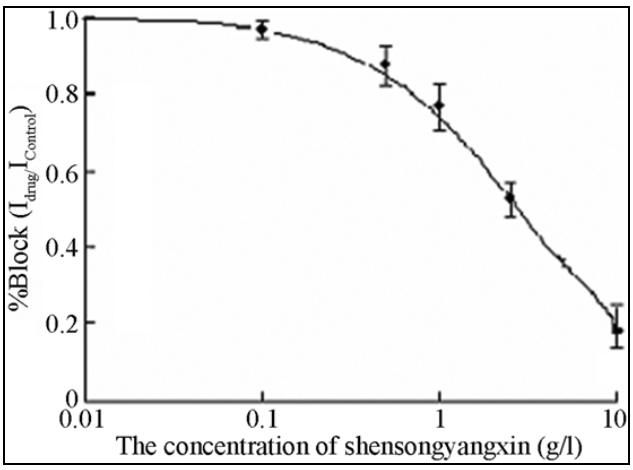

Fig. 1: Shensongyangxin concentration-dependent curve of fKv1.4ΔN. Holding potential was -80 mV, from which depolarization was performed till +50 mV within 5s. The concentrations of Shensongyangxin, 0.1, 0.5, 1, 5, 10g/L (n=5), were plotted on x-axis and channel blocking (%) was plotted on y-axis. IC50 of Shensongyangxin was 5 g/l.

Curve fitting employed formula as follows: f=Kd/(Kd+D), among which f, D and Kd represented fractional current, drug concentration and apparent dissociation constant or the half maximal inhibitory concentration (IC50), respectively. IC50 refers to the concentration of a drug that is required for 50% current inhibition, which reflects binding ability of drug to channel, i.e. the smaller the IC50, the higher the affinity

of drug to channel. IC50 of Shensongyangxin was 5g/l. In the following experiments, the concentration of 5g/l was used to study the effects of Shensongyangxin on fKv1.4ΔN channel.

Comparison of current curve between control and experimental groups and voltage-dependent curve

The current curve of fKv1.4ΔN channel was characterized by rapid activation and slow inactivation in fig. 2A; The current curve after reperfusion with 5g/l Shensongyangxin was shown in fig. 2B; the relationship of current and voltage was plotted in fig. 2C, in which outward current was noticed at command potential of -40 mV and current peaked at command potential of +50 mV. Current was significantly inhibited by the perfusion of 5g/l Shensongyangxin and elevated in a liner pattern along with the increase of stimulus voltage, which showed a typical voltage-dependent characteristic (n=5). When the command potential was +50 mV, the current reduced to (48.33±4.0)% of that in control group (Holding potential was -80 mV; command potential changed from -80 mV to +50 mV in 5 s with 10 mV interval; the recovery time was 20 s) (fig. 2).

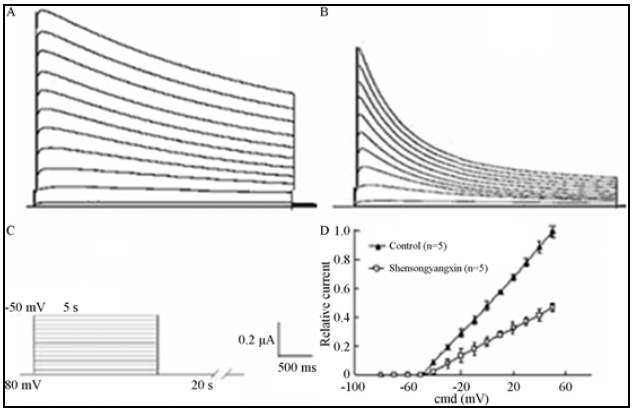

Fig. 2: fKv1.4ΔN current-voltage curve. (A) Current curve of fKv1.4ΔN (original record). (B) current curve after perfusion with 5g/l Shensongyangxin (original record). (C) Electrical stimulation program: Holding potential was -80 mV; command potential changed from -80 mV to +50mV in 5 s with 10 mV interval; the recovery time was 20 s. (D) Current- voltage curve in two groups: Current was significantly inhibited by Shensongyangxin perfusion and elevated in a liner pattern along with the increase of stimulus voltage, which showed a typical voltage-dependent characteristic.

Effects of shensongyangxin capsule on steady-state inactivation curve of fKv1.4ΔN channel

Holding potential was -80mV; depolarization was performed from -100mV to +50mV in 5 s with interval of 10mV in P1, followed by P2, in which stimulation with +50mV for 1 s was performed to induce tail current. fig. 3A and fig. 3B showed the current change of fKv1.4ΔN channel due to steady-state inactivation affected by Shensongyangxin; steady-state inactivation curve of Shensongyangxin was left shifted in experimental group compared with that of control group in fig. 3C. In addition, when the command potential was +50 mV, the inactivation time constants were (2962.56±175.35) ms and (304.13±36.22) ms in control and experimental groups ($P<0.05$, n=7).

Effects of shensongyangxin capsule on recovery of fKv1.4ΔN current after inactivation

Fig. 4 showed effects of Shensongyangxin perfusion on recovery after inactivation of fKv1.4ΔNchannel. The results indicated that Shensongyangxin perfusion slowed down the recovery of fKv1.4ΔN channel ($P<0.05$, n=5). The recovery time was (987±68.39) ms in control group and (1734.15±98.45) ms in experimental group ($P<0.05$, n=5). The condition was as follows: Holding potential was -90mV and P1 was +50 mV with duration of 5 s, followed by -120mV, deactivation potential with variable stimulation time (intervals were 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0 and 20.0 s); afterwards stimulation with +50 mV for 1 s was performed to induce tail current. The stimulation interval between every sweep program was 30 s to fully recover the channel.

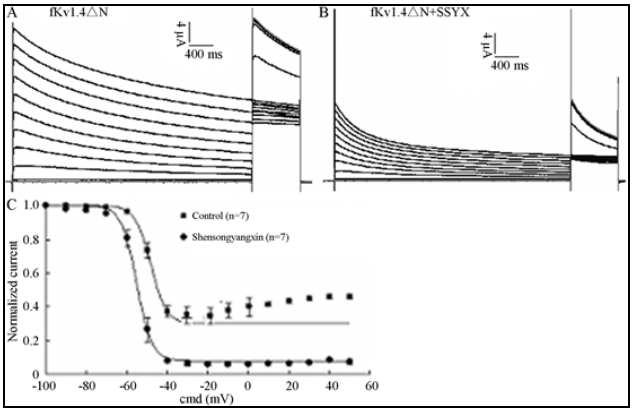

Fig. 3: (A) and (B) showed the current change of fKv1.4ΔN channel due to steady-state inactivation affected by Shensongyangxin. Holding potential was -80 mV; depolarization was performed from -100mV to +50 mV in 5 s with interval of 10mV in P1, followed by P2, in which stimulation with +50mV for 1 s was performed to induce tail current. (C) Effects of Shensongyangxin Capsule on steady-state inactivation curve of fKv1.4ΔN channel. Steady-state inactivation curve of Shensongyangxin was left shifted in experimental group compared with that of control group. The inactivation of c-type Kv1.4 channel accelerated.

DISCUSSION

Potassium channel Kv1.4 is widely distributed in mammals, which excites organs (such as heart and brain). The inactivation of potassium channel Kv1.4 includes inactivation of C-type and N-type Kv1.4 channels (Labro

et al., 2008). N-type inactivation inKv1.4 channels operates by a ball-and-chain mechanism, while C-type inactivation in Kv1.4 channels is induced by comformational change of S5, S6 and P-loop, which comprise external pore of the channel (Sahoo *et al*., 2013). C-type inactivation in Kv1.4 channels might result from the displacement of sulfhydryl and other groups near the lateral region of channel when the channels open, the changes of voltage sensor when the depolarization of voltage exceeded the threshold value and space structure changes of S6 and other peptide fragments (Bett *et al*., 2012; Herrera *et al*., 2005; Snyders, 1999).

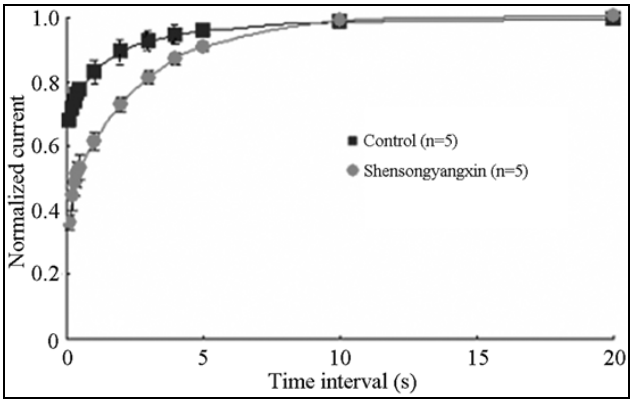

Fig. 4: Effects of Shensongyangxin perfusion on recovery after inactivation of fKv1.4ΔN channel: Holding potential was -90 mV and P1 was +50mV with duration of 5 s, followed by -120 mV, deactivation potential with variable stimulation time (time intervals were 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0 and 20.0 s); afterwards stimulation with +50 mV for 1 s was performed to induce tail current. Shensongyangxin perfusion slowed down the recovery of fKv1.4ΔN channel.

In our experiments, Shensongyangxin Capsule significantly inhibited peak current of fKv1.4ΔN channel, shifted I-V curve down and steady-state inactivation curve left, accelerated the inactivation of channel and slowed down the recovery of channel from inactivation. All of these could lead to 1-stage polarization change in action potential and APD (action potential duration) extension of subendocardially cardiac myocytes (Hoppe and Beuckelmann, 1998), which might be one of the underlying mechanisms for Shensongyangxin Capsule to resist arrhythmia. At the same time, inhibiting peak current of fKv1.4ΔN channel contributed to the reduction of cardiac Ito. Ito was considered as major current that caused the repolarization heterogeneity across myocardial wall, thus inhibition of Ito decreased the repolarization heterogeneity across myocardial wall, reduced the formation of micro-reentrant loop, inhibited phase 2 reentry and avoided occurance of arrhythmia such as torsades de pointes etc. (Li *et al*., 2012). Therefore, Shensongyangxin Capsule significantly inhibited arrhythmia.

C-type inactivation inKv1.4 channels is essentially the conformational change of channel protein, which results in closing external pore of channel and blocking outflow of potassium ion. Binding of Shensongyangxin to fKv1.4ΔN channel changes conformation of channel protein (including S5, S6 and p-loop), and inactivates C-type Kv1.4 channels (Labro *et al*., 2008; Imbrici *et al*., 2011). Our research displayed that I-V curve did not shift with consistent channel activation potential of -40 mV after drug application; the activation part on the curve showed a liner relationship either before or after drug application, which demonstrated that the drug neither had effect on activation of channels nor changed rectification properties of channels. The activation of channel actually correlated with movement of S4 voltage sensor. When the cell membrane was applied with depolarization potential, positively charged amino acids on S4 shifted in electric field, which made S4 transmembrane segments move in a spiral path, led to opening of 'activation gate' and generate current by outflow of potassium ions. We speculated that the effect of Shensongyangxin on fKv1.4ΔN channel had no relation with S4 due to Shensongyangxin did not change kinetic characteristics of activation.

Our study explored the effects of Shensongyangxin Capsule on Kv1.4ΔN channel with two-electrode voltage-clamp technique. Other research (Li *et al*., 2007) has reported that Shensongyangxin Capsule not only has effects on potassium channel, but also has retardation on sodium and calcium channels, which inhibits arrhythmia through many ways, links and targets. Therefore, it is of importance to investigate its mechanisms in ion channel level for discovering effective antiarrhythmic drugs with mild side effects.

REFERENCES

Bett GC, Lis A, Guo H, Liu M, Zhou Q and Rasmusson RL (2012). Interaction of the S6 proline hinge with N-type and C-type inactivation in Kv1.4 channels. *Biophys. J.,* **103**: 1440-1450.

Chen H, Zhang D, Chao SP, Ren JH, Xu L, Jiang XJ and Wang SM (2013). Comparison of the effects of antiarrhythmic drugs flecainide and verapamil on fKv1.4ΔN channel currents in Xenopus oocytes. *Acta. Pharmacol. Sin.,* **34**: 221-230.

Fan Z, Ji X, Fu M, Zhang W, Zhang D and Xiao Z (2012). Electrostatic interaction between inactivation ball and T1-S1 linker region of Kv1.4 channel. *Biochim. Biophys. Acta.,* **1818**: 55-63.

Fan Z, Zhang Z, Fu M, Qi Z and Xiao Z (2013). Effect of inserting charged peptide at NH (2)-terminal on N-type inactivation of Kv1.4 channel. *Biochim. Biophys. Acta.,* **1828**: 990-996.

Herrera D, Mamarbachi A, Simoes M, Parent L, Sauvé R, Wang Z and Nattel S (2005). A single residue in the S6

transmembrane domain governs the differential flecainide sensitivity of voltage-gated potassium channels. *Mol. Pharmacol.,* **68**: 305-316.

Hoppe UC and Beuckelmann DJ (1998). Modulation of the hyperpolarizationactivated inward current (If) by anti arrhythmic agents in isolated human atrial myocytes. *Nauny. Schmiedebergs Arch. Pharmacol.,* **358**: 635-640.

Imbrici P, D'Adamo MC, Grottesi A, Biscarini A and Pessia M (2011). Episodic ataxia type 1 mutations affect fast inactivation of K+ channels by a reduction in either subunit surface expression or affinity for inactivation domain. *Am. J. Physiol. Cell. Physiol.,* **300**: C1314-C1322.

Iwashita J, Kodama A, Konno Y, Abe T and Murata J (2013). Histone deacetylase induces accelerated maturation in Xenopus laevis oocytes. *Dev. Growth Differ.,* **55**: 319-329.

Labro AJ, Grottesi A, Sansom MS, Raes AL and Snyders DJ (2008). A Kv channel with an altered activation gate sequence displays both "fast" and "slow" activation kinetics. *Am. J. Physiol. Cell. Physiol.,* **294**: C1476-C1484.

Li N, Wu XF, Ma KJ, Wu YL and Pu JL (2007). The effect of Shensongyangxin capsule on potassium channel currents in isolated ventricular myocytes. *Chin. J. Difficult Complicated Cases.,* **6**: 133-137.

Li Q, Ma HJ, Song SL, Shi M, Ma HJ, Li DP and Zhang Y (2012). Effects of anandamide on potassium channels in rat ventricular myocytes: A suppression of I(to) and augmentation of K(ATP) channels. *Am. J. Physiol. Cell. Physiol.,* **302**: C924-C930.

Li X, Bett GC, Jiang X, Bondarenko VE, Morales MJ and Rasmusson RL (2003). Regulation of N-and C-type inactivation of Kv1.4 by pHo and K+: Evidence for transmembrane communication. *Am. J. Physiol. Heart Circ. Physiol.,* **284**: H71-H80.

Madeja M, Musshoff U and Speckmann EJ (1991). A concentration-clamp system allowing two-electrode voltage-clamp investigations in oocytes of Xenopus laevis. *J. Neurosci. Methods.,* **38**: 267-269.

Rasmusson RL, Morales MJ, Castellino RC, Zhang Y, Campbell DL and Strauss HC (1995). C-type inactivation controls recovery in a fast inactivation cardic K+ channel (Kv1.4) expressed in Xenopus oocytes. *J. Physiol.,* **489**: 709-721.

Sahoo N, Goradia N, Ohlenschläger O, Schönherr R, Friedrich M, Plass W, Kappl R, Hoshi T, Heinemann SH (2013). Heme impairs the ball-and-chain inactivation of potassium channels. *Proc. Natl. Acad. Sci. USA.,* **110**: E4036-E4044.

Snyders DJ (1999). Structure and function of cardiac potassium channels. *Cardiovasc. Res.,* **42**: 377-390.

Wu YL (2004). Luobing theory. Beijing: China Science and Technology Press, pp.280-283.

Wu YL, Gu CH, Gao XD, Xu GC and Yang LB (2007). The random double blind, multi-central clinical research on the therapeutic effect of Shensongyangxin capsule on ventricular premature beat in patients with coronary heart disease. *Chin. J. Difficult Complicated Cases,* **6**: 449-452.

Yoneto K, Li SK, Higuchi WI, Jiskoot W and Herron JN (1996). Fluorescence probe studies of the interactions of 1-alkyl-2-pyrrolidones with stratum corneum lipid liposomes. *J. Pharm. Sci.,* **85**: 511-517.