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K. Hill
C. B. Pénzes
B. G. Vértessy
Z. Szabadka
V. Grolmusz
É. Kiss

Amphiphilic Nature of New Antitubercular Drug Candidates and Their Interaction With Lipid Monolayer

K. Hill · C. B. Pénzes · É. Kiss (✉)
Laboratory of Interfaces and Nanostructures, Institute of Chemistry, Eötvös University, P.O. Box 32, 1518 Budapest 112, Hungary
e-mail: kissevak@ludens.elte.hu

B. G. Vértessy
Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary

Z. Szabadka · V. Grolmusz
Protein Information Technology Group, Institute of Mathematics, Eötvös University, Budapest, Hungary

Z. Szabadka · V. Grolmusz
Uratim Ltd., Nyíregyháza, Hungary

Abstract Tuberculosis remains a major problem throughout the world causing large number of deaths, more than that from any other single infectious disease [1]. The treatment of the chronic inflammatory caused by *Mycobacterium tuberculosis* (*Mtb*) requires prolonged chemotherapy often associated with unwanted side effects and developing resistant bacterium strains [2]. Introduction of new in silico identified drug candidates which are expected to be specific inhibitor of dUTPase [3] a vital enzyme of *Mtb* presents a novel approach in the combat with the disease. Three of those drug candidates – ligand 3, 4 and 69 – were compared in the present study considering their interfacial properties, polarity, amphipatic character and lipid affinity which are relevant in pharmaceutical function.

Langmuir monolayers were prepared from the ligands and their mixture with phospholipon as a simple model material of cell membrane. Analysis of the isotherms showed than ligand 3 and 44 presents significant affinity to the lipid building into the monolayer. The penetration ability of the drug candidates were also characterized by measuring the increase of surface pressure of the lipid monolayer following their injection to the subphase at two initial lipid densities. The results were in accordance with the order of log P_{app} values determined for the three compounds as well as with their dynamic surface activity although the highest difference amongst the three ligands was observed in the penetration ability which is of paramount importance in the selection of promising therapeutic agent.

Keywords Langmuir monolayer · Lipid affinity · Membrane model · Penetration of drug · Surface activity

Introduction

Pulmonary tuberculosis is a chronic respiratory transmitted inflammatory disease. One third of the human population of the world is infected. *Mycobacterium tuberculosis* (*Mtb*) causes 8 million new cases and nearly two million people die every year [4]. The problems and difficulties of the antitubercular therapy include the prolonged duration of treatment, the dose related drug toxicity and unwanted side effects like the damage of liver and kidney. The aim is to improve patient compliance and reduce harmful effects decreasing the duration of treatment. The emergence of drug resistant strains of *Mtb* has made the search for new drugs more urgent [3, 5, 6].

One of the new approaches to find more effective drugs is based on the inhibition of bacterial enzymes and hence killing the bacteria. A number of possible specific inhibitor of dUTPase, an enzyme of *Mtb* essential for cell viability [7] were identified by simulation methods [8, 9]. The selection, physico-chemical, biological, pharmaceutical characterization of these new drug candidates and

introduction of the best ones might be an effective means to treat tuberculosis infections in humans.

Drugs have to be transported through several membranes to reach the infected target cell therefore the interaction of drug with lipid membrane is in the focus of research. Among the membrane models the Langmuir monolayer of lipids is the most simplified but well defined system [10]. Although much less complex than biological membranes it is excellent model because various parameters like density, packing and nature of lipid as well as the subphase composition and temperature can been varied [11–13]. The monomolecular layer of oriented and close packed lipid molecules is prepared at water/air surface in a Langmuir balance. Quantitative information can be obtained on the influence of drugs on the stability, structure and permeability of lipid film as well as on drug's penetration behaviour. Affinity and compatibility of drug to the lipid membrane can be assessed analysing the area–surface pressure isotherms [11].

The monolayer technique became a widespread method in the last decades characterizing the molecular interaction between drug candidates and membrane forming lipids and other components [12, 14, 15]. Jablonowska and coworker studied the effect of the ibuprofen at various concentrations on dipalmitoyl-phosphatidylcholine, DPPC, monolayer [16]. The lipid layer was condensed at low concentration while higher amount of ibuprofen led to opposite effect perturbing the ordered structure of lipid monolayer which change is related to membrane function. Fa and coworkers obtained similar results with azithromycin which increased the fluidity and permeability of membrane layer [17]. The presence of cholesterol additive generally stabilizes the lipid layer hindering the drug penetration [18, 19]. The Langmuir technique applying functional monolayer components was used to reveal the mechanism of antifungal agent comparing toxic and less toxic derivatives [20].

The main component of lung surfactant mixture besides other important proteinous compounds is also a lipid, namely DPPC. Their role in inhibiting or improving the lung function was systematically investigated [21]. The understanding the mode of incorporation of drug into DPPC monolayer can help to design stable liposomes as drug carrying system [5, 22].

New drug candidates which are expected to be specific inhibitor of dUTPase [3, 23] a vital enzyme of *Mtb* were identified in silico [7, 8]. Three of those – ligand 3, 4 and 69 – were compared in the present study considering their interfacial properties, polarity, amphipatic character and lipid affinity which are relevant in pharmaceutical function. Langmuir monolayers were prepared from the ligands and their mixture with phospholipon as a simple model material of cell membrane. Interaction and compatibility with lipid is evaluated from the analysis of the isotherms. The penetration ability of the drug candidates were also characterized by measuring the increase of surface pressure of the lipid monolayer following their injection to the subphase at two initial lipid densities. The amphiphilicity of the three ligands were assessed using log P_{app} and dynamic surface activity values and related to the results of monolayer experiments.

Experimental

Materials

Phospholipon 100H containing 85 wt. % distearoyl-phosphatidylcholine DSPC and 15 wt. % dipalmitoyl-phosphatidylcholine DPPC, respectively was obtained from Nattermann GmbH (Germany).

The in silico identified selective inhibitor molecules of the dUTPase marked as ligand 3, 44 and 69 have chemical composition $C_{25}H_{38}N_4O$, $C_{25}H_{28}N_2O_5$ and $C_{15}H_{16}N_4O$, respectively. The important data of the three drug candidate compounds are given in Table 1.

Chloroform (purity > 99.8%) from Fisher Chemicals and methanol (purity ≥ 99.9%) from Sigma-Aldrich Kft. Hungary were used for preparing spreading solutions. Dichloromethane (purity ≥ 99.9%) from Spectrum-3D Kft. Hungary was used for cleaning the Langmuir trough. *n*-octanol from Reanal, Hungary, was used for log P_{app} determination. Double distilled water was checked by its conductivity (< 5 mS) and surface tension (> 72.0 mN/m at 23 ± 0.5 °C) values.

Methods

Determination of log P_{app}. The *n*-octanol/water partition coefficient (P) is the ratio of the concentration of the drug in *n*-octanol and water in equilibrium. The *n*-octanol and water in 1 : 1 volume ratio was shaken for 12 h to saturate both phases with each other. Then the drug was dissolved in the aqueous phase ($C_1 = 3 \times 10^{-5}$ M) and was shaken with equal volume of octanol phase for 1 h to achieve the partition equilibrium. Centrifugation was required to separate the two phases (2000 rpm, 10 min). After separation the absorbance of the aqueous phase was determined from

Table 1 Molecular data (molecular weight M, number of charges and molecular area A_m) of drug candidates from ZINC database [29] and the measured values: logarithm of apparent partition coefficient log P_{app} and static surface tension γ_{stat} (mN/m) of aqueous solution with concentration of 8×10^{-5} M

Drug candidates	M	Charges	A_m (A^2)	log P_{app}	γ_{stat} (mN/m)
Ligand 3	413.6	3	81.2	0.94 ± 0.05	70.0 ± 0.3
Ligand 44	438.5	2	156.2	0.89 ± 0.05	70.1 ± 0.5
Ligand 69	268.0	–	157.7	0.61 ± 0.05	71.6 ± 0.4

which the concentration was determined (C_2) using the calibration curve. Calibration curves were determined by absorbance at 227, 398 and 352 nm for ligand 3, 44 and 69, respectively. The drug concentration in octanol is $C_1 - C_2$.

$$\log P_{\text{app}} = \log([\text{drug}]_o/[\text{drug}]_{\text{w}}) = \log(C_1 - C_2)/C_2 \,,$$

where the $[\text{drug}]_o$ and $[\text{drug}]_{\text{w}}$ are the equilibrium concentrations of drug in octanol and water, respectively. Apparent partition coefficient P_{app} was obtained since pK values of the compounds were not taken into consideration for correction [24].

Static and Dynamic Surface Tensions. The surface tensions of drug solutions were determined with an accuracy of 0.1 mN/m by the axisymmetric drop shape analysis [25] using the OCA15+ instrument (Dataphysics, Germany). Drop of 10 µl of the aqueous solution of ligand 3, 44 and 69 with concentration of 8×10^{-5} M was formed on the tip of a Teflon coated capillary of a Hamilton syringe at a rate of 2 µl/s. Measuring adsorption at air/water surface the drop was immersed into a glass cuvette saturated with water vapour. All measurements were performed for each drug candidate as triplicate at 23 ± 0.1 °C. The surface tension values were reproduced with a scatter less then ±0.5 mN/m. The profile of capillary surface required to determine the surface tension is obtained by analysing the shape of the pendent drop using a CCD camera coupled to a video profile digitalizer board connected to a personal computer. Static surface tension was measured after 15 min. The instrument allows the programmed decrease or increase the volume/area of the pendent drop. After drop formation the volume of the drop was decreased slowly [26–28] with the rate of 0.1 µl/s, until the 50% of the initial area was reached. The surface tension response for the area decrease was recorded simultaneously with 120 frames/min frequency. From these data the dynamic surface tension as a function of surface area change was obtained.

Langmuir Film Experiments. The experiments were performed by using automated Langmuir balance ($18 \times 6 \times 0.6$ cm). The spread monolayer can be compressed by means of a movable barrier while the surface pressure and the area are continuously recorded. Surface pressure is measured tensiometrically with an accuracy of ±0.05 mN/m using a Wilhelmy plate made from chromatography paper (Whatman Chr1) connected to a force transducer. The surface pressure/area isotherm was recorded at a barrier speed of 10 cm^2/min at 23 ± 0.5 °C and there was no wait period between compression and expansion. For the monolayer studies the pure lipid and the pure drugs (0.1 g/l) as well as lipid–drug mixtures (5 : 1 molar ratio) were spread at water surface. The spreading solvent was 3 : 1 v/v chloroform/methanol mixture, 50 µl solution was applied dropwise by a Hamilton syringe to form the monolayer. Before compression the solvent was allowed to evaporate for 15 min. The compression/expansion isotherms were recorded five times consecutively. The trough was made of Teflon while the barrier from POM [14] and cleaned carefully with dichloromethane and bidistilled water.

In the first type of experiments isotherms of pure lipid and pure drug candidates then that of the two-component mixture monolayer were recorded. To get the mixed monolayer the components were premixed in the spreading solvent. The difference between the pure lipid and lipid–drug films were investigated to presume the incorporation of the drug into the lipid monolayer. In the second type of experiments penetration of drug into the lipid layer was detected by the change of surface pressure. As a first step pure lipid monolayer was formed and following one compression/expansion cycle the layer was compressed to a given value of surface pressure (15 and 20 mN/m). At that position the barrier was stopped and a fixed amount of aqueous solution of the drug was injected into the subphase to obtain a final drug concentration of 2×10^{-6} M. The change in surface pressure as the indicator of drug penetration was recorded as a function of time for one hour.

Results and Discussion

Amphiphilicity of Drug Candidates

The logarithm of *n*-octanol/water partition coefficient serves as a quantitative descriptor of lipophilicity (hydrophobicity) and is one of the key determinants of pharmacokinetic properties. Hydrophobicity affects drug adsorption, bioavailability, hydrophobic drug–receptor interactions, metabolism of molecules, as well as their toxicity. Partition coefficient is useful in estimating distribution of drugs within the body. Hydrophobic drugs with high partition coefficients are preferentially distributed to hydrophobic compartments such as lipid bilayers of cells while hydrophilic drugs (low partition coefficients) preferentially are found in hydrophilic compartments such as blood serum. The equilibrium organic and aqueous phases of *n*-octanol/water system contain 2.3 M of water and 4.5×10^{-8} M *n*-octanol, respectively. This is a widely accepted model for the estimation of distribution of a drug molecule to the cell membrane.

The solubility/amphiphilicity of the three drug candidates were characterized by partition experiment and determining their surface activity. These results and some other data of the ligands are summarized in Table 1. The log P_{app} values obtained indicated a medium hydrophobicity of the ligands. Considering the standard deviation of data ligand 44 and ligand 3 presented similar hydrophobicity while ligand 69 proved to be less hydrophobic although the difference is rather small.

Changing of water surface tension gives also information on the amphiphilic properties of dissolved drugs. If the molecules adsorb at air/water surface the surface

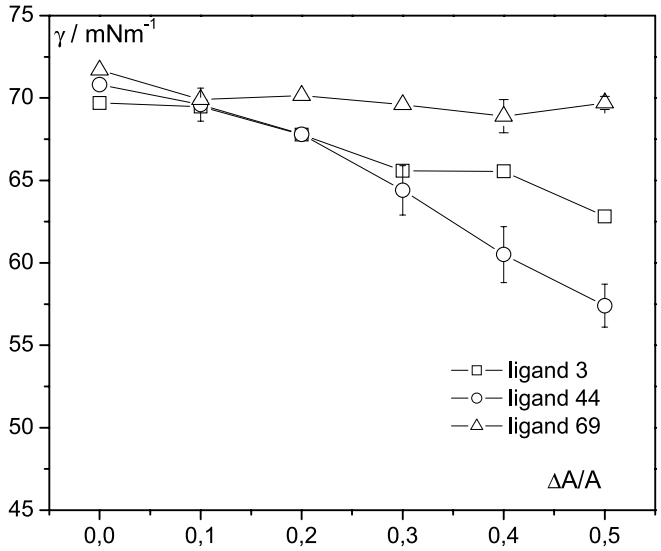

Fig. 1 Dynamic surface tension γ (mN/m) of aqueous solutions of ligand 3, 44 and 69 with a concentration of 8×10^{-5} M as a function of reduction of surface area $\Delta A/A$. (The scatter of surface tension values are shown when exceeding the size of symbol)

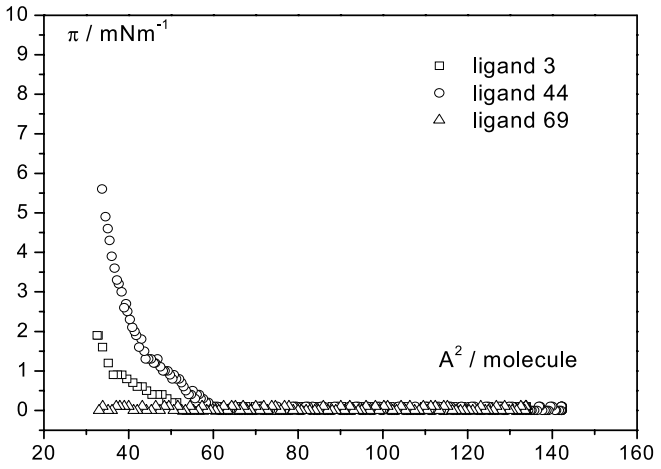

Fig. 2 Surface pressure π (mN/m) area A (A^2/molecule) isotherms for spread monolayer of pure ligand 3, 44 and 69

tension is reduced. There was detected neither static nor dynamic surface activity of any of the ligands at concentration used in the penetration experiment (2×10^{-6} M). Increasing the concentration 40 fold (8×10^{-5} M) small but significant reduction was obtained for ligand 3 and 44 (Table 1) in the static surface tension. Ligand 69 did not show surface active behaviour under the same circumstances. Measuring the dynamic surface tension of the same solutions much higher effect and difference between the three compounds were obtained. Dynamic surface tension as a function of area reduction of the drop was plotted in Fig. 1. A marked decrease of surface tension was observed from app. 30% of area reduction due to the considerable accumulation of the adsorbed ligand 3 and 44 molecules on the water surface. This decrease of surface tension maintains decreasing the area further with more pronounced effect for ligand 44. On the contrary, the surface tension remains almost unchanged in the presence of ligand 69. According to the surface tension measurements the increasing order of amphiphilicity of the drug candidates is ligand 69 < ligand 3 < ligand 44.

Monolayer Experiments

Isotherms of Pure Drug Candidates. Compression isotherm of spread layer determined with the Langmuir balance are plotted for the three ligands in Fig. 2. The shape of the isotherms reflect the instability of the monolayer of the pure ligands. A fraction – or in the case of ligand 69 almost the whole amount – of the spread molecules are probably squeezed out from the air/water surface during the compression. This result could be due to high water solubility of the drug candidates. A difference however, can be seen between the three ligands. A significant surface pressure was detected at high compression for ligand 3 while an even higher value for ligand 44.

Lipid + Ligand Monolayers. Isotherms of lipid + drug candidate mixed monolayers are shown in Fig. 3. The surface pressure/area isotherms are compared to the isotherm of pure lipid monolayer. The lipid : ligand molecular ratio was 5 : 1 in the spreading solution. Both the pure lipid and the mixed isotherms are presented in a form that the area corresponds to one lipid molecule, hence the shift of the isotherm directly indicates the presence of ligand in the mixed layer.

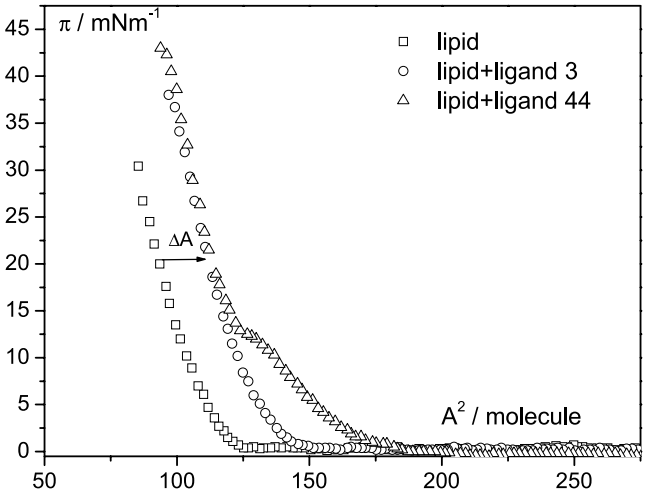

Fig. 3 Surface pressure π (mN/m) area A (A^2/molecule) isotherms for spread monolayer of pure lipid, lipid + ligand 3 and lipid + ligand 44 mixed films with lipid : ligand molar ratio of 5

The mixed isotherm overlaps with that of pure lipid in the case of ligand 69. From that we can conclude that there is no interaction between ligand 69 and lipid molecules, the ligand is most likely migrates into the aqueous subphase from the air/water surface. The mixed films with ligand 3 and 44 show the quite opposite behaviour. Their isotherms are shifted to higher area which is the consequence of the formation of mixed monolayer at the air/water surface where the ligand molecules between the lipid molecules occupy an additional area. Determining this additional area ΔA the amount of ligands present in the mixed film can be estimated at various surface pressures π. As a first approximation the highest molecular area of the ligands representing the planar orientation at the air/water surface were taken into consideration (Table 1). The results of this calculation expressed as lipid : ligand molecular ratio R are given in Table 2.

The behaviour of ligand 44 is similar to that of ligand 3 considering the affinity to lipid. At low surface pressures the molar composition of the two mixed layers are comparable. Lipid : ligand ratio $4 < R < 5$ was obtained in the expanded part of the isotherms. This is probably due to less compressed state of the mixed monolayer.

The section of the isotherm with high slope corresponds to compressed monolayer with close packed molecules (Fig. 3). Above surface pressure of 15 mN/m the molar area is getting nearly constant in this region. For ligand 3 the calculated molar ratios were in good accordance with the composition of the spreading solution characterized by lipid : ligand ratio of 5. This denotes that the ligand molecules remained in the lipid film during compression in spite of the solubility of the ligand in the subphase. That is the clear evidence for the ligand's affinity to lipid.

The R values for lipid films with ligand 44 are different at various compression states of the film. Above surface pressure of 10 mN/m $R \geq 7.5$ indicates that less ligand molecules are present at the air/water surface or the spread amount of molecules occupy less area. The former case is not probable because repeating the compression/expansion cycles overlapping isotherms could be detected. It is reasonable to assume that the structural change is related of the orientation of the ligand molecule in the lipid monolayer. The sign of this behaviour recognized as structural change was observed on the shape of the isotherm at $\pi = 12$ mN/m (Fig. 3). In order to estimate the possible orientation of ligand 44 molecules the calculated R values supposing the vertical orientation of the molecules to the air/water surface are also given in Table 2. The comparison reveals that the ligand 44 molecules are incorporated into the lipid layer in a way that occupy the area which corresponds an orientation between the planar and the vertical ones. The ligand 44 molecules can be tilted or partially submerged into the aqueous phase. Any of them occur the process was found reversible changing the compression of the monolayer and verifies the lipid–ligand interaction.

Table 2 Lipid to ligand ratios R in the mixed monolayers for ligand 3 and 44 assessed from the shift of the isotherms ΔA compared to that of pure lipid at various surface pressures π. Values in brackets were obtained supposing vertical orientation of ligand molecule in the lipid monolayer

π (mN/m)	Ligand 3 ΔA (A^2)	R	Ligand 4 ΔA (A^2)	R
5	20	4.1	38.0	4.2
10	18.3	4.4	32.5	4.8
15	16	5.1	20.8	7.5 (2.4)
18	15.6	5.2	19.5	8.0 (2.6)
20	15.2	5.3	18.7	8.3 (2.7)

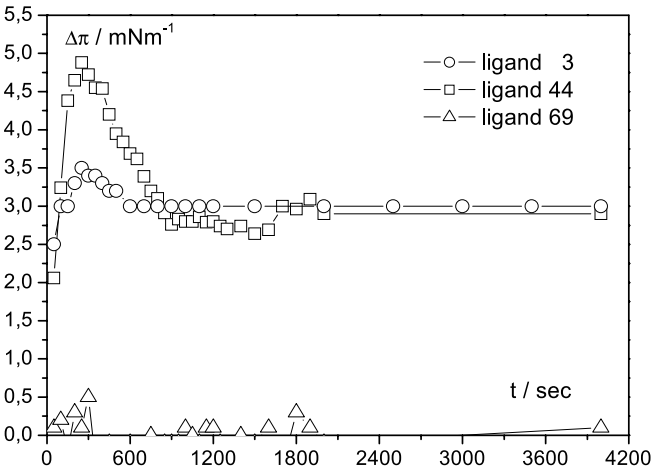

Fig. 4 Increase of surface pressure $\Delta\pi$ (mN/m) due to penetration of ligand 3, 44 and 69 into lipid monolayer with initial surface pressure of 15 mN/m as a function of time t (s)

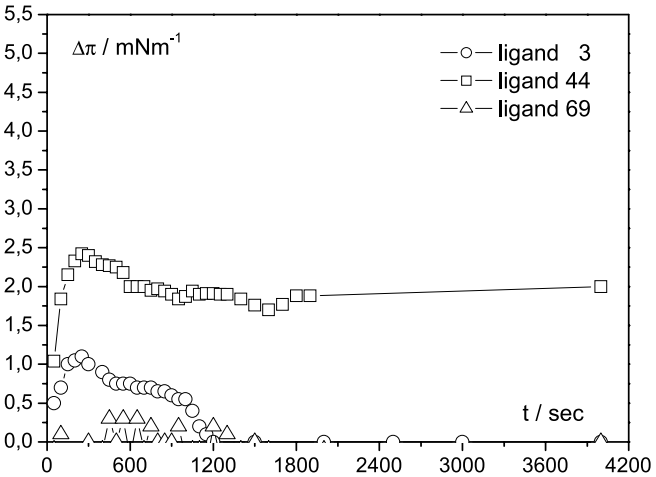

Fig. 5 Increase of surface pressure $\Delta\pi$ (mN/m) due to penetration of ligand 3, 44 and 69 into lipid monolayer with initial surface pressure of 20 mN/m as a function of time t (s)

Penetration. The lipid monolayer was compressed in the Langmuir balance to reach surface pressure of 15 and 20 mN/m and then the barrier was stopped. After the drug candidate solution had been injected into the subphase the change of surface pressure was recorded for an hour. The results of these penetration experiments were summarized in Figs. 4 and 5.

The fast increase of surface pressure observable in the first period is probably connected to the locally high concentration of ligands in the subphase. Therefore the static values developed after appr. 1200 s and maintained for more than an hour were applied for comparison. Considering the lipid film wih surface pressure of 15 mN/m the degrees of penetration are similar for ligand 3 and 44, presenting 3 mN/m as a static increase of surface pressure. The penetration however, into the more dense lipid layer with 20 mN/m is weaker. Nevertheless the ligand 44 produced static increase of surface pressure even of the lipid film with 20 mN/m reflecting the most pronounced affinity to lipid monolayer. The ligand 69 does penetrate neither the less nor the more dense monolayer exhibiting no affinity to lipid.

Conclusion

Three in silico identified drug candidates – ligand 3, 44 and 69 – were characterized and compared in the present work considering their hydrophobicity and membrane affinity. Octanol/water partiton as well as static and dynamic surface activity of the compounds were determined while the membrane affinity was studied in Langmuir film experiments. Results of the three kinds of measurements were in accordance concerning the hydrophobic/amphiphilic character of the three ligand molecules although, the most pronounced differences between the drug candidates were obtained in the dynamic surface tension measurements and investigating their interaction with lipid layers.

Despite the good water solubility of the molecules studied here we found considerable differences in their amphiphatic character and their affinity to lipid monolayer. Ligand 69 being the less hydrophobic showed no interaction with lipid, while the ligand 44 presented a substantial affinity to lipid monolayer being the most hydrophobic and most surface active compound.

It was demonstrated that amphiphilicity and affinity of a drug candidate to model membrane can be reasonably estimated using the Langmuir monolayer and penetration experiments in combination with the characterization of surface activity and hydrophobicity of the given compounds. These results might be promising for the future assessment whether the drugs possess ability to interact with the biomembrane models.

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