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Research article

Microplate assay for lipophilicity determination using intrinsic fluorescence of drugs: application to a promising anticancer lead, pyridoclax

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Abbreviations

CF	Carboxyfluorescein
CHOL	Cholesterol
DLS	Dynamic light scattering
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
EPC	Egg phosphatidylcholine
Kp	Partition coefficient
LUVs	Large unilamellar vesicles
SPC	Soybean phosphatidylcholine

1. Introduction

Pyridoclax was recently identified as the lead compound of an oligopyridine family, acting as a promising protein-protein interactions disruptor. Indeed, without being cytotoxic when administered as a single agent, pyridoclax is able to bind to Mcl-1. Hence, by sensitizing ovarian carcinoma cells to Bcl-x_L-targeting strategies, it induces apoptosis in ovarian, and also in lung, and mesothelioma cancer cells when it is administrated in combination with Bcl-x_L-targeting siRNA or Bcl-x_L targeting molecules such as ABT-737 or its orally available derivative ABT-263 (Navitoclax) (Gloaguen et al., 2015).

In drug discovery and development, it is fundamental to determine the physico-chemical properties of drugs that will influence their drugability. Considering that lipophilicity will directly influence the absorption, distribution, metabolism and elimination (ADME) processes of a drug as its pharmacodynamics profile, determination of this physicochemical property is a crucial step in course of a drug candidate development (Testa et al., 2000).

Lipophilicity is generally expressed as the *n*-octanol/water partition coefficient of the uncharged solute (log $P_{o/w}$). From the determination of many drug partition coefficients in isotropic biphasic *n*-octanol/water systems, large data banks have been constituted, now enabling useful *in silico* prediction of a drug's lipophilicity (Avdeef, 2003). However, the ability to precisely predict the behavior of drugs in biological systems from log $P_{o/w}$ has some limitations since partitioning in biological membranes is not governed by the same physicochemical forces.

In *n*-octanol/water systems, not all hydrophobic, H-bond, dipole-dipole and electrostatic interactions between drug and membrane can be considered (Austin et al., 1995).

Liposome model membranes are widely used as a better alternative (Peetla et al., 2009). Indeed, they offer both the outer polar surface and the hydrophobic core of natural

membranes, and it is possible to adjust their composition to exactly mimic membranes of interest.

Phosphatidylcholine (PC)-based Large Unilamellar Vesicles (LUVs) are often used as models since PC are major lipid components of cell membrane (Bourgaux and Couvreur, 2014), and LUVs offer a similar curvature to the cell membrane (Lucio et al., 2010). Cholesterol may be also included since it represents an important component of biological membranes, involved in membrane fluidity, dynamics, structuration (rafts), or membrane fusion (Ambike et al., 2011, Cooper, 1979, Dufourc, 2008, Simons and Ehehalt, 2002, Yang et al., 2016).

One advantageous method for the assessment of drug lipophilicity through the determination of its partition coefficient (Kp) using biomimetic membranes is based on spectrophotometry measurements. With this non-separative method, it is possible to explore the changes in the absorption parameters (molar absorptivity (ε), maximum wavelength (λ_{max}) of absorption) of the solute when it permeates from a polar to an apolar environment (Magalhães et al., 2010). Use of a microplate assay is particularly appealing, since in these conditions, the determination of Kp is rapid, not expensive, and assays being miniaturized, they require only small quantities of drug, that is very important to consider for a characterization work implied in the drug discovery step.

Nevertheless, to date, and according to our knowledge, all reported microplate assays have used ultraviolet (UV)-visible spectrophotometry.

Considering that pyridoclax is a fluorescent molecule maximum absorbing at 285 nm, and maximum emitting at 444 ± 2 nm in aqueous medium, it seems particularly appealing to use this intrinsic fluorescence to study its interactions with membranes.

So, we propose i). to determine Kp of pyridoclax by transposing a high-throughput microplate assay, ii). to study the influence of the nature of the phospholipids on the experimental values, iii). and to explore the feasibility of the use of intrinsic fluorescence for the Kp determination

in a miniaturized assay. Furthermore, as it is established that the use of biomimetic membranes permits also to highlight keys of the action mechanisms of molecules (Hendrich and Michalak, 2003, Pinheiro et al., 2013, Zepik et al., 2008), interactions between pyridoclax and biomimetic membranes were further studied by a liposome leakage assay.

2. Materials and methods

2.1. Materials

Soybean, Egg phosphatidylcholine (SPC and EPC, respectively) and 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were gifts from Lipoid GmbH (Ludwigshafen, Germany). Chlorpromazine hydrochloride, diclofenac sodium salt, progesterone, propranolol, cholesterol (CHOL), reagent-grade DMSO, carboxyfluorescein, Triton X-100, Sepharose® CL-4B, and 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TMSP) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Hepes buffer was obtained from Grosseron (Coueron, France). Sodium chloride was purchased from Carlo Erba (Val de Reuil, France). Indomethacin was obtained from Alfa Aesar (Thermo Fisher Scientific, Illkirch, France). Tripentone and Pyridoclax were synthesized according to the processes previously described (Gloaguen et al., 2015, Lisowski et al., 2004).

2.2. Preparation of liposomes

2.2.1. Formulation of liposomes

Liposomes were formulated according to the adapted method of the thin lipid film hydration (Bangham et al., 1965). Lipid solutions in chloroform/methanol (4:1) were evaporated under nitrogen flow, and left under vacuum for 3-4 h to form a lipid film. This thin lipid film was then hydrated in Hepes buffer (50 mM, NaCl 107 mM, pH 7.4), and vortexed. Rehydrated lipid suspension was subjected to 5 hot/cold cycles from liquid nitrogen to a 40°C water bath

(50°C for DPPC). At room temperature for SPC, EPC, and SPC:CHOL, or at 60°C for DPPC-based vesicles, the yielded multilamellar vesicles (MLVs) were then extruded 13 times with a mini-extruder (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA) through polycarbonate membranes with a pore diameter of 100 nm (Avanti Polar Lipids, Inc.) to form LUVs.

2.2.2. Characterization of liposomes by dynamic light scattering

The average hydrodynamic diameter associated with the polydispersity index (PDI) of the formulated LUVs were measured by dynamic light scattering (DLS) using a NanoZS® apparatus (Malvern Instruments SA, Worcestershire, UK). The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation, also using a NanoZS® apparatus. The measurements were performed in triplicate at 25°C, after a 1:100 dilution in the Hepes buffer after validation of the absence of the ionic strength influence on the measurements.

2.2.3. Dosage of liposomes by NMR spectroscopy

The lipid concentration of the formulated LUVs was quantified by ^{1}H NMR spectroscopy (Hein et al., 2016). ^{1}H NMR measurements were performed at ambient temperature for SPC, and EPC-based LUVS, or at 50°C for SPC:CHOL, and DPPC-based LUVs on a Bruker Avance III spectrometer (Bruker, Billerica, Massachusetts, USA) equipped with a 5 mm probehead operating at 400 MHz. The instrument's standard settings (90° pulse angle, 2.72 s acquisition time, 3 s relaxation delay, 15 ppm spectral width) were used. Locking and shimming was performed on the signal of the exchangeable deuterium atoms of the D_2O/CD_3OD mixtures. 64 scans were performed leading to a total acquisition time of 6 min 20 s. Data processing was performed with Topspin 3.5 After zerofilling to 64k data points, apodization (exponential and Gaussian functions, 1 Hz), Fourier transformation, phase- and

baseline correction, the peak areas were determined by integration. TMSP was used as an internal standard (δ = 0 ppm). For samples with increased amounts of water, presaturation of the water peak (δ = 4.79 ppm) was used to ensure that the dynamic range of the instrument was not exceeded during the measurement.

2.3. Determination of partition coefficient

2.3.1. Determination of partition coefficient by derivative UV-spectrophotometry

The partition coefficients (Kp) of the molecules were determined in presence of LUVs suspensions. 5 µL of each stock compound solution (750 µM in DMSO) was added to Hepes buffer (50 mM, 107 mM NaCl, pH 7.4) with increasing concentrations of phospholipids (0 to 900, 1000 or 4000 µM) to give a final volume of 250 µL. The corresponding reference solutions were identically prepared in absence of molecule. The microplate was incubated in a Biotek Synergy 2 microplate reader (Biotek, Colmar, France) at 37.0 °C or at 50.0 °C ± 0.1 °C for 1 hour with regular homogenization. The absorption spectra were then recorded using 1 nm wavelength interval in the 250-500 nm range at 37°C.

The corrected absorption spectra of compounds were obtained by subtracting the spectrum of the liposomal solution used at the same concentration. Spectra data recovery was performed using Microsoft® Excel® 2016. Second derivative spectra were calculated by using a second-order polynomial convolution of 9 points with GraphPad Prism (version 6.01, GraphPad Software, La Jolla, CA, USA) (Magalhães et al., 2010).

The Nernst partition coefficient in liposome (Ribeiro et al., 2010) is defined as:

$$Kp = \frac{\frac{n_{S,L}}{V_L}}{\frac{n_{S,W}}{V_W}} \quad (1)$$

where $n_{s,i}$ are the number of moles of solute present in the aqueous (i = W) and in the lipid (i = L) phases. From equation 1, the fraction of membrane-bound solute (X_L) can be related to Kp, at a given experimental concentration in phospholipids ([L]), by the following equation:

$$X_L = \frac{n_{S,L}}{n_{S,L} + n_{S,W}} = \frac{Kp\,.\,\gamma\,.\,[L]}{1 + Kp\,.\,\gamma\,.\,[L]} \quad (2)$$

where γ is the phospholipid molar volume.

Introducing D_t, the variation of the derivative value of the absorbance spectrum of the molecule at a given λ (nm) and a given concentration of phospholipids (M), X_L can also be defined as follows (Santos et al., 2003):

$$X_L = \frac{D_t - D_w}{D_l - D_w} \quad (3)$$

where D_l corresponds to the derivative value of the absorbance spectrum of the molecule in the lipid phase, and D_w is the derivative value of the absorbance spectrum of the molecule in the aqueous phase.

Upon phospholipid titration, by following the variation of D_t, which changes upon membrane insertion and for which the ensemble average is a linear combination of the contribution of the molecules in the aqueous (D_w) or on the lipid (D_l) phase, the value of Kp can be obtained. Thus, from equation 4, the Kp value of studied molecules were determined after a non-linear regression analysis and a graph plotting performed with GraphPad Prism:

$$D_t = D_w + \frac{(D_l - D_w).\,Kp.\,\gamma.[L]}{1 + Kp\,.\,\gamma.[L]} \quad (4)$$

where γ is the phospholipid molar volume, equal to 0.75 $L.mol^{-1}$ for SPC (Marsh, 2013), 0.69 and 0.70 $L.mol^{-1}$ for EPC and DPPC, respectively (Nunes et al., 2013). These values may be considered as sensibly stable, also in presence of 20 % (w/w) cholesterol as previously shown for DMPC or DPPC (Gallová et al., 2017, Greenwood et al., 2006). From that, no additional γ was considered for cholesterol in all cases. All experiments were performed in triplicate, by using 3 measurements each time. Data are presented as means ± standard deviation of the three experiments.

2.3.2. *Determination of partition coefficient by fluorescence spectrophotometry*

Once after measurements by UV-spectrophotometry, 100 µL of the well's content was transferred in a black microplate for fluorescence measurements. The microplate was incubated for 20 min in a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland) for measurements performed at 37°C, or in a Synergy 2 microplate reader controlled by Gen 5 software (Biotek, Colmar, France) for those performed at 50°C. Excitation wavelengths of 285 nm and 236 nm were chosen to record emission fluorescence spectra of pyridoclax (λ_{em} = [350-550 nm]), and of capsaicin (λ_{em} = [280-400 nm]), respectively. As for UV-measurements (Ribeiro et al., 2010), by following the variation with [L] of F_t, and after liposomal blank correction, the value of Kp was obtained from equation 5:

$$F_t = F_w + \frac{(F_l - F_w).\,Kp.\,\gamma.[L]}{1 + Kp\,.\,\gamma.[L]} \qquad (5)$$

where F_t is the fluorescence intensity of the fluorescence spectrum of the molecule at a given λ (nm) and a given concentration of phospholipids (L), F_l corresponds to the fluorescence intensity of the fluorescence spectrum of the molecule in the lipid phase, F_w is the fluorescence intensity of the fluorescence spectrum of the molecule in the aqueous phase, and γ is the phospholipid molar volume as previously described (see *Determination of partition coefficient by derivative UV-spectrophotometry*).

2.4. Liposomes leakage assay

Carboxyfluorescein (CF)-loaded LUVs were prepared by the same protocol than that used for blank LUVs, but after dissolution of 50 mM CF in the Hepes buffer used for the hydration step. The obtained LUVs were separated from possible unincorporated CF by passage through a Sepharose® CL-4B loaded (Sigma-Aldrich) column, using Hepes buffer as eluent. The LUVs size was assessed by DLS (NanoZS®, Malvern Instruments, Worcestershire, UK) after a 1:100 dilution in the Hepes buffer. The entrapment of CF was verified by dequenching of fluorescence after the addition of 20 % (v/v) Triton X-100 measured with a Synergy 2

microplate reader (Biotek, Colmar, France) equipped with the appropriate filters (λ_{ex} = 485/20 nm and λ_{em} = 528/20 nm). CF release assay was performed in a final volume of 200 µL, using 10 µM LUVs in Hepes buffer. Pyridoclax, solubilized in DMSO, was then added to the solution to reach a final concentration ranging from 1 to 20 µM. The fluorescence was recorded immediately (F_0) and for 3h at 25°C, using a Synergy 2 microplate at λ_{ex} 485/20 nm and λ_{em} 528/20 nm. It was compared with that measured at the end of the experiment after addition of 2 µL of 20% Triton X-100 solution to achieve complete liposome leakage (F_{max}). The percentage of CF release was calculated according to the following equation (Jimah et al., 2017) :

$$\% \text{ CF leakage } (t) = [(F_t - F_0) / (F_{max} - F_0)] \times 100 \quad (6)$$

where F_t was the fluorescence intensity at time t, F_0 the initial fluorescence intensity, and F_{max} the final fluorescence intensity after adding Triton X-100.

3. Results and discussion

3.1. Determination of partition coefficient by a miniaturized derivative UV-spectrophotometry method

By using a microplate protocol adapted from that initially described by Magalhaes *et al*., the determination of Kp has been undertaken for pyridoclax. Indeed, this miniaturized and simple method permits to rapidly provide partition coefficient values of drugs, and from that, it appears as well adapted to a drug discovery process. In the original microplate assay, hexadecylphosphocholine micelles were used to mimic the biomembranes (Magalhães et al., 2010). Nevertheless, considering that liposomes are a less restricted membrane tool than micelles (suitable only for substances with high ε and log Kp > 3) (Loureiro et al., 2018), we have chosen to transpose the microplate assay to LUVs.

LUVs were used as membrane models because they present adequate characteristics of cell membrane models. Indeed, they are formed of a simple bilayer unlike multilamellar or multivesicular vesicles, (MLVs or MVVs). Their composition is flexible. It is possible to vary physicochemical parameters like pH, buffer composition, without detrimental repercussion on their stability unlike single unilamellar vesicles (SUVs) (Akbarzadeh et al., 2013). Last, they are easier to produce and to manipulate than giant unilamellar vesicles (GUVs) (Bagatolli et al, 1999, Tamba et al, 2004). Taking into account these different properties, we have selected LUVs for experimental design of model membranes.

3.1.1. Preparation and characterization of liposomes

Soybean PhosphatidylCholine (SPC), Egg PhosphatidylCholine (EPC), DiPalmitoyl PhosphatidylCholine (DPPC), and mixed SPC/cholesterol (SPC/CHOL, 80:20, w/w) large unilamellar vesicles (LUVs) were produced by the classical thin hydration method, followed by an extrusion through polycarbonate filters (Fig. 1).

Their granulometric properties were similar, characterized by an average diameter about 130 nm, and homogeneous in size as indicated by the polydispersity index (PDI) values lower than 0.1 (Table 1). Only in the case of SPC/CHOL vesicles, the diameter was slightly lower, probably because of an increased packing of lipids due to cholesterol (Alves et al., 2017). In all cases, ζ potential values were neutral, in coherence with the chemical structure of the lipids used (Fig. 1).

For each LUVs formulation, the phospholipid concentration was precisely determined by a ^{1}H NMR (Hein et al., 2016). In the method we used, a defined volume of the prepared LUVs stock solution was first totally solubilized in deuterated chloroform, and a defined amount of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid (TMSP) was then added to the solution as internal standard (δ = 0 ppm). In these conditions, the signal at ca. 0.88 ppm, well separated from all other peaks, can be assigned to the terminal methyl groups of the phospholipid's fatty acid side chains. As this ^{1}H NMR method allows determination of total phospholipid content of lipid mixtures with different head groups, and fatty acid acyl side chains (Fig. 2.A), it was efficiently transposed to the different formulated LUVs, also originally, in presence of cholesterol (Fig. 2.B). The only requirement for LUVs formulated in presence of cholesterol or DPPC was to maintain a heating of samples at 50°C to avoid solubility issues. To obtain a more well-defined peak for the integration in presence of cholesterol, SPC/CHOL-based LUVs (80:20) were quantified using the C18 methyl group of cholesterol (δ = 0.67 ppm). So, this quantification method can be efficiently proposed in routine for characterization of LUVs of various compositions. Content in lipids [L] of various studied liposomal formulations are presented in Table 1. These experimental values have been further used in equation 4 for Kp determination (*see 3.1.3.*).

3.1.2. Molecules used as references

To establish the Kp method, we first used SPC-based LUVs and several drugs whose properties permit to cover a relatively large range of logP values. Molecules have been selected to have a suitable UV absorption, *i.e.* with a number of π bonds > 3 (Loureiro et al., 2018), and to be either bases (propranolol, chlorpromazine), neutral molecules (tripentone, progesterone), or acids (diclofenac, indomethacin) (Fig. 3).

All molecules were assayed at a fixed final concentration of 15 µM, since such a concentration provides UV signals, that were suitable for analysis, after derivatization of the UV-spectra too. Moreover, such a weak solute concentration is poorly molecule consumable, and permits also to minimize deviation from the Nernst conditions, *i.e.*, to ensure complete solubilization of the molecule in both phases, and to minimize solute own volume in the lipid phase. In order to avoid any precipitation in working intermediate solutions, compounds were directly added from a concentrated DMSO solution to increasing concentrations of SPC based-LUVs in Hepes buffer. It was checked that, in these conditions, the final percentage of DMSO, equal to 2% (v/v), didn't significantly impact calculated Kp values (data not shown). After recording the UV-spectra, the data management was performed from the calculation of the second-derivative spectrophotometer data, collected at defined wavelengths for each molecule in the presence of increasing LUVs concentration (Table 2). Indeed, the use of second-derivative spectra permits to eliminate the background caused by light scattering of phospholipids, and to identify an isobestic point at the vicinity of which a wavelength can be chosen to highlight the shift of observed D_t with increasing phospholipids concentration (Magalhães et al., 2010).

Experimental values determined for the 6 molecules appeared in accordance with those previously described in the literature (Table 2). The experimental log Kp values were in the same magnitude than log $D_{7.4}$ for neutral compounds. For the ionized molecules, log Kp were

higher than log $D_{7.4}$ with a difference from 0.3 to 3 log units, which is inversely correlated to the log Kp value. This may be explained by the fact, that, for the charged forms of the drugs at pH 7.4, the membrane partition of molecules is influenced by electrostatic forces between the positively charged propranolol or chlorpromazine and the negatively charged phosphate group of SPC, or between its positively charged ammonium group and the negatively charged diclofenac or indomethacin (Fig. 1,3).

3.1.3. Partition coefficient of pyridoclax determined by a miniaturized derivative UV-spectrophotometry method

Kp of pyridoclax was then determined by UV-spectrophotometry according to the validated protocol, by using a fixed concentration of drug (15 µM) and increasing concentrations of SPC-based LUVs at pH 7.4 and 37°C (Fig. 4). After recording the UV-spectra (Fig. 4.A), the data management was performed. The second-derivative spectra data highlighted three isobestic points (Fig. 4.B). The shift observed at λ_{max} of 306 nm with increasing concentration of phospholipids, particularly provided a clear indication that pyridoclax partitions from the aqueous phase to the LUVs as translated by sensitive differences in absorbance values. From the fitted non-linear regression curve obtained from the plot of the total membrane second-derivative intensity values (D_t) monitored at this λ_{max} of 306 nm (Fig. 4.C), and by applying equation 4, the partition coefficient of pyridoclax was found to be equal to 4.11 ± 0.09 (Table 3). This value is consistent with the experimental log P value of 4.4 previously reported, and experimentally defined by a chromatographic method (Groo et al., 2017). It is smaller than the calculated logP prediction of 5.83, performed using ChemAxon software (Marvin 16.1.4.0, 2016, ChemAxon (http://www.chemaxon.com/)). Thus, pyridoclax appears as a lipophilic drug, which is relative to the presence of four central pyridines in its scaffold, and that of a lateral styryl hydrophobic group, that reinforces its lipophilic character.

Considering that the most basic pKa value is 4.89 for pyridoclax (calculated using ChemAxon software), the molecule exists in a predominant unionized form at pH 7.4. So, the membrane partition of pyridoclax appears as mostly influenced by hydrophobic interactions rather than by electrostatic forces.

By keeping the protocol unchanged, Kp of pyridoclax was further assessed at pH 7.4 and at 37 or 50°C, in presence of LUVs formulated from SPC, EPC, DPPC, or mixed SPC/CHOL (80:20, w/w).

From results (Table 3), the partition coefficient values of pyridoclax appeared in the same magnitude order, *i.e.* log Kp ~ 4.0, if determined by using SPC or EPC based liposomes, at 37 or 50°C. Thus, SPC-LUVs could be considered as a biomimetic membrane model particularly suitable for drug discovery studies since this inexpensive raw material permits to characterize interactions of original drugs with membranes as other more expensive but more largely used raw materials do.

Moreover, results showed that, whatever the temperature was, the partition coefficient value was lower with DPPC membranes than with SPC-based LUVs, but slightly increased at 50°C. SPC is a phospholipids mixture, mainly composed of linoleic acid (approximatively 59-70 % according to the supplier), a bi-unsatured fatty acid (18:2). Although the fatty acid chain length is poorly different from that of DPPC (16:0), presence of two unsatured bonds can increase the membrane fluidity, and decrease the energy required for the drug to penetrate within the membrane (Stubbs and Smith, 1984). At both temperatures of experiments, SPC and EPC present a disordered liquid-crystalline phase since their phase transition temperature are -20 and -8 °C, respectively. On the contrary, at 37°C, *i.e.* below the DPPC Tm (41°C), the DPPC lipids are present in an ordered gel state, making the penetration of pyridoclax more difficult within the membrane. The fact that with membranes composed of SPC/CHOL (80:20, w/w), the partition coefficient of pyridoclax was smaller than with the SPC-based

LUVs indicates also that by increasing packing of lipids, the membrane becomes less accessible to pyridoclax. Indeed, it is known that the addition of cholesterol causes a decrease in membrane fluidity (Alves et al., 2017). In this case, the amount of drug able to partition into membranes being decreased, Kp value became directly affected and lowered.

Considering that drug-membranes interactions may be correlated with the permeabilization of the lipid bilayer, pyridoclax was assayed for its membrane-disruptive properties by carboxyfluorescein (CF) leakage from liposomes. CF-loaded LUVs, formulated from either SPC-, or SPC/CHOL (80:20, w/w) were used. In comparison with blank LUVs (Table 1), encapsulation of the fluorophore led to a slight increase in the average diameter of the liposomes, less pronounced in presence of cholesterol (Table 4). Nevertheless, in both cases, LUVs populations remain monodisperse in size, and present a surface potential close to zero (Table 4). Assays were performed by keeping their final phospholipid concentration (10 μM) unchanged in Hepes buffer. From Fig. 5, pyridoclax displayed a concentration-dependent membrane disruptive effect. Such appearance of lipid defects in the membrane upon exposure to pyridoclax could participate to the biophysical mechanisms contributing to the efficacy, and/or toxicity of this promising lead, as it was previously reported for other anticancer drugs (Engelk et al., 2001, Lucio et al., 2010). It appeared also the membrane permeabilization upon pyridoclax exposure was considerably decreased by employing the more rigid cholesterol-containing liposomes. As physiological membranes present variables proportions in cholesterol, the ability of pyridoclax to interpenetrate within them could differ according the cellular type (de Oliveira Andrade, 2016).

3.1.4. Partition coefficient of pyridoclax determined by a miniaturized fluorescence spectrophotometry method

Pyridoclax is a fluorescent molecule absorbing at 285 nm and emitting with a maximal intensity in the range 442-446 nm in aqueous medium. This intrinsic fluorescence originates from the presence of 4 pyridines in the scaffold, and is also influenced by the presence of a styryl group (Fig. 3.G) (Gloaguen et al., 2015). The same samples than those previously used for Kp determination of pyridoclax by UV measurement were then assayed by fluorescence spectrophotometry.

By increasing lipid concentration, the fluorescence signal intensity of pyridoclax significantly increased, and emission maxima progressively shifted from the range 442-446 nm towards 418 nm (Fig. 6.A). Such a shift of the emission maximum may reflect a molecule transition from polar (water) to a less polar environment (membrane), whereas an increase of fluorescence intensity may indicate immobilization of the compound within the membrane. Thus, it appeared that by measurement of the intrinsic fluorescence of the molecule, interactions between the drug and membranes could be highlighted. Only after blank correction, fluorescence intensity values measured at either 418, 442, or 446 nm were plotted in function of the phospholipid concentrations (Fig. 6.B). In all cases, Kp values were determined and appeared similar. Comparable results have been also obtained at a lower pyridoclax concentration (5 µM) and in another aqueous buffer such as PBS (results not shown). Assays have been performed by using SPC, EPC, DPP, or mixed SPC/cholesterol (80:20, w/w), at pH 7.4 and 37°C or 50°C. Whatever the conditions, the interactions between pyridoclax and membranes can be determined (Table 3). Log Kp values of pyridoclax defined by the fluorescence method appeared identical to those obtained by UV spectrophotometry, and effects of the composition or the fluidity of the membrane on the partition of the drug into these mimetic models can be also highlighted.

Only few studies report the follow in the fluorescence changes to determine drug-membrane interactions. Fluorescence anisotropy measurements have been proposed to investigate lipophilicity of fluorescent drugs, but light scattering by liposomes was considered as a hampering factor (Pallicer and Krämer, 2012). Fluorescence quenching measurements have been also carried out, but required use of an external fluorophore (Ferreira et al., 2005). Study of interactions of capsaicin with lipid bilayer membranes was performed using its intrinsic fluorescence but was realized thanks to a fluorescence spectrophotometer (Swain and Kumar Mishra, 2015).

Interestingly, we transposed our miniaturized fluorescence method to capsaicin (Fig. 3.H), and the partition coefficient was determined: Kp = 2,954 ± 619, corresponding to log Kp = 3.46. This value appeared coherent with that measured by the miniaturized derivative UV-spectrophotometry method: Kp = 6,394 ± 2,619, corresponding to log Kp = 3.79, confirming the interest of this approach.

From that, the microplate reader provides a rapid assay, by requiring only limited quantities of drugs, that is particularly appealing in drug discovery since available amounts of leads remain low at this drug development stage.

Moreover, this method can be advantageously proposed to confirm results obtained in UV. It also appears as an alternative sensitive and rapid approach to UV detection, in particular for substances with low molar absorption coefficient (Loureiro et al., 2018), and by avoiding the calculation of the second-derivative spectrophotometer data, it permits a simplified data management, and a gain in sensitivity.

4. Conclusion

Lipophilicity must be necessarily determined in drug discovery since this physicochemical property will directly influence the pharmacokinetics of a drug as its pharmacodynamics profile. Thanks to our analytical approach based on a microplate assay, and raw fluorescence spectrophotometry by taking advantage of drug intrinsic fluorescence, Kp was determined for pyridoclax. This rapid and sensitive method, transposable to other fluorescent drugs, appears particularly appealing for drug discovery since it permits to assess the lead partition coefficients by requiring only few drug amounts. Being applicable to liposomes with various phospholipid composition, it also permits to highlight impact of the membrane fluidity on drug-membranes interactions. This method can be advantageously proposed to confirm results obtained in UV. It also appears as an alternative sensitive and rapid approach to UV detection, in particular for intrinsic fluorescence gifted substances with low molar absorption coefficient.

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References

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S.W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M., Nejati-Koshki, K., 2013. Liposome: classification, preparation, and applications. Nanoscale Res. Lett. 8, 102. https://doi.org/10.1186/1556-276X-8-102

Alves, A.C., Ribeiro, D., Horta, M., Lima, J.L.F.C., Nunes, C., Reis, S., 2017. The daunorubicin interplay with mimetic model membranes of cancer cells: A biophysical interpretation. Biochim. Biophys. Acta Biomembr. 1859, 941–948. https://doi.org/10.1016/j.bbamem.2017.01.034

Ambike, A., Rosilio, V., Stella, B., Lepêtre-Mouelhi, S., Couvreur, P., 2011. Interaction of Self-Assembled Squalenoyl Gemcitabine Nanoparticles with Phospholipid−Cholesterol Monolayers Mimicking a Biomembrane. Langmuir 27, 4891–4899. https://doi.org/10.1021/la200002d

Austin, R.P., Davis, A.M., Manners, C.N., 1995. Partitioning of ionizing molecules between aqueous buffers and phospholipid vesicles. J. Pharm. Sci. 84, 1180–1183.

Avdeef, A., 2003. Absorption and drug development: solubility, permeability, and charge state. Wiley-Interscience, Hoboken, N.J.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238-IN27. https://doi.org/10.1016/S0022-2836(65)80093-6

Bourgaux, C., Couvreur, P., 2014. Interactions of anticancer drugs with biomembranes: What can we learn from model membranes? J. Controlled Release 190, 127–138. https://doi.org/10.1016/j.jconrel.2014.05.012

Cooper, R.A., 1979. Cholesterol on Membrane Fluidity and Cell, in: Normal and Abnormal Red Cell Membranes: Proceedings of the ICN-UCLA Symposium Held at Keystone, Colorado, March 1978. AR Liss, p. 135.

de Oliveira Andrade, L., 2016. Understanding the role of cholesterol in cellular biomechanics and regulation of vesicular trafficking: The power of imaging. Biomed. Spectrosc. Imaging 5, S101–S117. https://doi.org/10.3233/BSI-160157

Dufourc, E.J., 2008. Sterols and membrane dynamics. J. Chem. Biol. 1, 63–77. https://doi.org/10.1007/s12154-008-0010-6

Engelk, M., Bojarski, P., Bloss, R., Diehl, H., 2001. Tamoxifen perturbs lipid bilayer order and permeability: comparison of DSC, fluorescence anisotropy, laurdan generalized polarization and carboxyfluorescein leakage studies. Biophys. Chem. 90, 157–173.

Ferreira, H., Lúcio, M., Lima, J.L.F.C., Matos, C., Reis, S., 2005. Effects of diclofenac on EPC liposome membrane properties. Anal. Bioanal. Chem. 382, 1256–1264. https://doi.org/10.1007/s00216-005-3251-z

Gallová, J., Želinská, K., Balgavý, P., 2017. Partial molecular volumes of cholesterol and phosphatidylcholine in mixed bilayers. Eur. Pharm. J. 64, 1–3. https://doi.org/10.1515/afpuc-2017-0012

Gloaguen, C., Voisin-Chiret, A.S., Sopkova-de Oliveira Santos, J., Fogha, J., Gautier, F., De Giorgi, M., Burzicki, G., Perato, S., Pétigny-Lechartier, C., Simonin-Le Jeune, K., Brotin, E., Goux, D., N'Diaye, M., Lambert, B., Louis, M.-H., Ligat, L., Lopez, F., Juin, P., Bureau, R., Rault, S., Poulain, L., 2015. First Evidence That Oligopyridines, α-Helix Foldamers, Inhibit Mcl-1 and Sensitize Ovarian Carcinoma Cells to Bcl-x_L-Targeting Strategies. J. Med. Chem. 58, 1644–1668. https://doi.org/10.1021/jm500672y

Greenwood, A.I., Tristram-Nagle, S., Nagle, J.F., 2006. Partial molecular volumes of lipids and cholesterol. Chem. Phys. Lipids 143, 1–10. https://doi.org/10.1016/j.chemphyslip.2006.04.002

Groo, A.-C., De Pascale, M., Voisin-Chiret, A.-S., Corvaisier, S., Since, M., Malzert-Fréon, A., 2017. Comparison of 2 strategies to enhance pyridoclax solubility: Nanoemulsion delivery system versus salt synthesis. Eur. J. Pharm. Sci. 97, 218–226. https://doi.org/10.1016/j.ejps.2016.11.025

Hein, R., Uzundal, C.B., Hennig, A., 2016. Simple and rapid quantification of phospholipids for supramolecular membrane transport assays. Org. Biomol. Chem. 14, 2182–2185. https://doi.org/10.1039/c5ob02480c

Hendrich, A.B., Michalak, K., 2003. Lipids as a target for drugs modulating multidrug resistance of cancer cells. Curr. Drug Targets 4, 23–30.

Jimah, J.R., Schlesinger, P.H., Tolia, N.H., 2017. Liposome Disruption Assay to Examine Lytic Properties of Biomolecules 16.

Lisowski, V., Léonce, S., Kraus-Berthier, L., Sopková-de Oliveira Santos, J., Pierré, A., Atassi, G., Caignard, D.-H., Renard, P., Rault, S., 2004. Design, Synthesis, and Evaluation of Novel Thienopyrrolizinones as Antitubulin Agents. J. Med. Chem. 47, 1448–1464. https://doi.org/10.1021/jm030961z

Loureiro, D.R.P., Soares, J.X., Lopes, D., Macedo, T., Yordanova, D., Jakobtorweihen, S., Nunes, C., Reis, S., Pinto, M.M.M., Afonso, C.M.M., 2018. Accessing lipophilicity of drugs with biomimetic models: A comparative study using liposomes and micelles. Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci. 115, 369–380. https://doi.org/10.1016/j.ejps.2018.01.029

Lucio, M., Lima, J., Reis, S., 2010. Drug-membrane interactions: significance for medicinal chemistry. Curr. Med. Chem. 17, 1795–1809.

Magalhães, L.M., Nunes, C., Lúcio, M., Segundo, M.A., Reis, S., Lima, J.L.F.C., 2010. High-throughput microplate assay for the determination of drug partition coefficients. Nat. Protoc. 5, 1823–1830. https://doi.org/10.1038/nprot.2010.137

Marsh, D., n.d. Handbook of Lipid Bilayers. Boca Raton: CRC Press.

Nunes, C., Lopes, D., Pinheiro, M., Pereira-Leite, C., Reis, S., 2013. In Vitro Assessment of NSAIDs-Membrane Interactions: Significance for Pharmacological Actions. Pharm. Res. 30, 2097–2107. https://doi.org/10.1007/s11095-013-1066-8

Pallicer, J.M., Krämer, S.D., 2012. Evaluation of fluorescence anisotropy to assess drug–lipid membrane partitioning. J. Pharm. Biomed. Anal. 71, 219–227. https://doi.org/10.1016/j.jpba.2012.08.009

Peetla, C., Stine, A., Labhasetwar, V., 2009. Biophysical Interactions with Model Lipid Membranes: Applications in Drug Discovery and Drug Delivery. Mol. Pharm. 6, 1264–1276. https://doi.org/10.1021/mp9000662

Pinheiro, M., Nunes, C., Caio, J.M., Moiteiro, C., Lúcio, M., Brezesinski, G., Reis, S., 2013. The Influence of Rifabutin on Human and Bacterial Membrane Models: Implications for Its Mechanism of Action. J. Phys. Chem. B 117, 6187–6193. https://doi.org/10.1021/jp403073v

Ribeiro, M.M.B., Melo, M.N., Serrano, I.D., Santos, N.C., Castanho, M.A.R.B., 2010. Drug-lipid interaction evaluation: why a 19th century solution? Trends Pharmacol. Sci. 31, 449–454. https://doi.org/10.1016/j.tips.2010.06.007

Santos, N.C., Prieto, M., Castanho, M.A.R.B., 2003. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. Biochim. Biophys. Acta 1612, 123–135.

Simons, K., Ehehalt, R., 2002. Cholesterol, lipid rafts, and disease. J. Clin. Invest. 110, 597–603. https://doi.org/10.1172/JCI200216390

Stubbs, C.D., Smith, A.D., 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim. Biophys. Acta BBA - Rev. Biomembr. 779, 89–137. https://doi.org/10.1016/0304-4157(84)90005-4

Swain, J., Kumar Mishra, A., 2015. Location, Partitioning Behavior, and Interaction of Capsaicin with Lipid Bilayer Membrane: Study Using Its Intrinsic Fluorescence. J. Phys. Chem. B 119, 12086–12093. https://doi.org/10.1021/acs.jpcb.5b05351

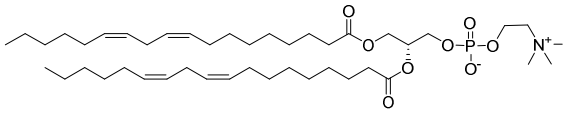
Testa, B., Crivori, P., Reist, M., Carrupt, P.-A., 2000. The influence of lipophilicity on the pharmacokinetic behavior of drugs: concepts and examples. Perspect. Drug Discov. Des. 19, 179–211.

Yang, S.-T., Kreutzberger, A.J.B., Lee, J., Kiessling, V., Tamm, L.K., 2016. The role of cholesterol in membrane fusion. Chem. Phys. Lipids 199, 136–143. https://doi.org/10.1016/j.chemphyslip.2016.05.003

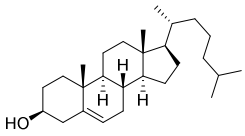
Zepik, H.H., Walde, P., Kostoryz, E.L., Code, J., Yourtee, D.M., 2008. Lipid Vesicles as Membrane Models for Toxicological Assessment of Xenobiotics. Crit. Rev. Toxicol. 38, 1–11. https://doi.org/10.1080/10408440701524519

Figures

A. soybean phosphatidylcholine (SPC)



B. cholesterol (CHOL)



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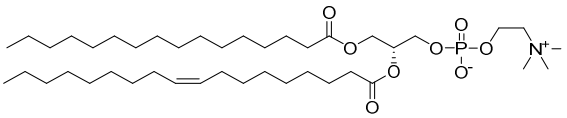


D. dipalmitoyl phosphatidylcholine (DPPC)

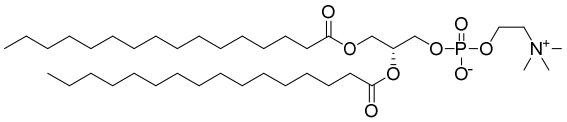
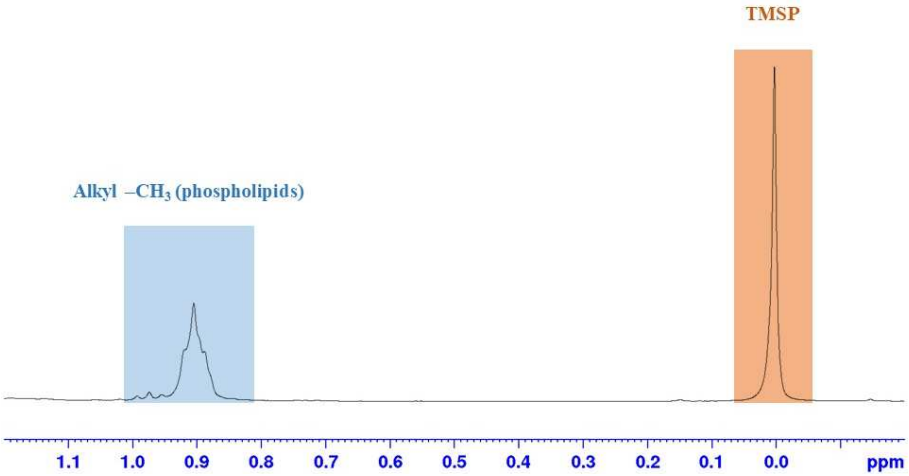


Fig. 1. Molecular structure of the lipids used for the formulation of the LUVs: **A.** SPC, **B.** cholesterol, **C.** EPC, **D.** DPPC.

A.



B.

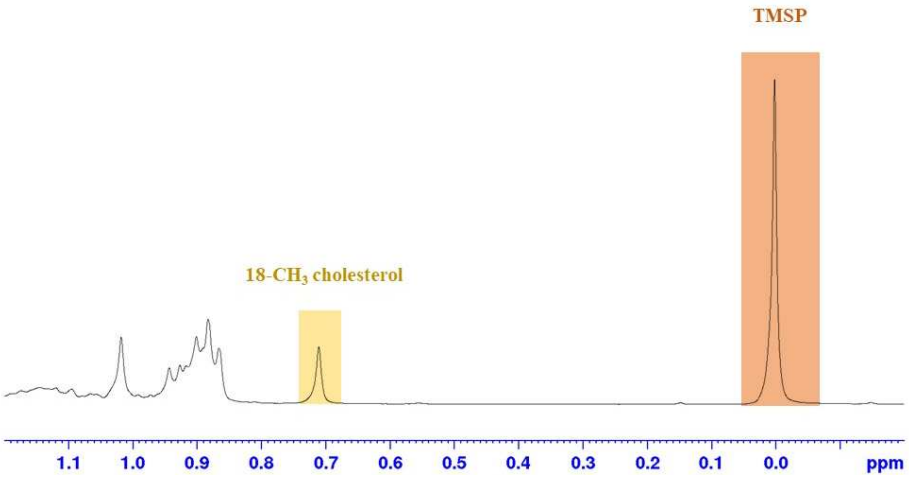


Fig. 2. ^{1}H NMR spectra of **A.** SPC-, and **B.** SPC-CHOL-based-LUVs.

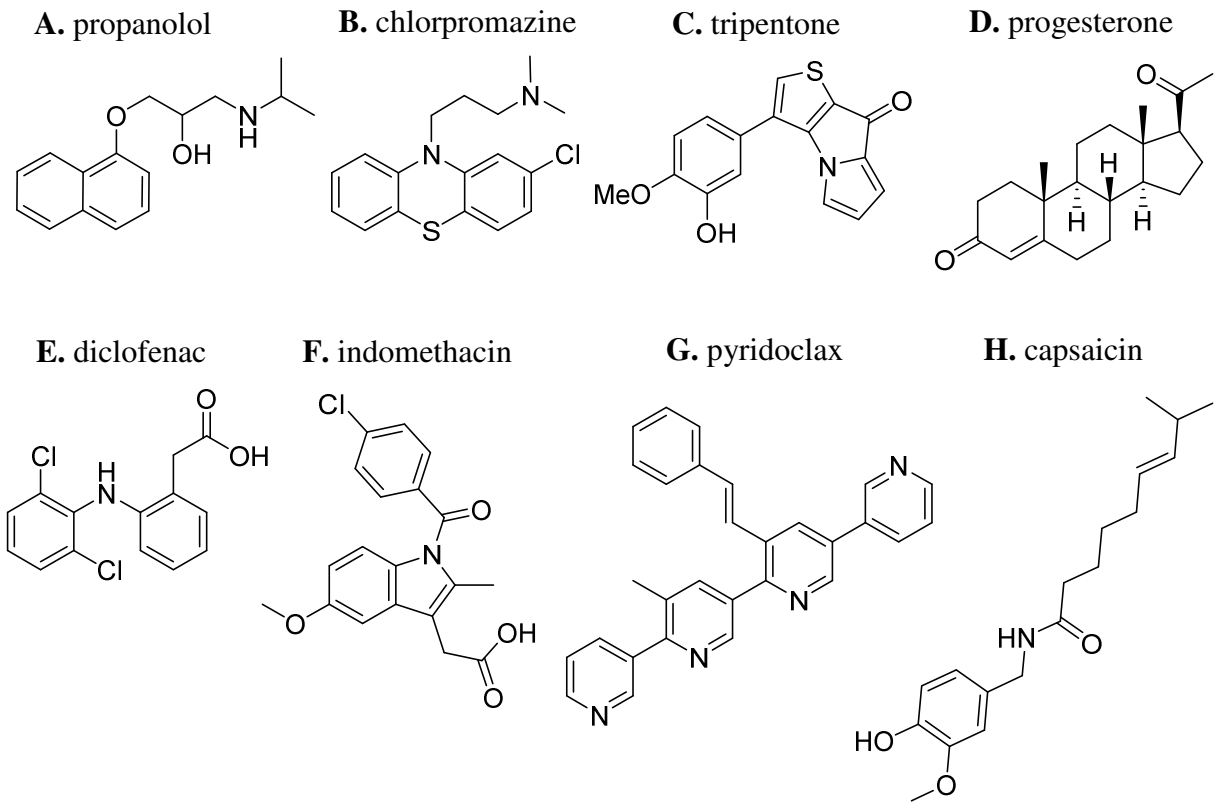
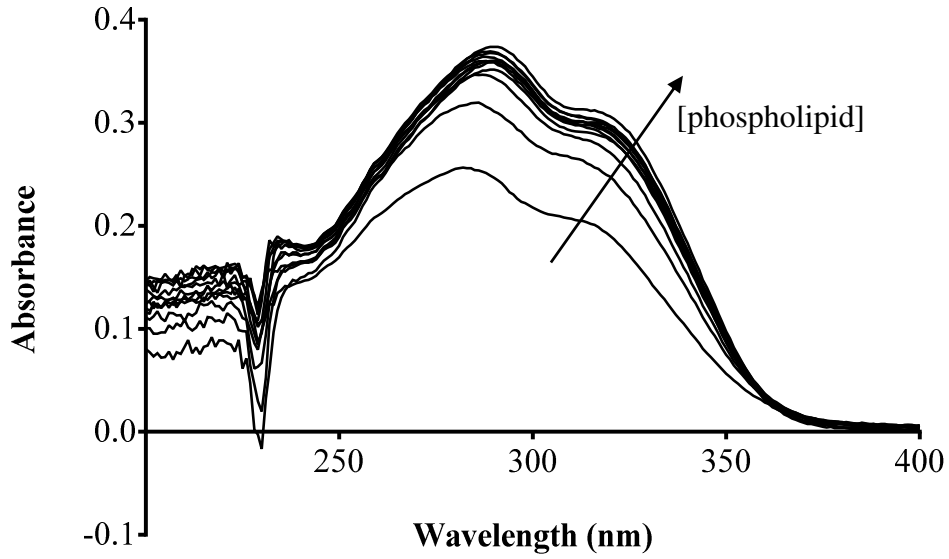
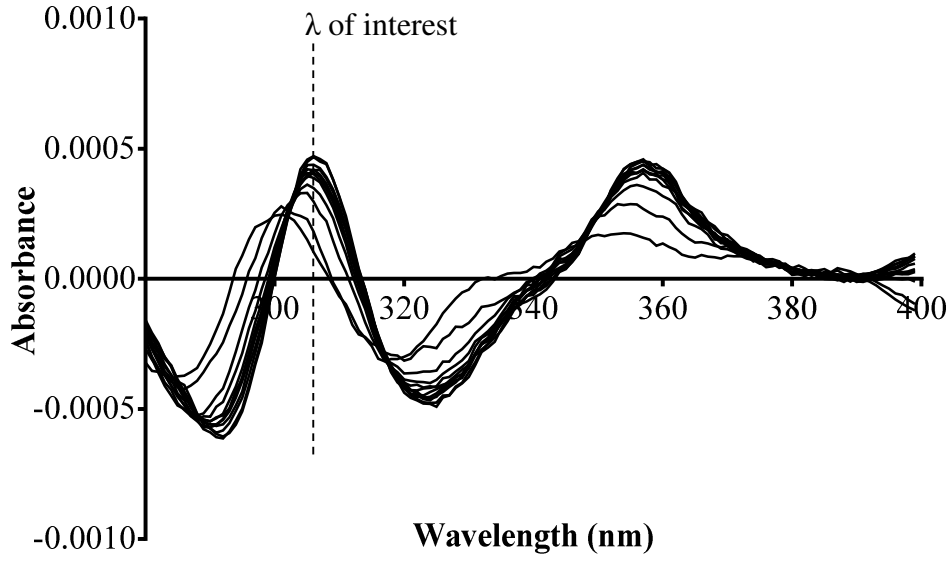


Fig. 3. Molecular structure of the tested molecules: **A.** propranolol, **B.** chlorpromazine, **C.** tripentone, **D.** progesterone, **E.** diclofenac, **F.** indomethacin, **G.** pyridoclax, **H.** capsaicin.

A.


0.4
0.3
0.2
0.1
0.0
-0.1
Absorbance
250
300
350
400
Wavelength (nm)
[phospholipid]

B.


0.0010
0.0005
0.0000
-0.0005
-0.0010
Absorbance
λ of interest
320
360
380
400
Wavelength (nm)

C.

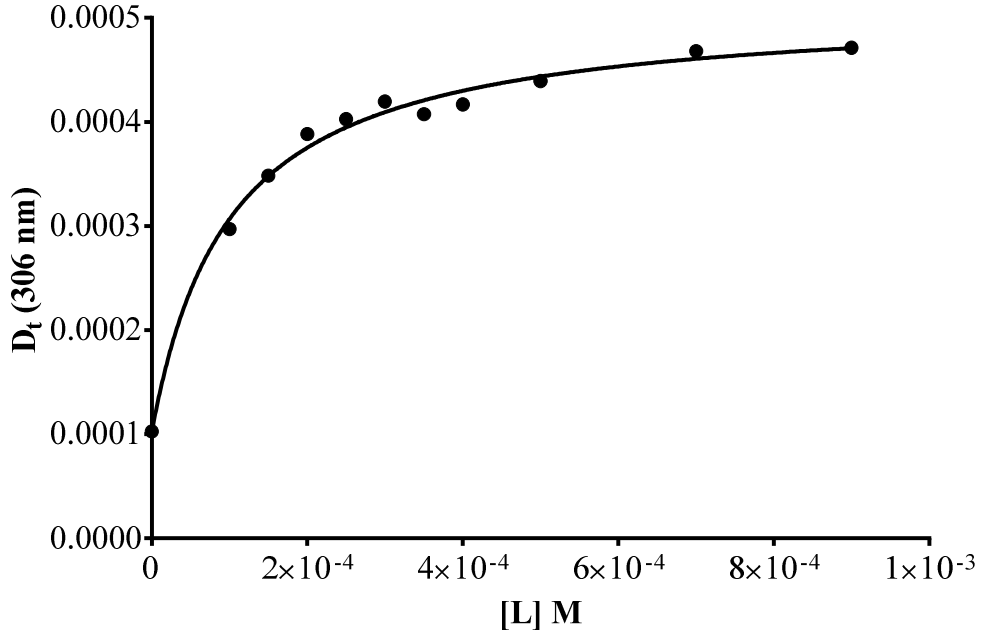


Fig. 4. **A.** Absorption spectra, and **B.** second-derivative spectra of pyridoclax (15 µM) incubated for 1h in the presence of increasing concentrations of phospholipids (SPC-LUVs: 0 to 900µM) in Hepes buffer (pH 7.4) at 37°C. After determination of the wavelength of interest at the vicinity of an isobestic point, D_t, the total membrane second-derivative intensity values were defined at λ=306 nm. **C.** D_t were then plotted in function of the concentration of phospholipids. From this fitted non-linear regression curve, Kp value of pyridoclax was determined using equation (1).

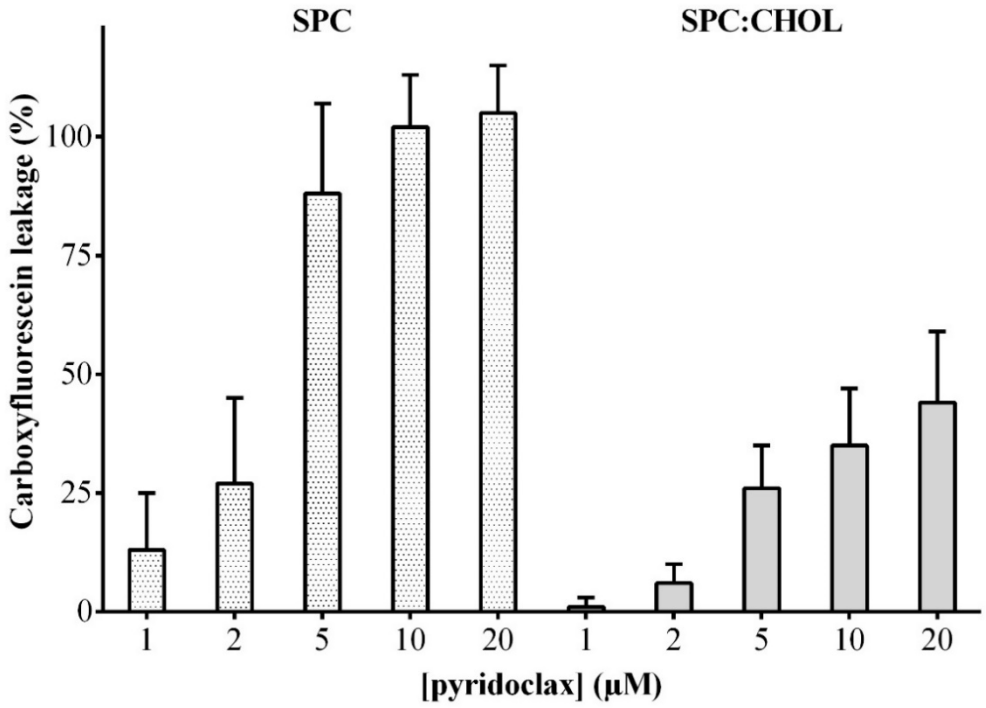
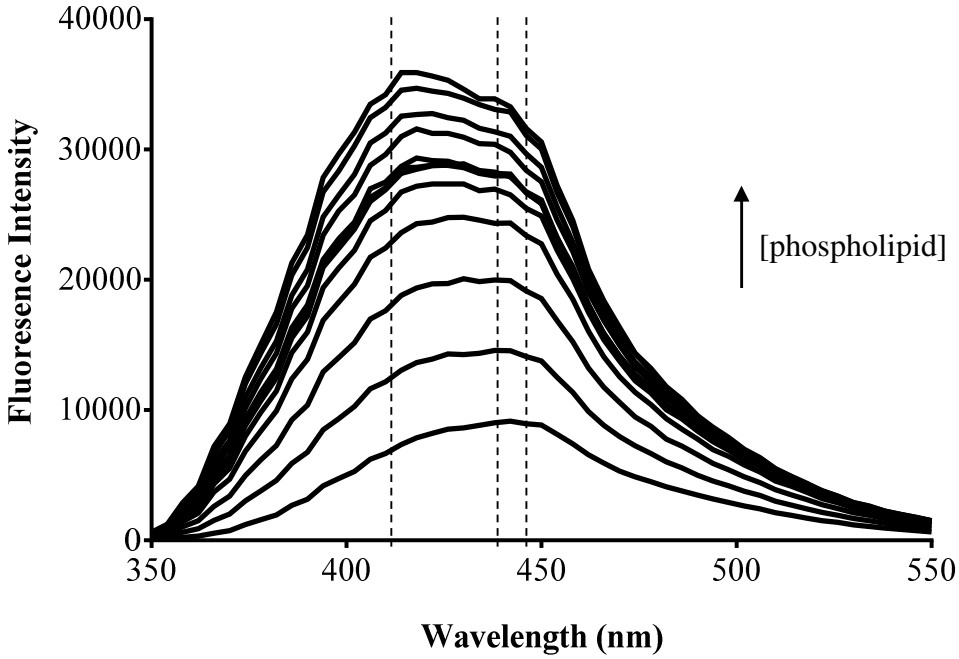


Fig. 5. Carboxyfluorescein leakage from SPC or SPC/cholesterol (80:20, w/w) liposomes (10 µM) as a function of pyridoclax concentration (1, 2, 5, 10, 20 µM) after 3h of incubation at 25°C. Each data point represents the mean of three determinations.

A.



B.

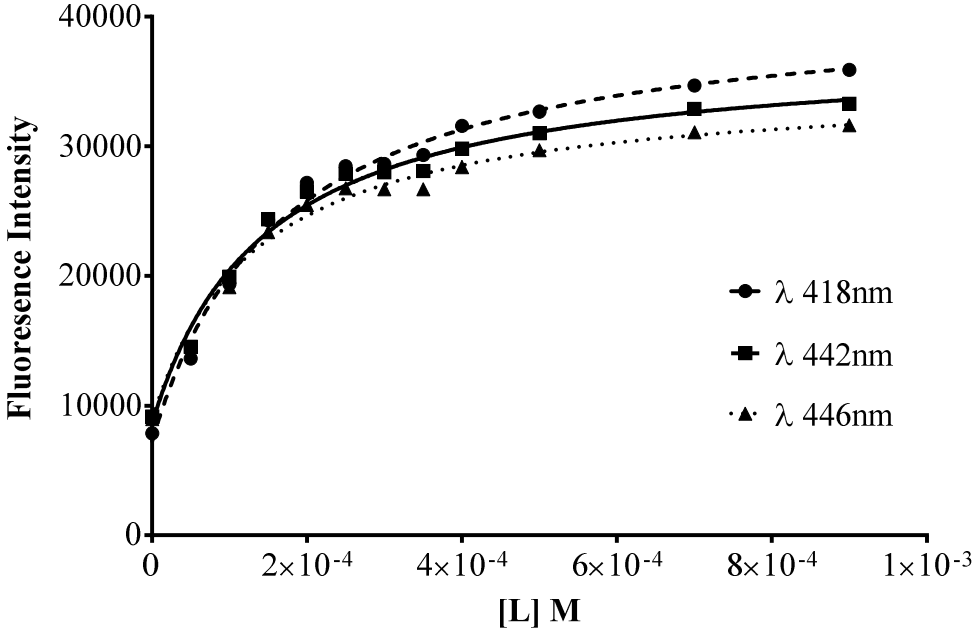


Fig. 6. **A.** Emission spectra of pyridoclax (15 μM, λ_{ex} = 285 nm) incubated in the presence of increasing concentrations of SPC-LUVs (0 to 900 μM) in Hepes buffer (pH 7.4) at 37°C. **B.** The analysis wavelengths for non-linear regression were either the maximum (418 nm), that

appeared during the experiments by increasing phospholipids concentration, or the emission wavelengths of the molecule (442-446 nm). From the fitted non-linear regression curve obtained by plotting fluorescence intensities, Kp value of pyridoclax was determined after applying equation (2).

Tables

Table 1. Characteristics of the LUVs (n = 3) determined directly at the end of the formulation process.

Composition	% (w/w)	Diameter (nm)	PDI	ζ potential (mV)	[C] (mM)
SPC	100	134.8 ± 1.6	0.064 ± 0.019	-2.1 ± 0.4	31.5 ± 0.2
EPC	100	134.0 ± 0.9	0.076 ± 0.004	-1.4 ± 0.4	29.8 ± 0.4
DPPC	100	129.7 ± 0.5	0.071 ± 0.009	0.6 ± 0.1	24.5 ± 0.1
SPC/CHOL	80:20	121.0 ± 0.5	0.062 ± 0.019	-3.1 ± 0.1	43.0 ± 0.8

Table 2. Partition coefficients (expressed as Kp, and log Kp) of the different molecules (15μM) using SPC-based LUVs as model membranes at pH 7.4 and 37°C (n=3), and compared with values (ref log Kp and ref log D) found in the literature, [i] (Alves et al., 2017),[ii] (Avdeef, 2003), [iii] (Castro et al., 2001), [iv] (Lucio et al., 2010), [v] (Pereira-Leite et al., 2013),[vi] (Tomasina, 2009). pKa and maximum emission wavelengths used for the analysis were also defined in this table.

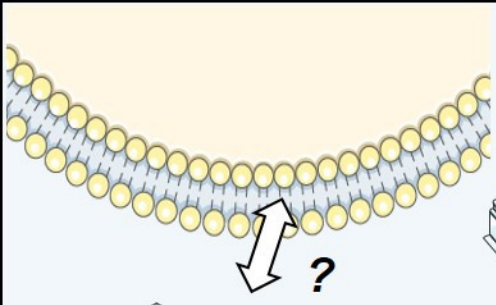
	Propranolol	Chlorpromazine	Tripentone[vi]	Progesterone	Diclofenac	Indomethacin
Kp	712 ± 312	5,828 ± 684	6,442 ± 799	1,432 ± 313	663 ± 106	1,244 ± 204
log Kp	2.73	3.77	3.81	3.16	2.79	3.09
ref log Kp	2.9[ii]	3.9[v]	-	3.5[i]	3.08[iv]	3.15[iii]
ref log $D_{7.4}$[ii]	1.41	3.45	3.94	3.48	1.30	0.68
pKa	9.42	9.30	9.17	-	4.15	4.50
Type	Base	Base	Neutral	Neutral	Acid	Acid
λ (nm)	309	271	336	273	285	297

Table 3. Partition coefficients (expressed as Kp, and log Kp) of pyridoclax (15µM) determined by using LUVs made of various lipids (SPC, EPC, DPP, or mixed SPC/cholesterol (80:20, w/w), at pH 7.4 and 37°C or 50°C (n=3).

	Kp		**Log Kp**	
	UV	Fluorescence	UV	Fluorescence
SPC (37°C)	13,164 ± 2,702	11,637 ± 3,785	4.11	3.97 (λ=418 nm) 4.03 (λ=442 nm) 4.07 (λ=446 nm)
EPC (37°C)	9,251 ± 625	10,596 ± 1,643	3.97	4.02
DPPC (37°C)	1,989 ± 314	1,450 ± 394	3.30	3.15
SPC/CHOL (37°C)	4,045 ± 284	4,290 ± 1,132	3.61	3.63
SPC (50°C)	9,450 ± 1,048	10,574 ± 1,058	3.97	4.02
EPC (50°C)	9,600 ± 3,297	7,104 ± 508	3.97	3.85
DPPC (50°C)	5,243 ± 1,684	4,230 ± 767	3.70	3.62

Table 4. Characteristics of the CF-loaded LUVs determined directly at the end of the formulation process (n=3).

Composition	**Diameter (nm)**	**PDI**	**ζ potential (mV)**
CF-SPC	143.1 ± 0.7	0.051 ± 0.023	-0.8 ± 0.2
CF-SPC/CHOL	135.5 ± 1.1	0.060 ± 0.004	-3.0 ± 1.4



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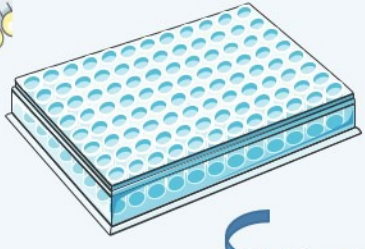
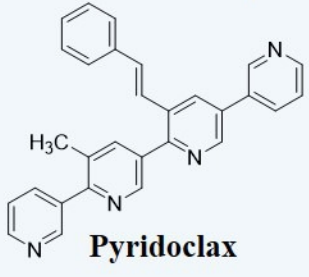


Kp determination for drug discovery



Pyridoclax