LIPOSOMAL CO-PERMEATION ASSAY REVEALS UNEXPECTED MEMBRANE INTERACTIONS OF COMMONLY PRESCRIBED DRUGS

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ABSTRACT

The permeation of small molecules across biological membranes is a crucial process that lies at essence of life. Permeation is involved not only in the maintenance of homeostasis at the cell level but also in the absorption and biodistribution of pharmacologically active substances throughout the human body. Membranes are formed by phospholipid bilayers that represent an energy barrier for the permeating molecules. Crossing this energy barrier is assumed to be a singular event and permeation has traditionally been described as a 1st order kinetic process, proportional only to the concentration gradient of the permeating substance. For a given membrane composition, permeability was believed to be a unary property dependent only on the permeating molecule itself. We provide experimental evidence that this long-held view might not be entirely correct. Liposomes were used in co-permeation experiments with a fluorescent probe, where simultaneous permeation of two substances occurred over a single phospholipid bilayer. Using an assay of six commonly prescribed drugs, we have found that the presence of a co-permeant can either enhance or suppress the permeation rate of the probe molecule, often more than two-fold in each direction. This can have significant consequences for the pharmacokinetics and bioavailability of commonly prescribed drugs when used in combination and provide a new insight into so-far unexplained drug-drug interactions, as well as changing the perspective on how new drug candidates are evaluated and tested.

INTRODUCTION

Membrane permeability and water/membrane partitioning coefficient are two key parameters determining the biodistribution and bioavailability of drugs. They affect the absorption of a drug upon administration (oral, transdermal, inhalation), its subsequent distribution in the body and accumulation in individual organs and tissues.

The rate at which a given molecule permeates across a membrane depends on the energy barrier represented by the tightly packed phospholipid bilayer. The structure of the lipid bilayer can be influenced by the presence of other, non-permeating molecules. This phenomenon is called permeability enhancement and has been studied extensively with regard to skin[19] or intestinal[20] permeability. Examples of simple permeation enhancers include ethanol, oleic acid, or dimethyl sulfoxide, but new enhancers and enhancement mechanisms are being actively investigated[21,22]. An opposite phenomenon – permeation retardation – remains rather unexplored although its biological and pharmacological implications can be just as important[23]. The ability to suppress the permeation rate of specific compounds could, for example, enable previously rejected drugs, which were found to be too "leaky" and thus unsuitable for liposomal formulation[24], to be revisited. Not being aware of permeation enhancement or permeation suppression caused by a medicinal substance that was not *a priori* meant to do so, could be problematic especially in the context of the so-called polypharmacy patients, who are simultaneously prescribed many (typically five or more) medicines simultaneously.

The permeability of a substance across a membrane of given composition has been traditionally assumed to depend only on the properties of the molecule itself (charge, lipophilicity, molar weight, etc.). In textbooks, permeation is described as 1st order kinetic process, proportional only to the concentration gradient of the permeating molecule alone. Experimental and computational permeation results have so far been interpreted in a way that assumed permeability to be a unary property. However, there is an increasing body of scientific literature pointing at potential drug-drug interactions in polypharmacy patients, many of whom are systematically over- or under-dosed due to significantly different bioavailability profiles when some drugs are prescribed in combination rather than alone[25]. Interestingly, such interactions were reported even for drugs that target very different metabolic pathways and that should not,

in theory, influence each other at the molecular target level. These phenomena could potentially be explained by considering permeability a binary (or higher order) property, i.e. by considering that the permeation rate of molecule A could also depend on the concentration of molecule B (or C, etc.). However, no direct experimental evidence for such collective permeation properties has been available so far, and in fact there was no method for reliably measuring co-permeation.

Experimental methods for studying membrane permeability and partitioning typically rely on measuring the concentration change of a single permeant in two macroscopic reservoirs separated by a planar membrane model. The permeation barrier can be formed synthetically from lipidic materials as in the PAMPA assay[1], assembled from living cells as in the Caco-2 permeability method[2], or collected from real tissues such as skin in the Franz diffusion cells[3]. The interpretation and cross-laboratory comparison of data obtained by the above-mentioned methods is complicated by the fact that permeation typically occurs across multiple lipid bilayers, whose exact count is rarely known or reported. Another common feature of the above methods is that the permeation area is limited to a few square cm, which means that very long measurement times are needed in the case of low-permeability substances. Therefore, significant efforts have been devoted also to the development of computational methods for determining membrane permeability and partitioning of individual molecules[4-7].

The problem of low surface area and unknown number of lipid bilayers can be overcome by replacing the macroscopic planar membrane analogue with liposomes. Liposomes are spherical molecular assemblies comprising a lipid bilayer enclosing an aqueous core. Their size and lamellarity can be fairly well controlled[8]. Liposomes are used as drug delivery vehicles thanks to their proven biocompatibility and tuneable properties. Examples of liposome-based drug formulations include Doxil®[9], or recent mRNA COVID-19 vaccines[10,11]. Not all molecules are directly suitable for liposomal encapsulation[12]. Too high or too low permeability prevents a drug from being reasonably retained and released from liposomes. Nevertheless, liposomes lend themselves as a tool for studying permeation and measuring permeability[13]. Methods based on detecting a pH change induced by the permeation of a weak base into liposomes[14], on pre-loading liposomes with engineered receptors whose fluorescence is quenched by the permeating molecule[15], or on the so-called immobilized liposome chromatography[16-18] have been reported.

Here, we present original co-permeation experimental data obtained by means of a new liposome permeation assay on a sample of six commonly prescribed drugs. The principle of the method is shown in Fig. 1. Our data reveal both positive and negative interactions of co-permeating molecules, providing the first direct evidence of collective permeation and partitioning behaviour that could have far-reaching consequences both for the prescription practices of existing drugs, and for the evaluation of new ones.

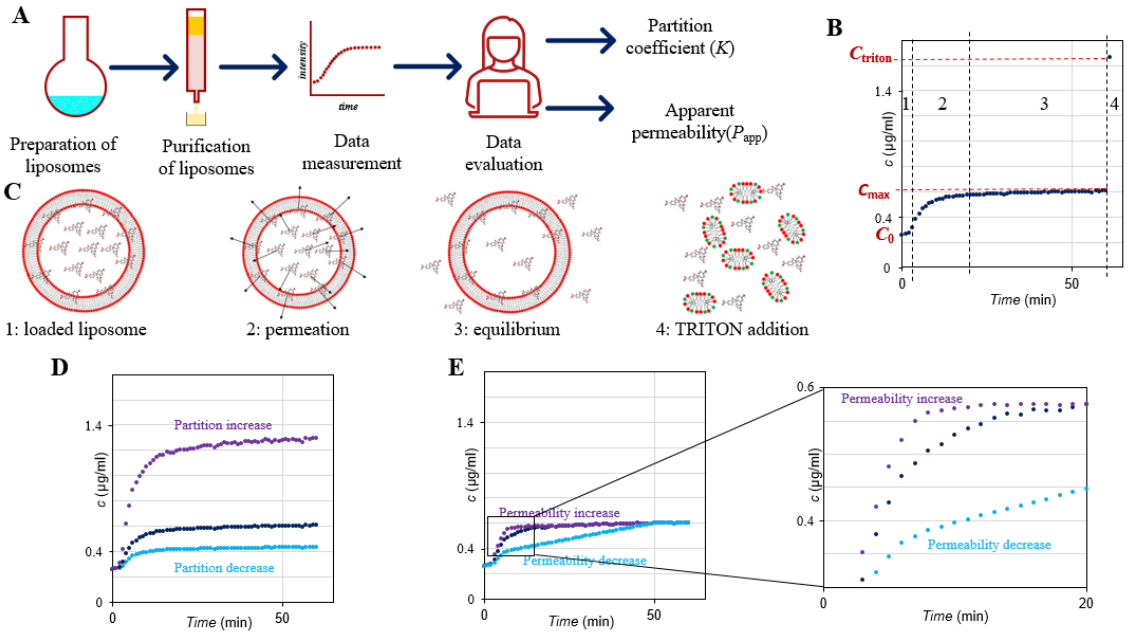


Figure 1: (A) Schematic representation of the liposomal co-permeation method. Liposomes were pre-loaded with a fluorescence probe (carboxyfluorescein, CF) and a co-permeant; after separating liposomes from the supernatant, the release kinetics into a fresh medium was induced by a temperature step; the release curve was evaluated by a mathematical model that provided two parameters: permeability and partitioning coefficient. These were then compared between single-component permeation and co-permeation. (B) Typical result of pure CF permeation, showing four stages. Stage 1: no release at room temperature; stage 1: permeation after heating to lipid bi-layer phase transition; stage 3: equilibrium between intra- and extra-liposomal concentration of the permeant; stage 4: dissolution of lipid bilayer by Triton, causing the release of membrane-bound permeant. (C) Schematic representation of phenomena that occur during each stage of the experiment. (D) Demonstration of positive and negative effect of a co-permeant on the partitioning coefficient (the dark blue symbols represent the original single-component permeation). (E) Demonstration of positive and negative effect of a co-permeant on permeability (the dark blue symbols represent the original single-component permeation, and the magnified section shows different slopes of the release curve).

RESULTS AND DISCUSSION

Single-component permeation measurement by liposomal assay

Dynamic light scattering (Fig. 2A) and TEM (Fig. 2B) analysis of purified liposomes containing encapsulated carboxyfluorescein (CF) as a fluorescent probe reveals that a population of liposomes with a mean particle size around 200 nm was prepared. At a lipid concentration of 5 mg/ml, the total surface area of such liposome is approximately 2 m^2/ml, which represents an increase by a factor of 10^4 compared to traditional permeation assays with planar membranes. The liposomes were colloidally stable; their zeta potential determined by electrophoretic light scattering was (−12.4 ± 1.3) mV. The negative surfaces charge is consistent with the fact that a negatively charged phospholipid DPPG was used as part of the membrane mix.

To utilise liposomes for permeation measurements, the temperature dependence of permeation rate had to be established first. A lipid bilayer can exist in the gel phase or in the liquid disordered phase, which differ dramatically in their permeation properties. The phase transition temperature of the three-component lipid bilayer with cholesterol, which was used in this work, has been previously shown[26] to be 41.5 °C. In a permeation assay, the liposomes should not be permeable at laboratory temperature, but it should be possible to start permeation by raising temperature. Three temperatures were investigated: 30 °C, 40 °C, and 50 °C. The experiment was run for 15 minutes. The time dependence of the relative amount of CF released (Fig. 2C) reveals that at 30 °C, which is safely below the phase transition temperature, there was no permeation throughout the measurement period. At the other extreme at 50 °C, which is well above the phase transition temperature, permeation was too rapid, and it would be inaccurate to evaluate permeability from only a few data points. A suitable temperature thus proved to be 40 °C, which was just below the phase transition but close enough for CF permeation to already occur at a reasonable rate. The measured CF release curve (time dependence of concentration over time taken from the inflexion point onwards) was regressed by an algebraic model, detailed in the Methods section. An excellent agreement between the model and experiment was obtained (Fig. 2D).

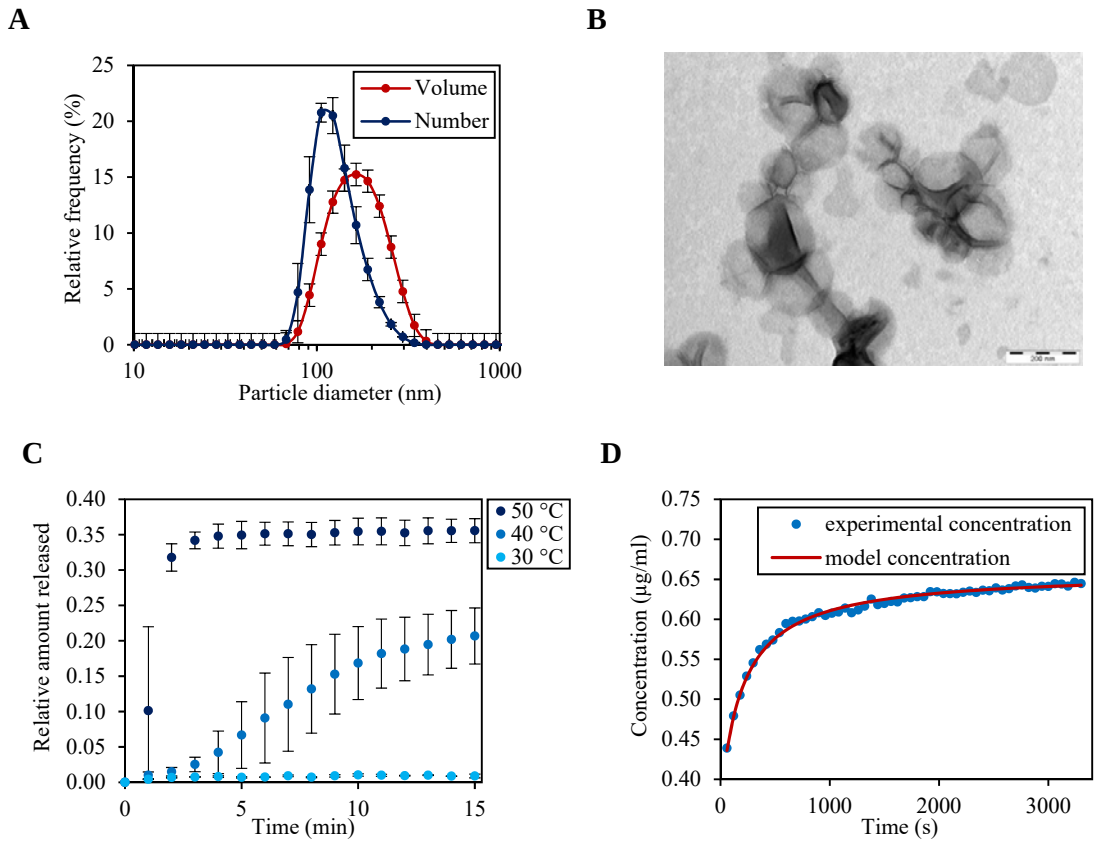


Figure 2: (**A**) Particle size distribution of liposomes with encapsulated CF, measured by dynamic light scattering. (**B**) TEM micrograph of the prepared liposomes. (**C**) Thermally induced release of encapsulated CF from liposomes at three different temperatures (the phase transition temperature of the used lipid bilayer is 41.5 °C). The data points are mean values and error bars indicate standard deviations (n = 3). (**D**) Comparison of CF release curve measured at 40 °C with regression by a mathematical model, which was used for evaluation permeability from the experimental data.

The liposomal permeability of CF in PBS medium had a value of $(1.4 \pm 0.4) \cdot 10^{-8}$ cm/s, which is consistent with previously reported values obtained from the COSMOPerm calculation ($\approx 10^{-8}$ cm/s)[4,27]. Furthermore, the partition coefficient was evaluated for this sample according to Eq. 3.2, which had a value of 1.6 ± 0.1. This value is again consistent with COSMOPerm calculation (≈1).

Direct observation of permeation enhancement mechanisms

The liposomal assay employed in this work allows direct observation of permeation enhancement in a single layer of phospholipids. Two well-known permeation enhancers with different enhancement mechanism were studied: ethanol and oleic acid. Permeation

enhancement was investigated at 30 °C, as no CF release occurred at this temperature under normal conditions. The effect of ethanol was investigated by stepwise addition of small quantities of ethanol (40 µl in each step) to a spectrophotometric cuvette containing a sample of liposomes containing CF. A stepwise release of CF from the liposomes was observed (Fig. 3A) after the addition of each ethanol aliquot. Ethanol is known to cause lipid extraction from the membrane and to subsequently form a second phase together with the extracted lipids[28]. After each ethanol addition, release from the affected liposomes was immediate but other liposomes remained intact as the added ethanol was bound with the extracted lipids. The increment of CF release in each step corresponds to the liposomes whose membrane integrity was disrupted by ethanol addition.

The second studied permeation enhancer was oleic acid. Oleic acid is known to work by a rather different mechanism than ethanol. Instead of irreversibly damaging liposomes, it incorporates itself into the membrane structure, slightly disrupts the ordered packing of the phospholipids, and makes the membrane more permeable to all molecules[29]. Even though the measured permeation was very slow (CF release occurred over 10 hours), permeability still increased from a limiting value close to zero to $6.3 \cdot 10^{-10}$ cm/s (Fig. 3B). The two permeation enhancement experiments demonstrate the ability of the liposomal assay to capture the effect of additional chemical species on the permeation rate of the fluorescent probe.

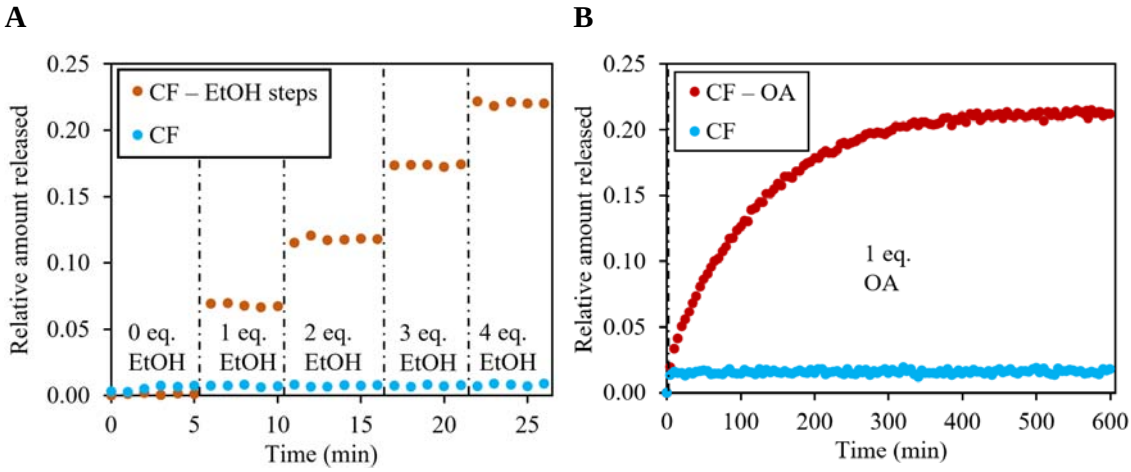


Figure 3: Experimentally measured dependence of the relative amount of CF released from liposomes on time at 30 °C. (**A**) Stepwise addition of ethanol into the system. (**B**) Addition of oleic acid. Note that the duration of the experiment was 600 min in the case of oleic acid. Blue data points represent the base case (only CF), red data points represent permeation in the presence of the permeation enhancer.

Membrane interactions revealed by co-permeation experiments

Having established that the liposome permeation assay makes it possible to directly observe permeation enhancement, we pose the question of whether commonly used pharmaceutical compounds might inadvertently modulate the membrane permeability and/or partitioning of another substance. A panel of 6 clinically approved drugs spanning all four Biopharmaceutics Classification System (BCS) classes[30] has been chosen for co-permeation experiments (Table 1). Based on their lipophilic/hydrophilic character, the drugs were incorporated into liposomes either by the aqueous route (i.e., dissolved in the hydration medium together with CF) or by the lipidic route (i.e., dissolved in chloroform and methanol together with the membrane lipids). For lipophilic compounds mildly solubile in water (HCTZ and NX), both loading methods were used (Table 1).

Table 1: Pharmaceutical compounds evaluated in co-permeation experiments, their properties and concentrations used. Note that CF concentration was 7.5 mg/ml in all cases.

Name and acronym	Indication	BCS Class	Properties	Liposome incorporation route and concentration
Ascorbic acid (ACS)	Essential vitamin	Class I	well soluble well permeable	aqueous (15 mg/ml)
Hydrochlorothiazide (HCTZ)	Hypertension	Class II	mildly soluble (0.72 mg/ml[31]) well permeable	lipidic & aqueous (0.5 mg/ml)
Kanamycin (KM)	Antibiotic	Class III	well soluble poorly permeable	aqueous (15 mg/ml)
Norfloxacin (NX)	Antibiotic	Class IV	mildly soluble (0.28 mg/ml [32]) poorly permeable	lipidic & aqueous (0.2 mg/ml)
Candesartan cilexetil (CC)	Hypertension	Class II	poorly soluble well permeable	lipidic (0.5 mg/ml)
Apixaban (APIX)	Anticoagulant	Class IV	poorly soluble poorly permeable	lipidic (0.5 mg/ml)

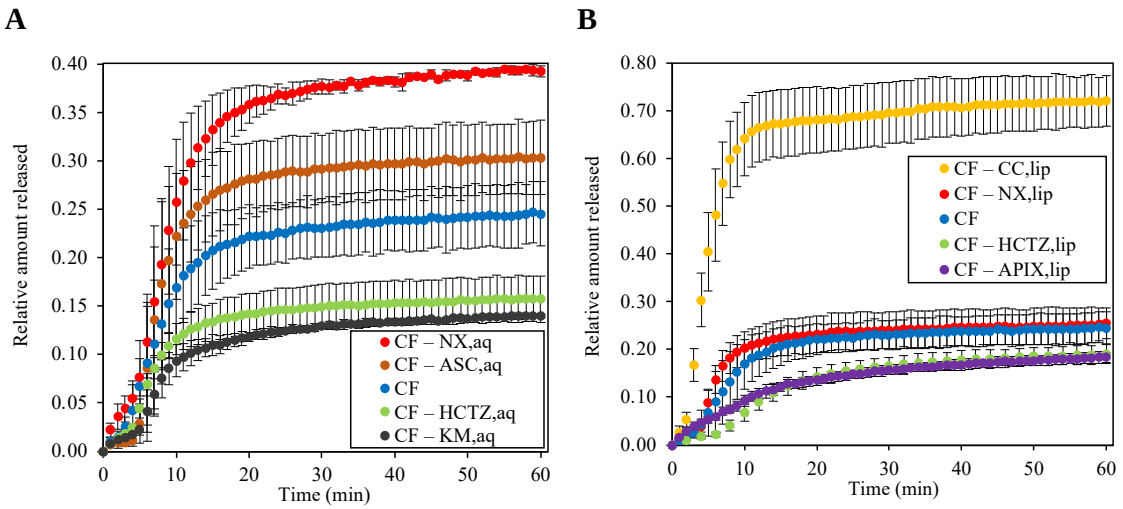


Figure 4: Relative amount of CF released as function time in binary co-permeation experiments conducted using liposomal assay at 40 °C. (**A**) Substances incorporated into liposomes by the aqueous route. (**B**) Substances incorporated into liposomes by the lipidic route. The permeation of CF alone is shown in both cases for reference. The acronyms of individual substances are given in Table 1. The data points are mean values, the error bars represent standard deviations (n = 3). Note the difference in the y-axis scale between cases (A) and (B).

Unexpected phenomena were observed during binary co-permeation experiments (Fig. 4). All investigated pharmaceutical substances (regardless of their molar weight, aqueous solubility, lipophilicity or BCS class) had a manifestable and sometimes very strong effect on CF permeation, although these substances are not *a priori* meant to act as permeation enhancers or retardants, and no such behaviour has been reported for them before. An increase in the asymptotic quantity released of CF was found for binary co-permeation with ASC_{aq}, NX_{aq}, and CC_{lip}, whereas a decease was found for $HCTZ_{aq}$, KM_{aq}, $HCTZ_{lip}$, and $APIX_{lip}$ (Fig. 4). Curiously, the increase in the relative amount released was caused by a pair of substances from exactly opposite BCS classes: ASC with high solubility and high permeability and NX with low solubility and low permeability. The same was true for the two substances that reduced the relative amount released: HCTZ with a low solubility and high permeability and KM with a high solubility and low permeability. These results suggest that solubility/permeability of the co-permeating substance alone is insufficient to determine its effect on the quantity released of the fluorescent probe. Clearly, both antagonistic and synergistic effects between the permeants exist, and these are sufficiently strong to change CF membrane partitioning 2-5x in both directions, and permeability up to 2x upwards and up to 6x downwards (Table 2). From the point of view of pharmacokinetics, such changes due to drug-membrane interaction could have dramatic therapeutic implications and could potentially lead to incorrect prescription and dosing

decisions, which are typically made on the assumption that each drug behaves as if it were in the patient's body alone. As no simple rule based on the BCS class can explain the experimental data, let us briefly consider the specific features of each permeant.

Table 2: Experimentally determined values of permeability and partition coefficient for CF alone and in co-permeation in binary mixtures with selected drugs added to the liposomal assay either by the aqueous or lipidic route.

Sample	Permeability (cm/s)	Partition coefficient
CF alone	$(1.4 \pm 0.4) \cdot 10^{-8}$	1.6 ± 0.1
CF–ASC_{aq}	$(2.4 \pm 0.7) \cdot 10^{-8}$	1.4 ± 0.2
CF–$HCTZ_{aq}$	$(1.5 \pm 0.3) \cdot 10^{-8}$	3.5 ± 0.5
CF–KM_{aq}	$(1.2 \pm 0.3) \cdot 10^{-8}$	3.9 ± 0.1
CF–NX_{aq}	$(2.3 \pm 0.5) \cdot 10^{-8}$	0.92 ± 0.04
CF–CC_{lip}	$(2.2 \pm 0.7) \cdot 10^{-8}$	0.32 ± 0.09
CF–$APIX_{lip}$	$(3.1 \pm 0.4) \cdot 10^{-9}$	1.9 ± 0.1
CF–$HCTZ_{lip}$	$(1.1 \pm 0.1) \cdot 10^{-8}$	2.7 ± 0.3
CF–NX_{lip}	$(2.2 \pm 0.2) \cdot 10^{-8}$	2.1 ± 0.3

Ascorbic acid (ASC) was added only by the aqueous route and caused CF permeability to be approximately doubled while the partition coefficient remained the same within the measurement error. Ascorbic acid is predominantly present in the anionic form (Table 3). Therefore, we suggest that this permeability increase can be influenced by the molecule charge. The negatively charged ASC molecules can locally increase the distances between the polar heads of the lipid molecules and therefore increase the permeation rate of CF through the membrane without affecting its partitioning coefficient. Thus, co-permeation with ASC has an enhancing effect on CF permeation.

Hydrochlorothiazide (HCTZ) and **Kanamycin** (KM) had the same effect on the permeation properties of CF (permeability remained the same within the measurement error, but the partition coefficient increased). Therefore, a similarity was sought between these substances. Both KM and HCTZ have ionizable NH_2 groups (Table 3), which allows both molecules to exist in a slightly positively charged form at the experimental pH 7.4. Either a change in the

membrane packing, or a temporary association with CF, could cause an increase of membrane partitioning. It should be noted that the increase in CF partitioning coefficient is different for HCTZ samples made by the aqueous and the lipid route (Table 2). This could be caused by the different amount of HCTZ remaining in the sample after liposome purification.

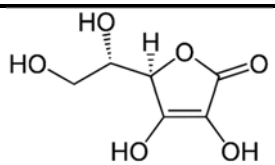
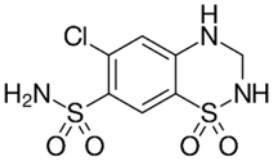
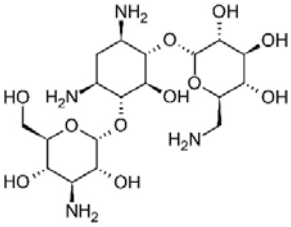
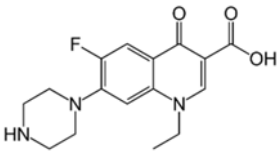
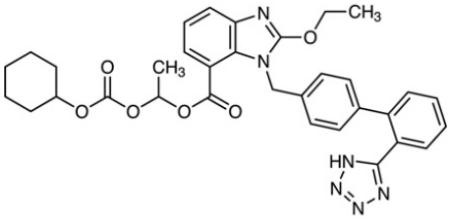
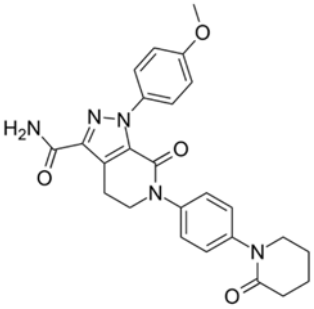
Norfloxacin (NX) again nearly doubled CF permeability, but the change of CF partition coefficient depends on the method of addition. At pH 7.4, NX is primarily a zwitterion, but since both the basic and acidic pKa is close to the used pH (7.4), there is a non-negligible amount of both anionic and cationic form. An approximate ratio of the three forms is zwitterion : anion : cation = 89 : 7 : 4. The anion can play the same role in increasing CF permeability as in the case of ASC described above. The difference in the partition coefficient for both ways of addition remains unclear.

Candesartan cilexetil (CC) occurs in a slightly negatively charged form, and the trend for enhancing CF permeability was confirmed, similarly to ASC and NX. Furthermore, there was a significant decrease in the partition coefficient. This may have been because CC is a very lipophilic and large molecule, which may have displaced CF from the membrane by its presence in the membrane during co-permeation. Consequently, the partition coefficient of CF was significantly reduced.

Apixaban (APIX) caused an approximately sixfold decrease in permeability for CF. This could be because APIX is an uncharged rigid molecule that may very distantly resemble sterols. Theoretically, it could incorporate into the membrane during co-permeation, increasing its rigidity and decrease its permeability for CF. Further correlative evidence for this hypothesis is the plot of the relative amount released. When CF is mixed with this substance, the curve has no inflexion point, as is the case for all mixtures with other substances. At the same time, however, its incorporation does not seem to affect the partition coefficient in any way, so its presence does not displace CF from the membrane.

A detailed mechanistic explanation of how each of the investigated substances might affect CF permeability and partitioning coefficient will be obtained by molecular dynamics simulations, but this is beyond the scope of the present Communication.

Table 3: Properties of substances used during co-permeation experiments with CF.

Substance	Molecule structure	pKa	Charge at pH 7.4
ASC		acidic 4.7	negative
HCTZ		basic 7.9	slightly positive
KM		basic 9.5	positive
NX		acidic 6.3 basic 8.8	prevalently zwitterion
CC		acidic 6.0	slightly negative
APIX		–	neutral

CONCLUSION

Using a novel permeation measurement methodology based on a liposomal assay, the permeation enhancement or suppression during co-permeation of two substances has been directly investigated for the first time. As a methodology validation after the selection of an appropriate temperature, two agents with known permeation enhancement properties due to membrane disruption were studied (ethanol and oleic acid). In the case of ethanol addition, a stepwise release of the permeant (CF) was observed. This was due to the extraction of lipids from the membrane by ethanol and the loss of membrane integrity in the affected the liposomes from which CF could leak out. Oleic acid worked on a different principle, which, due to its incorporation into the membrane, caused gradual permeation of CF even at 30 °C, i.e., well below the phase transition of the original membrane. A mathematical model of permeation enables the quantitative evaluation of permeability and the membrane partitioning coefficient of the permeant.

The liposomal permeation assay was then used for investigating the effect of six commonly prescribed pharmaceutical substances on permeability and partition coefficient during binary co-permeation experiments. The chosen substances are not meant to act as permeation modifiers and no such behaviour has been measured or reported for these molecules before. Unexpectedly, all six investigated substances were found to have a significant effect on the permeability and/or partitioning coefficient of the permeant. Depending on the substance, either enhancement or suppression of permeation was observed (by a factor of up to 6x). The membrane partitioning coefficient was influences by a factor of up to 5x, again both upwards and downwards depending on the co-permeant. There was no simple correlation between the BCS class of the investigated drug and its effect on permeation. Specific molecular interactions with the permeant (CF) and/the membrane lipids were therefore likely the cause of permeation modification in each case.

The liposomal co-permeation assay introduced in this Communication is fast and reproducible. The results indicate unexpected and previously unknown drug-membrane interactions that can have far-reaching consequences for the pharmacokinetics of commonly prescribed drugs in polypharmacy patients. As both permeability and the membrane partitioning coefficient can be upregulated or downregulated several times in a manner that is difficult to predict simply from

the molecular properties, this work highlights the need for a systematic screening of currently prescribed drugs for interactions at the permeation and biodistribution level, rather than at the metabolic level. The knowledge obtained in such co-permeation screening should then lead to better informed prescription and dosage decisions by physicians who so-far rely solely on single-molecule data.

METHODS

Materials

Phosphate-buffered saline in tablets (PBS), 5(6)-carboxyfluorescein (CF, >95%), norfloxacin (NX, >98%), cholesterol (>99%), kanamycin sulfate (KM), TRITON X-100® (laboratory grade), and oleic acid (OA, 90%) were purchased from Sigma-Aldrich s.r.o. Dipalmitoylphosphoglycerole (DPPG) and dipalmitoylphosphatidylcholine (DPPC) were purchased from Corden Pharma. Sodium hydroxide (NaOH, p. a.), ascorbic acid (ASC, p. a.), sodium chloride (NaCl, p. a.) phosphoric acid (H_3PO_4, >75%), and disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12\ H_2O$) were purchased from PENTA s.r.o. Chloroform (p. a.), and ethanol (EtOH, >99.8%) were purchased from Lach-Ner s.r.o. and methanol (>99.8%) was purchased from Fisher Scientific s.r.o. Hydrochlorothiazide (HCTZ), candesartan cilexetil (CC) and apixaban (APIX) were kindly provided by Zentiva k.s. All substances and materials were used as supplied and were not modified. Deionized water (Aqual 25, 0.07 μS/cm) was used in all experiments.

Preparation of liposomes

Liposomes were prepared by the standard lipid film hydration method. the mixture of phospholipids and cholesterol (8.1 mg DPPC, 1.1 mg DPPG, 0.9 mg cholesterol) was dissolved in 10 ml of methanol:chloroform solution (1:1 by volume). Subsequently, the solvent mixture was evaporated on a vacuum rotary evaporator (60 °C, gradually reducing the pressure from atmospheric to approximately 80 mbar). This process produced a dried lipid film which was subsequently dried in a desiccator for at least 3 hours (30 mbar).

The completely dried lipid film was then hydrated with 2 ml of aqueous medium (7.5 mg/ml of carboxyfluorescein solution in PBS, pH 7.4). The sample and the extruder (Avanti Mini Extruder) were heated to 69 °C for 10 minutes and the sample was then vortexed

to form polydisperse multilamellar liposomes. To increase the uniformity, the sample was extruded at least 21 times through a membrane with a pore size of 200 nm (at 69 °C).

The prepared liposomes were characterized. Particle size distribution was determined using dynamic light scattering (DLS), the zeta potential was determined using electrophoretic light scattering (ELS) (both Malvern Zeta sizer Nano-ZS), and by images from a transmission electron microscope (TEM – Jeol JEM-1010 – accelerating voltage 80 kV).

Encapsulation of co-permeants

The hydrophilic substances (ascorbic acid and kanamycin) and the mildly soluble lipophilic substances (hydrochlorothiazide and norfloxacin) were added to the hydration medium (solution CF in PBS) during lipid film hydration (aqueous addition route). Lipophilic substances (apixaban, candesartan cilexetil, hydrochlorothiazide and norfloxacin) were added during the first step of liposome preparation, i.e., they were mixed with the phospholipids and dissolved in a mixture of chloroform and methanol (lipid addition route). All samples were prepared in triplicates.

Purification of liposomes

All liposome samples were purified by size exclusion chromatography using PD Minitrap™ G-25 separation columns to separate the surrounding hydration solution from the liposomes themselves. In this way, 1 ml of purified liposome solution was collected. The principle of CF release kinetics measurement is based on the fluorescence quenching of concentrated CF. The intraliposomal CF does not fluoresce; its fluorescence increases sharply only upon dilution after release from the liposomes. For this reason, the hydration medium had to be separated from the liposomes before conducting any permeation experiments.

Permeation measurement

From a stock of purified liposomes, 60 µl was pipetted into a disposable cuvette and mixed with 1140 µl of PBS. Then the measurement (in triplicates for each sample) of CF permeation through the membrane was carried out in a fluorescence spectrophotometer (Cary Eclipse, Agilent) in which the sample was heated to the desired temperature (30 °C, 40 °C and 50 °C), which was kept constant throughout the measurement. The following settings were used: excitation wavelength 490 nm, emission wavelength 522 nm, excitation slit: 2.5 and 2.5, scan control: slow, detector voltage: medium, maximum intensity: 1000 a.u.. The time dependence

of the fluorescence intensity at constant temperatures was measured. At the end of the experiment, 5 µl of ten times diluted TRITON X-100® was added to cause total micellization of the system, thus releasing all previously unreleased CF. The mechanism of this micellization is shown in Fig. 1C and is based on molecular dynamics study[33]. The measured fluorescence intensity dependence on time was then converted to CF concentration using a calibration curve. The relative amount released of CF was then determined:

$$Relative\ amount\ released = \frac{c_{\mathrm{t,CF}} - c_{\mathrm{1,CF}}}{c_{\mathrm{triton,CF}} - c_{\mathrm{1,CF}}}, \quad \textbf{(1)}$$

where $c_{\mathrm{t,CF}}$ is the mass concentration of CF at a time t, $c_{\mathrm{1,CF}}$ is the CF mass concentration at the beginning of the measurement, and $c_{\mathrm{triton,CF}}$ is the final CF mass concentration after liposome micellization by the addition of TRITON X-100®. The partition coefficient was calculated from the mass balance using the relation:

$$K_{\mathrm{CF}} = \frac{c_{\mathrm{triton,CF}} - c_{\mathrm{fin,CF}}}{c_{\mathrm{fin,CF}}}, \quad \textbf{(2)}$$

where $c_{\mathrm{fin,CF}}$ is the asymptotic mass concentration of CF achieved by thermal release, i.e. the final concentration at the end of the experiment just before TRITON addition.

Permeation enhancers

For the study of permeation enhancers, CF-containing liposomes were prepared and purified as described above. For permeation enhancement by ethanol, 60 µl of purified liposomes with encapsulated CF was mixed with 1140 µl of PBS in a measuring cuvette. The samples were maintained at 30 °C. At approximately 5-minutes intervals, 40 µl of ethanol was added to the measuring cuvette from the top and the fluorescence intensity was measured by fluorescence spectrophotometry as described above. For permeation enhancement by oleic acid, the procedure was very similar to ethanol, only the volumes were different (50 µl oleic acid, 1090 µl PBS) and only one addition at the start of the experiment was done. The temperature was also 30 °C.

Model for permeability determination

The model was used for evaluating permeability from its definition, using the dependence of concentration on time:

$$j_{\mathrm{CF}} = P_{\mathrm{erm,CF}} \cdot \left(c_{\mathrm{liposome,CF}} - c_{\mathrm{t,CF}}\right), \quad \textbf{(3)}$$

where j_{CF} is a flux of the permeating substance, $P_{\mathrm{erm,CF}}$ is its permeability, and $c_{\mathrm{liposome,CF}}$ is the mass concentration of CF inside the liposomes. The previously mentioned $c_{\mathrm{t,CF}}$ was calculated

using the calibration curve from the measured fluorescence intensity over time. The unknown quantity $c_{\text{liposome,CF}}$, was obtained from the mass balance, considering that the total quantity of CF, which is known, is present in the liposomal lumen, in the membrane, in the bulk outside liposomes. We start with the basic expression:

$$c_{\text{liposome,CF}} = \frac{m_{\text{liposome,CF}}}{V_{\text{liposome}}}, \quad \textbf{(4)}$$

where $m_{\text{liposome,CF}}$ is the mass of CF inside the liposomes and V_{liposome} is the volume inside the liposomes in the measured sample, and combine it with the mass balance:

$$m_{\text{liposome,CF}} = m_{\text{total,CF}} - m_{\text{lipids,CF}} - m_{\text{t,CF}}, \quad \textbf{(5)}$$

where $m_{\text{total,CF}}$ is the total encapsulated mass of CF in the sample, $m_{\text{t,CF}}$ is the measured mass of CF in bulk outside liposomes at time *t*, and $m_{\text{lipids,CF}}$ is the mass of CF in the membrane, which is released only after TRITON addition and is be expressed as:

$$m_{\text{lipids,CF}} = m_{\text{total,CF}} - m_{\text{fin,CF}}. \quad \textbf{(6)}$$

Using this equation, eq. (3.5) becomes:

$$m_{\text{liposome,CF}} = m_{\text{fin,CF}} - m_{\text{t,CF}}, \quad \textbf{(7)}$$

where $m_{\text{fin,CF}}$ is the final mass of CF released during the experiment only by diffusion (i.e before TRITON addition). For the determination of $m_{\text{fin,CF}}$ and $m_{\text{t,CF}}$ it was necessary to use the conversion using the volume of the diluted sample (volume in the cuvette, V_{cuvette}) and a certain concentration ($c_{\text{j,CF}}$), which was evaluated from the measured intensity using a calibration curve:

$$m_{\text{j,CF}} = c_{\text{j,CF}} \cdot V_{\text{cuvette}} \quad \textbf{(8)}$$

Next, it was necessary to express V_{liposome} in (eq. 3.4) as follows:

$$V_{\text{liposome}} = V_{\text{liposome,1}} \cdot N_{\text{liposome}}, \quad \textbf{(9)}$$

where $V_{\text{liposome,1}}$ is the volume inside one liposome and N_{liposome} is the number of liposomes in the measured sample. $V_{\text{liposome,1}}$ could be determined with the following equation:

$$V_{\text{liposome,1}} = \frac{\pi}{6} \cdot d^3_{\text{liposome,in}}, \quad \textbf{(10)}$$

where $d_{\text{liposome,in}}$ is the inner diameter of the liposomes and was determined as follows:

$$d_{\text{liposome,in}} = d_{\text{liposome,volume}} - 2 \cdot d_{\text{membrane}}, \quad \textbf{(11)}$$

where $d_{\text{liposome,volume}}$ is the volume-weighted diameter measured by dynamic light scattering (Malvern Zetasizer) and d_{membrane} is 4.059 nm [34] for using the composition. In eq. 9, N_{liposome} was also expressed as:

$$N_{\text{liposome}} = \frac{m_{\text{lipid,sample}}}{m_{\text{lipid,1liposome}}}, \quad \textbf{(12)}$$

where $m_{\text{lipid,sample}}$ is the mass of lipids in the measured sample and $m_{\text{lipid,1liposome}}$ is the mass of lipids that form one liposome. The first mentioned was further modified to:

$$m_{\text{lipid,sample}} = c_{\text{lipid,sample}} \cdot V_{\text{sample}}, \quad \textbf{(13)}$$

where V_{sample} is the volume of the concentrated liposome sample, which is further diluted to the volume V_{cuvette} and measured. Furthermore, $c_{\text{lipid,sample}}$ (which is the mass concentration of lipids in this sample volume – V_{sample}) was determined as follows:

$$c_{\text{lipid,sample}} = \frac{m_{\text{lipid,column}}}{V_{\text{lipid,column}}}, \quad \textbf{(14)}$$

where $m_{\text{lipid,column}}$ is the mass of lipids to be purified on the column, which was 5 mg for all experiments and $V_{\text{lipid,column}}$ is the volume of the sample taken from the column, which was 1 ml for all experiments. From eq. 12, $m_{\text{lipid,1liposome}}$ was also modified:

$$m_{\text{lipid,1liposome}} = n_{\text{lipid,1liposome}} \cdot M_{\text{lipid}}, \quad \textbf{(15)}$$

where $n_{\text{lipid,1liposome}}$ is the molar amount of lipids that form a single liposome and M_{lipid} is the mean molar mass of the lipids used (DPPC, DPPG, Cholesterol):

$$M_{\text{lipid}} = \sum\left(x_{\text{lipid,l}} \cdot M_{\text{lipid,l}}\right), \quad \textbf{(16)}$$

where $x_{\text{lipid,l}}$ is the molar fraction of lipid *l* and $M_{\text{lipid,l}}$ is the molar mass of the lipid *l*. For the composition used and the molar ratio of lipids was M_{lipid} = 683.02 g/mol. From eq. 15, $n_{\text{lipid,1liposome}}$ was determined by definition:

$$n_{\text{lipid,1liposome}} = \frac{N_{\text{lipid,1liposome}}}{\mathrm{N_A}}, \quad \textbf{(17)}$$

where $\mathrm{N_A}$ is Avogadro's number and $N_{\text{lipid,1liposome}}$ is the number of lipids that form a single liposome, and it was determined as follows:

$$N_{\text{lipid,1liposome}} = \frac{2 \cdot A_{\text{liposome,1}}}{A_{\text{lipid,1}}}, \quad \textbf{(18)}$$

where $A_{\text{lipid,1}}$ is the area of one lipid, which is 47.9 Å^2 [35]. Since the liposome is made up of a lipid bilayer, it should be considered that the liposome has a double area (inner and outer). Therefore, the area of the liposome is multiplied by two. $A_{\text{liposome,1}}$ is the surface of one liposome through which the substance *i* permeates:

$$A_{\text{liposome,1}} = \pi \cdot \left(\frac{d_{\text{liposome,volume}} + d_{\text{liposome,in}}}{2}\right)^2 \quad \textbf{(19)}$$

The left side of eq. 3 has also been modified as follows:

$$j_{\mathrm{CF}} = \frac{\dot{m}_{\mathrm{through,CF}}}{A_{\mathrm{liposome,total}}} = \frac{m_{\mathrm{through,CF}}}{A_{\mathrm{liposome,total}} \cdot t}, \quad \textbf{(20)}$$

where $\dot{m}_{\mathrm{through,CF}}$ is the mass flow of CF passing through the liposomal membrane, $A_{\mathrm{liposome,total}}$ is the total surface of all liposomes in the measured sample, t is time, and $m_{\mathrm{through,CF}}$ is the mass of CF passing through the liposomal membrane:

$$m_{\mathrm{through,CF}} = m_{\mathrm{t,CF}} - m_{1,\mathrm{CF}}, \quad \textbf{(21)}$$

where $m_{1,\mathrm{CF}}$ is the initial mass of CF in the area around the liposomes before release and was determined by eq. 8. $A_{\mathrm{liposome,total}}$ was determined as follows:

$$A_{\mathrm{liposome,total}} = A_{\mathrm{liposome,1}} \cdot N_{\mathrm{liposome}}, \quad \textbf{(22)}$$

where $A_{\mathrm{liposome,1}}$ was determined from eq. 19 and N_{liposome} from eq. 12.

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Conflict of interest statement

The authors declare no conflict of interest, financial or otherwise.

Author contribution

K.O. – Methodology development, experimental investigation, mathematical model development, data analysis, manuscript writing.

M.B. – Methodology development, experimental investigation, mathematical model development, data analysis, manuscript writing

K.S. – Permeability data analysis, manuscript writing

A.Z. – Instrumental analytical method development, liposome preparation, manuscript writing

K.B. – Supervision, results interpretation, manuscript writing

F.S. – Project idea conception, funding acquisition, supervision, results interpretation, manuscript writing