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Binding of the small-molecule kinase inhibitor ruxolitinib to membranes does not disturb membrane integrity

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ABSTRACT

Ruxolitinib is a small-molecule protein kinase inhibitor, which is used as a therapeutic agent against several diseases. Due to its anti-inflammatory impact, ruxolitinib has also been considered recently for usage in the treatment of Covid-19. While the specific effects of ruxolitinib on Janus kinases (JAK) is comparatively well investigated, its (unspecific) impact on membranes has not been studied in detail so far. Therefore, we characterized the interaction of this drug with lipid membranes employing different biophysical approaches. Ruxolitinib incorporates into the glycerol region of lipid membranes causing an increase in disorder of the lipid chains. This binding, however, has only marginal influence on the structure and integrity of membranes as found by leakage and permeation assays.

1. Introduction

Protein kinases are a highly important protein family [1]. These proteins trigger the phosphorylation of free hydroxyl groups of specific amino acid residues, i.e. tyrosine, serine, or threonine, in order to modulate the activity of proteins which finally regulates many biological processes. Malfunctions, as an enhanced activity, of kinases, e.g. caused by mutations, are therefore the origin of several human diseases. In search of a convenient treatment of kinase-related diseases, inhibitors have been intensively investigated and applied to suppress the over-regulated enzyme activity. One group of those inhibitors is named small-molecule protein kinase inhibitors according to their size [2,3]. The majority of these drugs is used for the treatment of malignancies but in recent years they have also been applied to medicate diseases other than cancer.

Ruxolitinib is a small-molecule protein kinase inhibitor, which has mainly been used for the treatment of (i) myelofibrosis (a myeloproliferative neoplasm that is accompanied by the deposition of scar tissue in the bone marrow), (ii) Polycythaemia vera (a disease characterized by an overproduction of red blood cells), and (iii) acute graft-versus-host disease [4–6]. Ruxolitinib specifically inhibits Janus kinases (JAK) which among others play an important role in cytokine and inflammatory signaling pathways [7]. Due to this impact, ruxolitinib has also been considered recently for a usage in the treatment of Covid-19 since severe inflammations are concomitants of this world-widespread pandemic [8–10]. However, this therapeutic benefit is controversially discussed [9,11].

With regard to such an application, as in principle to the medical application of any drug, one important aspect is the interaction with (plasma) membranes since the latter are the first site of contact between cells and respective drugs. Notably, this process of drug-cell interaction has not been characterized in detail for many molecules which are therapeutically relevant. Concerning small-molecule protein kinase inhibitors, we have recently shown that the drugs sorafenib and regorafenib incorporate into lipid membranes resulting in a disturbance of the membrane organization [12]. Since for ruxolitinib the drug-membrane interaction has not been investigated so far, we have characterized its influence on lipid membranes by using different biophysical approaches.

2. Materials and methods

The materials and methods used are described in detail in the Supplementary Material.

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3. Results

3.1. Membrane orientation and localization of ruxolitinib by ^{1}H MAS NMR spectroscopy

^{1}H MAS NMR spectra of POPC membranes in the absence and in the presence of 20 mol% ruxolitinib exhibit well resolved ruxolitinib signals especially for the aromatic protons (Fig. S1). The peak assignment for ruxolitinib was obtained from SelleckChem (https://file.selleckchem.com/downloads/nmr/S137815-Ruxolitinib-INCB018424-hnmr-selleck.pdf) and standard ^{1}H–^{15}N-HMBC solution NMR experiments.

Insights into membrane orientation and localization of ruxolitinib were gathered by ^{1}H MAS NOESY NMR experiments; analyzing cross-relaxation rates between molecular groups of the phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and the inhibitor. Since the NOESY cross-relaxation rate δ_{ij} is strongly dependent on the distance between the respective protons, it can be used to determine a distribution function of the molecular groups of small molecules with respect to the lipid molecule in the membrane, as was shown before for other small molecules [12–16]. Fig. 1 exhibits the cross-relaxation rates between the indicated ruxolitinib protons and the individual lipid segments of the POPC membrane. Note, that due to overlaps of the acyl chain signal C-3 of POPC with the proton signals from ruxolitinib, a quantitative cross-relaxation analysis was not possible. The cross-relaxation rate between the proton H-3 of ruxolitinib and the β protons of POPC was dismissed due to the bad signal to noise ratio.

All proton signals indicate a mean location of the drug in the glycerol region of the membrane. Nevertheless, some small deviation between the different protons of ruxolitinib can be observed: The proton H-3 at the cyclopentane ring of ruxolitinib points towards the aqueous phase probably due to the neighbourhood to the polar triple-bonded nitrogen, while the protons H-1 and H-2 are located somewhat deeper in the membrane. The obtained broad distribution functions indicate high molecular disorder and mobility in the lipid membrane as it was already observed for other molecules [14,17]. Likewise, a rotation of the molecule within the membrane is probable.

3.2. Influence of ruxolitinib on membrane order measured by ^{31}P and ^{2}H NMR spectroscopy

The influence of the incorporation of ruxolitinib on the membrane organization was investigated by static ^{31}P and ^{2}H NMR spectroscopy. The ^{31}P NMR spectra in the presence of 20 mol% ruxolitinib (Fig. S2) exhibited the typical NMR powder pattern of lamellar liquid-crystalline bilayer membranes indicating that the membrane in the presence of the drug is still intact. Likewise, the chemical shift anisotropy of the two spectra, which is influenced by the mobility and orientation of the phosphatidyl head group, is in the presence of ruxolitinib (47 ppm) very similar to a pure POPC membrane (46 ppm). The same holds true if ruxolitinib is incorporated into a POPC/cholesterol membrane (Fig. S2). Note, that the somewhat different shape of the ^{31}P spectrum of POPC/cholesterol in the presence of ruxolitinib is caused by a deformation of spherical vesicles to an ellipsoidal shape due to the orientation-dependent diamagnetic susceptibility of lipid molecules, which can already occur for pure phospholipid membranes [18].

For investigating the effects of ruxolitinib on the hydrocarbon chain region of the membrane, the smoothed chain order parameters of the profiles were calculated from ^{2}H NMR spectra of the *sn*-1 chain deuterated POPC-d_{31}. In the presence of 20 mol% ruxolitinib, the order parameters are significantly decreased along the whole lipid chain (Fig. 2). This is also reflected in the lipid chain length L_c* (calculated according to Ref. [19]), which is decreased in the presence of ruxolitinib (10.1 Å) compared to a pure POPC membrane (11.0 Å). In a POPC/-cholesterol membrane, the addition of ruxolitinib counteracts the lipid condensation effect of cholesterol and leads to a large decrease in the order parameters, especially in the middle and lower chain region

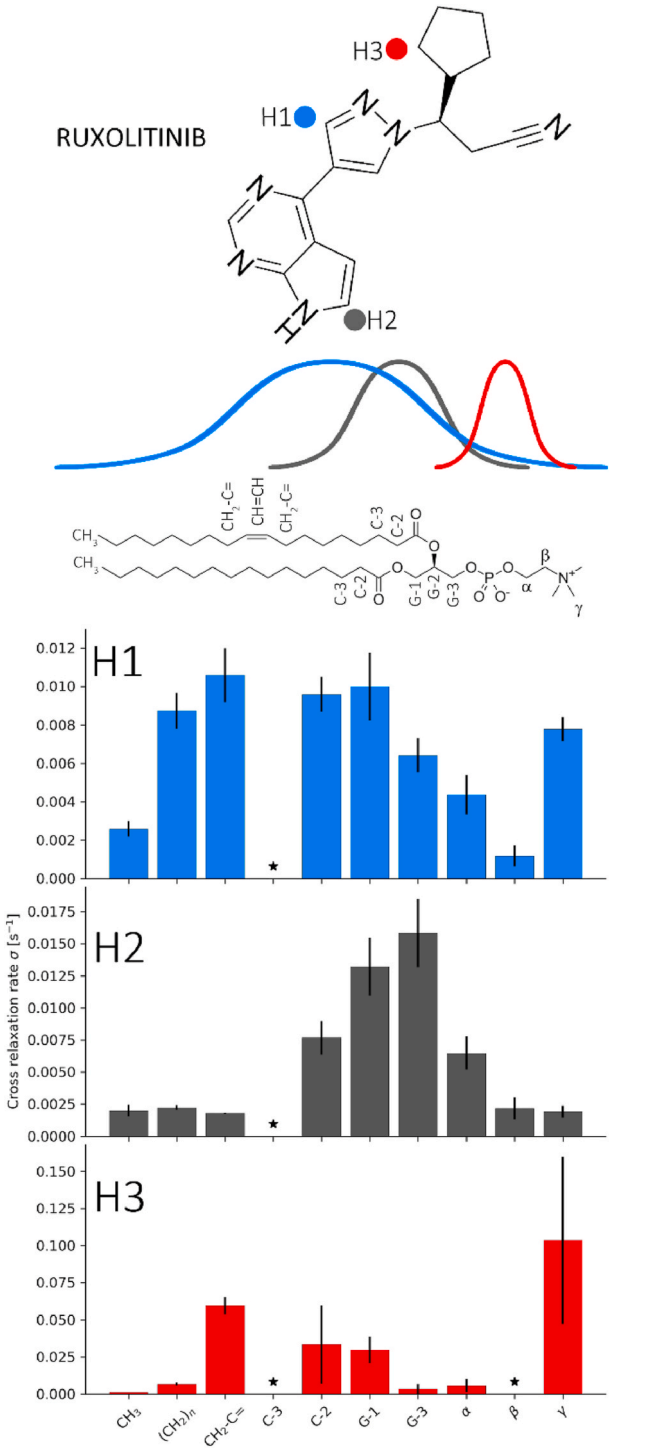

Fig. 1. Interaction of ruxolitinib with POPC membranes (Top) Chemical structure of ruxolitinib with colored designation of the protons used for NOESY analysis. (Bottom) NOESY cross-relaxation rates (s^{-1}) between respective protons of ruxolitinib (see top) and the individual molecular segments of POPC (see lipid structure). The colored lines reflect the distribution function of the respective protons of ruxolitinib within the membrane. Note, that due to overlaps of the acyl chain signal C-3 of POPC with signals from ruxolitinib, a quantitative cross-relaxation analysis was here not possible. The cross-relaxation rate between the proton H-3 of ruxolitinib and the β protons of POPC was dismissed due to bad signal to noise.

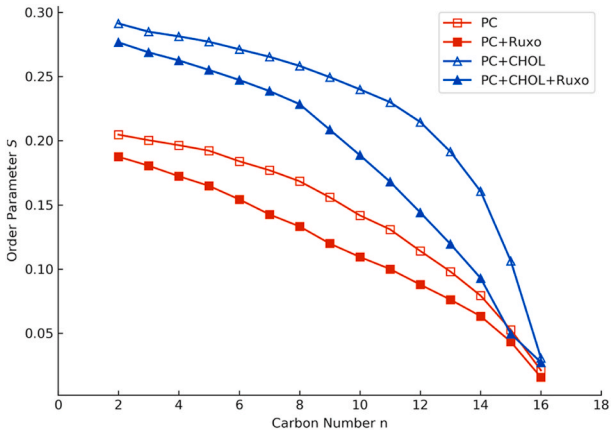

Fig. 2. Influence of ruxolitinib on the order parameter of fatty acyl chains The effect of 20 mol% ruxolitinib (closed symbols) on the ^{2}H NMR order parameter of the *sn-1* chain of POPC-d_{31} in a pure POPC-d_{31} (red) membrane as well as in a POPC-d_{31}/cholesterol (blue, molar ratio 4:1) membrane was estimated. For comparison the order parameter for the respective membranes without ruxolitinib are shown (open symbols). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 2). The lipid chain length is decreased to 12.1 Å in the presence of ruxolitinib compared to 13.4 Å for a POPC/cholesterol membrane. Therefore, we conclude that the addition of ruxolitinib significantly increases the mobility and decreases molecular orders of the lipid chains.

3.3. *Influence of ruxolitinib on membrane structure/integrity*

Furthermore it was investigated whether the membrane incorporation of ruxolitinib as shown by the NMR measurements has an influence on membranes. For this purpose we employed different assays which characterize the membrane integrity. The experiments were performed on large unilamellar vesicles (LUVs) of POPC or POPC/cholesterol using a lipid to drug ratio up to 2 : 1. First, the influence on the membrane permeation of dithionite was measured by following the reduction-kinetics of the fluorescent lipid 1-palmitoyl-2-(12-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) upon addition of the drug (see Supplementary Material). For POPC LUVs, we found a small but significant increase (*t*-Test, $P \leq 0.05$) of the transmembrane permeation of dithionite in the presence of ruxolitinib (Fig. 3A). In contrast, no significant effect of the drug on dithionite permeation was observed for POPC/cholesterol LUVs. Second, measuring the leakage of the water-soluble fluorophore 6-carboxyfluorescein (CF) from LUVs showed that after addition of ruxolitinib the release kinetics of CF was similar to that of control vesicles, i.e. in the absence of the drug (Fig. 3B). Third, the fluorescence lifetime of NBD-PC in LUVs was determined. We observed no differences of the average lifetime in the absence and the presence of ruxolitinib (Fig. 3C).

4. Discussion

The JAK kinase inhibitor ruxolitinib is a small-molecule protein kinase inhibitor FDA approved for the treatment of several diseases (see Introduction). Due to its anti-inflammatory impact, it has been proposed for the treatment of Covid-19-associated cytokine-induced inflammatory processes recently. For understanding the molecular mechanism(s) of the efficacy of ruxolitinib, its specific influence on the respective proteins/enzymes is of great importance. However, the investigation of the drug's interaction with membranes is also of high relevance in order to characterize (i) the general impact of the drug on plasma membranes, (ii) its cellular uptake mechanism, and (iii) the cause of side effects. This

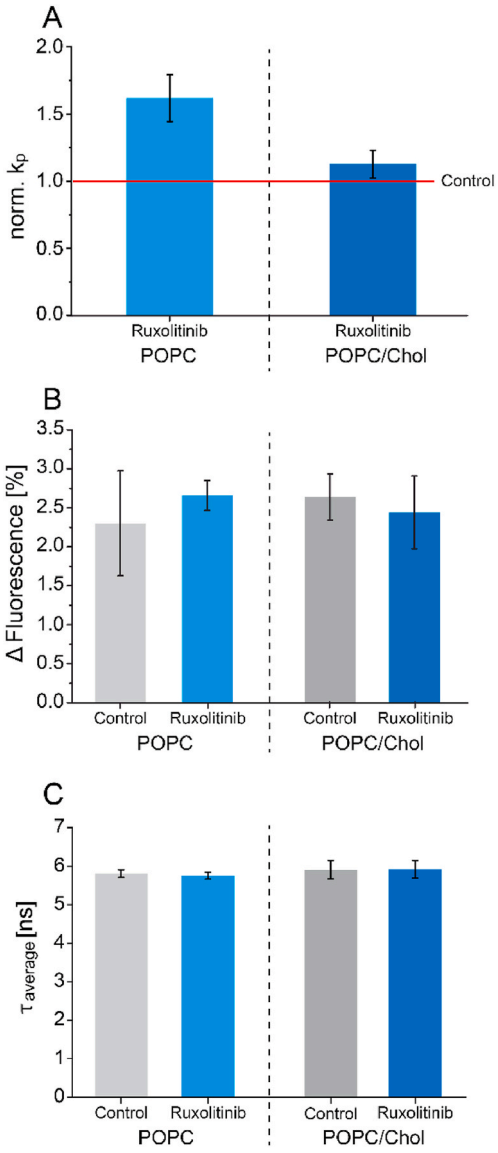

Fig. 3. Influence of ruxolitinib on membrane structure The experiments were done with POPC and POPC/cholesterol (molar ratio 4:1) LUVs. (A) The rate constants (k_P) for dithionite permeation across vesicles each containing 0.5 mol% NBD-PC were determined in the presence of ruxolitinib and normalized to those measured in the absence of the drug (control) measured at 37 °C. The data represent the mean ± SE of at least 6 (POPC) and 11 (POPC/cholesterol) independent samples. (B) The CF leakage from LUVs in the absence and in the presence of ruxolitinib measured at 37 °C and the calculation of leakage degree (percentage of Δ fluorescence) was performed as described in the Supplementary Material. The values represent the mean ± SD (>3 samples). (C) NBD fluorescence lifetimes of LUVs containing 0.5 mol% NBD-PC were measured (λ_{ex} = 467 nm, λ_{em} = 540 nm) without or with ruxolitinib at room temperature. The average fluorescence lifetime (τ_{av}) was calculated as described in the Supplementary Material. The values represent the mean ± SD of 2 independent samples each measured seven times. The molar lipid/drug ratio was 2:1 for all measurements.

aspect of cellular effects has not been studied for ruxolitinib so far. Therefore, we investigated its interaction with lipid membranes.

The analysis of the MAS NMR NOESY spectra revealed that ruxolitinib molecules incorporate into the lipid bilayer of vesicles in the upper chain/glycerol region. The broad distribution function of ruxolitinib reflects a high mobility within the membrane with regard to molecular rotation and movement along the membrane normal. This dynamic behavior might indicate the drug's disposition for a passive

cellular uptake mechanism. Accordingly, the drug may permeate across plasma membranes by passive diffusion followed by release into the cytosol. However, so far it has not been shown how ruxolitinib enters cells. Hence, also a combination of passive transmembrane movement and processes of endocytosis seems to be likely.

Using a couple of approaches, we investigated the influence of membrane binding of ruxolitinib on membrane structure and dynamics. ^{2}H NMR measurements revealed that the presence of ruxolitinib molecules entail a decrease of the lipid chain order with the largest effect in the middle and lower tail region of the membrane for both POPC and POPC/cholesterol membranes. This is in agreement with a position of ruxolitinib molecules in the glycerol region as indicated by the NOESY measurements. Since ruxolitinib molecules act as "spacer" between the POPC molecules, their presence results in an increased mobility of the hydrocarbon lipid chain underneath the drug molecules. Additionally, the influence of ruxolitinib on membrane permeation of dithionite was determined. The diffusion of the anion dithionite across intact lipid membranes is very slow. Disturbances of membrane structure, e.g. mediated by the impact of membrane-binding molecules, may cause an accelerated membrane permeation of dithionite, which is reflected in a faster decay of the NBD fluorescence intensity (see Supplementary Material) [20]. For ruxolitinib, we observed no influence on the rate constants of dithionite permeation across POPC/cholesterol membranes, whereas for POPC LUVs a 1.6-fold increase of dithionite permeation was found. The latter result implicates some small influence of ruxolitinib on POPC membranes by slightly affecting their integrity. However, for other small-molecule protein kinase inhibitors a much larger impact on dithionite permeation was described [12]. Next, we studied whether ruxolitinib may increase the leakage of CF from LUVs. An increased leakage reflects a significant deterioration of membrane structure e.g. caused by the formation of pores [21]. Adding ruxolitinib to CF-loaded LUVs, a slow leakage similar to control vesicles was observed for POPC and POPC/cholesterol vesicles. Moreover, the fluorescence lifetime of NBD-PC was determined serving as a sensitive parameter for the environmental conditions around the NBD moiety at a molecular level [22]. Ruxolitinib had no influence on the lifetime of the fluorescent lipid, indicating an unchanged environment of the NBD-group. The data of the latter three assays show, that while the molecular order of the membrane hydrocarbon core is significantly affected by ruxolitinib as shown by ^{2}H NMR, membrane structure stays intact in the presence of even high ruxolitinib concentrations which is in agreement with the observed line shapes of the ^{31}P NMR spectra.

The drug concentrations used in this study (up to 0.4 mM) are higher than those clinically measured, since the plasma concentration of ruxolitinib following an administration of 100 mg is about 10 μM [23]. However, regarding to an interaction with membranes, we note that not the absolute drug concentrations, but the relative drug to lipid ratio has to be considered (see below). Moreover, the binding and accumulation of drug molecules in the plasma membrane may result in larger local concentrations also under physiological conditions.

With regard to its influence on cells, a few studies have investigated the (cytotoxic) impact of ruxolitinib. Briglia and coworkers observed that ruxolitinib causes some hemolysis of red blood cells at a drug concentration of 25 μM [24]. Unfortunately, the authors gave no information on the cell concentration used in their experiments from which the effective molar ratio of drug to membrane lipids could be calculated. However, the hemolysis measured after 48 h was very low (below 2%). In another study, very low concentrations of ruxolitinib (5 nM) caused a significant increase of damaged cells in cell lines after 48 h measured by propidium iodide staining. Also this study did not provide data about the cell numbers used in order to calculate the amount of lipids of the plasma membranes [25]. This impact of ruxolitinib was found not to be related to a direct influence on the plasma membrane but to a triggering of apoptotic pathways. This was proven by a ruxolitinib-mediated exposure of the lipid phosphatidylserine on the outer plasma membrane of cells. Notably, a similar effect of ruxolitinib was observed in red blood cells [24]. Our data on lipid membranes show that ruxolitinib while inserting into the membrane and increasing lipid disorder does not cause a substantial loss of membrane integrity. Therefore we assume, that ruxolitinib even at large concentrations does not disturb plasma membranes nonspecifically which might affect cell viability.

Author contributions

Holger A. Scheidt: designed the study, wrote the manuscript. and Peter Müller: designed the study, wrote the manuscript. Markus Fischer: performed the experiments, analyzed the data. Meike Luck: performed the experiments, analyzed the data. Maximilian Werle: performed the experiments. All authors have read and approved the manuscript.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100838.

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