CHIMs are versatile cholesterol analogs mimicking and visualizing cholesterol behavior in lipid bilayers and cells

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Cholesterol is an essential component of cellular membranes regulating the structural integrity and fluidity of biological bilayers and cellular processes such as signal transduction and membrane trafficking. However, tools to investigate the role and dynamics of cholesterol in live cells are still scarce and often show limited applicability. To address this, we previously developed a class of imidazolium-based cholesterol analogs, CHIMs. Here we confirm that CHIM membrane integration characteristics largely mimic those of cholesterol. Computational studies in simulated phospholipid bilayers and biophysical analyses of model membranes reveal that in biologically relevant systems CHIMs behave similarly to natural cholesterol. Importantly, the analogs can functionally replace cholesterol in membranes, can be readily labeled by click chemistry and follow trafficking pathways of cholesterol in live cells. Thus, CHIMs represent chemically versatile cholesterol analogs that can serve as a flexible toolbox to study cholesterol behavior and function in live cells and organisms.
Biological membranes are composed of different types of lipids, phospholipids, sphingolipids, and cholesterol, and integral as well as associated proteins. They can adopt a vast array of configurations and are highly dynamic in nature, allowing rapid lateral diffusion and thus assembly and disassembly of their principal components. This activity enables membranes to fulfill highly versatile biological functions that range from constituting a mere scaffold and diffusion barrier to forming a two-dimensional platform for biochemical reactions.

Cholesterol is an important component of biological membranes. It regulates the cell membrane’s structural integrity and fluidity and plays a crucial role in mediating signal transduction and membrane trafficking processes. However, the exact mode of action of cholesterol in these events is still not fully understood and tools to study cholesterol-mediated processes in an unperturbed biological environment are still limited and often suffer from major disadvantages. Of prime importance for accessing cholesterol’s cellular functions is the possibility to visualize its subcellular distribution with high spatiotemporal resolution, for instance, by employing fluorescence microscopy techniques. As cholesterol does not show intrinsic fluorescence, modifications of the sterol backbone introducing a fluorescent moiety or fluorescent, and fluorescently labeled cholesterol derivatives, mostly either modified at the C2 position in a highly versatile manner, e.g., by introducing an azide linker to which a fluorophore outside of the membrane and therefore is predicted to not affect the proper membrane integration of the cholesterol backbone. Due to its unique design CHIM addresses many drawbacks of established cholesterol analogs; it should enable the visualization of cholesterol distribution in live cells without the need for cell fixation and should allow a flexible on-demand attachment of any fluorophore of choice or other functional groups (e.g., photoswitches, crosslinkers, or biotin labels) that could be used for the investigation and manipulation of the native cholesterol membrane environment. Our comprehensive characterization shows that CHIMs mimic cholesterol behavior in cellular membranes and can functionally replace endogenous cholesterol in the model organism C. elegans. Furthermore, combined computational, biophysical, and biochemical analyses reveal that membrane integration and dynamics of the chemically versatile CHIM derivatives can be controlled by chemical modification and membrane composition. Therefore, we envision that this highly modifiable class of cholesterol analogs will serve as important tools and flexible platforms for the investigation of cholesterol-dependent membrane processes.

**Results**

Characterization of CHIM within different bilayer environments via extensive molecular dynamics simulations. One of cholesterol’s most prominent characteristics is its ability to order alkyl chains of glycerol- and sphingolipids, thereby regulating the fluidity and packing density of membranes. To investigate whether CHIM shares this fundamental property of natural cholesterol we analyzed its ordering abilities in molecular dynamics (MD) simulations by calculating the median of the DPPC chain order parameter in DPPC/sterol bilayers, containing 30 mol% of CHIM, cholesterol (CHOL) or a mix of both with ratios of 1:4 and 1:1 CHIM to CHOL, respectively (Fig. 2a). In the MD simulations we focused on a comparison between cholesterol and CHIM to characterize the effect of changing the hydroxyl group to an imidazolium salt residue. The linker introduced in CHIM-L, the derivative used for cell labeling experiments (see below), is unlikely to affect the membrane integration properties of CHIM in a significant manner as revealed by analyzing a slightly modified CHIM-L (Supplementary Note and Supplementary Fig. 1). The amount of 30 mol% sterol was chosen to have a strong impact of these moieties on bilayer properties and because it resembles the amount of CHOL found in natural plasma membranes (PMs). To analyze the phase behavior, we simulated temperatures from 20 °C above to 20 °C below phase transition of the pure DPPC bilayer system (Fig. 2a, left panel); based on the nonlinear increase of average order parameters of the DPPC/CHOL mixtures at roughly S = 0.7, bilayers with an average order parameter of S > 0.7 were defined as ordered, otherwise as disordered. Based on the phase behavior of pure DPPC bilayers within the simulations, the data can be separated into two distinct temperature regimes: a high-temperature regime (red, T > 47 °C) and a low-temperature regime (blue, T < 47 °C) with the black curve showing simulations at 47 °C (Fig. 2a, left panel). In the low-temperature regime and at CHIM/CHOL ratios of 1:1 or lower, the presence of CHIM only slightly affected the bilayer structure, while exceeding amounts of CHIM precluded the transition to an ordered phase. In the high-temperature regime, the DPPC bilayers containing only CHIM exhibited the same order as the pure DPPC bilayer. The linear dependence of the order parameter on the CHOL fraction suggests that in these conditions CHIM does not act as CHOL with respect to generating order in the DPPC bilayer.

CHIM’s distinct behavior within the two regimes may be related to its position within the bilayer. In DPPC/CHIM mixtures, the sterol ring of CHIM was found to be closer to the bilayer headgroup region than that of cholesterol (Supplementary Fig. 2). This position, derived from the density profiles of reference atoms (phosphor of phospholipids and C3 carbon of cholesterol and analogs) (Supplementary Fig. 3), was significantly changed when cholesterol was introduced and, already in a 1:1 CHIM/CHOL mixture, CHIM was tucked towards the acyl chain.
region. In contrast, natural cholesterol’s position remained unaffected up to a 1:1 CHOL/CHIM ratio. The alignment of CHIM’s sterol ring position with that of cholesterol’s sterol ring when mixing both sterols connotes CHIM’s similarity to CHOL in such an environment.

CHIM’s ordering capabilities in lipid bilayers are mainly affected by its charge. The charge of CHIM is one significant difference to the structure of natural cholesterol and we hypothesized that it presumably is dominant in the above-described effects on order. At low concentrations, these charge effects are likely without relevance and thus CHIM behaves similar to cholesterol in these conditions. To examine potential charge effects, we performed simulations of the same binary mixtures (DPPC/CHIM) with an artificially charge-neutralized moiety termed CHIM-0 (Supplementary Fig. 4). The ordering capability of CHIM-0 was found to be far stronger than that of CHIM (Fig. 2a, right panel). In the low-temperature regime, the formation of an ordered phase was preserved, whereas in the high-temperature regime the ordering capability was approximately half that of CHOL. The finding that uncharged CHIM-0 maintained CHOL’s properties with respect to the overall phase behavior of DPPC bilayers is supported by the observation that CHOL and CHIM-0 share similar positions within bilayers of DPPC (Supplementary Fig. 2).

In native PMs negatively charged lipids comprise a small but significant proportion of the overall lipids. As these could affect CHIM’s properties by shielding its positive charge in the head region, we also simulated CHIM’s membrane integration in bilayers containing the negatively charged phospholipid POPS and employed phospholipid (PL) mixtures of DPPC/POPS with 20% of either CHIM, CHOL or mixtures of CHIM/CHOL (Fig. 2b). The impact of CHIM-0 on the DPPC order in DPPC/POPS/CHIM-0 bilayers was similar to that observed in the respective DPPC/CHIM-0 bilayers indicating that the presence of POPS had basically no impact on CHIM-0 properties (see right parts of panels a and b in Fig. 2). In the case of DPPC/POPS/CHIM bilayers, however, POPS indeed showed an effect, resulting in CHIM’s properties to become more closely related to those of natural cholesterol. Unlike in the DPPC bilayers without POPS, CHIM does not disrupt the order in the low-temperature regime and even pure CHIM had an ordering effect in the high-temperature regime, indicating that CHIM has ordering capabilities itself, albeit somewhat weaker than CHOL. Since the DPPC/POPS bilayer containing only CHIM behaves very similar to that containing only CHIM-0, the impact of CHIM’s charge appears to be largely compensated by POPS. Moreover, the behavior of the 1:4 system linearly interpolates between the pure CHIM and pure CHOL systems, suggesting that the CHOL-like properties of CHIM are intrinsic and not modified by the simultaneous presence of CHOL. Finally, the position of CHIM in the DPPC/POPS bilayers was similar to the position in the bilayers without POPS (Supplementary Fig. 2). Thus, POPS seems to be able to stabilize the headgroup region of CHIM in phospholipid bilayers containing higher concentrations of CHIM.

CHIM shows segregation behavior similar to that of cholesterol in DPPC:DLiPC bilayers. Next, we investigated CHIM’s membrane phase segregation behavior in comparison to that of natural cholesterol, which preferentially assembles into liquid-ordered domains together with saturated phospholipids. Therefore, we first simulated the dynamic behavior of a preformed DPPC patch in a DPPC:DLiPC bilayer without sterol molecules. We recorded snapshots of the initial and final configurations at different temperatures after simulation times of at least 500 ns and determined the fraction of DPPC neighbors overall PL neighbors as a function of simulation time (Supplementary Fig. 5a and 5b). Fractions of 0.9 indicate complete segregation of DPPC and DLiPC, while fractions of 0.4 indicate random mixing for this setup of a 4:6 DLiPC:DPPC ratio. At temperatures above 47 °C, complete and rapid mixing of the two lipid species was observed, whereas at 27 °C the DPPC patch was stable with the fraction of DPPC neighbors remaining at roughly 0.8 (segregation) even after 1.5 µs simulation time. At 37 °C the DPPC neighbor fraction slowly decreased to roughly 0.6, indicative of a slow mixing process. Due to the overall patch stability, a temperature of 27 °C was chosen to further investigate the impact of CHIM and/or CHOL on the segregation behavior. Specifically, we compared three different sets of randomly distributed sterols: pure CHOL (M1), pure CHIM (M3), and a 1:4 mixture of CHIM/CHOL (M2) with each bilayer containing a total of 16 mol% sterols (Fig. 3a). Already a qualitative evaluation of the lipid segregation behavior revealed different characteristics within the three sets. A strong deformation of the initial DPPC patch was observed for the pure CHIM system (M3), while the DPPC patch in the pure CHOL systems (M1) remained stable. Moreover, in bilayer M1, most cholesterol molecules were located within or near the DPPC patch, while in M3, the CHOL molecules were mostly statistically distributed within the bilayer. In the membrane simulation of a CHIM/CHOL mixture (M2), the DPPC patch, though slightly more deformed than in the pure CHOL
system, remained intact and segregation between DPPC and DLiPC was preserved.

The quantification of the DPPC lipid environment in such bilayers revealed a change of CHIM’s segregation behavior depending on the CHIM/CHOL ratio (Fig. 3b, top). A slight mixing starting from complete segregation with DPPC neighbor ratios of 0.9 (dashed line) was visible in all simulation setups. In the case of pure CHIM, the relative number dropped to 0.65, indicating an ongoing mixing of DPPC and DLiPC, while in M1 and M2, the relative number ended at appr. 0.8. This shows that a modest presence of CHIM does not alter the stability of the DPPC patch. In the mixed system the DPPC enrichment is the same around CHIM and CHOL being in both cases significantly above random mixing and, within the margin of error, similar to the case of pure CHOL (Fig. 3b, bottom). In contrast, for the pure CHIM system a random distribution is observed.

Thus, in agreement with the analysis of the order parameters, even in the case of uncharged PL bilayers CHIM behaves very similar to natural cholesterol in ordered regimes (i.e., in DPPC rich regimes at low temperatures) as long as it is the minority component as compared to natural cholesterol.

The partitioning of CHIM-L in model membranes mimics that of cholesterol as revealed by fluorescence microscopy and lipid–protein interaction studies. Based on the thorough computational characterization, we next investigated experimentally...
to what extent CHIM exhibits cholesterol-like behavior in biological membranes. First, the phase partitioning of CHIM was analyzed in both, artificial and cell-derived model membranes. In these and the following experiments we chose CHIM-L as derivative because it could be labeled with fluorescent dyes by Cu-free click chemistry at a site residing outside of the bilayer (Fig. 1). On the one hand, we employed giant unilamellar vesicles (GUVs) with known and adjustable lipid composition, and on the other hand, giant plasma membrane vesicles (GPMVs) resembling the lipid composition of the cellular plasma membrane (PM). The presence of fluorescently labeled CHIM-L precluded the formation of GUVs with the simple non-charged lipid composition DLiPC:DPPC:CHIM-L (60:40:20), likely due to CHIM-L integration of GUVs with the simple non-charged lipid composition of the cellular plasma membrane (PM). The hand, giant plasma membrane vesicles (GPMVs) resembling the with known and adjustable lipid composition, and on the other

Cholesterol-like properties of CHIM-L were also analyzed by assessing protein-membrane interactions known to be affected by cholesterol. Here, we focused on the peripheral phospholipid-binding protein annexin A2 (AnxA2), which interacts with membranes in a cooperative manner in the presence but not absence of cholesterol. As revealed by QCM-D (Quartz Crystal Microbalance with Dissipation) measurements employing bilayers containing negatively charged phospholipids, CHIM could faithfully replace cholesterol in triggering cooperativity in the AnxA2-membrane interaction (Supplementary Fig. 6). The nonlinear regression of the respective binding isotherms as a function of AnxA2 concentration showed a sigmoidal shape typical for positive cooperativity (n_Hill = 1.88 ± 0.13, R² = 0.98). This cooperative binding, most likely reflecting a rearrangement of lipids upon interaction with AnxA2, is not seen when the same bilayer devoid of CHIM or cholesterol is employed in the binding experiments.

CHIM is incorporated into cellular membranes and can visualize the dynamic distribution of cholesterol. As CHIM-L behaves similarly to cholesterol in model membranes, we next tested whether this is also observed in the membrane system of live cells. We first concentrated on caveolae, which represent cholesterol-rich PM invaginations functioning as mechanosensors and cellular signaling regulators. Cholesterol is recruited to such raft-like areas by interaction with the prime protein constituent caveolin 1 (Cav1) and serves a structural role as removal of cholesterol causes disruption and flattening of caveolae. As the levels of cholesterol or sphingolipids rise, caveolae are internalized, indicating that caveolae can sense the lipid composition. To assess whether CHIM-L shows an enrichment in PM caveolae that is typical for endogenous cholesterol, we chose MDCK (Madin–Darby canine kidney) type II cells, a well-established model known to possess abundant caveolae. We found that fluorescently labeled CHIM-L added to the culture medium was readily integrated into the PM and enriched in regions partially also positive for Cav1-mCherry (Fig. 4a). CHIM-L’s preferential PM association could also be visualized by ectopic intracellular expression of the cholesterol-binding probe D4H that preferentially labels the cholesterol-rich PM (Fig. 4b). Upon internalization of caveolae and other cholesterol-rich PM structures, natural cholesterol is delivered to the cell’s endosomal system (Supplementary Fig. 7). To address whether CHIM-L follows the same internalization route, we used markers for early and late endosomes, Rab5 and Rab7, and analyzed the intracellular distribution of fluorescently labeled CHIM-L, observed after longer incubation times, in comparison to these markers. To extend our analysis to primary cells, intracellular trafficking studies were carried out in endothelial cells derived from umbilical cord veins (HUVECs). We found that internalized CHIM-L colocalizes with both endosomal markers (Supplementary Figs. 8 and 9). Moreover, this colocalization followed internalization kinetics, which are in line with a trafficking of CHIM-L through first early (Rab5) and then late endosomes (Rab7).

Interestingly, the arrangement of CHIM-L in the endocytic organelles appeared to change as it was present in the limiting membrane of early endosomes but seemed to concentrate in the internal lumen of late endosomes. The accumulation of CHIM-L in the lumen of late endosomes/lysosomes was seen even more pronounced when cholesterol egress from these organelles was blocked by the drug U18666A, which inhibits the cholesterol transport protein Niemann Pick type C1 (NPC1) (Fig. 4c and Supplementary Fig. 10). This again reflected the behavior of natural cholesterol.

Cholesterol and caveolar components, in particular Cav1, also associate with lipid droplets (LDs), cellular reservoirs of fatty acids and cholesterol that are characterized by a hydrophobic core containing neutral lipids and cholesterol esters and a surrounding phospholipid monolayer. Interestingly, internalized CHIM-L also colocalized with the LD marker Bodipy 493 (Fig. 4d and Supplementary Fig. 11) suggesting that CHIM-L, as a cholesterol analog that cannot be esterified, is also transported to LDs. We also compared cell labeling by CHIM-L to that of other labeled cholesterol derivative, BODIPY-cholesterol, which has often been used in such type of experiments. BODIPY-cholesterol when given for 60 min at room temperature, also stained the plasma membrane of HeLa cells albeit less prominently than AF405-labeled CHIM-L (Fig. 4e). Moreover, BODIPY-cholesterol, again similar to natural cholesterol and CHIM-L, accumulated in the lumen of late endosomes/lysosomes when cells were treated overnight with the drug U18666A (Supplementary Fig. 10). Various reports have also shown that BODIPY-cholesterol exhibits cholesterol-like properties in model membranes such as GPMVs, thus extending the similarity to CHIM-L. However, it should be noted that BODIPY-cholesterol shows a lower degree of plasma membrane staining and a more pronounced intracellular distribution as compared to CHIM-L (Fig. 4e), possibly due to the fact that the BODIPY label is located in the acyl chain (i.e., inside the bilayer) and thereby precludes a very efficient membrane incorporation. In the CHIM-L, on the other hand, the fluorescent dye is attached outside of the membrane and thus most likely does not interfere with a proper bilayer incorporation of the sterol backbone.

Previous MD simulations have also suggested that BODIPY-cholesterol can be considered a proper cholesterol analog as its membrane integration does not affect the membrane structure
even though it exhibits significantly higher tilt angles than cholesterol. This discrepancy might be due to the very low concentration of the BODIPY-cholesterol used in the simulation setup, which could allow for a buffering of the BODIPY-cholesterol effects in the otherwise unperturbed surroundings. Thus, these findings cannot be compared to our results on the CHIM effects on phospholipid order parameters that used much higher CHIM concentrations in the simulations. The high cholesterol similarity of CHIM as compared to BODIPY-cholesterol is also supported by an assessment of the tilt angle distribution of CHIM in relation to the cholesterol angle. Even in the pure DPPC/CHIM bilayers CHIM’s average tilt angle is only slightly increased and CHIM’s tilt becomes identical to that of cholesterol in the 1:4 CHIM/CHOL mixture (Supplementary...
transcriptionally active SREBP fragments, which in turn activate the transcriptional control of cholesterol biosynthesis. With time (2–3 days), CHIM-L is most likely metabolically degraded and/or externalized and endogenous cholesterol levels are restored. We also assessed whether this sensing of CHIM-L concentrations (1, 5, and 10 µM) first caused a drop and then a restoration of total cholesterol levels (Fig. 5a). This suggests that cells sense the presence of CHIM-L in their membranes and adjust their total cholesterol levels accordingly. With time (2–3 days), CHIM-L is most likely metabolically degraded and/or externalized and endogenous cholesterol levels are restored. We also assessed whether this sensing of CHIM-L extends to the transcriptional control of cholesterol biosynthesis. Therefore, we analyzed the proteolytic cleavage of the sterol regulatory element-binding protein (SREBP) that liberates transcriptionally active SREBP fragments, which in turn activate transcription of target genes involved in cholesterol synthesis. Low cellular cholesterol levels that are sensed by the SREBP cleavage activating protein SCAP trigger cleavage and transcriptional activation whereas high cholesterol levels prevent cleavage by retaining the SCAP/SREBP complex in the ER[35]. HeLa cells were treated with CHIM-L at high concentrations (10 µM) and the SREBP signal in immunoblots was compared to that of cells either left untreated or treated with cholesterol or U18666A as a means to lower ER cholesterol levels. Supplementary Figure 13 shows that similar to cholesterol, CHIM-L appears to slightly reduce the proteolytic cleavage of SREBP.

CHIM can functionally replace cholesterol in an animal model. The above data indicate that CHIM-L behaves similarly to cholesterol in mammalian cells. Therefore, we asked next whether CHIM-L could replace cholesterol in a whole animal. The nematode Caenorhabditis elegans represents an excellent model for studying different aspects of sterol function on the level of a whole organism, because, in contrast to mammals, C. elegans is a cholesterol auxotroph[36]. Cholesterol is essential for development and fertility and its depletion during C. elegans development induces loss of both motility and muscle mass together with a reduction of life expectancy[47]. Besides being a membrane component, cholesterol is involved in hormonal signaling during development and dauer formation[38–40]. In the laboratory, worms are grown in the presence of 5 mg/l of cholesterol[41]. Therefore, the feeding of CHIM-L instead of cholesterol can reveal whether CHIM-L can functionally replace natural sterols during development of the nematode.
To address this aspect, we grew worms for three generations in media containing either cholesterol, CHIM-L, or no-added sterols. Consistent with previous reports, in the absence of dietary cholesterol, only 46% of the F1 generation worms reached adulthood and developed into fertile species, whereas this fraction increased to 78% in animals grown in the presence of CHIM-L (Fig. 5b). In the F2 generation, however, animals grown without cholesterol or in the presence of CHIM-L failed to develop into adults. The development of the F1 into adult worms demonstrates that CHIM-L can functionally replace cholesterol in membranes. We assume that the developmental arrest in the F2 generation is due to the inability of CHIM-L to function as a precursor for steroid hormones and thus inappropriate hormonal signaling. Nevertheless, our data indicate that CHIM-L can take over cholesterol functions in the membranes of a living animal.

Discussion
Herein, we introduce imidazolium-based cholesterol analogs and report that they show cholesterol-like behavior in model and cellular membranes and can functionally replace cholesterol in the development of the F1 generation in C. elegans. Moreover, we conducted in-depth MD simulations to examine the influence of steric differences between CHIM and CHOL as well as charge effects introduced by CHIM’s positive charge. We found that for sterol mixtures of 1:4 CHIM/CHOL, CHIM has no perturbing influence and even shows similar ordering and condensing characteristics as cholesterol. Similar ratios are likely to be met in cells loaded with CHIM or CHIM-L following the protocols used herein as the cells seem to be able to adjust their endogenous cholesterol content, which dropped following CHIM treatment, most likely to maintain the total cholesterol/CHIM homeostasis (Fig. 5a). We could also show that at physiological temperatures the membrane perturbing effects of CHIM can be largely attributed to its positive charge. They mainly take effect at concentrations above 15 mol% and are only observed in an otherwise uncharged lipid environment and hence are not relevant in cellular membranes. Importantly, the similarity to cholesterol is markedly increased when negatively charged lipids are present. This is corroborated by the GUV and GPMV experiments that showed no disturbing effect of CHIM-L when negatively charged phospholipids were included (GUV) or were naturally present (GPMV). Moreover, the GUV and GPMV experiments revealed that CHIM-L preferentially integrates into Lo membrane domains, thus exhibiting a phase separation behavior similar to that of natural cholesterol. This experimental finding is again in line with the computer simulations of a preformed DPPC/CHOL/CHIM domain in bilayers containing also DLiPC, where CHIM, in low concentrations, shows similar segregation behavior as cholesterol even without countercharged lipids. Together, the findings indicate that CHIM can integrate into biological membranes containing negatively charged lipids in a cholesterol-like manner, and it can be specifically labelled without perturbing its cholesterol-like membrane integration and partitioning.

Further improvement of our cholesterol probe is expected and will be driven by the MD simulations. For instance, the positive charge in CHIM could be removed chemically by generating a cholesterol analog bearing a polar, but uncharged imidazole headgroup instead of the permanently charged imidazolium salt. Alternatively, the design of an imidazolium salt with a more shielded permanent charge, for instance by choosing bulkier N-substituents, could represent an approach to reduce the undesired effect of charge on CHIM’s membrane integration. However, in this case, the supposedly beneficial partial shielding of the positive charge versus unfavorable, potentially disturbing interactions of the bulky headgroup with the membrane and the possible loss of CHIM’s cholesterol-resembling amphiphilic structure would need to be carefully balanced.

Due to CHIM’s cholesterol-like nature it is readily integrated into cellular membranes in a manner most likely resembling natural cholesterol, and within cells, it follows the trafficking routes of the native counterpart. It is present in caveolae, which are known to contain high levels of cholesterol, and upon internalization is found in Rab5- and Rab7-positive compartments that also function as destinations for internalized natural cholesterol. The intracellular cholesterol distribution can be visualized by the cholesterol-binding sensor mCherry-D4H ectopically expressed following transfection. mCherry-D4H is present in the cytosolic leaflet of the plasma membrane or of internal organelles. When expressed in CHIM-L treated cells mCherry-D4H shows a substantial but not full colocalization with the cholesterol analog. This supports the conclusion that CHIM-L resides in cholesterol-rich membranes/membrane domains but also suggests that not all cholesterol membrane pools are accessible to CHIM-L. Interestingly, upon overload, some CHIM-L most likely also accumulates in lipid droplets. As CHIMs cannot be esterified this suggests that the charged imidazolium ring does not preclude incorporation into this organelle. Future experiments, like isolating and analyzing CHIM-L-loaded lipid droplets, have to address this point.

All studies in model and cellular membrane complemented with the in-depth MD simulations predict that CHIM can function as a substitute for cholesterol in biological membranes and thus can be applied to study cholesterol trafficking and behavior in live cells following specific labeling at a site not affecting CHIM’s proper membrane integration. The functional equivalence as a membrane component is further corroborated by our finding that CHIM supports growth and development of an animal (C. elegans), which is cholesterol auxotroph and is reliant on exogenous addition of cholesterol in the diet.

In sum, we have shown that imidazolium-based cholesterol analogs (CHIMs) exhibit cholesterol-like behavior and can substitute natural cholesterol in native biological membranes and living cells. We, therefore, envision that this highly modifiable class of cholesterol analogs will serve as an important tool and flexible platform for the investigation of cholesterol-dependent membrane processes.

Methods
Synthesis of cholesterol analogs CHIM and CHIM-L. Cholesterol analogs CHIM and CHIM-L were synthesized as previously described. The synthesis was performed starting from commercially available cholesterol that was converted into the corresponding 2,3-cholestadiene in five steps. Subsequent formation of the respective imidazole, with either paraformaldehyde or 6-azidohexanal, and N-methylation yielded the herein investigated imidazolium-based cholesterol analogs CHIM and CHIM-L.

Structure and parameters of the cholesterol analog. A relaxed structure of the cholesterol analog (CHIM) was obtained using Avogadro after an energy minimization with GaFF. This structure was used as input for the CHARMM General Force Field (CgenFF) program version 1.0.0 to obtain parameters for the CgenFF version of 3.0.13. Each parameter is provided with a penalty value to receive an estimate of its fitness. The atoms in CHIM were found to have an agreeable analog in already existing molecules in CgenFF, as visible through low penalty values. Only the nitrogen atoms and the attached methyl groups showed a slightly higher penalty, hence the fitness of these parameter values for this part of the molecule was further analyzed. As the best analogous molecule already parameterized in CgenFF is 7-methyleneguanosine, we compared those parameters to those of CHIM. We found that the overlap is sufficient for the bonded parameters. For the analysis of the fitness of partial charges, we received similar results following the common CgenFF procedure of parameterization. Therefore, and to avoid over-parameterization, we decided to use the CgenFF program parameters. To analyze charge effects, we additionally added a CHIM version with a zero net charge (CHIM-0, Supplementary Fig. 2) by rescaling the partial charges of all atoms within the imidazolium ring (excluding those charges of the alkyl
System setup and analysis. All initial bilayers containing random mixtures of DPPC, DLiPC and cholesterol were prepared using the CHARMM-GUI web-based graphical interface. To add CHIM to the configurations, cholesterol molecules were reconstructed to fit the relaxed CHIM template structure. The patched configurations were constructed from a mixture of DLiPC and CHOL, where CHOL again was replaced by CHIM and all center DLiPC molecules were reconstructed to DPPC to obtain a 2:3 ratio of DPPC:DLiPC. The resulting net charges of the systems were neutralized by exchanging random water molecules with either potassium or chloride anions in the system. In the experiments the counterion of CHIM was iodide due to its optimized synthesis pathway. In the simulations though, to avoid artifacts, we chose chloride as counterion as it is well established in the CHARMM force-field set and most commonly used. Since the aqueous biological environment most probably enables rapid dissociation and anion exchange, we don’t expect the choice of counterion to largely affect the properties of the CHIM analog.

Calculation of the chain order parameter S employed a being the angle that is spanned by every second carbon atom in the alkyl chains and the average leaflet tilt. The average tilt angle is defined as the average between the vectors from the last to the first last carbon atoms of phospholipid chains and the bilayer normal (z-axis of the simulation box).

To identify the position of CHIM or cholesterol the difference between the average positions of the carbon atom attached to cholesterol’s hydroxyl group or the respective carbon atom in CHIM to the average z positions of the PL’s phosphoatomic central bilayer was used. All visualizations of the system configurations were created using VMD software.

Molecular dynamics simulations. All simulations were conducted using the CHARMM36 force field25 and Gromacs–2018 software package26,27. The systems were equilibrated using the 6-step established CHARMM-GUI parameter set, gradually decreasing restraints on lipid headgroup positions and chain dihedral angles (Supplementary Table 4). In the case of the pure DPPC bilayer, two additional 50 ns equilibration steps with position restraints of 1000 kJ/mol on the lipid head groups were performed to minimize leaflet intercalation. The first 50 ns were cutoff of each production run to avoid non-equilibrium statistics. A list of all systems with lipid composition and trajectory lengths is provided in Supplementary Tables 5, 6 and 7.

The TIP3P model was used as water model. The Nose-Hoover algorithm was used to maintain the temperature with a constant of 1 ps, coupling bilayer and solvent separately. To maintain the pressure at 1 bar the Parrinello-Rahman barostat was used with a semiisotropic coupling scheme, a coupling constant of 5 ps−1, and a time constant of 0.1 ps. The Lennard-Jones potential was used with a real-space cutoff of 1.2 nm58. The Lennard-Jones potential was cut off at 12 Å. The systems were equilibrated using the 6-step established CHARMM-GUI parameter set, gradually decreasing restraints on lipid headgroup positions and chain dihedral angles (Supplementary Table 4). In the case of the pure DPPC bilayer, two additional 50 ns equilibration steps with position restraints of 1000 kJ/mol on the lipid head groups were performed to minimize leaflet intercalation. The first 50 ns were cutoff of each production run to avoid non-equilibrium statistics. A list of all systems with lipid composition and trajectory lengths is provided in Supplementary Tables 5, 6 and 7.

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