A novel alkyne cholesterol to trace cellular cholesterol metabolism and localization

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Abstract  Cholesterol is an important lipid of mammalian cells and plays a fundamental role in many biological processes. Its concentration in the various cellular membranes differs and is tightly regulated. Here, we present a novel alkyne cholesterol analog suitable for tracing both cholesterol metabolism and localization. This probe can be detected by click chemistry employing various reporter azides. Alkyne cholesterol is accepted by cellular enzymes from different biological species (Brevibacterium, yeast, rat, human) and these enzymes include cholesterol oxidases, hydroxylases, and acyl transferases that generate the expected metabolites in vitro and in vivo assays. Using fluorescence microscopy, we studied the distribution of cholesterol at subcellular resolution, detecting the lipid in the Golgi and at the plasma membrane, but also in the endoplasmic reticulum and mitochondria. In summary, alkyne cholesterol represents a versatile, sensitive, and easy-to-use tool for tracking cellular cholesterol metabolism and localization as it allows for manifold detection methods including mass spectrometry, thin-layer chromatography/flourography, and fluorescence microscopy.—Hofmann, K., C. Thiele, H-F. Schött, A. Gaebler, M. Schoene, Y. Kiver, S. Friedrichs, D. Lütjohann, and L. Kuerschner. A novel alkyne cholesterol to trace cellular cholesterol metabolism and localization. J. Lipid Res. 2014. 55: 583–591.

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Cholesterol is the major sterol of mammalian cell membranes (1). Beyond its role for discrete membrane structures such as caveolae (2) or microdomains (3), cholesterol also specifically interacts with numerous proteins (4, 5). Cholesterol metabolites are involved in signaling (6), transport (7), and lipid storage (8). Dysregulation of cholesterol levels causes a wide range of human diseases (9–11). Hence, mammalian cells tightly regulate sterol biosynthesis, turnover, and transport (1, 7, 12). To follow the cellular fate of cholesterol by fluorescence microscopy, various fluorescent analogs (13–16), sterol binding toxins (17), and anti-cholesterol antibodies (18) have been used. Isotope-labeled cholesterol (1) and isotope-labeled toxins (19) have been employed to follow metabolism or organization of cholesterol in membranes, respectively. A probe suitable for conveniently tracing both cholesterol metabolism and localization would be of great value.

With the advent of bioorthogonal chemistry (20), including click chemistry (21), the sensitive and specific detection of compounds containing azido groups or terminal alkynes has become possible. Lipids containing an alkyne moiety have been used to trace their metabolism and distribution (22–26) and to monitor protein lipidation (27–30) or protein-lipid interaction (31). Thus far, no alkyne-bearing analog of cholesterol has been used to trace cholesterol in mammalian cells.

Here we report on the synthesis and use of alkyne cholesterol to study cholesterol metabolism by in vitro assays, and in vivo by using various cells. These data are supplemented by cellular localization studies of the probe.

MATERIALS AND METHODS

Filipin and azide-PEG3-biotin conjugate were obtained from Sigma. Borondifluorodipyrromethene (Bodipy)-cholesterol [23-(dipyrrometheneboron-difluoride)-24-norcholesterol] was from Avanti. Deuterated d6-cholesterol, cholesterol-26,26,26,27,27,27-2H6, and 4-cholestene-3-one were from C/D/N Isotopes and Steraloids, respectively. LD540 was described before (32). Antibodies against calnexin (Stressgen, SPA-860), EGFR (Epitomics, 2235-1), GM130 (Epitomics, 1837-1), and HSP60 (Stressgen, SPA-806) were used.

Abbreviations:  ALA, 5-aminolevulinic acid; ALDH, alcohol dehydrogenase; CSM, complete synthetic medium; CTL, cholestatrienol; DHE, dehydroergosterol; ER, endoplasmic reticulum; OPC, oligodendrocyte precursor cell.

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Plasmids

pmRFP1-mito codes for the native presequence (MLRAALSTARRGPRLSRKLL) of rat alcohol dehydrogenase (ALDH) followed by the first 17 amino acids of ALDH (SAAATSAPNPQPEV), a linker (6 amino acids; LVVPAT), and mRFP1. pmRFP1-Golgi and pmRFP1-endoplasmic reticulum (ER) are described elsewhere (35). All constructs were sequence-verified and added to the lab database.

Chemical synthesis

The syntheses of alkynyl cholesterol (Fig. 1) and the azido-reporter molecules are described in the supplementary text. Data from analyses of alkynyl cholesterol by TLC, MS, and NMR spectroscopy are provided (supplementary Fig. 1).

Generation of a Δhem1 yeast strain. A Saccharomyces cerevisiae strain lacking HEM1 (delta-aminolevulinic acid synthase) was created (34). For that, the strain W305 (a gift from Simon Alberti: ade2-1; trp1-1; ura3-1; leu2-3,112; his3-11,15; can1-100) was transformed with the deletion plasmid pEAGa-KanMX6 (35) containing the targeted sequence of hem1 (hem1::KanMX6), introduced after performing a PCR using the following primers (GGT GTC CTT CAA TTA TCA CAG TAC TTA GGT TTT TTT TTC AGT CCG ATC CCC CCG GGG TTA ATT AA and AAA TTC CTT GTA CCT CTA TCT CAG CCC ATC CAT ATA TTG GGT GAA TTC GAG CTC GTT TAA AC). The genotype of the resulting Δhem1 strain was verified by PCR.

Mammalian cell culture. Human A172 glioblastoma cells (ATCC®; CRL-1620) were maintained in DMEM (Gibco) containing 10% (v/v) FCS. For primary cultures P0 to P2 Wistar rats (Charles River) were euthanized. The cortex was trypsinized at 37°C and 5% (v/v) CO2. After performing a PCR using the following primers (GTT GTC CTT CAA TTA TCA CAG TAC TTA GGT TTT TTT TTC AGT CCG ATC CCC CCG GGG TTA ATT AA and AAA TTC CTT GTA CCT CTA TCT CAG CCC ATC CAT ATA TTG GGT GAA TTC GAG CTC GTT TAA AC). The genotype of the resulting Δhem1 strain was verified by PCR.

Yeast culture. Cells (Δhem1) were maintained in complete synthetic medium (CSM) (MP Biomedicals) containing 0.01% (w/v) 5-aminolevulinic acid (ALA) and 290 µg/ml G418. For feeding experiments, cells were transferred to CSM containing G418 to deplete cellular ALA pools and precultured for 12 h (34). This starter culture was split into equal aliquots and the growth media were supplemented with 0.5% (v/v) Tween80, 5.6 mg/ml methionine, and 30 µM sterol or ALA. Cells were incubated aerobically with shaking at 30°C and growth was monitored using spectroscopy (optical density at 600 nm) for 22 h.

Sterol labeling. A172 cells were grown in 3 cm dishes in DMEM containing FCS or delipidated FCS (38). Lipids were delivered by supplementing the respective fresh growth media with sterol from an ethanol stock solution to a final concentration of 10 µg/ml.

Cell collection and lipid extraction for TLC analysis. Cells incubated with sterols were washed twice with PBS containing 10 mg/ml fatty acid-free BSA (Applichem) and once with PBS. Cells were scraped in 155 mM ammonium acetate and lipid extraction was performed as described (39), but the pH of the aqueous phase was not adjusted.

Click reaction, lipid analysis by TLC, and imaging of TLC plates. The dry lipid pellet was redissolved by addition of 7 µl chloroform, followed by addition of 30 µl click reaction mixture (5 µl of 44.5 mM 3-azido-7-hydroxycoumarin, 500 µl of 10 mM [acetonitrile], CuBF4 in acetonitrile, 2 ml ethanol). Click reaction, TLC separation, and imaging were performed as described (24). Lipid quantification by fluorometry and colorimetry was performed using a GelPro analyzer (Media Cybernetics) or ImageGaugeV3.3 software (Fuji), respectively.

Lipid analysis by mass spectrometry. Details are provided in the supplementary text.

Cholesterol oxidase assay (in vitro). Purified cholesterol oxidase from Brevibacterium sp. (Sigma, C8868) was dissolved in phosphate buffer (50 mM KH2PO4 (pH 7.5), 1 mg/ml lipid-free BSA) and stored in aliquots at −80°C. Incubation time, buffer composition, and the amount of enzyme in the assay were optimized to ensure linearity of the reaction rate in the kinetic studies. Alkynyl cholesterol was incubated at 25°C with 2 ng enzyme in glass vials containing a total volume of 100 µl phosphate buffer supplemented with 0.1% (v/v) Triton X-100 while shaking (1,100 rpm) for 5 min. The reaction was stopped with 500 µl chloroform/methanol 3:1 (v/v), the vials centrifuged (500 g, 5 min), and the lower phase transferred to a reaction tube. The aqueous phase was washed with 200 µl chloroform and the combined chloroform phases were evaporated. Lipids were redissolved in 7 µl chloroform and reacted as above, but using a triple-concentrated click reaction mixture (15 µl of 44.5 mM 3-azido-7-hydroxycoumarin, 500 µl of 10 mM [acetonitrile], CuBF4 in acetonitrile, 2 ml ethanol). Detection and quantification of the signal from TLC plates, as well as the calculation of the Michaelis-Menten constant, Km, were performed as described previously (25).

Cholesterol oxidase assay (in vivo). Cells incubated for 16 h in medium containing delipidated serum and equal amounts of alkynyl cholesterol and deuterated δ9-cholesterol (total 10 µg/ml) were chased in medium lacking lipids for 1 h to achieve a steady-state distribution of the provided sterols. Samples were either fixed for 1 h using glutaraldehyde to arrest cellular lipid transport or left untreated. All samples, living or fixed, were incubated with cholesterol oxidase in 310 mM sucrose and 0.5 mM Na-phosphate (pH 7.5) at 37°C for 30 min (40) before lipid extraction. Extracts were divided, deuterated sterols were quantified by MS, and alkynyl sterols were quantified by MS and also by TLC.
Click reaction, lipid detection by fluorescence microscopy. Cells incubated with medium containing delipidated FCS and 10 µg/ml alkyne cholesterol for 16 h were washed in PBS and fixed using 3.7% formalin in buffer A (0.1 M HEPES/KOH (pH 7.4)) for at least 16 h. Cells were washed once with 155 mM ammonium acetate and twice with buffer A. For the detection, 50 µM of the azido-reporter were dissolved in prewarmed buffer A and added to the samples. The click reaction was initiated by addition of 2 mM [acetonitrile]CuBF₄ in acetonitrile (final 2% acetonitrile) and performed at 43°C for 30 min while gently agitating. Samples were extensively washed using buffer A. If applying, samples were further incubated with fluorescent streptavidin, LD540, or 100 µg/ml freshly dissolved filipin, and mounted.

Filipin binding assay. Liposomes from pure POPC were prepared by the reverse-phase evaporation method using ether and PBS solvents. The UV-absorbance of filipin in PBS containing the above liposomes was recorded before and after addition of 125 µM sterol or ethanol carrier to the mix using a Shimadzu UV-2450PC spectrophotometer.

Subcellular fractionation. Cells were washed, scraped, homogenized (EMBL cell cracker; 7 strokes, 16 µm clearance), and centrifuged to obtain a postnuclear supernatant. The postnuclear supernatant was loaded onto a continuous sucrose gradient and centrifuged to obtain a postnuclear supernatant. The postnuclear supernatant was loaded onto a continuous sucrose gradient and centrifuged to obtain a postnuclear supernatant. The postnuclear supernatant was loaded onto a continuous sucrose gradient and centrifuged to obtain a postnuclear supernatant. The postnuclear supernatant was loaded onto a continuous sucrose gradient and centrifuged to obtain a postnuclear supernatant.

Microscopy. Epifluorescence microscopy was performed using a Zeiss Observer.Z1 microscope (Carl Zeiss) equipped with a Plan-Apochromat 63× (1.40 NA) DIC and Fluar 40× (1.30 NA) and a Photometrics CoolSnap K4 camera. If applying, optical sectioning was performed using the apotome mode. The light source was a Polychrome V 150 W xenon lamp (Till Photonics). All images were processed employing Fiji (42) and Adobe Photoshop 6.0 (Adobe) software. Projections of z-stacks were calculated by summarizing corresponding pixel values.

RESULTS

Alkyne cholesterol rescues sterol auxotroph yeast

To track cellular metabolism, we have synthesized an analog of cholesterol, alkyne cholesterol (Fig. 1, supplementary Fig. I). The alkyne moiety comprises the terminal part of the side chain and thus results in an analog of pronounced structural similarity to natural cholesterol. To test the bio-compliance of alkyne cholesterol, we generated a sterol auxotroph yeast strain lacking the hem1 gene (34). Under heme-depleted conditions, these cells are unable to synthesize sterols and depend on exogenous sources to meet the various demands and enable growth. We provided ergosterol, cholesterol, alkyne cholesterol, or ALA, the metabolic product of Hem1p, delta-aminolevulinic acid synthase to these cells and measured their growth (Fig. 2A). Alkyne cholesterol, as well as the natural sterols and ALA rescued the auxotroph, while an analog carrying a Bodipy-fluorophore in the side chain did not allow for rapid cell expansion (Fig. 2A). In our experiments yeast cells propagated by cell division, not fusion (43), and enzymatically converted alkyne cholesterol to the corresponding esters during growth (Fig. 2B). These data illustrate that alkyne cholesterol can substitute natural sterol in these cells.

Alkyne cholesterol mimics cholesterol in in vitro and in vivo enzymatic assays

Next, we assayed the conversion of alkyne cholesterol to the corresponding cholest-4-en-3-one by purified cholesterol oxidase using our detection routine for alkyne lipids (24), recently optimized for in vitro assays (25). Cholesterol oxidase from Brevibacterium sp. accepted alkyne cholesterol as a substrate and the enzymatic reaction followed Michaelis-Menten kinetics (Fig. 3). The kinetic constant, Kₘ, was determined as 19.4 µM by nonlinear regression. These enzymatic measurements were complemented by studies in living mammalian cells analyzing the conversion of alkyne cholesterol to alkyne cholesterol esters. Cultured human A172 glioblastoma cells were incubated with alkyne cholesterol and analyzed for cellular alkyne metabolites by click reaction and TLC (Fig. 4). Cells growing in regular culture medium took up the alkyne cholesterol and its cellular levels saturated after 5 h of incubation (Fig. 4A). Also, cells incubated in medium with delipidated FCS reached a plateau in alkyne cholesterol content between 5 and 16 h, but contained only half the amount of alkyne cholesterol esters after 16 h compared with cells in regular medium. At this time, the latter had esterified nearly half of the alkyne cholesterol. The total content of alkyne sterols, free and esterified, increased throughout the experiment for both feeding conditions. The rate of esterification in the different growth media was studied in more detail in pulse-chase experiments (Fig. 4B). After pulse-incubation of 1 h with alkyne cholesterol, hardly any esters could be detected. Cells chased in regular medium for up to 24 h progressively converted the absorbed alkyne cholesterol into esters reaching final levels of nearly 70%, a level that could not be further increased by supplementing the chase media with oleic acid to drive sterol ester formation. On the contrary, cells in delipidated medium accumulated levels of 4% or, in the presence of increased oleic acid levels, of 18% of alkyne cholesterol esters. The biosynthesis of sterol esters was also studied in primary cells of the rat brain (supplementary Fig. II). When incubated with alkyne cholesterol in the respective growth medium, astrocytes (panel A) and hippocampal neurons (panel B) were found to generate alkyne cholesterol esters to different extents, while oligodendrocytes (panel C) did not accumulate detectable amounts during the course of the experiments.
After 20 h, astrocytes showed about 10-fold higher levels of alkyne cholesterol esters than neurons. After extended incubation times, oligodendrocytes, neurons, and astrocytes contained unidentified alkyne cholesterol metabolites, some of which potentially correspond to alkyne oxysterols synthesized by these cells (6).

To investigate the influence of alkyne cholesterol on cellular sterol balance, cells were incubated with alkyne cholesterol, cholesterol, or carrier in medium with delipidated FCS for 48 h and cellular sterols were analyzed by MS (Table 1). Compared with controls without any sterol supplement, cells showed reduced levels of latho-, lano-, and 24-dihydrolanosterol, all important precursors in cellular sterol biosynthesis, upon incubation with alkyne cholesterol. This finding is expected, as cholesterol feeding results in a downregulation of its endogenous synthesis. The cellular levels of cholestanol, an enzymatic product of cholesterol, followed the changes of the cholesterol concentration under the different feeding conditions. When feeding alkyne cholesterol, our experimental conditions yielded a ratio of alkyne cholesterol to cholesterol of 1:4.2. The total amount of cholesterol, originating from exogenous and endogenous sources, was increased if the medium was supplemented with either sterol. We also determined the cellular levels of oxysterols under the different feeding conditions and compared wild-type cells with cells transiently overexpressing cholesterol-24-hydroxylase CYP46A1 (Table 2). Increased amounts of the CYP46A1 enzyme resulted in a profound accumulation of the 24-hydroxylation products of both cholesterol and alkyne cholesterol that were identified by GLC-MS (supplementary Fig. III). Basal levels of 24-OH-alkyne cholesterol were also detectable in wild-type cells, indicating a basal expression of CYP46A1 in these cells.

**Alkyne cholesterol and cellular metabolites can be visualized by fluorescence microscopy**

An important feature of alkyne cholesterol is its applicability to microscopy. Using the same analog, this allows for parallel investigations on cholesterol metabolism and distribution. To localize alkyne cholesterol by fluorescence microscopy in fixed cells, we developed a staining protocol employing different azido reporters. Importantly, the use of these detection agents allows for a great choice of bright photostable fluorophores with different spectral properties. In our microscopy studies, we detected either cellular alkyne cholesterol after click reaction with an azide coupled to a fluorescent Bodipy-dye (Fig. 5A) or an azide coupled to biotin and subsequent incubation with a fluorescent streptavidin-conjugate (Fig. 5B). With both approaches, we observed intense alkyne cholesterol staining at the plasma membrane and internal organelles. Parallel probing for free sterol using the UV-excitable filipin in a separate channel is possible and full channel separation could be achieved (Fig. 5C, D). Compared with cells in delipidated medium without any sterol supplement, the incubation with 10 µg/ml alkyne cholesterol increased the

**Fig. 2.** A yeast sterol auxotroph (*hem1*) is rescued by and converts alkyne cholesterol. Before the experiments, the cellular pools of ALA were depleted by preincubating the cells in CSM lacking sterols or ALA. Subsequently cells were cultured in CSM supplemented with Tween80 as a source for oleic acid and 0.03 mM sterol or ALA. A: Cells were shaken aerobically at 30°C and growth was monitored for 22 h. The optical density at 600 nm (OD$_{600}$) is plotted over time. B: Cellular alkyne sterols were analyzed by TLC after incubation with cholesterol (lane 1) or alkyne cholesterol (lane 2) for 22 h. The sterol auxotroph yeast metabolically converts alkyne cholesterol (cholesterol ester or CE). X denotes unidentified lipids, potentially oxysterols, for which we have not yet synthesized an according lipid standard.

**Fig. 3.** Purified cholesterol oxidase converts alkyne cholesterol into the corresponding alkyne cholest-4-en-3-one. Michaelis-Menten kinetics were measured and the reaction rate directly (main) or double-reciprocally (insert) plotted against the substrate (S) concentration. Line graphs show the reaction rates calculated from the Michaelis-Menten equation using nonlinear (main: $K_m = 19.4$ µM) or linear regression (insert). The experiment was performed in triplicate; error bars are shown in the direct plot only.
lipids of washed cells were extracted and analyzed by TLC for fluorescent metabolites, which were identified by comigrating lipid standards. Relative amounts of cellular alkyne cholesterol esters (CE) and alkyne cholesterol (chol) were determined by fluorescent metabolites, which were identified by comigrating lipid standards. Relative amounts of cellular alkyne cholesterol esters (CE) and alkyne cholesterol (chol) were determined by fluorography; total phosphatidylycerol was determined colorimetrically from the same TLC plates after charring. Asterisks denote lipid amounts below the detection limit. ori, origin. B: After 1 h of incubation, cells were washed twice with the respective medium containing delipidated BSA and incubated with fresh chase medium supplemented or not with 100 μM oleic acid for the indicated chase times. Lipids were analyzed as above. Data are mean ± range, n = 2 for solid colors, or single determinations.

Table 1. Analysis of sterol metabolites in glioblastoma cells

| Condition          | Alkyne Cholesterol (μg/mg) | Cholesterol (μg/mg) | Lanosterol (μg/mg) | 24β-Hydroxy-lanosterol (μg/mg) | Lathosterol (μg/mg) | Desmosterol (μg/mg) | Cholestanol (μg/mg) |
|--------------------|---------------------------|--------------------|-------------------|-------------------------------|-------------------|--------------------|--------------------|
| No supplement      | ND                        | 13.80 ± 2.32       | 25.23 ± 3.82      | 9.52 ± 4.63                   | 0.148 ± 0.002     | 0.038 ± 0.006      | 0.055 ± 0.016      |
| Plus alkyne chol   | 3.04 ± 0.23               | 12.74 ± 0.92       | 12.07 ± 3.03      | 7.36 ± 4.15                   | 0.074 ± 0.015     | 0.021 ± 0.002      | 0.044 ± 0.009      |
| Plus cholesterol   | ND                        | 19.98 ± 2.43       | 8.64 ± 0.62       | 4.27 ± 0.85                   | 0.074 ± 0.021     | 0.028 ± 0.007      | 0.170 ± 0.125      |

Glioblastoma cells were cultivated in medium containing delipidated FCS and supplemented with alkyne cholesterol, cholesterol, or carrier. Cellular sterol lipids were analyzed by GLC-MS. chol, cholesterol; ND, not determined. Data are mean ± SEM, n = 3.

Fig. 4. Cellular acyltransferases synthesize alkyne cholesterol esters from alkyne cholesterol in A172 glioblastoma cells. Cells were grown in medium containing regular FCS (full) or delipidated FCS (DL) for 16 h prior to incubation with the respective medium containing 12 μM of alkyne cholesterol. A: After the indicated times, lipids of washed cells were extracted and analyzed by TLC for fluorescent metabolites, which were identified by comigrating lipid standards. Relative amounts of cellular alkyne cholesterol esters (CE) and alkyne cholesterol (chol) were determined by fluorography; total phosphatidylycholine was determined colorimetrically from the same TLC plates after charring. Asterisks denote lipid amounts below the detection limit. ori, origin. B: After 1 h of incubation, cells were washed twice with the respective medium containing delipidated BSA and incubated with fresh chase medium supplemented or not with 100 μM oleic acid for the indicated chase times. Lipids were analyzed as above. Data are mean ± range, n = 2 for solid colors, or single determinations.

DISCUSSION

To trace the cellular metabolism and localization of cholesterol, the alkyne analog presented here offers several advantages over cholesterol radioisotopes. Its handling is easier, as specialized laboratories and official permissions are not needed. Because experimental samples involving alkyne cholesterol are free of radioactivity, they are easily...
TABLE 2. Analysis of oxysterols in glioblastoma cells

|野型 | 无补充 | 增加丙炔胆固醇 | 胆固醇 | + CYP46A1 | 无补充 | 增加丙炔胆固醇 | 胆固醇 |
|---|---|---|---|---|---|---|---|
|24-氧胆固醇 | 4.17 ± 0.28 | 1.28 ± 0.15 | 2.70 ± 0.82 | 9.68 ± 3.14 | 3.06 ± 1.29 | 10.11 ± 5.26 |
|比值: 24-氧-丙炔 cholesterol ×10^3/4-氧-胆固醇 | ND | 14.23 ± 6.67 | ND | ND | 24.17 ± 3.90 | ND |

Glioblastoma cells were cultivated in medium containing delipidated FCS and supplemented with alkyne cholesterol, cholesterol, or carrier. Cells transiently expressing CYP46A1 were also examined. Cellular sterol lipids were analyzed by GLC-MS. chol, cholesterol; ND, not determined. Data are mean ± SEM, n = 3.

Compatible with modern analysis methods, Alkyne cholesterol and other alkyne lipids can be detected from lipid extracts by MS and, as we showed before for alkyne fatty acids, by TLC and fluorography with high sensitivity after click reaction with a reporter azide (24). When designing the cholesterol analog, we chose to implement the small alkyne moiety at the terminus of the side chain. This allows for a minimal impact on the overall biophysical and biological properties, as all important features of the cholesterol molecule are preserved (45) and introduction of heteroatoms (46) is avoided. From the structural aspect, alkyne cholesterol shares its high similarity to cholesterol with the fluorescent dehydroergosterol (DHE) and cholesta-trienol (CTL) (13–15, 47). Both these probes contain additional double bonds in the sterol rings and in CTL, but not DHE, the unmodified side chain of cholesterol is preserved. While for many applications alkyne cholesterol will prove superior, experiments requiring a probe with an unchanged side chain especially will benefit from CTL as a complementary tool.

To validate our alkyne cholesterol probe, we employed various biological systems. Alkyne cholesterol is accepted by enzymes from different biological species (Brevibacterium, yeast, rat, human), and these enzymes include cholesterol oxidases, hydroxylases, and acyl transferases. We have demonstrated the use of alkyne cholesterol in vitro and in vivo experiments and followed its enzymatic conversion to various metabolites. Also, alkyne cholesterol allowed for the survival and proliferation of a yeast strain vitally dependent on exogenous sources of sterols. A related yeast strain that also auxotroph for exogenous sterol has been used before for validation of DHE (13).

A major advantage of the alkyne cholesterol analog is its applicability to microscopy. After a click reaction employing a group of versatile reporter azides and bright photostable fluorophores, alkyne cholesterol was conveniently localized at subcellular resolution. As alkyne cholesterol binds to filipin, a popular cholesterol probe, an additional detection option is available. In a localization experiment using filipin, or both filipin and click detection, we detected profound sterol labeling of the plasma membrane in accordance with the notion that the majority of cellular cholesterol is located there (48). Additionally, a significant costaining in the perinuclear region was observed. As the intensity of the filipin signal, especially at internal organelles, increased upon alkyne cholesterol feeding, we argue that filipin also interacts with alkyne cholesterol in cells, in agreement with our in vitro binding data. These cellular pools of cholesterol have been imaged in many studies employing filipin, DHE, CTL (13–15, 47), and BCθ, a derivative of the sterol binding toxin perfringolysin O (17, 18, 49). Using alkyne cholesterol and fluorescently labeled marker proteins, we confirmed positive costaining of the Golgi, ER, and mitochondria. Supportive subcellular fractionation analysis was performed to complement the imaging experiments and demonstrate equality of alkyne and normal cholesterol localization. Thus far, ER and mitochondria labeling have not been highlighted in microscopy studies using filipin or DHE. Contrarily, BCθ did not label the ER in an electron microscopy analysis (49). It is currently unclear whether the addition of alkyne cholesterol to cells lifts the overall sterol levels, e.g., at the ER above a certain threshold (50, 51), that if met otherwise, would allow for labeling by BCθ also. A dependence of the toxins’ interaction on the cholesterol levels has been reported (18, 50). Filipin is known to fail to label some sterol-containing membranes (52), and the sub-optimal spectral properties of DHE and CTL (47) might prevent detection of lower levels of sterol in specific cellular membranes. However, the ER is known to contain an important finely-tuned pool of cholesterol (51). Our findings on mitochondrial cholesterol in turn are supported by MS experiments that show considerable amounts of ergosterol or cholesterol in mitochondria from the yeast Pichia pastoris (53) and in mitochondria from mouse brains (54), respectively.

In summary, the novel alkyne cholesterol presented here proves useful for tracking cellular cholesterol metabolism and localization. It represents a versatile, sensitive, and easy-to-use tool allowing for manifold modes of detection such as MS, TLC/fluorography, and fluorescence microscopy. Despite several important advantages, microscopy studies using alkyne cholesterol are not free of limitations. As applying to all analogs, great attention must be paid to determine whether a certain probe represents a suitable tool for the specific scientific question addressed. One obvious concern when localizing alkyne cholesterol and metabolites by microscopy using our protocol is the need for an additional chemical detection reaction involving copper ions. While alkyne cholesterol is metabolically converted to the respective sterol esters that are typically stored in lipid droplets, the copper ions will unlikely be able to catalyze the click reaction with a reporter azide within the hydrophobic core of these organelles. Accordingly, staining of lipid droplets in our micrographs is likely...
Sterol tracing by alkyne cholesterol remains accessible by our imaging method. As the current protocol includes a fixation step, it does not allow for lipid tracking in living cells. However, this limitation will likely be overcome in the near future.

Importantly, this only applies to microscopy, not to assays on cholesterol ester metabolism, and as cholesterol esters usually contribute very little to the total cellular sterol content, the majority of the cellular cholesterol remains accessible by our imaging method. As the current protocol includes a fixation step, it does not allow for lipid tracking in living cells. However, this limitation will likely be overcome in the near future.

Fig. 5. Alkyne cholesterol distribution can be studied by fluorescence microscopy. Cells incubated with alkyne cholesterol or carrier in delipidated medium for 16 h were fixed and probed for sterols. Alkyne cholesterol was detected after click reaction using azides coupled to a Bodipy-dye (BDY) (A, C), or biotin in combination with Alexa-488-streptavidin (A488) (B, D). Cellular sterols were costained by filipin (C, D). The micrographs show individual slices from a z-stack (A, B), or epifluorescence images (C, D) depicting the signals of click reporter (green) and filipin (magenta) in merged images with colocalization resulting in a white appearance. The individual channels are shown as inserts (C, D); the full z-stacks of (A, B) are provided as supplementary Movies I and II, respectively. Bars, 20 μm.
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Fig. 6. Co-localization analysis of alkyne cholesterol and fluorescent organelle marker proteins. Cells transiently expressing marker proteins were incubated with alkyne cholesterol in delipidated medium for 16 h, fixed, and probed for alkyne cholesterol by a click reaction using an azide coupled to biotin. Al- exa-488-streptavidin was used for detection. Epifluorescence micrographs depict the signals of click reporter (green) and marker proteins (magenta) in merged images with colocalization resulting in a white appearance. The individual channels are shown as gray scale images. Bars, 20 μm.
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