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Specific Imaging of Intracellular Lipid Droplets using a Benzothiadiazole Derivative with Solvatochromic Properties

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Abstract

Altered lipid metabolism and extensive lipid storage in cells has been associated with various medical disorders, including cancer. The development of fluorescent probes that specifically accumulates in lipid deposits are therefore of great interest in order to study pathological processes that are linked to dysregulated lipogenesis. In the present study, we present a small fluorescent benzothiadiazole dye that specifically stains lipid droplets in living and fixated cells. The photophysical characterization of the probe revealed strong solvatochromic behaviour, large Stokes shifts and high fluorescent quantum yields in hydrophobic solvents. In addition, the fluorophore exhibits a nontoxic profile and a high signal to noise ratio in cells (i.e. lipid droplets vs. cytosol), which make it an excellent candidate for studying lipid biology using confocal fluorescent microscopy.

Introduction

Intracellular lipid droplets (LDs) are lipid-rich spherical organelles that mainly consist of triglycerides (TAG) and cholesterol esters (CE) enclosed by a phospholipid monolayer with associated proteins.¹⁻⁵ Besides being energy reservoirs, and providing building blocks for membrane synthesis, LDs are dynamic bodies with imperative cellular assignments ranging from membrane trafficking, fusion, and involvement in signal transduction pathways.⁶⁻¹⁰ Thus, the interest in LDs has emerged during recent years due to their association with pathological processes in diseases such as cancer, obesity, diabetes and inflammatory disorders.¹¹⁻¹⁴ The development of fluorophores that specifically assembles in lipid deposits are therefore of great importance for studying dysregulated lipogenesis on a cellular level. Several lipid droplet specific dyes have been reported in the literature, such as Nile Red and various BODIPY derivatives.¹⁵⁻¹⁹ However, Nile Red also stains other hydrophobic structures in the cell, resulting in low LD selectivity and unfavourable signal-to-noise ratio.²⁰⁻²¹ On the contrary, BODIPY fluorophores have shown high selectivity toward LDs. However, they usually display small Stokes shifts that can give background artifacts due to light scattering originating from the excitation source.²² Nevertheless, numerous efforts have been performed in order overcome these issues and this has further resulted in alternative LD-specific fluorophores with beneficial photophysical properties.²³⁻²⁷

In our search for fluorescent benzothiadiazole derivatives useful in studying cellular changes associated with malignant transformation, we came across an interesting compound that showed specific accumulation in intracellular lipid droplets. Hence, in this paper we investigate a fluorescent benzothiadiazole dye, hereinafter referred to as **LD-BTD1** (Lipid Droplet-Benzothiadiazole 1), which could be used to explore pathological processes associated with extensive intracellular lipid storage. Notably,

this compound has recently been reported in the literature, including synthesis, crystal structure determination and optical properties in dichloromethane.²⁸ However, in the present study, we have characterized the photophysical properties for the derivative in solvents with different polarity and also investigated its specific localization in fibroblasts and melanoma cells using confocal fluorescence microscopy.

Results and Discussion

The benzothiadiazole (BTD) moiety has a strong electron-withdrawing capacity, and it is known for its favorable photophysical properties, such as large Stokes shifts, high signal-to-noise ratios and red-shifted optical profiles.²⁹ The BTD unit has therefore extensively been used in polymeric materials for various light technology applications, primarily in organic light emitting diodes (OLEDs) and high performance solar cells.³⁰ However, the use of BTDs in other research areas has rapidly been developed. For example, in the field of fluorescent cell microscopy, small synthetic modifications (e.g. introduction of different substituents) on the BTD scaffold have shown to generate fluorophores with unique properties in terms of photophysics, cell permeability and specific organelle accumulation.²⁹ For instance dyes that exclusively assemble in mitochondria, the cytosol, the nucleus (due to e.g. binding to double-stranded DNA) or in lipid-based structures.³¹⁻³⁵

Synthesis: In the present study, we have combined the electron-withdrawing benzothiadiazole scaffold with an electron-donating substituent (dimethylaminophenyl), resulting in a push-pull fluorophore.²⁸ In a push-pull system, the LUMO is mainly localized on the acceptor moiety whereas the HOMO is centered on the donor fragment. This allows for individual tuning of the HOMO and LUMO energies, resulting in a low HOMO-LUMO gap, and thus a red-shifted absorption.³⁶ As depicted in Scheme 1, the benzothiadiazole derivative (**LD-BTD1**) was prepared in 96% yield using a palladium mediated Suzuki reaction. Accordingly, 4-bromo-2,1,3-benzothiadiazole and 4-(dimethylamino)phenylboronic acid were cross-coupled in the presence of PEPSSI-IPr as catalyst and K₂CO₃ as base in Toluene/MeOH (1:1).

Scheme 1. Reagents and conditions: 4-(dimethylamino)phenylboronic acid, PEPSSI-IPr, K₂CO₃, Toluene/MeOH (1:1), 80 °C, overnight.

Cellular Studies: Initially, we wanted to explore the utility of **LD-BTD1** as a fluorophore for cell imaging using confocal fluorescence microscopy. Human normal fibroblasts from the skin (AG01518) and a malignant transformed cell line originating from melanocytes in the skin (malignant melanoma, SK-MEL-28) were used in the study. **LD-BTD1** staining of cultured cells, both living and fixated, resulted in a punctate staining pattern (Figure 1A and Supplementary Figure S1). The staining was more intense in the melanoma cell line compared to normal fibroblasts, although staining was also seen in the fibroblasts when using a higher gain (Supplementary Figure S2). The evident difference in fluorescence intensity was quantified by flow

cytometry, showing that the median fluorescence intensity in melanoma cells was significant higher compared to the intensity in the fibroblasts (Figure 1B-D), which is well in accordance with the microscopy data.

Figure 1. Human fibroblasts and melanoma cells incubated with **LD-BTD1** (10 μM) for 24 h. A) Confocal microscopy images of cells stained with **LD-BTD1** (seen in green; ex 458 nm, em 465-682 nm) and DAPI (nuclei staining seen in blue; ex 405, em 410-453). Scale bar 10 μm . Staining was performed on living cells, which were fixated directly after the incubation (prior to the microscopy analysis). Panels B and C show representative histograms from flow cytometry analysis of **LD-BTD1** in fibroblasts and melanoma cells, respectively. D) Quantification (median fluorescence intensity) of the data presented in B and C. Results are presented as mean + SD; n=4. * denotes statistical significant difference ($p \leq 0.05$).

To further explore the potential of **LD-BTD1** as a fluorophore for cell imaging, the cell viability during prolonged incubation (72 h) was investigated using the MTT-assay.³⁷ No signs of toxicity could be seen in the cell cultures after incubation with **LD-BTD1**, as cell morphology was indifferent from controls (Figure 2A). In addition, no significant alteration in cell viability was observed (Figure 2B), suggesting that **LD-BTD1** is non-toxic at 10 μM and possible to use in living cells for prolonged

times. However, staining was also analyzed at lower concentrations, which revealed a detection limit of approximately 0.5 μ M (Supplementary figure S3).

Figure 2. Cell viability data of human fibroblasts and melanoma cells incubated with **LD-BTD1** (10 μ M). A) Phase-contrast images of cells treated with **LD-BTD1** for 48 h (scale bar 40 μ m). B) Cell viability, measured by the MTT assay, after treatment with **LD-BTD1** for up to 72 h. Results are expressed as % of corresponding DMSO control and presented as mean+ SD; n = 4.

The punctate intracellular pattern for **LD-BTD1** was further investigated using co-staining with different markers for major cellular organelles. The results did not show any apparent co-localization with mitochondria, lysosomes, early endosomes or autophagosomes (Supplementary Figure S4). However, specific staining of hydrophobic compartments (including LDs) using Nile Red showed an equivalent staining pattern as for **LD-BTD1** (Supplementary Figure S5). Notably, co-localization analysis of **LD-BTD1** and Nile Red is not possible since their excitation and emission spectra overlap (Supplementary Figure S6) and thereby cannot be distinguished by fluorescence microscopy. Instead, **LD-BTD1** staining was combined with an antibody towards adipophilin. Adipophilin, also known as perilipin2 (PLIN2) or adipose differentiation-related protein (ADRP), is a protein that associate with the lipid droplet surface and is therefore widely utilized as a protein marker for LDs.³⁸ As

shown in Figure 3, we found that **LD-BTD1** and adipophilin co-localized in the cells. Thus, in panels to the right (Figure 3, merged and zoomed) adipophilin staining is seen in the periphery of **LD-BTD1**-positive dots, which is well in concordance with adipophilin being localized on the surface of the LDs. These results indicate that **LD-BTD1** accumulates in LDs in cultured cells and further suggests that it preferentially stains the core of the LD, where the hydrophobic triglycerides and cholesterol esters are found.

In general, LDs can virtually be found in any cell. However, many cell types exhibit only a few, small lipid droplets under normal conditions, while others (such as adipocytes or hepatocytes) have a higher content due to active lipid synthesis and storage.³⁹ A low level of LDs is a reasonable explanation for the relative weak staining of **LD-BTD1** seen in fibroblasts (Figure 1A). Further, upregulated lipogenesis is a common characteristic in a number of human carcinomas, which might explain the higher staining intensity observed in the malignant melanoma cell line.⁴⁰⁻⁴¹ In order to endorse the LD specific staining of **LD-BTD1**, we performed experiments on human isolated adipocytes (Supplementary figure S7), which often contain one single large LD.¹ **LD-BTD1** staining of adipocytes revealed staining that corresponded to a large LD constituting the vast majority of the cell area and displacing the nucleus to the periphery (Supplementary Figure S7), which is well in concordance with previous reports.⁴²⁻⁴³

Figure 3. Fluorescent microscopy images of **LD-BTD1** (10 μ M, 24 h) and the lipid droplet associated protein adipophilin (PLIN-2) in fibroblasts and melanoma cells. Staining was performed on living cells, which were fixated directly after the incubation and further used for immunocytochemistry. Panels to the left show **LD-BTD1** (seen in green; ex 458 nm, em 484-707 nm) or the anti-PLIN-2 antibody (seen in red; ex 595 nm, em 600-734 nm). The panels to the right show merged images in order to emphasize the co-localization between **LD-BTD1** and the adipophilin antibody. Scale bar 10 μ m.

Supplementation of the cell culture media with fatty acids (e.g. oleic acid) is known to enhance the number and size of LDs, due to the stimulation of the fatty acid receptor FFAR4.^{38,44} These dynamic features of LDs can be visualized using a fluorophore that specifically accumulates in lipid deposits. As shown in Figure 4, the increased quantity of LDs upon the addition of oleic acid could clearly be monitored by

enhanced fluorescence intensity, both in fibroblasts and melanoma cells, using contemporary staining with **LD-BTD1**. Notably, the dynamics of LDs has recently been studied in living cells using a benzothiadiazole derivative with a pendant oleic acid tail.³⁵ However, our results demonstrate that the small **LD-BTD1** derivative (in the absence of a fatty acid substituent) is a suitable fluorophore for studying dynamic changes in LD biology. Altered lipid metabolism and extensive lipid storage, represented by enhanced numbers of LDs, have been described in several neoplastic diseases and are considered as a hallmark of cancer aggressiveness and poor prognosis.^{12,41,45} Besides the accumulation in cancer cells, LD alterations are frequently observed in infections and other inflammatory disorders.¹² Therefore, **LD-BTD1** is a potential LD dye that could be used in preclinical studies of cancer as well as in the study of inflammatory diseases.

Figure 4. **LD-BTD1** staining (seen in green; ex 458 nm, em 465-682 nm) in fibroblasts and melanoma cells after lipid supplementation with oleic acid (150 μ M, 24h). Cell nuclei are stained with DAPI (seen in blue; ex 405 nm, em 410-453 nm). Scale bar 10 μ m. Staining was performed on living cells, which were fixated directly after the incubation (prior to the microscopy analysis).

Photophysical Characterization: In order to rationalize the selective emission of **LD-BTD1** in cells, the photophysical properties of **LD-BTD1** were explored by UV-vis absorption and emission spectroscopy. In hexane, **LD-BTD1** exhibits an absorption maximum at 420 nm ($\epsilon=7100 \text{ M}^{-1}\text{cm}^{-1}$), a fluorescence maximum at 511 nm (Figure 5) and a high fluorescence quantum yield ($\phi_F = 0.66$). The brightness (α extinction coefficient * fluorescence quantum yield) is proportional to the fluorescence output per chromophore and can be used as a value of merit between fluorescent probes. Consequently, the brightness of **LD-BTD1** in hexane could be described as moderate due to its relatively low extinction coefficient. However, the highest priority for cellular microscopy applications is to acquire a large signal-to-noise ratio between the studied species and the background. Factors that can affect the signal-to-noise ratio include: 1) the concentration ratio of the probe between the cellular feature under study and the surroundings, 2) the difference in the fluorescence quantum yield of the probe between the cellular target under study and the surroundings, and 3) the magnitude of the Stokes shift of the fluorophore, as to avoid scattered light from the excitation source.

Figure 5. Molar absorptivity (blue) and normalized emission spectra (green, exc.430 nm) of **LD-BTD1** in hexane.

To give a rough estimate of the partitioning equilibrium between **LD-BTD1** in water and lipid droplets, the octanol-water partition constant, K_{ow} , was measured. The K_{ow} was determined to 2000, which corresponds to $\log P = 3.3$. However, due to the relative difficulty in measuring high K_{ow} values, this should be viewed as a lower estimate. The K_{ow} value can be used for direct estimation of the concentration ratio of **LD-BTD1** between a lipid feature and the cytosol. Thus, when using **LD-BTD1** as a fluorescent probe in a cellular experiment, the concentration ratio between the lipid feature and the cytosol is expected to be greater than 2000. To give an idea on the difference on the photophysical properties of **LD-BTD1** in a hydrophobic and lipid-rich environment compared to the cytosol, the general solvatochromic behavior of **LD-BTD1** was investigated in a series of solvents with various polarity index. Absorption and fluorescence maxima (Supplementary Figure S8 and S9), Stokes shift values and fluorescence quantum yields for **LD-BTD1** in solvents of different polarity are presented in Table 1. **LD-BTD1** exhibits large solvatochromic shifts with increasing solvent polarity from hexane ($\lambda_{E, max}=511$ nm) to DMSO ($\lambda_{E, max}=759$ nm). A Lippert plot (Supplementary Figure S10) was used to rationalize the solvent effect on spectral shifts for **LD-BTD1**. The linearity of this plot indicates that the solvent polarity is the dominant reason for spectral shifts, and thus, that the dipole moment of the ground and excited state differs significantly.⁴⁶ Specific solvent effects were also investigated by the addition of a small amount of water to **LD-BTD1** dissolved in DMF (Supplementary Figure S11). Increasing amount of water (0.5-10%) did not result in significant spectral changes, except for emission quenching. This excludes specific solvent effects, such as hydrogen bonding, as a reason for the observed spectral shifts. Furthermore, the spectroscopic properties in water were also investigated. However, 10% DMSO in water was used for the measurements due to the low solubility in water. Despite of increasing the polarity with a quite large percentage, considerable blue-shifts were seen in the absorption and emission spectra (Supplementary Figure 12), suggesting that the dye is aggregating in an aqueous environment.

Table 1. Absorption and fluorescence maxima, Stokes shift values and fluorescence quantum yields for **LD-BTD1** in solvents of different polarity.

Solvent	$\lambda_{A,max}$ (nm)	$\lambda_{E,max}$ (nm)	$\lambda_{E,max} - \lambda_{A,max}$ (nm)	ϕ_F
Hexane	420	511	91	0.66
Toluene	429	578	149	0.71
Diethyl ether	420	583	163	0.66
THF	429	633	204	0.38
Octanol	431	635	204	0.02
Methanol	423	709	286	< 0.01
DMF	429	713	284	0.04
DMSO	440	759	319	0.03

The fluorescent quantum yields of **LD-BTD1** were found to decrease from 70 % to less than 1 % going from low to high polarity solvents (Table 1). However, comparing solvents of similar polarity but with different capability for hydrogen bonding, such as THF and octanol, indicates that hydrogen bonding increases quenching of the excited state by contributing to the non-radiative relaxation process. This has also been earlier been observed in other benzothiadiazole containing fluorophores.⁴⁷⁻⁴⁹ The two orders of magnitude difference of the fluorescence quantum yield between a hydrophobic and hydrophilic environment results in a high contrast ratio between **LD-BTD1** being present in different cellular environments. Furthermore, the contrast ratio between the surroundings depends on the concentration ratio between the environments times the difference in fluorescence quantum yield. The observed contrast ratio at equilibrium between **LD-BTD1** being present in the cytosol compared to a hydrophobic environment is thus expected to exceed 2×10^5 (i.e. the concentration multiplied with the difference in fluorescence quantum yield). Also, for microscopy studies, the relative high Stokes shift (even in hydrophobic solvents such as hexane) is advantageous, since it makes removal of scattered light from the excitation source straightforward. Furthermore, the pH dependence (pH 2-12) of **LD-BTD1** was investigated, which showed that the emission was not significantly changed in a wide range (pH 4-12) (Supplementary Figure S13). However, a reduced intensity was observed at pH 2, likely due to protonation of the benzothiadiazole.⁵⁰⁻⁵¹ Importantly, the shape of the emission curve was unchanged at pH 4-12 and we therefore suggest that the compound is stable under these conditions.

Conclusion

In summary, we have described the synthesis, photophysical characterization and confocal fluorescence microscopy studies of a small nontoxic benzothiadiazole derivative (**LD-BTD1**) that specifically stains intracellular lipid droplets. The fluorophore exhibits strong solvatochromic behavior in solvents with increasing polarity, resulting in Stokes shifts ranging from 91 nm (in hexane) to 319 nm (in DMSO). The localization of **LD-BTD1** in the lipophilic environment of LDs can be rationalized by its preferred partition in hydrophobic solvents in comparison with an aqueous solution ($K_{ow} = 2000$). In addition, the fluorescence quenching in water and the high quantum yields in hydrophobic solvents (e.g. toluene $\phi_F = 0.71$) further enlarges the perceived contrast between the LDs and the cytosol resulting in low to none background. We have further demonstrated that **LD-BTD1** clearly can reflect the higher abundance of LDs in melanoma cancer cells in comparison to normal fibroblasts. It can also be used to study lipid dynamics in cells, which were shown using fatty acid supplementation. Thus, we foresee that **LD-BTD1** can be used as an

attractive fluorescent dye for exploring the role of extensive lipid storage and dynamics that are related to pathological processes in cancer and inflammatory disorders.

Experimental Procedures

General

All reagents and solvents were of analysis or synthesis grade and were used without further purification unless otherwise indicated. ^1H and ^{13}C NMR were recorded on a Varian 300 instrument (Varian Inc., Santa Clara, CA, USA) (at 300 and 75 MHz, respectively) in CDCl_3 . Chemical shift is reported in ppm with the solvent residual peak as reference; CDCl_3 (δ_{H} 7.26, δ_{C} 77.0). The reaction was monitored by thin-layer chromatography (TLC), on silica plated (Silica gel 60 F₂₅₄, E. Merck) aluminium sheets, detecting spots by UV (254 and 365 nm). Flash chromatography was performed using Merck Silica Gel 60 (40-63 μm). High-resolution mass data (ES-ToF) were obtained from the Division of Mass spectrometry, Department of Chemistry, Imperial College London. Absorbance was measured using a Cary 5000 (Varian) or Lambda 650 (Perkin Elmer) spectrophotometer. Fluorescence measurements were done using a Spex Fluorolog (JY Horiba) spectrofluorometer. Hexane, toluene, diethyl ether, THF and DMF were dried using an MBraun MB SPS-800 solvent purification system. All other chemicals were used without further purifications.

Synthesis of 4-(4-dimethylaminophenyl)-2,1,3-benzothiadiazole (LD-BTD1).

PEPSSI-IPr (8.0 mg, 0.011 mmol) was added to a solution of 4-bromo-2,1,3-benzothiadiazole (0.123 g, 0.57 mmol), 4-(dimethylamino)phenylboronic acid (0.189 g, 1.15 mmol) and K_2CO_3 (0.237 g, 1.72 mmol) in Toluene/MeOH 1:1 (16 mL). The reaction was heated at 80 °C for 2 h, was then allowed to reach room temperature and was further stirred overnight. The reaction mixture was added dropwise to water (50 mL) and the solution was extracted with Ethyl acetate (1 x 30 mL). The organic layer was washed with water (2 x 30 mL), concentrated under reduced pressure and the residue was further recrystallized from MeOH/water to obtain a red solid. Purification using column chromatography (100 % Toluene to 10% Ethyl acetate in Toluene) gave the pure product as an orange powder (0.141 g, 96%). ^1H NMR (300 MHz, CDCl_3) δ 3.04 (s, 6H), 6.85-6.91 (m, 2H), 7.60-7.67 (m, 2H), 7.86-7.92 (m, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 40.4, 112.3, 118.9, 125.2, 125.8, 129.8, 130.0, 134.7, 150.6, 153.7, 155.8. HRMS (ES-ToF) $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{14}\text{N}_3\text{S}$, 256.0903. Found, 256.0915.

Cell lines and culture conditions

Normal human skin fibroblasts (AG01518; passages 12-24; Coriell Institute, Camden, NJ, USA) and malignant melanoma cells SK-MEL-28 (HTB-72; ATCC, Manassas, VA, USA) were cultured in Eagle's minimum essential medium (EMEM) GlutaMAX, supplemented with 50 IU/ml penicillin-G, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 10 % fetal bovine serum (all from Gibco, Paisley, UK). Cells were kept in humidified air with 5% CO_2 at 37 °C. The day before experiments, cells were seeded to an approximate 50% confluence. For microscopic analysis cells were seeded on glass coverslips No 1.0.

Staining of cells

The day after seeding, cells were stained with 10 μ M **LD-BTD1** in complete cell culture media for indicated times. Notably, the staining solution (10 μ M) was prepared from a 1.5 mM stock solution of **LD-BTD1** in DMSO immediately prior to use, which correlates to a final DMSO concentration of < 1%. In all experiments a corresponding DMSO control was run in parallel. For staining of fixed cells, cells were first fixed in 4% paraformaldehyde (PFA; 20 min, 4 °C), rinsed three times with PBS and thereafter incubated with **LD-BTD1** (500 nM, 30 min; room temperature). For microscopic evaluation, the cells were rinsed three times with PBS, fixed in 4% paraformaldehyde (PFA; 20 min, 4 °C) and mounted using Vectashield with or without DAPI (Vector Laboratories, Burlingame, CA, USA) for visualization of the cell nuclei.

Isolation of primary human adipocytes

The study was approved by the Regional Ethics Board at Linköping University, Sweden (00-398) and has been carried out in accordance with the Declaration of Helsinki. During elective surgery at the University Hospital in Linköping, Sweden, a piece of subcutaneous abdominal fat tissue was excised and immediately treated by collagenase (type 1, Worthington, NJ, USA) digestion in modified Krebs–Ringer solution (0.12 M NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄) to isolate adipocytes.⁵² The cells were incubated overnight in the same solution mixed with DMEM containing 7% albumin, 200 nM phenylisopropyladenosine, 20 mM HEPES, 50 UI/ml penicillin, 50 μ g/ml streptomycin, pH 7.40, at 37°C. **LD-BTD1** was added to a final concentration of 10 μ M. The day after, the adipocytes were washed in PBS, fixed in PFA, added to poly-L-lysine coated coverslips and mounted with DAPI.

Viability Analysis

The reducing capacity of cell cultures was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Sigma-Aldrich).³⁷ This method is widely used to assess cytotoxicity and cell viability, and it is currently thought that the amount of MTT formazan is proportional to the number of living cells. Cells were incubated with 0.5 mg/ml MTT for 2 h at 37°C. Then, the MTT solution was removed and the formazan product was dissolved in DMSO. The absorbance was measured at 550 nm with a VICTOR™ X Series Multiple Plate Reader (PerkinElmer, Waltham, MA).

Flow cytometry

For flow cytometry, the stained cells were detached by trypsinization, washed with PBS, filtrated and analysed using a Gallios flow cytometer (BeckmanCoulter, Gallios™, USA). The fluorescence was measured by using a 488 nm laser, using 595SP and 575BP filters (FL2). Ten thousand cells were collected for each sample and the data was analyzed using the software Kaluza.

Co-staining with organelle markers

For staining of mitochondria, probe-stained cells were incubated with MitoTracker Orange CMTMRos (150 nM, 30 min, 37° C; Molecular Probes, Eugene, OR). Cells were then fixed in 4% PFA (20 min, 4°C). For immunostaining, stained cells were after fixation permeabilized with 0.1% saponin (Sigma-Aldrich) in PBS containing 5 % fetal bovine serum (20 min, room temperature) and incubated for 2 h at room

temperature with the monoclonal mouse primary antibodies: lysosome-associated membrane protein 2 (LAMP-2, 1:50; Southern Biotech, Birmingham, AL), early endosomal antigen-1 (EEA-1, 1:400; Sigma-Aldrich), LC3B (1:100, Novus Biologicals, Littleton, CO, USA) or adipophilin (ADRP; 1:50; Abcam, Cambridge, UK). This step was followed by incubation with the appropriate secondary antibodies conjugated to Alexa Fluor 594 (1:400, Molecular Probes) for 1 h. For staining of lipid droplets, fixed cells were stained with 0.1 $\mu\text{g/ml}$ Nile Red in 150 mM NaCl, 10 min at room temperature. Cells were mounted using Vectashield. For microscopic analysis of the samples, an inverted LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany) was used. Cells stained with **LD-BTD1** and DAPI were analyzed using lasers at 458 and 405 nm, respectively, and the emission settings were 465-682 nm and 410-453 nm. The emission settings for **LD-BTD1** in the co-localization experiments were 484-707 nm, except for the co-localization with mitochondria were 466-545 nm were used. Nile Red fluorescence was collected with the optimal excitation and emission settings, excitation 458 nm and emission 484-707 nm, for imaging of LDs over hydrophobic membranes (seen when using a more red-shifted wavelength).³⁸ Cell cultures stained with Alexa594 conjugate antibodies were analyzed using a 595 nm laser for excitation and the emission was collected at 600-734 nm). Mitotracker Orange CMTMRos was excited at 550 nm and the emission settings were 555-750 nm. A plan-Apochromat 633/1.40. Oil DIC 60 X M27 objective was used for all imaging.

Lipid supplementation

Accumulation of lipid droplets were stimulated by adding 150 μM oleic acid-albumin (Sigma-Aldrich, St. Louis, MO, USA) to the cell culture medium and the cells were then incubated 24 h together with **LD-BTD1**.

Statistical analysis

All experiments were repeated four times and the results are presented as the means and standard deviations of independent samples. Data were statistically evaluated using student's t-test (comparison of two groups) or ANOVA. P values ≤ 0.05 were considered to be significant. All calculations were done using SPSS Statistics 24.

Quantum yield determination

Fluorescent quantum yields (ϕ_F) of **LD-BTD1** in different polarity solvents were measured relative to fluorescein in 20 mM NaOH (aq) ($\phi_F = 0.93$).⁵³ The excitation wavelength for the quantum yield measurements was chosen so that the absorbance was around 0.02-0.04 and so it was possible to record the whole emission spectra of both the sample and the reference.

Octanol-water partitioning

The octanol-water partition constant (K_{ow}) was determined by dissolving **LD-BTD1** in a 1:1 mixture of octanol and water by shaking vigorously and then left to separate to two phases. After separation, 2 ml samples were taken from both phases. To obtain equivalent solvent conditions, 2 ml water and 5 ml methanol was added to the octanol sample and 2 ml octanol and 5 ml methanol was added to the water sample. Thus, the final solvent mixture of water:octanol:methanol was 2:2:5 for both samples. The octanol-water partition constant was then determined from the absorption spectra. The solubility of **LD-BTD1** in water is low and to be able to compare the absorbance value of the two phases, the absorbance spectra for the water phase was measured in a

1 cm cuvette and the octanol phase in a 1 mm cuvette. Notably, the measured absorbance for the water phase was close to the sensitivity limit of the instrument, and the reported octanol/water partition coefficient is therefore best viewed as a lower estimate.

pH dependence

A small amount of **LD-BTD1** was dissolved in DMSO and the resulting solution was diluted to a final DMSO concentration of 5% using buffers with different pH (pH 2 and 4: Glycine·HCl buffer, 50 mM; pH 6 and 8: phosphate buffer, 25 mM; and pH 10 and 12: Glycine·OH buffer, 50 mM). Emission spectra were collected using a Tecan Sapphire 2 micro plate reader (Tecan Group Ltd., Männerdorf, Switzerland). All measurements were repeated in triplicates and the excitation wavelength used was 450 nm.

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Supporting information

Supplementary data including NMR spectra, additional fluorescent microscopy pictures and photophysical characterization spectra can be found in the online version at doi...

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