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Small-Molecule Targeting of the Mitochondrial Compartment with an Endogenously Cleaved Reversible Tag

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Targeted accumulation of chemically unaltered compounds within the mitochondrial compartment has not yet been achieved. Here we describe a reversible tag that is endogenously cleaved after mitochondrial accumulation has occurred. Specifically, we have reversibly tagged α-lipoic acid with a triphenylphosphonium moiety that is cleaved by the physiologically contained mitochondrial aldehyde dehydrogenase (ALDH-2). This reversibly tagged compound activates the lipoic acid-sensitive pyruvate dehydrogenase complex, and this results in increased glucose oxidation. We observed a reduction in ROS accumulation after preincubation with the reversibly tagged compound, whereas untagged or irreversibly tagged compounds either had no effect on ROS formation or rather caused increased oxidative stress, respectively. Lastly, the cytotoxicity of the reversibly tagged compound is less than that of the irreversibly tagged compound. Overall, reversible tagging combines decreased tag-related cytotoxicity with increased bioactivity, and this potentially provides a novel concept in mitochondrial pharmacology.

Introduction

Mitochondria are the main sites of cellular energy production. Different substrates, including pyruvate and fatty acids, are degraded to provide electrons that are transferred along the electron transport chain (ETC). The energy thereby released is used to pump protons from the matrix into the intermembrane space and to create a chemo-electric gradient. This is exploited to drive ATP synthetase, which eventually generates ATP.[1] Premature release of electrons from the ETC leads to reduction of molecular oxygen and the formation of reactive oxygen species (ROS). Enzymatic systems such as superoxide dismutase, catalase and glutathione peroxidase mitigate ROS at an early stage. This is complemented by endogenous antioxidants such as glutathione, tocopherols and ascorbic acid, which are able to scavenge ROS or further intermediates and to prevent damage to macromolecules.

Excessive generation of ROS cannot be balanced, however, and this impairs mitochondrial functions and causes DNA damage and protein oxidation.[2] In particular, the enzyme complexes along the ETC are compromised. The result is a vicious cycle in which electron leakage, enhanced formation of ROS and subsequent damage to mitochondrial DNA (mtDNA) lead to defective proteins and progressive mitochondrial dysfunction.[3] ROS scavengers have been covalently conjugated to lipophilic cations in the form of triphenylphosphonium (TPP) tags. Since TPP carries a positive charge, mitochondria-targeted antioxidants pass through the phospholipid bilayers of cellular and mitochondrial membranes and accumulate in mitochondria by factors of a hundredfold, driven by the membrane potential.[4] To curb oxidative stress, vitamin E, ubiquinol, lipoic acid (LipAc) and polyphenols, as well as mimetics of superoxide dismutase and glutathione peroxidase, have been conjugated to TPP and successfully targeted towards mitochondria.[5–10]

In each of these compounds, the antioxidant is firmly bound to the TPP tag and exhibits properties—including antioxidant activity, intra-mitochondrial distribution and metabolism—that are different from those of the original, naturally occurring molecule. As one example, a covalently bound TPP-conjugated α-lipoyl derivative has been successfully introduced into mitochondria, but failed to reduce oxidative stress.[7] Another study described a cleavable thiol-modified tag that is reduced by the cytosolic glutathione pool to release mRNA-interacting peptide nucleic acid (PNA) molecules.[11]

Here we present a new concept focused on a biocleavable linkage of the lipophilic tag and the molecule to be shuttled into mitochondria. We screened the literature for mitochondrial enzymes capable of cleaving tagged bioactive molecules, found that mitochondrial aldehyde dehydrogenase (ALDH-2) had a rather promiscuous esterase activity and therefore designed a reversibly bound LipAc derivative (revMitoLipAc) to test this concept (Scheme 1 B).

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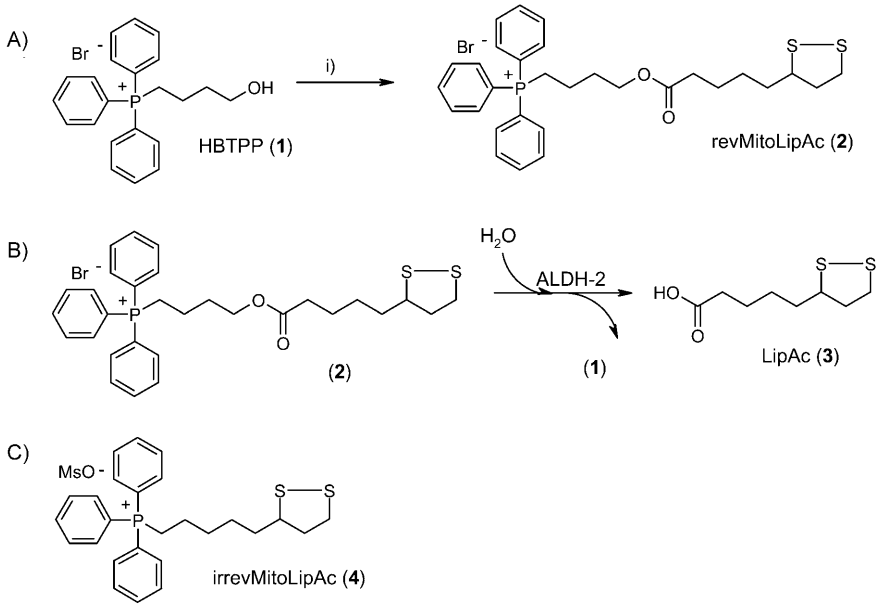

Scheme 1. A) Chemical synthesis of mitochondrially targeted lipoic acid (revMitoLipAc). i) α-Lipoic acid, DMAP, DCC, 24 h. B) Enzymatic release of lipoic acid through the action of mitochondrial aldehyde dehydrogenase (ALDH-2). C) Irreversibly tagged lipoic acid (irrevMitoLipAc) designed by Brown et al.

Results

Synthesis

The synthesis of revMitoLipAc (**2**) was carried out by condensation of (4-hydroxy-butyl)-triphenylphosphonium bromide (HBTPP, **1**) with LipAc (**3**) with DMAP as activator reagent (Scheme 1 A). Oxidized **2** was purified by flash chromatography and obtained in 45% yield. For cell imaging experiments, the thiol moieties of both LipAc (**3**) and revMitoLipAc (**2**) were reduced by sodium borohydride and the compounds were treated with the thiol-specific fluorescence label monobromobimane (see the Supporting Information).[12]

The irreversibly tagged LipAc irrevMitoLipAc (**4**, Scheme 1 C) was obtained by a multistep procedure as described by Brown et al. (see the Supporting Information).[7]

Intramitochondrial accumulation and cleavage

The results of our approach suggest the fast accumulation of revMitoLipAc within the mitochondrial matrix, followed by enzymatic hydrolysis of the lipoyl ester by mitochondrial ALDH-2 (EC1.2.1.3). RevMitoLipAc (**2**) was incubated with isolated mitochondria from pig liver and enzymatic hydrolysis was monitored by thin-layer chromatography (TLC). After incubation the mitochondria were extracted with organic solvents ($CHCl_3$/MeOH 8:2 *v*/*v*) and TLC examination was carried out. The cleavage reaction was complete within 60 min (see the Supporting Information). No cleavage took place when the incubation was performed in the presence of benomyl, a previously established specific inhibitor of mitochondrial ALDH-2.[13–15] In a separate experiment, the release of free LipAc was monitored and quantified by HPLC–UV detection. After 60 min approximately 60 mol% of the revMitoLipAc (**2**) had been converted into LipAc (Figure 1 A, black bars), whereas 40 mol% was not detected, due either to incomplete extraction or to subsequent metabolism into LipAc metabolites, such as dimethyl lipoic acid,[16] which could not be detected. Again, no cleavage reaction occurred when mitochondria were pre-incubated with benomyl (250 μM; Figure 1 A, white bars).

Cell culture experiments were performed to detect the accumulation of mitochondrially targeted LipAc in a physiological system. HepG2 cells were incubated with revMitoLipAc (**2**), LipAc (**3**) and the irreversibly tagged LipAc (irrevMitoLipAc, **4**) for 2 h. Cells were then harvested for subcellular fractionation. The mitochondrial fraction was analysed by ESI (+ve mode) mass spectroscopy, and showed intact **2** (*m*/*z* 523) and the cleavage product of the ester hydrolysis (**1**, *m*/*z* 335, Figure 1 B). Hydrolysed free LipAc (**3**) was not observed, presumably due either to its rapid conversion

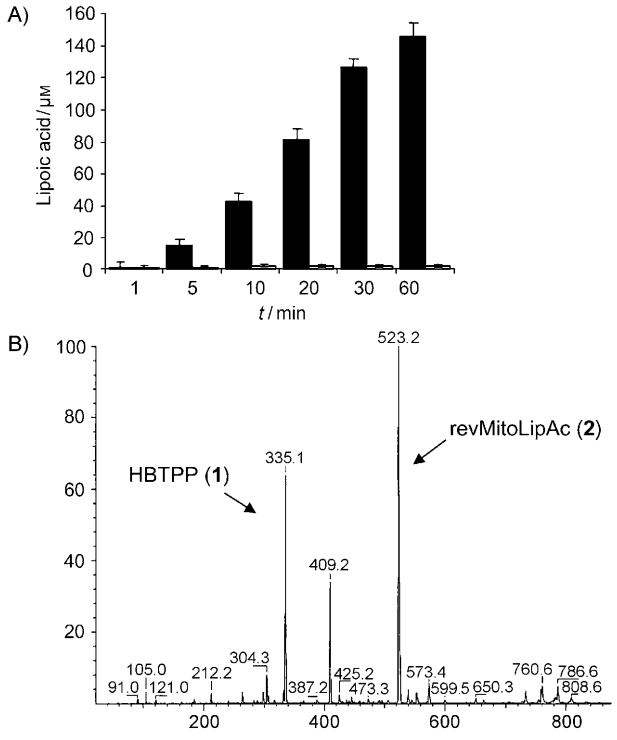

Figure 1. A) Mitochondrial hydrolysis of revMitoLipAc (240 μM), analysed by HPLC after incubation of isolated mitochondria with revMitoLipAc for the depicted time intervals. White bars indicate incubations with benomyl and black bars indicate incubations without this ALDH-2-inhibitor. B) ESI (+ve mode) mass spectrum of a mitochondrial extract of HepG2 cells treated with revMitoLipAc (100 μM) for 2 h.

into metabolites and secretion into the cytoplasm or to its incorporation into lipoylated proteins. Incubation with irrevMitoLipAc (**4**) also showed significant accumulation in the mitochondrial fraction, represented by abundance of its positively charged mass ion (*m/z* 437; data not shown). Interestingly, no intra-mitochondrial accumulation was observed after application of LipAc (**3**); this suggests strict regulation of its intra-mitochondrial metabolism.

In a third approach we used monobromobimane-labelled derivatives of revMitoLipAc (**2**) and LipAc (**3**) to visualize the distribution within HepG2 cells.[12] The cells were co-incubated with MitoTracker Red, a mitochondrion-specific fluorescence dye, and with bimane-labelled **2** or **3** for 1 h and observed by fluorescence microscopy. MitoTracker Red rapidly accumulated within mitochondria (Figure 2 A). Bimane-labelled revMitoLipAc (**2**) also accumulated in the cells with the same sub-cellular localization (Figure 2 B). A digital overlay of both images revealed colocalization of MitoTracker Red with bimane-labelled **2** (Figure 2 C). Interestingly, bimane-labelled LipAc did not enter the cell (image not shown).

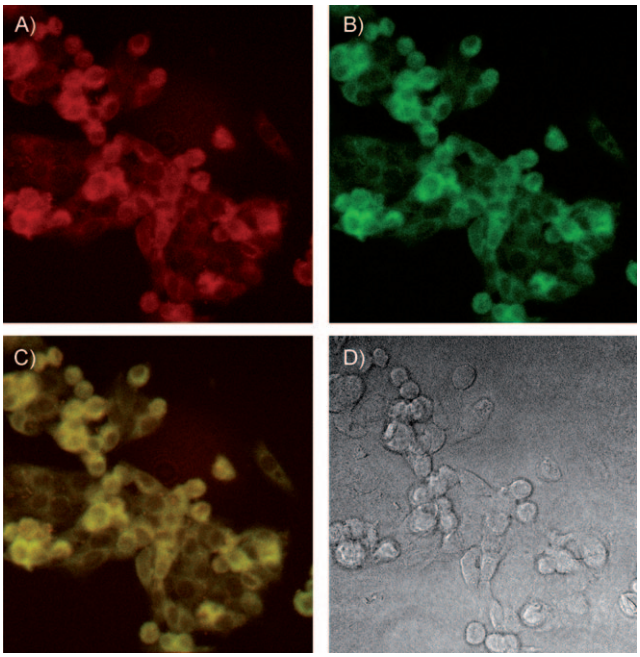

Figure 2. Fluorescence imaging of bimane-labelled revMitoLipAc in HepG2 cells (1 μM, 1 h). A) MitoTracker Red fluorescence, B) Bimane-labelled revMitoLipAc fluorescence. C) Overlay of (A) and (B) fluorescence. D) Phase contrast.

Biological activity

LipAc is a known cofactor for pyruvate dehydrogenase (PDH) activity. Because we forced mitochondrial importation of LipAc through the application of revMitoLipAc, we could expect activation of the PDH complex; this activation should be accompanied by an increased flux of pyruvate oxidation to acetyl-CoA and at the same time by a decrease in lactate production. Dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (PDK) and thus an activator of PDH, induced increased overall glucose utilization together with reduced lactate generation.[17,18] We observed that compound **2** modulated glucose metabolism in the same manner (see the Supporting Information).

In addition, isolated intact mitochondria were incubated with LipAc and with revMitoLipAc, and PDH activity was measured by a published procedure.[19] DCA and 3-fluoropyruvate (3-FP), a direct inhibitor of PDH, were used as controls. Free LipAc significantly reduced PDH activity (0.15 $\mu mol\,min^{-1}\,\mu g^{-1}$ vs. 0.17 $\mu mol\,min^{-1}\,\mu g^{-1}$, $p<0.01$; Figure 3), whereas revMitoLipAc induced activity (0.21 vs. 0.17 $\mu mol\,min^{-1}\,\mu g^{-1}$, $p<0.05$). For revMitoLipAc, we could not distinguish between PDH activation through inhibition of PDK and direct activation of PDH due to a surplus of liberated LipAc.

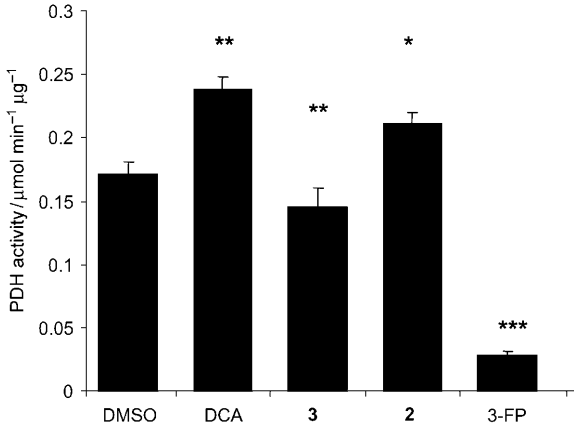

Figure 3. PDH activities of isolated mitochondria incubated for 2 h at 37 °C with dichloroacetate (DCA, 5 mM), lipoic acid (**3**, 5 μM), reversibly tagged lipoic acid (**2**, 5 μM) and 3-fluoropyruvate (3-FP, 5 mM) with subsequent measurement of activity. Data are expressed as means of four individual experiments ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to DMSO and PDH activity is given in μmol of pyruvate consumed min^{-1} μg of $protein^{-1}$.

Most of the mitochondrially targeted antioxidants significantly reduced ROS in vitro and in vivo. Therefore, we incubated HepG2 cells with free LipAc, revMitoLipAc and HBTPP and measured mitochondrial ROS generation. Mitochondria were loaded with the nonfluorescent dye MitoTracker CM-H2XROS, which selectively accumulates within mitochondria and subsequently oxidized to a fluorescent chromophore, as repeatedly demonstrated in the past.[20–22] Free LipAc did not reduce the fluorescence of the redox-sensitive probe at 1 μM concentration (Figure 4 A). At higher concentrations (10 μM), LipAc had significantly elevated endogenous ROS levels after 12 h. In contrast, reversibly targeted LipAc (revMitoLipAc) protected mitochondria from endogenous ROS at 1 μM and 10 μM concentrations. HBTPP (**1**) did not appear to be responsible for any reduction of ROS. The antioxidative effects of revMitoLipAv (**2**) are rather small and have to be handled with care because the MitoTracker fluorescent dye is potentially capable of interfering with the mitochondrial permeability transition, and so may influence the ROS production or fluorescence determination.[23,24]

LipAc is known to reduce levels of exogenously induced ROS in vitro and in vivo at concentrations ranging between

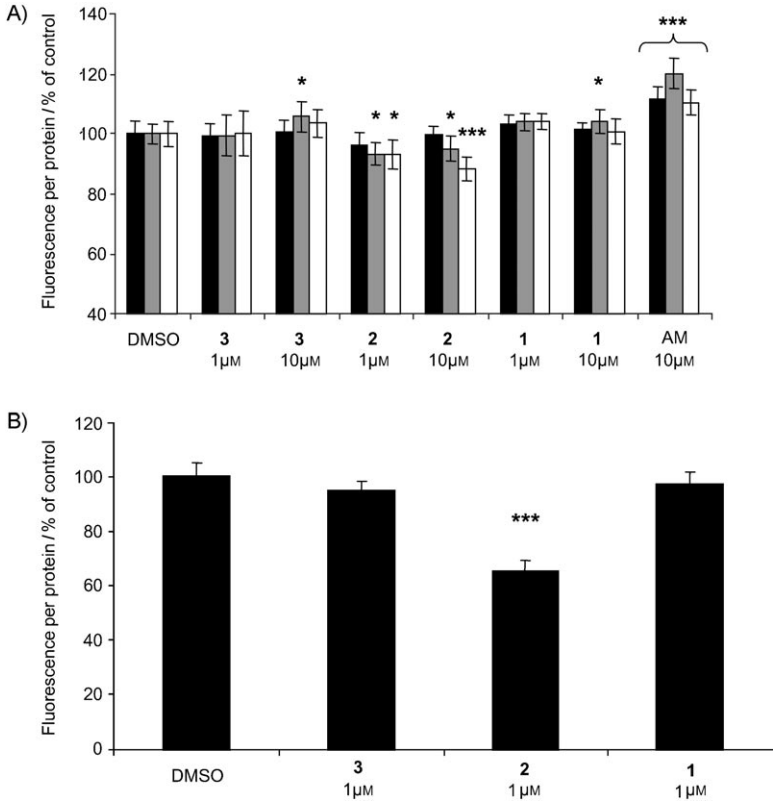

Figure 4. A) Endogenous mitochondrial ROS formation in HepG2 cells after different incubation time intervals. Cells were treated with lipoic acid (**3**), reversibly tagged lipoic acid (**2**), the TPP tag **1** and antimycin (AM) as positive control. ROS were quantified by treatment with MitoTracker CM-H2XROS. Black bars represent 4 h treatment, grey bars 12 h treatment and white bars 24 h treatment. Data are expressed as means of $n = 8 \pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to DMSO. B) Accumulation of exogenously added ROS , 4 h post-treatment with *tert*-butylhydroperoxide (TBHP, 50 μM). ROS were quantified by treatment with dichlorofluoresceine (DCF). Data are expressed as means of $n = 6 \pm$ SD, *** $p < 0.001$.

100 and 500 μM.[25–27] We compared **2** with its hydrolysis products LipAc (**3**) and HBTPP (**1**) for their potential to scavenge external ROS, here induced by *tert*-butylhydroperoxide (TBHP, 50 μM; Figure 4B). Quantification of cytosolic ROS formation was essentially by the method of Wang and Joseph.[28] The antioxidant effect of free LipAc (1 μM) was not seen when HepG2 cells were incubated for 4, 12 or 24 h prior to TBHP treatment (data shown for 4 h only). However, cells treated with the reversibly targeted compound revMitoLipAc (**2**) and subsequently challenged by TBHP were significantly protected from oxidative stress (40% versus control, $p < 0.001$). These results suggest that the application of revMitoLipAc is of advantage in comparison with application of LipAc. Moreover, in this respect revMitoLipAc is superior to irrevMitoLipAc because the latter compound did not protect cells from oxidative damage either by hydrogen peroxide or by *tert*-butylhydroperoxide.[7] The mitochondrial accumulation and subsequent cleavage of LipAc by revMitoLipAc enhances antioxidant defence and mitigates the impact of ROS.

Comparison of differentially tagged LipAc derivatives

A direct comparison of revMitoLipAc (**2**) and irrevMitoLipAc (**4**) revealed a significant reduction in cell growth when hepatocytes were treated with **4** at a concentration of 10 μM (15% reduction at 12 h, 30% at 24 h and 60% at 48 h, $p < 0.001$; Figure 5A). When **4** (1 μM) was applied, cells growth was diminished after 24 h, but the cells had recovered after 48 h.

In a previously published study by Brown et al., the decrease in protein content of each well after treatment with **4** was accompanied by an apoptotic appearance of the cells, presumably due to mitochondrial ROS formation.[7] These results are in line with our observations that **4** produced a most pronounced increase in endogenous ROS formation relative to revMitoLipAc (**2**) as depicted in Figure 5B. Whereas cells seemed to recover from initial stress induced by irrevMitoLipAc (**4**, 1 μM), endogenous ROS concentration nevertheless increased over time when treated with **4** (10 μM). We postulate that irrevMitoLipAc (**4**) acted as a pro-oxidant within mitochondria and finally induced apoptosis.

We further investigated the mitochondrial membrane potential $\Delta\Psi_m$, which is a reliable indicator of mitochondrion-dependent apoptosis quantifiable by applying the fluorescence dye JC-1. In the presence of a high $\Delta\Psi_m$ the dye forms so-called JC-1 aggregates with an emission wavelength of 590 nm.[29] We measured the effects of the mitochondriotropic compounds **1**, **2** and **4** (Figure 6). The mitochondrial tag (**1**) and revMitoLipAc (**2**) showed either no influence or even a positive influence (**1** at 10 μM) on $\Delta\Psi_m$. In contrast, irrevMitoLipAc (**4**) had reduced $\Delta\Psi_m$ after 1 h of treatment; this is in line with the results observed in Figure 5.

Overall, reversible tagging combines decreased tag-related cytotoxicity with increased bioactivity, and potentially provides a novel concept in mitochondrial pharmacology.

Discussion

Lipophilic TPP-tagged molecules (TPPMs) have been successfully used to target mitochondria in the past.[6–8] Because mitochondria are the main site of endogenous ROS generation and thus prone to oxidative damage, TPPMs with antioxidant activity have been used to reduce oxidative stress. Ubiquinol, vitamin E or LipAc moieties have been covalently bound to TPP tags. Mitochondrion-targeted tagged coenzyme Q10 (MitoQ) has been shown to reduce mitochondrial ROS in vitro and in vivo and is currently used in phase II studies for the treatment of Parkinson's disease, Friedreich's ataxia and chronic hepatitis C infections.[30] In contrast, the mitochondrion-targeted LipAc irrevMitoLipAc, first described by Brown and colleagues,[7] failed to reduce oxidative load within mitochondria. The authors showed that irrevMitoLipAc was reduced by less than

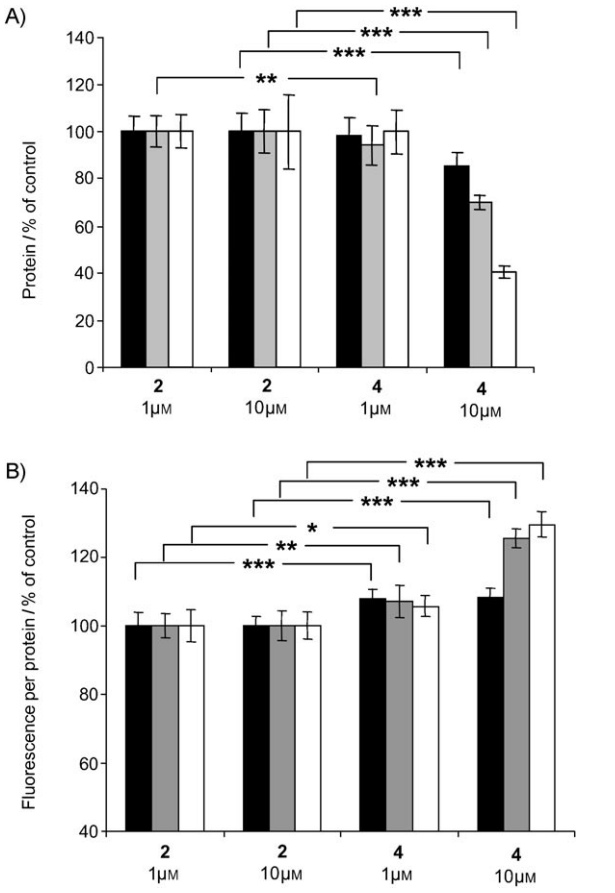

Figure 5. A) Direct comparison of hepatocyte protein content after treatment with reversibly tagged lipoic acid (**2**) and irreversibly tagged lipoic acid (**4**). Black bars represent 12 h treatment, grey bars 24 h treatment and white bars 48 h treatment. B) Direct comparison of endogenous ROS formation in HepG2 cells after treatment with revMitoLipAc and irrevMitoLipAc. Black bars represent 4 h treatment, grey bars 12 h treatment and white bars 24 h treatment. RevMitoLipAc values were set to 100%. ROS were quantified by treatment with dichlorofluoresceine (DCF). All data are expressed as means of $n = 8 \pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

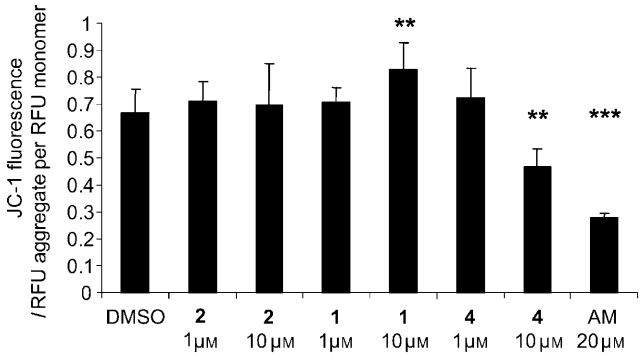

Figure 6. Influence of revMitoLipAc (**2**), HBTPP (**1**) and irrevMitoLipAc (**4**) on mitochondrial membrane potential ($\Delta\Psi_m$) measured by JC-1 fluorescence (RFU aggregate/RFU monomer). HepG2 cells were treated with **2**, **1** or **4** for 1 h, and fluorescence of JC-1 monomers ($\lambda_{em} = 535$ nm) and aggregates ($\lambda_{em} = 590$ nm) was measured. The quotient of fluorescence values correlates with $\Delta\Psi_m$. Antimycin A (AM, 20 μM) was used as positive control. All data are expressed as means of $n = 6 \pm$ SD, ** $p < 0.01$, *** $p < 0.001$ relative to DMSO.

10% and that this compound could not be reduced by mitochondrial thioredoxin reductase (Trx), because it was *S*-methylated and thus did not act as a mitochondrial antioxidant.[7]

Here we describe a new class of TPPMs that feature reversibly bound TPP tags cleavable by mitochondrial ALDH-2. The mitochondrial isoform of ALDH-2 displays three enzymatic activities. The enzyme is responsible for the detoxification of ethanol and has been shown to activate the vasodilating drug nitroglycerine (glyceryl trinitrate, GTN) by its reductase activity.[31,32] In addition, the enzyme is capable of hydrolysing ester bonds.[33–35] In the current study, this property was used to liberate LipAc from a tagged LipAc derivative (revMitoLipAc) that accumulates within mitochondria. Co-incubation with benomyl, a previously established inhibitor of ALDH-2, prevented ester hydrolysis. In addition, we used electrospray ionisation mass spectroscopy (ESI +ve mode) to demonstrate that revMitoLipAc was taken up into the cytosol and accumulated within the mitochondria of HepG2 cells. While we could find revMitoLipAc and the hydrolysed TPP moiety (**1**), we could not detect either free LipAc or its main metabolites mono- and dimethyl lipoic acid within these mitochondria (data not shown). We postulate that free LipAc (**3**) was either rapidly converted into metabolites and subsequently secreted into the cytoplasm, or incorporated into lipoylated proteins. Further studies will have to address these possibilities.

Colocalization of a bimane-labelled derivative of revMitoLipAc (**2**) with MitoTracker Red in HepG2 cells revealed rapid accumulation of revMitoLipAc within mitochondria. We propose that revMitoLipAc escapes unspecific cytosolic esterases because of its extremely fast mitochondrial accumulation. Although we cannot entirely rule out contributions to the hydrolytic process by mitochondrial esterases other than ALDH-2, the effect of the inhibitor benomyl appears to point to ALDH-2 as being most prominently involved. Future work with small interfering RNA targeted against ALDH-2 could potentially help to clarify this question, although on the other hand it has no immediate impact either on the efficacy of the novel method described here or on the specific compounds analysed in this study.

Because we observed strong activation of PDH complex activity, we assume that liberated LipAc underwent fast metabolic conversion into lipoylated proteins. Pyruvate dehydrogenase complex has been shown to be modulated by free LipAc (LipAc).[36,37] Although we observed a significant reduction in PDH activity for LipAc (5 μM), our results are in line with the previous studies. The tagged LipAc **2**, however, significantly enhanced PDH complex activity at comparable low concentrations (5 μM). It should be mentioned that phosphonium-tagged compounds in general might accumulate by factors of up to 500–1000-fold within mitochondria. If hydrolysis of revMitoLipAc by ALDH-2 is assumed, intra-mitochondrial concentrations of free LipAc could reach up to 5 mM. We further postulate that liberated LipAc is transferred to lysine residues of the PDH complex as has been found for thiamine pyrophosphate in a similar experimental setup.[19]

Comparably little is known about mitochondrial metabolism of LipAc in mammals.[38–40] Homozygous murine embryos lack-

ing mitochondrial lipoate synthetase (*Lias*) died before day 9.5 of embryonic development.[41] Unexpectedly, supplementation of heterozygously *Lias*-deficient (*Lias*$^{+/-}$) mothers with LipAc during pregnancy failed to prevent the prenatal deaths of homozygous knock-out embryos; this suggests that free passage of LipAc into the embryonic mitochondria is restricted. Heterozygous *Lias*$^{+/-}$ mice show a diminished hepatic LipAc content and are especially sensitive to lipopolysaccharide-induced oxidative stress.[42] The irreversibly tagged LipAc **4** described by Brown and colleagues was used to overcome this obstacle; however, the compound failed to reduce oxidative stress within the cell. In fact, **4** showed cytotoxic effects in Jurkat cells at concentrations as low as 2.5 μM.[7]

Pro-apoptotic and pro-oxidative activity has been observed for free LipAc on application of this compound in high concentrations (1 mM) in HT-29 cells,[43] or at 200–500 μM in HepG2 and FAO cells[44] or 3T3L1 adipocytes.[45] In contrast, here we report antioxidant activity of revMitoLipAc at 1–10 μM concentrations for all time periods analysed. In addition, revMitoLipAc acted as an antioxidant even at a low, pharmacologically relevant concentration (1 μM) when cells were challenged by *tert*-butylhydroperoxide (TBHP). We assume that revMitoLipAc liberates LipAc within the mitochondria and that free LipAc is subsequently reduced by mitochondrial thioredoxin reductase.[46] We compared the influences of the tagged LipAcs **2** and **4** on cell growth, ROS accumulation and mitochondrial membrane potential $\Delta\Psi_m$ (Figures 5 and 6). All parameters point to a pro-oxidative and pro-apoptotic activity of irrevMitoLipAc (**4**), especially at higher concentrations (10 μM).

To the best of our knowledge, here we report for the first time a biocleavable transport system for the transfer of bioactive compounds into the mitochondrial inner matrix. The system takes advantage of the intrinsic ALDH-2 activity to hydrolyse the shuttle and to liberate the desired compound. In this way, we overcome the tightly regulated transport of nutrients, drugs and other bioactive compounds into the mitochondrial compartment by the use of a reversibly bound mitochondriotropic tag.

Experimental Section

General information: All chemicals were used as received from the supplier. Tetrahydrofuran, 4-dimethylaminopyridine, dicyclohexyl-carbodiimide, triphenylphosphonium hydrobromide, antimycin A and benomyl were obtained from Sigma–Aldrich (Schnelldorf, Germany). Diethyl ether, methanol and dichloromethane were obtained from Roth (Karlsruhe, Germany). DL-α-Lipoic acid was obtained from Fluka (Schnelldorf, Germany). IrrevMitoLipAc was synthesized by published procedures (see the Supporting Information for a brief description).[7] TLC analysis was performed on Silica Gel 60 F254 coated plates from Merck (Darmstadt, Germany). ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 and 75.47 MHz, respectively, with a Bruker (Bremen, Germany) AMX 300 instrument in $CDCl_3$ as solvent and with TMS as internal standard. Phosphoric acid (30%) was used as external standard for ^{31}P NMR. Electrospray ionisation mass spectrometry (ESI-MS) was performed in positive ionisation mode with a MAT 95 XL-Trap instrument (Thermoquest Finnigan, Bremen, Germany).

Synthesis of revMitoLipAc (2): DL-lipoic acid (**3**, 824 mg, 4 mmol), (4-hydroxybutyl)triphenylphosphonium bromide (**1**, 1.4 g, 3.4 mmol)[47] and 4-dimethylaminopyridine (432 mg, 3.6 mmol) were dissolved in dichloromethane (20 mL) and the mixture was cooled in an ice bath. Then, dicyclohexylcarbodiimide (887 mg, 3.4 mmol) was added. The solution was stirred overnight and a white precipitate formed. The filtrate was concentrated and applied to a silica gel column ($CHCl_3$/MeOH 8:1) to give **2** (920 mg, 45% yield) as a pale yellow gum. TLC analysis ($CHCl_3$/MeOH 8:1) R_f 0.36; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.894$–7.624 (m, 15H; Ar-H), 4.081 (t, 2H; CH_2O), 3.955 (m, 2H; CH_2P^+), 3.532 (m, 1H; CHS), 3.101 (m, 2H; CH_2S), 2.412 (m, 1H; CH_aC), 2.162 (t, 2H; CH_2CO), 2.039 (m, 4H; $2CH_2$) 1.911 (m, 1H; CH_bC), 1.720–1.381 ppm (m, 6H; $3CH_2$); ^{13}C NMR ($CDCl_3$): 172.14, 135.25, 134.13, 129.65, 119.00, 61.82, 55.15, 41.24, 37.55, 35.64, 29.55, 27.72, 27.34, 23.30, 22.41, 22.13 ppm; ^{31}P NMR: $\delta = 25.55$ ppm; HRMS (ESI+): *m/z* calcd for $C_{30}H_{36}O_2PS_2$: 523.1894 $[M+H]^+$; found: 523.1907 $[M+H]^+$.

Cell culture experiments: HepG2 cells were maintained in RPMI 1640 medium (Pan-Biotech, Aidenbach, Germany) containing FBS (10% *v/v*) and antibiotic–antimycotic solution [1% *v/v*, penicillin (100 U mL^{-1}), streptomycin (10 μg mL^{-1})] at 37 °C under a humidified atmosphere containing CO_2 (5%). Hepatocytes were grown to 80% confluence on 15 cm cell culture dishes. For experimental procedures, cells were rinsed with PBS, trypsinized and transferred to a Falcon tube. After centrifugation, cells were quantified, diluted with medium and seeded either into 96-well microtiter plates (20000–40000 per well) or into cell culture dishes (8×10^6 per dish) for the isolation of mitochondria. After 24 h, stock solutions of substances were diluted in prewarmed RPMI medium to desired concentrations. To obtain a uniform solvent concentration, an equal volume of DMSO was also added to the medium of control samples. Cells were then incubated with prepared solutions for certain periods of time at conditions depicted.

Preparation of mitochondrial fractions: Mitochondria were isolated from HepG2 cells by the method of Lai et al.[48] Cells were grown to confluence and incubation procedures were conducted as described above. After incubation, cells were washed twice with cold PBS, scraped into ice-cold sucrose buffer [10 mL, sucrose (250 mM), Tris (5 mM), ethylene glycol tetraacetic acid (2 mM), pH 7.4; STE buffer] and stored at 4 °C. Samples were centrifuged (490 *g*, 10 min) and supernatants were carefully decanted. Cells were then resuspended in STE buffer (10 mL) and transferred to a Dounce homogenizer; this allowed a gentle but efficient cell disruption. The sample was transferred to a glass vessel and manually homogenized with a glass pestle for seven strokes. Subsequent centrifugation (1000 *g*, 10 min) pelleted the cell debris. The supernatant, which contained mitochondrial fractions, was filtered into a new tube through gauze to retain insoluble particles. Supernatants were pooled and centrifuged (11000 *g*, 10 min) to pellet the mitochondrial fraction. This was gently resuspended in a small volume of buffer with use of a fine paintbrush in order to avoid organelle destruction by shear stress that could be caused by use of a pipette. To remove undesired cytosolic fractions, the washing step was repeated twice. Mitochondria were resuspended in a small volume of buffer and immediately used for assay procedures. The protein concentration was determined by Bradford's method.[49]

Cleavage of mitochondrially targeted LipAc (2): RevMitoLipAc (**2**) was incubated with energized mitochondria from pig liver and enzymatic hydrolysis was monitored either by thin-layer chromatography (TLC; see the Supporting Information) or HPLC. Mitochondrial fractions (50 μg protein), prepared from porcine liver or human HepG2 cells as outlined above, were mixed with STE buffer

(200 μL) and revMitoLipAc was added to give a final concentration of 230 μM. Samples were incubated in a thermomixer (37 °C) for defined time intervals. The reaction was stopped with an equal volume of an aqueous acetonitrile solution (65%, *v/v*) and the sample was acidified to pH 3. Samples were intensely vortexed for 1 min to extract LipAc and were then centrifuged (10800 *g*, 1 min) to pellet mitochondria debris. Supernatant was carefully transferred, filtered (0.2 μm) and analysed by reversed-phase HPLC. In brief, we used a reversed-phase Kromasil C18 column (5 μm 250× 4.0 mm) with a mixture of acetonitrile and water (65:35 *v/v*, 0.1% acetic acid pH 4.0) as eluent and a detection wavelength of 267 nm. LipAc eluted at 10.0 min. To demonstrate that revMitoLipAc is hydrolysed by mitochondrial ALDH-2, the mitochondrial fractions were pre-incubated for 30 min with the specific ALDH inhibitor benomyl (250 μM).

Pyruvate dehydrogenase activity assay: To measure the activity of pyruvate dehydrogenase complex (PDH), crude mitochondrial HepG2 cell extracts were prepared as described. Mitochondria were thawed, centrifuged (10400 *g*, 10 min) and carefully resuspended in a small volume of cold STE buffer until the pellet was completely dissolved. The protein concentration was determined and adjusted to 1–2 μg protein per μL with buffer. A spectrophotometric assay based on previous protocols was performed with slight modifications.[19] The assay was based on the indirect quantification of NADH generated by the E3 subunit of PDH and subsequent reduction of *p*-iodonitrotetrazolium violet (INT) to a purple coloured formazan, the increasing concentration of which was monitored at 500 nm. Absorption values are proportional to the quantity of NADH generated and pyruvate consumed.

Mitochondria (25 μg protein) were mixed with Soerensen buffer (pH 7.6, 200 μL) that contained INT (0.6 mM) and essential PDH cofactors [NAD^+ (2.5 mM), TPP^+ (0.2 mM), CoA (0.2 mM), Mg^{II} (1 mM)]. To disrupt the outer mitochondria membrane and to solubilize PDH, the buffer also contained the detergent Triton X-100 (0.1% *v/v*). Addition of pyruvate started the reaction. Addition of the PDH inhibitor fluoropyruvate and omission of pyruvate provided a blank value for monitoring of the background signal originating from initially present NADH or non-specific NAD^+-dependent enzymatic reactions. To provide a positive control and to enhance PDH activity, the PDK inhibitor dichloroacetate (DCA) was added to the assay buffer (5 mM). The reaction kinetics were then continually monitored at 37 °C for 60 min.

Determination of ROS: To assess the total cellular amount of reactive oxygen species, the DCF assay was conducted essentially as previously described.[50] In brief, HepG2 cells were loaded with 2,7-dihydrodichlorofluorescein diacetate (H2-DCF-DA; 100 μM final concentration) in RPMI medium for 30 min at 37 °C under an atmosphere containing CO_2 (5%). H2-DCF-DA is a nonfluorescent fluorescein derivative that emits fluorescence after oxidation to DCF-DA. Fluorescence intensity is directly proportional to intracellular ROS formation. After loading with H2-DCF-DA, the cells were washed with PBS and test compounds were added for the time periods depicted in Figures 4B and 5B). Fluorescence intensity was read immediately (0 h) and after different incubation periods at 37 °C under CO_2 atmosphere (5% or 4 L min^{-1} within the fluorimeter). Fluorescence intensity was measured with a NOVOstar (BMG Labtech, Offenburg, Germany) microplate fluorimeter at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

A similar setup was used for the determination of mitochondrially derived ROS. Cells were loaded with MitoTracker CM-H2XROS (Molecular Probes/Invitrogen, Germany) and treated as described above. Fluorescence intensity was measured at an excitation wavelength of 543 nm and an emission wavelength of 590 nm.

Fluorescence microscopy: HepG2 cells were seeded onto round coverslips in twelve-well microtiter plates and allowed to grow for 24 h. The cells were coincubated with bimane-labelled revMitoLipAc and MitoTracker Red (Molecular Probes/Invitrogen, Germany) for 1 h. Cells were washed with PBS and coverslips were mounted on glass slides for microscopy. Images were taken on an Axiovert 100 LSM 410 laser scanning microscope (Zeiss, Germany). The bimane fluorescence was excited with an argon UV laser at 364 nm and fluorescence images were taken at 488 nm. MitoTracker Red fluorescence was excited at 543 nm and emission images were recorded at 575–640 nm. All acquisition parameters were kept constant during the imaging process.

JC-1 staining: HepG2 cells were seeded in a 96-well microtiter plate and maintained overnight. The staining solution was prepared by dilution of a JC-1 stock solution (10 mM, Molecular Probes) 1:1000 with PBS. Cells were incubated with **1**, **2**, **4** and antimycin A (20 μM) under an atmosphere containing CO_2 (5%) at 37 °C for 1 h. The medium was removed, and the cells were washed and incubated with the JC-1 staining solution for 1 h. Fluorescence was measured with an Optima microplate fluorimeter (BMG Labtech, Offenburg, Germany) at 535 nm for JC-1 monomers and at 590 nm for JC-1 aggregates.

Statistical analyses: Data obtained from experiments were analysed to determine statistical differences between certain samples. Means and standard deviations were calculated with Microsoft Excel. Statistical analysis was performed with SPSS 15.0. Results are depicted as most significant (*** $p < 0.001$), highly significant (** $p < 0.01$) and significant (* $p < 0.05$).

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