Photocleavable activity-based acid glucosylceramidase probes

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Photochemical properties of photocleavable activity-based acid glucosylceramidase probes

3.1 Introduction

Photocleavable protective groups and linkers are widely used in chemistry, biology and biomedicine [1]. In the context of synthetic organic chemistry, photocleavable protective groups are used to mask a compound's functional site that can be unveiled at the desired moment, for instance, to allow further elaboration of the involved functional group in a chemical synthesis route. In terms of (chemical) biology research, photocleavable groups are for instance used to activate a bio-responsive group in a spatiotemporal manner so that ensuing biological processes in which the functional group is involved can be studied (see for example [2]). In such experiments, the intensity, duration and field of the irradiation can be tuned, allowing to uncage compounds locally in a controlled manner [3]. However, insufficient penetration of tissues by light limits applications of photocaged compounds [1]. Also, DNA damage as a possible side effect of irradiation has to be considered in medical applications or experiments with living organisms, while degradation of endogenous molecules may occur in experiments on a cellular level [4–6]. Still, the advantages often outweigh these downsides and therefore a wide variety of photo cages and photocleavable groups have been developed, which are catalogued in several reviews [1,7].

When describing the nature of a photocleavable group, two parameters are often considered. The first parameter is the molar extinction coefficient ε ($M^{-1}cm^{-1}$), which describes how much light is absorbed in a 1 cm deep 1 M solution. The second parameter is the uncaging quantum yield φ_u that gives the efficiency with which a photocleavable molecule upon excitation is cleaved. Multiplying ε with φ_u gives the uncaging efficiency, which is the probability per photon leading to a photocleavage event.

The *ortho*-nitrobenzyl group was first described as a "photosensitive protective group" in 1966 [8] and is cleaved with light in the UV-A range (315-400 nm) (Figure 3.1A) [9,10]. *Ortho*-nitrobenzyl has a molar extinction coefficient of 260 $M^{-1}cm^{-1}$ [11] and the photochemical reaction does not proceed to completion. The upon photo-irradiation formed *ortho*-nitrosobenzaldehyde reacts further into azobenzene-2,2-dicarboxylic acid, which acts as an "internal light filter" that quenches the reaction [8]. *Ortho*-nitrophenethyl (Figure 3.1A) was found to be a good alternative for *ortho*-nitrobenzyl because it converts into the less reactive *ortho*-nitrosoacetophenone that does not intervene with the photochemical reaction [8,12].

The reaction mechanism of photoreactive groups based on the *ortho*-nitrobenzyl scaffold has been extensively studied and reviewed [7,13,14] and is summarized in Figure 3.1B

on the example of an *ortho*-nitrophenethyl compound. To tweak the properties of this reaction, both the aromatic ring and the benzylic position of the photocleavable group can be modified. Manipulations on the aromatic ring allow for tuning absorbance efficiency [7,15], absorbed wavelength [7,15], as well as solubility [7]. Substitution on the benzylic position may prevent the formation of reactive products like the *ortho*-nitrosobenzaldehyde [12] and may also improve the uncaging quantum yield φ_u of the photocleavable group [7].

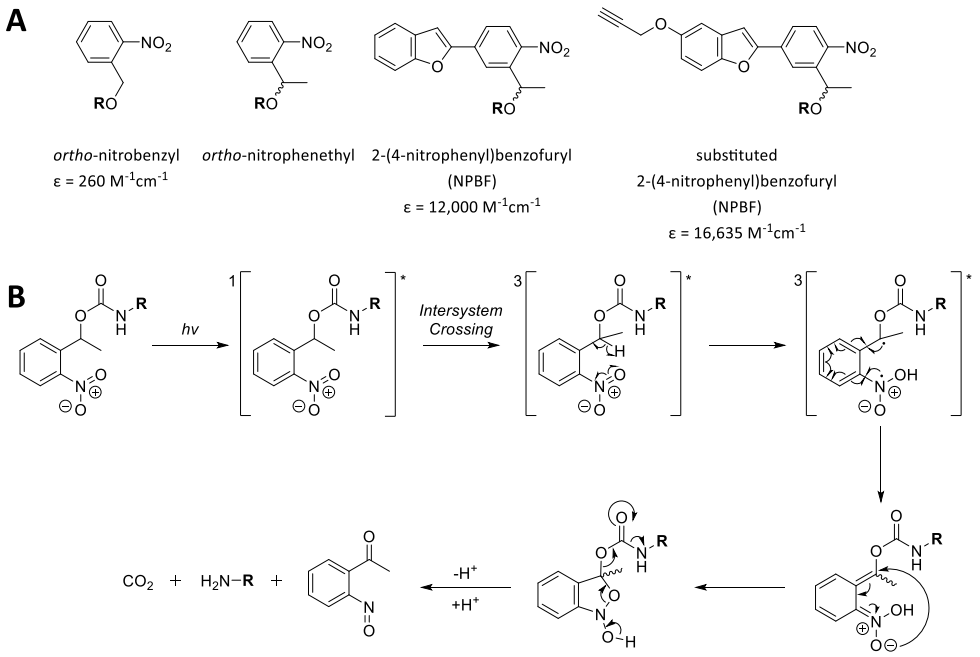


Figure 3.1 The *ortho*-nitrobenzyl group as a photocleavable group. A) Structures of the photocleavable groups that led to the development of the photocleavable linker used in this thesis. The molar extinction coefficient ε for *ortho*-nitrobenzyl and NPBF are given for R = H. The molar extinction coefficient ε for the substituted NPBF is given for R = Boc-Lysine-OMe [16]. B) Mechanism of the photochemical reaction of an *ortho*-nitrophenethyl as a protection group on amine **R**. Irradiation of the photoactive group leads to an excited singlet state, which relaxes into an excited triplet state. Hydrogen is transferred from the benzylic position to the nitro group, followed by radical rearrangement. From here, a labile five-membered ketal ring is formed, which collapses to give the nitroso product with the concomitant release of carbamic acid. The carbamic acid further collapses into CO_2 and the free amine **R**.

Based on the *ortho*-nitrobenzyl group, the 2-(4-nitrophenyl)benzofuryl (NPBF, Figure 3.1A) group was developed in the lab of Kobayashi [15]. Their goal was to develop a

photocleavable protection group that could not only be deprotected by one-photon irradiation but also by two-photon irradiation. Furthermore, its molar extinction coefficient of 12,000 $M^{-1}cm^{-1}$ [15] is 46 times higher than that of *ortho*-nitrobenzyl, while it forms the less reactive *ortho*-nitrosoacetophenone as one of the cleavage products. Its maximum absorbance is 364 nm [15], but it can also be activated at higher wavelengths up to 420 nm [2]. By adding a second substituent to the benzofuryl moiety of NPFB, a photocleavable linker was obtained (Figure 3.1A substituted NPBF) [2]. Depending on this substituent, the linker has an even higher molar extinction coefficient (ε = 16,635 $M^{-1}cm^{-1}$ for an attached propargyl group) than the monosubstituted NPFB, while the absorbance maximum changes only by a few nanometers [16].

Although the photochemical cleavage product *ortho*-nitrosoacetophenone does not interfere with the photochemical reaction itself, side reactions in biological settings have been reported where the formed nitroso ketone inhibited the enzyme of interest [9], most likely promoted by the still-applied irradiation. This side reaction could be prevented by the addition of dithiothreitol (DTT), leading to cyclisation of the cleaved product (Figure 3.2) [17,18]. Barth *et al.* however, also stress the possibility of the covalent interaction of *ortho*-nitrosoacetophenone with monofunctional thiols [17]. Besides side reactions with thiols, Rinnová *et al.* also identified products formed by the reaction of *ortho*-nitrosoacetophenone with amines under various conditions [19].

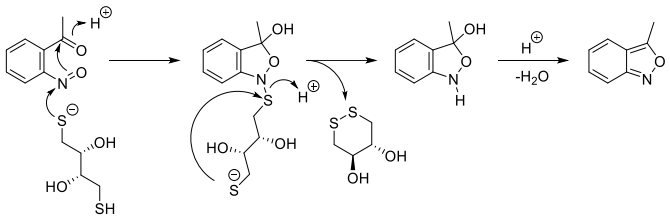


Figure 3.2 Mechanism of *ortho*-nitrosoacetophenone with DTT as proposed by Barth *et al.* [17].

Chapter 2 of this thesis described the synthesis of photocleavable activity-based probes (ABPs) **1** and **2** (structures in Figures 3.3A and B), featuring substituted NPFB as a photocleavable linker. These compounds ought to function as enrichment probes, targeting lysosomal acid glucosylceramidase (GBA1) by binding covalently to the active pocket of the enzyme. Chapter 2 further described the ability of compounds **1** and **2**, as well as compound **5** as a non-photoreactive control compound (Figure 3.3C), to potently and selectively inhibit GBA1. Following the reaction within the GBA1 active site to form a covalent and irreversible enzyme-ABP adduct, GBA1 (and possibly also GBA1-

associated proteins) is projected to be enriched through biotin-streptavidin pull-down, after which the thus captured protein content is foreseen to be released from the streptavidin-coated beads by photocleavage. Experiments towards this ABP-capture-photorelease of GBA1 from biological samples are discussed in Chapter 4.

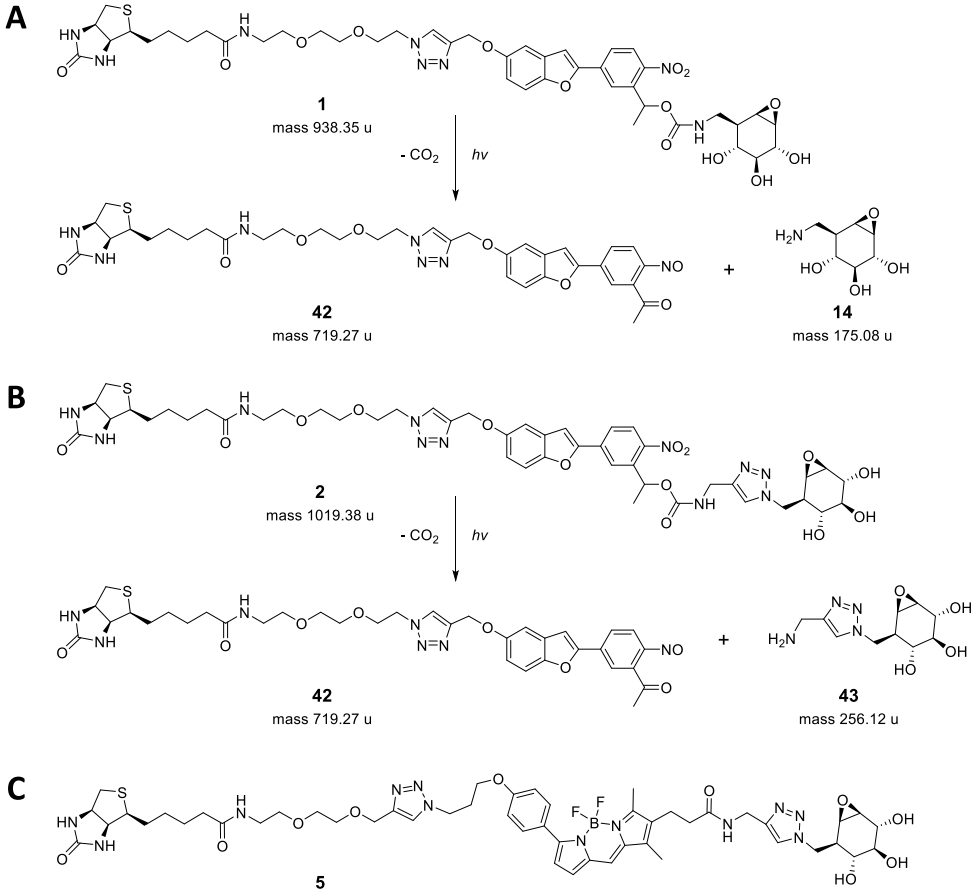


Figure 3.3 The ABPs of this work and their expected photocleavage products. A) Compound **1** is expected to give *ortho*-nitrosoacetophenone **42** and cyclophellitol **14**. B) Compound **2** cleaves also into *ortho*-nitrosoacetophenone **42** and cyclophellitol **43**. C) Structure of non-photoresponsive ABP **5**.

In this chapter, the results of studies on the photoreactive properties of ABPs **1** and **2** are presented. Figures 3.3A and B show the products expected to be formed when compounds **1** and **2** are irradiated with light at a wavelength of 375 nm. In both instances, the formation of *ortho*-nitrosoacetophenone **42** is anticipated. Compound **1**

would also yield 6-deoxy-6-aminocyclophellitol **14** (glucopyranose numbering), while compound **2** would give triazole-functionalized cyclophellitol **43**.

In this chapter, the photochemical reactions of compounds **1** and **2** were investigated both before and after the reaction with GBA1. Next, the photochemical reaction was tested in full cell lysates, the potential side reactions of the photocleavable linker were monitored and the effect of irradiation on cell lysates was assessed. Further, it was tested whether compounds **1** and **2** can pass the cell membrane of living cells.

3.2 Results and Discussion

Having confirmed that ABPs **1** and **2** are potent GBA1 inhibitors (see Chapter 2), their photoreactive properties were tested as the first objective of the research described here. First, the photoconversion of compounds **1** and **2** into their respective photocleaved products (see Figure 3.3) was measured and quantified. For this, the compounds were dissolved in an H_2O/MeC/^{t}BuOH mix (1:1:1), after which the samples were irradiated at a wavelength of 375 nm for varying times and the results were analysed by LC-MS (Figure 3.4). The results were filtered for the specific mass of the starting material (compounds **1** or **2**) and for biotin-containing cleavage product **42**, as well as for the wavelength range of 350 nm to 500 nm. This wavelength range encompasses the linker's characteristic emission wavelength (364 nm) [15] and the emission wavelength of the photoreaction product (420 nm) [16]. Photochemical reaction products **14** and **43** could not be identified with this method, possibly due to their high polarity. The efficiency of the photochemical reactions was therefore only quantified by the amount of product **42** formed.

Compound **1** was found to be completely consumed within 5 to 10 min of irradiation with 375 nm, while the signal of compound **2** already disappeared after 5 min. In the mass-filtered results (Figures 3.4A and B), conversion from the starting compound **1** highlighted in blue and compound **2** in green to product **42** in orange could be visualized. In the wavelength-filtered results (Figures 3.4C and D), again conversion from starting materials to product **42** could be observed, but also the formation of potential side products that were detected in the wavelength range of 350 to 500 nm.

The conversion from compounds **1** and **2** to product **42** was quantified as depicted in Figures 3.4E and F, by integration of the respective peaks of three independent experiments filtered by wavelength. Starting materials **1** and **2** always appeared as a single peak and could easily be integrated. For the integration of desired product **42**, only the peak containing mostly the correct mass was used for quantification (as described in Supplementary Figure 3.1C). For both compounds **1** and **2**, the decay of the starting material was comparably fast and reproducible. Formation of product **42**, however, varied from one experiment to the next and was therefore depicted in separate lines for each of the three measurements. Formation of product **42** from compound **1** ranged from 22% to 114% yield, while for compound **2**, a range from 37% to 89% was measured. Low conversion to product **42** always coincided with the formation of a variety of side products and even the formation of only side products occurred in some experiments, which were then not used for quantification.

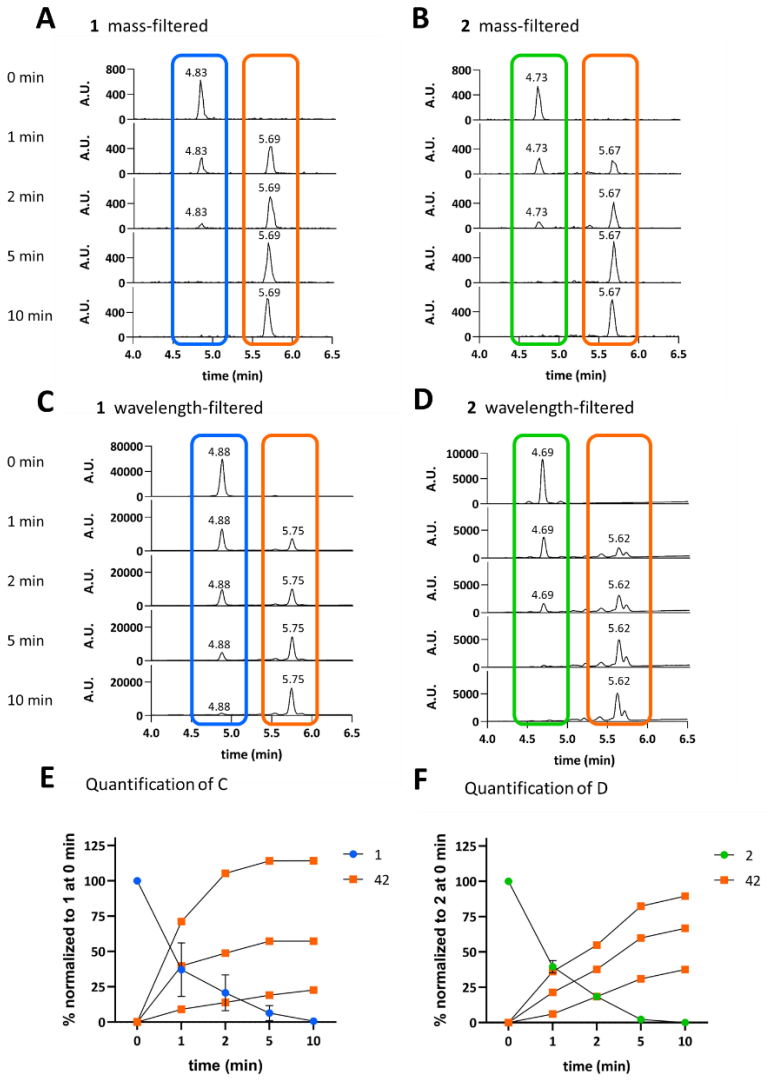


Figure 3.4 LC-MS measurements of the photochemical reaction of ABPs **1** (blue) and **2** (green) over time into product **42** (orange). Compounds **1** and **2** were dissolved in H_2O/MeCN/^{t}BuOH (1:1:1), irradiated at a wavelength of 375 nm for the indicated times and then measured by LC-MS. A) and B) show a measurement of ABPs **1** and **2**, respectively, filtered by the mass of compounds **1** or **2** and product **42**. C) and D) show a measurement filtered by wavelengths from 350 nm to 500 nm. E) and F) show the quantification of the wavelength-filtered results of three independent experiments for compounds **1** and **2**, respectively. The area under the curve of starting material and product peaks was integrated and the values were normalized for the area under the curve of compounds **1** or **2** at 0 min, respectively.

For all three experiments with each compound, reaction conditions such as solvents, starting material concentration and irradiation time were kept constant and the cause of the variation in product formation could not be identified with the applied method. It should also be noted that when the integrated area of product **42** and all suspected side products were summed up, full conversion from the starting material could still not be observed in some measurements (Supplementary Figures 3.1A and B).

Considering the high reactivity of *ortho*-nitrosoacetophenone with nucleophiles, three of the side products formed in the LC-MS experiment could be identified in experiments with high side product formation (Supplementary Figures 3.1C and D). Compounds **44** – **46** (See Supplementary Figure 3.1E for chemical structures) could all be formed by a nucleophilic attack on the nitroso group of product **42** by the thioether of biotin, leading to oxidation of biotin and ring closure of the nitroso ketone as suggested by Barth *et al.* (Supplementary Figure 3.1F) [17]. Product **44** contains the fused 5-membered ring, product **45** the oxidized biotin and product **46** has both new features. Since products **42** and **45** have the same mass, a differentiation between the two was not possible by the applied method. Side product **47** (Supplementary Figure 3.1E) could be explained by dimerization of the nitroso group as also observed before by Barth *et al.* [17].

Next, the efficiency of the photochemical reaction was tested following the reaction of the photocleavable ABPs **1** and **2** with GBA1, either as purified recombinant GBA1 (Cerezyme) or with GBA1-containing cell lysate (Figure 3.5). Samples with DMSO instead of ABP were taken along as a background control, while ABP **5** served as a non-photoreactive control compound. For each compound, two samples were first incubated with GBA1 for 30 min at 37°C, then one sample was kept in the dark as the unirradiated control ("-") while the other one was irradiated for 15 min at 375 nm ("+"). Next, samples were denatured with sample buffer and the protein content of these samples was resolved by SDS PAGE. The gel-separated proteins were blotted onto a membrane and the membrane was incubated with antibodies against GBA1 (red signal in merge) and biotin (blue signal) and then imaged. When the GBA1 and biotin signals overlapped, the photocleavable bond was still intact, while when the biotin signal disappeared it could be assumed that the linker was cleaved (see Figure 3.5A for a graphical depiction).

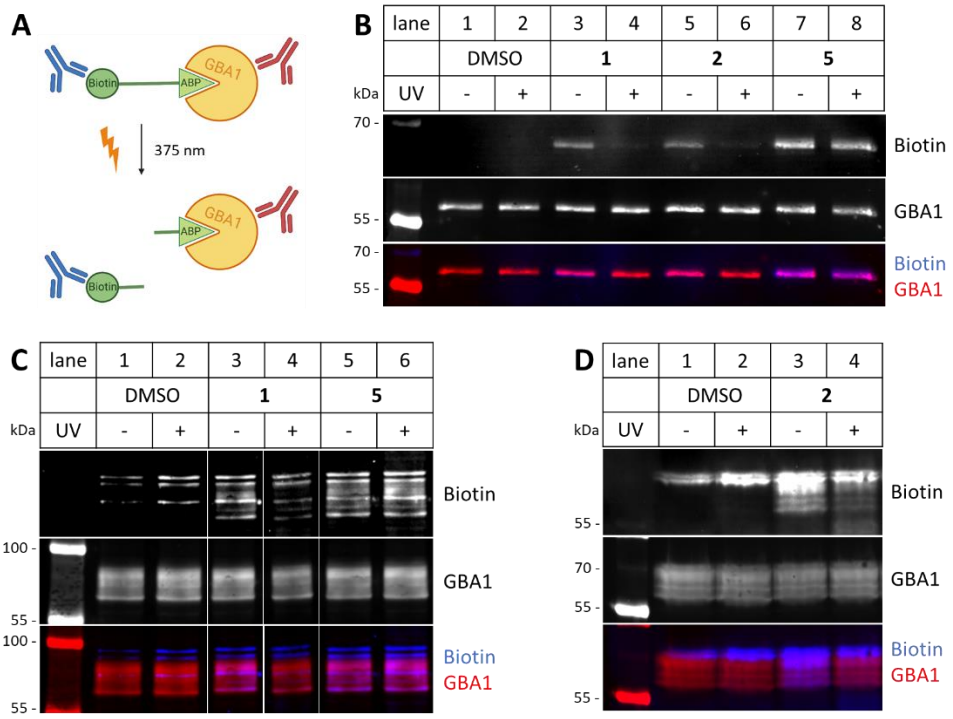


Figure 3.5 Western blots of photochemical reaction of probes **1** and **2** when bound to GBA1. Per probe, two GBA1-containing samples were incubated for 30 min at 37°C with either DMSO (negative control) or ABPs **1**, **2** or **5** (positive control) at 1 µM concentration. Then, UV "-" samples were kept in the dark, while samples with a UV "+" were irradiated for 15 min at 375 nm. Samples were denatured with sample buffer, followed by SDS PAGE and the gel was blotted over on a nitrocellulose membrane. Membranes were treated with antibodies against biotin and against GBA1. A) Graphical explanation of the expected antibody signals. When the sample is not irradiated and the probe is intact, both, the anti-GBA1 and the anti-biotin antibody are bound to the same complex and their signals, therefore, overlap on gel. When the complex is irradiated, the probe is cleaved and the small biotin moiety does not run at the same height on SDS PAGE as GBA1 anymore. Therefore, in the irradiated sample, only an anti-GBA1 signal is expected. B) Photocleavage of ABPs **1** and **2** tested with Cerezyme. DMSO and ABP **5** were taken along as controls. The gel is representative of a total of three gels. C) and D) Compounds **1** and **2** tested in fibroblast lysate (7 mg/mL total protein concentration) with DMSO and ABP **5** taken along as controls. These results are representative of three independent experiments.

Figure 3.5B shows the experiment performed with Cerezyme. The DMSO-treated samples did not give any biotin signal but only a GBA1 signal, as expected. For all ABPs, a clear biotin signal could be observed for non-irradiated "-" samples (lanes 3, 5, 7)

overlapping with the GBA1 signal, indicating that the compounds labelled GBA1 reliably. Largely abolished biotin signal in irradiated "+" samples of ABPs **1** and **2** (lanes 4 and 6) suggest a successful photochemical cleavage of the two compounds, while the biotin signal of non-cleavable ABP **5** remained present upon irradiation (lane 8). Quantification of the biotin signals of three comparable experiments was performed. On average, the biotin signal of the irradiated ABP **1** sample proved to be about 15% of the intensity of the unirradiated sample (compare lanes 4 and 3), while for ABP **2**, the irradiated sample had 20% of the intensity of the unirradiated sample (lanes 6 and 5). Biotin signals stemming from experiments involving ABP **5** had similar intensities for both experiments. Cleavage of the photoreactive linker in compounds **1** and **2**, when reacted with GBA1, proved, therefore, to be about equally efficient, whereas non-cleavable ABP **5** proved as expected resistant to treatment with 375 nm light, also following reaction with GBA1.

The photocleavage event could also be successfully demonstrated in fibroblast lysate (Figures 3.5C and D). Here, the DMSO samples (lanes 1 and 2 in both gels), showing cell lysate without any added probes, came in handy to differentiate between physiologically biotinylated proteins in the lysate and GBA1 labelled by compounds **1**, **2** and **5**, which appeared to be on a similar height on the gel. In the non-irradiated "-" samples of ABPs **1** and **2** (lane 3 in Figures 3.5C and D, respectively) an additional biotin signal overlaying with the GBA1 signal could be visualized, indicating that the compounds bound to GBA1 in the cell lysate. In the irradiated "+" samples treated with ABP **1** and **2** (lane 4 in Figures 3.5C and D, respectively), the biotin signal was less prominent, revealing that the photochemical reaction of the NPFB linker could also be performed in the presence of a lysate. Regardless of irradiation, samples treated with ABP **5** (lanes 5 and 6 in Figure 3.5C) again showed stable GBA1 labelling with biotin.

It should be noted that the visualization of the photochemical reaction in cell lysate was difficult to reproduce: A protein concentration of at least 7 mg/mL was necessary to show any results, however, was no guarantee for a successful experiment. In the experiments that did not show the expected effect, instead of a decrease in the biotin signal in the irradiated "+" samples, the biotin signal was greatly enhanced and spread over the entire lane of the sample. This smearing effect was found exclusively when performing the experiment with HEK293T cell lysate (Figure 3.6). Here, the biotin bands in the non-irradiated "-" samples treated with ABPs **1** or **2** (lanes 3 and 5) could barely be detected, whereas the samples treated with **5** (lanes 7 and 8) helped as an orientation point to see where the biotin signal should be visible (between 55 and 70 kDa). In the irradiated "+" samples, however, the biotin signal covered a big part of the lane when

the sample was treated with compound **1** (lane 4) and an even stronger smearing effect was visible when the sample was treated with compound **2** (lane 6). Since this smearing effect was never visible in samples treated with ABP **5**, it could be assumed that it was caused by the photochemical linker of cleavable ABPs **1** and **2**.

A possible explanation for the enhanced biotin signal in the irradiated samples could be a reaction between cleaved photochemical product *ortho*-nitrosoacetophenone **42** with proteins of the lysate as suggested by Barth *et al.* [17]. Free thiols [17] and amines [19] of proteins could interact with the nitroso ketone. In the non-irradiated samples, only the fraction of compounds **1** or **2** that was bound specifically to their target GBA1 would be visualized, while unbound probe would run at the bottom line of the gel and its signal would not be detected. In the irradiated sample, however, all of compounds **1** or **2** converted into photochemical product **42** had the chance to interact with the proteins of the lysate, enhancing the biotin signal compared to the biotin signal in the unirradiated sample.

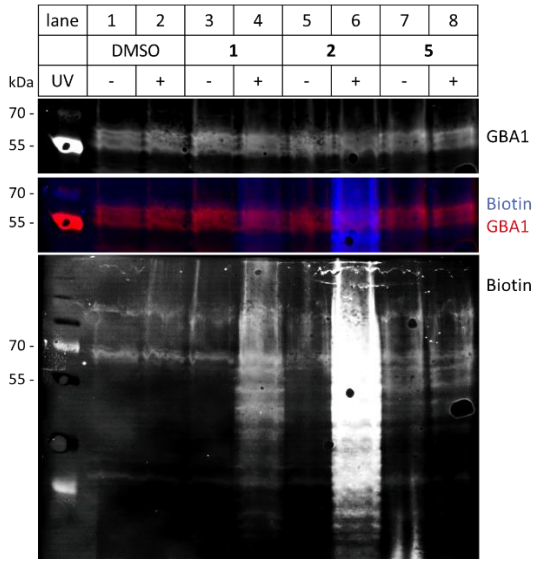
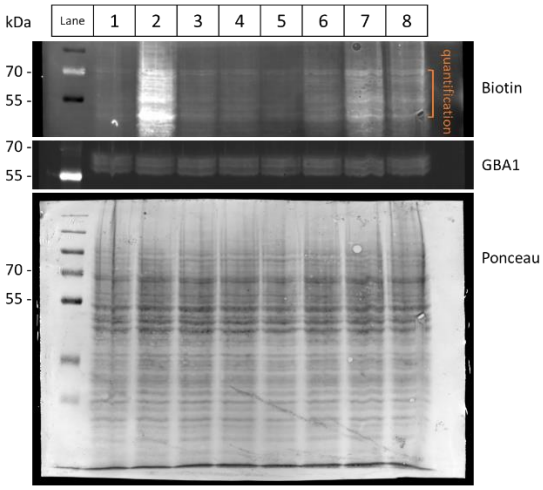


Figure 3.6 Effect of photocleavage of ABPs **1** and **2** in HEK293T cells (20 mg/mL total protein concentration). DMSO and ABP **5** were taken along as control. All samples were treated with a compound concentration of 1 µM and incubated for 30 min at 37°C. Samples with a UV "-" were kept in the dark, while samples with a UV "+" were irradiated for 15 min at 375 nm. Samples were then denatured with sample buffer, protein content was separated by SDS PAGE and then blotted on a membrane. The membrane was treated with antibodies against biotin and against GBA1. These results are representative of three independent experiments.

To get a better insight into this effect of enhanced biotin signal in the irradiated samples, the experiment was repeated with compound **2**, with samples where the order of the protocol was changed slightly (Figure 3.7). The biotin signal was quantified from roughly 50 to 70 kDa (as indicated in the biotin gel, results in the last column of the table). For the samples of lanes 1 and 2, the conditions used in the experiment of Figure 3.6 were repeated. Again, the non-irradiated sample (lane 1) showed no biotin signal associated with GBA1, while the irradiated sample (lane 2) had a strong smearing biotin signal. Since lane 2 had the strongest biotin signal in this blot, its intensity was set to 100% for quantification and all other lanes were reported relative to it. Sample 1 had therefore a biotin signal intensity of 5%.



Lane	Pretreatment	Mix	Incubate	Irradiation I	Cooking	Irradiation II	Intensity biotin lane (%)
1	-	Lysate + 2	30 min at 37 °C	-	SB, 5 min at 95 °C	-	5
2	-	Lysate + 2	30 min at 37 °C	10 min	SB, 5 min at 95 °C	-	100
3	-	Lysate + 2	30 min at 37 °C	-	SB, 5 min at 95 °C	10 min	14
4	-	Lysate + 2	30 min at 37 °C	-	Colourless SB 5 min at 95 °C	10 min	13
5	Lysate + Colourless SB; 5 min at 95 °C	+ 2	30 min at 37 °C	10 min	-	-	9
6	2, 10 min UV	+ Lysate	30 min at 37 °C	-	SB, 5 min at 95 °C	-	20
7	2, 10 min UV	+ Lysate	30 min at 37 °C	10 min	SB, 5 min at 95 °C	-	53
8	2, 60 min UV	+ Lysate	30 min at 37 °C	10 min	SB, 5 min at 95 °C	-	27

Figure 3.7 The effect of photocleavage of ABP **2** in HEK293T cell lysate. Compound **2** and HEK293T cell lysate (5 mg/mL total protein concentration) were treated according to the table. Irradiation was performed at 375 nm for the indicated time. Protein content was separated by SDS PAGE and then blotted on a membrane. The membrane was treated with antibodies against biotin and GBA1 and with Ponceau staining for total protein. The intensity of the biotin signal was quantified in the indicated section of the biotin blot and intensity is given in % relative to the intensity of lane 2 as 100%.

For the samples of lanes 3 and 4, the lysate was incubated with compound **2** and then cooked with sample buffer before the sample was irradiated. To exclude that the bromophenol blue used in the sample buffer affected irradiation, sample 3 was cooked in bromophenol blue-containing sample buffer, while sample 4 was cooked in transparent sample buffer. Both lanes showed a comparable low intensity in biotin signal of 14% and 13%. Irradiation caused only little side reactions with proteins of the lysate, most likely because cleaved product **42** interacted with omnipresent DTT as an ingredient of the sample buffer rather than with proteins of the lysate. As described in the literature, DTT has been used in the past to prevent side reactions of *ortho*-nitrosoacetophenone with proteins [17]. To exclude that the low biotin signal intensity of samples 3 and 4 was due to impairment of the photocleavable linker by cooking it in sample buffer, for the sample of lane 5, the lysate was first cooked with sample buffer, then compound **2** was added and the mixture was irradiated. For this sample, again the biotin signal intensity was low at 9%, confirming that the ingredients of the sample buffer prevented side reactions of cleaved product **42** with the lysate.

For the samples of lanes 6, 7 and 8, compound **2** was irradiated before it was incubated with the lysate. For samples 6 and 7, compound **2** was irradiated for 10 min, while for sample 8, compound **2** was irradiated for 60 min to guarantee full conversion of the starting material to the cleaved product **42** (and potential side products). After incubation of irradiated compound **2** with the lysate, sample 6 was kept in the dark while samples 7 and 8 were irradiated for another 10 min. For sample 6, a biotin signal intensity of 20% was measured, four times higher than that of unirradiated sample 1. This result indicates that cleaved product **42** could interact with proteins in the lysate, even without additional irradiation. With additional irradiation in the presence of lysate, the unspecific biotin signal in lane 7 had an intensity of 53% and in lane 8 only an intensity of 27%, indicating an interaction of the photocleavable linker with the lysate. These lower intensities compared to sample 2 could be explained by the reaction of product **42** with itself during the first irradiation time, as already observed in the experiment of Figure 3.4. The longer this first irradiation, the more product **42** could react already and the lower the biotin signal intensity.

Even though the GBA1 bands in the Western blots (Figures 3.5 and 3.6) showed no obvious influence of irradiation on the protein, an experiment to visualize the influence of 375 nm light on fibroblast and HEK293T cell lysate was performed. Pure cell lysate (Figure 3.8) or cell lysate incubated with 1 µM ABP **2** (Supplementary Figure 3.2, protein concentration of all lysates at 4 mg/mL) was irradiated for various time points between 0 and 30 min. The irradiated samples were denatured with sample buffer, run on an SDS

PAGE, blotted onto membranes and the membranes were stained with antibodies against GBA1 and biotin and with Ponceau as a general protein stain. Irradiation had no visible effect neither on fibroblast nor on HEK293T cell proteins as the intensity of the protein signals stayed constant for all time points. Only in the HEK293T lysate incubated with **2**, the known effect of increased biotin signal upon irradiation could be detected again (Supplementary Figure 3.2B, biotin lane). An effect of irradiation on antibody-protein interaction could therefore be excluded. Still, protein modifications undetectable by this method could occur; possible modifications could be undetectable by denaturing the proteins when preparing the samples for SDS PAGE or simply did not influence the binding of the antibodies or staining with Ponceau. Even though the influence of irradiation on cell lysates could not be ruled out, it could be assumed that irradiation did not influence the cell lysate in a way that interferes with the experiments performed in this thesis.

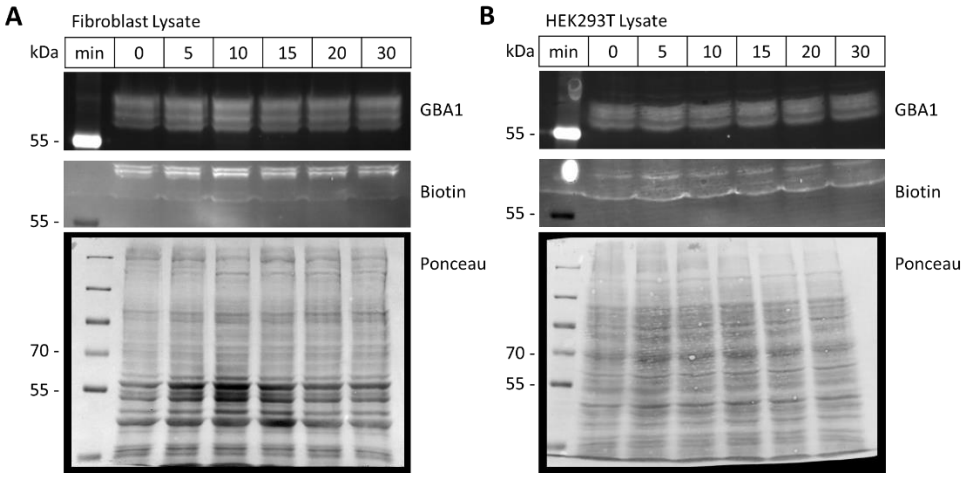


Figure 3.8 Effect of irradiation on cell lysate (4 mg/mL total protein concentration). The cell lysate was irradiated for the indicated time with 375 nm light, then denatured with sample buffer, separated by SDS PAGE and blotted onto a membrane. Membranes were treated with antibodies against biotin and against GBA1, as well as with Ponceau staining to visualize total protein. A) Pure fibroblast cell lysate and B) pure HEK293T cell lysate.

To assess whether compounds **1** and **2** could be exploited in *in cellulo* experiments, their membrane permeability was tested. Therefore, fibroblasts grown to a confluent cell layer were incubated either with DMSO, ABPs **1** or **2** at various concentrations for 5 hours (Figure 3.9). Cells were then harvested and lysed in the presence of GBA1-inhibitor MDW941 [20], a cyclophellitol-based ABP tagged with a Bodipy (see Supplementary

Figure 3.3 for the chemical structure) to label all GBA1 that was not inhibited by compounds **1** or **2**. Samples were separated by SDS PAGE, followed by blotting the proteins onto a membrane, which was incubated with Streptavidin Alexa Fluor 488 to visualize the biotin moieties of **1** and **2** (Figure 3.9A). No additional biotin signal could be detected when comparing the samples with the DMSO-only incubated samples (lane 1) that showed the physiologically biotinylated proteins, indicating that neither ABP **1** (lanes 2-4) nor ABP **2** (lanes 5-6) were able to pass the cellular membranes to label GBA1 within the timeframe of this experiment. The Bodipy channel confirmed this result, showing that independent of the concentration of compounds **1** and **2**, GBA1 was equally labelled with MDW941 in all samples.

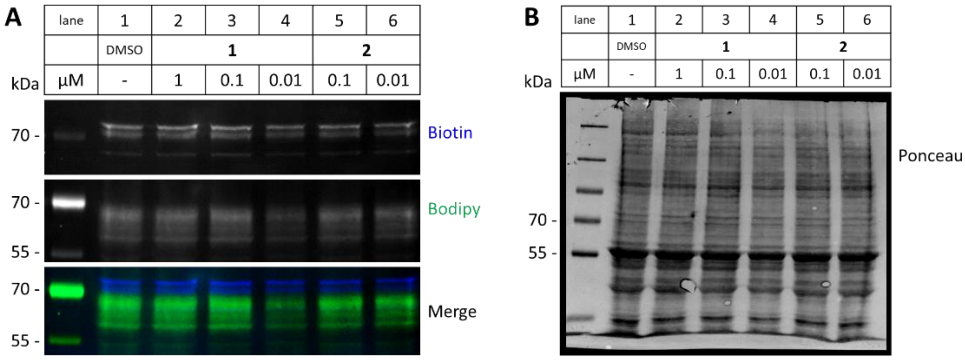


Figure 3.9 Incubation of fibroblasts with compounds **1** and **2** to test their membrane permeability. Cells were first incubated with the indicated concentrations of the compounds, then during lysis with 10 µM of specific GBA1 inhibitor MDW941 tagged with a Bodipy. A) Western blot with staining for biotin to detect ABPs **1** and **2**, which were both not visible. The Bodipy channel shows GBA1 that was not occupied by the ABPs during *in vivo* incubation. The bottom gel shows a merge of the two above. B) Ponceau staining of the same gel serves as a loading control.

3.3 Conclusion

In this chapter, the photochemical properties of photocleavable ABPs **1** and **2** were studied under various conditions. By analysing the photochemical reaction of **1** and **2** with LC-MS, it could be demonstrated that both compounds were fully consumed within a maximum irradiation time of 10 min when the compounds were irradiated at a wavelength of 375 nm. However, quantification of the reaction showed that the yield of the formation of expected product **42** varied greatly. Some of the formed side products could be identified by their mass. The photochemical reaction was also tested in the presence of GBA1. When the compounds were incubated with purified recombinant GBA1 and the complex was irradiated, for ABP **1** about 85% was cleaved and for ABP **2** this percentage was about 80%. A successful photochemical reaction could also be demonstrated in fibroblasts, while artefacts made the reaction undetectable in HEK293T cells. Here, cleaved product **42** most likely bound non-specifically to a range of proteins in the lysate. The effect of irradiation on proteins in the cell lysate was tested by irradiation of fibroblast and HEK293T cell lysate for up to 30 min at 375 nm and no alterations on the stained proteins could be detected. All these results lead to the conclusion that both, ABPs **1** and **2**, can be reliably cleaved in a biological setting when irradiated at a wavelength of 375 nm and that this irradiation does not have further influence on the experiment itself. Formation of expected product **42** could be observed on LC-MS, however, various side products were detected as well and the conditions under which these form still need to be investigated. When the photochemical reaction was quantified on gel, however, the loss of biotin signal was satisfying. In an *in vitro* experiment, neither **1** nor **2** were able to cross the cell membrane in detectable amounts, indicating that the compounds should only be used to incubate with lysed cells. Compound **5** could be reliably used as a non-cleavable control compound that tagged GBA1 with its Bodipy.

3.4 Experimental

3.4.1 Material

Custom-made photochemical reaction lamp

Irradiation was performed with a custom-made irradiation set-up. LEDs were High Power Single Chip (H2A1 Series, 1 W) from Roithner Lasertechnik GmbH. According to [16], the power output was 3 mW/cm^2 for the 375 nm LED and 13 mW/cm^2 for the 420 nm LED.

Antibodies

Primary Antibodies:
Anti-biotin Streptavidin Alexa 488 (S-32354) by Invitrogen, diluted 1:3000 in 5% BSA in TBS/T.
Anti-biotin Streptavidin/HRP (P0397) by Dako, diluted 1:10,000 in 5% BSA in TBS/T.
Anti-GBA mouse (8E4), house-made, diluted 1:1000 in 5% BSA in TBS/T.

Secondary Antibody:
Anti-mouse Alexa 647 donkey (A-31571) by Invitrogen, diluted 1:10,000 in 5% BSA in TBS/T.

3.4.2 Methods

LCMS-experiment photochemical reaction (Figure 3.4 and Supplementary Figure 3.1)

1 mM of ABP **1** or **2** in DMSO was diluted to a final concentration of 10 µM for **1** or 30 µM for **2** in an H_2O/MeCN/^{t}BuOH mixture (1:1:1). Samples of 100 µL of this mixture were irradiated in a black flat-bottom 96-well plate for 0, 1, 2, 5 or 10 min with a wavelength of 375 nm, pipetted over into dark-glass LC-MS vial and measured on an LCQ Fleet (Thermo Fisher Scientific) ion-trap spectrometer (ESI+) coupled to an Ultimate 3000 UHPLC system (Thermo Fisher Scientific) equipped with a C18 Gravity column (Nucleodur, 4.6 mm x 50 mm, 3 µm particle size, Macherey-Nagel) equipped with buffers A: H_2O, B: MeCN and C: 1% aq. TFA. Results were analysed using Xcalibur and either filtered for a wavelength of 350-500 nm or the mass of the start material or product. Quantification was performed by integrating the area under the peak

for the wavelength-filtered data. For each experiment the area of the starting material at 0 min was set to 100% and other values were calculated relative to that. Three experiments were performed for both compounds.

Cell culture

Cells were kept in culture at 37 °C at a 7% CO_2 atmosphere. The medium was refreshed twice a week, once by simply exchanging the medium and once by splitting the cells. Normal human dermal fibroblasts (referred to as "fibroblasts") were kept in DMEM/F12 with added fetal bovine serum (FBS), penicillin-streptomycin and glutamate. HEK293T cells were kept in DMEM with added newborn bovine serum (NBS), penicillin-streptomycin and glutamate. To split the cells, they were washed once with PBS, trypsinized for 1 min at 37 °C, resuspended in medium and spun down at 300 rcf for 5 min. The supernatant was discarded, cells resuspended in medium and seeded back into their bottle. Fibroblasts were seeded 0.5 x 10^6 cells per T75 bottle and HEK293T cells were seeded 2 x 10^6 cells per T75 bottle to grow confluent within a week.

Cell pellet preparation

Cells were washed with PBS, trypsinized for 1 min at 37 °C, resuspended in medium and spun down at 300 rcf for 5 min. The supernatant was discarded, the cells resuspended in PBS, a sample was taken for cell counting and the cells were spun down again at 300 rcf for 5 min. The supernatant was discarded, the cells were resuspended in PBS, distributed over 1.5 mL Eppendorf tubes and spun down at 5000 rcf for 5 min. The supernatant was discarded and pellets were shock-frozen with liquid N_2 and stored at -80 °C.

Cell lysate preparation

A cell pellet was suspended with two times its volume of lysis buffer (150 mM McIlvaine Buffer pH 5.2 + 0.1% (v/v) TX-100 + 1x protease Inhibitor cocktail (Roche)), vortexed well and incubated on ice for 15 min. The pellet was then vortexed well again and stored for another 15 min on ice. The suspension was vortexed again, and then three times shock-frozen with liquid N_2 and thawed. The protein concentration was determined using Bradford Assay (see procedure below).

Bradford assay for the determination of the protein concentration

The Protein Assay Dye Reagent Concentrate (Bio-Rad) was diluted 1:4 with MilliQ H_2O and 100 µL per well was used in a transparent flat-bottom 96-well plate. 1 µL of BSA solution (concentrations from 2.5 mg/mL down to 0.078 mg/mL) was added per well in duplicates for the calibration curve and 1 µL of lysate per well in triplicates. Various dilutions were made of cell lysates with an expected protein concentration higher than 2 mg/mL. The 96-well plate was shaken for 1 min before reading out on an iMark Microplate reader (Bio-Rad). Protein concentrations were calculated with the blotted calibration curve.

Photochemical reaction with recombinant GBA1 (Cerezyme) (Figure 3.5B)

10 µL Cerezyme mix (85.26 fmol Cerezyme in 150 mM McIlvaine Buffer pH 5.2 + 0.1% (v/v) TX-100 + 0.2% (w/v) NaTc as assay buffer) was incubated with 5 µL ABP-mixture (3 µM of ABPs **1**, **2** or **5** in assay buffer was used if not noted otherwise in the results). Thus, samples containing ABPs at 1 µM final concentration were incubated for 30 min at 37 °C in the dark and controls were incubated with DMSO in assay buffer (vol DMSO = vol ABP stock). Samples chosen for irradiation were irradiated for 15 min at a wavelength of 375 nm with the custom-made lamp, while the other samples were kept in the dark, all at room temperature. 4 µL of 5x Laemmli Buffer was added to all samples and incubated for 5 min at 95 °C. Samples can be stored in the dark at -20 °C but were usually used right away to load 15 µL on a 10% acrylamide gel (29% acrylamide + 1% bis by Bio-Rad). 4 µL of PageRuler Prestained Protein Ladder (Thermo Fisher) was used as protein ladder. The gel was run for 180 min at 120 V in the dark and afterwards, blotted on a 0.2 µm nitrocellulose membrane (Trans-Blot Turbo Pack, Bio-Rad) with a Trans-Blot Turbo Transfer System (Bio-Rad). For all washing and incubation steps, the membrane was kept in the dark. The membrane was washed two times for 10 min with TBS/T, then blocked for 30 min with 5% BSA in TBS/T. Incubation of the membrane with the primary antibodies was always performed overnight at 4 °C in 5% BSA in TBS/T in dilution with the respective antibody (see the materials section). The membrane was then washed three times for 10 min with TBS/T and, if applicable, incubated with the secondary antibodies for 1 hour at room temperature diluted 1:10,000 in 5% BSA in TBS/T. The membrane was again washed three times for 10 min with TBS/T and once for 10 min with TBS and afterwards imaged on a Typhoon FLA 9500 scanner (GE Healthcare) at the excitation wavelengths of the antibodies' fluorescent label. Quantification of the bands was performed with ImageQuant Tl 1D v8.1. The background was subtracted with the Rolling Ball Method (radius 106). Values of two

experiments were taken and the uncleaved band of each respective compound was set to 100% to calculate the decrease in the band's intensity due to photocleavage.

Photochemical reaction in a cell lysate (Figures 3.5C and D and Figure 3.6)

10 µL cell lysate (cell line and protein concentration as indicated in Results and Discussion) was incubated with 5 µL ABP-mixture (3 µM of ABPs **1**, **2** or **5** in 150 mM McIlvaine Buffer pH 5.2 + 0.1% (v/v) TX-100 as assay buffer was used if not noted otherwise in the results). Thus, samples containing ABPs at 1 µM final concentration were incubated for 30 min at 37 °C in the dark and control lysate was incubated with DMSO in assay buffer (vol DMSO = vol ABP stock). Samples chosen for irradiation were irradiated for 15 min at 375 nm with the custom-made lamp while the other samples were kept in the dark, all at room temperature. 4 µL of 5x Laemmli Buffer was added to all samples and incubated for 5 min at 95 °C. Samples can be stored in the dark at -20 °C but were usually used right away to load 15 µL on a 10% acrylamide gel (29% acrylamide + 1% bis by Biorad). 4 µL of PageRuler Prestained Protein Ladder (Thermo Fisher) was used as a protein ladder. The gel was run for 15 min at 90 V, then for 120 min at 120 V in the dark and afterwards, blotted on a 0.2 µm nitrocellulose membrane (Trans-Blot Turbo Pack, Bio-Rad) with a Trans-Blot Turbo Transfer System (Bio-Rad). For all washing and incubation steps, the membrane was kept in the dark. The membrane was washed two times for 10 min with TBS/T, then blocked for 30 min with 5% BSA in TBS/T. Incubation of the membrane with the primary antibodies was always performed overnight at 4 °C in 5% BSA in TBS/T in dilution with the respective antibody (see the materials section). The membrane was then washed three times for 10 min with TBS/T and, if applicable, incubated with the secondary antibodies for 1 hour at room temperature diluted 1:10,000 in 5% BSA in TBS/T. The membrane was again washed three times for 10 min with TBS/T and once for 10 min with TBS and afterwards imaged on a Typhoon FLA 9500 scanner (GE Healthcare) at the excitation wavelengths of the antibodies' fluorescent label.

Photochemical reaction and its side reactions in cell lysate (Figure 3.7)

10 µL HEK293T cell lysate (5 mg/mL total protein concentration) and 5 µL ABP-mixture (30 µM of ABP **2** in 150 mM McIlvaine Buffer pH 5.2 + 0.1% (v/v) TX-100. Final concentration is 10 µM) were used. Samples were treated according to the table in Figure 3.7. Samples chosen for irradiation were irradiated for the indicated time at 375 nm with the custom-made lamp, while the other samples were kept in the dark, all at room temperature. 4 µL of 5x Laemmli Buffer (as sample buffer, either with or without

Bromophenol Blue, as indicated) was added to all samples and incubated for 5 min at 95 °C if indicated. Samples were used right away to load 15 µL on a 10% acrylamide gel (29% acrylamide + 1% bis by Biorad). 4 µL of PageRuler Prestained Protein Ladder (Thermo Fisher) was used as a protein ladder. The gel was run for 3 h at 90 V in the dark and blotted on a 0.2 µm nitrocellulose membrane (Trans-Blot Turbo Pack, Bio-Rad) with a Trans-Blot Turbo Transfer System (Bio-Rad). For all washing and incubation steps, the membrane was kept in the dark. The membrane was washed two times for 10 min with TBS/T, then blocked for 30 min with 5% BSA in TBS/T. Incubation of the membrane with the primary antibodies was always performed overnight at 4 °C in 5% BSA in TBS/T in dilution with the respective antibody (see the materials section). The membrane was then washed three times for 10 min with TBS/T and, if applicable, incubated with the secondary antibodies for 1 hour at room temperature diluted 1:10,000 in 5% BSA in TBS/T. The membrane was again washed three times for 10 min with TBS/T and once for 10 min with TBS and afterwards imaged on a Typhoon FLA 9500 scanner (GE Healthcare) at the excitation wavelengths of the antibodies' fluorescent label. Quantification of the bands was performed in the indicated area with ImageQuant Tl 1D v8.1. The background was subtracted with the Rubber Band Method and all values were given relative to the value of lane 2 as 100%.

Effect of irradiation on cell lysate (Figure 3.8 and Supplementary Figure 3.2)

HEK293T cell lysate was diluted with PBS or PBS with ABP **2** to a total protein concentration of 4 mg/mL and a total ABP concentration of 1 µM Samples containing ABP were incubated for 30 min at 37 °C. 15 µL per sample were pipetted into a 96-well plate and irradiated with a Capro Box (Caprotec) at 350 nm and 4 °C for the indicated times. After the irradiation time, samples were pipetted over into a tube and stored on ice until all samples were irradiated. To each sample, 5 µL 4x Laemmli Buffer was added and the samples were cooked for 5 min at 95 °C. 4 µL of PageRuler Prestained Protein Ladder (Thermo Fisher) and 15 µL of each sample were loaded on a 10% acrylamide gel (29% acrylamide + 1% bis by Biorad) and the gel was run at 90 V for 15 min and at 120 V for 2 h in the dark. Afterwards, the gel was blotted on a 0.2 µm nitrocellulose membrane (Trans-Blot Turbo Pack, Bio-Rad) with a Trans-Blot Turbo Transfer System (Bio-Rad). For all washing and incubation steps, the membrane was kept in the dark. The membrane was washed two times for 10 min with TBS/T, then blocked for 30 min with 5% BSA in TBS/T. Incubation of the membrane with 8E4 anti-GBA1 antibody (mouse, homemade) diluted 1:1000 in 5% BSA in TBS/T was performed overnight at 4 °C. The membrane was then washed three times for 10 min with TBS/T and incubated with the Anti-mouse Alexa Fluor 647 (Invitrogen, A31571) diluted 1:10,000 in 5% BSA

in TBS/T for 1 hour at room temperature. The membrane was then washed again three times for 10 min with TBS/T and once for 10 min with TBS and afterwards imaged on a Typhoon FLA 9500 scanner (GE Healthcare) with the 647 nm laser setting. The membrane was then incubated with Streptavidin Alexa Fluor 488 (Invitrogen, S-32354) diluted in 1:10,000 in 5% BSA in TBS/T overnight at 4 °C, washed three times for 10 min with TBS/T and once for 10 min with TBS and afterwards imaged again on a Typhoon FLA 9500 scanner (GE Healthcare) with the 488 nm laser setting. The membrane was then incubated for 1 h in Ponceau S Staining (Gelman instrument company), washed with demi water and imaged on the ChemiDoc MP (Bio-Rad).

In vivo incubation with 1 and 2 (Figure 3.9)

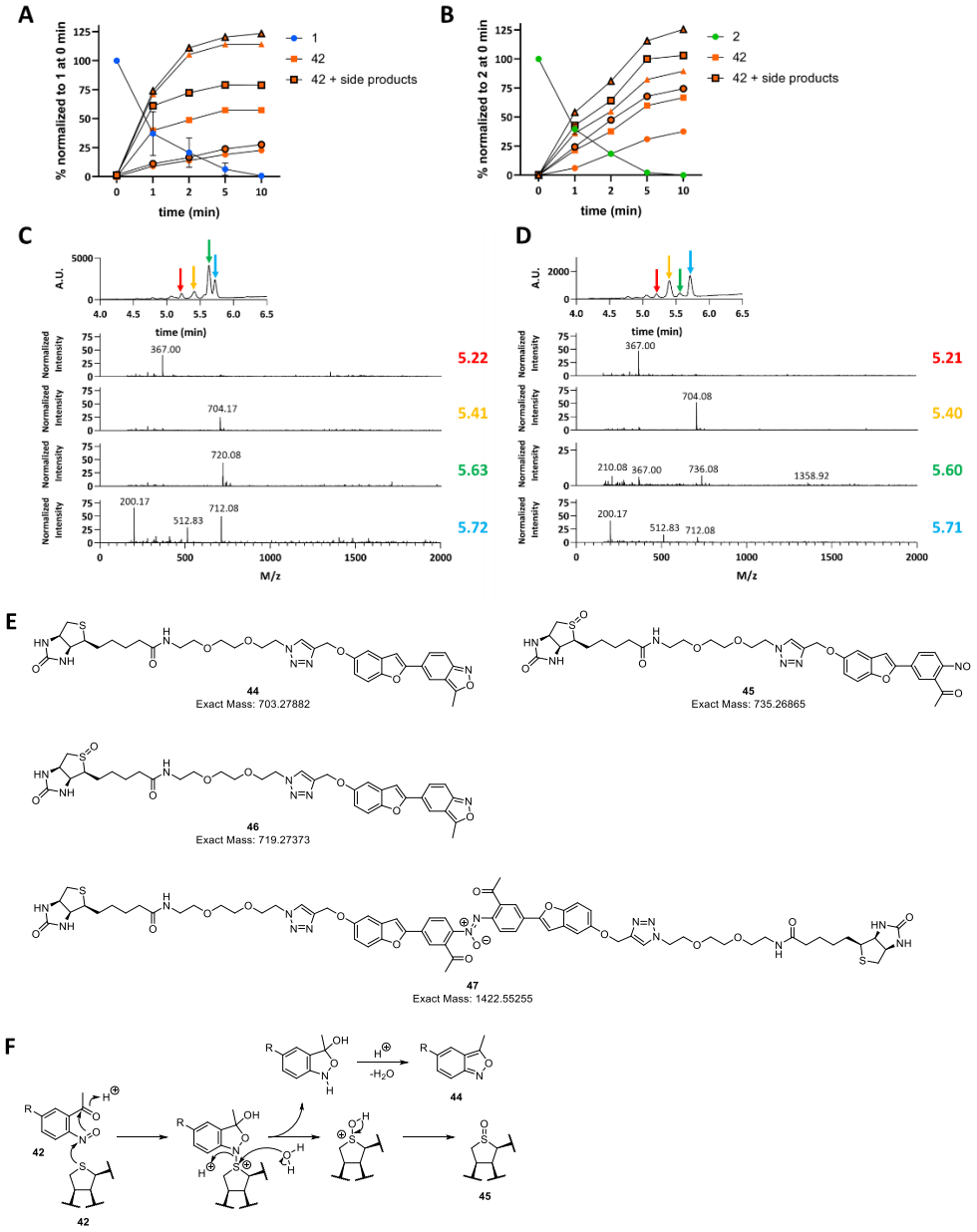
Fibroblasts were seeded out in a 6-well plate with 0.3 Mio cells per well and let grow for 48 hours. Cells were then incubated with 2 mL of medium with either DMSO (2 µL), ABPs **1** or **2** (both at indicated concentration) added and incubated for 5 hours at 37 °C at a 7% CO_2 atmosphere. Cells were then washed with 1 mL PBS, incubated with 0.5 mL 0.05% trypsin in PBS/EDTA for 1 min at 37 °C, resuspended in 1 mL medium and spun down at 300 rcf for 5 min. The pellets were washed twice with PBS and then 20 µL of lysis buffer (150 mM McIlvaine Buffer pH 5.2 + 0.1% (v/v) TX-100 + 1x protease Inhibitor cocktail (Roche)) with 10 µM MDW941 (GBA1-inhibitor with Bodipy [20]) was added to each pellet. The samples were incubated on ice for 30 min and then three times frozen with liquid N_2 and thawed. 6.6 µL of 4x Laemmli Buffer was added and the samples were cooked for 5 min at 95 °C. 4 µL of PageRuler Prestained Protein Ladder (Thermo Fisher) and 15 µL of each sample were loaded on a 7.5% acrylamide gel (29% acrylamide + 1% bis by Biorad) and the gel was run at 90 V for 2.5 h in the dark. Afterwards, the gel was blotted on a 0.2 µm nitrocellulose membrane (Trans-Blot Turbo Pack, Bio-Rad) with a Trans-Blot Turbo Transfer System (Bio-Rad). For all washing and incubation steps, the membrane was kept in the dark. The membrane was washed two times for 10 min with TBS/T, then blocked for 30 min with 5% BSA in TBS/T. Incubation of the membrane with Streptavidin Alexa Fluor 488 (Invitrogen, S-32354) diluted 1:3000 in 5% BSA in TBS/T was performed overnight at 4 °C. The membrane was then washed three times for 10 min with TBS/T and once for 10 min with TBS and afterwards imaged on a Typhoon FLA 9500 scanner (GE Healthcare) at 488 nm and 532 nm for the Streptavidin Alexa Fluor 488 and MDW941's own fluorescence, respectively. The membrane was then incubated for 1 h in Ponceau S Staining (Gelman instrument company), washed with demi water and imaged on the ChemiDoc MP (Bio-Rad).

3.5 References

1. J.M. Silva, E. Silva, R.L. Reis, "Light-triggered release of photocaged therapeutics - Where are we now?" *J. Control Release* **2019**, *298*, 154-176.
2. E. Maurits, M.J. van de Graaff, S. Maiorana, *et al.*, "Immunoproteasome inhibitor–doxorubicin conjugates target multiple myeloma cells and release doxorubicin upon low-dose photon irradiation," *J. Am. Chem. Soc.* **2020**, *142*, 7250-7253.
3. G.C.R. Ellis-Davies, "Caged compounds: photorelease technology for control of cellular chemistry and physiology," *Nat. Methods* **2007**, *4*, 619-628.
4. A. Svobodova, D. Walterova, J. Vostalova, "Ultraviolet light induced alteration to the skin," *Biomed. Pap.* **2006**, *150*, 25-38.
5. K. Kim, H. Park, K.M. Lim, "Phototoxicity: its mechanism and animal alternative test methods," *Toxicol. Res.* **2015**, *31*, 97-104.
6. Y. Matsumura, H.N. Ananthaswamy, "Toxic effects of ultraviolet radiation on the skin," *Toxicol. Appl. Pharmacol.* **2004**, *195*, 298-308.
7. P. Klán, T. Šolomek, C.G. Bochet, *et al.*, "Photoremovable protecting groups in chemistry and biology: reaction mechanisms and efficacy," *Chem. Rev.* **2013**, *113*, 119-191.
8. J.A. Barltrop, P.J. Plant, P. Schofield, "Photosensitive protective groups," *Chem. Commun.* **1966**, 822.
9. J.H. Kaplan, B. Forbush, J.F. Hoffman, "Rapid photolytic release of adenosine 5'-triphosphate from a protected analog: utilization by the sodium:potassium pump of human red blood cell ghosts," *Biochemistry* **1978**, *17*, 1929-1935.
10. J. Engels, E.J. Schlaeger, "Synthesis, structure, and reactivity of adenosine cyclic 3',5'-phosphate-benzyltriesters," *J. Med. Chem.* **1977**, *20*, 907-911.
11. A. Hasan, K.P. Stengele, H. Giegrich, *et al.*, "Photolabile protecting groups for nucleosides: synthesis and photodeprotection rates," *Tetrahedron* **1997**, *53*, 4247-4264.
12. Y. V. Il'ichev, M.A. Schwörer, J. Wirz, "Photochemical reaction mechanisms of 2-nitrobenzyl compounds: methyl ethers and caged ATP," *J. Am. Chem. Soc.* **2004**, *126*, 4581-4595.
13. J.E.T. Corrie, A. Barth, V.R.N. Munasinghe, D.R. Trentham, M.C. Hutter, "Photolytic cleavage of 1-(2-nitrophenyl)ethyl ethers involves two parallel pathways and product release is rate-limited by decomposition of a common hemiacetal intermediate," *J. Am. Chem. Soc.* **2003**, *125*, 8546-8554.
14. T. Šolomek, S. Mercier, T. Bally, C.G. Bochet, "Photolysis of *ortho*-nitrobenzylic derivatives: the importance of the leaving group," *Photochem. Photobiol. Sci.* **2012**, *11*, 548-555.
15. N. Komori, S. Jakkampudi, R. Motoishi, *et al.*, "Design and synthesis of a new chromophore, 2-(4-nitrophenyl)benzofuran, for two-photon uncaging using near-IR light," *Chem. Commun.* **2016**, *52*, 331-334.
16. M.J. van de Graaff, "*Synthesis of Chemical Tools to Study the Immune System,*" Thesis, Leiden University, **2023**.

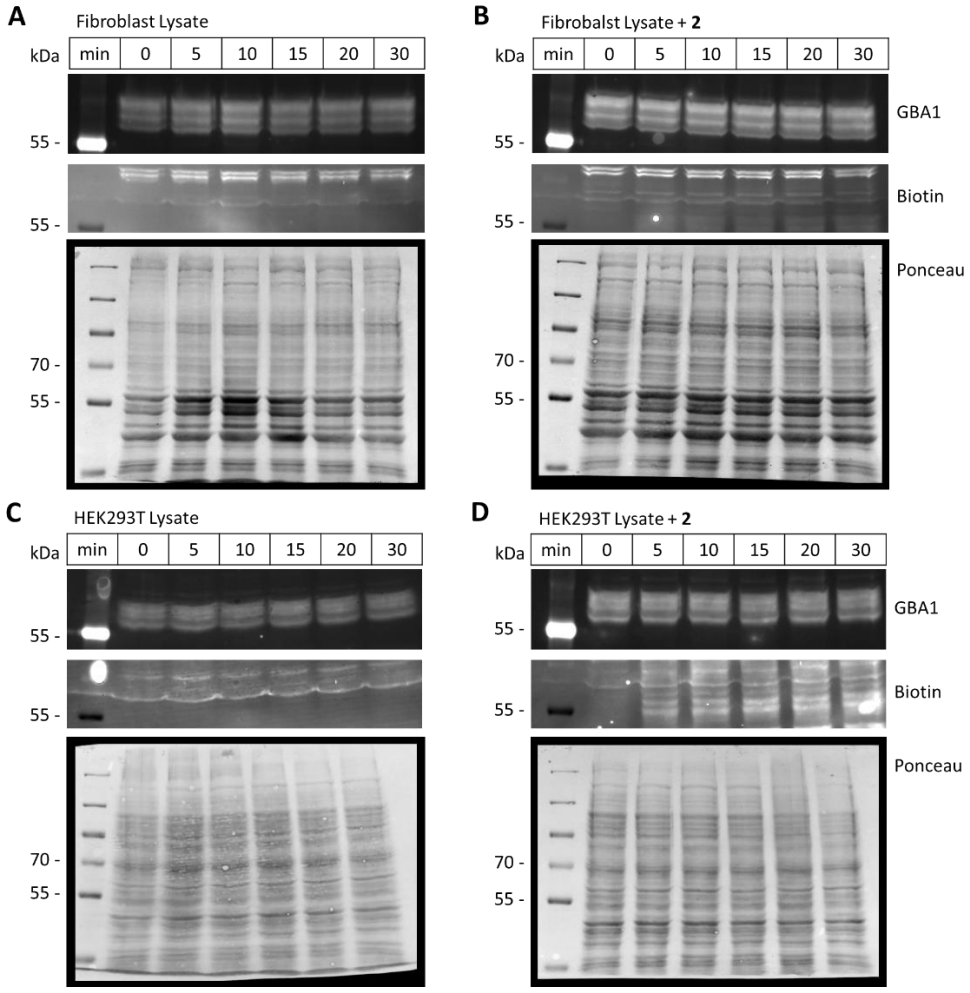
17. A. Barth, J.E.T. Corrie, M.J. Gradwell, *et al.*, "Time-resolved infrared spectroscopy of intermediates and products from photolysis of 1-(2-nitrophenyl)ethyl phosphates: reaction of the 2-Nitrosoacetophenone byproduct with thiols," *J. Am. Chem. Soc.* **1997**, *119*, 4149-4159.
18. J.W. Walker, G.P. Reid, J.A. McCray, D.R. Trentham, "Photolabile 1-(2-nitrophenyl)ethyl phosphate esters of adenine nucleotide analogs. Synthesis and mechanism of photolysis," *J. Am. Chem. Soc.* **1988**, *110*, 7170-7177.
19. M. Rinnová, M. Nováková, V. Kašicka, J. Jirácek, "Side reactions during photochemical cleavage of an alpha-methyl-6-nitroveratryl-based photolabile linker," *J. Pept. Sci*. **2000**, *6*, 355-365.
20. M.D. Witte, W.W. Kallemeijn, J. Aten, *et al.*, "Ultrasensitive *in situ* visualization of active glucocerebrosidase molecules," *Nat. Chem. Biol*. **2010**, *6*, 907-913.

3.6 Supplementary Information

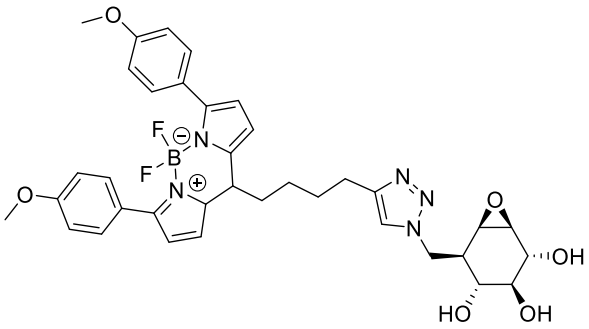


Supplementary Figure 3.1 LC-MS measurement of photochemical reaction of compounds **1** and **2**. Compounds **1** and **2** were dissolved in H_2O/MeCN/^{t}BuOH (1:1:1), irradiated at a wavelength of 375 nm for the indicated times and then

measured by LC-MS. A) and B) Quantification of wavelength-filtered results of three independent experiments for the decay of compounds **1** and **2**, respectively, and the formation of desired product **42** and side products. The area under the curve was integrated and the values were normalized for the area of ABPs **1** or **2** at 0 min, respectively. Integration of pure product **42** is indicated by fully orange symbols, while the integral of product **42** plus side products for the same measurement is indicated by the same symbol with a black border. C) UV-filtered (350 – 500 nm) measurement of compound **2** after 10 min irradiation. Masses of indicated peaks show desired product **42** (mass: 720 u) and side products. For the quantification of **42**, only peak 5.63 (green arrow) was integrated. D) UV-filtered (350 – 500 nm) measurement of compound **2** after 10 min irradiation without the formation of desired product **42**. E) Structures of potential side products found in C and D. Compound **44** could be assigned to signals m/z = 704.17 and m/z = 704.08 in C and D, respectively. Compound **45** could be assigned to signal m/z = 736.08 in D and compound **46** to signal m/z = 720.08 in C, as it has the same mass as product **42**. Compound **47** could be assigned to signal m/z = 712.08 in C with z = 2 ionization. F) Suggested mechanism for the formation of side products **44** – **46** based on the mechanism suggested by Barth *et al*. [17].



Supplementary Figure 3.2 Effect of irradiation on cell lysate (4 mg/mL total protein concentration). The cell lysate was irradiated for the indicated time with 375 nm light then denatured with sample buffer, separated by SDS PAGE and blotted onto a membrane. Membranes were treated with antibodies against biotin and against GBA1, as well as with Ponceau staining to visualize total protein. A) Pure fibroblast cell lysate. B) fibroblast cell lysate incubated with 1 μM of ABP **2** for 30 min at 37 ˚C. C) Pure HEK293T cell lysate. D) HEK293T cell lysate incubated with 1 μM of ABP **2** for 30 min at 37 ˚C.



Supplementary Figure 3.3 Structure of GBA1-inhibitor MDW941 with a cyclophellitol warhead and a BODIPY as the detectable group.