Photoisomerization Neutralizes Vasoconstrictive Activity of a Heme Degradation Product

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ABSTRACT: Delayed cerebral ischemia (DCI) caused by cerebral vasospasm is the leading determinant of poor outcome and mortality in subarachnoid hemorrhage (SAH) patients, but current treatment options lack effective prevention and therapy. Two substance families of heme degradation products (HDPs), bilirubin oxidation end products (BOXes) and propentdyopents (PDPs), are elictors of pathologic cerebral hypoperfusion after SAH. Z-configured HDPs can be photoconverted into the corresponding E-isomers. We hypothesize that photoconversion is a detoxification mechanism to prevent and treat DCI. We irradiated purified Z-BOXes and Z-PDPs with UV/Vis light and documented the Z→E photoconversion. E-BOX A slowly reisomerizes to the thermodynamically favored Z-configuration in protein-containing media. In contrast to vasoconstrictive Z-BOX A, E-BOX A does not cause vasoconstriction in cerebral arterioles in vitro and in vivo in wild-type mice. Our results enable a critical assessment of light-induced intrathecal photoconversion and suggest the use of phototherapy to prevent and cure HDP-mediated cerebral vasospasms.

INTRODUCTION

Subarachnoid hemorrhage (SAH) is a severe and life-threatening medical condition with an overall incidence of 9 per 100 000 per year1 and occurs upon aneurysmal bleeding or traumatic cranial injury. The early location of the bleeding focus with aneurysm treatment and the interventional release of intracranial pressure, e.g., via external ventricular drainage (EVD), are mandatory to limit the volume of tissue damage. However, about 50% of the survivors of the initial hemorrhagic event develop a secondary ischemic period, which is caused by delayed arterial vasospasm in the perihematomal space. The vasospasm onset is most likely between 3 and 21 days post-SAH with a peak incidence between day 5 and day 7 after the bleeding event.2 Importantly, the vasospasms may occur in both the larger arteries preceding the brain parenchyma and the microvessels,3 which lead to microthrombosis,4 perfusion deficits, and delayed cerebral ischemia (DCI).3 Since the measurement of a vasospastic blood flow acceleration via Doppler sonography is limited to larger vessels, it is mostly the clinical appearance that leads to the diagnosis of DCI, which is to be confirmed via contrast-enhanced MR or CT perfusion imaging.5,6 The consequences of DCI include transient and persistent neurological deficits and an unfavorable outcome in general with an increased six-month mortality rate up to 60%.1 Although DCI is in a clear causal relation to a preceding bleeding event, the molecular pathogenesis of delayed vasospasm represents a multifactorial network. It includes heme-mediated depletion of vasodilatory nitric oxide (NO), vasoconstrictive hemolysate compounds, humoral factors such as serotonin, oxidative stress-mediated inflammation, and an impaired neurovascular coupling.7,8 The current treatment options for SAH-induced vasospasm and DCI focus on the application of vasodilative agents like nimodipine, the hemodynamic management, including hypertonia and euvolemia, or invasive interventions such as balloon angioplasty or intra-arterial spasmolysis.9 However, the achieved effects of contemporary therapy are not sufficient because they neither provide causal prophylaxis to block the formation of mediators nor offer a therapy, which eliminates vasoconstrictive effectors. Higher-order heme degradation products (HDPs) (Figure 1), such as the monopyrrolic bilirubin oxidation end products (BOXes) and propentdyopents (PDPs), arise upon non-enzymatic break-down of the tetrpyrrole heme, biliverdin, and bilirubin.10–12 Inflammatory processes and further conditions...
with elevated concentrations of reactive oxygen species are likely to increase their production.13,14

Among these HDPs, the regioisomers Z-BOX A and Z-BOX B as well as the regioisomer pairs Z-PDP A1/2 and Z-PDP B1/2 (Figure 1) were recently shown to cause vasoconstriction of cerebral arteries in different in vivo and in vitro models.15,16 These Z-BOXes and Z-PDPs were quantified in the cerebrospinal fluid of SAH patients, resulting in nanomolar concentrations in patients with SAH, whereas these compounds were hardly traceable in the control group without intracranial bleeding.17 This suggests that the compound class of HDPs has a high potential to influence the development of vasospasms after SAH. Their mode of action includes the inhibition of BKCa potassium channels in vascular smooth muscle cells and contribution of astrocytes and the complete neurovascular unit.15,16 Nevertheless, despite this evidence for the contribution of these heme-derived substance classes, a causal prophylaxis or therapy concerning these effectors of vasospasm is still missing.

In analogy to the open-chain tetrapyrrole precursors biliverdin and bilirubin,17 these higher-order degradation products may exist in two configurations, i.e., with Z- and E-configurations at the exocyclic double bonds (Figure 1). It is striking that state-of-the-art analytical determination with high-performance liquid chromatography coupled to mass spectrometry only detected the more stable Z-BOXes and Z-PDPs in human specimens so far.12,14,15

The (neuro-)toxicity of bilirubin is linked to its chemical structure in the all-Z-configurations, exhibiting high lipophilicity and, therefore, poor excretablity. The successful application of phototherapy converts the bulk part of Z-bilirubin to bilirubin with an E-configuration, which is far less toxic and has an increased water solubility and excretablity.17,18 We assume that also the detrimental effects of Z-configured HDPs, such as Z-BOXes and Z-PDPs, occurring after SAH may be treated via irradiation to generate the corresponding E-isomers with potentially less or even without toxicity and faster excretion.

In the initial description of BOXes, the use of UV-irradiation to reduce the amount of such vasoconstrictors was already mentioned.10 However, the proposed photodegradation of the BOX A and BOX B isomers to methyl vinyl maleimide could not be confirmed so far. The first evidence of photoisomerization was obtained in the course of the total syntheses of Z-BOXes19,20 and while investigating the stability of serum samples during preanalytical sample preparation.21

The aim of this work is to study the physicochemical and biological feasibility of a phototherapeutic treatment to prevent and/or cure cerebral HDP-mediated vasospasms after subarachnoid hemorrhage. We therefore studied the formation of E-HDPs via photoconversion of the naturally occurring Z-isomers, evaluated suitable irradiation conditions, clarified the chemical structure and tested the stability, established protocols for the isolation, and finally studied the vasoactive potency of the representative photoconversion product E-BOX A in comparison to the naturally formed Z-HDPs in mouse in vitro and in vivo.

RESULTS

Wavelength-Dependent Photoconversion of Z-Configured Heme Degradation Products BOXes and PDPs with UV/Vis Light. To find the optimal conditions for a targeted photoconversion of Z-configured BOXes and PDPs, we investigated the influence of the deployed wavelengths on E-isomer formation in aqueous solutions. The wavelength range was modified by using different K-edge glass filters in the light path, which led to a sharp cut-off transmission of light above the indicated wavelength (Supporting Information Figure S1). Especially with UV light, we observed a decrease in the total peak areas, which is due to the already reported, overlaying photodegradation.21 The following considerations focus on the peak area ratio of the E- to Z-isomers and thus exclude the analysis of photodegradation.

Measurements of the peak area ratios of E-BOX A/B and Z-BOX A/B after a 10 min irradiation period of pure Z-BOXes yielded a 50:50 ratio of the E/Z-isomers when the irradiation wavelength cut-offs were between 275 and 345 nm (Figure 2). At a wavelength cut-off of 360 nm, the peak areas of E-BOXes were slightly lower with ~30%, whereas above a wavelength cut-off of 485 nm, no photoconversion of Z-BOX A and Z-BOX B to the corresponding E-isomers was detected. When Z-PDPs were irradiated likewise, the peak area ratios of E-PDPs to Z-PDPs were below 40% E (Figure 2B). In contrast to BOXes, the ratio of E- to Z-isomers of PDPs is decreasing already at wavelength cut-offs between 275 and 345 nm. Irradiation wavelength above 360 nm did not cause photoconversion of Z-PDPs. To foster interpretation of the peak area/wavelength diagrams, we provide UV/Vis absorbance spectra (Supporting Information Figure S2). Taken together, a fast and substantial (>40%) conversion of Z-BOXes and Z-PDPs to their corresponding E-isomers is achieved with a wavelength of up to 345 nm. Irradiation with a wavelength cut-
off of 350 nm is only sufficient for the photoconversion of Z- to E-BOXes.

**Preparative Isolation of E-BOX A.** To characterize the physicochemical properties of E-BOX A and E-PDPs as well as to provide the compounds for individual biological testing, we studied their preparative formation and purification via analytical and semipreparative high performance liquid chromatography (HPLC). We established an irradiation procedure with a Xenon arc lamp and carried out the photoconversion from saturated BOXes solutions in 3 mL of acetonitrile. E-BOX A and E-BOX B were isolated after a 12 min irradiation period of synthetic Z-BOX A and Z-BOX B using nonacidiﬁed isocratic (17% acetonitrile/water) liquid chromatography on the octadecyl column material. Due to a several-minute diﬀerence in retention time (Figure 3), E-BOX A (11.3 min retention time) was easily separated from Z-BOX A (28.0 min). By this method, about 50% of E-BOX A and B was produced from the corresponding Z-isomers. It was not possible to achieve a higher conversion ratio in favor of the E-BOX isomers, as longer irradiation time led to photo-degradation and, therefore, a decreased yield. After removal of the solvent in an evacuated centrifuge, we investigated the stability of the E-fractions, resulting in only 0.6% of Z-BOX B after 10 d at room temperature. We characterized the E-BOXes with HR-MS and NMR and found that chemical shifts are in accordance with the earlier published data of the hitherto proposed E-BOX A structure.19

**Structural Evidence for E-BOX A as a Major Photoconversion Product of Z-BOX A.** To prove the conﬁguration of the assumed E-BOX A, we crystallized the HPLC-puriﬁed photoconversion product of Z-BOX A from methanol as colorless prisms for structural determination. The results from X-ray diﬀraction conﬁrm that the photoproduct exhibits the molecular backbone of BOX A with E-conﬁgurations of the exocyclic double bond (Figure 4; C₁₄H₁₆N₂O₂, 178.19 g/mol; for crystal data, see Supporting Information). The remaining bond lengths and angles of E-BOX A and Z-BOX A are comparable.

The major impact that results from the isomerization of the exocyclic double bond is the altered spatial orientation of the acetamide side chain with respect to the heterocyclic pyrrole ring (Figure 4B). While the carbamide function of the side chain of Z-BOX A is close enough to form a strong intramolecular hydrogen bond to the pyrrole nitrogen, this interaction and concomitant steric ﬁxation are not possible in E-BOX A. In turn, E-BOX A exhibits more polar molecular moieties outwards and facilitates dipolar intermolecular interactions with other molecules and polar solvents. Thus, E-BOX A forms three-dimensional planes (Supporting Information Figure S3), whereas Z-BOX A may only form a two-dimensional spread (Supporting Information Figure S4). Z-BOX A represents a nearly complete planar structure (with

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*Figure 2. Relative peak areas of the Z- and E-isomers of BOXes (A) and PDPs (B) in relation to the transmission cut-oﬀ wavelengths after irradiation of Z-isomers.*

*Figure 3. Analysis of BOX isomers using liquid chromatography coupled to mass spectrometry. (A) Preparative HPLC separation of E-BOX A, E-BOX B, Z-BOX A, and Z-BOX B after photoconversion of Z-BOXes; the UV absorption at 280 nm is shown. (B, C) Ultra performance liquid chromatography-tandem mass spectrometer (UHPLC-MS) proﬁles of E-BOX A and E-BOX B over after preparative HPLC fractionation (t = 0 day) and after 10 days at room temperature in the dark; the ion trace of BOXes, m/z = 179.0815 [M + H]+ ± 5 ppm, is shown.*
the exception of the vinyl group). In contrast, the carbamide function of E-BOX A is rotated (∼33° torsion angle) toward the plane that contains the pyrrole ring and the exocyclic double bond (Figure 4B).

We recorded the 1H NMR spectrum (Supporting Information Figure S5) and confirmed the identity of the crystallized structure with experimental and computed data on the so far only proposed E-BOX A structure. With an elution time of 4.5 min in analytical reversed-phase chromatography, E-BOX A interacts much less with the used octadecylsilyl column material than Z-BOX A, which elutes at 6.5 min. E-BOX A was stable as a solid for more than 4 months when kept protected from light at room temperature, whereas E-BOX B was less stable and slowly reisomerized to the thermodynamically favored Z-isomer.

Preparation and Stability of E-PDP Isomers. In analogy to the photoconversion method to generate E-BOXes, we set up an irradiation procedure for 0.3 mL aliquots of saturated aqueous PDP solutions that were obtained from oxidative bilirubin degradation and subsequent solid-phase extraction with 20% acetonitrile/water elution. After an irradiation time of 30 min, the maximum amount of around 20% of newly formed isomers was reached. UHPLC-MS analysis revealed that six new PDP isomers were formed (Figure 5), suggesting that besides the four corresponding E-PDP isomers, two additional isomeric structures were formed.

Although the chromatographic separation of the isomers was established, the isolation of these six photoconversion products of Z-PDPs proved to be very difficult as the formed E-PDPs readily reisomerized to the corresponding Z-isomers. We applied different solvent mixtures (neutral acetonitrile/water or buffered with ammonium formate buffer at pH 4, or buffered with ammonium carbonate at pH 8; water/methanol), gradient elution programs, and tested the effects of switching the chromatography column from (lipophilic) reversed-phase to (hydrophilic) normal phase. Attempts to isolate the pure PDP photoproducts failed as the removal of the solvent from chromatographic separation revealed the instability of these structures, which was associated with the reconversion to the initially used Z-PDPs in the range of 2–20% even when the fractions were freeze-dried immediately after elution (see the Supporting Information).

With the knowledge that irradiation leads to equilibria between the Z- and E-isomers, we irradiated the isolated fractions of PDP photoconversion to gain further insight about which photoconversion product would correspond to the individual Z-PDP A1/2 or Z-PDP B1/2 isomers. Thus, we structurally assigned four of the six new compounds to their related Z-PDPs (Supporting Information Figure S6).

To evaluate whether the photoconversion products of PDPs exhibit the E-configuration at the exocyclic double bond, we oxidized these fractions with hydrogen peroxide and assessed the E/Z-configuration of the formed BOXes via UHPLC-MS (Supporting Information Figure S7). The oxidation of two PDP isomers led to E-BOX A, and the oxidation of one further PDP photoisomer led to the formation of E-BOX B. This is a strong indication that these PDP photoisomers exhibit an E-configuration at the exocyclic double bond.
Taken together, E-BOX A was generated via photoconversion and isolated via semipreparative HPLC as a stable compound when protected from light. E-BOX B, E-PDPs, and two additional isomeric PDP structures that formed during irradiation were generated likewise but could not be isolated due to the fast conversion to their Z-configured precursors.

**E-BOX A Does Not Induce Morphological Alterations in Cultured Cells but Reisomerizes to Z-BOX A in Cell Culture.** To assess whether purified E-BOX A causes

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**Figure 6.** Impact of E-BOX A on cell morphology in vitro and reisomerization in protein-containing media. (A) Morphological effects of E-BOX A and Z-BOX A treatments on HepG2 cells compared to control conditions. Scale bar: 25 μm. (B) Residual fraction of E-BOX A in relation to the initial amount (t = 0 h; 100%) after incubation in different biochemical milieus at 37 °C in the dark after 6, 24, and 48 h. While E-BOX A is stable in water and protein-free, physiologic salt solution for 48 h, the addition of 1% human serum albumin (HSA; a representative for serum proteins present in CSF of SAH patients) causes a time-dependent decrease of the E-BOX A content in favor of Z-BOX A. In CSF of SAH patients and in serum samples of healthy humans, E-BOX A reisomerizes faster to its Z-isomer. Data are presented as mean ± standard deviation (SD).

**Figure 7.** Vasoactive effect of the E-BOX A isomer on intracortical arterioles in vitro and on pial arterioles in vivo. (A) Time course of the normalized diameter of preconstricted, intraparenchymal arterioles after superfusion with Z-BOX A or E-BOX A (5 μmol/L, N = 6) in Slo1-WT mice. (B) Percentage arteriolar diameter changes after 90 min Z-BOX A or E-BOX A superfusion in slices of Slo1-WT mice. In line diagrams, the bold lines represent the mean ± SD (gray and pink regions). Thin lines correspond to the diameter time course of single experiments. Dot plots are shown as mean ± SD. Labels above the plots represent comparisons between the BOX A-induced diameter change and the control group. (C) Time course of the normalized diameter of pial arterioles compared between Z-BOX A and E-BOX A (100 μmol/L, N = 5). Bold lines represent the mean value ± SD (gray and pink regions). (D) Summary of percentage diameter changes after Z-BOX A or E-BOX A application. Dot plots are presented as mean ± SD. Data points correspond to the diameter values of single experiments. Statistical comparison between the E-BOX A time slots (120 min versus 180 min) refers to paired samples. ***P < 0.001; **P < 0.01; and *P < 0.05. ns = nonsignificant.
morphological alterations of cultured cells as described for Z-BOX A in HepG2 cells, we tested E-BOX A, Z-BOX A (positive control), and 0.25% DMSO in growth medium as vehicle control (negative control) according to a recently published protocol. After an incubation period of 24 h, Z-BOX A induced the formation of spherical cell bodies, whereas its photoconversion product E-BOX A did not cause any morphological alterations in comparison to cells treated with the vehicle control (Figure 6A). When the compound solutions in the cell culture medium were stored for 24 h prior to administration, part of the E-BOX A-treated cells exhibited the spherical morphotype seen in Z-BOX A-treated cells. We assessed the post-experimental E/Z-isomer purity of both treatments using HPLC-MS/MS. While the Z-BOX A solution still contained only the Z-isomer, the E-BOX A solution exhibited only a residual fraction of ~3% E-BOX A and a high concentration of the Z-isomer. We therefore hypothesized that E-BOX A had reisomerized in the course of the experiment. To test this hypothesis, we assessed the isomeric purities of the individual treatments via the peak area ratios of E-BOX A and Z-BOX A. After 24 h, only 68.9 ± 0.03% and after 48 h 45.3 ± 6.52% residual E-BOX A was detected in the cell culture supernatants (N = 3).

**E-BOX A Reisomerizes to Z-BOX A in Protein-Rich Environments.** With the knowledge that E-BOX A may reisomerize to Z-BOX A at 37 °C in the presence of human cells in the cell culture medium and without further light exposure, we asked whether and how long E-BOX A would be stable in (bio-)chemical milieu relevant for the production of BOXes and possible elimination via the systemic circulation. We therefore spiked different media as well as human specimens with E-BOX A and assessed the resulting isomer content of E-BOX A after incubation for up to 48 h at 37 °C. E-BOX A was fully stable (>99% of initial E-isomer) in water and artificial cerebrospinal fluid (aCSF, physiologic saline solution) over the 48 h incubation time in the dark. Compared with this, the E-BOX A content in 1% human serum albumin (HSA)/aCSF, a matrix resembling even pathologically elevated protein concentrations, was significantly reduced through the formation of 14% Z-BOX A within the first 6 h of incubation and 51% after 48 h. We further investigated the reisomerization in human CSF samples withdrawn from SAH patients via EVD as well as in serum samples of healthy humans. To exclude errors of the measured E-BOX A and Z-BOX A values by potentially pre-existing BOXes in the samples or due to the formation of BOXes via in vitro degradation of heme/hemoproteins, we incubated and analyzed additional aliquots of the samples without E-BOX A spiking. The patient samples did not contain BOXes at the beginning of the experiment and further did not exhibit any production BOXes during incubation. When E-BOX A-spiked human CSF samples and serum samples were incubated at 37 °C, the decrease of the E-BOX A content was even stronger compared to the samples in 1% HSA/aCSF. After an initial reduction to 86 and 92%, respectively, after 6 h post-incubation, the residual relative amount of E-BOX A after 48 h was only ~14%.

**Photoconversion of Z-BOX A to E-BOX A Neutralizes Vasoconstrictive Activity in Mouse In Vitro and In Vivo.** Following our hypothesis that the structural differences of E-BOX A compared to Z-BOX A would result in an altered impact on the vascular diameter in cerebral arterioles, we tested the vasoactivity of E-BOX A in comparison to the naturally occurring Z-BOX A in both in vitro and in vivo (Figure 7A,B). For in vitro experiments, coronal brain slices of 350 μm thickness, comprising the occipital and parietal cortex, were cut using a microtome and continuously superfused with aCSF during the optical imaging. Due to the lack of intravascular blood pressure and blood flow in acute brain slices, the physiological resting tone was simulated using the NO synthase inhibitor L-NAME. A previous study demonstrated that the superfusion of mouse brain slices with L-NAME did not change the mean arteriolar diameter (−4.8 ± 5.4%; N = 8) during the application period of 90 min. Application of 5 μmol/L Z-BOX A resulted in the reduction of the mean arteriolar diameter (N = 6; −13.2 ± 2.5%; P < 0.01) compared to the L-NAME control. When the brain slices were superfused with 5 μmol/L E-BOX A (−5.6 ± 5.8%; N = 6), E-BOX A was not vasoactive in the brain slice model and the diameter change differed significantly from the Z-BOX A conditions (P < 0.05). We assessed the ratio of Z- to E-BOX A with UHPLC-MS and found only 0.5 ± 0.2% Z-BOX A at the beginning of the experiment and a minimal increase to 0.8 ± 0.2% after 90 min.

We further evaluated the in vivo vasoactivity of the photoconversion product E-BOX A in comparison to the naturally occurring and pretested isomer Z-BOX A (Figure 7C,D). Therefore, an acute cranial window of 2.5 mm in diameter over the parietal and occipital cortex was implemented in intact animals. After a 10 min baseline scan, a 100 μmol/L solution of E-BOX A was repeatedly injected with small puffs of around 5 nL volume in 5 min intervals over 180 min. During this time, the selected vessel was scanned every 30 min for 10 min to monitor potential changes in the arteriolar diameter. Joerk and Ritter et al. demonstrated that equally concentrated Z-BOX A (N = 5) significantly narrowed pial arterioles to −17.0 ± 7.2% of the baseline level after an application period of 120 min. The diameter decrease was significantly different in comparison to the aCSF control group (N = 4; P < 0.01). In contrast, the diameter did not change upon application of E-BOX A (N = 5), reached −3.5 ± 4.8%, and were not significantly different from the control group treated with aCSF (P = 0.25; Figure 7C). In contrast, the vasoresponse between the Z-BOX A and E-BOX A treatment group was significantly different (P < 0.01; Figure 7D). Since the mean arteriolar diameter tended to decrease from 90 to 120 min under E-BOX A treatment, we extended the recording time to 180 min. During this additional period, the indicated decrease of the mean diameter was preceded and led to a final diameter change of −7.5 ± 6.0% of the baseline level without a significant difference to the 120 min level. In summary, E-BOX A did not induce arteriolar vasoconstriction in vitro and in vivo in clear contrast to its corresponding Z-isomer.

**DISCUSSION**

Z-BOXes and Z-PDPs, the hitherto most widely investigated higher-order heme degradation products, retain the Z-configuration of the exocyclic double bonds from their biological precursors, heme, bilirubin, and biliverdin. Accordingly, in studies investigating the amount of these naturally occurring HDPs in CSF, serum/plasma, and bile, exclusively Z-configured BOXes and PDPs were detected. While recent in vivo and in vitro studies gave evidence that Z-configured HDPs induce a manifest arteriolar vasoconstriction in cerebral arterioles, there was so far no data on the biological effects of E-configured HDPs. If an interventional photoconversion of Z-configured heme degradation products...
should be used for the prevention and therapy of such hemolysate compound-mediated DCI in SAH patients, the following three basic requirements must be fulfilled. The photoconversion products must (1) not be vasoactive or toxic, (2) have physicochemical properties that allow their adequate elimination, and (3) be stable long enough to be excreted.

**Conditions of Photoconversion.** To examine whether these preconditions are fulfilled, we first investigated the photoconversion process of Z-BOXes and Z-PDPs. While in the course of the discovery, the light sensitivity of BOXes was attributed to photodegradation to methyl vinyl maleimide,\textsuperscript{10} we herein confirmed assumptions from HPLC-MS/MS and NMR experiments\textsuperscript{19,21} that the prior process at short irradiation times is indeed $Z \rightarrow E$ photoisomerization. We further demonstrate the same reactivity in favor of $Z \rightarrow E$ photoisomerization for the PDP A1/2 and PDP B1/2 isomers.

In regard to maximizing the fraction of $Z$-isosomers converted to their corresponding $E$-isosomers via irradiation, the light of the UV-A spectrum is optimal and results in conversions of $\sim$50% for BOXes and $\sim$20% for PDPs at 10 or 30 min irradiation periods (Figures 3 and 5). As seen with the biological precursor bilirubin,\textsuperscript{22} it is important to include co-occurring photodecomposition into the considerations on the ideal wavelength range for $Z \rightarrow E$ photoconversion. The energy input of photochemical reactions is inversely proportional to the irradiation wavelength ($E = h\nu/\lambda$, with $E =$ energy, $h =$ Plank constant, $\nu =$ speed of light). Photoisomerization of the exocyclic double bond of $Z$-BOX A succeeds already with UV-A light, i.e., the lower-energy wavelengths range (315–380 nm) of UV light (Figure 2), resembling photochemically mild conditions. The light energy of the photons is needed to cross the activation energy barrier of the exocyclic $\pi\pi$-bond, whereas the energy difference between $Z$-BOX A and $E$-BOX A is only about 36 kJ/mol.\textsuperscript{15} When $Z$-BOX A is irradiated with shorter wavelength light, also further bonds, particularly within the conjugated double bond system, will be excited (see the UV absorbance spectrum)\textsuperscript{19} and may lead to a variety of further parallel reactions, including the break-up of the molecule (photodegradation). Such often unspecified reactions will consequently decrease the yield of the desired $Z \rightarrow E$ isomerization, which was shown earlier during an 8 h irradiation of $Z$-BOX A with sunlight, which also includes UV-B light.\textsuperscript{21} Thus, the wavelength ranges in the upper UV-A light should be used to maximize the fraction converted via the desired photoisomerization process even though this may result in slightly longer irradiation periods. In addition, exposure to longer-wavelength light may drastically reduce potential side effects,\textsuperscript{23} e.g., tissue damage, which also strongly depends on the way, the envisaged prophylactic or therapeutic irradiation is implemented. To avoid such detrimental side effects, neither irradiation of the whole body via the skin nor of CSF inside the body, e.g., via fiber optics, seem appropriate. We rather propose to photoconvert vasocostrictive Z-HDPs via UV-A irradiation by means of extracorporeal apheresis and consequent recirculation of the cleared CSF. Photoconversion of Z-HDPs also slowly succeeds with ambient light, which includes near UV-A wavelength. The long-wavelength absorbance shoulder of $Z$-BOX A reaches even beyond 400 nm (Supporting Information Figure S2A), which makes in particular $Z$-BOX A photoconvertible with lower-energy light. Since photoconversion of the HDP precursor bilirubin may be markedly more efficient in situ through bypassing thermodynamic barriers via protein binding,\textsuperscript{24} further in vitro and in vivo studies are encouraged.

**Stability of E-BOXes and E-PDPs.** It was not possible to achieve a complete $Z \rightarrow E$ photoconversion of BOXes and PDPs as the reaction reaches equilibria, where also $E$-configured compounds are photoconverted back to the precursors with the $Z$-configuration. Additionally, the purified $E$-isosomers reisomerize thermally to the $Z$-configuration, which is congruent to density functional theory calculations for BOX A, which showed that the $Z$-configuration of the exocyclic double bond is thermodynamically favored ($\sim$35 kJ/mol) compared to the $E$-configured isomeric structure.\textsuperscript{19} We found that the thermal stability of the individual $E$-configured HDPs differs substantially. While $E$-BOX A was stable for several months at room temperature in the dark, $E$-BOX B started to slowly reisomerize during chromatographic purification and isolation. Due to a faster reisomerization, it was not possible to isolate pure $E$-PDP isomers, which contained at best $\sim$2% of the corresponding $Z$-isosomers after chromatography. To monitor the concentrations of $E$- and $Z$-configured HDPs in situ, HPLC/MS analytics may be coupled with recently developed techniques of CSF microdialysis.\textsuperscript{24}

We demonstrate the labile $E$-configuration of the dipyrrolic photoconversion products via biomimetic oxidative degradation with hydrogen peroxide to the corresponding $E$-BOX A and $E$-BOX B structures, respectively. Using the thermal reisomerization, we further assign four $E$-PDP isomers to their structural classes PDP A/B, which differ from each other in the position of the methyl and vinyl side chains (Figure 1).

**Cell Culture Experiments and Reisomerization of E-BOX A.** $Z$-BOX A alters the spread and brick-shaped morphology of different cell culture models for hepatocytes by inducing a spherical morphology with condensed cell bodies.\textsuperscript{14} $E$-BOX A does not induce this detrimental condensation of cell bodies. This finding suggests different pharmacodynamic properties of $E$- and $Z$-BOX A. The naturally occurring $Z$-PDP isomer pairs, Z-PDP A1/2 and Z-PDP B1/2, also do not cause this morphological alteration (Supporting Information Figure S8). Based on the observation that cells incubated with $E$-BOX A slowly began to switch to the spherical morphology, we revealed that also $E$-BOX A reisomerizes to its $Z$-isomer in biological systems. However, this $E \rightarrow Z$ reconversion is much slower than that of the $E$-PDP isomers. The thermodynamically driven process is impeded by an activation energy barrier\textsuperscript{19,20} and includes presumably unspecified interactions of $E$-BOX A with proteins, which may interact with $E$-BOX A, resulting in an accelerated conversion. While $E$-BOX A is stable for 48 h in water and aCSF in the dark, the presence of serum proteins in CSF and other body fluids enables a slow reisomerization reaction as shown with CSF of SAH patients and serum of healthy humans. The thermodynamic reisomerization has several implications. First, it provides a conclusive explanation that no $E$-configured HDPs were found in human specimens. Second, in regard to a prophylactic or therapeutic application of photoconversion, it is important that the generated $E$-isomers can sufficiently be withdrawn from the perivascular space to diminish the elicitation of vasocostriction after the reisomerization. In the case of $E$-BOX A, the strongly increased hydrophilicity and stability of $>85\%$ for 6 h in CSF of SAH patients leave a time window for redistribution and elimination. The part of $E$-BOX A reallocated from the site of action to the bloodstream might enter the efficient pathway
of active hepatobiliary excretion. Although the elimination pathways for the other HDPs investigated herein still remain unknown, photoconversion may be beneficial to convert the Z-HDPs at the site of action and to achieve at least a reduction of locally high concentrations of the detrimental Z-isomers. Since reisomerization of E-BOX A only takes place when the molecules get in contact with proteins/peptides, we propose a catalytic (but presumably unspecific) mechanism that lowers the energy barrier for the formal flip of the exocyclic double bond.

**Structure of E-BOX A.** The stability of E-BOX A allowed its crystallization and X-ray diffraction examination to obtain the precise molecular structure. Due to the changed steric situation of E-BOX A, intramolecular hydrogen bonding, a key feature of Z-BOX A, cannot be sustained in the E-configuration. Consequently, two polar moieties, the acetamide group and the pyrrole N–H functionality, become fully available for intermolecular interactions. Hence, all hydrophilic groups of the molecule are oriented outwardly, which leads to a substantially increased hydrophilicity (confirmed in the markedly earlier elution in reversed-phase chromatography) and makes E-BOX A much less likely to retain in lipophilic compartments such as cell membranes or myelin tissue. Augmented polar intermolecular interactions also become obvious from the crystal structure of E-BOX A, where the individual molecules develop meshed structures (Supporting Information Figure S3), whereas Z-BOX A forms only ribbon-like patterns (Supporting Information Figure S4). Moreover, the photochemical break-up of the intramolecular hydrogen bond of Z-BOX A abrogates the enforced coplanarity of the pyrrole ring and the acetamide group, which exhibit a dihedral angle of about 33° in E-BOX A. As a consequence of the markedly altered steric and distribution of functional groups, E-BOX A may interact distinctly different with the molecular targets of its Z-isomer.

**In Vitro and In Vivo Testing of E-BOX A in Mouse Models.** In contrast to Z-BOX A, E-BOX A does not cause vasoconstriction in cerebral arterioles in vitro and in vivo. This proves that the structural alterations drastically influence the vasoconstrictive potency and further pinpoints a fine-tuned recognition of these small molecule effectors. Given that the photoconversion neutralizes the detrimental vasoconstrictive properties of BOX A, E-BOX A fulfills the central requirement for the feasibility of a preventive and therapeutic photoconversion of Z-BOX A. Interestingly, we observed a tendency to a slight vascular narrowing in the in vivo experiments after an initial 90 min treatment with E-BOX A. This trend continued until the end of the extended observation time of 3 h without reaching a steady state. In the light of our cell culture experiments and in particular the protein-triggered reisomerization of E-BOX A to Z-BOX A, it is conclusive that fractions of the applied E-BOX A reisomerized upon contact with the mouse CSF and brain tissue. This entails a gentle increase of Z-BOX A concentrations, which subsequently explain the smooth but constant vascular narrowing. By making use of the results of E-BOX A reisomerization in protein-rich biological matrices, we interpolate a conversion of E-BOX A to Z-BOX A of approximately 7% in 3 h. With respect to recently published data on the dose–response characteristics of Z-BOX A, this concentration of about 2 μmol/L would still be sufficient to cause a comparable vasoconstriction in the same assays. Since also Z-configured BOX B and PDP A1/2 and B1/2 contain analogous structural elements, particularly the exocyclic double bond, we anticipate that the photoconversion of these effectors leads to a similar neutralization of vasoconstrictive impact.

**CONCLUSIONS**

In conclusion, photoconversion of HDPs affects the intramolecular cohesion and alters the positioning of functional groups. At the example of BOX A, we demonstrate that beyond the markedly altered physicochemical properties, photoconversion to the E-isomer particularly changes the pharmacodynamics of the molecule and neutralizes the detrimental vasoconstrictive properties. In light of our findings, an irradiation-based conversion of the naturally occurring Z-HDPs to the transiently stable E-HDPs e.g. via extracorporeal apheresis seems to be a feasible option to prevent and treat HDP-mediated hypoperfusion in SAH patients. Our findings encourage further investigations on such irradiation-based treatment options for DCI.

**EXPERIMENTAL SECTION**

**Preparation and Quantification of BOXes and PDPs.** Z-BOXes and Z-PDPs were prepared and purified from in vitro degradation of Z,Z-bilirubin with hydrogen peroxide and from chemical synthesis according to the published protocols. The E-isomers of BOXes and PDPs were prepared via irradiation of the corresponding Z-isomers. Solutions of Z-BOX A, Z-BOX B, Z-PDP A1/2, and Z-PDP B1/2 in acetonitrile/water were exposed for 0.5–60 min to the light of a xenon arc lamp or sunlight at which different K-edge glass filters (SCHOTT, Jena, Germany) were introduced to modulate the irradiation wavelength range (Supporting Information Figure S1). The used filter set is given as an identifier and absorption edge wavelength: WG7 (275 nm), WG3 (335 nm), WG5 (310 nm), WG9 (345 nm), GG13 (360 nm), GG14 (485 nm), OG3 (575 nm), and RG1 (590 nm). After irradiation, the samples were analyzed via HPLC-MS/MS and integrated peak areas were compared.

E-BOX A and B were purified via isocratic high-performance liquid chromatography (HPLC) based on a published protocol but with 17% (v/v) acetonitrile/water and a flow rate of 5 mL/min.

Crystal structure determination and crystal data are reported in the Supporting Information.

The peak areas of Z- and E-BOX A, BOX B, PDP A1/2, and PDP B1/2 were assessed via (ultra) HPLC coupled to (high resolution) mass spectrometry using the described procedures.

**Reisomerization of E-BOX A in Different Chemical Environments and Human Specimens.** Aliquots of HPLC-purified aqueous E-BOX A (5 μL of 100 μmol/L) were added to a final concentration of 5 μmol/L to water, artificial cerebrospinal fluid (aCSF), 1% human serum albumin in aCSF, human CSF, and human serum (N = 3 independent samples each plus three technical replicates of each sample) and incubated under light exclusion at 37 °C. Human CSF and serum samples without E-BOX A addition served as a control for the potential generation of BOXes by the oxidation of heme, biliverdin, or bilirubin. After 6, 24, and 48 h of incubation, one technical replicate of each biological replicate was quenched and extracted (protein precipitation) by 10 min vortex mixing with 200 μL of acetonitrile followed by 10 min
centrifugation at 16.100g. The supernatant was transferred to brown glass vials and kept at 4 °C in an autosampler until measurement via HPLC-MS/MS. The samples of the supernatants from cell culture experiments (see below) were taken after 24 h and 48 h of incubation time at 37 °C in the dark and analyzed likewise.

**Human Specimens.** Human serum and human cerebrospinal fluid (CSF) from EVD were withdrawn with written consent after approval by the ethical committee of the Jena University Hospital (3256-09/11, 3548-08/12). The study was not preregistered.

**Animals.** All animal procedures were approved by the local regulatory authority (Thüringer Landesamt für Verbraucherschutz; registration numbers 02-040/12, and TWZ-030-2017) and carried out in accordance with the Federation of European Laboratory Animal Science Associations, the German Federal Institute for Risk Assessment and the ARRIVE guidelines. In vitro experiments were performed on acute brain slices from randomly assigned male and female S101 WT mice (26) at postnatal day 20–31 (N = 20; P0 is defined as the day of birth). For in vivo studies, adult male and female S101 WT at P70-200 (body weight: 20–35 g) were used (N = 14) in experiments during the daytime. Animals were obtained by crossing heterozygous S101 mice. All mice had a genetic background of FVB/NJ, were imported from the ULAR Diagnostic Services and Rodent QA (Philadelphia), and were genotyped in the second postnatal week. Animals had ad libitum access to food and water. Up to five mice were housed in environmental enrichment cages with a soft filter top.

**Brain Slice Preparation, Optical Imaging, and Diameter Calculation In Vitro.** Acute mouse brain slice preparation was already reported. The slice samples were illuminated using differential interference contrast video microscopy (Eclipse FN1, Nikon Instruments, Amsterdam, Netherlands) in combination with a Hamamatsu camera, a PCI-RTV24 acquisition board, driven by the software ViewCreatorPro (ADLINK Technology, Taipei, Taiwan). Arterioles located in layer II/III of the parietal cortex and running perpendicular to the cortical surface were selected as structures of interest. The brain slice recording and arteriolar diameter calculation was performed as recently reported.

**Cranial Window Implantation and Two-Photon Laser Scanning Microscopy of Cerebral Arterioles.** In intact animals, pial arterioles were illuminated by two-photon laser scanning microscopy after acute implantation of a cranial window according to a recently published standard protocol. To minimize animal suffering during the in vivo experiment, animals were pretreated with the analgesic drug meloxicam (1 mg/kg) and anaesthetized with iso-flurane (Fluorane Landesamt für Verbraucherschutz; registration numbers 02-040/12, and TWZ-030-2017). The slice samples were prepared with the analgesic drug meloxicam (1 mg/kg) and anaesthetized with iso-flurane allows a precise control of the respiratory rate and anesthetic state. Before the scalp incision, the skin of the head was infiltrated by the subcutaneous application of 2% lidocaine. To differentiate arterioles from veins, the fluorescent dye Alexa 633 was puffed applied in the subarachnoid space via glass micropipettes (resistance: 1.0–1.5 MΩ, puff length: 100 ms, pressure: 1 bar) penetrating the dura mater. E-BOX A (100 µmol/L) was locally applied by the same procedure as Alexa 633 (<100 µm distance to the arteriolar vessel wall of interest). Fluorescence excitation at 810 nm was provided by a tunable Ti:Sapphire laser (Chameleon Ultra II, Coherent, Santa Clara, California) using a 20X water immersion objective. Changes in the diameter of pial arterioles were monitored at 5 Hz (MPScan). After optical recording, animals were killed by decapitation under deep iso-flurane anesthesia. No animals died during the experiments. The study was exploratory and had no exclusion criteria.

**Vessel Diameter In Vivo.** We selected pial arterioles on the brain surface with an intraluminal diameter ranging between 30 and 80 µm. Owing to intrinsic regulated vasomotions, pial arterioles were monitored over 10 min for each measurement point to subtract out these variations. In detail, the medium arteriolar diameter was calculated as the distance between the opposite labeled vessel walls at the level of the largest diameter and at three defined locations along the vessel course in the field of view. Therefore, we used an orthogonal projection of the current image stack provided by ImageJ (version 1.48, Wayne Rasband, National Institutes of Health).

**Cell Culture.** To study the impact of E-BOXes and PDP isomers on cell morphology with regard to a cell culture model established for Z-BOXes, HepG2 cells (DSMZ, Braunschweig, Germany; authenticated by short tandem repeats profiling; twelve passages at the maximum) were incubated overnight to attach (30,000 cells/well of a 96-well plate). The cells were treated (N = 3 independent cell culture preparations) either with the freshly prepared individual home degradation products at 500 µmol/L in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum for 24 h or with treatment solutions that were kept for 24 h at 37 °C before the 24 h incubation. Morphological changes were monitored via light microscopy.

**Statistical Analysis.** The animal experiments were single-blinded. The experimenter was masked to the applied BOX A compounds. Within the experimental groups, animals were identified by numbers and a random number generator selected the animals to be treated next. In the case of in vitro experiments, the parameter N represents the number of animals tested. Because only one arteriole per animal was always monitored, N also represents the number of arterioles. For in vivo experiments, N represents the total number of animals. The assessed number of animals used per group was set to N = 7 (in vitro) or N = 5 (in vivo). In dot plots, all marked line values represent means ± standard deviation (SD). Line diagrams depicted the mean ± 95% SD. Group comparisons were performed using Student’s t-test for two independent samples or one-way analysis of variance (ANOVA) for more than two groups with the Bonferroni test applied to correct for multiple comparisons. To check the robustness of the results with regard to parametric test assumptions, the nonparametric equivalent of the parametric test was also applied. As the results did not differ substantially, the description is given as parametric test results. With regard to the animals, no sample calculation was performed. An unadjusted P ≤ 0.05 was considered statistically significant. Data were gathered, statistically analyzed, and displayed using ImageJ (version 1.48, Wayne Rasband, National Institutes of Health), Microsoft Excel (Microsoft Office 2010, Redmond, Washington), SigmaPlot 14 (Systat, Erkrath, Germany), and OriginPro 9.1 (OriginLab, Northampton, Massachusetts).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01698.
Transmission spectra of K-edge glass filters employed to adjust the wavelength range of irradiation; UV/Vis absorbance spectra of Z-BOX A and B as well as of Z-PDP A1, Z-PDP B1, Z-PDP A2, and Z-PDP B2 in aqueous solution; E-BOX A forms meshed structures with a three-dimensional spread; Z-BOX A forms ribbon-like structures with a two-dimensional spread; $^1$H NMR spectrum of E-BOX A in deuterated acetonitrile; irradiation of single PDPs and assessment of newly formed E-isomers by LC-MS; oxidation of isolated E-PDPs to E-BOXes; morphological effects of Z-PDP A1/2 and Z-PDP B1/2 treatments on HepG2 cells compared to control conditions; time-line diagram of the in vivo animal experiments (PDF)

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This manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors kindly acknowledge Franziska Röstel, Hannes Richter, and Dr. Marcel Kahnes for technical assistance and financial support by the German Research Foundation within the framework of FOR1738. A.J. and O.W.W. acknowledge funding by the Research Program, “Else Kröner-Forschungskolleg AntiAge”.

## ABBREVIATIONS

aCSF, artificial cerebrospinal fluid; BOX, bilirubin oxidation end product; CSF, cerebrospinal fluid; DCI, delayed cerebral ischemia; EVD, external ventricular drainage; HSA, human serum albumin; HDP, heme degradation product; (U)HPLC, (ultra) high-performance liquid chromatography; PDP, propentylendopent; MS, mass spectrometry; SAH, subarachnoid hemorrhage; SD, standard deviation

## REFERENCES

(1) Steiner, T.; Juvela, S.; Unterberg, A.; Jung, C.; Forsting, M.; Rinkel, G. European Stroke Organization guidelines for the management of intracranial aneurysms and subarachnoid haemorrhage. Cerebrovasc. Dis. 2013, 35, 93–112.
(2) Corsten, L.; Raja, A.; Guppy, K.; Roitberg, B.; Misra, M.; Alp, M. S.; Charbel, F.; Debrun, G.; Ausman, J. Contemporary management of subarachnoid hemorrhage and vasospasm: the UIC experience. Surg. Neurol. 2001, 56, 140–150.
(3) Uhl, E.; Lehmburg, J.; Steiger, H. J.; Messmer, K. Intraoperative detection of early microvasospasm in patients with subarachnoid hemorrhage by using orthogonal polarization spectral imaging. Neurosurgery 2003, 52, 1307–1317.
(4) Giller, C. A.; Giller, A. M.; Landreneau, F. Detection of emboli after surgery for intracerebral aneurysms. Neurosurgery 1998, 42, 490–494.
(5) Korbakis, G.; Prabhakaran, S.; John, S.; Garg, R.; Conners, J. J.; Bleck, T. P.; Lee, V. H. MRI Detection of Cerebral Infarction in Subarachnoid Hemorrhage. Neurocrit. Care 2016, 24, 428–435.
(6) Rodriguez-Regent, C.; Hafa, M.; Turc, G.; Ben Hassen, W.; Edjlali, M.; Sermet, A.; Laquay, N.; Trystram, D.; Al-Sharief, F.; Meder, J. F.; et al. Early quantitative CT perfusion parameters variation for prediction of delayed cerebral ischemia following aneurysmal subarachnoid hemorrhage. Eur. Radiol. 2016, 26, 2956–2963.
(7) Balbi, M.; Koide, M.; Wellman, G. C.; Plesnila, N. Inversion of neurovascular coupling after subarachnoid hemorrhage in vivo. J. Cereb. Blood Flow Metab. 2017, 37, 3625–3634.
(8) Dreier, J. P.; Drenckhahn, C.; Woltz, J.; Major, S.; Offenhauser, N.; Weber-Carstens, S.; Wolf, S.; Strong, A. J.; Vajkoczy, P.; Hartings, J. A.; et al. Spreading ischemia after aneurysmal subarachnoid hemorrhage. Acta Neurochir. Suppl. 2013, 115, 125–129.
(9) Vivancos, J.; Gilo, F.; Frutos, R.; Maestre, J.; Garcia-Pastor, A.; Quintana, F.; Roda, J.; M.; Ximenez-Carrillo, A.; et al. Clinical management guidelines for subarachnoid haemorrhage. Diagnosis and treatment. Neurologia 2014, 29, 353–370.
(10) Kranc, K. R.; Pyne, G. J.; Tao, L.; Claridge, T. D.; Harris, D. A.; Cadoux-Hudson, T. A.; Turnbull, J. J.; Schofield, C. J.; Clark, J. F. Oxidative degradation of bilirubin produces vasoactive compounds. Eur. J. Biochem. 2000, 267, 7094–7101.
(11) Ritter, M.; Neupane, S.; Seidel, R. A.; Steinbeck, C.; Pohnert, G. In vivo and in vitro identification of Z-BOX C—a new bilirubin oxidation end product. Org. Biomol. Chem. 2018, 16, 3553−3555.

(12) Ritter, M.; Seidel, R. A.; Bellstedt, P.; Schneider, B.; Bauer, M.; Gorls, H.; Pohnert, G. Isolation and Identification of Intermediates of the Oxidative Bilirubin Degradation. Org. Lett. 2016, 18, 4432−4435.

(13) Clark, J. F.; Sharp, F. R. Bilirubin oxidation products (BOXes) and their role in cerebral vasospasm after subarachnoid hemorrhage. J. Cereb. Blood Flow Metab. 2006, 26, 1223−1233.

(14) Seidel, R. A.; Claudel, T.; Schleser, F. A.; Ojha, N. K.; Westerhausen, M.; Nietzsche, S.; Sponholz, C.; Cuperus, F.; Coldewey, S. M.; Heinemann, S. H.; et al. Impact of higher-order heme degradation products on hepatic function and hemodynamics. J. Hepatol. 2017, 67, 272−281.

(15) Joerk, A.; Ritter, M.; Langguth, N.; Seidel, R. A.; Freitag, D.; Herrmann, K. H.; Schaeigen, A.; Ritter, M.; Gunther, M.; Sommer, C.; et al. Propendyopents as Heme Degradation Intermediates Constrict Mouse Cerebral Arterioles and Are Present in the Cerebrospinal Fluid of Patients With Subarachnoid Hemorrhage. Circ. Res. 2019, 124, e101−e114.

(16) Joerk, A.; Seidel, R. A.; Walter, S. G.; Wiegand, A.; Kahnes, M.; Klopfleisch, M.; Kirmse, K.; Pohnert, G.; Westerhausen, M.; Witte, O. W.; et al. Impact of heme and heme degradation products on vascular diameter in mouse visual cortex. J. Am. Heart Assoc. 2014, 3, 1−13.

(17) Jasprova, J.; Dal Ben, M.; Vianello, E.; Goncharova, I.; Urbanova, M.; Vyroubalova, K.; Gazzin, S.; Tiribelli, C.; Sticha, M.; Cerna, M.; et al. The Biological Effects of Bilirubin Photoisomers. PLoS One 2016, 11, No. e0148126.

(18) Watchko, J. F.; Tiribelli, C. Bilirubin-induced neurologic damage—mechanisms and management approaches. N. Engl. J. Med. 2013, 369, 2021−2030.

(19) Klopfleisch, M.; Seidel, R. A.; Gorls, H.; Richter, H.; Beckert, R.; Imhof, W.; Reiher, M.; Pohnert, G.; Westerhausen, M. Total synthesis and detection of the bilirubin oxidation product (Z)-2-(3-ethenyl-4-methyl-5-oxo-1,5-dihydro-2H-pyrrol-2-ylidene)ethanamide (Z-BOX A). Org. Lett. 2013, 15, 4608−4611.

(20) Seidel, R. A.; Schowtka, B.; Klopfleisch, M.; Kuhl, T.; Weiland, A.; Koch, A.; Gorls, H.; Imhof, D.; Pohnert, G.; Westerhausen, M. Total synthesis and characterization of the bilirubin oxidation product (Z)-2-(4-ethenyl-3-methyl-5-oxo-1,5-dihydro-2H-pyrrol-2-ylidene)-ethanamide (Z-BOX B). Tetrahedron Lett. 2014, 55, 6526−6529.

(21) Seidel, R. A.; Kahnes, M.; Bauer, M.; Pohnert, G. Simultaneous determination of the bilirubin oxidation end products Z-BOX A and Z-BOX B in human serum using liquid chromatography coupled to tandem mass spectrometry. J. Chromatogr. B 2015, 974, 83−89.

(22) Goncharova, I.; Jasprova, J.; Vitek, I.; Urbanova, M. Photoisomerization and oxidation of bilirubin in mammals is dependent on albumin binding. Anal. Biochem. 2015, 490, 34−45.

(23) Kielbassa, C.; Reza, L.; Epe, B. Wavelength dependence of oxidative DNA damage induced by UV and visible light. Carcinogenesis 1997, 18, 811−816.

(24) Thelin, E. P.; Nelson, D. W.; Ghatan, P. H.; Bellander, B. M. Microdialysis Monitoring of CSF Parameters in Severe Traumatic Brain Injury Patients: A Novel Approach. Front. Neurol. 2014, 5, 159.

(25) Kilkenny, C.; Browne, W. J.; Cuthill, I. C.; Emerson, M.; Altman, D. G. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010, 8, No. e1000412.

(26) Meredith, A. L.; Thorneloe, K. S.; Werner, M. E.; Nelson, M. T.; Aldrich, R. W. Overactive bladder and incontinence in the absence of the BK large conductance Ca2+−activated K+ channel. J. Biol. Chem. 2004, 279, 36746−36752.