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Water-Soluble Cuprizone Derivative: Synthesis, Characterization, and in Vitro Studies

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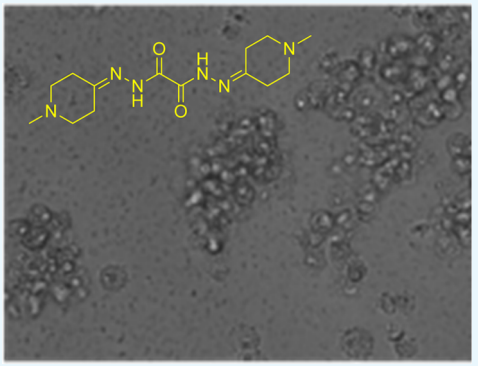
Supporting Information

ABSTRACT: The cuprizone mouse model is one of the most accepted model systems for the investigation of oligodendrocyte degeneration, a process critically involved in the pathogenesis of diseases such as multiple sclerosis or schizophrenia. In order to substitute the in vivo experiments by in vitro approaches, the amine derivative BiMPi is introduced as a water-soluble alternative to cuprizone. Regarding superoxide dismutase activity, toxicity for oligodendrocytes, and disturbance of mitochondrial membrane potential, BiMPi shows similar in vitro effects as is observed in vivo for cuprizone.



INTRODUCTION

Cuprizone **1** has been first described in 1946 as a metal ion chelator, which is able to interact with various transition metal ions showing a high specificity for copper ions even at low concentration.[1] Since then, it has been used for the determination of trace copper content within metals and alloys.[2] However, more than 60 years later, the copper coordination chemistry of cuprizone is still not well understood (Figure 1).[3]

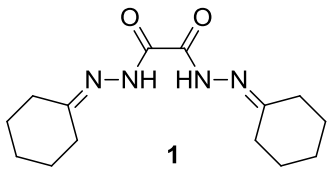


Figure 1. Cuprizone.

In 1972, it has been realized that oral treatment of mice with cuprizone results in oligodendrocyte degeneration.[4] Since then, the cuprizone mouse model has been established as one of the most important in vivo models for the investigation of the pathology of multiple sclerosis and other diseases that involve oligodendrocyte pathology, such as schizophrenia.[5] The mechanisms by which cuprizone exhibits its toxicity within the central nervous system are not understood in detail. However, it seems likely that by complexation of copper ions, it disturbs mitochondrial functions and inhibits copper-dependent detoxifying enzymes such as superoxide dismutase (SOD).[6] These disturbances in cellular homeostasis result in increased levels of oxidative stress, neuroinflammation, and the loss of myelin-producing oligodendrocytes.[7] So far, cuprizone is almost exclusively used for in vivo studies in mice. In vitro, cuprizone toxicity is limited to primary mature oligodendrocytes.[8] Despite its low toxicity, a second major drawback of cuprizone for the in vitro use is its low solubility in nontoxic solvents such as water or cell culture medium (Figure 2).

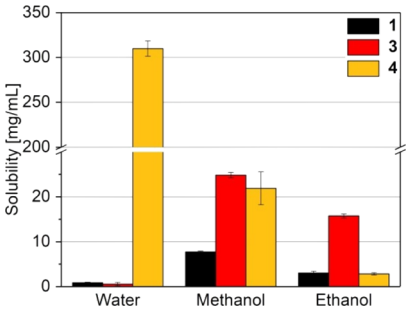


Figure 2. Solubility of cuprizone derivatives **1**, **3**, and **4** in three different solvents at 37 °C.

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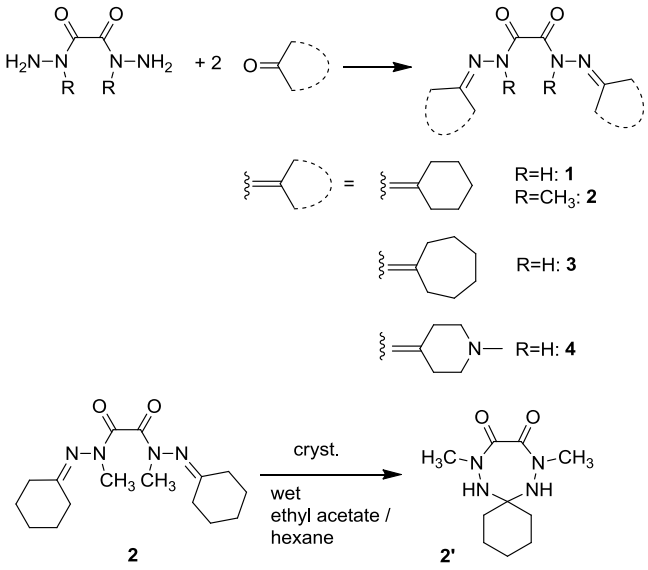
In this study, we describe slight modifications of the cuprizone side-chain substituents to find water-soluble analogues, which can be easily tested in vitro with water-based enzymes and cell culture systems and thus may substitute some mice experiments with the nonwater-soluble cuprizone (Scheme 1).

Scheme 1. Preparation of Cuprizone and Its Derivatives



RESULTS AND DISCUSSION

Cuprizone and its derivatives **3** and **4** are easily prepared by condensation of oxalylic acid bishydrazide with the corresponding ketone. Compounds **1**−**4** are characterized by standard analytical techniques.

The crystal structure of cuprizone has previously been described in the literature (Figure 3a).[3c]

The *N*,*N*-dimethyl derivative of cuprizone is prepared from *N*-methyl cyclohexylidene hydrazine[10] and oxalic acid dichloride and can be characterized by spectroscopic techniques. However, the C═N bond of this compound is rather labile and upon crystallization from wet solvents the partly hydrolyzed cyclic product **2′** is obtained (Figure 3b). In comparison to the methylated **2**, the nonmethylated derivatives **3** and **4** are more stable.

In parent cuprizone **1**, a twist is observed at the hydrazine unit with a C−N−N═C torsion angle of 68°. In contrast to this, the cycloheptyl- (**3**, Figure 3c) as well as the methylpiperidinyl-substituted (**4**, Figure 3d) analogues show a corresponding torsion angle of 155°. As expected, the C═O groups in the center of **1**, **3**, and **4** are orientated anti to each other because of dipole effects of the carbonyl and of C−NH. Hydrogen bonding motifs in **3** and **4** are similar and expected N−H···O type, whereas cuprizone **1** shows corresponding N−H···N motifs without involving oxygen atoms as acceptors. The cyclic derivative **2′** has, of course, a different hydrogen bonding pattern, also because of different locations of N−H donors. The hydrogen bonding motifs for **1**, **2′**, **3**, and **4** are shown in the Supporting Information of the present paper.

In addition to the structural elucidation, solubility tests were carried out at 37 °C with the cuprizone derivatives **1**, **3**, and **4**. All three compounds show moderate solubility in organic solvents such as methanol or ethanol with the highest solubility observed for the least polar cycloheptyl-based **3**. However, in water, extraordinary solubility differences are observed for the apolar compounds **1** and **3**, which are virtually insoluble compared to **4** (Figure 3). The water/*n*-octanol partition coefficient log P can be estimated using Cambridge soft ChemBioDraw Ultra 12.0 to be 2.31 for **1** and −0.19 for **4**.

Cuprizone **1** and BiMPi **4** form blue-colored complexes upon addition of copper(II) ions to a water solution of the bishydrazones with absorption maxima at λ = 640 nm for the cuprizone copper(II) complex and λ = 588 nm for the BiMPi complex with the latter being much more intense in color.

The high solubility in water makes the *N*-methyl piperidyl cuprizone derivative (**4**, BiMPi) an ideal candidate for the in vitro evaluation of its interaction with enzymes and oligodendroglial cell lines (Table 1).

Table 1. Solubility of 1, 3, and 4 in Different Solvents at 37 °C

	cuprizone **1**	**3**	BiMPi **4**
water	0.9[a]	0.6[a]	309.9[a]
methanol	7.8[a]	24.9[a]	21.9[a]
ethanol	3.1[a]	15.8[a]	2.9[a]

[a]mg mL^{-1}.

Therefore, BiMPi **4** was tested for toxic effects on the murine oligodendroglial cell line OliNeu and the rat oligodendroglial

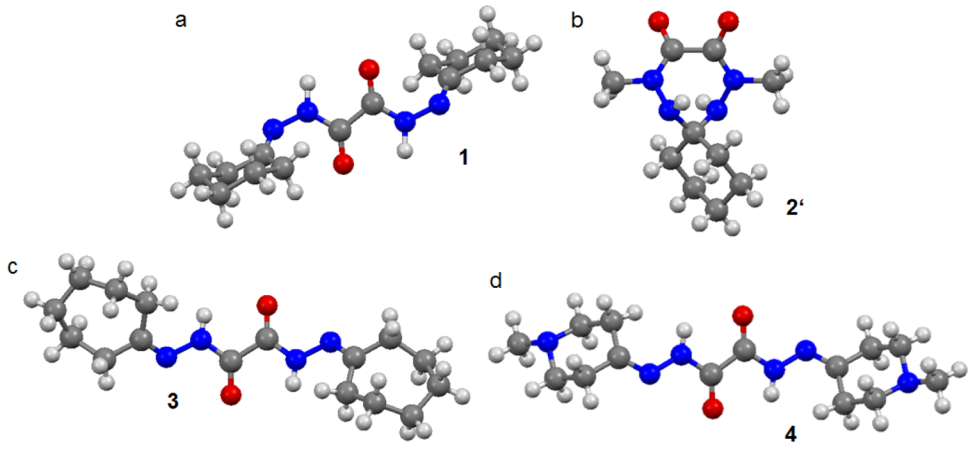


Figure 3. Molecular structures of (a) cuprizone **1**[3c] and its analogues (b) **2′**, (c) **3**, and (d) **4** (BiMPi) in the crystal.

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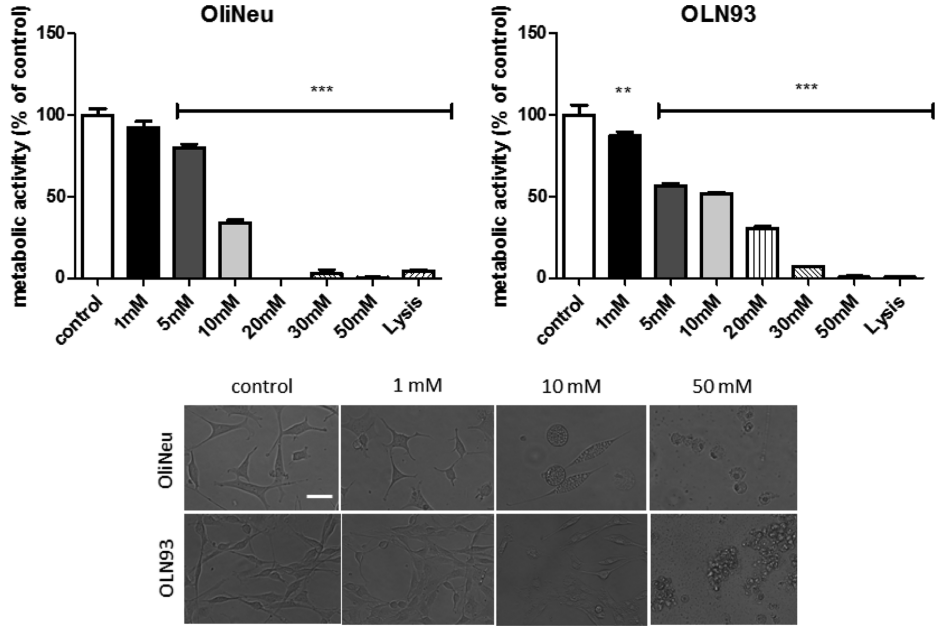

Figure 4. Dose-dependent toxicity of BiMPi in two oligodendroglial cell lines (bar plots). Images show the morphology of both cell lines after 24 h incubation with different concentrations of BiMPi. Scale bar: 20 μm; $^{**}p < 0.01$; $^{***}p < 0.001$.

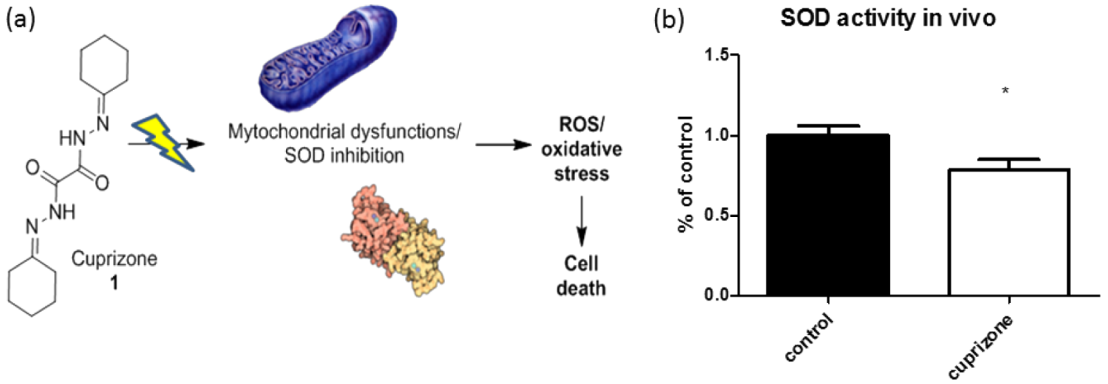

Figure 5. In vivo effects of cuprizone. In (a), the proposed mechanism of action of cuprizone is presented. (b) Shows SOD activity in the brain tissue of control mice and animals that received 0.2% cuprizone for 5 weeks.

cell line OLN93 by applying cell viability assays. As shown in Figure 4 (bar charts), treatment of these cells with BiMPi for 24 h resulted in a dose-dependent cytotoxic effect that was accompanied by severe morphological changes (microscopic images in Figure 4). This toxic effect was not selective for oligodendrocytes as shown in Figure S15 for primary rat astrocytes. In 2013, Bénardais and colleagues[9a] showed a selective toxicity of cuprizone (dissolved in 50% ethanol) to mature oligodendrocytes at very low concentrations ranging from 25 to 500 μM. However, they failed to induce toxic effects in other cells such as premature oligodendrocytes, microglia, astrocytes, and SH-SY5Y cells. This selective vulnerability can be explained by the unique physiology of mature oligodendrocytes including high lipid synthesis, high metabolic rate, high intracellular iron, and high susceptibility to oxidative stress[9b] (Liessem-Schmitz et al., 2018). Because the toxic effects of solvents such as ethanol and methanol obscure cuprizone toxicity at higher concentrations, BiMPi is a valuable substitute for the use of cuprizone in vitro, especially when working with oligodendrocyte precursor cell lines.

In a next step, we investigated in detail whether the mechanisms causing this toxicity can be compared to cuprizone effects in vivo. Therefore, the following end points were assessed: disturbance of the mitochondrial membrane potential, inhibition of the SOD activity, and induction of oxidative stress responses in oligodendrocytes.

In Figure 5a, the proposed mechanism of action of cuprizone in vivo is summarized: by binding copper, cuprizone may impair copper-dependent enzymes such as mitochondrial complex IV and SOD,[11] thus leading to higher levels of reactive oxygen species and oxidative stress,[12] finally resulting in cellular death. As an example, reduced SOD activity in the brain of cuprizone-fed animals is shown in Figure 5b.[13]

The in vivo effects of cuprizone are mimicked by BiMPi in vitro as indicated by depolarization of mitochondrial membranes and reduced SOD activity in OliNeu cells in response to BiMPi (Figure 6a). The mitochondrial complex IV inhibitor sodium azide served as a positive control for mitochondrial depolarization measures.[14] Another well-described cuprizone effect in vivo is the suppression of the expression of typical oligodendroglial genes coding for proteins such as the

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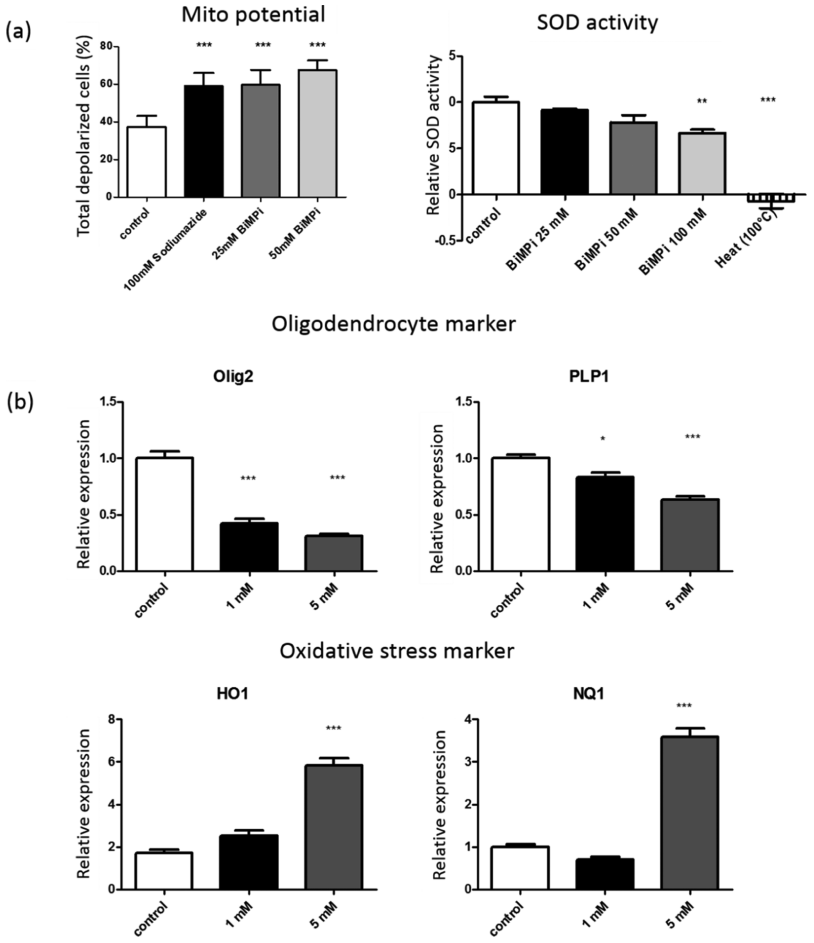

Figure 6. BiMPi effects in vitro. In (a), the impact of BiMPi on the mitochondrial membrane potential of OliNeu cells is shown in the left bar plot. In the right bar plot, the inhibitory effects of BiMPi on the SOD activity are summarized. (b) shows the gene expression of oligodendrocyte markers Olig2 and PLP1 and the oxidative stress-related genes HO1 and NQ of OliNeu cells in response to BiMPi. $*p < 0.05$; $***p < 0.001$.

transcription factor Olig2 and the myelin protein PLP1.[15] In contrast, the expression of antioxidative genes such as heme oxygenase 1 and NAD(P)H dehydrogenase (NADPH) is increased by cuprizone in vivo.[8] These effects were mimicked in vitro in OliNeu cells treated with low concentrations (1 and 5 mM) of BiMPi.

CONCLUSIONS

In summary, our data highlight the water-soluble cuprizone derivate BiMPi as a novel and useful tool to in vitro study cell-type specific responses in the context of demyelinating diseases and mitochondrial dysfunctions. Because of the different polarity of BiMPi, we do not expect similar pharmacokinetics as the nonpolar model cuprizone shows. However, because of its water solubility, it can act as a "model for the model" to be used in vitro and to reduce the number of animal experiments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02523.

Experimental procedures and characterization of the compounds and details of the X-ray structural analyses as well as of the biochemical tests (PDF)

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Notes

The authors declare no competing financial interest.
Animals were housed under standard laboratory conditions according to the Federation of European Laboratory Animal Science Association's recommendations. The procedures were approved by the Review Board for the Care of Animal Subjects of the district government (North Rhine-Westphalia; Germany; file no.: 84-02.04.2017.A117) and performed according to international guidelines on the use of laboratory mice.

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ABBREVIATIONS

BiMPi $N'1,N'2$-bis(1-methylpiperidin-4-ylidene)-oxalohydrazide

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