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Synthesis and biological evaluation of 8-hydroxy-2,7-naphthyridin-2-ium salts as novel inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)^{†‡}

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By analogy with the natural product chelerythrine, which has been identified as an inhibitor of both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), we prepared a small series of 8-hydroxy-2,7-naphthyridin-2-ium salts. Spectroscopic analyses allowed us to elucidate the zwitterionic nature of 2,7-naphthyridin-1(7*H*)-ones, the neutral state of 8-hydroxy-2,7-naphthyridin-2-ium salts. Among the tested compounds, we identified dual inhibitors of AChE and BChE as well as an inhibitor showing a preferential inhibition of AChE over BChE. By *in vitro* characterization in combination with docking studies, we were able to identify structural features that influence the biological activity of 8-hydroxy-2,7-naphthyridin-2-ium salts.

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1. Introduction

The enzyme family of cholinesterases (ChEs) catalyses the hydrolysis of choline-based esters to terminate cholinergic signal transmission.¹ There are two different types of cholinesterases, which were termed according to their substrate preferences: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). AChE is mainly found in chemical synapses of the central and peripheral nervous systems and in the membranes of erythrocytes, while BChE is primarily present in the blood. However, to some extent, BChE can be found in the CNS as well.² In contrast to AChE and its well-known function as a pivotal regulator of cholinergic neurotransmission, BChE is widely viewed as a “backup enzyme” for AChE and a general-purpose metabolizer of bioactive esters in the diet. Yet, recent reports highlight the role of BChE as a co-regulator of acetylcholine levels in the human brain as well as its influence on the control of aggression and social stress by hydrolysing the peptide hor-

mone ghrelin.^{3,4} A dysregulation of cholinergic signal transduction has been associated with several diseases like myasthenia gravis,⁵ postoperative ileus,⁶ bladder dysfunction,⁷ glaucoma,⁸ and Alzheimer's disease (AD).⁹ The hallmarks of AD are the formation of amyloid β (A β) plaques, neurofibrillary tangles, and a drastic decrease in hippocampal and cortical levels of acetylcholine.¹⁰ A multitude of evidence indicate that the pathological accumulation of A β in the CNS leads to various damaging events, which finally cause neuronal destruction, cholinergic deficit, and decreased cognitive function. To counteract the deprived cholinergic neurotransmission by blocking the inactivation of acetylcholine, the inhibition of AChE is a highly relevant therapeutic approach for the symptomatic treatment of AD. In the mid-1990s, the development of AChE inhibitors (AChEIs) resulted in the approval of three drugs (donepezil (1, Fig. 1), rivastigmine, and galantamine) for the treatment of mild-to-moderate AD. While the carbamate rivastigmine, classified as a “pseudo-irreversible AChEI”, forms a covalent adduct with the serine residue of the catalytic triad of AChE, donepezil and galantamine are reversible AChEIs that compete with acetylcholine for AChE binding.¹¹ To date, these drugs represent the first line pharmacotherapy for the treatment of mild-to-moderate AD. In addition to the disease alleviating effects of AChEIs due to an amplification of the deprived cholinergic activity, various studies demonstrated that AChEIs are also able to prevent A β oligomerization, at least to some extent.¹² AChE is considered as the main cholinesterase to target in the AD scenario, however, there are studies that highlight the role of BChE in the progression of AD.^{13–15} These studies

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Fig. 1 Chemical structures of the approved AChEI donepezil hydrochloride (1) and the dual ChEI chelerythrine (2) in comparison with the generic structure of 8-hydroxy-2,7-naphthyridin-2-ium chloride (3).

indicate that in the course of disease progression, the AChE activity decreases while the activity of BChE remains unaffected or is even elevated.^{13,14} Since BChE is able to compensate for an inhibition of AChE, at least to a certain extent,¹⁶ the inhibition of BChE seems to be an attractive therapeutic concept in AD as well. Several potent and selective BChE inhibitors have been developed over the last decade.^{15,17–21} However, none of these BChEIs has received the approval for AD treatment yet. Notably, the approved AD drug rivastigmine is a dual inhibitor of AChE and BChE,²² while donepezil and galantamine show a high selectivity for AChE.^{23,24} However, efforts were made either to achieve BChE inhibition of galantamine and donepezil by a chemical modification of their structures or to discover novel chemotypes as dual ChEIs.^{25–30} We have identified natural products with an isoquinoline scaffold as ChEIs, among these is chelerythrine (2, Fig. 1), a dual inhibitor of AChE and BChE. Moreover, chelerythrine was shown to prevent A β aggregation and to dis-aggregate already formed A β aggregates.³¹

Kinetic analyses revealed that chelerythrine binds to both the catalytic site and the peripheral anionic site (PAS) of AChE.³¹ By analogy with chelerythrine with its quaternary ammonium group that is linked by three carbons to an oxygen, we aimed to develop 8-hydroxy-2,7-naphthyridin-2-ium salts (3) as potential inhibitors of AChE and BChE. The synthesis of the highly fluorescent 8-hydroxy-2,7-naphthyridin-2-ium salts, as well as their conjugated bases, the 2,7-naphthyridin-1(7H)-ones, has already been published,^{32–35} however, no distinct biological activity has been attributed to this chemotype. Instead, the formation of 8-hydroxy-2,7-naphthyridin-2-ium salts was utilized as an operating principle of several fluorescence-based assay methods to detect nicotinamide and its analogues.^{34,36–38}

2. Results and discussion

2.1. Design of the 8-hydroxy-2,7-naphthyridin-2-ium salts as ChEIs

Since natural products of the 8-methoxyisoquinoline group, e.g. chelerythrine (2), palmatine, and berberine, have been

identified as ChEIs,³¹ we designed 8-hydroxy-2,7-naphthyridin-2-ium salts (3) as potential inhibitors of AChE and BChE, based on their similarity to the 8-methoxyisoquinoline scaffold. The binding mode of 8-methoxyisoquinolines is unknown, because these compounds have not been co-crystallized with ChEs yet. Therefore, we aimed to generate structurally different 8-hydroxy-2,7-naphthyridin-2-ium salts to gain first insights into structural motifs that influence the biological activity. According to the synthetic procedure (see below), the residues at positions 2 and 6 of the 2,7-naphthyridin-2-ium scaffold could be easily modified by using different *N*¹-alkylated nicotinamide derivatives and enolizable ketones, respectively, as starting materials for the formation of the 8-hydroxy-2,7-naphthyridin-2-ium salts. By analogy with the *N*-benzylated AChEI donepezil (1), we synthesized a number of compounds that bear a benzyl moiety in position 2 of the 2,7-naphthyridin-2-ium scaffold. Docking of substituted 8-hydroxy-2,7-naphthyridin-2-ium salts and comparison with the binding mode of donepezil at AChE showed that bulky aromatic rings (e.g. naphthyl) at position 6 show favourable interaction with Trp279 that is a part of the peripheral anionic site (PAS).

2.2. Synthetic procedures and physicochemical characterization

The synthesis of the 8-hydroxy-2,7-naphthyridin-2-ium salts started with the *N*-alkylation of nicotinamide (4) with ethyl iodide or benzyl chloride to give the pyridinium salts (5a–b) in 29% and 71% yield, respectively.^{39,40} *N*¹-Methyl nicotinamide was purchased from a commercial vendor. The 8-hydroxy-2,7-naphthyridin-2-ium salts were formed by the reaction of *N*¹-alkylated nicotinamide derivatives with enolizable ketones, according to the general procedure published by Ukawa-Ishikawa *et al.*³⁴ In an alkaline milieu at 0 °C, this one-pot synthesis was initiated by the nucleophilic attack of a deprotonated ketone to position 4 of the pyridinium salt to form 1,4-dihydropyridines with the general structure of 6. Notably, 1,4-dihydropyridines were not isolated. Upon the addition of formic acid and refluxing, the 8-hydroxy-2,7-naphthyridin-2-ium salts were formed by intramolecular condensation and oxidation in yields ranging from 2–33% (Scheme 1). All the synthesized compounds were characterized by IR, ¹H NMR, ¹³C NMR and mass spectrometry (for detailed information see the ESI[†]). UV-vis and fluorescence properties, including excitation and emission maxima ($\lambda_{\text{exc}}^{\text{max}}$ and $\lambda_{\text{em}}^{\text{max}}$), absorption coefficient (ϵ), relative fluorescence intensity (FI) and fluorescence quantum yield (Φ_{F}) are summarized in Table S1.[†] Since there is contrasting information on the chemical structure of the main product of the reaction between *N*¹-alkylated nicotinamide derivatives with enolizable ketones, we put efforts into the structural elucidation of this chemotype. In 1947, Huff *et al.* postulated the nucleophilic attack of deprotonated methylketones in position 2 of the pyridinium salts resulting in 1,6-naphthyridin-5(1H)-ones (7, see Fig. 2).⁴¹ However, Kröhnke falsified this finding

Scheme 1 Synthesis of 8-hydroxy-2,7-naphthyridin-2-ium salts **3a-h**.

by melting point analyses, which clearly indicated that deprotonated methylketones attack N^1 -alkylated nicotinamide derivatives in position 4, finally leading to 2,7-naphthyridin-1(7H)-ones (**8a**).³² Even though Kröhnke's findings have already been verified by Birkofer *et al.* in the mid-1950s,⁴² it is surprising that up to the year 2009 several publications^{36,38} adopted the disproven structure of Huff *et al.* Our spectroscopic data (see the ESI[†]) strongly supports the formation of 2,7-naphthyridines.

However, we think that the basicity as well as the spectroscopic properties of the uncharged 2,7-naphthyridin-1(7H)-ones (**8a**) can be much better explained by the zwitterionic resonance structure (**8b**), 2,7-naphthyridin-2-ium-8-olate. During the purification process, we observed distinct acid-base properties of the final products, which were either precipitated from an acidified aqueous layer by the addition of KOH to pH = 11 or from ethanol by adjusting to a pH value of 2. Thus, we aimed to characterize these acid-base properties in more detail. By recording the UV-vis spectra at different pH values (Fig. S1[†]), we were able to determine a pK_a value of 8.7 for compound **3a** (see the ESI[†]), which is consistent with an

acid-base equilibrium between imidic acid **3** and its conjugate base **8b**. This assumption could be further supported by NMR spectroscopy. In the 1H NMR spectra of the uncharged as well as the positively charged forms, dissolved in DMSO, the signals for the protons in positions 1 and 3 of the 2,7-naphthyridine scaffold are highly downfield shifted (>8.80 ppm) and nearly identical (see Table 1 and Fig. S2[†]). This can be explained best by the existence of a positively charged nitrogen atom in position 2 of the zwitterionic and the positively charged state. For either aqueous or DMSO solution, we therefore suggest the existence of a pH-dependent equilibrium, which is dominated by the uncharged 2,7-naphthyridin-2-ium-8-olate (**8b**) and the positively charged 8-hydroxy-2,7-naphthyridin-2-ium ions (**3**).

2.3. Biological characterization of 8-hydroxy-2,7-naphthyridin-2-ium salts as ChEIs

To assess the inhibitory effects of the synthesized 8-hydroxy-2,7-naphthyridin-2-ium chlorides on AChE and BChE, the compounds were tested in an absorption-based hydrolase activity assay published by Karlsson *et al.*⁴³ All compounds were initially tested at a concentration of 10 μ M in parallel against horse serum BChE and electric eel AChE using the conditions described in our earlier contributions.^{43,44} The control inhibitor in all the trials is physostigmine (typically tested at 10 μ M). Results are presented in Table 2. For further characterization, we selected the compounds that displayed more than 75% inhibition against any of the target enzymes at 10 μ M to continue with dose-response experiments. The individual IC_{50} curves and the corresponding IC_{50} values are shown in Fig. 3. The *in vitro* inhibition data reveals that the benzylated compounds **3c** and **3e** act as dual inhibitors of both cholinesterases. As can be seen in Table 2, a benzyl moiety in position 2 of the 2,7-naphthyridine scaffold is of utmost importance for ChE inhibition. Furthermore, a naphthyl moiety in position 6 of the 2,7-naphthyridine scaffold seems to gain both BChE and AChE inhibition. These findings are consistent with the predicted binding modes of **3e** for AChE and BChE (see chapter 2.4 and Fig. 4). Moreover, our inhibition data indicates that BChE inhibition seems to be more sensitive to modifications at the 2,7-naphthyridine scaffold, compared to AChE inhibition. Tetracyclic **3f** inhibits AChE more potently than BChE.

Fig. 2 Presentation of some chemotypes that have been proposed as the main products of the reaction of N^1 -alkylated nicotinamide derivatives with enolizable ketones in comparison with our suggestion (**8b**) for the uncharged state. **7** was taken from ref. 36, 38 and 41, **8a** from ref. 32, 33 and 37, **3** from ref. 34 and 42, and **9** from ref. 42.

Table 1 Chemical shifts in the 1H NMR spectra of compound **3b**

Protons	Chemical shifts of the protonated state [ppm]	Chemical shifts of the deprotonated state [ppm]
–OH	12.67	No signal
H-1	9.66	9.65
H-3	8.96–8.91	8.87–8.82
H-4	8.21	8.13
H-5	7.24	7.22

Table 2 *In vitro* inhibition of BChE and AChE by 8-hydroxy-2,7-naphthyridin-2-ium salts **3a–h** at a concentration of 10 μM

Cpd	Chemical structure	BChE (inh% $\pm$ SD)	AChE (inh% $\pm$ SD)
3a		4.1 $\pm$ 2.9	51.6 $\pm$ 3.6
3b		3.9 $\pm$ 2.7	45.9 $\pm$ 5.9
3c		83.8 $\pm$ 1.5	76.4 $\pm$ 2.3
3d		34.5 $\pm$ 4.7	59.5 $\pm$ 2.0
3e		98.6 $\pm$ 0.3	97.1 $\pm$ 1.8
3f		8.5 $\pm$ 4.3	77.3 $\pm$ 2.1
3g		n.d. ^a	n.d. ^a
3h		1.7 $\pm$ 3.2	48.5 $\pm$ 2.5

^a n.d.: not detected.

2.4. Docking results

In order to rationalize the determined biological activities, docking of the inhibitors was carried out using the program Glide SP. The crystal structures of *Electrophorus electricus* AChE (EeAChE) in the apo form and *Torpedo californica* AChE (TcAChE) in complex with donepezil (PDB ID 1EEA, 1EVE) were selected for docking and rescoring studies.⁴⁵ Both enzymes share an identical binding pocket with 100% sequence identity. Since the TcAChE structure shows a better resolu-

tion and was solved in complex with donepezil (PDB ID 1EVE) this protein structure was used for docking and rescoring. As an example, the predicted binding mode of the most potent inhibitor **3e** is shown in Fig. 4A. For the docking experiments we used the protonated state of our ligand, since the pK_a determination for the model compound **3a** ($\text{pK}_a = 8.7$) has clearly shown that this is the predominant state under physiological conditions ($\text{pH} = 7.4$) as well as under assay conditions ($\text{pH} = 8.0$). The quaternary nitrogen of **3e** adopts the same position as the protonated amino group of donepezil inside the binding pocket allowing cation- π interactions with the surrounding aromatic residues (Trp84 and Phe330). The terminal phenyl ring of **3e** occupies the same region as the phenyl ring of donepezil and interacts with Trp84 and Phe330 of the catalytically active site (CAS, Fig. 4B). The naphthyl moiety of **3e** interacts with Trp279 through π - π interactions and with Ileu287 through van der Waals interaction in the peripheral pocket. A hydrogen bond between the phenol OH of **3e** and a conserved water molecule (bound to Tyr121) further stabilizes the inhibitor position (Fig. 4A). All compounds under study show a similar binding mode to that described for **3e**. The docking of the more rigid compound **3f** shows that it interacts in a similar way to AChE making the relevant interactions with Tyr121 and Trp279 (Fig. S3[†]). Also the quaternary nitrogen adopts the same position as that observed for **3e**. Substitution with smaller moieties on positions 2 and 6 resulted in less favourable

Fig. 3 IC_{50} curves and IC_{50} values for the inhibition of BChE and AChE, respectively.

Fig. 4 Predicted binding modes of **3e** for AChE and BChE. A) Predicted binding mode of **3e** (colored cyan) for TcAChE. Water molecules are shown as red balls. Hydrogen bond interactions are represented as dashed lines. B) Comparison between the **3e** docking pose for TcAChE and the co-crystallized inhibitor donepezil (colored orange). The surface of the binding pocket is shown and colored according to the hydrophobicity (hydrophilic = magenta, hydrophobic = green). C) Predicted binding mode of **3e** (colored cyan) for BChE. D) Comparison between the **3e** docking pose and the co-crystallized inhibitor benzyl pyridinium-4-methyltrichloroacetimidate (colored orange). The same color scheme as in Fig. 4B.

interaction energies compared to **3e**. For the docking to BChE, the crystal structure of the human enzyme in complex with benzyl pyridinium-4-methyltrichloroacetimidate was chosen due to the structural similarity of the co-crystallized inhibitor to the 8-hydroxy-2,7-naphthyridin-2-ium salts and due to the absence of a crystal structure of horse serum BChE (Fig. 4C).⁴⁶

The interaction of the benzyl pyridinium part of **3e** with the lower part of the BChE binding pocket is similar to that observed for the co-crystallized inhibitor (Fig. 4D). A salt bridge between Asp70 and the quaternary nitrogen as well as a hydrogen bond between the phenol of **3e** and one of the water molecules located in the BChE crystal structure is observed. The terminal naphthyl group is located at the entrance of the pocket nearby the residues Pro285 and Ser287 (Fig. 4C). In the case of **3f**, which inhibits BChE less potently than AChE, the docking model for BChE shows that the rigid molecule is not able to adopt the favourable orientation (Fig. S4†). Due to the missing benzyl group, the compound cannot be “anchored” in the correct position in the large BChE binding pocket. The salt bridge between the quaternary nitrogen of **3f** and Asp70, cation- π interaction with Tyr332, as well as

π - π interaction with Trp82 are not present due to the absence of the benzyl group. For experimental details see the ESI.†

3. Conclusions

In this study, we designed and prepared a small series of 8-hydroxy-2,7-naphthyridin-2-ium salts as potential ChEIs by analogy with the natural product chelerythrine, known as a dual inhibitor of AChE and BChE. By spectroscopic analyses we were able to elucidate the zwitterionic nature of 2,7-naphthyridin-1(7H)-ones, the neutral form of 8-hydroxy-2,7-naphthyridin-2-ium salts. Among the synthesized compounds, we identified **3e** and **3e** as dual inhibitors of AChE and BChE as well as tetracyclic **3f** inhibiting AChE more potently than BChE, which is a promising starting point for subsequent selectivity studies. These are the first biological activities that are attributed to 8-hydroxy-2,7-naphthyridin-2-ium salts. By *in vitro* characterization rationalized by docking studies, we gained first insights into ChE inhibition by using 8-hydroxy-2,7-naphthyridin-2-ium salts, which allowed us to identify structural features that influence inhibitor potency. Furthermore, the intrinsic fluorescence of the 8-hydroxy-2,7-naphthyridin-2-ium salts may allow their use as molecular tools for esterase biochemistry and biology.

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