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Tacrine-Silibinin Codrug Shows Neuro- and Hepatoprotective Effects *in Vitro* and Pro-Cognitive and Hepatoprotective Effects *in Vivo*

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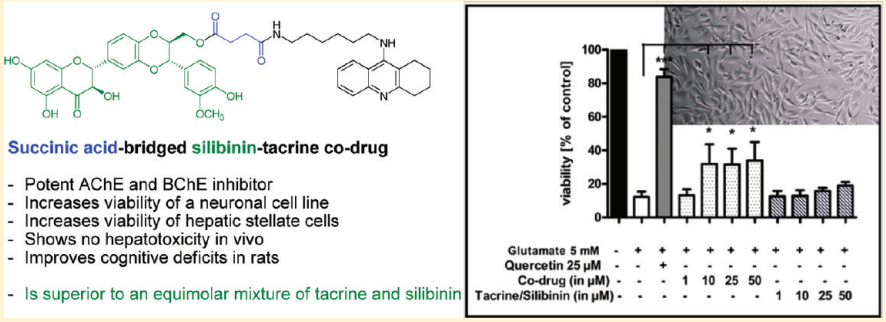
Supporting Information

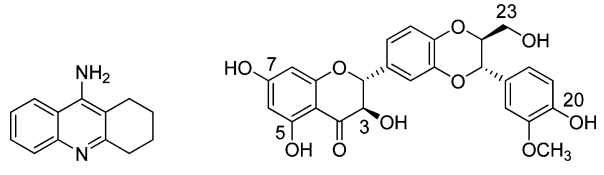


ABSTRACT: A codrug of the anti-Alzheimer drug tacrine and the natural product silibinin was synthesized. The codrug's biological and pharmacological properties were compared to an equimolar mixture of the components. The compound showed potent acetyl- and butyrylcholinesterase inhibition. In a cellular hepatotoxicity model, analyzing the influence on viability and mitochondria of hepatic stellate cells (HSC), the toxicity of the codrug was markedly reduced in comparison to that of tacrine. Using a neuronal cell line (HT-22), a neuroprotective effect against glutamate-induced toxicity could be observed that was absent for the 1:1 mixture of components. In subsequent *in vivo* experiments in rats, in contrast to the effects seen after tacrine treatment, after administration of the codrug no hepatotoxicity and no induction of the cytochrome P450 system were noticed. In a scopolamine-induced cognitive impairment model using Wistar rats, the codrug was as potent as tacrine in reversing memory dysfunction. The tacrine−silibinin codrug shows high AChE and BChE inhibition, neuroprotective effects, lacks tacrine's hepatotoxicity *in vitro* and *in vivo*, and shows the same pro-cognitive effects *in vivo* as tacrine, being superior to the physical mixture of tacrine and silibinin in all these regards.

INTRODUCTION

Alzheimer's disease (AD) is the most prominent form of dementia in the world. Despite huge efforts and numerous successes in investigating the pathophysiology of AD, the disease is still incurable.[1] The number of approved drugs is extremely limited to only three acetylcholinesterase (AChE) inhibitors (the moderately active drugs rivastigmine, donepezil and galantamine) and one NMDA antagonist (memantine). The most potent AChE inhibitor tacrine (Chart 1), albeit clinically effective like all AChE inhibitors,[2] was withdrawn from the market due to its dose-dependent hepatotoxicity.[3] Because of the clinical effectiveness of AChE inhibitors in general and the high potency of tacrine in particular, this structure has been widely and successfully used in medicinal chemistry for application in hybrid or multitarget compounds in order to combine its potent AChE inhibition with other pharmacological properties by covalently connecting tacrine to other pharmacologically active structures,[4] such as CB_1 receptor antagonists and an M_1 agonist to name just two recent examples.[5,6]

Chart 1. Structures of the Anti-Alzheimer Drug and AChE Inhibitor Tacrine (1) and the Hepatoprotective Natural Product Silibinin (2)



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The hybrid approach was also applied by us and co-workers to specifically target the problem of tacrine's liver toxicity. For this reason, we synthesized chemically stable hybrids with the

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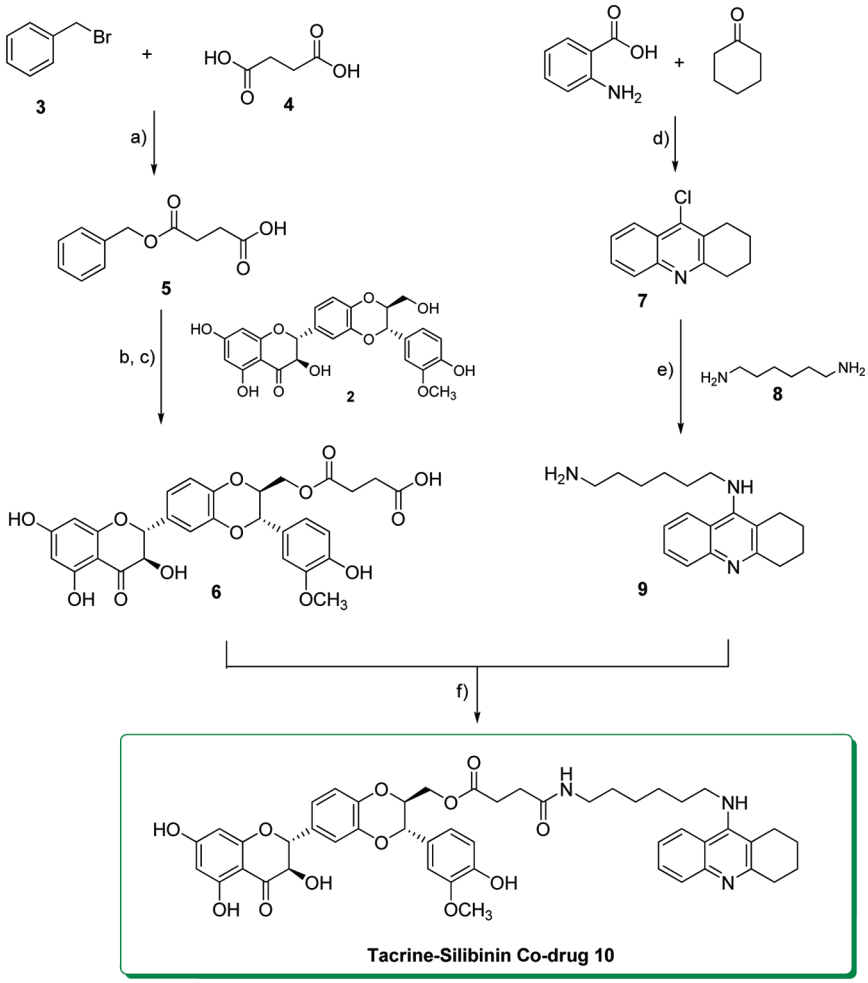
Scheme 1. Synthesis of Tacrine–Silibinin–Codrug 10[a]



[a]Reagents and conditions: (a) Et_3N, THF, reflux, 16 h; (b) DIAD, Ph_3P, anhydrous THF, N_2, 16 h, 0 to 20 °C; (c) EtOH, 5% Pd/C, H_2, 50 °C; (d) $POCl_3$, reflux, N_2; (e) 1-heptanol, 140 °C; (f) EDCI, HOBt, anhydrous DMF, 16 h, rt.

natural product ferulic acid.[7,8] These compounds were potent AChE inhibitors and antioxidants, but *in vivo* improvement of cognition assessed by scopolamine-induced cognitive impairment in rats using a radial maze showed low activity.[8b] Another approach used the combination of tacrine with nitric oxide donating moieties: here, both the *in vitro* and *in vivo* properties are promising, although the strong vasorelaxant properties of NO-donors might cause problems *in vivo*.[9]

Driven by the positive results of combining tacrine with other biologically active moieties to improve its overall pharmacological properties, in this work we focused on the natural product and flavonolignan silibinin in order to specifically target tacrine's hepatotoxicity. Silibinin (also known as silybin; Chart 1) is one of the main components of the silymarin complex, a standardized mixture obtained from the fruits of *Silybum marianum* (L.) GAERTN. (syn. *Carduus marianus* L., Asteraceae) commonly known as milk thistle. It represents a diastereomeric mixture of silybin A (2*R*,3*R*,2′*R*,3′*R*) and silybin B (2*R*,3*R*,2′*S*,3′*S*), only the structure of the latter is used in this article for better illustration. Various pharmacological properties have been assigned to silibinin in recent years including anti-inflammatory and anticancer activities.[10] Interestingly, also neuroprotective properties have been attributed to silymarin and silibinin derivatives, which makes it an interesting component for anti-Alzheimer drug candidates.[11,12] The main medicinal use of silymarin (since antiquity) and silibinin is therapeutic intervention in liver diseases.[13] Despite the fact that *in vitro* results are generally positive, clinical studies are controversial, probably because of the variable composition of silymarin preparations applied leading in consequence to different doses of the biologically active compounds.[13]

We aimed at synthesizing a compound consisting of a tacrine-based AChE inhibiting part covalently connected to silibinin. In contrast to previous work based on ferulic acid, the compound should not contain a stable (amide) bond between the components,[7−9] but a more labile ester bond enabling the AChE inhibiting part to penetrate the blood–brain barrier (BBB) and therefore show central nervous system (CNS) activity (Scheme 1), a codrug approach. Biological properties of the codrug structure (in a hybrid structure the components are connected in a chemically stable way[14]) should be compared to an equimolar mixture of tacrine and silibinin in order to check whether the stable connection provides additional benefits (pharmacokinetically and kinetically) due to a chemical connection as was recently shown for silibinin galloyl esters with regard to antiangiogenic activity.[15] The question whether a chemical connection offers advantages over a physical mixture of components or may actually lead to disadvantages is an open

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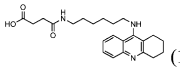
Table 1. Inhibition of AChE and BChE (IC_{50} and pIC_{50} Values ± SEM) by Codrug 10 and Tacrine Moiety-Containing Intermediates and Putative Metabolites, Respectively, and Selectivity Expressed as the Ratio of Resulting IC_{50} Values

Compound	IC_{50}, nM (pIC_{50} ± S.E.M.)[a]		Selectivity ratio IC_{50}(AChE)/IC_{50}(BChE)
	AChE[b]	BChE[c]	
Galantamine	640[16] (6.20 ± 0.05)	8400[16] (5.08 ± 0.03)	0.08[16]
Tacrine (**1**)	15.6 (7.34 ± 0.06)	3.2 (8.29 ± 0.08)	4.9
Silibinin (**2**)	5.5% inhibition at 10^{-4} M	3.1% inhibition at 10^{-4} M	-
6-Amino-hexamethylene tacrine (**9**)	39.0 (7.41 ± 0.04)	2.0 (8.69 ± 0.05)	19.5
 (**11**)	101.9 (6.99 ± 0.04)	361.3 (6.44 ± 0.05)	0.3
Co-drug (**10**)	53.9 (7.27 ± 0.02)	49.7 (7.30 ± 0.02)	1.1

[a]Data are the means of at least three determinations. pIC_{50} = −log IC_{50}. [b]AChE from electric eel. [c]BChE from equine serum.

question that has to be worked out for every set of hybrid or codrug molecules, although the number of examples with additional benefits is overwhelming.[4−6,14,16]

After the synthesis of a suitable codrug, several biological and pharmacological properties were assessed *in vitro*: AChE inhibition, cytotoxicity, and cytoprotection toward a neuronal cell line, and toxicity toward a hepatic cell line (and therefore putative resulting hepatoprotection). Additionally, the codrug, the components, and the mixture, separately, were evaluated *in vivo* in rats for hepatotoxicity or hepatoprotection and a possible interaction with the biotransformation capacity of the liver, as assessed by the influence on the cytochrome P450 (CYP) system. Experiments were performed at two different dosages, the dosage used in the subsequent behavioral pharmacology experiments and at a dosage equimolar to the highest tolerated dose of tacrine. Furthermore, *in vivo* procognitive effects of the compounds were evaluated. We used a method applying scopolamine and an eight-arm radial maze as an accepted protocol in behavioral pharmacology for assessing the antidementive potential of compounds acting directly or indirectly at the muscarinic acetylcholine receptor, such as AChE inhibitors or allosteric modulators.[6,8b,17]

RESULTS AND DISCUSSION

Chemistry. The tacrine−silibinin codrug **10** is the combination of two parts: silibinin hemisuccinate (**6**) and 6-aminohexamethylene tacrine (N^1-(1,2,3,4-tetrahydroacridin-9-yl)hexane-1,6-diamine **9**). The latter tacrine-spacer compound (**9**) was synthesized by alkylation of 1,6-hexanediamine (**8**) with 9-chlorotetrahydroacridine (**7**).[7,18,19] To generate the selective esterification of succinic acid (**4**) with the only primary hydroxyl group of silibinin (**2**), a Mitsunobu reaction between silibinin (**2**) and monobenzyl succinate (**5**) was applied.[20] The latter ester was synthesized using succinic acid (**4**) and benzyl bromide (**3**) (Scheme 1). The chemical shift in ^{1}H NMR of the hydrogen atoms at position 23 changes upon esterification according to what had been described in the literature.[20]

Two alternative synthetic routes were also investigated to access the codrug structure, yet all failed due to the poor solubility of both silibinin and tacrine derivatives (see Supporting Information for detailed procedures and description). First, tacrine-spacer-succinamide was synthesized (so connecting the tacrine part of the codrug with succinic acid in the first step), but the amino acid-like structure made it a poorly soluble compound even in THF and consequently led to the failure of the Mitsunobu reaction with silibinin. Second, silibinin (**2**) was partially protected by trityl groups according to the method described in the literature.[21] In the subsequent reaction with either succinic anhydride or succinic acid, no specific reaction at the primary hydroxyl group occurred because of the poor reaction selectivity between aliphatic and aromatic hydroxyl groups. The compound that formed during the preceding protection step was actually 7,20-ditrityl silibinin instead of 5,7-ditrityl silibinin reported in the literature as confirmed by ^{1}H NMR (see Supporting Information for details).[21]

Cholinesterase Inhibition. The cholinesterase inhibition of the codrug and its tacrine moiety-containing components were evaluated using the colorimetric Ellman assay (Table 1).[22] Two cholinesterases exist in the human body: acetylcholinesterase (AChE; 3.1.1.7), the target of the approved anti-Alzheimer drugs,[2b] and butyrylcholinesterase (BChE, 3.1.1.8), the exact functions of which are not yet fully understood.[23] Several findings indicate that BChE can compensate for the lack of AChE appearing in later stages of AD. Cognitive performance of aged rats was improved and the amount of β-amyloid peptide was lowered by selective BChE inhibition making its inhibition potentially therapeutically desirable.[23b,24]

We used AChE (EC 3.1.1.7, type VI−S, from electric eel) and BChE (EC3.1.1.8, from equine serum) in the Ellman assay, and selectivity was expressed as the ratio of IC_{50}(AChE)/IC_{50}(BChE).

As expected, silibinin (**2**) is not able to inhibit significantly either AChE or BChE even at a concentration of 100 μM. The codrug (**10**) loses quite a bit of inhibitory activity at BChE (16-fold lower) and moderately at AChE (3.5-fold lower). Nevertheless, the codrug is still a two-digit nanomolar inhibitor leading to high inhibition concomitantly at both ChEs (Table 1). The two tacrine moiety-containing spacers were also tested. With respect to the tacrine−hexamethylene amine (**9**), analogues with lower and higher spacer lengths had been synthesized and tested before and represent one-digit nano-

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molar inhibitors at AChE and at BChE.[7,9] The primary amine compound **9** shows an IC_{50} value of 39 nM at AChE and 2.0 nM at BChE, therefore acting at the same range as the parent compound tacrine (**1**), and these values are in accordance with the inhibition data of analogues with similar spacer lengths (Table 1).[7,9] The tacrine hexamethylene amine (**9**) connected to succinic acid as a hemi amide (compound **11**) shows much weaker inhibition values with IC_{50}(AChE) = 102 nM and IC_{50}(BChE) = 361 nM (Table 1). For this compound, some minor selectivity toward AChE is achieved, but in general, for all compounds tested selectivity differences are moderate. Compound **11** is probably the initial hydrolysis product *in vivo* through cleavage of the ester bond to silibinin. Despite the inhibitory activity decrease, also this compound might at least contribute to the codrug's *in vivo* profile. All tacrine derivatives investigated for their enzyme inhibition show at least similar activity at AChE (ranging from 16 to 102 nM); therefore, no indication can be given for the structure of the compound being procognitively active *in vivo* (see Behavioral Studies).

In summary, the codrug itself and its putative cleaved parts (metabolites) are potent AChE and BChE inhibitors in the submicromolar to the nanomolar range.

Neuroprotection. It is well established that oxidative stress, the formation of reactive oxygen species (ROS), and subsequent neurotoxicity are key processes in the pathophysiology of AD.[25] Silymarin, silibinin, and chemical derivatives have been described to possess neuroprotective properties.[11,12] The results obtained are not unambiguous though. Silymarin increases glutathione (GSH) level and enhances superoxide dismutase activity.[12] However, it turned out that derivatives (oxidized forms, alkenylated and amidated ones) are more active than the parental silibinin in terms of radical scavenging properties.[11] Unambiguous structure–activity relationships for silibinin derivatives are difficult to describe, but it seems that lipophilicity increased the radical scavenging properties as well as the cell viability of PC-12 cells (as a model for neuronal cell death) measured in a MTT test, albeit the flavonol quercetin used as a positive control was always more potent, especially with regard to the parental silibinin.[11a] We therefore wanted to investigate whether and to what extent tacrine, silibinin, silibinin physically mixed with tacrine (equimolar mixture), and the synthesized codrug (**10**) exhibit a cytotoxic or especially cytoprotective potential in a neuronal cell line and quantify putative differences.

The HT-22 mouse hippocampal cell line represents an established *in vitro* model system for studying oxidative stress induced neurotoxicity caused by glutamate.[26] Two pathways of glutamate toxicity have been described: an excitotoxic one mediated by the ionotropic glutamate receptors via cell death through Ca^{2+} influx and ROS accumulations and a nonreceptor mediated "oxidative" pathway.[27−29] The latter oxidative glutamate toxicity is initiated by extracellular glutamate in high concentrations that inhibits cystine (as oxidized form of cysteine) uptake mediated by the cystine/glutamate antiporter system leading to intracellular cysteine and therefore glutathione depletion, which induces ROS accumulation and cell injuries.[28] HT-22 hippocampal cells lack these ionotropic glutamate receptors, and therefore, the glutamate-mediated excitotoxicity cell death pathway can be excluded.[29] In this neuronal oxidative stress induced toxicity (oxytosis), extracellular glutamate in high concentrations decreases intracellular glutathione and therefore leads to ROS production.[30] Administration of antioxidants such as vitamin E or flavonoids can effectively prevent oxidative neuronal death in this cell line.[31]

Figure 1A shows the influence of different doses of codrug **10** on the viability of hippocampal HT-22 cells measured by a

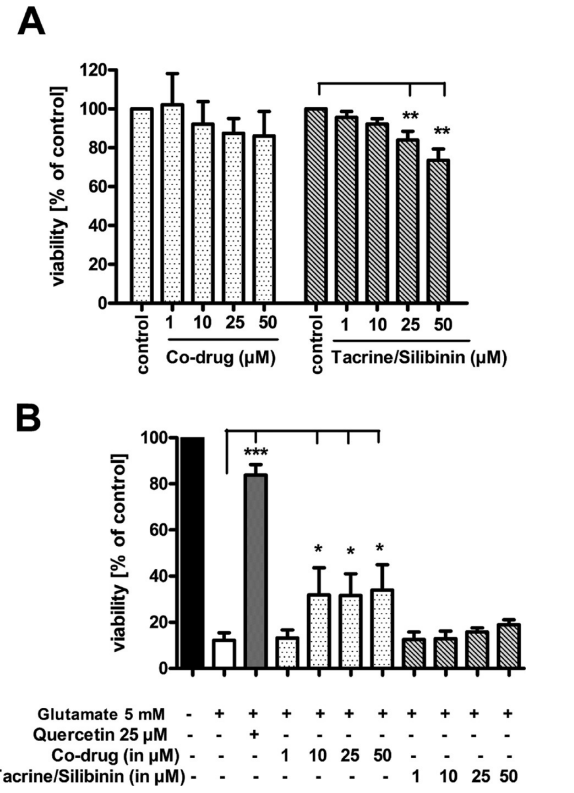

Figure 1. Evaluation of the neurotoxicity (A) and neuroprotection (B) of codrug **10** and an equimolar mixture of tacrine (**1**) and silibinin (**2**) against glutamate induced oxidative stress on HT-22 cells. Data were subjected to one-way ANOVA followed by Dunnett's multiple comparison post-test using GraphPad Prism 4 Software (levels of significance $*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

MTT test[16] in comparison to the equimolar mixture of tacrine and silibinin. The tacrine/silibinin mixture showed a significant, albeit very moderate, neurotoxicity starting from a concentration of 25 μM. Both tacrine and silibinin showed very weak neurotoxicity starting from a concentration of 50 μM (cf. Figure A in the Supporting Information). The mixture does not show lower neurotoxicity compared to the individual components; in contrast, the toxicities showed an additive effect. For the codrug, even at the highest concentration tested (50 μM), no neurotoxicity was observed (Figure 1A).

Figure 1B shows the effect of the tacrine/silibinin mixture and the codrug on the reversal of glutamate-induced neurotoxicity at different concentrations using quercetin as the (potent) positive control. All concentrations of the mixture tested did not show any effect even at a concentration of 50 μM, the individual components did not show a neuroprotective effect either (cf. Figure A in the Supporting Information). Albeit intensively discussed in the literature, we could not observe a neuroprotective effect of silibinin in this assay.[11,12] A weak, but similarly low effect was observed for silibinin on PC-12 cells.[11a] In contrast to these data, the codrug showed a significant increase in cell viability after exposure to glutamate (Figure 1B). Although glutamate's neurotoxic effect could not be reversed to the extent reached by 25 μM Quercetin, already

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at a concentration of 10 μM of the codrug a significant increase in cell viability was mediated (Figure 1B). Interestingly, this value seems to represent the highest achievable increase in cell viability since further increase of the concentration up to 50 μM did not increase cell viability (Figure 1B). The mechanism of neuroprotection seems to differ from the one of quercetin, or alternatively, quercetin is able to activate several mechanisms of antioxidant and neuroprotective activity.[31]

The codrug does not show the neurotoxic effects determined by the unchanged viability of hippocampal HT-22 cells and shows at low concentrations a significant neuroprotective effect (albeit lower than the one of the flavonol quercetin) that could not be observed for the physical mixture of tacrine and silibinin.

***In Vitro* Hepatoprotection.** To investigate a possible hepatoprotective effect of the codrug and the tacrine/silibinin mixture compared to tacrine, the compounds' effect on human hepatic stellate cells (HSC) was investigated.[32] First, the influence of test compounds on the viability of HSC was determined by a crystal violet assay. Crystal violet is a basic dye and is used to stain cell nuclei. The photometrically measured intensity of the dye directly correlates with the number of cells.[33] HSC were incubated with different concentrations of the respective test compounds and stained with crystal violet, and the absorbance was measured photometrically after treatment. Whereas tacrine and the equimolar mixture had a significant impact on the cell number starting at a concentration of 75 μM, the codrug showed no significant toxicity up to 200 μM (cf. Figure B in the Supporting Information).

For a more precise analysis, we applied fluorescence microscopy and examined the influence on cell number and mitochondria. Microscopical data confirmed the results of the crystal violet assay, being even more sensitive. As can be seen in Figure 2 (cf. also Figure C in the Supporting Information),

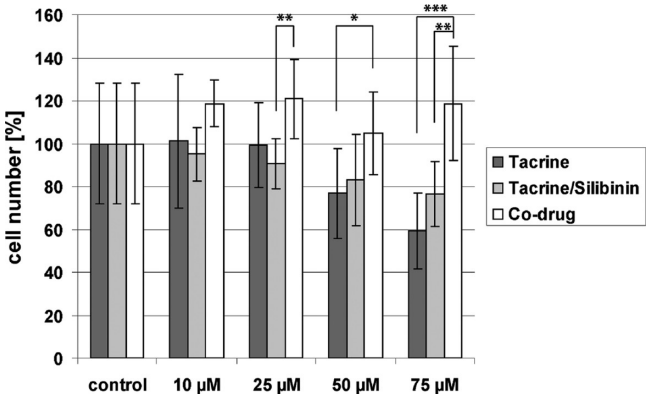

Figure 2. Comparison of cell numbers determined by microscopy after treatment of HSC with different concentrations of tacrine, an equimolar mixture of tacrine and silibinin, and codrug (**10**). Data were subjected to one-way ANOVA followed by Tukey's multiple comparison post-test using GraphPad Prism 4 Software (levels of significance $*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

tacrine decreases the amount of cells already starting at a concentration of 50 μM with higher concentrations being more hepatotoxic.

An interesting observation can be made when the influence of the codrug is compared with the tacrine/silibinin mixture (Figure 2): Statistically significant differences can already be observed from a concentration of only 25 μM. The mixture shows lower cell viability and therefore higher cytotoxicity than the codrug. At concentrations where tacrine is toxic, also the mixture exhibits toxicity. Therefore, the codrug is greatly superior to the equimolar mixture in being nontoxic even at the highest concentration tested.

Additionally, we investigated the impact of the compounds on the mitochondria of HSC, as mitochondrial dysfunction is an important mechanism of hepatotoxicity[34] and is discussed to be involved in tacrine-induced liver toxicity.[35] One such mechanism is the onset of mitochondrial permeability transition caused by opening of permeability transition pores in the inner mitochondrial membrane. This pore opening causes mitochondrial depolarization, uncoupling, and large amplitude swelling and can lead to both necrotic and apoptotic cell death.[36,37] For mitochondrial analysis, mitochondria were stained after the treatment of cells with different concentrations of tacrine, tacrine/silibinin, and codrug with a mitochondria specific dye that followed the determination of mitochondrial fluorescence intensity (Figure 3 and Supporting Information, Figure C).

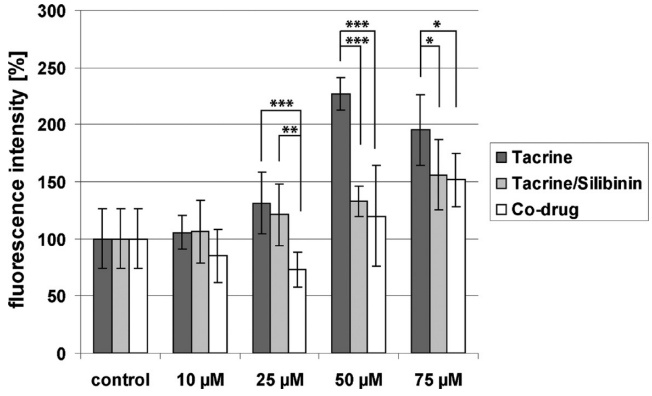

Figure 3. Fluorescence intensity of mitochondria after treatment of HSC with different concentrations of tacrine, an equimolar mixture of tacrine and silibinin, and codrug (**10**). Data were subjected to one-way ANOVA followed by Tukey's multiple comparison post-test using GraphPad Prism 4 Software (levels of significance $*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

Mitochondrial fluorescence intensity dramatically increases after the treatment of cells with tacrine at 50 μM to a value of 220% of the control (Figure 3). This increase in fluorescence intensity indicates a swelling of mitochondria and therefore mitochondrial dysfunction, which can lead to necrotic or apoptotic cell death. This effect is significantly lower for the tacrine/silibinin mixture at 50 and 75 μM. The codrug shows the same effect as the mixture at these concentrations. In terms of the hepatotoxicity indicated by increased mitochondrial fluorescence intensity in HSC, both the physical mixture and the codrug show greatly decreased toxicity compared to tacrine. Regarding the effect of the codrug at 25 μM, both tacrine and the tacrine/silibinin mixture show significantly higher fluorescence intensity, again indicating a superior effect of the codrug over the physical mixture of compounds (Figure 3).

Hepatotoxicity of tacrine and hepatoprotective activity of silibinin have been demonstrated before on HepG2 cells.[38] Our studies revealed also pronounced cyto- and mitotoxicity of tacrine on HSC. The mitotoxic effect is significantly lower for both the physical mixture of tacrine/silibinin and the codrug (Figure 3). Already at lower concentrations, the codrug's cytoprotective effect is more pronounced than the one of the

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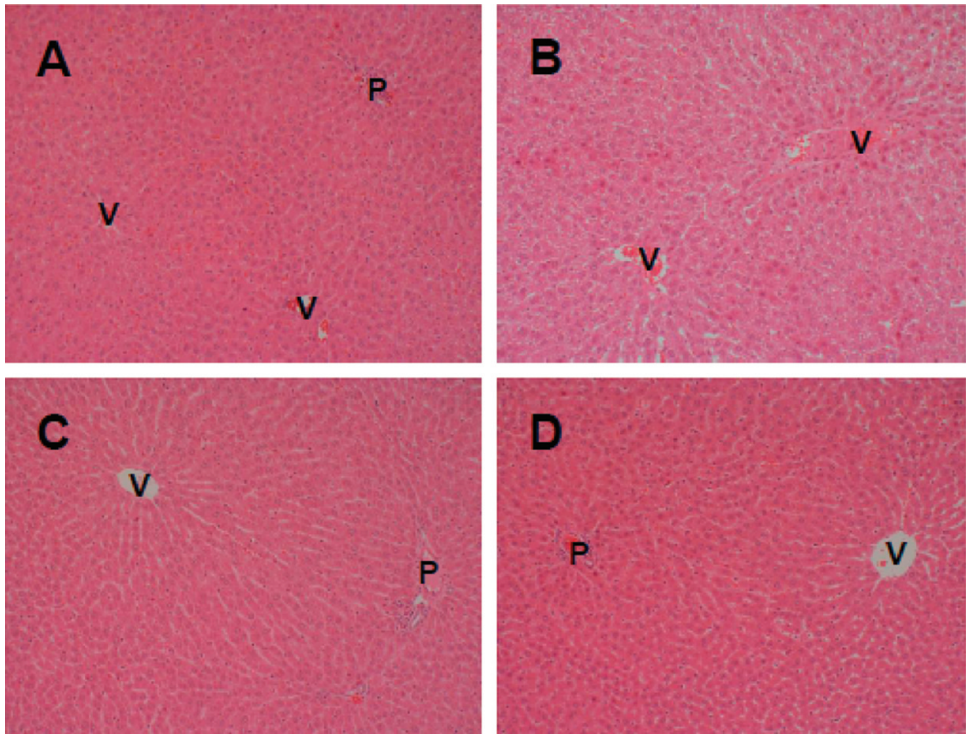

Figure 4. Histomorphological appearance of the liver of a male control rat (A) and of male rats 24 h after treatment with 6 μmol/100 g body weight tacrine (B), tacrine plus silibinin (C) or codrug (**10**) (D), respectively. H&E; original magnification: ×200. V, central vein; P, portal field.

mixture (Figure 2). Here, the mixture was only moderately superior to tacrine's toxicity, whereas the codrug did not show any toxicity even at the highest concentration tested.

***In Vivo* Studies on Hepatotoxicity/Hepatoprotection.** First, investigations were carried out on female rats and at a dosage of the compounds of 2 μmol/100 g body weight, i.e., on the same gender and at the same dosage used in the subsequent behavioral pharmacology experiments. Second, experiments were performed at a dosage equimolar to the highest tolerated dose of tacrine, 6 μmol/100 g body weight, in male rats, because of the higher cytochrome P450 content and activity in the livers of male than of female rats [and thus a higher biotransformation of tacrine (and probably also of the other compounds) into (reactive) metabolites].

Histopathological Changes. In a histopathological experimental setting in which doses of 2 μmol/100 g body weight corresponding to a therapeutic dosage of tacrine and 6 μmol/100 g body weight corresponding to the highest tolerated dosage of tacrine were tested, livers of control and of silibinin treated rats (results not shown) displayed normal histomorphology (Figure 4A). After tacrine administration, however, especially after the higher dosage in male rats, liver cell apoptosis and distinct fatty degeneration of the hepatocytes were observed (Figure 4B). This hepatotoxic effect of tacrine is well-known from the literature both from animal experiments as well as from clinical experience in humans.[3,39,40] As expected from literature data on the hepatoprotective effects of silymarin and silibinin,[41,42] simultaneous administration of silibinin prevented the toxic effects of tacrine (Figure 4C). In contrast to tacrine, after treatment of the rats with the codrug at both dosages, no adverse histomorphological changes were observed (Figure 4D).

Liver Tissue Oxidative State. The tissue content of reduced glutathione was significantly enhanced both after silibinin, tacrine plus silibinin, or codrug treatment. This effect was dosage dependent and amounted to 10% or 15%, respectively. However, the tissue content of oxidized glutathione was elevated after the administration of tacrine or of tacrine plus silibinin by 10% at the lower dosage (2 μmol/100 g b. wt.) and by 15% at the higher dosage (6 μmol/100 g b. wt.), respectively, but not following silibinin or codrug treatment. Consequently, the ratio of reduced/oxidized glutathione was significantly diminished after tacrine administration but increased after codrug administration and at the higher dosage also after silibinin treatment (Figure 5).

At the lower dosage in female rat treatment with silibinin, tacrine plus silibinin, or codrug, a reduction in tissue content of lipid peroxidation products of about 15% to 25% was observed. After the higher dosage in male rats, a remarkable increase in the values by about 140% was caused by tacrine treatment. After simultaneous application of tacrine plus silibinin, this effect was less pronounced. In contrast to these findings, no increase in tissue content of lipid peroxidation products was observed after silibinin or codrug treatment (Figure 6).

These results are in line with the histopathological findings and point to an increased oxidative stress after administration of tacrine that could partially be reversed by the simultaneous treatment with silibinin. In line with the results from the *in vitro* experiments and the histopathological findings, antioxidative and hepatoprotective effects were observed not only with silibinin but (at least to a similar extent) also with the codrug.

Behavioral Studies. In order to determine how the *in vitro* cholinesterase inhibition translates into behavioral effects, we applied a cognition assay using rats in an eight-arm radial maze.[6,8b,9b,43a] In this model, deteriorated information acquisition resulting in amnesia is reversibly induced by administration of the muscarinic acetylcholine receptor (M) antagonist scopolamine.[43b] This effect can be reversed

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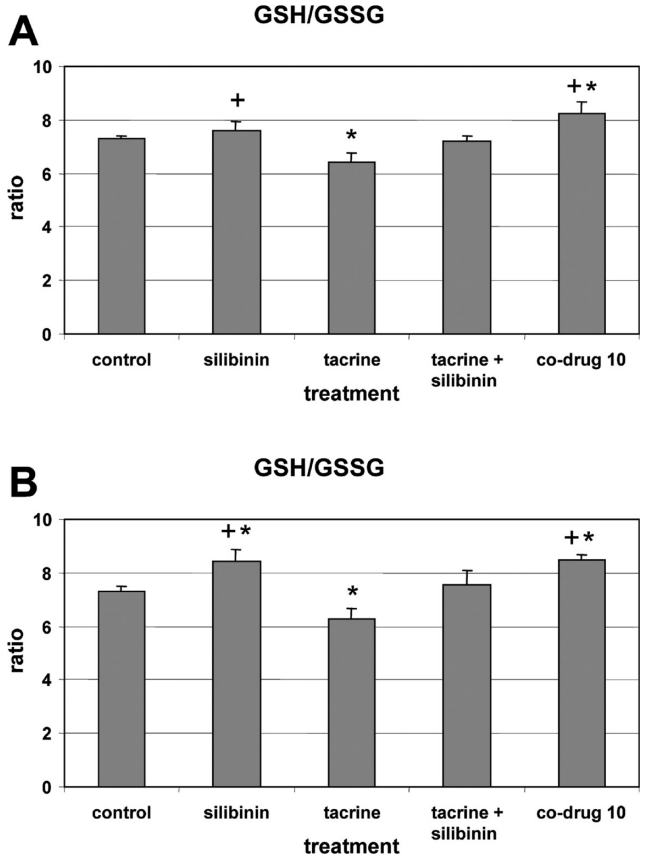

Figure 5. Ratio of the tissue content on reduced glutathione (GSH) and oxidized glutathione (GSSG) in the livers of female control rats and of female rats 24 h after treatment with 2 μmol/100 g body weight (A; therapeutic dosage) and in the livers of male control rats and of male rats 24 h after treatment with 6 μmol/100 g body weight (B; highest tolerated dosage of tacrine) silibinin, tacrine (**1**), tacrine/silibinin, or codrug (**10**), respectively. For statistical analysis, the Mann–Whitney test ($p \leq 0.05$) was applied. Arithmetic means ± SEM, n = 5–8; *, $p \leq 0.05$ vs control; +, $p \leq 0.05$ vs tacrine.

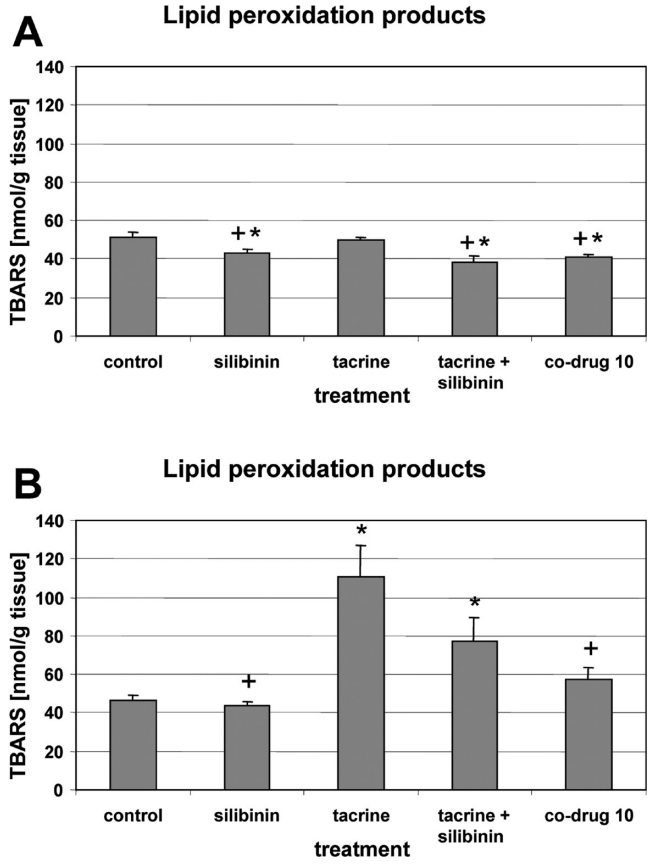

Figure 6. Tissue content on lipid peroxidation products as determined by thiobarbituric acid reactive substances (TBARS) in the livers of female control rats and of female rats 24 h after treatment with 2 μmol/100 g body weight (A; therapeutic dosage) and in the livers of male control rats and of male rats 24 h after treatment with 6 μmol/100 g body weight (B; highest tolerated dose of tacrine) silibinin, tacrine, tacrine plus silibinin, or codrug (**10**), respectively. For statistical analysis, the Mann–Whitney test ($p \leq 0.05$) was applied. Arithmetic means ± SEM, n = 5–8; *, $p \leq 0.05$ vs control; +, $p \leq 0.05$ vs tacrine.

effectively by the administration of M_1 (ortho- and allosteric) agonists or AChE inhibitors, such as tacrine.[9b,17] In our setting, various behavioral parameters can be investigated reflecting cognitive deficits and their compound-induced attenuation, such as the time to find food pellets or the number of pellets not found as well as the number of errors made by the rats while investigating the radial maze.[6,9b,43]

Figure 7 shows the effect of tacrine, silibinin, the equimolar mixture, and the codrug on scopolamine-induced amnesia measured as the number of food pellets not found plus the number of errors made in an eight-arm radial maze. Scopolamine induces cognitive deficits after 20 min, effects that fade after 2 h due to metabolism and excretion of scopolamine. This well-described effect is not altered by silibinin, but coadministration of tacrine significantly reduces the number of errors made which corresponds to an improvement of working memory. As expected, the same effect is observed for the equimolar mixture of tacrine and silibinin. The codrug shows the same pro-cognitive effect as tacrine (Figure 7), a statistically highly significant effect since 18 experiments were performed with 5 rats for each experiment. Therefore, despite slightly lower *in vitro* AChE and BChE inhibition and unknown metabolism of the codrug, the compound's *in vivo* efficacy is as high as that for tacrine.

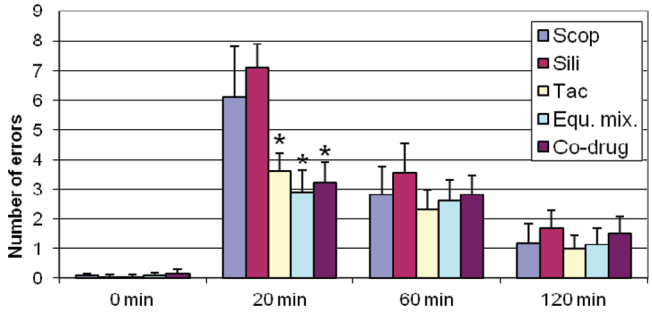

Figure 7. Influence of tacrine, silibinin, equimolar mixture of tacrine and silibinin, and codrug **10** (each 2 μmol in 1 mL/100 g b. wt.) on scopolamine (0.05 mg/100 g b. wt.) induced impairment of working memory in adult rats measured in an eight-arm radial maze. Arithmetic means ± SEM, n = 18 per group. Maximal run-time: 10 min. Parameter: errors made plus pellets not found after 10 min. Student's t test was used to assess significant differences (level of significance from scopolamine *p > 0.05).

CONCLUSIONS

A codrug of the natural product silibinin and the AChE inhibitor tacrine was synthesized, and its biological and

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pharmacological properties were investigated *in vitro* and *in vivo* and compared to an equimolar mixture of these two compounds. The codrug is a slightly less potent AChE and BChE inhibitor than tacrine. It did not show neurotoxic effects on a hippocampal cell line but exhibited a (saturable) neuroprotective effect against glutamate induced oxidative stress which could not be observed for the physical mixture. Since tacrine is a known hepatotoxic agent (the reason for withdrawal from the market), and silibinin was described as a hepatoprotective agent, the influence of both the codrug **10** and the physical mixture was compared to tacrine's effects both *in vitro* and *in vivo*. In hepatic stellate cells (HSC), the mixture did not exhibit a significant improvement over tacrine with respect to cell number; the codrug though was not cytotoxic even at high concentrations. Increase of mitochondrial fluorescence intensity, which was induced by tacrine, was used as an indicator of mitochondrial dysfunction and therefore hepatotoxic effect. Both the mixture and the codrug caused a significantly lower increase in mitochondrial fluorescence intensity than tacrine. Investigating the effects of tacrine, silibinin, the physical mixture of both, and the codrug on rats *in vivo* verified and enlarged the data obtained *in vitro*: Histopathologically, tacrine's toxicity was less pronounced for the mixture and completely absent for the codrug. This finding was further fostered by determination of the ratio GSH/GSSH, which was lowered by tacrine, stayed the same for the mixture, but was even increased for the codrug. Furthermore, the amount of lipid peroxidation products formed after tacrine administration was lowered at high tacrine dosage with coadministration of silibinin; again the codrug showed even superior effects. Co-administration of tacrine and silibinin slightly diminished the induction of CYP isoforms observed for tacrine, but the codrug exhibited no influence on the CYP enzymes (cf. Supporting Information for detailed presentation of results).

In an *in vivo*-model investigating scopolamine-induced deficits of working memory in rats, their memory was improved by administration of both tacrine (**1**) and the codrug (**10**) to the same extent.

Two important issues have not been addressed yet: the oral bioavailabilty of the codrug, which might be lower than the one of the components' mixture, and the investigation about which compounds/metabolites are actually responsible for hepatoprotection and the pro-cognitive effects and which time scale describes their formation. Quite an intensive investigation into stability especially in plasma is necessary for that, and with regard to oral bioavailabilty, further modifications of the chemical structure or pharmaceutical formulation might be necessary, which has not been the scope of this work. Nevertheless, this work has shown that a codrug represents a chemical entity in its own regard with superior pharmacological properties compared to the mixture of components.

Taken together, the silibinin−tacrine codrug exhibits pronounced pro-cognitive effects just like tacrine, but it lacks tacrine's therapy-limiting hepatotoxic effects completely both *in vitro* and *in vivo*, and additionally shows neuroprotective properties. This superior behavior could only be observed for the stable chemical connection within a codrug but was either less pronounced or absent for the physical equimolar mixture of tacrine and silibinin.

EXPERIMENTAL SECTION

Chemistry. *General Methods for Synthesis.* Melting points are uncorrected and were measured in open capillary tubes, using a Barnstead Electrothermal IA9100 melting point apparatus. ^{1}H and ^{13}C NMR spectral data were obtained from a Bruker Advance spectrometer (300 and 75 MHz, respectively). TLC was performed on silica gel on aluminum foils with a 254 nm fluorescent indicator (Fluka) or aluminum oxide on TLC-PET foils with a 254 nm fluorescent indicator (Fluka). For detection, iodine vapor or UV light (254 nm) were used. ESI-MS samples were analyzed using electrospray ionization ion-trap mass spectrometry in nanospray mode using a Thermo Finnigan LCQ Deca. The CHN analyses were undertaken using Perkin-Elmer Elemental Analyzer PE2400CHNS. For column chromatography, silica gel 60, 230−400 mesh (Merck) was used. In addition to mass spectrometry and NMR, purity was evaluated by high resolution mass and the following HPLC system (confirming purity ≥95%).

System 1. Analytical HPLC using a VWR HITACHI L-2130 pump coupled to a VWR HITACHI column oven L-2350, and L-2455 diode array detector. The solvents were as follows: (A) water + 0.05% trifluoroacetic acid and (B) acetonitrile + 0.05% trifluoroacetic acid; flow, 0.4 mL/min. Column Hibra 125-4 Purospher STAR RP-18e (3 μm) at 20 °C, detecting at 247 nm; solvent A from 100 to 60% for 25 min, then 60% for 10 min, and 100% for 5 min.

System 2. Analytical HPLC using a VWR HITACHI L-2130 pump coupled to a VWR HITACHI column oven L-2350, and L-2455 diode array detector. The solvents were as follows: (A) water + 0.1% formic acid and (B) 95% acetonitrile + 5% water + 0.1% formic acid; flow, 0.4 mL/min. Column Hibra 125-4 Purospher STAR RP-18e (3 μm) at 40 °C, detecting at 287 nm; solvent A from 80 to 0% for 30 min, then 0% for 10 min, and 80% for 5 min.

*N^1-(1,2,3,4-Tetrahydroacridin-9-yl)hexane-1,6-diamine (**9**).* The mixture of 9-chloroacridine **7**[7,18,19] (0.218 g, 1 mmol), hexamethylenediamine (0.23 g, 2 mmol), and catalytic amount of sodium iodide in 1-pentanol (10 mL) was warmed to reflux in a 160 °C oil bath for 24 h. Then another portion of hexamethylenediamine (0.23 g, 2 mmol) was charged, and the warming was continued for another 24 h during which time the reaction mixture was protected by nitrogen. After the reaction, the solvent was removed in vacuum, and then the crude product was purified by column chromatography, using chloroform/methanol/ammonia = 100:15:2 as the eluent system. The final product was yielded as a brown oil (0.22 g, 73%). ESI-MS: 298.4 *m/z* $[M + H]^+$. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.18−1.62 (m, 8H), 1.82−1.88 (m, 4H, $CH_2(CH_2)_2CH_2$ (tacrine)), 2.59−2.64 (m, 4H, $CH_2(CH_2)_2CH_2$ (tacrine)), 2.99−3.00 (m, 2H, $NH(CH_2)_5CH_2NH_2$), 3.41−3.44 (m, 2H, $NHCH_2(CH_2)_5NH_2$), 3.92 (br, 1H, NH), 7.26−7.29 (m, 1H, arom), 7.46−7.50 (m, 1H, arom), 7.84−7.90 (dd, 2H, *J* = 8.50 Hz, arom) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 23.20, 23.42, 25.21, 27.10, 27.26, 32.20, 34.00, 34.38 (8C, $NHCH_2(CH_2)_4CH_2NH_2$, $CH_2(CH_2)_2CH_2$ (tacrine)), 42.48 ($NH_2CH_2(CH_2)_5NH$), 49.90 ($NHCH_2(CH_2)_5NH_2$), 116.24, 120.58, 123.27, 124.05, 128.78, 129.03, 147.74, 151.27, 158.76 ppm. HR-MS: $C_{19}H_{29}N_3$ Calcd.: 298.2278 *m/z* $[M + H]^+$. Anal.: 298.2276 *m/z* $[M + H]^+$. HPLC purity (system 1), 96.53%; retention time, 21.65 min.

*4-(Benzyloxy)-4-oxobutanoic Acid (**5**).* A mixture of succinic acid **4** (8 g, 0.068 mol), benzyl bromide **3** (8.92 mL, 0.074 mol), and triethylamine (10.4 mL, 0.074 mol) in tetrahydrofuran (20 mL) was refluxed for 5 h. Ethyl acetate was added to the mixture and extracted with 15% sodium carbonate aqueous solution (3 × 30 mL). The combined aqueous phases were washed with diethyl ether (30 mL), acidified with 4 N HCl to pH 1−2, and extracted with ethyl acetate (4 × 30 mL). The organic phases were combined, washed with brine (50 mL), dried over anhydrous sodium sulfate, and concentrated under vacuum. Compound **5** was yielded as a white solid (9.3 g, 66%). M.P.: 56−58 °C. ESI-MS: 207.1 *m/z* $[M - H]^-$. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.71 (s, 4H, $CO(CH_2)_2CO_2H$), 5.18 (s, 2H, $PhCH_2O$), 7.38 (s, 5H, arom) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 28.91, 28.95 ($CO(CH_2)_2CO_2H$), 66.69 (PhCH2O), 127.71, 128.24, 128.33, 128.44,

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128.61 (arom CH), 140.64 (arom CCH_2), 172.07 ($CH_2O_2C(CH_2)_2CO_2H$), 178.26 (CO_2H) ppm.

*4-(((2S,3S)-3-(4-Hydroxy-3-methoxyphenyl)-6-((2R,3R)-3,5,7-trihydroxy-4-oxochroman-2-yl)-2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy)-4-oxobutanoic Acid (**6**).* To the precooled solution of silibinin **2** (1 g, 2 mmol), compound **5** (0.8 g, 4 mmol), and triphenylphosphine (1.4 g, 5.2 mmol) in dry tetrahydrofuran (10 mL) was added dropwise the solution of diisopropyl azodicarboxylate (1.02 mL, 5.2 mmol) in dry tetrahydrofuran (10 mL) in an ice bath. The mixture was then allowed to warm to room temperature gradually after the addition, and it was stirred overnight at room temperature. The mixture was concentrated after the reaction. The residue was dissolved in ethanol (20 mL), and Pd/C (5%, 0.1 g) was added. The reaction mixture was placed under hydrogen and stirred vigorously at 50 °C for 4 h. The catalyst was filtered off, and the filtrate was concentrated under vacuum. The residue was purified via column chromatography by using dichloromethane/methanol 7:1 as the eluent system. Compound **6** was yielded as a colorless oil (500 mg, 40% over two steps). ESI-MS: 581.1 m/z $[M - H]^-$. ^{1}H NMR (MeOD-d_4, 300 MHz) δ: 2.53−2.56 (m, 4H, $COCH_2CH_2CO_2H$), 3.82 (s, 3H, OCH_3), 3.91−3.95 (m, 1H, C(10)*H*), 4.17−4.24 (m, 2H, C(23)H_2O), 4.40−4.44 (d, 1*H*, C(2)H, J = 11.61 Hz), 4.77−4.81 (m, 1H, C(11)*H*), 4.88−4.92 (d, 1H, C(3)*H*, J = 11.60 Hz), 5.88−5.92 (m, 2H, C(6)*H*, C(8)*H*), 6.82−7.08 (m, 6H, arom) ppm. ^{13}C NMR (MeOD-d_4, 75 MHz) δ: 30.27, 30.43 ($CH_2O_2C(CH_2)_2CO_2H$), 56.73 (OCH_3), 64.33 (H_2C(23)), 73.74 (*C*(3)), 77.23 (*C*(10)), 77.86 (*C*(11)), 84.53 (*C*(2)), 96.70 (*C*(8)), 97.65 (*C*(6)), 101.92 (*C*(4a)), 112.08 (*C*(13)), 116.57 (*C*(16)), 117.90 (*C*(18)), 118.08 (*C*(21)), 121.86 (*C*(15)), 122.49 (*C*(22)), 128.83 (*C*(14)), 131.75 (*C*(17)), 145.05 (*C*(19), *C*(12a)), 148.46 (*C*(20)), 149.29 (*C*(16a)), 164.40 (*C*(8a)), 165.26 (*C*(5)), 168.78 (*C*(7)), 174.08 (CO_2CH_2), 177.05 (CO_2H), 198.24 (O=*C*(4)) ppm.

*((2S,3S)-3-(4-Hydroxy-3-methoxyphenyl)-6-((2R,3R)-3,5,7-trihydroxy-4-oxochroman-2-yl)-2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methyl 4-oxo-4-(6-(1,2,3,4-tetrahydroacridin-9-ylamino)hexylamino)butanoate Hydrochloride (Codrug **10**).* The solution of silibinin hemisuccinate **6** (400 mg, 0.69 mmol) in the mixture of *N*,*N*-dimethylfomamide (10 mL) and toluene (20 mL) was concentrated under vacuum until all toluene was removed. Then, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (140 mg, 0.72 mmol) and 1-hydroxybenzotriazole (50 mg) were added to the above solution. The resulting solution was stirred at room temperature for 0.5 h. In the mean time, a solution of **9** (205 mg, 0.69 mmol) in a mixture of *N*,*N*-dimethylformamide (10 mL) and toluene (20 mL) was also concentrated under vacuum until all toluene was removed. The resulting solution was added to the above solution dropwise at room temperature. The reaction solution was stirred overnight under nitrogen. Afterward, the solvent was removed under vacuum. The residue was dissolved in chloroform/methanol 6:1 (10 mL) and was acidified with HCl/iPrOH (5 M, 2 mL). The resulting solution was concentrated. The residue was purified via column chromatography using chloroform/methanol 6:1 as the eluent system. The target compound **10** was yielded as yellow solid (350 mg, 59%). M.P.: 135−138 °C. ESI-MS: 862.4 m/z $[M + H]^+$. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.26−1.91 (m, 12H, $CONHCH_2(CH_2)_4CH_2NH$, $CH_2(CH_2)_2CH_2$ (tacrine)), 2.42−2.47 (t, 2H, $CH_2(CH_2)_2CH_2C{=}N$ (tacrine), J = 6.38 Hz), 2.58−2.65 (m, 4H, $CH_2O_2C(CH_2)_2CONH$), 2.85 (s, 1H, C(3)O*H*), 2.95−2.97 (m, 2H, $CH_2(CH_2)_2CH_2C{=}N$ (tacrine), 3.07−3.12 (t, 2H, $CONHCH_2(CH_2)_5NH$, J = 7.00 Hz), 3.50−3.66 (m, 2H, $CONH(CH_2)_5CH_2NH$), 3.85 (s, 3H, OCH_3), 3.92−3.97 (m, 1H, C(10)*H*), 4.20−4.27 (m, 2H, C(23)H_2O_2C), 4.35−4.39 (d, 1H, C(2)*H*, J = 11.5 Hz), 4.80−4.89 (m, 2H, C(3)*H*, C(11)*H*), 5.82−5.89 (m, 2H, C(6)*H*, C(8)*H*), 6.82−7.08 (m, 6H, arom (silibinin)), 7.47−7.51 (m, 1H, arom (tacrine)), 7.72−7.76 (arom (tacrine)), 8.22−8.24 (m, 1H, arom (tacrine)) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 24.69, 25.76, 27.49, 29.96, 30.05, 32.48, 32.90, 33.15, 34.06, 34.33, 42.95, 51.97 (12C, $CONH(CH_2)_6NH$, $CH_2(CH_2)_2CH_2$ (tacrine), $O_2CCH_2CH_2CONH$)), 59.54 (OCH_3), 66.92, 67.16, 76.25, 80.24, 86.88, 86.98, 99.37, 99.75, 103.98, 104.05, 114.30, 115.75, 119.22, 120.02, 120.47, 102.76, 123.83, 124.35, 125.17, 128.79, 128.94, 131.01, 136.36, 143.27, 143.42, 147.56, 147.59, 151.09, 151.90, 154.76, 159.89, 166.67, 176.26, 176.43 (2C, $O_2CCH_2CH_2CONH$), 199.81(C(4)) ppm. HR-MS: $C_{48}H_{51}N_3O_{12}$ Calcd.: 862.3546 m/z $[M + H]^+$ Anal.: 862.3547 m/z $[M + H]^+$. HPLC purity (system 2), 96.06%; retention time, 15.46 min.

EXPERIMENTAL PROCEDURES FOR THE PHARMACOLOGICAL INVESTIGATIONS

Acetyl- and Butyrylcholinesterase Inhibition Assay. AChE (E.C.3.1.1.7, Type VI−S, from electric eel) and BChE (E.C.3.1.1.8, from equine serum) were used, and the assay was performed as previously described (cf. Supporting Information).[6,16]

Neurotoxicity and Protection Assay. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to previously described procedures.[16,44,45] Briefly, cells were seeded in 96-well plates at a density of 5×10^3 per well and cultured for 24 h. Subsequently, cells were incubated for another 24 h either with medium, compounds, or solvent only in the absence (neurotoxicity assay) or presence (neuroprotection assay) of 5 mM glutamate (monosodium-L-glutamate, Merck, Darmstadt, Germany). Quercetin (Sigma, Steinheim, Germany) in a concentration of 25 μM served as the positive control in the neuroprotection assay. All compounds were dissolved in DMSO and diluted with fresh medium. DMSO concentration in final dilutions was ≤0.1%. MTT (Sigma, Steinheim, Germany) solution (4 mg/mL in PBS) was diluted 1:10 with the medium, and the mixture was added to the wells after the removal of previous medium. The plates were then incubated for further 3 h. Afterward, supernatants were removed, and 100 μL of sodium dodecyl sulfate (10%) was added to the wells. Absorbance at 560 nm was determined on the next day with a multiwell plate reader (Tecan, Crailsheim, Germany). Results of cell viability are expressed as the percentage to untreated control cells.

Determination of Hepatotoxicity by Crystal Violet Assay. Cells were seeded in 96-well plates at a density of 5×10^3 per well and cultured for 24 h. Then cells were treated with medium, medium with solvent (0.1% DMSO), or with test compounds, namely, silibinin (PhytoLab, Vestenbergsgreuth, Germany), tacrine, an equimolar mixture of silibinin and tacrine, respectively, and the codrug in different concentrations (1−200 μM) for another 24 h. Subsequently, the medium was carefully removed, and the cells were incubated with 50 μL of crystal violet solution (0.5% in 20% methanol) per well for 10 min at room temperature. Staining solution was aspirated, and cells were washed three times with ultra pure water. After drying plates overnight, 100 μL of sodium citrate buffer (EtOH + 0.1 M sodium citrate (1:1, v/v)) was added to each well and absorbance of the solution was determined at 560 nm using a multiwell plate reader (Tecan, Crailsheim, Germany).

Fluorescent Microscopy and Image Analysis. For fluorescence microscopic analysis, cells were seeded in 96-well plates at a density of 5×10^3 per well and cultured for 24 h. Then cells were treated for another 24 h either with medium, medium with solvent, or with test compounds. Then, the cells were incubated with a 1:2000 dilution of MitoTracker Red CMXRos (Molecular Probes, Invitrogen) for 25 min at 37 °C. Subsequently, nuclei were stained with Hoechst33342 (bisBenzimide H 33342 trihydrochloride, Sigma-Aldrich, 20 mM, 1:1200) for 5 min. Afterward, cells were fixed with 3% paraformaldehyde for 30 min at room temperature and then shortly treated with permeabilization buffer (PBS with 0.1% Triton X-100) to improve signal-to-noise ratio.

Automatic image acquisition was carried out using a Carl Zeiss Axio Observer (Carl Zeiss, Göttingen, Germany) with Software AxioVision 4.8.1 (Carl Zeiss MicroImaging, Germany), motorized stage, AxioCam HRm, a Plan-Neofluar 10× objective, and appropriated filters for the fluorescent dyes.

Image analysis was done automatically by the ASSAYbuilder Physiology Analyst software. Cell nuclei were identified by the software and used to automatically detect and count the cells. The mean fluorescence intensity of detected mitochondria spots was measured within a defined ring mask over and around the nucleus.

Statistical Analysis (*in Vitro* Hepatoprotection and Neuroprotection). In the cell-based assays, results are presented as the mean ± SD and refer to untreated control cells which were set as 100% values. If not mentioned otherwise, experiments were carried out with three parallels and repeated independently at least three times. Statistical analysis was performed using GraphPad Prism 4 Software. Levels of significance: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

***In Vivo* Studies on Hepatotoxicity/Hepatoprotection and on the Interaction Capacity with the Cytochrome P450 System.** For the two experimental settings used (2 and 6 μmol/100 g body weight), the rats were randomly assigned to different treatment groups (n = 5−8 each): (1) control rats, receiving the solvent 0.9% NaCl i.p., (2) silibinin treated rats, (3) tacrine treated rats, (4) rats given the mixture of tacrine and silibinin, and (5) codrug **10** treated rats. All substances were given i.p. Twenty-four hours after drug administration, the animals were sacrificed, and the livers were removed for histological and biochemical investigations. For details concerning the biochemical and histological investigations, see Supporting Information.

Behavioral Studies. We examined whether the codrug could improve spatial memory impairment induced by scopolamine using performance in an eight-arm radial maze (RAM).[43a]

Each arm (44 cm L, 30 cm H, 14 cm W) radiated from an octagonal platform that served as a starting point. A food cup (3 cm diameter) was located at the end of each arm. The entire arms and food cups were painted gray and placed in a dark and calm room. Animal behavior was monitored by a video camera. Image analysis and pattern recognition from the monitor were performed by a VideoMot 2 program (video tracking, motion analysis, and behavior recognition system) provided by TSE Systems (Bad Homburg, Germany). The computerized recording systems were located in the same room.

After a short handling period in which the rats were in close contact with the laboratory staff, three rats experienced free movement and feeding in the RAM once a day for 5 days to adapt to the maze. The baits (Dustless Precision Rodent Pellets, Bilaney Consultants Ltd., Sevenoaks, Kent, U. K.) were scattered in all arms. After handling and adaptation of the rats to the maze, food was restricted to reduce the rat's body weight by 10%.

Training trials: Each rat was placed once daily in the center of the RAM to visit all eight arms and eat all reward food baits in each food cup. Each trial was performed until the rat entered and ate all the pellets in the eight arms, or made 16 errors (re-entry into an arm that has been previously visited), or 10 min elapsed.

Memory impairment by scopolamine was induced in rats trained to the criteria of trials at three consecutive days. Before the administration of substances, a control run was performed. Thus, each animal was its own control. The following parameters were registered 20, 60, and 120 min after scopolamine: number of errors and time needed to visit all arms (total exploration time). The trial was finished after either the rat had eaten all pellets, 10 min had elapsed, or 16 errors were made.

Experimental groups: (1) 0.05 mg scopolamine/100 g b.wt. (scop); 5 min after scop administration coadministration of (2) 2 μmol silibinin (sili), (3) 2 μmol tacrine (tac), (4) 2 μmol equimolar mixture of tacrine and silibinin (equ. mix.), and (5) 2 μmol codrug (codrug)/100 g b. wt. for each coadministration. Solutions were injected intraperitoneally (i.p.).

■ ASSOCIATED CONTENT

Supporting Information

Experimental and spectral data of synthetic precursors; detailed information about cytochrome P450 isoform expression and activity; and information on cell culture and cell lines, statistics, information on animals, biochemical and histological procedures, and statistics of behavioral studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This article is dedicated to Professor John L. Neumeyer on the occasion of his 82nd birthday.

■ ABBREVIATIONS USED

ACh(E), acetylcholine(esterase); AD, Alzheimer's disease; BBB, blood−brain barrier; BChE, butyrylcholinesterase; CAS, catalytic anionic site; CNS, central nervous system; CYP, cytochrome P450; DCM, dichloromethane; DIAD, diisopropyl azodicarboxylate; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; HOBt, hydroxybenzotriazole; HSC, hepatic stellate cells; M receptor, muscarinic acetylcholine receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SAR, structure−activity relationship; TBARS, thiobarbituric acid reagible substances; TFA, trifluoroacetic acid; THF, tetrahydrofuran

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