Design, synthesis and *in vitro* evaluation of indolotacrine analogues as multi-target-directed ligands for the treatment of Alzheimer's disease

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Abstract

In this study, novel indolotacrine analogues have been designed, synthesized and evaluated as potential drugs for the treatment of Alzheimer's disease. Based on a multi-target-directed ligand approach, novel compounds have been designed to act simultaneously as cholinesterase and monoamine oxidase (MAO) inhibitors. Prepared compounds were also evaluated for their antioxidant, cytotoxic, hepatotoxic and permeability (Blood-Brain Barrier penetration) properties. Indolotacrine **9b** (9-methoxy-2,3,4,6-tetrahydro-1*H*-indolo[2,3-*b*]quinolin-11-amine) showed the most promising results in the *in vitro* assessment being a potent inhibitor of acetylcholinesterase (IC_{50} = 1.5 μM), butyrylcholinesterase (IC_{50} = 2.4 μM) and monoamine oxidase A (IC_{50} = 0.49 μM) and a weak inhibitor of monoamine oxidase B (IC_{50} = 53.9 μM). Although its cytotoxic (IC_{50} = 5.5 ± 0.4 μM) and hepatotoxic (IC_{50} = 1.22 ± 0.11 μM) profile is not as good as the standard 7-methoxytacrine (IC_{50} = 63 ± 4 μM and IC_{50} = 11.50 ± 0.77 μM respectively), the overall improvement in the inhibitory activities and potential to cross blood-brain barrier make indolotacrine **9b** a promising lead compound for further development and investigation.

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by progressive and irreversible cognitive impairment and memory loss.[1] Despite enormous efforts the aetiology of AD has not yet been elucidated and the disease remains incurable.[2] According to current knowledge, beta-amyloid (Aβ) aggregates,[3] τ-protein phosphorylation,[4] oxidative stress[5] and deficits of acetylcholine (ACh)[6] are considered to play significant roles in AD pathophysiology.

The cholinergic hypothesis asserts that the decreased level of ACh in the brain leads to cognitive and memory deficits and that sustaining or recovering the cholinergic function should therefore result in amelioration of the symptoms.[7–9] Accordingly, current AD therapy is mainly based on ACh esterase inhibitors (AChEIs), which are able to increase ACh levels in cholinergic synapses. To date, the number of approved drugs is limited to three AChEIs (rivastigmine, donepezil and galantamine) and a NMDA antagonist (memantine). However, these drugs cannot prevent or cure the disease but afford only symptomatic treatment.[9,10]

The ''one-target, one-compound'' paradigm has been highly successful in the past for many common diseases because their underlying molecular mechanisms were understood, allowing biologists to define the key target for a particular disease. Once the target was identified, medicinal chemists strategically designed a molecule to interact selectively with such a target, with a potential drug as the outcome. However, it is apparent that this target-based approach does not always guarantee success. Drugs directed to a single target might not always modify complex multifactorial diseases such as AD, even if they act in the way they are expected to proceed.[11] It is now widely accepted that a more effective therapy would result from the use of multipotent compounds able to intervene simultaneously in the different pathological events underlying the aetiology of AD.[12,13]

Monoamine oxidase (MAO; EC 1.4.3.4) is another important target that was considered for the treatment of AD because some symptoms of AD are due to alterations in the dopaminergic, serotoninergic and other monoaminergic neurotransmitter systems.[14,15] Moreover, MAO catalysed oxidative deamination gives arise to production of hydrogen peroxide and, consequently, reactive oxygen species that have also been implicated in the progress of AD.[16] MAO inhibitors (MAOI) should increase monoaminergic neurotransmission and reduce reactive oxygen species formation, both effects potentially valuable for the treatment of AD.[13,15] Thus, in this context, multipotent molecules able to simultaneously bind both ChEs and MAOs have been investigated.[17–20]

The aim of this preliminary communication was to develop novel multi-target-directed ligands (MTDLs) acting primarily as MAO and cholinesterase inhibitors. For this purpose we chose structural motifs contained in previously described MAO and/or ChEIs and incorporated them into the scaffold of the novel compounds. Two distinct series of molecules have been designed. The first series containing 2-aminoindole-3-carbonitrile structural scaffold (further referred as *"indole"* series; compounds **4a**-**c** and **8c**) employs an indole ring, that is a structural core in several MAOIs or dual-acting compounds targeting both MAO and ChEs, such MBA236 (Figure 1),[21,22] and the *β*-aminonitrile motif found in some previously identified MAOI.[23] Compounds **4b**,**c** also contain the propargylamine moiety, which is an essential part of many neuroprotective, irreversible MAOIs (Figure 1).[24] Originally, only compounds **4a**,**b** had been designed, however, during the synthesis of **4b** a side-product **4c** was isolated. Because of the low yield obtained, **4c** was tested only for its inhibitory activity against MAO. Compound **8c** was synthesized later on to explore, whether the *N*-allyl or *N*-propargyl substitution on the amino group in position two is important for MAO inhibition

and also to validate the importance of the phenolic group for the antioxidant activity of other compounds in the series (discussed later in the text).

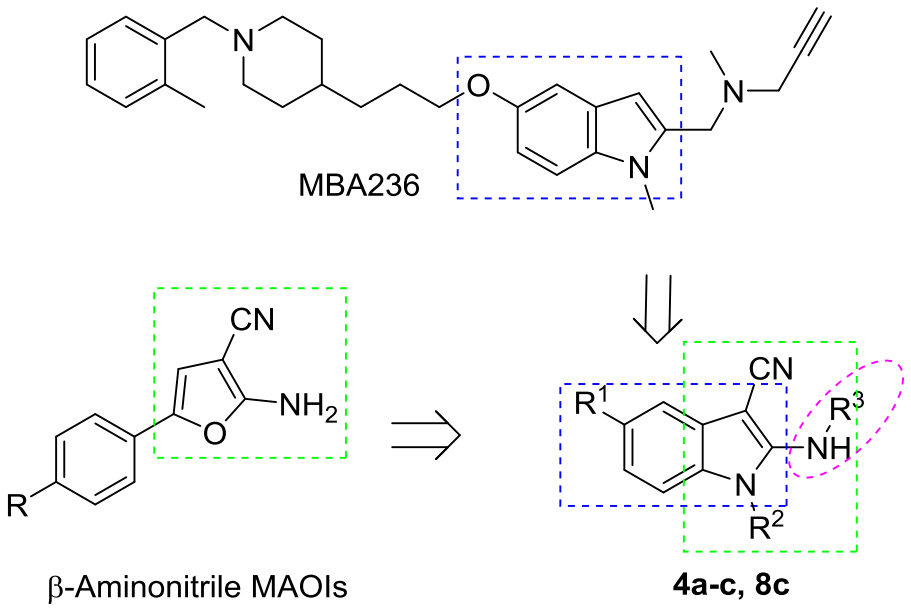


Figure 1: Design of *indole* series.

The second series was then designed employing the 2,3,4,6-tetrahydro-1*H*-indolo[2,3-*b*]quinolin-11-amine structural scaffold (described hereafter as the *"indolotacrine"* series; compounds **9a**,**b** and **13**) to improve the unsatisfactory anti-ChE activity of the *indole* series. For this purpose the 2-aminoindole-3-carbonitrile scaffold of the *indole* series was fused with the structure of potent ChEI tacrine or 7-methoxytacrine (7-MEOTA). Moreover, the resulting indolotacrines also resemble *β*-carboline alkaloids (e.g. harmine), which are known MAOI (Figure 2).[25,26] Since compound **4c** with *N*-propargyl substitution in position 1 was found to be the most potent MAOI within the indole series, it was decided to preserve this potentially favourable motif when designing compound **13** with benzyl substitution analogous to former *N*-propargyl moiety.

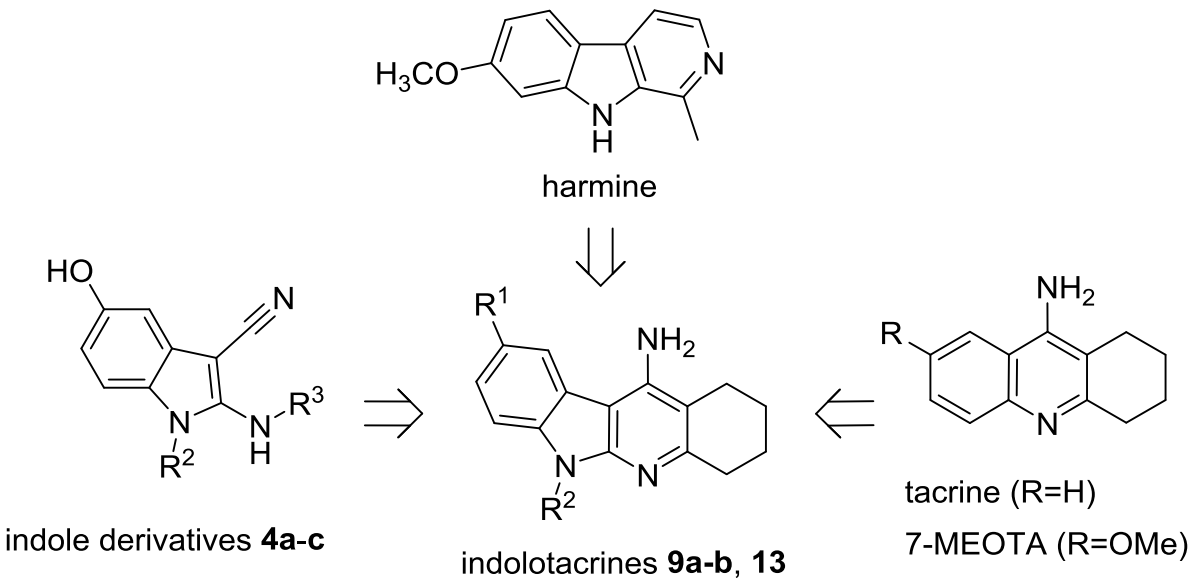
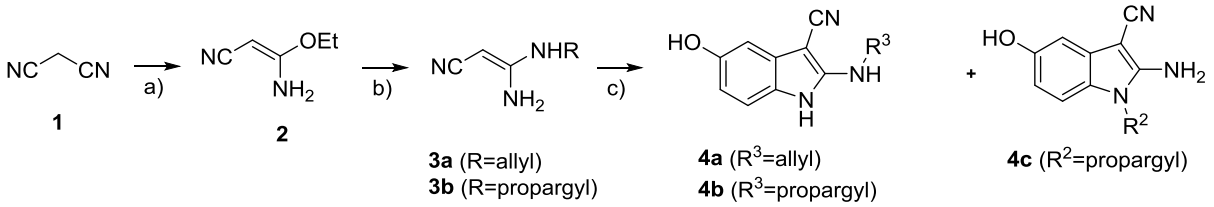


Figure 2: Design of *indolotacrine* series.

5-Hydroxy-1*H*-indole-3-carbonitrile derivatives **4a**-**c** were prepared in three steps (Scheme 1). At first malononitrile (**1**) was treated with ethanol in diethyl ether saturated with HCl (g) to obtain 3-amino-3-ethoxyacrylonitrile (**2**). In next step acrylonitrile **2** was treated with the corresponding alkylamine to give *N*-alkylated 3,3-diaminoacrylonitriles **3**. Lastly, diaminoacrylonitriles **3** were treated with *p*-benzoquinone to give 2-(alkylamino)-5-hydroxy-1*H*-indole-3-carbonitriles **4a**,**b**.[27] Moreover, a by-product, whose structure was assigned as the alkylated in position 1 (**4c**), was also isolated from the reaction of 3-amino-3-(prop-2-yn-1-ylamino)acrylonitrile (**3b**).



1 **2**

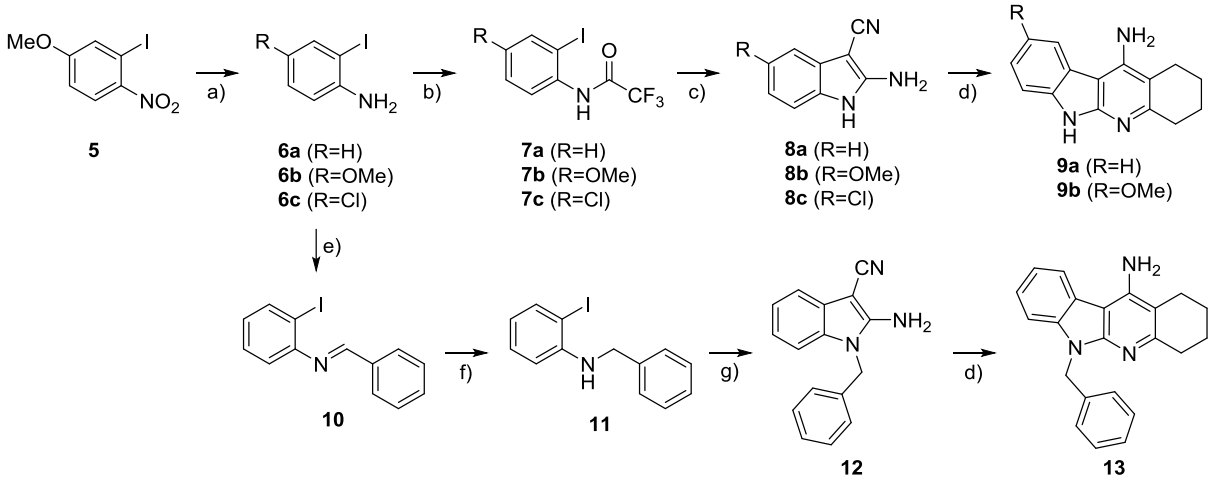
3a (R=allyl)
3b (R=propargyl)

4a (R^3=allyl)
4b (R^3=propargyl)

4c (R^2=propargyl)

Scheme 1: Synthesis of indole series (**4a – 4c**). *Reagents and conditions*: a) HCl, EtOH, Et_2O, 0˚C – rt, 4h, 18%; b) alkylamine, EtOH, rt, overnight, 61-77%; c) *p*-benzoquinone, EtOH, rt, 1h, 10-22%.

Indole **8c** and indolotacrines **9a**,**b** were prepared in two to four steps using a similar synthetic approach (Scheme 2). The synthesis of compound **9b** started from commercial 2-iodo-4-methoxy-1-nitrobenzene (**5**) which was reduced using Fe powder and ammonium chloride to the corresponding aniline derivative **6b**. Intermediates **6a** and **6c** were obtained as a commercial chemicals. Further, the synthesis proceeded identically for three compounds. Corresponding 2-iodoaniline derivatives **6a – 6c** were treated with trifluoroacetic anhydride to give the trifluoracetamides **7a** - **7c**, which were then used for the copper iodide catalysed cyclization with malononitrile to obtain corresponding indole derivatives **8a** - **8c**.[28] Finally, indolotacrines **9a** and **9b** were prepared using microwave-assisted Friedländer reaction[29] of corresponding indole **8a – 8b** with cyclohexanone.



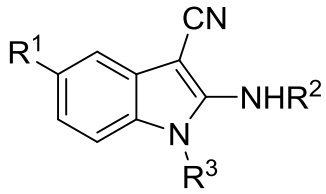
Scheme 2: Synthesis of indolotacrines **9a**, **9b**, **13** and indole **8c**. *Reagents and conditions*: a) Fe, NH_4Cl, MeOH/H_2O (3:1), 50˚C, 2h, 79%; b) trifluoroacetic anhydride, Et_3N, THF, -7˚C – rt, overnight, 97-99%; c) malononitrile, L-proline, K_2CO_3, CuI, DMSO/H_2O (1:1), 60˚C, overnight, 48-90%; d) cyclohexanone, $AlCl_3$, 1,2-dichloroethane, microwave irradiation, 95˚C, 2h, 16-54%; e) benzaldehyde, MeOH, rt, overnight, 97%; f) $NaBH_3CN$, AcOH/MeOH, 0˚C – rt, overnight, 75%; g) malononitrile, picolinic acid, K_2CO_3, CuI, DMSO, microwave irradiation, 90˚C, 12h, 26%.

Indolotacrine **13** was prepared by a slightly different synthetic procedure in four steps (Scheme 2). Firstly, 2-iodoaniline **6** was treated with benzaldehyde to give imine **10**, which was then reduced to corresponding amine **11** using $NaBH_3CN$. In next step cyclization of amine **11** with malononitrile gave indole **12.**[30] In final step the Friedländer reaction[29] of **12** with cyklohexanone gave indolotacrine **13**.

For the biological evaluation, all final products (Figure 3) were transformed into better water-soluble hydrochlorides by stirring them in diethyl ether saturated with HCl (g).

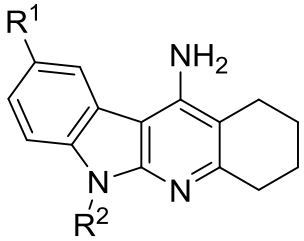
Figure 3: a) Indole and b) indolotacrine analogues prepared in this study.

a)



4a R^1=OH, R^2=allyl, R^3=H
4b R^1=OH, R^2=propargyl, R^3=H
4c R^1=OH, R^2=H, R^3=propargyl
8c R^1=Cl, R^2=H, R^3=H

b)



9a R^1=H, R^2=H
9b R^1=OMe, R^2=H
13 R^1=H, R^2=benzyl

Both series were assayed *in vitro* for their inhibitory activity against membrane-bound MAO A and MAO B (Table 3). All the *indoles* were potent and unselective MAOIs, with **4c** being the best inhibitor of both isoenzymes in the series. Indoles **4a**-**c** were evaluated for irreversible inhibition and, unexpectedly, none of compounds showed significantly lower IC_{50} value after 30 min pre-incubation with enzyme, despite compounds **4b**,**c** have the *N*-propargylamine moiety, which is present in many known irreversible MAOIs (e.g. deprenyl, clorgyline or rasagiline). This could be due to the change in the electron density on the triple bond of the *N*-propargyl motif, as its connecting nitrogen atom is here part of the aromatic system, in contrast to the known irreversible inhibitors where the *N*-propargylamine moiety is separated from the aromatic system usually by an alkyl linker. Alternatively, steric hindrance from the carbonitrile substituent could prevent the generation of the reactive intermediate or its modification of the enzyme. Based on this finding, we decided to investigate whether the *N*-allyl or *N*-propargyl substitution is necessary for MAO inhibition and so we synthesized compound **8c**. Evaluation revealed that indole **8c** devoid of any *N*-alkyl substitution on the amino group at position 2 retained the inhibitory activity at level similar to other indoles, showing that the propargyl moiety does not contribute to the binding.

Indolotacrine **9b** retained the inhibitory activity for both MAO isoenzymes, however, **9a** inhibited only MAO A and **13** with an extra *N*-benzyl substitution showed no inhibition of either MAO isoenzyme. It could be assumed that the extended spatial size of **13** would prevent entry into the active site of MAO enzymes.[31] In addition, compound **9a** was tested for inactivation of MAO A but it showed the expected reversible mode of inhibition. Unlike the unselective indole analogues, indolotacrines **9a**,**b** both exerted some selectivity towards MAO A inhibition with **9b** being the most potent MAO A inhibitor among all the compounds tested (IC_{50} = 0.49 μM). Standards tacrine and 7-MEOTA showed only moderate activity being poorer inhibitors of both MAO isoenzymes than the indolotacrine **9b**.

Table 3: Inhibition of MAO A and MAO B.

Compound	MAO A IC_{50} ± SD (μM)	MAO B IC_{50} ± SD (μM)	SI[b]	MAO A 30'[c] IC_{50} ± SE (μM)	MAO B 30'[c] IC_{50} ± SE (μM)
4a	2.32 ± 0.26	2.02 ± 0.56	0.9	1.78 ± 0.33	10.86 ± 0.78
4b	1.32 ± 0.12	1.70 ± 0.40	1.3	1.80 ± 0.56	2.48 ± 0.32
4c	0.68 ± 0.08	1.62 ± 0.35	2.4	0.45 ± 0.03	0.87 ± 0.10
8c	2.80 ± 0.40	3.89 ± 0.02	1.4	-	-
9a	11.40 ± 1.10	> 100	8.8	30.0 ± 1.9	-
9b	0.49 ± 0.05	53.90 ± 10.70	110.0	-	-
13	> 100	> 100	-	-	-
tacrine	14.07 ± 1.47	317.2 ± 201.0	22.5	-	-
7-MEOTA	7.10 ± 0.03	98.61 ± 14.63	13.9	-	-

[a] IC_{50} and SD/SE values were obtained as a mean of 2 independent measurements

[b] selectivity index = IC_{50} MAO B / IC_{50} MAO A

[c] IC_{50} values after 30 min pre-incubation of enzyme with inhibitor

All final compounds with the exception of **4c** (which was a by-product of synthesis and, due to low yield, was tested only for MAO inhibition) and **8c** (prepared subsequently to enhance SAR on MAO inhibition and antioxidant activity) were assayed *in vitro* for their inhibitory activity against human recombinant acetylcholinesterase (AChE) and human plasma butyrylcholinesterase (BChE) (Table 4).

No significant inhibitory activity against AChE or BChE was detected for *indoles* **4a**,**b**. Both compounds exerted only poor inhibition of AChE in high micromolar range and were found to be inactive against BChE at the highest concentration tested (50 μM). A possible explanation for this observation is that compounds **4a**,**b** lack the structural complexity of other indoles or indanes, which are capable of ChE inhibition (e.g., the extra *N*-benzylpiperidine moiety present in donepezil, ASS234 and MBA236 or the carbamate moiety of ladostigil).[22] Conversely, indolotacrines **9a**,**b** were found to be potent unselective inhibitors of both ChE enzymes with IC_{50} values in low micromolar range, and compound **13** was a selective BChEI. None of the compounds were better than tacrine, but compound **9b** was a better inhibitor of both ChEs compared to 7-MEOTA. IC_{50} values obtained for standard inhibitors tacrine and 7-MEOTA were in good agreement with previously published results.[32]

Table 4: Inhibition of AChE and BChE.

Compound	AChE IC_{50} ± SEM (μM)	BChE IC_{50} ± SEM (μM)	SI[b]
4a	319.2 ± 15.9	> 1000	3.1
4b	101.9 ± 5.4	> 1000	9.8
9a	11.6 ± 0.6	4.7 ± 0.1	0.4
9b	1.5 ± 0.1	2.4 ± 0.1	1.6
13	> 1000	1.09 ± 0.07	0.001
tacrine	0.32 ± 0.01	0.088 ± 0.001	0.3
7-MEOTA	10.0 ± 1.0	17.6 ± 0.8	1.8

[a] IC_{50} and SEM values were obtained as a mean of 3 independent measurements

[b] selectivity index = IC_{50} BChE / IC_{50} AChE

Additionally, as ROS are likely to play a part in the development and progression of AD,[33] the compounds were evaluated for their antioxidant activity using DPPH assay (Table 5). Indoles **4a**,**b** showed promising antioxidant properties, similar to the standard *N*-acetylcysteine and only slightly weaker than trolox. We hypothesized that this could be due to the presence of phenolic group, which is a key structural motif common of many antioxidants.[34] To prove this assumption we synthesized compound **8c**, where the phenolic group was replaced with chlorine. Evaluation showed, in good correlation with our hypothesis, that indole **8c** exerts more than 20 times weaker antioxidant activity compared to phenolic compounds **4a** and **4b**. Neither the indolotacrines nor tacrine or 7-MEOTA showed any significant antioxidant activity, which is not surprising, as they all lack the phenolic group responsible for this activity as demonstrated for the indoles. Introduction of the phenolic moiety therefore presents a possible improvement of the indolotacrine compounds for the future.

Table 5: Antioxidant activity and cytotoxicity of prepared compounds.

Compound	Antioxidant activity EC_{50} ± SEM (μM)	Cytotoxicity IC_{50} ± SEM (μM)
4a	37.86 ± 5.01	> 1000
4b	25.82 ± 1.35	> 1000
8c	731.70 ± 27.17	113 ± 29
9a	> 5000	13.0 ± 1.4
9b	> 5000	5.5 ± 0.4
13	3827.0 ± 227.1	7.0 ± 0.7
tacrine	> 5000	248 ± 11
7-MEOTA	> 5000	63 ± 4

N-acetylcystein	27.91 ± 1.82	-
trolox	16.20 ± 0.42	-

[a] EC_{50}/IC_{50} and SEM values were obtained as a mean of 3 independent measurements

Next, the cytotoxicity of the compounds was evaluated using the MTT assay on the CHO-K1 cell line (Table 5). *Indoles* were found to possess very low toxicity with IC_{50} values above the measurable range (>1000 μM) in case of **4a**,**b** and at high microlomolar range for **8c**. All indolotacrines exerted similar level of cytotoxicity with IC_{50} values around 10 μM. Standards 7-MEOTA and tacrine were both found to be less toxic, with tacrine being the least toxic compound among the series *in vitro*. This could be considered quite a surprising result as it is known that *in vivo* tacrine is more toxic than 7-MEOTA.[35]

Assuming that the principal target of tacrine toxicity *in vivo* is liver, we decided to evaluate tacrine and 7-MEOTA together with the most promising indolotacrine **9b** for their hepatotoxicity on the HepG2 cell line using the MTT assay (Table 6).[36] Compound **9b** was found to be more hepatotoxic compared to 7-MEOTA and tacrine. As in cytotoxicity evaluation, tacrine showed lower *in vitro* hepatotoxicity compared to 7-MEOTA, which is at odds with the *in vivo* results.[35] A possible explanation for this peculiarity is that the hepatoxicity is not caused by tacrine itself but by its metabolites, products of cytochrome P450 oxidation.[37] Therefore it is hard to conclude about the compounds' toxicity *in vivo* (e.g. **9b**) based on the results of *in vitro* testing and these cytotoxicity and hepatotoxicity assessments have, in this case, only generally informative character.

Table 6: Hepatotoxicity evaluation.

Compound	**Hepatotoxicity** IC_{50} ± SEM (μM)
9b	1.22 ± 0.11
tacrine	17.28 ± 0.76
7-MEOTA	11.50 ± 0.77

[a] IC_{50} and SEM values were obtained as a mean of 3 independent measurements

Penetration across the blood-brain barrier (BBB) is an essential property for compounds targeting the CNS and should always be considered during the drug development. In order to predict passive BBB penetration, modification of the parallel artificial membrane permeation assay (PAMPA) has been used based on reported protocol.[38] As summarized in Table 7, it is obvious that compound **9b** has a high potential to be available in the CNS. Data obtained for the new compound were correlated to standard drugs, where CNS availability is known and also reported using the PAMPA assay.[38] Our data show high resemblance with previously reported penetrations as well as with a general knowledge about the availability in the CNS of such standard drugs.

Table 7: Prediction of blood-brain barrier penetration of **9b** and reference compounds.

Compound	BBB penetration estimation	
	P_e ± SEM (10^{-6} cm s^{-1})	CNS (+/-)[b]
9b	6.6 ± 0.65	(+)
donepezil	7.3 ± 0.9	(+)
rivastigmine	6.6 ± 0.5	(+)
tacrine	5.3 ± 0.19	(+)
testosterone	11.3 ± 1.6	(+)
chlorpromazine	5.6 ± 0.6	(+)
hydrocortisone	2.85 ± 0.1	(+/-)
piroxicam	2.2 ± 0.15	(+/-)
theophyline	1.07 ± 0.18	(-)
atenolol	1.02 ± 0.37	(-)

[a] P_e and SEM values were obtained as a mean of 4 independent measurements

[b] (+) (high BBB permeation predicted) P_e (10^{-6} cm s^{-1}) > 4.0

(-) (low BBB permeation predicted) P_e (10^{-6} cm s^{-1}) < 2.0

(+/-) (BBB permeation uncertain) P_e (10^{-6} cm s^{-1}) = 2.0 – 4.0

In summary, in this work we have reported design, synthesis and *in vitro* evaluation of series of indoles and series of indolotacrine hybrid analogues as potential drugs for the treatment of AD. The novel compounds were designed as MTDLs targeting primarily ChEs and MAOs. In addition to ChE and MAO inhibition, the biological evaluation also involved determination of antioxidant, cytotoxic and hepatotoxic properties and permeability prediction (BBB assay). The most promising compound, indolotacrine **9b**, was found to be a potent inhibitor of AChE (IC_{50} = 1.5 μM), BChE (IC_{50} = 2.4 μM) and MAO A (IC_{50} = 0.49 μM) and weak inhibitor of MAO B (IC_{50} = 53.9 μM). The inhibitory activity of **9b** against ChEs and MAOs seems quite well balanced, and thus has potential for the desired simultaneous multi-target directed action *in vivo*, but the optimal balance of inhibitory ability against each target in AD is still not known.[39] The cytotoxic and hepatotoxic profile of **9b** are slightly inferior to the standards, tacrine and 7-MEOTA, but the overall improvement in the enzymatic inhibitory activities and potential to cross BBB make indolotacrine **9b** a promising lead compound for further development and investigation.

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Keywords

Alzheimer's disease, cholinesterase, cytotoxicity, inhibitors, monoamine oxidase, multi-target-directed ligands (MTDLs)

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Based on multi-target-directed ligand approach series of novel compounds have been designed to act simultaneously as cholinesterase and monoamine oxidase inhibitors. The most promising compound, indolotacrine **9b**, was found to be a potent inhibitor of both cholinesterases and monoamine oxidase A and moderately potent inhibitor of monoamine oxidase B.